Rearing temperature's effect on the growth performance, nutrient utilization and immune response of growth hormone transgenic female triploid Atlantic salmon (*Salmo salar*)

by

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ABSTRACT

This thesis examined the effect of rearing temperature on AquAdvantage Salmon (AAS; growth hormone transgenic female triploid Atlantic salmon) reared at 10.5°C, 13.5°C, 16.5°C from first feed to 1500 g. Fish reared at 16.5°C reached 1500 g in the shortest amount of time, however, thermal-unit growth coefficient (TGC) results showed that salmon reared at 10.5°C exhibited higher growth rates for most of the trial. Through analysis of whole-body and fillet samples at various growth stages, it was determined that rearing temperature significantly influenced composition, deposition rates and retention efficiencies of numerous nutrients. For example, AAS reared at 16.5°C diverted lipid storage away from muscle tissue into their viscera and were less efficient at retaining important nutrients, such as omega-3 fatty acids. By assessing the antiviral immune gene expression response of fish injected with polyriboinosinic polyribocytidylic acid (pIC), it was concluded that temperature significantly influenced immune responses of AAS. Based on transcript expression responses, as measured by real-time quantitative polymerase chain reaction (qPCR) analysis, it appeared that a more robust immune response was mounted by fish reared at 10.5°C compared to 16.5°C. Collectively, these results help establish foundational knowledge that can guide future research to optimize commercial rearing of AquAdvantage Salmon.

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LIST OF ABBREVIATIONS

°C	Degree centigrade
µL	Microlitre
ω	Omega
AAS	AquAdvantage Salmon
actb	Beta-actin
AGD	Amoebic gill disease
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists' Society
ARA	Arachidonic acid (20:4n-6)
BCWD	Bacterial cold water disease
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CT	Cycle threshold
D	Nutrient deposition
d	Day
DHA	Docosahexaenoic acid (22:6n-3)
dsRNA	Double-stranded ribonucleic acid
ef1a	Elongation factor 1 alpha
eif3d	Eukaryotic translation initiation factor 3 subunit D
eif3s6	Eukaryotic translation initiation factor 3 subunit 6

EPA	Eicosapentaenoic acid (20:5n-3)
FCR	Feed conversion ratio
FI	Feed intake
gDNA	Genomic deoxyribonucleic acid
g	Grams
GH	Growth hormone
h	Hour
hpi	Hours post-injection
HPLC	High performance liquid chromatography
HSI	Hepatosomatic index
IFN	Interferon
ifng	Interferon-gamma
isg15a	Interferon-stimulated gene 15a
IP	Intraperitoneal
IRF	Interferon regulatory factor
ISAV	Infectious salmon anaemia virus
ISG	Interferon-stimulated gene
k	Fulton's condition factor
kg	Kilogram
L	Litre
LCDV	Lymphocystis disease virus
lgp2; alias *dhx58*	RNase helicase lgp2
m	Metre

mg	Milligram
min	Minute
mL	Millilitre
MPa	Megapascal
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
MUFA	Mono-unsaturated fatty acid
mxb	Interferon-induced GTP-binding protein of myxovirus resistance b
NCBI	National Center for Biotechnology Information
N_f	Final nutrient composition
ng	Nanogram
N_i	Initial nutrient composition
NRQ	Normalized relative quantity
NTC	Non-template control
NTG	Non-transgenic
no-RT	No reverse transcriptase
p	P-value
pabpc1	Polyadenylate-binding protein 1
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance

pIC	Polyriboinosinic polyribocytidylic acid
PIT	Passive integrated transponder
polr2	RNA polymerase II
PRR	Pathogen recognition receptor
psi	Pound-force per square inch
PUFA	Poly-unsaturated fatty acid
QC	Quality control
qPCR	Real-time quantitative polymerase chain reaction
r^2	Correlation coefficient
RAS	Recirculating aquaculture system
RE	Retention efficiency
RLR	Retinoic acid-inducible gene I-like receptor
rpl32	60S ribosomal protein 32
rsad2; alias *viperin*	Radical S-adenosyl methionine domain containing protein 2
SAV	Salmonid alphavirus
SEM	Standard error of the mean
SIMPER	Similarity percentages
SNP	Single nucleotide polymorphism
ssRNA	Single-stranded ribonucleic acid
stat1b	Signal transducer and activator of transcription 1b
CT_{max}	Thermal critical maxima
tlr3	Toll-like receptor 3
TG	Transgenic

TGC	Thermal-unit growth coefficient
T_i	Mean daily water temperature
US EPA	United States Environmental Protection Agency
VHSV	Viral hemorrhagic septicemia virus
VNN	Viral nervous necrosis
VSI	Viscerosomatic index
W_f	Final fish body weight
W_i	Initial fish body weight
x*g*	Times gravitational force

LIST OF APPENDICES

CO-AUTHORSHIP STATEMENT

The research described in this thesis was performed by Eric H. Ignatz, with guidance from Drs. Matthew L. Rise[a] and Jillian D. Westcott[b]. Eric Ignatz was responsible for conducting the experiments, collecting and analysing data, and writing the thesis. However, several others were instrumental in the completion of this research as well.

Dr. André Dumas[c] provided training on how to analyse the compositional data collected in Chapter 2 and calculate nutrient deposition rates and retention efficiencies. Dr. Dumas also edited and provided feedback on the second chapter of this thesis. Dr. Tillmann J. Benfey[d] assisted in overall study design of both Chapter 2 and Chapter 3, provided training on how to conduct plasma cortisol ELISA analysis, and helped edit both manuscripts for publication. Dr. Tiago S. Hori[c,e] also aided in study design development for both data chapters, helped train on qPCR analysis, and provided feedback for Chapters 2 and 3. Dr. Laura M. Braden[f,g] was involved in training of statistical methods, as well as qPCR analysis, and assisted in editing of manuscripts. C. Dawn Runighan[f] helped supervise the experiments and played a large role in study design development. Dr. Albert Caballero-Solares[a] provided training on multivariate statistics that were performed in the pIC experiment, as well as assisted in editing of Chapter 3. Dr. Mark D. Fast[g] aided with qPCR analysis and provided feedback on the manuscript prepared from the third data chapter. Drs. Jillian Westcott and Matthew Rise supervised throughout the entire program and both helped edit the thesis.

Authorship for the publication derived from **Chapter 2** is: Eric H. Ignatz, André Dumas, Tillmann J. Benfey, Tiago S. Hori, Laura M. Braden, C. Dawn Runighan, Matthew L. Rise, Jillian D. Westcott. This manuscript was submitted to the journal *Aquaculture*. Authorship for the manuscript prepared from **Chapter 3** is: Eric H. Ignatz, Laura M. Braden, Tillmann J.

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CHAPTER 1: General Introduction

1.1 Introduction

As growth of the global fisheries sector has stagnated, the aquaculture industry recently became the primary source for seafood (Cai and Leung, 2017; FAO 2018). By the early 2020s however, world seafood demand is expected to increase by 47 million tonnes and expansion in global aquaculture production is projected to meet only 40% of this requirement (Cai and Leung, 2017). With this shortcoming, fish prices are expected to increase to reduce the demand-supply gap (Cai and Leung, 2017). Anthropogenic climate change is also hypothesized to have a large negative impact on the seafood industry (Barange et al., 2018). Changes in water temperature, acidification, oxygen-level and depth are expected to have long-term effects on aquaculture production (Barange et al., 2018). With increasing temperature, comes increased risk of disease, parasitic infection and harmful algal blooms (Barange et al., 2018). There is also the increased likelihood of natural disasters, such as floods or storms, that are a danger to farm infrastructure (Barange et al., 2018). Within the Atlantic salmon (*Salmo salar*) aquaculture industry, Norway and Chile have been identified as being the most vulnerable to climate change impacts due to their high production levels (Barange et al., 2018). Canada, which produces approximately 191,000 metric tonnes in total aquaculture production, worth almost $1.4 billion (CAD), would also face the same challenges (DFO, 2018). For these reasons, producers are investigating alternative options to open-ocean systems and are beginning to invest into land-based closed containment operations.

Recirculating aquaculture systems (RAS) are becoming a popular option for producers as clean water resources become increasingly limited. This is because RAS require reduced amounts of make-up water (Kristensen et al., 2009) and produce small volumes of effluent that can more easily be treated to reduce waste output (Sharrer et al., 2010). Developments in

denitrification systems, sludge thickening technologies and ozone utilization have all contributed to improvements in waste management and water use reduction in RAS (Martins et al., 2010). RAS also afford more control over the rearing environment, including rearing temperature, reduce pathogen risk as biosecurity protocols are more easily implemented, permit fish production closer to major markets, and reduce the risk of farm escapees interacting with wild populations (Summerfelt et al., 2001; Summerfelt and Vinci, 2008). Economic analysis suggests that both production and operating costs between RAS and traditional ocean net pens can be similar when raising Atlantic salmon to harvest size of 4 - 5 kg (Liu et al., 2016). While capital costs of RAS models are approximately 80% higher than that of net pens, a higher premium may be charged for RAS-raised salmon in niche markets, resulting in similar profit margins for the two systems (Liu et al., 2016). Additional economic analysis is required, though, to definitively differentiate between production systems. Atlantic salmon grown in freshwater RAS display similar growth rates and feed conversion compared to net pen salmon during smolt rearing and full grow-out to harvest, and fish pathogens and parasites that would commonly be found in net pens were not detected in RAS studies (Terjesen et al., 2013; Davidson et al., 2016). Therefore, RAS are suitable in helping the aquaculture industry expand to help meet global production demand with minimal environmental impact.

1.2 Genetic modification in aquaculture

Genetic modification, the process of altering the genetic makeup of an organism, is one strategy employed in North America to enhance aquaculture species to exhibit desired traits. AquaBounty Technologies is an aquaculture company that utilizes transgenic genetic modification in the rearing of their AquAdvantage Salmon (AAS) in land-based freshwater RAS.

Their fish is a growth hormone (GH) transgenic (TG) triploid female Atlantic salmon. AquaBounty gained approval to sell their AAS product in the United States in 2015 (US-FDA, 2017), with subsequent approval in Canada received in 2016 (Health Canada, 2016), making it the world's first-ever genetically-modified animal approved for human consumption. While investigation into GH transgenesis, the process of introducing recombinant DNA to elicit a new and desirable trait in an organism, in Atlantic salmon and coho salmon (*Oncorhynchus kisutch*) dates back several decades (Devlin et al., 1994; Du et al., 1992), the GH TG Atlantic salmon involved in the current research were from a specific line. This line is called EO-1α, in which there is a single copy of a specific construct (opAFP-GHc2) consisting of an ocean pout (*Macrozoarces americanus*) antifreeze protein promoter and the coding sequence of a Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone gene (Yaskowiak et al., 2006, 2007). The transgene is expressed in almost all tissues of Atlantic salmon suggesting there is a lack of tissue-specificity from the antifreeze promoter (Hobbs and Fletcher, 2008). This allows for enhanced growth characteristics as GH is widely expressed, however no additional freeze resistance traits are reported in Atlantic salmon with the transgene present (Hobbs and Fletcher, 2008). Previous research involving AquaBounty's salmon demonstrated that, when compared to non-transgenic (NTG) siblings, juvenile (~10 g) TG fish reared at ~12.6°C exhibited more than twice their growth rate and feed consumption (Cook et al., 2000a). Furthermore, at 13°C, TG pre-smolts (< 55 g) had higher oxygen consumption rates than NTG salmon (Cook et al., 2000b). At a later stage (~100 g), mixed sex (female and male) populations of GH TG triploid Atlantic salmon reached target body weight in approximately 40% of the time it took their NTG siblings, and demonstrated lower feed conversion ratios and higher nitrogen retention efficiencies when reared in a flow-through system at approximately 10°C (Tibbetts et al., 2013). While more feed was

consumed daily compared to NTG counterparts, they required 25% less feed overall to reach the same target weight due to their shortened production cycle (Tibbetts et al., 2013). Additionally, GH TG salmon at ~30 g maintained or exceeded their enhanced growth performance when fed a high plant protein, fish meal-reduced diet (Ganga et al., 2015).

Information on what effect genetic modification has on host immune function is sparse. However, research suggests that GH transgenesis decreases immune response in transgenic fish compared to their conventional counterparts. For example, during a challenge with a bacterial pathogen, *Aeromonas salmonicida,* GH TG coho salmon reared at ~10°C were more susceptible to furunculosis than wild-type NTG fish (Kim et al., 2013). Another study, comparing diploid GH transgenic and wild-type coho salmon found that, when injected with either polyriboinosinic polyribocytidylic acid (pIC) or peptidoglycan (to elicit antiviral or antibacterial responses, respectively), immune biomarker gene expression response in the muscle tissue was attenuated in transgenic fish (Alzaid et al., 2018). The authors hypothesized that enhanced growth rate impacts normal crosstalk among growth and immune systems (i.e. affects energy allocation), negatively influencing host response to pathogens (Alzaid et al., 2018). GH TG coho have higher levels of hematocrit, hemoglobin and erythrocytes, but lower numbers of leukocytes compared to wild-type NTG coho salmon (Kim et al., 2013). These differences may help explain the aforementioned reports of decreased immune responses in GH TG coho compared to wild-type salmon.

1.3 Triploid salmonids

Salmonid genomes are recognized as pseudotetraploid, as this family underwent a whole-genome duplication event, followed by partial re-diploidization (Allendorf and Thorgaard, 1984;

Davidson et al., 2010; Xu et al., 2013; Lien et al., 2016). However, throughout this thesis, the conventional Atlantic salmon genome will be referred to as diploid, and where the second polar body was retained, triploid. Triploids are beneficial to the aquaculture industry, as they offer an effective, albeit not 100% guaranteed, option for reproductive sterility and genetic containment (Benfey, 2016). However, there are multiple methods to induce sterility in salmonid fish, and there are advantages and disadvantages to each. Surgical removal of the gonads, exposure to high-energy radiation, androgen treatment and genetic modification are all potential options, but due to high cost, time, and marketing constraints, none of these are currently viable options for commercial production of sterile fish. For these reasons, triploidy remains the only feasible option for the aquaculture sector (Benfey, 2016).

Triploidy can be induced by either duplicating the maternal genome, duplicating the paternal genome, or by crossing a tetraploid parent with a diploid parent (Benfey, 2016). Currently, duplicating the female parent's genome is the simplest and most common method for inducing triploidy (Benfey, 2016). Immediately following fertilization of the egg, there is a brief window of opportunity when there are two sets of maternal chromosomes and one paternal set present (Benfey, 2016). It is during this time that the second stage of meiotic division can be blocked, and instead of the second maternal chromosome set exiting the egg as the second polar body, it can be retained (Benfey, 2016). This process leads to an increase in nuclear volume and cell size to accommodate the extra genetic material (Benfey, 1999). Utilizing pressure or thermal shock is a simple and effective method of retaining the second polar body (Benfey, 2016).

For Atlantic salmon, the industry standard (such as in Tasmania) is a 5 min pressure shock at 65.5 MPa (9500 psi) starting 300°C-min after fertilization (Benfey, 2016). For Arctic

charr (*Salvelinus alpinus*), this same pressure treatment starting at 210 °C-min post-fertilization resulted in 100% triploidy, as determined by measurement of erythrocyte DNA content using a flow cytometer (Chiasson et al., 2009), and for brook charr (*Salvelinus fontinalis*), 5 min at 65.5 MPa starting at 29.5 min after fertilization was confirmed to successfully induce triploidy (O'Donnell, MacRae et al., 2017). Triploidy induction in rainbow trout (*Oncorhynchus mykiss*) has been achieved with 62 MPa for 8 min (Weber et al., 2013). Chinook, coho and sockeye (*Oncorhynchus nerka*) salmon, as well as brown trout (*Salmo trutta*), have all seen success in triploid induction, however, limited research on induction methods has been dedicated to these species (Benfey, 2016).

Crossing a tetraploid parent with a diploid is meant to produce 100% triploids with fewer negative impacts on embryonic development (Weber et al., 2014). The production of tetraploid finfish is not perfected, though, due to problems of low survival and fertility (Weber et al., 2014). Poor egg quality and suboptimal sperm size (too large to fit into a diploid micropyle), especially during first generation of tetraploids, have hindered to the use of tetraploids to generate triploids (Weber and Hostuttler, 2012). However, advancements make it feasible to create tetraploids and produce intercross-triploids when reproducing with diploids in rainbow trout (Weber and Hostuttler, 2012).

The most practical way of assessing the success of the triploidization process is through measurement of erythrocyte DNA content or size (Benfey, 2016). Flow cytometers or particle size analyzers are machines for analysis to determine relative DNA content or cell size, respectively (Allen Jr, 1983; Benfey, 2016), but blood smears analyzed by microscopy can also be used to determine triploidy based on cell and nuclear volume (Benfey et al., 1984).

1.3.1 Comparison of diploid and triploid growth performance

Triploidy negatively impacts GH TG salmon growth performance, as evidenced by decreased weight gain in both triploid Atlantic and coho salmon compared to diploid counterparts (Leggatt et al., 2012; Tibbetts et al., 2013). While studying juvenile GH TG Atlantic salmon, researchers found hepatic transcripts differentially expressed between fast- and slow-growing triploid families (Xu et al., 2013). Genes including *apoa1*, *apoa4*, *b2m*, *fadsds6*, *ftm*, and *gapdh*, which are collectively involved in metabolism, iron homeostasis, oxygen transport, and immune- or stress-related responses, were found to be up-regulated in fast-growing triploids (Xu et al., 2013). These growth-associated genes can help identify candidates for further single nucleotide polymorphism (SNP) marker development (Dunham et al., 2014). SNPs could provide marker-assisted selection of diploids that give rise to fast-growing triploid GH TG salmon.

During the early rearing of Arctic charr, diploids outperformed triploids in terms of weight gain and condition factor (Chiasson et al., 2009). However, during grow-out to market size of 1 kg, while condition factor equated to that of diploids, triploids still weighed significantly less than diploids (Chiasson et al., 2009). It was noted that the diploid fish were beginning to show signs of sexual maturation at harvest and if the study were to continue past 1 kg, it was hypothesized that the growth performance of the triploids would equal or surpass that of the diploids (Chiasson et al., 2009). In Atlantic salmon, the weight of late alevin and fry stages of diploids exceeded that of their triploid siblings, however, at the parr stage, the weight of triploids equalled that of diploids (Nuez-Ortín et al., 2017). Based on these results, it appears that the growth performance of triploid salmonids is influenced by their developmental stage.

Additionally, a comparison between rainbow trout tetraploid/diploid intercross-triploids, pressure shocked induced-triploids and diploids demonstrated differences in growth performance (Weber et al., 2014). After 2 years, intercross-triploids weighed significantly more than induced-triploids and diploids, which weighed similarly at the end of the trial (Weber et al., 2014). Condition factor was lowest amongst the intercross-triploids, and the presence of higher amounts of vertebral compression in induced-triploids was thought to contribute to their higher condition factor (Weber et al., 2014). Condition factor is generally lower in triploids than in diploids, as triploids are consistently longer fish at a given weight (Benfey and Sutterlin, 1984). Furthermore, family-specific differences were prevalent between ploidy types, with a greater correlation between performance in intercross-triploids and their diploid siblings being noted (Weber et al., 2014). This suggests a greater potential to improve growth performance based on selection of diploid parents (Weber et al., 2014). Additionally, strain-related differences between diploids and triploids are apparent. For example, a study comparing Atlantic salmon diploids of three strains [Mowi, commercial St. John River (SJR), wild SJR] versus triploids of two (Mowi, Cascade), reported growth differences between ploidies (Sacobie et al., 2012). While the Mowi strain of triploids demonstrated comparable growth to diploids within strain (with a lower condition factor), the Cascade strain of triploids did not perform as well as the two SJR diploid strains (Sacobie et al., 2012).

Rearing environment has also been shown to impact growth performance of triploid salmonids. When diploid and triploid Atlantic salmon were reared in a communal environment, triploids exhibited reduced growth (Friars et al., 2001). It has been hypothesized that diploids outcompete triploids for feed as triploids are known to be less aggressive (Benfey, 1999), and so rearing the two separately may be advantageous for producers (Friars et al., 2001). Further

research has shown that triploids in a mixed ploidy tank had a harvest weight 29.6% lower than that of triploids raised independent of diploids (Taylor et al., 2014).

1.3.2 Comparison of diploid and triploid nutrition

The specific nutrient requirements of triploid salmonids are yet to be determined (Benfey, 2011). While no commercial feed chart for triploid salmonids exists, in North America they are currently being fed according to diploid salmon charts. It has been hypothesized that Atlantic salmon triploids have a higher dietary requirement for protein and amino acids (Smedley et al., 2016), and it has been proposed that triploids require a higher dietary digestible protein to digestible energy ratio compared to diploids (Tibbetts et al., 2013; Ganga et al., 2015). Current practices typically involve triploids being fed commercial diets prepared for diploid salmon which may not meet these requirements. Atlantic salmon triploids have a significantly lower relative gut length, pyloric caeca number and intestinal mass compared to diploids (Peruzzi et al., 2015). The differences seen in gut morphology could influence digestive efficiency and therefore should be considered when formulating a diet for triploids (Peruzzi et al., 2015). Due to the higher occurrence of cranial and skeletal deformities in triploid Atlantic salmon compared with diploids, researchers have investigated supplementation of higher amounts of protein and phosphorus to minimize deformities and promote growth (Burke et al., 2010; Smedley et al., 2016), and likewise histidine is supplemented to reduce the occurrence of cataracts (Taylor et al., 2015). The ability of triploid fish to absorb and utilize nutrients may be impacted by differences in cellular morphology, increased heterozygosity, and decreased cellular surface-to-volume ratio (Piferrer et al., 2009).

Differences in the body composition of triploid Atlantic salmon have been detected, with higher gross energy and lipid levels and lower moisture and ash levels compared to their diploid counterparts (Burke et al., 2010). While significantly higher whole-body lipid concentrations were found at the parr stage of triploid Atlantic salmon compared to diploids, the fatty acid profile remained broadly unchanged by ploidy (Nuez-Ortín et al., 2017). The increase in lipid deposition is thought to be attributed to the increased cell size in triploids and the consequent increased lipid storage capacity (Johnston et al., 1999; Nanton et al., 2007). A significant reduction in flesh colour intensity has been evidenced in triploids, which may be attributable to the decreased surface area-to-volume ratio and/or the binding affinity of triploid cells, affecting uptake of pigment (Smedley et al., 2016). Additionally, an increase in texture score (e.g. firmness, as determined by maximum shear force) has also been demonstrated (Smedley et al., 2016), which may account for the decrease in fillet redness as these traits are thought to be inversely correlated as muscle fibre structure influences flesh redness (Bjørnevik et al., 2004). Interestingly, no differences in digestibility of nutrients such as protein or lipid have been detected between triploid and diploid Atlantic salmon (Burke et al., 2010; Tibbetts et al., 2013; Ganga et al., 2015).

1.3.3 Comparison of diploid and triploid immune and stress responses

There is a paucity of information related to the immune response of triploid or GH TG salmonids to pathogens compared to conventional diploids. In terms of response to vaccination and subsequent bacterial infection, triploid and diploid Atlantic salmon showed no differences in vaccine adhesion scores around the injection site when injected with a commercial furunculosis vaccine (Chalmers et al., 2016) as determined by the Spielberg Scale (Midtlyng et al., 1996).

This contrasts with previous work that reported an increase in vaccine adhesion scores in triploid salmon compared to diploids when injected with a multivalent vaccine (Fraser et al., 2014). Chalmers et al. (2016) demonstrated that in vaccinated Atlantic salmon exposed to *A. salmonicida*, ploidy did not influence complement activity, antibody response or survival, indicating that the vaccine was equally effective between ploidies. Another study involving the bacterial infection of unvaccinated diploid and triploid rainbow trout detected minimal differences in survival between ploidies after exposure to *Flavobacterium psychrophilum*, the etiological agent of bacterial cold water disease (BCWD) (Weber et al., 2013). Regarding parasitic infection, diploid and triploid Atlantic salmon infected with *Neoparamoeba perurans*, the causative agent of amoebic gill disease (AGD), did not demonstrate differences in terms of how the disease manifested, the severity of gill damage, or complement and anti-protease activities (Chalmers et al., 2017). Similarly, no differences in infestation level were detected between triploid and diploid Atlantic salmon exposed to sea lice (*Lepeophtheirus salmonis*) (Frenzl et al., 2014).

In a study of susceptibility to pancreatic disease, mortality was similar in diploid and triploid Atlantic salmon fry exposed to SAV sub-type 1 through either intraperitoneal (IP) injection, bath immersion, or cohabitation (Herath et al., 2017). However, a significantly higher viral RNA copy number was noted in the liver of diploid fry in the cohabitation group, and diploids displayed higher levels of myocardial and pancreatic degeneration compared to triploids (Herath et al., 2017). In another study in which diploid and triploid Atlantic salmon post-smolts were injected with SAV sub-type 3, viral load increased at a slower rate in triploids with 19% and 56% at 14 and 21 days post-exposure, respectively, compared to 82% and 100% seen in diploids (Moore et al., 2017). Researchers hypothesized that the slower accumulation could

delay the onset of pancreatic disease or aid in the avoidance an outbreak as triploids may be able to mount a more robust immune response to SAV (Moore et al., 2017).

Ploidy manipulation may also influence stress response in salmonids, but research is not conclusive. For example, in brook charr after acute handling, diploids and triploids demonstrated similar short-term increases in plasma cortisol and hematocrit levels, with a gradual increase in plasma glucose levels (Biron and Benfey, 1994). In a subsequent study, after acute handling, similar increased plasma cortisol, hematocrit and glucose levels were reported between diploid and triploid brook charr and rainbow trout (Benfey and Biron, 2000). Only minor differences were found between triploids and diploids, other than significantly lower resting total blood cell concentrations (erythrocytes and leukocytes) in triploids, which did not appear to have a significant impact on triploid stress response (Benfey and Biron, 2000). In Atlantic salmon, no differences in plasma cortisol or lactate were detected between triploids and diploids after confinement stress in either freshwater parr or saltwater smolts (Sadler et al., 2000). In contrast, one-year-old GH TG triploid Atlantic salmon exposed to the stress of week-long fasting or hypoxia had stress response indicators (e.g. blood sodium concentrations) that peaked to higher levels and took longer to return to pre-stress levels when compared to conventional salmon (Cnaani et al., 2013). More research is required to determine what effect, if any, triploidy has on salmonid immune and stress responses.

1.4 Impact of rearing temperature on finfish

The effect of temperature on Atlantic salmon growth is well documented (Johnston and Saunders, 1981; Koskela et al., 1997; Imsland et al., 2014). Growth modeling indicates that growth rate increases with temperature in most fish species until a critical limit is reached,

beyond which it will decrease (Jobling, 1997). Reports of the optimal temperature for growth performance in diploid Atlantic salmon vary. Handeland et al. (2008) purported that the optimal growth temperature for Atlantic salmon increases with fish size, recommending the early rearing of post-smolts at 12.8°C with an increase to 14.0°C when the salmon reach 150 g. In laboratory systems, greater weight gain can be achieved at approximately 16°C in diploid Atlantic salmon than at lower temperatures (Jensen et al., 2015), but optimal growth efficiency, in terms of feed conversion ratios and nutrient utilization rates, is reported to be lower, near 13°C (Hevrøy et al., 2013). However, triploid Atlantic salmon likely have a lower thermal optimum than diploids (Atkins and Benfey, 2008), as they are less tolerant to high temperature and low oxygen conditions (Sambraus et al., 2017, 2018). Triploid Atlantic salmon raised in sea cages demonstrated a preferred rearing temperature of 16.5-17.5°C when oxygen levels were favourable (> 60% saturation), avoiding surface waters above 20.1°C and areas with dissolved oxygen less than 35% of air saturation (Stehfest et al., 2017). GH transgenesis does not appear to impact the thermal critical maxima (CT_{max}) in coho salmon when compared to wild-type and domesticated counterparts (Chen et al., 2015), so it may not affect thermal optima in this species. GH TG diploid Atlantic salmon, reared at a temperature of 16°C under 24-h light did not experience smolt development inhibition as NTG salmon did (Saunders et al., 1998). It was suggested that GH TG salmon growth could be maximized under higher temperature and longer photoperiod conditions which would add to their production benefits (Saunders et al., 1998).

In terms of CT_{max} between ploidies, no differences were reported in brook charr in under-yearlings and yearlings (Benfey et al., 1997). However, under hypoxic conditions, diploid and triploid brook charr exhibited differences in CT_{max} (Ellis et al., 2013). The results of two CT_{max} experiments contradict one another, as in the first, CT_{max} was lower in triploids than diploids, but

the opposite was found in the second experiment (Ellis et al., 2013), therefore further research is required to elucidate the true mechanisms involved. In rainbow trout, only minor differences were determined between triploids and diploids in response to hypoxia at temperatures between 20°C and 27°C (Benfey & Devlin, 2018). Research has shown that, while initial oxygen uptake at ~16°C does not differ between ploidies in brook charr, the rate of decline in uptake is slower in triploids (O'Donnell et al., 2017). The slower rate of decline results in higher final oxygen uptake rates, resulting in higher standard metabolic rates, implying reduced aerobic scope in triploids (O'Donnell et al., 2017). These data contradict previous work which reported triploids having a lower metabolic rate than diploids (Stillwell and Benfey, 1996).

Temperature is also known to influence viral pathogenicity and host immune responses of diploid teleost fish. For instance, Avunje et al. (2012) showed that both viral and host responses differ based on rearing temperature in viral hemorrhagic septicemia virus (VHSV)-infected diploid olive flounder (*Paralichthys olivaceus*). Viral gRNA copy number, a proxy for the level at which viral replication has occurred, peaked earlier in flounder reared at 20°C and declined at significantly faster rates than in fish reared at 15°C (Avunje et al., 2012). Concurrently with viral copy number, antiviral gene expression increased more rapidly and to higher levels in the 20°C group compared to 15°C (Avunje et al., 2012). Transcriptome sequencing based differential expression (DE) analysis of head kidney tissue of diploid olive flounder reared at either 13°C or 20°C and infected with VHSV revealed hundreds of DE genes between temperature groups related to metabolism, virulence factors, adhesion and several immune signalling pathways (Hwang et al., 2018; Jeong et al., 2018). Previous research demonstrated that mortality is lower in diploid olive flounder infected with VHSV reared at 20°C than at 14°C (Sano et al., 2009).

However, the impact of rearing temperature on virulence is specific to the type of virus, as evidenced by increased temperature correlating with increased mortality in diploid zebra fish (*Danio rerio*) infected with betanodavirus, causing viral nervous necrosis (VNN) (Binesh, 2014). Similarly, diploid Senegalese sole (*Solea senegalensis*) infected with betanodavirus at 16°C, 18°C and 22°C experience increased mortality with increasing rearing temperature (Souto et al., 2015). Following IP injection of inactivated lymphocystis disease virus (LCDV) in diploid olive flounder reared at 9°C, 15°C, 21°C or 26°C, it was found that the optimal temperature to immunize was 21°C, as surface immunoglobulin positive cells reached higher levels faster than the other temperature groups (Xu et al., 2011). VHSV replication has also been shown to be temperature-dependent, with infection inhibited at 26°C, as evidenced in a walleye (*Sander vitreus*) cell line (WE-cfin11f) (Vo et al., 2015). In Atlantic cod (*Gadus morhua*), elevated temperature (16°C compared with 10°C) appeared to accelerate the spleen antiviral transcript expression response to pIC (Hori et al., 2012). In rainbow trout, temperature modulated mortality after exposure to infectious pancreatic necrosis virus (IPNV) (Dorson & Torchy, 1981). Juvenile trout reared at 10°C exhibited a more rapid spike in mortality (reaching almost 100%), while fish infected with IPNV at 16°C experienced the same initial mortality responses, however, rates quickly decreased and mortality did not surpass 40% (Dorson & Torchy, 1981). In juvenile trout exposed to VHSV, virus persistence was found inversely proportional to rearing temperature, with undetectable viral levels found 8 days post-injection (dpi) at 15°C and 20°C, meanwhile, it took 35 dpi for levels to drop at 10°C (Jørgensen, 1982). Rainbow trout fingerlings IP-injected with epizootic haematopoietic necrosis virus (EHNV) at 19 to 21°C all perished within 10 dpi, yet, mortality was delayed in fish reared at 8 to 10°C, with a few surviving until the end of the 63-day trial (Whittington & Reddacliff, 1995). While research demonstrates that

rearing temperature directly impacts viral pathogenesis in diploid fish, there is a paucity of information available on triploids in relation to temperature-mediated antiviral response.

1.5 Research objectives

The overall objective of this research was to determine the effect of rearing temperature on growth performance, nutrient utilization and immune response of GH TG triploid female Atlantic salmon. This was accomplished by evaluating weight gain, morphometric data, feed conversion, whole-body and fillet nutrient composition, and fillet colour at various stages as salmon were grown from first-feeding to 1500 g at 10.5°C, 13.5°C and 16.5°C. AAS at 800 g were pIC-injected to induce an antiviral response at the same three rearing temperatures, with sampling and data collection at multiple time points post-injection. Using real-time quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) analyses, target biomarker head kidney gene expression and plasma cortisol levels were obtained, respectively. It is anticipated that knowledge from these studies can aid in developing rearing strategies for commercial production of AAS in freshwater RAS accounting for the growth rate, nutrient efficiency and antiviral response at each temperature.

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CHAPTER 2: Growth performance and nutrient utilization of growth hormone transgenic female triploid Atlantic salmon (*Salmo salar*) reared at three temperatures in a land-based freshwater recirculating aquaculture system

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2.1 Abstract

This study examined the effect of rearing temperature on growth performance and nutrient utilization of AquAdvantage Salmon (growth hormone transgenic female triploid Atlantic salmon) reared in a land-based freshwater recirculating aquaculture system (RAS) at three temperatures (10.5°C, 13.5°C, 16.5°C) from first-feeding to 1500 g. All fish were fed the same commercial diets, which were analysed for proximate, amino acid, fatty acid and mineral composition, and daily feed consumption was recorded for the calculation of feed conversion ratios (FCRs). Whole-body samples were collected at 300, 500, 800 and 1500 g for proximate composition, and fillet samples were collected at 500, 800 and 1500 g for amino acid, fatty acid, and mineral composition. Fillet yield, astaxanthin concentration and DSM SalmoFan™ colour were determined at 1500 g. Results indicated that fish reared at 16.5°C required more feed per unit of biomass gain and were less efficient at retaining important nutrients, such as omega-3 fatty acids, compared to salmon reared at 10.5°C and 13.5°C. With higher visercosomatic index (VSI) and condition factor (k) scores at 16.5°C, these fish also diverted more dietary energy to lipid deposition in the viscera instead of muscle tissue compared to the lower temperature treatments. Elevated temperature also resulted in lighter fillet colouration compared to salmon reared at 13.5°C, and fish reared at 16.5°C exhibited lower fillet yields than the other temperature groups. Fish reached the end target weight (1500 g) in 36 and 93 fewer days when reared at 16.5°C compared to 13.5°C and 10.5°C, respectively. However, thermal-unit growth coefficient (TGC) results indicated that growth rate was higher in fish reared at 10.5°C compared to 16.5°C for the majority of the trial. Correspondingly, it is preferable to rear AquAdvantage salmon at either 10.5°C or 13.5°C in freshwater RAS. The information generated from this

study is the first to provide insight into the relationships between rearing temperature and nutrient utilization in AquAdvantage Salmon for commercial application.

2.2 Introduction

Growth hormone (GH) transgenic (TG) female triploid Atlantic salmon (*Salmo salar*), commercially referred to as AquAdvantage Salmon (AAS), are a reproductively sterile alternative to conventional farmed diploid salmon that reach target body weight in approximately 40% of the time it takes their non-transgenic (NTG) siblings, and demonstrate lower feed conversion ratios (FCRs) and higher nitrogen retention efficiencies when reared in a flow-through freshwater system at approximately 10°C (Tibbetts et al., 2013). While more feed is consumed on a daily basis compared to NTG counterparts, they require 25% less feed overall to reach the same target weight due to their shortened production cycle (Tibbetts et al., 2013). Additionally, these fish can maintain or even exceed their enhanced growth performance even when fed a diet substituting high amounts of plant protein for fish meal (Ganga et al., 2015).

Salmonid genomes are recognized as pseudotetraploid, as this family underwent a whole-genome duplication event, followed by partial re-diploidization (Allendorf and Thorgaard, 1984; Davidson et al., 2010; Xu et al., 2013; Lien et al., 2016). However, to improve the readability of this chapter, the conventional Atlantic salmon genome will be referred to as diploid, and where the second polar body was retained, triploid. Several putatively associated genes have been found in relation to fast growth in triploid juvenile GH TG salmon (Xu et al., 2013), which can help identify candidates for further single nucleotide polymorphism (SNP) marker development (Dunham et al., 2014). SNP markers can potentially assist in breeding program selection, as triploids see a high variance in growth and tolerance to ploidy manipulation (Friars et al., 2001). Some research suggests that all-male stocks of triploid Atlantic salmon reared separately from diploids could outperform female triploids for growth (Taylor et al., 2014). However, a decrease in flesh quality may result as males typically display more pre-harvest gonadal development

compared to females (Taylor et al., 2014). The gonadosomatic index of Atlantic salmon female triploids is approximately 7.7% that of diploids, and triploid ovaries appear underdeveloped, with few oocytes present (Benfey and Sutterlin, 1984). Furthermore, they demonstrate fewer incidences of skeletal deformities compared to triploid males (Taylor et al., 2013). For these reasons, all-female populations of Atlantic salmon triploids are preferred.

The specific nutrient requirements of triploid salmonids, including GH TG triploid salmonids, are yet to be determined (Benfey, 2011). As a result, triploids are fed according to diploid salmon feed charts in North America. This is problematic when it has been theorized that Atlantic salmon triploids have a higher dietary requirement for protein and amino acids (Smedley et al., 2016). Furthermore, differences in gut morphology in Atlantic salmon triploids could influence digestive efficiency, thus requiring consideration when formulating a diet for triploids (Peruzzi et al., 2015). Due to the higher occurrence of cranial and skeletal deformities in triploids, researchers have also investigated supplementation of higher amounts of protein and phosphorus to minimize deformities and promote growth (Burke et al., 2010; Smedley et al., 2016), and likewise histidine is supplemented to reduce the occurrence of cataracts (Taylor et al., 2015).

Research on the impact of rearing temperature on various aspects of triploid salmonid production characteristics is limited. However, it has been purported that salmonid triploids have lower thermal optima than diploids, as they have higher metabolic rates at lower temperatures, and lower rates at higher temperatures, than diploids (Atkins and Benfey, 2008), which may explain reports of higher mortality in triploids at chronically elevated temperatures (Sambraus et al., 2017). Furthermore, vertebral deformities are more prevalent in Atlantic salmon reared at 16°C compared to those reared at 10°C (Ytteborg et al., 2010) and salmon

reared at 18°C exhibited a higher prevalence of lower jaw deformities compared to those reared at 14°C (Amoroso et al., 2016). Temperature is also known to impact the gut microbiota of Atlantic salmon (Cantas et al., 2011), with increasing temperature correlating to an increase in the number and diversity of bacteria present, which can impact the digestibility of nutrients (Neuman et al., 2014). Rearing of conventional Atlantic salmon in land-based RAS at 15°C to 16°C is shown to be biologically and technologically feasible from post-smolt to market-size (Davidson et al., 2016), while it has been recommended that triploid Atlantic salmon be raised at a temperature less than 15°C (Hansen et al., 2015; Sambraus et al., 2017, 2018). However, prior to the current study, no such recommendation regarding rearing temperature was available for AAS.

The purpose of this experiment was to determine the impact of rearing temperature on AquAdvantage Salmon growth performance and nutrient utilization in freshwater RAS. This was accomplished by evaluating weight gain, morphometric data, feed conversion and whole-body and fillet nutrient composition at various stages as salmon were grown from first feed to 1500 g at 10.5°C, 13.5°C and 16.5°C. This report is the first investigation of the effect of rearing temperature on these aspects of AAS commercial production.

2.3 Materials and methods

2.3.1 Experimental animals and rearing conditions

All fish used in this study were St. John River (New Brunswick, Canada) origin. Fish were hatched and reared in a freshwater land-based RAS facility at AquaBounty Canada, Prince Edward Island, Canada. The animals were handled and cared for in accordance with the Canadian Council on Animal Care's Guidelines on the Care and Use of Fish in Research,

Teaching and Testing (Canadian Council on Animal Care, 2005). The study was approved by the Animal Care Committees of both AquaBounty Canada (Animal Care Protocol [ACP] ABC-ACC-013) and Memorial University (ACP 17-03-JW).

AAS eggs were produced from a specific TG line (termed EO-1α) containing a gene construct (opAFP-GHc2) comprised of an ocean pout (*Macrozoarces americanus*) antifreeze protein promoter and the coding sequence of a Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone gene (Yaskowiak et al., 2006, 2007). A single homozygous TG sex-reversed neomale (i.e., functionally masculinized genetic female) was crossed with three NTG females, producing all female TG offspring. Fertilized eggs were pooled before shocking via hydrostatic pressure to induce triploidy. Transgene status and zygosity of the broodstock were confirmed by polymerase chain reaction (PCR) and quantitative PCR, respectively, using validated procedures developed by AquaBounty. Ploidy status of the offspring was verified through flow cytometry, demonstrating that pressure-shock was 99.5% effective in producing triploids.

All eggs were incubated in fresh water at 2.0-3.0°C (lower temperatures were used to delay time of hatching) and survival to hatching was calculated as ~71.7%. At approximately 600 degree-days, yolk-sac fry were moved from their incubators into 1.5 m^3 cylindrical fibreglass tanks, each containing a 320 L insert designed for early rearing which was later removed when fish reached an average weight of 4 g. Approximately 8,100 yolk-sac fry were distributed amongst 9 tanks (n = 900 per tank) held at 13°C and introduced to feed over a period of 14 days. After an acclimation period of two weeks following the establishment of exogenous feeding, tank temperatures were increased or decreased over a period of 24 hours to meet the experimental conditions. De-gassed and oxygenated fresh water from a well (<1 g/L salinity) was supplied to each tank in a RAS (10–20% make-up daily) and dissolved oxygen was

measured daily and maintained at >9.0 mg/L. Water temperature was maintained at 10.5°C, 13.5°C and 16.5°C (± 0.5°C) in triplicate tanks and recorded twice daily. Fish were maintained on constant light (mimicking AquaBounty's commercial practices) and fed daily to meet predicted requirements using automatic feeders. Feed rations and pellet sizes were adjusted as required to meet the demands of the fish, which was accomplished by manually monitoring any excess feed collected during tank flushing and tracking average fish weights per tank, respectively. Daily feed consumption was measured by weighing the amount of feed added to each of the feeders and collecting excess feed pellets in order to calculate feed intake and feed conversion ratios. All nine tanks were fed the same lots of commercial Atlantic salmon diet (Skretting, St. Andrew's, New Brunswick, Canada). The gross nutrient composition of the feed (on an as-fed basis) was 3-8% moisture, 47-59% crude protein, 15-26% lipid and 6-11% ash. A full report of diet composition and a summary of the weight stages at which they were fed is provided in Appendix I. Dead or moribund salmon were removed daily, and visual fish health examinations were conducted on all fish collected with any abnormal findings that could impact fish welfare noted (i.e. fin erosion, skin abrasions, etc.). Fish weight (g), fork length (cm) and general appearance were monitored on at least a monthly basis from a sample of a 100 fish per tank, or all fish in a tank if there were less than 100. As fish in each rearing temperature reached an average weight of 400 g, passive integrated transponder (PIT) tags (Avid Canada Corp., Calgary, Alberta, Canada) were inserted into their peritoneal cavities. Tank densities were controlled by scheduled reductions in fish numbers, culling (by convenience) a predetermined biomass of fish to maintain densities below 50 kg/m^3. During periods of handling or sampling, fish were starved for a minimum of 24 h prior to being anesthetized using tricaine

methansulfonate (MS-222) (Syndel Canada, British Columbia) at a dose of 0.1 g/L, buffered with sodium bicarbonate.

2.3.2 Sample collection

Whole-body samples of fish were obtained by euthanizing fish with buffered anesthetic (MS-222) at a dose of 0.4 g/L when fish averaged 300, 500, 800 and 1500 g in weight. A convenience sampling method was employed, such that the first salmon identified that fell within the desired weight range (±10% from the sampling target weight) was selected at each growth stage. This process was repeated until 4 fish per tank (n = 12 per treatment) were obtained for each growth stage. Whole-body samples were maintained on ice following euthanasia and subsequently stored at -70°C until shipped to BioFoodTech/PEI Analytical Laboratories (Charlottetown, Prince Edward Island, Canada) for proximate composition analysis.

Fillet samples containing dorsal and ventral muscle with bone and skin, were collected from the fish's right side and placed in plastic bags as fish held at each temperature reached average weights of 500, 800 and 1500 g; convenience samples of 10 fish per tank (n = 30 per treatment) were taken at 500 g, 7 fish per tank (n = 21 per treatment) at 800 g and 4 fish per tank (n = 12 per treatment) at 1500 g. Fillets were maintained on ice and subsequently stored at -70°C until shipped to SickKids (Toronto, Ontario, Canada), VALORĒS (Shippagan, New Brunswick, Canada) and Exova (Surrey, British Columbia, Canada) for compositional analysis (amino acid, fatty acid, and mineral, respectively), as these companies have all been used in the past for nutritional analysis by AquaBounty. At the 1500 g weight point, four additional fillets were collected via convenience sample per tank (n = 12 per treatment) and scored using the DSM SalmoFan™ colour chart (DSM, 2019), the industry-recognized method for visual assessment of

the degree of pigmentation in salmon flesh. For this assessment, fillets were placed into a white Styrofoam container with plain white paper lining the bottom and a white 100W halogen light bulb hanging 31.5 inches directly above the individual fillets to be assessed. Two trained technical staff independently scored each fillet to determine its level of pigmentation; individual scores were assigned to describe the colouration along the lateral line and peripheral edges of the fillet. A final score for each sample was determined by calculating the average colouration across the fillet. Samples were wrapped in aluminum foil and stored in plastic bags at -70°C until shipped to VALORĒS for astaxanthin concentration determination. Additionally, liver and viscera weight were measured at 800 and 1500 g, and fillet weight was measured at 1500 g.

A sample of each lot of feed utilized throughout the study was collected at the time of opening and stored at -20°C until proximate, amino acid, fatty acid and mineral composition analyses were conducted.

2.3.3 Analytical techniques

Proximate composition analyses were conducted on the whole bodies of individual fish and commercial feed samples according to the Association of Official Analytical Chemists (AOAC) Official and American Oil Chemists' Society (AOCS) methods: dry matter, 16 h at 105°C (AOAC, 2012; method 930.15); ash, a minimum of 6 h at 600°C (AOAC, 2012; method 942.05); nitrogen (AOAC, 2012; method 990.03; crude protein = N×6.25), and lipid (AOCS, 2017; method Am 5-04). All fillet samples destined for either amino acid, fatty acid and mineral composition, or astaxanthin composition, were pooled by tank before analysis. Feed samples were analyzed individually. Sample preparation prior to determining amino acid content consisted of drying the sample at 100-102°C for 18 h to determine moisture content and

subsequently freeze drying a portion of the sample and defatting it via Soxhlet extraction using hexane. Amino acid analysis was performed on a Waters ACQUITY UPLC System using a modified and combined version of established methods (Bidlingmeyer et al., 1984, 1987; Heinrikson and Meredith, 1984; Cohen and Strydom, 1988). Fatty acid composition of fillet and feed samples was measured by direct transesterification using acetyl chloride under controlled heating conditions. Fatty acids were separated and quantified via gas chromatography using a flame ionization detector following a modification of the method of Lepage and Roy (1984). Mineral content was determined using a modified method of the United States Environmental Protection Agency (US EPA), Metals and Trace Elements by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (US EPA, 2000; method 6010C). For fillet astaxanthin analysis, following crude fat extraction, a portion of the sample was purified via solid-phase extraction using a silica column. The pigments retained by the silica were eluted using butylated hydroxytoluene (BHT)-containing acetone. Following the evaporation of the solvent, the pigments were dissolved in ether and cold-saponified using methanolic potassium hydroxide (KOH). The pigment solution was rinsed three times using a saline solution to stop the reaction. The pigment-containing organic phase was recovered and evaporated to dryness, then re-dissolved in the mobile phase for High Performance Liquid Chromatography (HPLC) analysis. The chromatographic separation was achieved using a 150 mm silica column with a hexane-acetone mixture. Pigment quantification was completed using a calibration curve.

2.3.4 Standard growth performance indicators

Standard growth performance indicators were measured, including FCR (Equation 1), Fulton's condition factor (k, Equation 2), hepatosomatic index (HSI, Equation 3) and viscerosomatic index (VSI, Equation 4), using the following equations:

$$FCR = \frac{Dry\ matter\ feed\ intake\ (g)}{Fish\ weight\ gain\ (g)} \quad \text{Equation 1.}$$

$$k = \frac{Fish\ weight\ (g)}{(Fish\ fork\ length\ [cm])^3} \times 100 \quad \text{Equation 2.}$$

$$HSI = \left(\frac{Liver\ weight\ (g)}{Fish\ weight\ (g)}\right) \times 100 \quad \text{Equation 3.}$$

$$VSI = \left(\frac{Viscera\ weight\ (g)}{Fish\ weight\ (g)}\right) \times 100 \quad \text{Equation 4.}$$

Growth rate was calculated using the thermal-unit growth coefficient (TGC) equation, with the exponent 0.209 used for fish up to 25 g in size (Equation 5) and the standard 1/3 for fish above that threshold (Equation 6) (Iwama & Tautz, 1981; Cho, 1992; Dumas et al., 2007b), as it is suspected, based on historical data from AquaBounty, that GH TG Atlantic salmon have different growth stanzas:

$$TGC = \left(\frac{W_f^{0.209} - W_i^{0.209}}{\sum_{i=1}^{n} T_i}\right) \times 1000 \quad \text{Equation 5.}$$

$$TGC = \left(\frac{W_f^{1/3} - W_i^{1/3}}{\sum_{i=1}^{n} T_i}\right) \times 1000 \quad \text{Equation 6.}$$

where W_f and W_i are final and initial fish body weight (g), respectively, n is the number of days recorded from W_i, and T_i is mean daily water temperature (°C).

The results of the compositional analysis were used to calculate nutrient deposition (D) rates [mg $(°C \cdot d)^{-1}$] using the following equation:

$$D = \frac{(N_f \times W_f) - (N_i \times W_i)}{\sum_{i=1}^{n} T_i}$$ Equation 7.

where N_f and N_i are the final and initial nutrient compositions on a percentage as-is basis, respectively, W_f and W_i are the final and initial weights of the whole fish or fillet, respectively, n corresponds to the number of days between the initial and final measurements, and T_i is mean daily water temperature (°C) (Dumas et al., 2007a).

Nutrient retention efficiencies (%) were calculated for each tank using the following equation:

$$E = \frac{(N_f \times W_f) - (N_i \times W_i)}{(N_d \times F) \div n}$$ Equation 8.

where N_f and N_i are the final and initial nutrient compositions of the whole fish or fillet on an as-is basis, respectively, W_f and W_i are the final and initial weights of the whole fish or fillet, respectively, N_d is the nutrient composition of the diet on a percentage as-is basis, F is the total feed intake for the tank, and n is the number of fish in the tank (Azevedo et al., 2005).

2.3.5 Data analysis

Statistical comparisons of treatment means were performed by two-way ANOVA in R Studio (R Studio Team, 2015; v.3.5.2), testing the influence of temperature and size-interval on growth performance indicators and nutrient utilization factors. Tukey's post-hoc tests were performed to determine differences between groups where significant differences were detected. Replicate tank means were compared first to confirm the absence of tank effects, after which data from replicate tanks were pooled for further analyses. Multiple linear regression, performed in Microsoft Excel, was used to test the relationship between fish weight, body lipid content, rearing temperature, lipid intake, and dietary protein-to-lipid ratio. Predictive equations for body

lipid and lipid intake were created based on results. Statistical differences were considered significant at $p < 0.05$.

2.4 Results

2.4.1 Growth performance

The average weight of AAS up to 1500 g reared at three temperatures (10.5°C, 13.5°C, 16.5°C) is shown in Figure 2-1. At the start of the trial, fry averaged 0.51 g in weight, based on batch weighing. While fish grew at all three temperatures, weight began to differ significantly among treatments within two months post-first feeding ($p < 0.001$), with the fastest weight gain at 16.5°C. Fish reached an average weight of 1500 g in 382 days at 16.5°C, followed by 418 and 475 days for fish reared at 13.5°C and 10.5°C, respectively. TGC results (Table 2-1) indicate that during the first three months post-first-feeding, salmon at 16.5°C had higher growth rates than salmon at 10.5°C ($p < 0.001$); however, after five months this pattern reversed, with fish at 10.5°C consistently having higher TGCs ($p < 0.01$).

Growth performance was assessed at 800 and 1500 g (Table 2-2). TGC differed among temperature treatments. During the 500-800 g growth interval, fish reared at 10.5°C exhibited higher growth rates than those reared at 13.5°C and 16.5°C ($p < 0.001$), while during the 800-1500 g growth interval, fish reared at 10.5°C and 13.5°C exhibited higher growth rates compared to those reared at 16.5°C ($p < 0.01$). From 500 to 800 g, feed intake was higher in the 16.5°C treatment ($p < 0.05$) compared to the lower two temperatures, while intake significantly increased with temperature in all three treatments from 800 to 1500 g ($p < 0.05$). FCR was also influenced by rearing temperature, as 500-800 g and 800-1500 g fish reared at 10.5°C had lower FCRs ($p < 0.01$) compared to fish reared at 16.5°C. Fish reared at 13.5°C also exhibited lower

FCRs for the 800-1500 g growth interval compared to those reared at 16.5°C ($p < 0.01$). During both growth intervals, salmon reared at 16.5°C exhibited higher k and VSI values compared to those reared at 10.5°C ($p < 0.01$). HSI was higher within the 13.5°C treatment group than both other temperatures at 800 g ($p < 0.05$), and HSI was highest among both the 13.5°C and 16.5°C treatments at 1500 g compared to 10.5°C at 1500 g ($p < 0.05$).

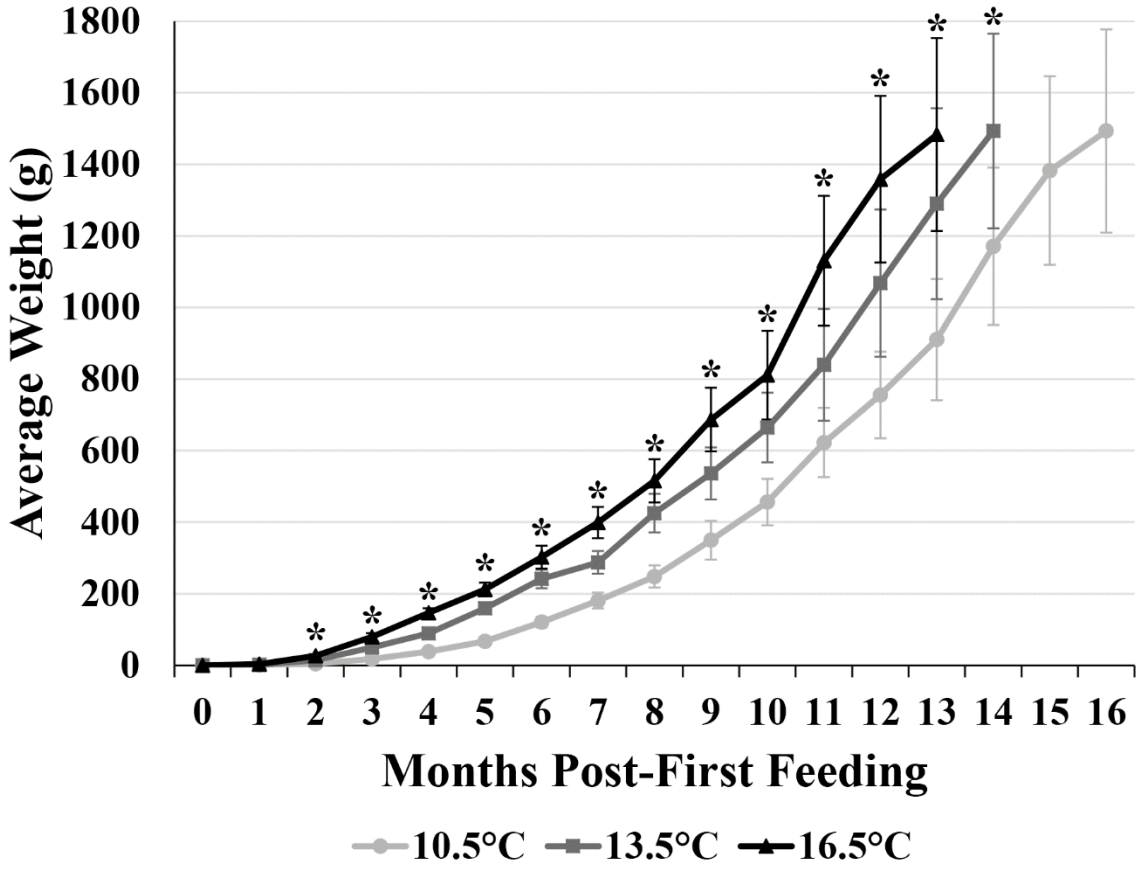

Figure 2-1. Average weight of AquAdvantage Salmon reared at three temperatures up to 1500 g. Error bars represent plus and minus one standard deviation from the mean weight. Asterisks denote the months where average fish weight differed significantly ($p < 0.05$) among treatments.

Table 2-1. Thermal-unit growth coefficient (TGC) of AquAdvantage Salmon held at three rearing temperatures calculated on a month-to-month basis post-first feeding. Corresponding cumulative degree days (°C·d) are provided alongside the mean TGC. Mean values are given with their standard errors (±SEM). Lower-case letters denote significance between different temperatures at the same time point ($p < 0.05$). Dashes (-) are inserted after a treatment reached the target weight of 1500 g and final data measurements were collected on those fish.

	TGC								
	10.5°C			13.5°C			16.5°C		
Month Post-First Feeding	°C·d	Mean	(±SEM)	°C·d	Mean	(±SEM)	°C·d	Mean	(±SEM)
1	514	0.41[a]	0.025	614	0.60[b]	0.005	718	0.71[c]	0.008
2	834	1.08[a]	0.039	1011	1.43[b]	0.011	1212	3.03[c]	0.042
3	1160	1.36[a]	0.006	1415	3.14[c]	0.027	1719	2.57[b]	0.064
4	1467	2.21[b]	0.051	1802	1.85[a]	0.111	2179	1.92[ab]	0.022
5	1797	2.28[b]	0.086	2226	2.46[b]	0.035	2646	1.64[a]	0.016
6	2158	2.44[c]	0.072	2696	1.70[b]	0.031	3186	1.36[a]	0.067
7	2434	2.60[c]	0.040	2987	1.04[b]	0.035	3624	1.51[a]	0.011
8	2736	2.08[b]	0.006	3454	2.29[b]	0.086	4078	1.46[a]	0.091
9	3106	2.04[b]	0.136	3929	1.27[a]	0.050	4652	1.40[a]	0.028
10	3387	2.32[c]	0.030	4314	1.68[b]	0.052	5095	1.16[a]	0.098
11	3778	2.14[c]	0.030	4792	1.39[b]	0.016	5677	1.78[a]	0.063
12	4065	1.97[b]	0.041	5161	2.05[b]	0.027	6118	1.43[a]	0.008
13	4353	2.02[c]	0.069	5557	1.73[b]	0.025	6565	1.21[a]	0.030

14	4709	2.38[b]	0.013	5838	1.93[a]	0.111	-	-	-
15	5013	1.36	0.028	-	-	-	-	-	-
16	5175	1.78	0.008	-	-	-	-	-	-

Table 2-2. Thermal-unit growth coefficient (TGC), feed intake (FI), feed conversion ratio (FCR), condition factor (k), hepatosomatic index (HSI), and viscerosomatic index (VSI) of AquAdvantage Salmon held at three rearing temperatures and assessed at 800 and 1500 g. TGC and FCR values at 800 and 1500 g are for growth intervals starting at 500 and 800 g, respectively. Mean values are given with their standard errors (±SEM). Upper- and lower-case letters denote significant differences between weights at the same temperature or between temperatures at the same weight, respectively ($p < 0.05$).

Variables	Treatments					
	10.5°C		13.5°C		16.5°C	
	Mean	(±SEM)	Mean	(±SEM)	Mean	(±SEM)
800 g						
TGC	2.31^{b}	0.11	1.45^{Aa}	0.04	1.37^{a}	0.04
FI	4.47^{Aa}	0.10	4.51^{Aa}	0.07	5.13^{Ab}	0.12
FCR	0.81^{a}	0.01	0.87^{ab}	0.03	0.94^{b}	0.01
k	1.25^{Aa}	0.01	1.26^{Aa}	0.01	1.31^{Ab}	0.01
HSI (%)	0.91^{a}	0.01	0.97^{Ab}	0.01	0.91^{Aa}	0.01
VSI (%)	6.96^{a}	0.10	7.29^{Aa}	0.10	7.82^{Ab}	0.09
1500 g						
TGC	2.09^{b}	0.05	1.86^{Bb}	0.06	1.43^{a}	0.02
FI	6.01^{Ba}	0.07	6.86^{Bb}	0.03	7.31^{Bc}	0.12
FCR	0.86^{a}	0.01	0.87^{a}	0.01	1.01^{b}	0.02
k	1.32^{Ba}	0.01	1.36^{Bb}	0.01	1.40^{Bb}	0.02
HSI (%)	0.94^{a}	0.02	1.07^{Bb}	0.03	1.00^{Bb}	0.03
VSI (%)	7.08^{a}	0.10	8.38^{Bb}	0.10	8.86^{Bb}	0.18

2.4.2 Whole body & fillet composition

Whole-body proximate composition data are shown in Table 2-3. Percent dry matter was significantly higher for fish at 300, 500 and 1500 g within the 13.5°C and 16.5°C treatments ($p < 0.01$) compared to fish of equal size reared at 10.5°C. This pattern corresponded with higher lipid percentages in 300 and 500 g fish at 13.5°C and 16.5°C ($p < 0.01$) compared to fish of equal size at 10.5°C, and percent lipid was highest ($p < 0.001$) in 1500 g fish reared at 16.5°C compared to both other temperature groups. Only at the 800 g sampling point was percent lipid not significantly different among rearing temperatures. At 10.5°C and 13.5°C, percent lipid significantly increased with weight ($p < 0.05$). At 16.5°C, percent lipid was not significantly different at the 300 and 500 g stages, however, it did increase at each of the next two weight stages ($p < 0.001$). Percent protein was not significantly different among rearing temperatures except for 500 g salmon reared at 13.5°C and 16.5°C having higher values compared to fish reared at 10.5°C ($p < 0.05$). Percent ash remained constant throughout the trial, with no significant differences among weight groups or rearing temperatures.

Total omega-3 (ω3) fatty acids, docosahexaenoic acid (DHA, 22:6ω3), eicosapentaenoic acid (EPA, 20:5ω3), lysine, methionine, histidine, phosphorus, and calcium percentages in fillets were determined when fish reached an average weight of 500, 800 and 1500 g within each rearing temperature (Table 2-4). These nutrients were chosen for analysis based on their importance to product marketability and/or triploid metabolism. Percent total ω3 fatty acids remained stable across all sizes and rearing temperatures. Rearing temperature had no significant effect on percent DHA or EPA, however, at 16.5°C both were significantly lower ($p < 0.05$) in 1500 g fish compared to 800 g fish. When expressed as percent of total fatty acids (Appendix II), total ω3, DHA and EPA all decreased ($p < 0.01$) at each weight stage, with

differences ($p < 0.05$) based on rearing temperature. However, it is noteworthy that these differences in contribution to total fatty acids did not equate to differences in fillet composition (Table 2-4). Rearing temperature did not affect percent lysine, but values were higher at 1500 g than at 800 g within the 10.5°C treatment group ($p < 0.05$). Rearing temperature of 16.5°C resulted in higher percent methionine in 500 and 800 g fish ($p < 0.05$) compared to the 10.5°C group, but temperature did not affect percent methionine in 1500 g fish. Percent methionine did not differ among fish size groups at 10.5°C, but was significantly higher at 800 g compared to 500 g and 1500 g in the 13.5°C treatment group ($p < 0.05$), and significantly higher at both 500 and 800 g compared to 1500 g in the 16.5°C treatment group ($p < 0.01$). There were no significant differences in percent histidine among fish weight or temperature groups. Percent phosphorus was unaffected by rearing temperature within size groups but was significantly higher at 500 and 800 g than at 1500 g in all temperature groups ($p < 0.001$). Finally, percent calcium remained constant throughout the experiment.

Table 2-3. Whole-body proximate composition (% as-is basis) for dry matter (DM), protein, lipid, and ash of AquAdvantage Salmon held at three rearing temperatures and assessed at 300, 500, 800 and 1500g. Mean values are given with their standard errors (±SEM) and n-value. Upper- and lower-case letters denote significant differences between weights at the same temperature or between different temperatures at the same weight, respectively ($p < 0.05$).

Variables	Treatments								
	10.5°C			13.5°C			16.5°C		
	Mean	(±SEM)	n	Mean	(±SEM)	n	Mean	(±SEM)	n
300 g									
% DM	32.72^{Aa}	0.20	12	34.15^{Ab}	0.15	12	34.9^{Ab}	0.19	12
% Protein	17.48^{AB}	0.11	12	17.42^{A}	0.09	12	17.49^{A}	0.12	12
% Lipid	11.77^{Aa}	0.14	12	13.43^{Ab}	0.12	12	14.33^{Ac}	0.18	12
% Ash	1.99	0.02	12	2.07	0.06	12	2.02	0.05	12
500 g									
% DM	33.51^{Aa}	0.15	12	35.36^{Bb}	0.12	12	35.86^{ABb}	0.23	12
% Protein	17.18^{Aa}	0.12	12	17.78^{ABb}	0.09	12	18.31^{Bb}	0.09	12
% Lipid	13.02^{Ba}	0.15	12	14.51^{Bb}	0.13	12	14.26^{Ab}	0.19	12
% Ash	2.14	0.06	12	2.03	0.04	12	2.22	0.15	12
800 g									
% DM	35.74^{B}	0.15	12	36.27^{B}	0.18	10	36.10^{B}	0.34	13
% Protein	17.79^{B}	0.11	12	17.59^{AB}	0.12	10	17.60^{A}	0.17	13
% Lipid	15.11^{C}	0.13	12	15.68^{C}	0.25	10	15.78^{B}	0.26	13
% Ash	2.20	0.03	12	2.10	0.07	10	2.01	0.04	13

1500 g									
% DM	36.70^{Ba}	0.18	12	38.02^{Cb}	0.17	12	38.95^{Cb}	0.34	12
% Protein	17.89^{B}	0.11	12	18.11^{B}	0.11	12	17.98^{AB}	0.18	12
% Lipid	16.22^{Da}	0.18	12	16.81^{Da}	0.22	12	18.22^{Cb}	0.34	12
% Ash	2.01	0.02	12	2.01	0.04	12	2.01	0.04	12

Table 2-4. Fillet composition (% as-is basis) for total omega-3 fatty acid (Σ ω3), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), lysine (Lys), methionine (Met), histidine (His), phosphorus (P), and calcium (Ca) of AquAdvantage Salmon held at three rearing temperatures and assessed at 500, 800 and 1500 g. Mean values are given with their standard errors (±SEM) (*n*=3). Upper and lower-case letters denote significant differences between weights at the same temperature or between temperatures at the same weight, respectively ($p < 0.05$).

Variables	Treatments					
	10.5°C		13.5°C		16.5°C	
	Mean	(±SEM)	Mean	(±SEM)	Mean	(±SEM)
500 g						
% Σ ω3	1.13	0.049	1.27	0.025	1.27	0.078
% DHA	0.55	0.022	0.68	0.010	0.68^{AB}	0.043
% EPA	0.21	0.009	0.24	0.004	0.23^{AB}	0.013
% Lys	1.07^{AB}	0.050	0.94	0.079	1.03	0.090
% Met	0.27^{a}	0.007	0.29^{Aab}	0.008	0.41^{Bb}	0.050
% His	0.41	0.014	0.48	0.033	0.47	0.024
% P	0.93^{B}	0.018	0.91^{B}	0.009	0.92^{B}	0.015
% Ca	0.08	0.003	0.10	0.002	0.10	0.002
800 g						
% Σ ω3	1.24	0.051	1.19	0.053	1.40	0.155
% DHA	0.56	0.023	0.59	0.024	0.71^{B}	0.078
% EPA	0.23	0.010	0.22	0.010	0.25^{B}	0.029
% Lys	0.82^{A}	0.043	0.95	0.032	1.02	0.088

% Met	0.22[a]	0.007	0.45[Bb]	0.003	0.42[Bb]	0.068
% His	0.47	0.023	0.47	0.012	0.48	0.019
% P	0.89[B]	0.030	0.90[B]	0.015	0.90[B]	0.006
% Ca	0.09	0.023	0.09	0.006	0.09	0.003
1500 g						
% Σ ω3	1.09	0.059	1.15	0.008	1.06	0.024
% DHA	0.47	0.024	0.54	0.008	0.52[A]	0.008
% EPA	0.20	0.011	0.20	0.002	0.18[A]	0.005
% Lys	1.15[B]	0.005	1.08	0.014	1.18	0.017
% Met	0.22	0.005	0.20[A]	0.010	0.20[A]	0.007
% His	0.42	0.028	0.43	0.031	0.39	0.008
% P	0.74[A]	0.012	0.73[A]	0.026	0.76[A]	0.021
% Ca	0.05	0.017	0.06	0.016	0.08	0.023

2.4.3 Nutrient utilization

Utilizing the whole-body and fillet compositional data, nutrient deposition rates [mg $(°C \cdot d)^{-1}$] and retention efficiencies (%) were calculated for three growth periods: period 1 (300-500 g), period 2 (500-800 g), and period 3 (800-1500 g). Results for whole-body protein, lipid and ash for all three growth periods are shown in Table 2-5. Protein deposition was higher amongst fish reared at 10.5°C compared to 16.5°C ($p < 0.01$) and increased significantly from each period to the next at this temperature ($p < 0.001$). Protein deposition for the 13.5°C and 16.5°C treatments increased during period 3 compared to the previous two periods ($p < 0.001$). Lipid deposition followed a similar trend, with the exception that rates increased at every growth period amongst all three rearing temperatures ($p < 0.05$). Lipid deposition was highest at the lower two temperatures during period 1 compared to 16.5°C ($p < 0.05$), highest in the 10.5°C treatment during period 2 over both other temperatures ($p < 0.0001$), and highest in 10.5°C and lowest in 16.5°C with significant differences between each temperature group for period 3 ($p < 0.05$). Only during period 2 was there a difference in ash deposition among temperatures, with the 10.5°C treatment exhibiting the highest rates ($p < 0.05$). Ash deposition was highest at all three temperatures within period 3 ($p < 0.05$). Protein retention efficiencies were higher in salmon reared at 10.5°C and 13.5°C than at 16.5°C during period 1 ($p < 0.001$) and decreased across all treatments during period 2 ($p < 0.05$), with the 10.5°C treatment group remaining significantly higher than the other two ($p < 0.05$). Within the third growth period, protein retention efficiencies increased in the 13.5°C treatment ($p < 0.01$), comparable to the efficiency levels of the 10.5°C treatment group. As with protein, lipid retention efficiencies were higher at 10.5°C and 13.5°C than at 16.5°C during growth period 1, however, efficiencies decreased thereafter across all rearing temperatures ($p < 0.05$) and remained at lower levels for the

remainder of the trial. Efficiency of ash retention was unaffected by rearing temperature, however, levels did decrease from the first growth period to the last for fish reared at 10.5°C ($p < 0.05$).

Total ω3, DHA, EPA, lysine, methionine, histidine, phosphorus and calcium nutrient deposition rates and retention efficiencies were calculated from fillet samples collected during growth periods 2 and 3 (Table 2-6). Total ω3, DHA and EPA deposition rates were not affected by temperature during period 2, but all were significantly lower at 16.5°C than at 10.5°C or 13.5°C during period 3 ($p < 0.05$), and all three had lower deposition rates at 16.5°C during period 3 compared to period 2 ($p < 0.05$). Lysine deposition increased from period 2 to period 3 in both the 10.5°C and 13.5°C treatment groups ($p < 0.05$) and were higher at 10.5°C than at 13.5°C or 16.5°C during period 3 ($p < 0.05$). Rates of methionine deposition were higher at 13.5°C than 10.5°C during growth period 2 ($p < 0.05$), however, levels were lower during the final stage of the trial compared to period 2 in both the 13.5°C and 16.5°C treatment groups ($p < 0.05$), and below those of the 10.5°C treatment group during period 3 ($p < 0.05$). No differences in histidine deposition rates were found during the second growth period, however, during growth period 3, histidine deposition rates were significantly higher in salmon reared at 10.5°C compared to those reared at 16.5°C ($p < 0.05$). Phosphorus deposition increased in the 10.5°C treatment group as the fish grew ($p < 0.05$) but decreased within the 16.5°C treatment group ($p < 0.05$). During growth period 3, fish reared at 10.5°C exhibited higher phosphorus deposition rates compared to the other treatments ($p < 0.01$). No differences were found for calcium deposition among growth periods or rearing temperatures.

In terms of nutrient retention efficiencies, results for total ω3, DHA and EPA were consistent with utilization rates, i.e., significantly decreased during the final growth period

compared to period 2 at 16.5°C ($p < 0.01$), but with no other growth effects or any temperature effects in either growth period. Lysine retention efficiencies increased within the 10.5°C treatment over time ($p < 0.001$) but were otherwise unaffected by temperature or growth. Just as methionine deposition rates were higher in fish reared at 13.5°C compared to fish reared at 10.5°C during the second growth period, so were methionine retention efficiencies ($p < 0.05$) and, similarly, levels decreased ($p < 0.05$) during the third growth period in the 13.5°C and 16.5°C treatment groups. Histidine retention efficiencies remained unchanged by temperature and growth period. Phosphorus retention efficiencies were higher at 16.5°C than 10.5°C ($p < 0.05$) during growth period 2, but this temperature effect was reversed in growth period 3 ($p < 0.05$). Furthermore, phosphorus retention efficiencies were lower in period 3 than period 2 at both 13.5°C and 16.5°C. Neither rearing temperature nor growth period affected calcium retention efficiencies.

Table 2-5. Nutrient utilization: whole-body deposition rates and retention efficiencies (RE) of protein (Protein RE), lipid (Lipid RE) and ash (Ash RE) of AquAdvantage Salmon held at three rearing temperatures and assessed across three growth periods. Mean values are given with their standard errors (±SEM; n=3). Different upper- and lower-case letters indicate the effect was significant between periods at the same temperature or between temperatures during the same period, respectively ($p < 0.05$).

Variables	Treatments					
	10.5°C		13.5°C		16.5°C	
	Mean	(±SEM)	Mean	(±SEM)	Mean	(±SEM)
Period 1 (300 - 500 g)						
Protein [mg (°C·d)$^{-1}$]	55.95^{Ab}	1.54	51.27^{Aab}	0.31	47.15^{Aa}	0.97
Lipid [mg (°C·d)$^{-1}$]	50.80^{Ab}	2.98	44.89^{Ab}	1.46	33.70^{Aa}	1.08
Ash [mg (°C·d)$^{-1}$]	8.02^{A}	0.61	5.52^{A}	0.06	6.14^{AB}	1.39
Protein RE (%)	56.69^{Bb}	2.85	55.46^{Cb}	1.69	41.59^{Ba}	1.82
Lipid RE (%)	158.12^{Bb}	5.32	149.76^{Bb}	6.13	91.63^{Ba}	3.88
Ash RE (%)	55.33^{B}	5.23	40.60	1.11	37.22	9.42

Period 2 (500 - 800 g)

Protein [mg (°C·d)$^{-1}$]	90.01^{Bb}	2.05	57.38^{Aa}	1.10	51.00^{Aa}	0.97
Lipid [mg (°C·d)$^{-1}$]	87.35^{Bb}	1.91	58.13^{Ba}	1.10	58.87^{Ba}	3.74
Ash [mg (°C·d)$^{-1}$]	10.98^{ABb}	0.30	7.48^{Aa}	0.75	5.07^{Aa}	0.29
Protein RE (%)	44.68^{Ab}	1.32	36.06^{Aa}	0.84	33.48^{Aa}	1.40
Lipid RE (%)	80.15^{A}	3.44	67.44^{A}	1.42	71.32^{A}	5.15
Ash RE (%)	37.60^{AB}	1.04	32.53	3.66	22.90	1.22

Period 3 (800 - 1500 g)

Protein [mg (°C·d)$^{-1}$]	124.65^{Cc}	2.29	112.70^{Bb}	0.48	85.14^{Ba}	0.61
Lipid [mg (°C·d)$^{-1}$]	121.54^{Cc}	1.84	109.37^{Cb}	2.08	98.04^{Ca}	2.22
Ash [mg (°C·d)$^{-1}$]	12.31^{B}	0.45	11.33^{B}	0.87	9.28^{B}	0.54
Protein RE (%)	46.04^{A}	0.83	46.55^{B}	0.40	39.19^{AB}	0.63
Lipid RE (%)	82.88^{A}	1.14	83.39^{A}	1.67	83.38^{AB}	3.12
Ash RE (%)	31.36^{A}	0.81	32.26	2.42	29.52	2.14

Table 2-6. Nutrient utilization: fillet omega-3 (Σ ω3), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), lysine (Lys), methionine (Met), histidine (His), phosphorus (P) and calcium (Ca) deposition rates and retention efficiencies (RE) (Σ ω3 RE, DHA RE, EPA RE, Lys RE, Met RE, His RE, P RE and Ca RE, respectively) of AquAdvantage Salmon held at three rearing temperatures and assessed across two growth periods. Mean values are given with their standard errors (±SEM) (*n*=3). Different upper- and lower-case letters indicate the effect was significant between periods at the same temperature or between temperatures during the same period, respectively ($p < 0.05$).

Variables	Treatments					
	10.5°C		13.5°C		16.5°C	
	Mean	(±SEM)	Mean	(±SEM)	Mean	(±SEM)
Period 2 (500 - 800 g)						
Σ ω3 [mg (°C·d)$^{-1}$]	3.31	0.13	2.12	0.29	3.44^{B}	0.60
DHA [mg (°C·d)$^{-1}$]	1.34	0.06	0.90	0.14	1.61^{B}	0.30
EPA [mg (°C·d)$^{-1}$]	0.59	0.03	0.39	0.05	0.63^{B}	0.11
Lys [mg (°C·d)$^{-1}$]	0.69^{A}	0.11	1.92^{A}	0.40	2.19	0.75
Met [mg (°C·d)$^{-1}$]	0.31^{a}	0.07	1.40^{Bb}	0.04	0.94^{Bab}	0.47
His [mg (°C·d)$^{-1}$]	1.34	0.18	0.91	0.17	1.07	0.17
P [mg (°C·d)$^{-1}$]	1.79^{A}	0.12	1.74	0.05	1.88^{B}	0.04
Ca [mg (°C·d)$^{-1}$]	0.27	0.15	0.16	0.04	0.18	0.02
Σ ω3 RE (%)	33.41	1.30	27.06	3.57	45.99^{B}	8.59
DHA RE (%)	69.88	3.75	59.35	8.81	111.32^{B}	21.80
EPA RE (%)	18.65	0.85	15.32	2.00	26.27^{B}	5.00
Lys RE (%)	8.30^{A}	1.26	28.97	5.55	35.01	12.39

Met RE (%)	14.77^{a}	3.63	82.96^{Bb}	3.64	59.62^{Bab}	29.86
His RE (%)	35.10	5.05	30.03	5.91	36.90	6.19
P RE (%)	32.65^{a}	2.88	40.00^{Bab}	1.33	45.23^{Bb}	2.07
Ca RE (%)	3.46	1.87	2.58	0.57	2.94	0.34
Period 3 (800 - 1500 g)						
Σ ω3 [mg (°C·d)$^{-1}$]	4.06^{b}	0.35	3.45^{b}	0.26	1.14^{Aa}	0.62
DHA [mg (°C·d)$^{-1}$]	1.62^{b}	0.11	1.45^{b}	0.14	0.48^{Aa}	0.30
EPA [mg (°C·d)$^{-1}$]	0.73^{b}	0.06	0.57^{b}	0.05	0.16^{Aa}	0.12
Lys [mg (°C·d)$^{-1}$]	6.64^{Bb}	0.25	3.92^{Ba}	0.05	3.02^{a}	0.37
Met [mg (°C·d)$^{-1}$]	0.97^{b}	0.02	-0.41^{Aa}	0.07	-0.32^{Aa}	0.24
His [mg (°C·d)$^{-1}$]	1.60^{b}	0.36	1.13^{ab}	0.18	0.51^{a}	0.05
P [mg (°C·d)$^{-1}$]	2.52^{Bb}	0.23	1.60^{a}	0.17	1.15^{Aa}	0.10
Ca [mg (°C·d)$^{-1}$]	-0.03	0.24	0.07	0.11	0.15	0.12
Σ ω3 RE (%)	30.47	2.38	28.92	2.05	10.81^{A}	5.81
DHA RE (%)	62.76	4.05	63.01	5.72	23.54^{A}	14.52
EPA RE (%)	17.13	1.17	14.85	1.23	4.85^{A}	3.40
Lys RE (%)	59.22^{B}	2.77	39.10	0.64	33.38	3.60
Met RE (%)	33.70	1.11	-15.98^{A}	2.51	-14.01^{A}	10.46
His RE (%)	30.95	6.74	24.64	4.02	12.42	1.27
P RE (%)	34.13^{b}	3.46	24.29^{Aab}	2.59	19.49^{Aa}	2.01
Ca RE (%)	-0.29	2.28	0.77	1.11	1.74	1.34

2.4.4 Fillet colour & yield

Fillet samples collected from 1500 g fish were weighed for the measurement of fillet yield, visually assessed to determine the degree of pigmentation, and subsequently quantified for total astaxanthin concentration (Table 2-7). Average fillet yields were significantly higher ($p < 0.01$) for salmon reared at 10.5°C and 13.5°C than at 16.5°C (by 7% and 4%, respectively). The 13.5°C treatment group exhibited darker colouration than the 16.5°C treatment ($p < 0.01$). No differences in total astaxanthin concentration were detected among groups.

Table 2-7. Fillet yield, total astaxanthin concentration and DSM SalmoFan™ colour scoring of fillet samples collected as AquAdvantage Salmon reached an average weight of 1500 g. Mean values are given with their standard errors (±SEM). Different lower-case letters indicate the effect was significant between temperatures ($p < 0.05$).

Variables	Treatments								
	10.5°C			13.5°C			16.5°C		
	Mean	(±SEM)	n	Mean	(±SEM)	n	Mean	(±SEM)	n
1500 g									
Fillet yield (%)	59.27^{b}	0.88	24	56.59^{b}	0.93	24	52.11^{a}	0.99	24
Total astaxanthin (µg / g)	4.17	0.33	3	3.83	0.35	3	4.10	0.21	3
DSM SalmoFan™ score	28.00^{ab}	0.17	12	28.35^{b}	0.12	12	27.44^{a}	0.29	12

2.4.5 Regression models

Although increased growth was achieved at 16.5°C, the higher amount of lipid deposited in the viscera was an undesirable outcome for commercialization. The relative whole-body lipid (BL) content of AAS was approximately 2% higher at 16.5°C than 10.5°C. The BL (%) of these salmon can be predicted ($r^2 = 0.940$) using the following equation based on multiple linear regression results:

$$BL\ (\%) = 12.082(\pm 0.849) + 0.203(\pm 0.040)T + 0.020(\pm 0.003)LI - 0.073(\pm 0.022)PL \quad \text{Equation 9.}$$

where T and LI are rearing temperature (°C) and lipid intake (g/fish), respectively, and PL is the dietary protein-to-lipid ratio (expressed as % of diet). Numbers in parentheses represent standard errors. All coefficients were significant ($p < 0.01$). Body weight (BW, g/fish) was not a significant predictor of BL ($p = 0.4201$) and was thus excluded from the multiple regression analysis. Rearing temperature and dietary PL data can be obtained easily, but LI requires data that may not always be available in a commercial setting. Therefore, another equation was developed to estimate LI ($r^2 = 0.986$) using two significant predictors, namely BW ($p < 0.0001$) and PL ($p < 0.0001$):

$$LI = 1.179(\pm 7.922) + 0.112(\pm 0.005)BW + 1.484(\pm 0.288)PL \quad \text{Equation 10.}$$

Rearing temperature was not a significant predictor of LI ($p = 0.115$) and was not considered in the equation.

2.5 Discussion

The effect of temperature on growth of Atlantic salmon is well documented (Johnston and Saunders, 1981; Koskela et al., 1997; Imsland et al., 2014). Data modeling indicates that growth rate increases with temperature in most fish species until a critical limit is reached, beyond which it decreases (Jobling, 1997). Handeland et al. (2008) purported that the optimal growth temperature for diploid Atlantic salmon increases with fish size, recommending the early rearing of post-smolts at 12.8°C with an increase to 14.0°C when they reach 150 g. In laboratory systems, greater weight gain can be achieved at approximately 16°C in diploid Atlantic salmon than at lower temperatures (Jensen et al., 2015), but optimal growth efficiency, in terms of feed conversion ratios and nutrient utilization rates, is reported to be lower, near 13°C (Hevrøy et al., 2013). However, triploid Atlantic salmon likely have a lower thermal optimum than diploids (Atkins and Benfey, 2008), as they are less tolerant to high temperature and low oxygen conditions (Sambraus et al., 2017, 2018). GH transgenesis does not appear to impact the critical thermal maximum in coho salmon (*Oncorhynchus kisutch*) when compared to wild-type and domesticated counterparts (Chen et al., 2015), so it may be hypothesized that it may not affect thermal optima in this species (although this requires further study). The current study sought to determine rearing temperature's effect on AAS in relation to growth performance and nutrient utilization in order to help improve commercial rearing strategies.

In the current study, fish reared at 16.5°C reached the sampling target weights in a significantly shorter period of time compared to fish at 13.5°C and 10.5°C. However, they exhibited lower TGCs throughout the trial compared to fish reared at 10.5°C, suggesting that rate of change in weight gain per degree-day was not constant within the temperature range tested in this study. This assumption deserves further study and suggests that the exponents used in the

TGC model need to be validated in AAS reared in freshwater RAS. Also in the present study, FCRs increased with temperature, similar to previous reports in diploid Atlantic salmon post-smolts (Kullgren et al., 2013). Research has shown that feed intake increases with increasing temperature in diploid Atlantic salmon from 11°C to 17°C, after which it begins to decrease at higher temperatures (Koskela et al., 1997), which supports the results from the current study. Furthermore, in the present study, condition factor and VSI were higher at 16.5°C than at 10.5°C, suggesting that the study fish deposited more fat in their viscera than in their muscle tissue. Higher condition factors at elevated temperature were also noted by Koskela et al. (1997) in salmon reared at 19°C compared to those at 11°C and 15°C, but lower values were reported at 23°C. Additionally, studies in salmonids have shown a direct link between liver storage of glycogen and triglycerides and their required usage during periods of feed restriction or developmental maturation, with greater HSIs indicating more storage of these energy molecules (Arndt et al., 1996; Silverstein et al., 1997). HSI was consistently high amongst fish reared at 13.5°C in this study which could be advantageous over the other rearing temperatures if feed restriction were to occur, however it may negatively divert dietary nutrients away from muscle growth of the fish in a typical production setting. All these factors together suggest that rearing AAS at 16.5°C from first feeding up to 1500 g may not be beneficial.

Whole-body protein content of fish is thought to be determined mainly by fish size, while lipid content is more greatly affected by both endogenous (i.e. genetic background) and exogenous (i.e. diet and environmental conditions) factors (Shearer, 1994). A consistent effect of temperature was not detected on either protein or body ash content in AAS. In contrast, relative content of body lipid and dry matter increased as the trial progressed with the highest content observed in fish reared at 16.5°C. Body lipid concentrations were previously found

positively correlated with rearing temperature in market weight conventional Atlantic salmon (Ørnholt-Johansson et al., 2017). Interestingly, there is also a strong positive correlation between lipid content and condition factor (Herbinger and Friars, 1991), which supports the present results for AAS reared at 16.5°C. Earlier work on juvenile GH TG Atlantic salmon reared at 11.8°C demonstrated higher lipid digestibility compared to NTG counterparts reared at 8.2°C; however, their body lipid levels were found to be lower than in NTG salmon (Tibbetts et al., 2013). The authors hypothesized that GH TG fish oxidize more lipid absorbed from their diet to help better meet the demands of their metabolism compared to NTG fish (Tibbetts et al., 2013). Only GH TG females were utilized in the current trial and nutrient digestibility was not measured, nor were NTG salmon included, which limits comparisons to previous trials conducted by AquaBounty.

The results of the macronutrient deposition rates and retention efficiencies illustrate a broader effect of temperature than what was seen from whole-body proximate composition. Body protein and lipid deposition rates and retention efficiencies indicate that rearing AAS at 16.5°C is not advantageous. Lipid retention efficiency was above 100% in the first weight period at the lower two temperatures, which is likely achieved by the fish converting glucose from the breakdown of excess amino acids into fatty acids (Salmerón, 2018; Horn et al., 2019; Li et al., 2019). With retention efficiencies above 100% or below 0%, it is also possible that these values are the result of experimental error or variance. The protein retention efficiency results of this study, which varied from 41.59-56.69%, are similar to those reported in Tibbetts et al. (2013) and Ganga et al. (2015), however, lipid retention was higher in the current study; this may be attributed to the fact that fish were considerably larger in the current study compared to the two previously described studies.

Relative total ω3 fatty acid, DHA and EPA levels in fillets were unaffected by rearing temperature. Deposition rates and retention efficiencies of ω3 fatty acids (total, as well as DHA and EPA) decreased during the 800 to 1500 g growth period compared to the previous growth period at 16.5°C but were unchanged at the other two temperatures. Previous research on juvenile diploid Atlantic salmon suggests that elevated rearing temperature affects ω3 fatty acid metabolism (Norambuena et al., 2015). The pattern of higher retention efficiencies of DHA compared to EPA in the current study is supported by previous findings (Stubhaug et al., 2007; Sanden et al., 2011; Ytrestøyl et al., 2015), and DHA retention efficiencies above 100% have also been reported (Rosenlund et al., 2016). However, triploid salmonids metabolize fatty acids differently than diploids, as has been observed in rainbow trout (*Oncorhynchus mykiss*) (Manor et al., 2014) and Atlantic salmon (Murray et al., 2018). In the latter study, triploids were found to have significantly lower levels of total lipid, mono-unsaturated fatty acids (MUFAs) and PUFAs in the muscle, however, when fatty acid content was normalized for total lipid content, triploids were shown to have higher total ω3, arachidonic acid (ARA), EPA and DHA compared to diploids (Murray et al., 2018). While nutrient deposition rates and retention efficiencies of ω3 fatty acids did not significantly influence the overall composition of the fillet in the current trial, it would be important to maximize ω3 delivery to muscle tissue in AAS in order to minimize any negatively associated temperature effects.

Essential amino acid profiles, deposition rates and retention efficiencies in the fillet were affected by rearing temperature. For example, methionine was present in a higher percentage in the fillets of fish reared at 13.5°C and 16.5°C at 500 and 800 g, and salmon at 13.5°C also exhibited higher fillet methionine deposition rates and retention efficiencies during the 500 to 800 g growth period compared to those reared at 10.5°C. Methionine deposition rates decreased

during the final stage of the trial (i.e., dropping below zero) suggesting that fish reared at 13.5°C and 16.5°C mobilize dietary methionine faster than it could be assimilated, as it was either being poorly digested and/or was deficient in the diet. Previous research has shown that increasing crystalline DL-methionine by approximately 17% in the diet can improve methionine intake and apparent energy digestibility in post-smolt diploid Atlantic salmon (Espe et al., 2011). Collectively, these results suggest that AAS reared at temperatures above 10.5°C may require additional methionine in their diet when they reach 800 g. The feed provided at 800 g 0.51% (as-fed) methionine, but increasing this amount by 17% to 0.60% could help address the decreased rates of nutrient deposition at 13.5°C and 16.5°C. In the current study, rearing temperature did not affect the percent of lysine or histidine in fillets, but deposition rates were higher in fillets of AAS salmon reared at 10.5°C compared to 16.5°C during the 800 to 1500 g growth period, further emphasizing that temperature and growth stage should be considered when formulating diets for AAS. Mineral composition of the fillet remained consistent throughout the trial, with no statistically significant differences found among rearing temperature treatments, but all groups experienced the same decrease in percent phosphorus at the 1500 g stage. Phosphorus and calcium may have been deposited in areas other than the fillet, such as in the form of hydroxyapatite in the skeletal system, but this was not quantified in the current experiment. Furthermore, the current results demonstrate that diets developed for AAS should also be tailored based on weight stage and rearing temperature.

DSM SalmoFan™ is the standard colour reference used for visual assessment of the degree of pigmentation in salmon flesh (Alfnes et al., 2006); values range from 20 to 34, from lightest to darkest in colour, respectively (DSM, 2019), with darker pigmentation being more attractive to consumers (Lerfall et al., 2017b). Most fillets in the Norwegian market score

between 25 and 27 (Alfnes et al., 2006). In the present study, average scores of fillets of 1500 g fish were higher than Norwegian farmed salmon, suggesting it may be beneficial to further investigate if lower amounts of dietary astaxanthin are required for AAS than for conventional farmed Atlantic salmon. If true, this could equate to substantial savings for the producer as astaxanthin is an expensive and necessary feed ingredient (Solymosi et al., 2015). Lerfall et al. (2017a) demonstrated that, harvesting at 1600 g, Atlantic salmon reared at 15°C in a saltwater system had increased astaxanthin concentration compared to those reared at 5°C and 10°C, regardless of ploidy. This contrasts with the current study results where no differences in astaxanthin concentration were found among rearing temperatures. It was also reported that triploid salmon, regardless of rearing temperature, exhibited on average paler fillets after frozen storage compared to their diploid counterparts, however the difference was unlikely to be recognized by the human eye (Lerfall et al., 2017a). These aspects of elevated rearing temperature and triploidy require further investigation to determine if mitigation strategies can be developed to improve fillet quality.

Research has suggested that feeding diploid Atlantic salmon a high-protein diet will increase condition factor, feed efficiency and slaughter yield, while decreasing muscle fat and VSI (Dessen et al., 2017; Weihe et al., 2019). Testing a high protein diet with AAS at elevated rearing temperature could help reduce the amount of lipid deposited in the viscera and improve their use of dietary lipid through greater deposition in the fillet. As the dietary protein-to-lipid ratio was a significant predictor of body lipid content in the current study, further investigation of this factor is important. Consideration into what protein sources are used in diet formulation should be made, as TG Atlantic salmon exhibit different abilities to digest and retain nutrients supplied from diets high in plant protein compared to NTG salmon (Ganga et al., 2015). AAS

share close similarities to conventional Atlantic salmon but, through their growth hormone transgenesis and ploidy manipulation, there are changes in their metabolism and responses to temperature that remain unknown. Future research should focus on tailoring diets to meet the unique demands of these fish in order to maximize their growth performance and nutrient utilization.

2.6 Conclusions

The results from this study indicate that AAS reared at 16.5°C used more feed per unit of biomass gain and were less efficient at retaining nutrients, such as ω3 fatty acids, and pigment, than their counterparts reared at 10.5 and 13.5°C. Furthermore, salmon reared at 16.5°C allocated more dietary energy to lipid storage in their viscera instead of muscle growth than fish reared at the other temperatures and had lower fillet yields. For these reasons, it is recommended that AAS are reared below 16.5°C in freshwater RAS. While fish reared at 16.5°C did reach target weight in the least number of days, a time-cost analysis needs to be conducted to ascertain at which point the benefits of enhanced weight gain are outweighed by the negative effects of poorer nutrient usage. This is the first report measuring the impact of rearing temperature on the growth performance and nutrient utilization of GH TG triploid female Atlantic salmon. This information can be used to inform future commercial production strategies for AAS.

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CHAPTER 3: Impact of rearing temperature on antiviral immune response of growth hormone transgenic female triploid Atlantic salmon (*Salmo salar*)

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3.1 Abstract

AquAdvantage Salmon (growth hormone transgenic female triploid Atlantic salmon) are a faster-growing alternative to conventional farmed diploid Atlantic salmon. To investigate optimal rearing conditions for their commercial production, a laboratory study was conducted in a freshwater recirculating aquaculture system (RAS) to examine the effect of rearing temperature (10.5°C, 13.5°C, 16.5°C) on their antiviral immune and stress responses. When each temperature treatment group reached an average weight of 800 g, a subset of fish were intraperitoneally injected with either polyriboinosinic polyribocytidylic acid (pIC), a viral mimic, or an equal volume of sterile phosphate-buffered saline (PBS). Blood and head kidney samples were collected before injection and 6, 24 and 48 hours post-injection (hpi). Transcript abundance of 7 antiviral biomarker genes (*tlr3, lgp2, stat1b, isg15a, rsad2, mxb, ifng*) was measured by real-time quantitative polymerase chain reaction (qPCR) on head kidney RNA samples. Plasma cortisol levels from blood samples collected pre-injection and from pIC and PBS groups at 24 hpi were quantified by ELISA. All genes tested were significantly upregulated by pIC at all three temperatures, except for *tlr3*, which was only upregulated in the 10.5°C treatment. Target gene activation was generally observed at 24 hpi, with transcript levels decreasing by 48 hpi in pIC-injected fish. Although a high amount of biological variability in response to pIC was evident across all treatments, rearing temperature significantly influenced transcript abundance and/or fold-changes comparing time- and temperature-matched pIC- and PBS-injected fish for several genes (e.g. *tlr3, lgp2, stat1b, isg15a, rsad2* and *ifng*) at 24 hpi. As an example, significantly higher fold-changes of *rsad2*, *isg15a* and *ifng* were found in fish reared at 10.5°C when compared to 16.5°C. Rearing temperature and treatment did not significantly affect circulating cortisol. Multivariate analysis concluded that each rearing temperature

influenced antiviral immune response. Further research is required to ascertain whether the differences noted in this study reflect a more robust immune response at 10.5°C compared to 16.5°C, or if immune regulation was accelerated at elevated temperature and these responses were not captured within the time-points chosen for this study. Still, the present experiment provides novel insight into the relationship between rearing temperature and antiviral immunity in AquAdvantage Salmon.

3.2 Introduction

Growth hormone (GH) transgenic (TG) female triploid Atlantic salmon (*Salmo salar*), referred to commercially as AquAdvantage Salmon (AAS), are a reproductively sterile alternative to conventional diploid farmed salmon. These fish can reach 300 g approximately 40% faster than non-transgenic (NTG) counterparts (Tibbetts et al., 2013), are ~25% more feed efficient (Tibbetts et al., 2013), and can maintain or improve their enhanced growth performance when fed a diet with high inclusion rates of plant protein (Ganga et al., 2015).

As with conventional farmed Atlantic salmon, AAS are susceptible to disease, including viral infections. Several of the pathogenic viruses in Atlantic salmon aquaculture are RNA viruses such as viral hemorrhagic septicemia virus [VHSV; negative-sense single-stranded RNA (ssRNA)], salmonid alphavirus (SAV; positive-sense ssRNA) and infectious salmon anaemia virus (ISAV; negative-sense ssRNA) (Lang et al., 2009). These viruses can cause considerable economic losses in the salmonid aquaculture industry (Collet, 2014) and commercial vaccines are either not available, or when they do exist, may not be 100% efficacious (Dhar et al., 2014). For these reasons, it is important to investigate the nature of viral infection and the subsequent host-response. Polyriboinosinic polyribocytidylic acid (pIC), a dsRNA pathogen-associated molecular pattern (PAMP), can provide information on host response to dsRNA viruses, as well as ssRNA and DNA viruses (as replication or symmetrical transcription of these pathogens also creates dsRNA) (Akira et al., 2006), and is a useful proxy to study antiviral response.

There is, however, a general paucity of information on the immune response of triploid or GH TG salmonids to pathogens or PAMPs. Salmonid genomes are recognized as pseudotetraploid, as this family underwent a whole-genome duplication event ~80 million years ago, followed by partial re-diploidization (Allendorf and Thorgaard, 1984; Davidson et al., 2010;

Xu et al., 2013; Lien et al., 2016). However, to improve the readability of this chapter, the conventional Atlantic salmon genome will be referred to as diploid, and where the second polar body was retained, triploid.

Adhesion scores, measuring visible damage or presence of lesions, were not different between diploid and triploid Atlantic salmon following injection with a commercial furunculosis vaccine (Chalmers et al., 2016) as determined by the Spielberg Scale (Midtlyng et al., 1996). This contrasts with previous work that reported an increase in adhesion scores in triploids (Fraser et al., 2014). With respect to pathological responses during infection, there appears to be no difference between diploids or triploids. For example, after exposure to *Aeromonas salmonicida* ploidy did not influence complement activity or antibody response, and no differences in survival were observed between groups, demonstrating equal efficacy between diploids and triploids (Chalmers et al., 2016). Similarly, Weber et al. (2013) found minimal differences in survival of diploid or triploid rainbow trout (*Oncorhynchus mykiss*) after exposure to *Flavobacterium psychrophilum*, the etiological agent of bacterial cold water disease (BCWD). Regarding parasitic infections, there were no differences in disease progression, severity or host response between diploid and triploid Atlantic salmon infected with *Neoparamoeba perurans*, the causative agent of amoebic gill disease (AGD) (Chalmers et al., 2017). Likewise, following a sea louse (*Lepeophtheirus salmonis*) infestation challenge, no differences in infection level were found between triploid and diploid Atlantic salmon (Frenzl et al., 2014).

In contrast, there appears to be a difference in how diploid and triploid salmon respond during viral infection. For example, mortality rates were similar between diploid and triploid Atlantic salmon fry exposed to SAV sub-type 1 through either intraperitoneal injection (IP), bath immersion, or cohabitation. However, higher viral RNA copy number in the liver of diploid fry

in the cohabitation group was reported and diploids also displayed higher levels of myocardial and pancreatic degeneration compared to triploids (Herath et al., 2017). Furthermore, viral load accumulated at a slower rate in triploids compared to diploids after injection with SAV sub-type 3 (Moore et al., 2017). Researchers hypothesized that the slower accumulation could delay the onset of pancreatic disease or help avoid an outbreak (Moore et al., 2017).

Research suggests GH transgenesis may impair immune function in salmon. For example, compared to wild-type NTG coho salmon (*Oncorhynchus kisutch*), diploid GH TG counterparts have higher basal levels of hematocrit, hemoglobin and erythrocyte, but lower leukocyte numbers (Kim et al., 2013). Correspondingly, during a challenge with *A. salmonicida*, GH TG coho salmon were more susceptible to furunculosis (i.e. faster disease progression and death) than wild-type NTG coho (Kim et al., 2013). Another study comparing diploid GH transgenic and wild-type coho salmon found that, when injected with either pIC or peptidoglycan (to elicit antiviral or antibacterial responses, respectively), transcript expression of immune-related genes in the muscle tissue was attenuated in TGs (Alzaid et al., 2018). The authors of Alzaid et al. (2018) hypothesized that enhanced growth rate impacted energy allocation between growth and immune systems, negatively influencing host response to pathogens. With the contrasting effects of triploidy and GH transgenesis, it is unknown what effect both factors have on AAS compared to conventional farmed Atlantic salmon.

Further, temperature influences viral pathogenicity and host immune responses of teleost fish. For instance, Avunje et al. (2012) demonstrated that viral and host responses differed based on rearing temperature in VHSV-infected diploid olive flounder (*Paralichthys olivaceus*). Viral gRNA copy number coinciding with antiviral gene expression peaked earlier in flounder reared at 20°C and both factors declined at significantly faster rates than fish reared at 15°C (Avunje et

al., 2012). Transcriptome sequencing of diploid olive flounder reared at either 13°C or 20°C and infected with VHSV revealed 1366 differentially expressed genes between temperature groups related to metabolism, adhesion and several immune signalling pathways in addition to virulence factors (Hwang et al., 2018; Jeong et al., 2018).

The impact of rearing temperature on virulence is specific to the type of virus, as evidenced by increased temperature correlating with increased mortality in diploid zebrafish (*Danio rerio*) infected with betanodavirus (the etiological agent of viral nervous necrosis - VNN) (Binesh, 2014). Similarly, in diploid Senegalese sole (*Solea senegalensis*) infected with betanodavirus at 16°C, 18°C and 22°C, mortality increased with elevating temperature (Souto et al., 2015). Following IP injection of inactivated lymphocystis disease virus (LCDV) in diploid olive flounder reared at 9°C, 15°C, 21°C or 26°C, it was determined that the optimal temperature for immunization was 21°C as surface immunoglobulin positive cells reached higher levels in less time than in the other temperature groups (Xu et al., 2011). VHSV replication has also been shown to be temperature-dependent as evidenced in a walleye (*Sander vitreus*) cell line (WE-cfin11f) (Vo et al., 2015). In Atlantic cod (*Gadus morhua*), elevated temperature (16°C compared with 10°C) appears to accelerate the spleen antiviral transcript expression response to pIC (Hori et al. 2012a). Comparing rearing environments of 16°C and 10°C with juvenile rainbow trout, mortality was significantly lower at elevated temperature following exposure to infectious pancreatic necrosis virus (IPNV) (Dorson & Torchy, 1981). Furthermore, viral persistence was found inversely proportional to temperature in juvenile trout after bath infection with VHSV, with fish reared at 10°C taking 27 days longer to reach undetectable viral levels compared to those at 15°C and 20°C (Jørgensen, 1982). Conversely, in an experiment where rainbow trout fingerlings were infected with epizootic haematopoietic necrosis virus (EHNV) by

IP injection, fish reared at ~20°C all perished within a short period, while survivors remained at the end of the trial at ~9°C (Whittington & Reddacliff, 1995). Collectively, these studies suggest that it is important when producing any aquaculture species to understand how selected rearing temperatures may impact their antiviral immune response.

The objective of this experiment was to determine the effect of rearing temperature on the antiviral immune and stress response of AAS raised in a freshwater RAS. AAS at 800 g were pIC-injected to induce an antiviral response at rearing temperatures of 10.5°C, 13.5°C or 16.5°C, with sampling and data collection at multiple time points post-injection. Using real-time quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) analyses, target immune-relevant transcript expression and plasma cortisol results were obtained, respectively. This is the first report of the impact of rearing temperature on the antiviral immune response of AAS and helps establish a baseline of knowledge that can guide future research and industrial application.

3.3 Materials and methods

3.3.1 Experimental animals and rearing conditions

All fish used in this study were of St. John River origin and were hatched and reared at AquaBounty Canada, Prince Edward Island, Canada. The animals were handled and cared for in accordance with the Canadian Council on Animal Care's Guidelines on the Care and Use of Fish in Research, Teaching, and Testing (Canadian Council on Animal Care, 2005). The study was approved by the Animal Care Committees of both AquaBounty Canada (Animal Care Protocol ABC-ACC-013) and Memorial University of Newfoundland (17-03-JW).

AAS were produced from a specific TG line (termed EO-1α) containing a gene construct (opAFP-GHc2) consisting of an ocean pout (*Macrozoarces americanus*) antifreeze protein promoter and the coding sequence of a Chinook salmon (*Oncorhynchus tshawytscha*) GH gene (Yaskowiak et al., 2006, 2007). A single homozygous TG sex-reversed neomale (i.e., functionally masculinized genetic female) was crossed with three NTG females, producing all-female TG offspring. All fertilized eggs were pooled together before subjection to hydrostatic pressure shock to induce triploidy, making the fish reproductively sterile (Benfey et al., 1988; AquaBounty Technologies, Inc., 2010). Transgene status and zygosity of the broodstock were confirmed by polymerase chain reaction (PCR) and quantitative PCR, respectively, using validated procedures developed by AquaBounty. Ploidy status of the offspring was verified through flow cytometry (Allen Jr, 1983; AquaBounty Technologies, Inc., 2010) on 200 homogenized eyed eggs, estimating that pressure shocking was 99.5% effective in inducing triploidy.

AAS were grown from first feeding fry (starting weight of ~0.51 g) in 1.5 m^3 tanks at 10.5°C, 13.5°C and 16.5°C (± 0.5°C) in triplicate. De-gassed and oxygenated fresh water from a well (<1 g/L salinity) was supplied to each tank in a RAS (10–20% make-up daily) and dissolved oxygen was measured daily and maintained at > 9.0 mg/L. Fish were maintained on a 24-h photoperiod (to match AquaBounty's commercial production) and fed using automatic feeders before the start of the trial. Passive integrated transponder (PIT) tags (Avid Canada Corp., Alberta, Canada) were inserted into the peritoneal cavity of each fish as they reached an average weight of 400 g. At an average weight of 800 g, a sample of fish from each set of tanks was intraperitoneally (IP) injected with either pIC (Sigma-Aldrich, Ontario, Canada), a synthetic dsRNA that elicits a potent antiviral response (Caballero-Solares et al., 2017), or an equal

volume of sterile phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Massachusetts, USA) for generation of time-matched control fish. During periods of fish handling where sampling was not required, fish were anesthetized using tricaine methanesulfonate (MS-222) (Syndel Canada, British Columbia, Canada) at a dose of 0.1 g/L, buffered with an equal amount of sodium bicarbonate.

3.3.2 Experimental design

Figure 3-1 provides an overview of the experimental design for this study. Fish were taken off feed at least 24 h in advance of sampling or injection event, and feeding was not resumed until all sampling was completed. As salmon at each rearing temperature reached an average weight of 800 g, three fish per tank (n = 9 per temperature group) were selected for sampling (by convenience) and assigned as 0 h controls (i.e. unstimulated). Immediately following that sampling, fish were removed from each tank and anesthetized (0.1 g/L buffered MS-222) until a total of 12 salmon per tank were identified as weighing between 762.5 and 837.5 g. A narrow weight range was chosen to limit the possible confounding effect of fish weight. IP injections of pIC (750 µL; ~9.36 µg pIC/g fish) or PBS were alternated as fish that met the weight criterion were selected (n = 18 per injection group). Fish weighing 762.5 – 800 g were injected with 7.31 mg of pIC and those weighing 800 – 837.5 g were injected with 7.69 mg of pIC. PBS injections were of equal volume (750 µL). Colour-coded flag tags (Pentair, Florida, USA) were then inserted into the dorsal region of all injected fish to distinguish between fish injected with pIC and PBS. At 6 h post-injection (hpi), 24 hpi and 48 hpi, two pIC-injected fish and two PBS-injected fish were sampled (by convenience) per tank (n = 6 per injection treatment at each time point).

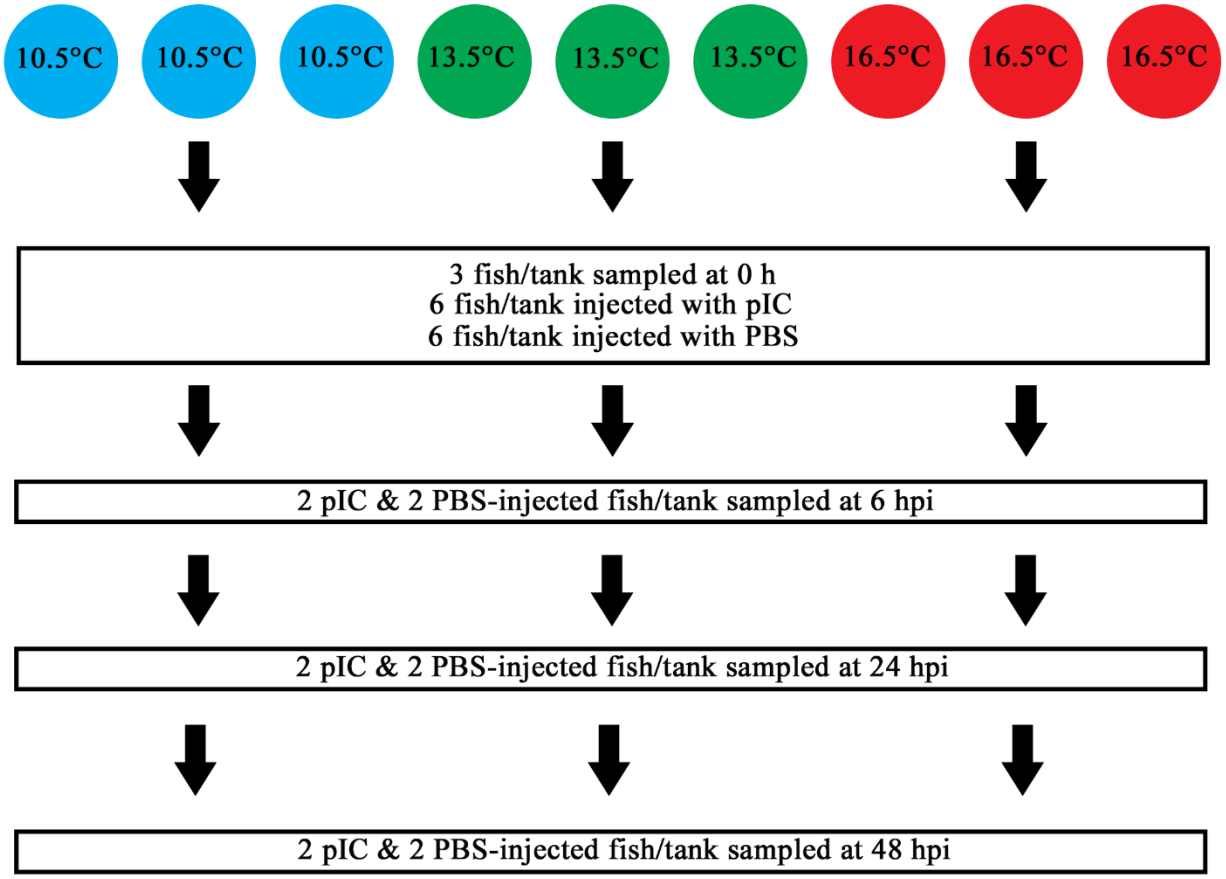

Figure 3-1. Overview of the experimental design used to investigate the impact of rearing temperature on the immune and stress responses of AquAdvantage Salmon.

3.3.3 Sample collection

At any sampling time point, fish were selected (by convenience sampling) and euthanized by cranial concussion followed by cervical dislocation. This method was performed to limit the effects of handling stress on the results of the study, particularly regarding the cortisol response. Blood was obtained within 90 sec following euthanasia, placed on ice, and transported to the lab for centrifugation within 10 min. Samples were centrifuged at 13,000 x*g* for 5 min at 4°C, after which the plasma was collected and stored at -70°C until subsequent processing. Head kidney samples (~ 50 mg) taken approximately 0.5 cm inferior from the most cranial portion of the kidney using standard aseptic techniques and preserved in 1 mL of RNA*later*™ kept on ice, then stored at 4°C overnight; supernatant was then removed and samples stored at -70°C until RNA extraction.

3.3.4 RNA extraction & purification

RNA extractions were performed on randomized samples using the RNeasy® Mini Kit and RNase-Free DNase Set (Qiagen, Ontario, Canada) for both on-column digestions of DNA during RNA purification and DNase digestion in solution. All procedures were completed according to the manufacturers' instructions with small adjustments made based on guidelines found in the Qiagen Purification of Total RNA Using the RNeasy® Fibrous Tissue Mini Kit Protocol. RNA quantity and quality were assessed using spectrophotometry and 0.7% agarose gel electrophoresis, respectively. Only high-quality RNA samples with distinct 18S/28S rRNA bands and satisfactory A260/280 and A260/230 ratios were used for qPCR analysis. A total of 6 out of 133 samples were excluded from the final analysis as they did not meet these criteria after

multiple attempts at RNA purification. The remainder of DNase-treated RNA had A260/280 above 2.0 and all samples had A260/230 above 1.8, except for 2 samples.

3.3.5 cDNA synthesis

cDNA was synthesized from 1 µg of high-quality DNase I-treated RNA using the iScript™ cDNA synthesis kit (Bio-Rad, Quebec, Canada) following the manufacturer's instructions. No reverse transcriptase (no-RT) controls were created from pooling the RNA of randomized samples (n = 10 per pool) to aid in assessing gDNA contamination levels during qPCR quality control (QC) testing. After cDNA synthesis was complete, an aliquot from every individual sample was pooled to assess qPCR primer pair amplification efficiencies. Based on amplification results from QC testing, cDNA was diluted in nuclease-free water (Invitrogen, Ontario, Canada) at 1:5 and 1:25 concentrations before qPCR analysis for lower and higher expressed transcripts, respectively.

3.3.6 qPCR analysis

Seven antiviral biomarker transcripts were chosen for qPCR analysis based on previous study results demonstrating response to pIC in Atlantic salmon head kidney samples (Caballero-Solares et al., 2017). Transcripts were also chosen to represent different aspects of the interferon (IFN) pathway to better capture overall antiviral response: pattern recognition receptors (PRRs) (*tlr3*, *lgp2*), signal transduction and transcription (*stat1b*), immune effectors (*isg15a*, *rsad2*, *mxb*) and a cytokine (*ifng*). Target and normalizer transcript primers were taken from previous studies (Caballero-Solares et al., 2017; Fast et al., 2006; Hixson et al., 2017; Jones et al., 2007; Skugor et al., 2008; Xue et al., 2015) except for the *tlr3* primer set which was designed using NCBI's

Primer-Blast (Ye et al., 2012). Primers for *tlr3* were identified by the GenBank accession number and using *Salmo salar* (taxid: 8030) as the organism and ensuring product size was below 200 bp. Every primer set was quality-assessed using a cDNA pool derived from equal volume (and input RNA) contribution from each sample to test amplification efficiencies (Pfaffl, 2001) using a 5-fold, 6-point dilution series in triplicate. Gel electrophoresis also confirmed single products and amplicon size (88-189 bp) of each primer pair. A single peak was observed for each dissociation curve, no gDNA contamination was found in no-RT controls and amplification efficiencies ranged between 93-109%. All information related to the primers utilized in this study is shown in Table 3-1.

qPCR amplification was performed using a SsoAdvanced™ Universal SYBR® Green Supermix qPCR kit (Bio-Rad) and the manufacturer's instructions with a CFX-384 thermal cycler (Bio-Rad). Master mix was loaded using an Aurora automated plate dispenser with VERSAware 10.v.1.2.48 software, and samples were plated in triplicate using an electronic Sartorius Picus® pipette. The total reaction volume was 11 µL and comprised of 5 µL SYBR® Green Supermix, 4 µL nuclease-free water, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), and 1 µL of diluted cDNA template (2 or 10 ng input RNA, depending on dilution concentration). The PCR program consisted of one cycle of 95°C for 30 sec, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and then a dissociation stage (65°C to 95°C at 0.5°C increments reading fluorescence every 5 sec). Each gene was run on a single, independent 384-well plate, which included no-template (NTC) controls.

Raw expression profiles were imported into qbase+ (Biogazelle) (Hellemans et al., 2007), and technical replicates falling outside of ±0.5 cycle threshold (CT) from two close replicates, were removed. A total of 7 normalizer genes were evaluated (*eif3d*, *rpl32*, *ef1a*, *pabpc1*, *polr2*,

actb, *eif31*). From these *eif3d* and *rpl32* were chosen as the final study normalizers, based on their having less than one CT difference in average CT between all samples and the highest stability (mean geNorm M value and coefficient of variation of 0.548 and 0.192, respectively). Normalized relative quantities (NRQs) (Vandesompele et al., 2002) were calculated using amplification efficiencies for each primer pair (Table 3-1), which were then $\log_2$ transformed in Microsoft Excel.

Table 3-1. qPCR primers utilized in the study.

Gene Name (GenBank Accession Number)	Nucleotide sequence (5'-3')	Amplification Efficiency[a] (%)	r^2	Amplicon size (bp)	Source
Toll-like receptor 3 (*tlr3*) (XM_014195262)	F: GCACAGTTGTTTGGGTCATCT	100.9	0.993	135	This study
	R: ACATTTGGATCTTTGGCGGC				
RNase helicase lgp2 (*lgp2*, alias*dhx58*) (BT045378)	F: TCCAAGACCCGTAAAAGCAC	98.2	0.991	189	Caballero-Solares et al. 2017
	R: GGTGGAGATCAGGAGGTTGA				
Signal transducer and activator of transcription 1b (*stat1b*) (BT048927)	F: GTTCAGGATGCAGAGCATGA	100.5	0.998	109	Caballero-Solares et al. 2017
	R: CCATCCCATTCACCTCTTGT				
Interferon-stimulated gene 15a (*isg15a*) (BT049918)	F: AAAGTGGCCACAACAAAGCAG	93.4	0.997	140	Caballero-Solares et al. 2017
	R: ATAGGAGCGGGCTCCGTAATC				
Radical S-adenosyl methionine domain containing protein 2 (*rsad2*, alias *viperin*) (BT047610)	F: ACCATTTTACCCGACAGTGC	102.7	0.995	183	Caballero-Solares et al. 2017
	R: TCCCCAAGAAATCACCTCTG				
Interferon-induced GTP-binding protein of myxovirus resistance b (*mxb*) (BT044881)	F: ACGCACCACTCTGGAGAAAT	108.3	1.000	184	Caballero-Solares et al. 2017
	R: CTTCCATTTCCCGAACTCTG				
Interferon-gamma (*ifng*) (AJ841811)	F: CCGTACACCGATTGAGGACT	108.3	0.995	133	Caballero-Solares et al. 2017
	R: GCGGCATTACTCCATCCTAA				
Eukaryotic translation initiation factor 3 subunit D (*eif3d*) (GE777139)[b]	F: CTCCTCCTCCTCGTCCTCTT	96.6	0.995	105	Caballero-Solares et al. 2017
	R: GACCCCAACAAGCAAGTGAT				
60S ribosomal protein 32 (*rpl32*) (BT043656)[b]	F: AGGCGGTTTAAGGGTCAGAT	96.8	0.974	119	Xue et al. 2015
	R: TCGAGCTCCTTGATGTTGTG				

Elongation factor 1 alpha (*ef1a*) (NM_001141909)[c]	F: GTGGAGACTGGAACCCTGAA R: CTTGACGGACACGTTCTTGA	100.0	0.998	155	Jones et al. 2007
Polyadenylate-binding protein 1 (*pabpc1*) (EG908498)[c]	F: TGACCGTCTCGGGTTTTTAG R: CCAAGGTGGATGAAGCTGTT	96.1	0.994	108	Caballero-Solares et al. 2017
RNA polymerase II (*polr2*) (CA049789)[c]	F: TTCTGAAAGACCCCCAAGTG R: AGCTCGCTGATGAGGTCAGT	103.5	0.999	145	Hixson et al. 2017
Beta-actin (*actb*) (KU885449)[c]	F: CAACTGGGACGACATGGAGA R: AGTGAGCAGGACTGGGTGCT	99.2	0.998	88	Fast et al. 2006
Eukaryotic translation initiation factor 3 subunit 6 (*eif3s6*) (CX040383)[c]	F: GTCGCCGTACCAGCAGGTGATT R: CGTGGGCCATCTTCTTCTCGA	101.8	0.998	92	Skugor et al. 2008

[a] Amplification efficiencies were calculated using a 6-point 1:5 dilution series starting with an equal mixture of cDNA from every sample

[b] Normalizer gene chosen for this study

[c] Normalizer gene tested, but ultimately not chosen for this study

3.3.7 Plasma cortisol analysis

To free cortisol from its binding proteins, previously collected plasma samples were diluted 10-fold in buffer (0.1 M phosphate, containing 0.1% BSA, 0.4 M sodium chloride, 1 mM EDTA, and 0.01% sodium azide) and then incubated in a 70°C water bath for 1 h, followed by centrifugation at 15,000 x*g* for 15 min. Supernatants were collected and frozen at -70°C until analysis using a monoclonal antibody ELISA kit (Cayman Chemical, Michigan, USA), following the manufacturer's specifications. The plates were read at 420 nm on a microplate reader (Molecular Devices SpectraMax® M5) using SoftMax® Pro v6.4 software. Only samples from the 0 h and 24 h (both pIC- and PBS-injected) time points were analysed, as the ELISA testing was performed after target transcript qPCR analysis from the current study had shown that target transcript activation occurred by 24 hpi. Samples were run in triplicate at two different concentrations (1:10, 1:100). Non-specific binding wells, maximum binding wells and a 6-point standard curve, all run in triplicate, were included on each 96-well plate. Performing a 4-parameter logistic fit with the standard curve results gave r^2 values of 0.988 or higher for each plate.

3.3.8 Statistical analysis

NRQs and plasma cortisol concentrations were analyzed by three-way, two-way and one-way analysis of variance (ANOVA) comparing interactions between time points (0, 6, 24 and 48 hpi), rearing temperatures (10.5, 13.5 and 16.5°C) and injection types (PBS and pIC). Tukey's post-hoc tests were performed where significant effects of a variable were detected. Similarity percentages (SIMPER) (Clarke, 1993), permutational multivariate analysis of variance (PERMANOVA) and principal coordinates (PCoA) analyses were completed using PRIMER v7

software to explore differences between pIC-injected temperature groups at 24 hpi. Generation of hierarchical clustering and correlogram diagrams, together with ANOVA testing, were performed in R (version 3.5.2) (R Studio Team, 2015). Statistical differences were considered significant at $p < 0.05$.

Two outliers were removed from the qPCR data set: one pIC- and one PBS-injected fish at 24 hpi that were reared at 13.5°C. Grubbs' tests performed in GraphPad revealed that the pIC-injected fish was a significant outlier in five of seven genes, while the PBS-injected fish was a significant outlier for three of seven genes.

3.4 Results

3.4.1 Target gene expression in response to pIC injection

The expression of seven targeted antiviral biomarker genes is shown in Figures 3-2 to 3-8. Only fish reared at 10.5°C showed signs of response at 6 hpi in *lgp2* (9-fold; Figure 3-2). All genes, apart from *tlr3* (Figure 3-3), were found to be upregulated across all three rearing temperatures at 24 hpi. The magnitude in response varied considerably among genes at 24 hpi, with the highest average fold-change differences found in *isg15a* (2076-fold at 10.5°C; Figure 3-4) and the lowest significant pIC response seen with *tlr3* (3-fold at 10.5°C; Figure 3-3). Temperature was observed to affect average fold-changes (pIC versus time- and temperature-matched PBS) in *lgp2* (10.5°C: 97-fold; 13.5°C: 20-fold; 16.5°C: 32-fold), *isg15a* (10.5°C: 2076-fold; 13.5°C: 1272-fold; 16.5°C: 426-fold), *stat1b* (10.5°C: 62-fold; 13.5°C: 21-fold; 16.5°C: 47-fold; Figure 3-5), *rsad2* (10.5°C: 298-fold; 13.5°C: 98-fold; 16.5°C: 66-fold; Figure 3-6) and *ifng* (10.5°C: 59-fold; 13.5°C: 31-fold; 16.5°C: 13-fold; Figure 3-7), with significantly higher fold-change values at 10.5°C compared to 13.5°C in all these cases and 10.5°C was also

higher compared to 16.5°C in *isg15a*, *rsad2* and *ifng*. No differences in fold-change among rearing temperatures were observed for *mxb* (10.5°C: 100-fold; 13.5°C: 88-fold; 16.5°C: 77-fold; Figure 3-8).

In addition to the effect of rearing temperature on fold-change (pIC versus PBS), differences in NRQ based on rearing temperature at 24 hpi were also detected in *lgp2*, *tlr3*, *isg15a*, *stat1b* and *ifng*. Transcript levels at 10.5°C were higher than at 13.5°C for *tlr3*, than at 16.5°C for *isg15a*, and than both 13.5°C and 16.5°C for *lgp2*, whereas those of *ifng* were downregulated with elevated temperature (i.e., 10.5°C > 13.5°C > 16.5°C). Fish at both 10.5°C and 13.5°C exhibited an attenuated response by 48 hpi in *lgp2* (10.5°C: 8-fold), *isg15a* (10.5°C: 171-fold; 13.5°C: 12-fold), *stat1b* (10.5°C: 16-fold; 13.5°C: 13-fold), *rsad2* (10.5°C: 33-fold; 13.5°C: 15-fold), *ifng* (13.5°C: 5-fold) and *mxb* (10.5°C: 34-fold; 13.5°C: 23-fold). No response (relative to PBS-control) was detected in fish reared at 16.5°C in any of the genes at 48 hpi, and none of the three temperature groups demonstrated a response in *tlr3* at that time point. No significant differences were detected between any of the fold-changes calculated for the 10.5°C and 13.5°C treatments at 48 hpi.

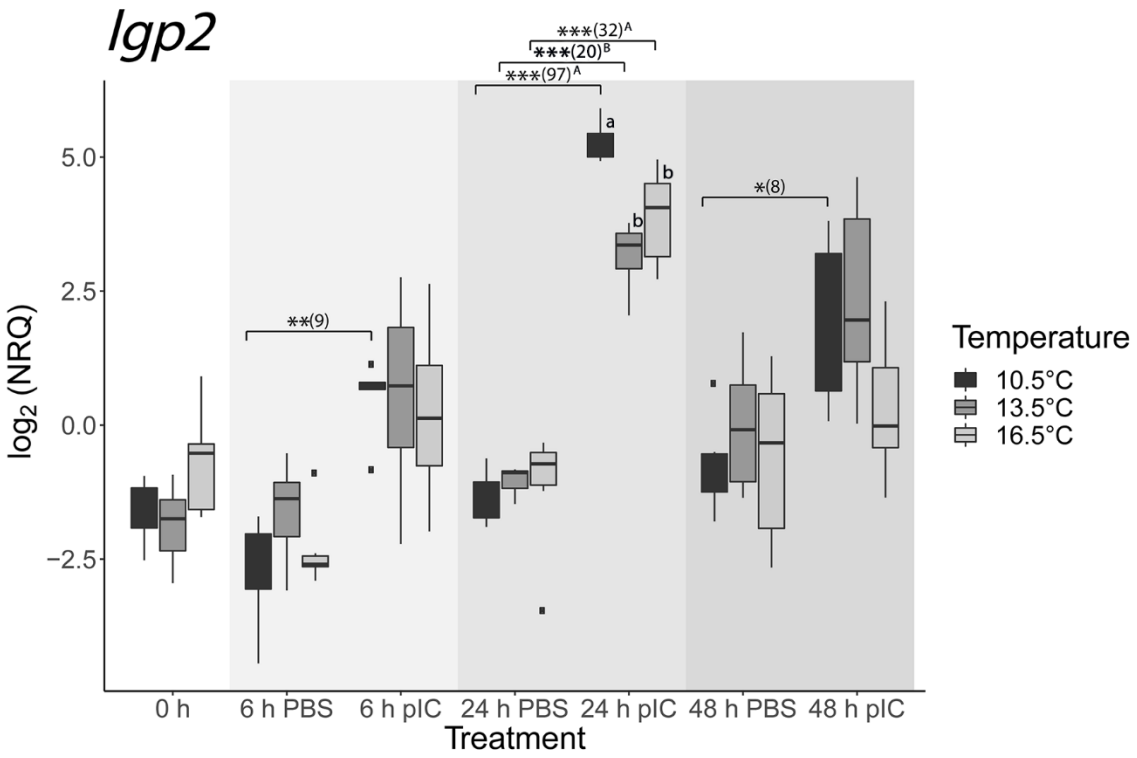

Figure 3-2. Effect of rearing temperature on RNase helicase lgp2 (*lgp2*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and hours (h) post-injection are indicated by asterisks (*, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$). Differences by one-way ANOVA are denoted by lower-case letters between temperatures within PBS- or pIC-injected groups at any specific time point ($p < 0.05$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments, with upper-case letters signifying significant differences between temperatures ($p < 0.05$).

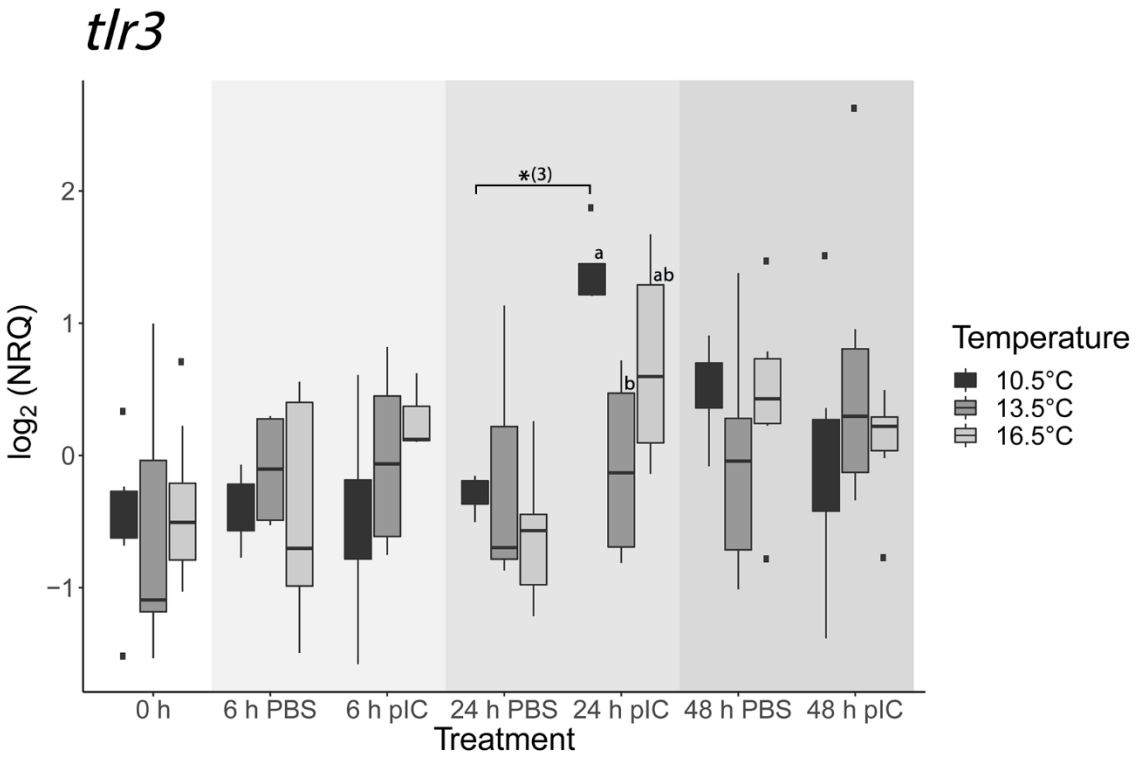

Figure 3-3. Effect of rearing temperature on Toll-like receptor 3 (*tlr3*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and hours (h) post-injection are indicated by asterisks (*, $p < 0.05$). Differences by one-way ANOVA are denoted by lower-case letters between temperatures within PBS- or pIC-injected groups at any specific time point ($p < 0.05$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments.

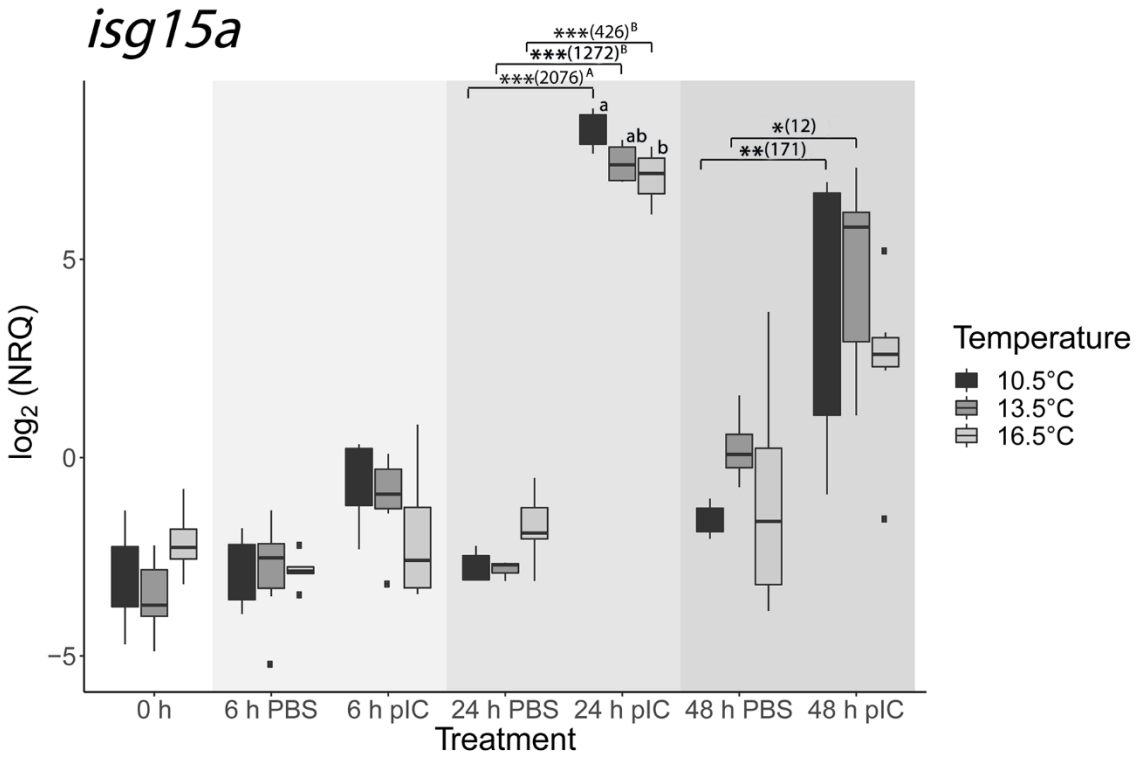

Figure 3-4. Effect of rearing temperature on interferon-stimulated gene 15a (*isg15a*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and time (h) post-injection are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Differences by one-way ANOVA are denoted by lower-case letters between temperatures within PBS- or pIC-injected groups at any specific time point ($p < 0.05$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments, with upper-case letters signifying significant differences found between temperatures ($p < 0.05$).

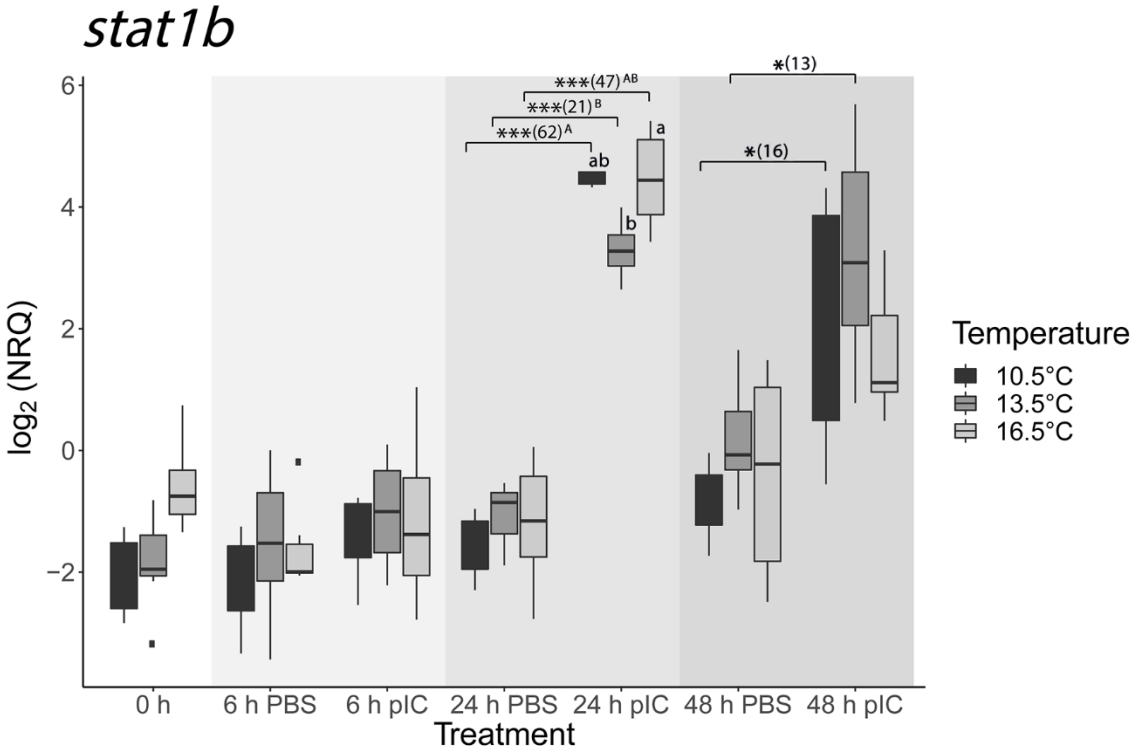

Figure 3-5. Effect of rearing temperature on signal transducer and activator of transcription 1b (*stat1b*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and hours (h) post-injection are indicated by asterisks (*, $p < 0.05$; ***, $p < 0.001$). Differences by one-way ANOVA are denoted by lower-case letters between temperatures within PBS- or pIC-injected groups at any specific time point ($p < 0.05$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments, with upper-case signifying significant differences between temperatures ($p < 0.05$).

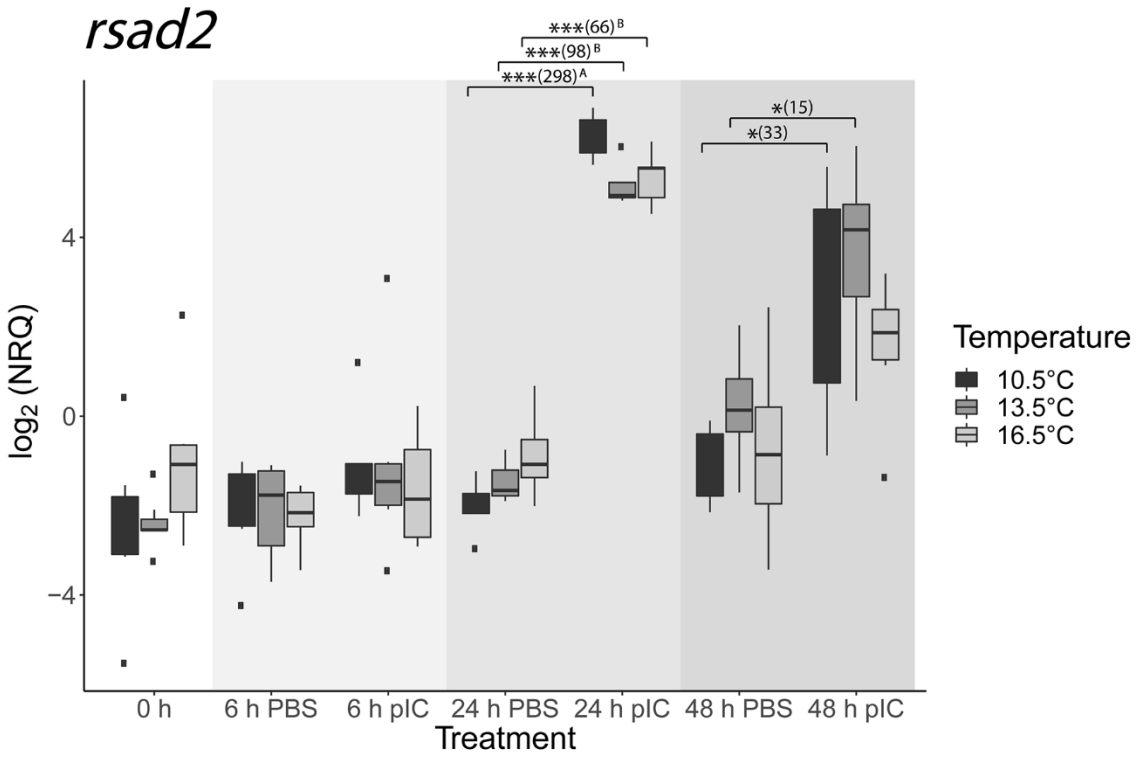

Figure 3-6. Effect of rearing temperature on radical S-adenosyl methionine domain containing protein 2 (*rsad2*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and hours (h) post-injection are indicated by asterisks (*, $p < 0.05$; ***, $p < 0.001$). Differences by one-way ANOVA are denoted by lower-case letters between temperatures within PBS- or pIC-injected groups at any specific time point ($p < 0.05$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments, with upper-case letters signifying significant differences between temperatures ($p < 0.05$).

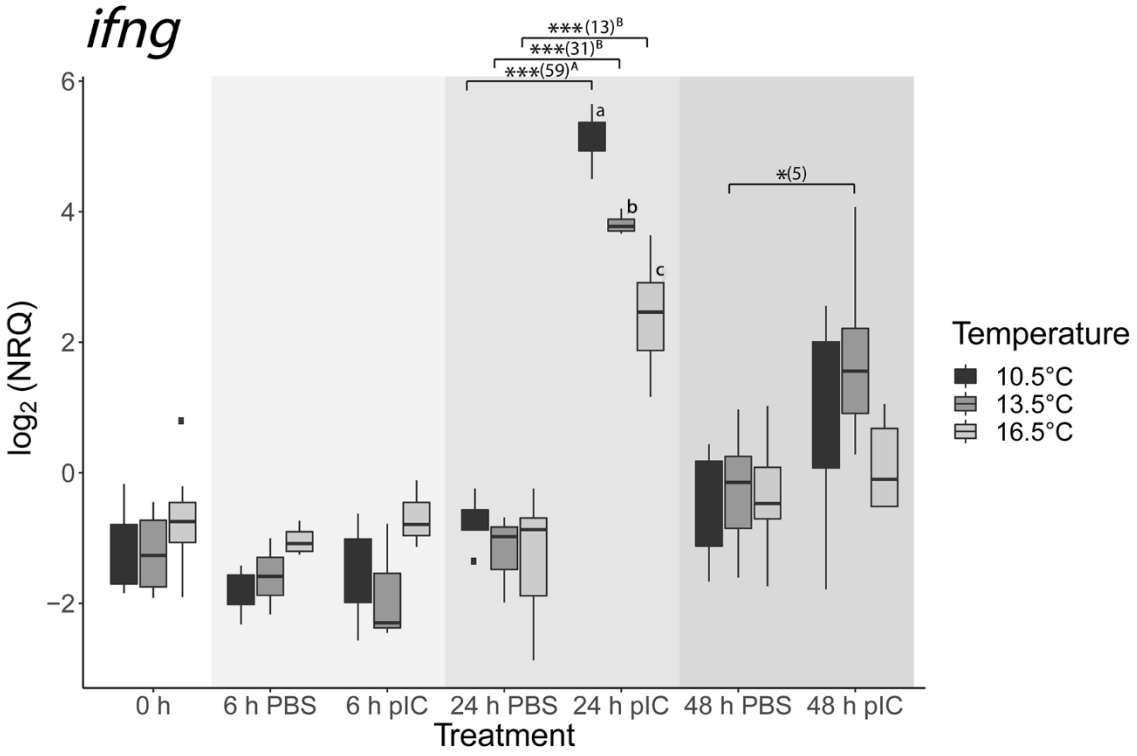

Figure 3-7. Effect of rearing temperature on interferon gamma (*ifng*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and hours (h) post-injection are indicated by asterisks (*, $p < 0.05$; ***, $p < 0.001$). Differences by one-way ANOVA are denoted by lower-case letters between temperatures within PBS- or pIC-injected groups at any specific time point ($p < 0.05$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments, with upper-case letters signifying significant differences between temperatures ($p < 0.05$).

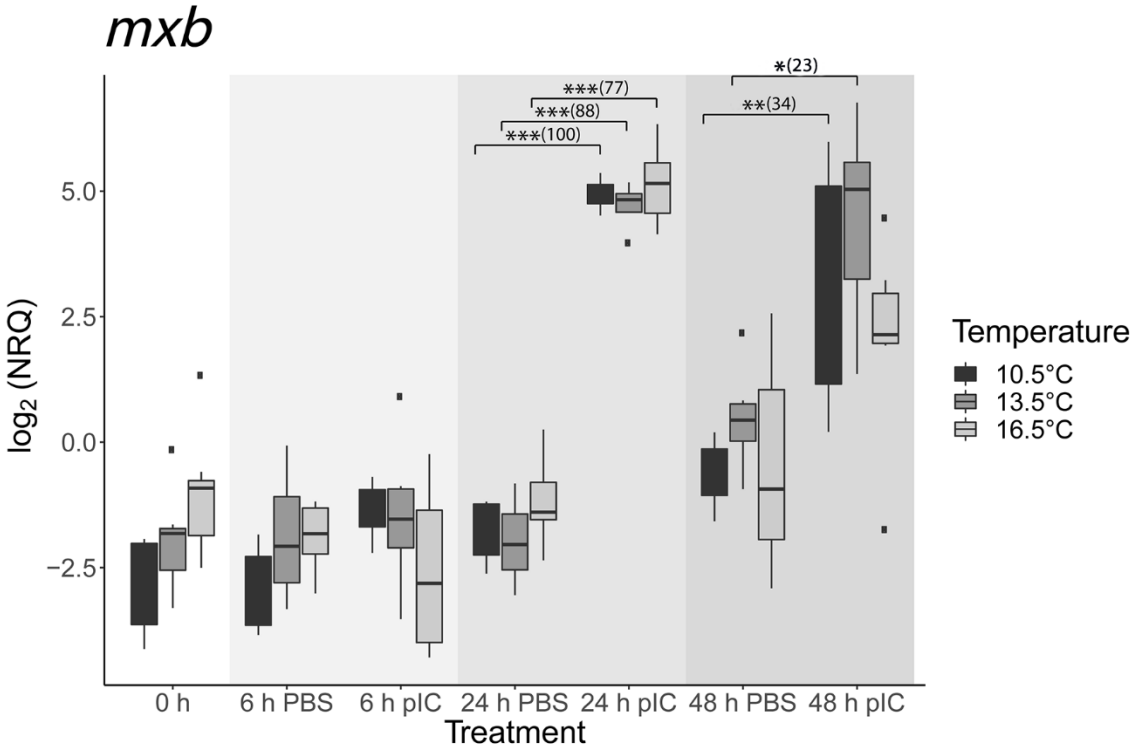

Figure 3-8. Effect of rearing temperature on interferon-induced GTP-binding protein of myxovirus resistance b (*mxb*) transcript levels following immunostimulant (pIC) injection. Significant differences by two-way ANOVA and subsequent post-hoc tests between PBS controls and pIC-injected fish at a given temperature and hours (h) post-injection are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Numbers in parentheses next to asterisks denote mean fold-change values between time- and temperature-matched pIC and PBS treatments.

3.4.2 Plasma cortisol concentration in response to pIC injection

Plasma cortisol concentrations of non-injected fish at 0 h alongside PBS- and pIC-injected fish at 24 hpi are shown in Figure 3-9. Plasma cortisol averaged (± SE) 1.60 ± 0.40, 8.67 ± 4.01 and 5.67 ± 3.96 ng mL^{-1} at 10.5°C, 13.5°C and 16.5°C, respectively, in non-injected fish. In PBS-injected fish, concentrations averaged 1.67 ± 0.92, 2.50 ± 1.50 and 4.33 ± 0.71 ng mL^{-1} at 10.5°C, 13.5°C and 16.5°C, respectively. Furthermore, in pIC-injected fish, plasma cortisol levels were 9.70 ± 2.32, 2.75 ± 1.33 and 8.67 ± 3.12 ng mL^{-1} at 10.5°C, 13.5°C and 16.5°C, respectively. No significant differences were found for comparisons of rearing temperature, treatment, or time point by 3-way, 2-way or 1-way ANOVA.

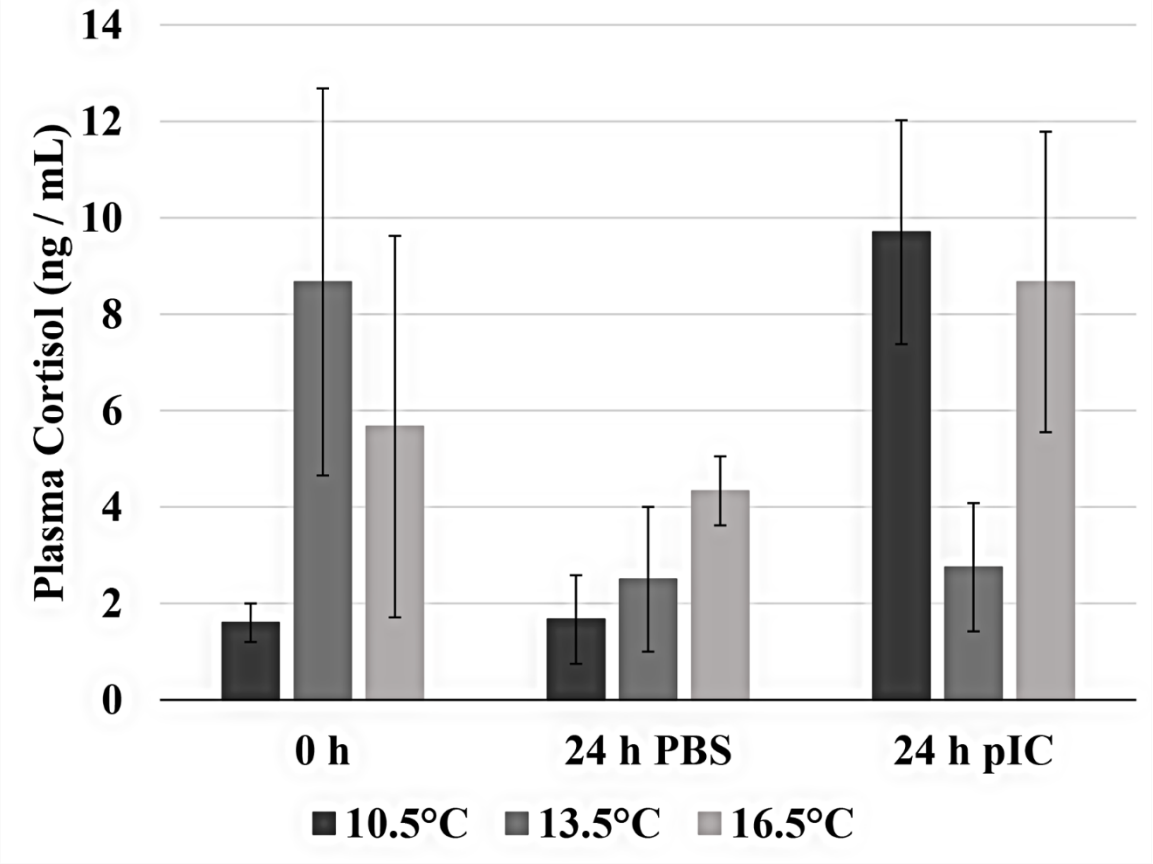

Figure 3-9. Effect of rearing temperature on plasma cortisol concentrations before and after immunostimulant (pIC) injection. Error bars denote standard error.

3.4.3 Multivariate analysis

Hierarchical clustering with input from all samples segregated target genes into two major clusters (Figure 3-10). The first cluster (Cluster I) was comprised of *stat1b, lgp2, ifng* and *tlr3*, representing pattern recognition, signal transduction and transcription, and cytokine aspects of the IFN pathway. The second cluster (Cluster II) consisted of *mxb, rsad2* and *isg15a*, which are all classified as immune effectors. Clustering based on individual fish split into 4 major clusters (Figure 3-10). Cluster 1 consisted of 63.6% pIC-injected fish at 24 hpi and 36.4% 48 hpi pIC-injected fish. The second cluster was comprised of 5.0% non-injected salmon, 15.0% pIC-injected salmon at 6 hpi, 45.0% PBS-injected salmon at 48 hpi and 35.0% pIC-injected salmon at 48 hpi. Cluster 3 contained 38.5% non-injected fish, 38.5% PBS-injected fish at 6 hpi and 23.0% pIC-injected fish at 6 hpi. The fourth cluster included 34.0% non-injected salmon, 17.0% pIC-injected salmon at 6 hpi, 27.7% PBS-injected salmon at 24 hpi, 17.0% PBS-injected salmon at 48 hpi and 4.3% pIC-injected salmon at 48 hpi. When examining the rearing temperature of these fish, a similar distribution across clusters 3 and 4 was found. However, cluster 1 was comprised of 21.7% fish reared at 10.5°C, 34.8% fish reared at 13.5°C and 43.5% fish reared at 16.5°C, while cluster 2 contained 45.0% salmon at 10.5°C, 35.0% salmon at 13.5°C and 20.0% salmon at 16.5°C.

The degree of correlation among the expression profiles of all seven target genes along with plasma cortisol results and phenotypic measurements such as condition factor (k), viscerosomatic index (VSI) and hepatosomatic index (HSI) in pIC-injected fish at 24 hpi are shown in Figure 3-11A. A significant positive correlation was found between *mxb, lgp2, rsad2, stat1b* and *tlr3* genes and also between *ifng, isg15a, lgp2* and *rsad2*. No significant correlations were found between plasma cortisol concentrations or any of the phenotypic measurements with

any of the target genes. The PCoA in Figure 3-11B illustrates comparable results where all the antiviral biomarker genes' eigenvectors had the greatest influence on PCO1, while cortisol, HSI and k contributed more so to PCO2. The PCoA diagram also revealed that samples clustered by rearing temperature. Sample numbers were uneven at this time point; two of the 10.5°C group samples and one 13.5°C sample were removed from the study as they did not meet RNA purification quality standards. As previously stated, a second fish from the 13.5°C group was removed as it was identified as an outlier.

SIMPER analysis using that same dataset at 24 hpi in pIC-injected salmon revealed there was a 39.10% average dissimilarity between the 10.5°C and 13.5°C temperature groups with, in degree of decreasing magnitude, *tlr3*, *lgp2*, *rsad2*, *stat1b*, VSI, *isg15a* and HSI contributing the most to the differences (Appendix III). Comparing the 13.5°C and 16.5°C groups, *stat1b*, k, *tlr3*, VSI, *ifng*, *mxb* and HSI made the greatest contributions to the 42.93% average dissimilarity. Lastly, *ifng*, *isg15a*, VSI, *rsad2*, *lgp2*, *tlr3* and k were found to make the greatest contributions to the 35.83% average dissimilarity observed between the 10.5°C and 16.5°C temperature groups. PERMANOVA analysis further showed a significant ($p < 0.05$) difference in every pairwise comparison between rearing temperatures.

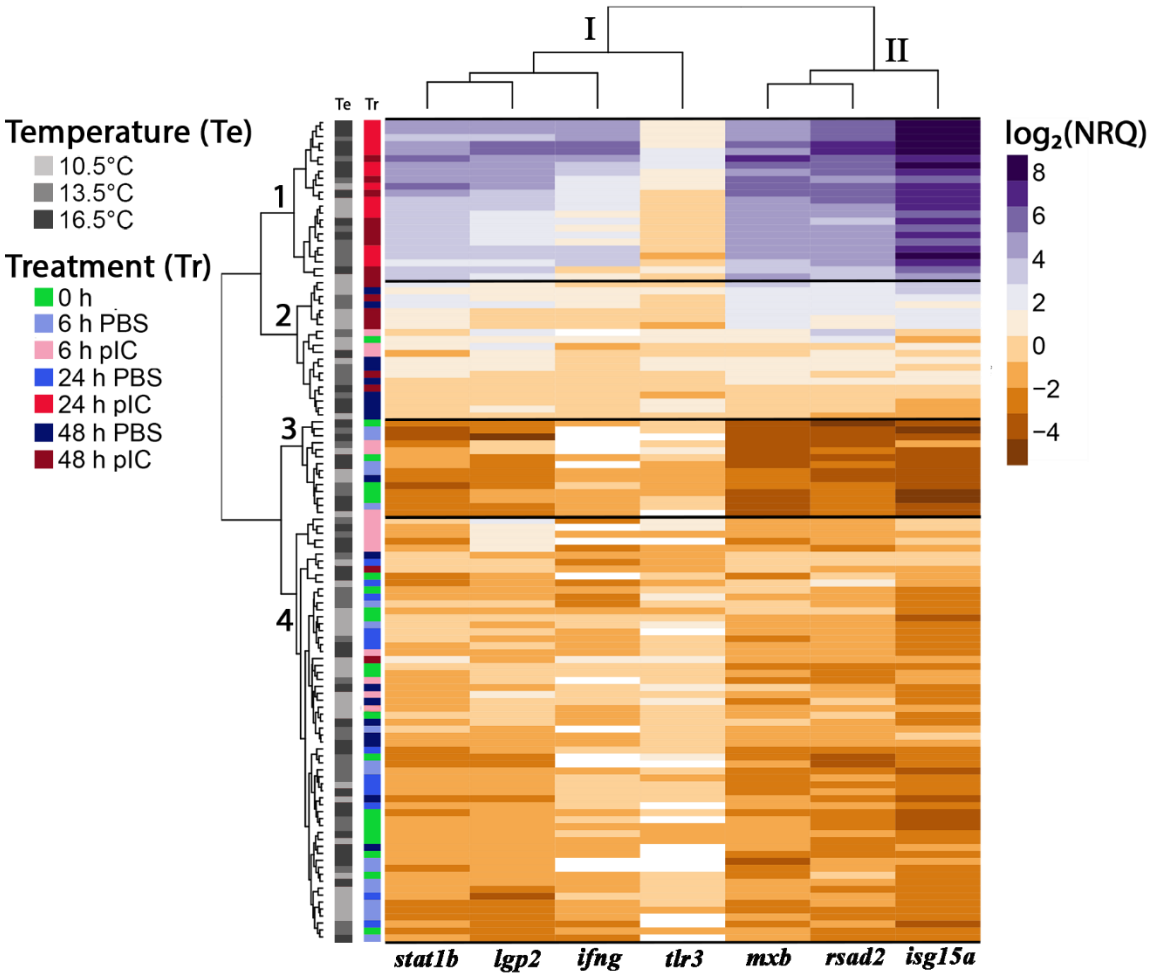

Figure 3-10. Hierarchical clustering of $\log_2$ Normalized Relative Quantities (NRQs) for targeted immune markers. Gene names are labeled on the base of the figure, while hierarchical clustering of the columns is based on individual samples, with rearing temperature and injection treatment denoted by colour along the vertical axis.

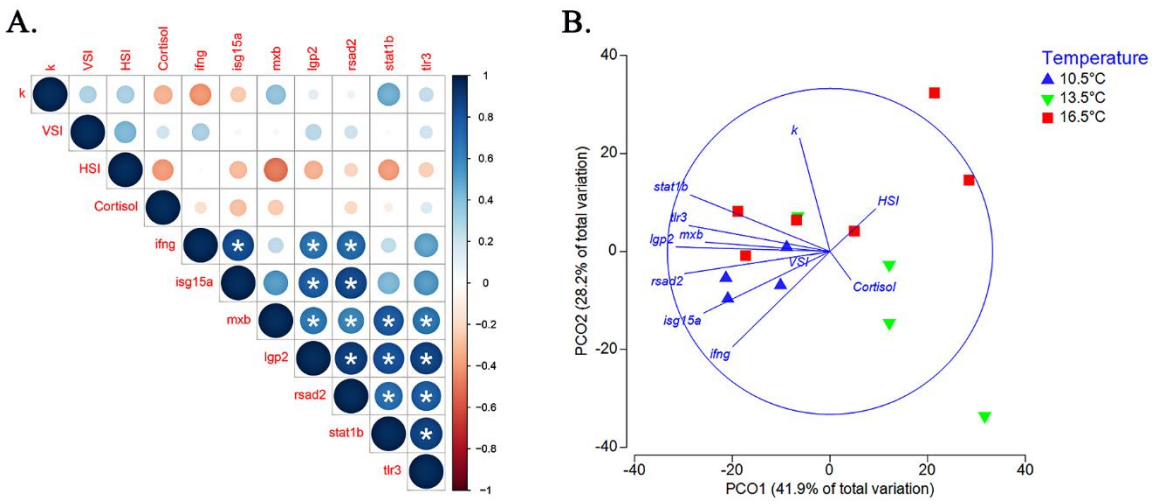

Figure 3-11. A) Correlogram of expression profiles for all genes of interest alongside plasma cortisol results and phenotypic measurement data amongst all fish injected with pIC and sampled 24 hpi. The coloured scale shows degree of correlation ranging from r = -1.00 (red) to r = 1.00 (blue). The size of the coloured circle indicates its significance level and circles containing asterisks are significant at $p < 0.05$. B) Principal coordinates analysis (PCoA) of same multivariate dataset from fish injected with pIC and sampled 24 hpi illustrating distinction between rearing temperatures in their phenotypic characteristics and immune response.

3.5 Discussion

The purpose of this experiment was to investigate the immune and stress responses of AAS when reared at different temperatures after injection with a viral mimic. The results of this study showed that temperature significantly influenced target antiviral biomarker gene expression. As a PRR, *tlr3* is responsible for recognizing dsRNA and inducing type I interferon (IFN) production (Akira et al., 2006), resulting in viral replication inhibition (Noppert et al., 2007). Only within the 10.5°C group was *tlr3* activation observed in the current study. Temperature-mediated TLR responses during viral infection have been described for olive flounder (*Paralichthys olivaceus*) reared at 15°C or 20°C, with disruption of *tlr2* and *tlr7* coinciding with higher mortality and significant upregulation of *lgp2, isg15* and *mx* at the lower temperature (Avunje et al., 2013). No differences were found between temperature treatments in *tlr3* expression (Avunje et al., 2013), which is not reflected in the results of the current study which suggests further study of *tlr3*-mechanisms in antiviral response is required. Retinoic acid-inducible gene I-like receptors (RLRs), including *lgp2*, are known to play an important role in fish antiviral immunity (Chang et al., 2011). In a rainbow trout cell line (RTG-2), exposure to synthetic or viral dsRNA induced *lgp2* and *mx* expression (Chang et al., 2011). This has also been demonstrated after exposure to VHSV in olive flounder where significant upregulation of *lgp2*, *mx* and *isg15* was associated with delayed cytopathic effects (Ohtani et al., 2010). Furthermore, Signal Transducer and Activator of Transcription (STAT) family molecules are responsible for signalling type I and II IFNs. In Atlantic salmon, *stat1* has been characterized and found to have a response to viral infection similar to that of mammalian orthologues (Collet et al., 2008; Skjesol et al., 2010, 2014; Collins et al., 2014). Multiple paralogues exist for *stat1*, *isg15* and *mxb*, but all were shown responsive to pIC in Atlantic salmon previously (Caballero-

Solares et al., 2017). Selection of paralogues for the current study was made based on which version formerly demonstrated the most consistent, highest fold-change differences between pIC- and PBS-injected salmon (Caballero-Solares et al., 2017). The differential *lgp2* and *stat1b* expression in the current study could impact the effectiveness of viral recognition and consequent cell signalling between temperature treatments.

The IFN signalling pathway activates the expression of interferon-stimulated genes (ISGs), such as *isg15a*, *rsad2*, and *mxb*, whose function is to limit viral replication and infectivity (Poynter and DeWitte-Orr, 2016). These are well characterized in teleosts and appear to share function and homology with mammalian counterparts (Poynter and DeWitte-Orr, 2016). For example, in mammals, ISG15 has cytokine activity and binds to intracellular proteins (Bogunovic et al., 2013). ISG15 may have a similar function in Atlantic salmon, as it was found in the supernatants of pIC-injected salmon leucocytes (Røkenes et al., 2007). From IFN signaling, ISG15 is one of the most predominant proteins produced (Røkenes et al., 2007); this explains why fold-change differences between PBS- and pIC-injected fish in the current trial were considerably higher than other target genes. As another ISG, *rsad2* is capable of using a variety of both direct and indirect antiviral mechanisms to impact viral replication (Helbig and Beard, 2014). In Atlantic cod isolated macrophages, *rsad2* expression after pIC induction was inhibited by 2-Aminopurine, Chloroquine, SB202190, and Ruxolitinib, signifying that TLR-recognition and signal transducers downstream of the TLR pathway may activate the expression of *rsad2* (Eslamloo et al., 2019).

In a rainbow trout cell line exposed to VHSV as well as low and high molecular weight pIC, comparing the latter two treatments, *mx1* and *mx2* were more highly expressed with the high molecular weight pIC (Poynter and DeWitte-Orr, 2015). This suggested that induction rates

of ISGs are pIC length-dependent, and longer dsRNA molecules induce a more robust response (Poynter and DeWitte-Orr, 2015). The pIC in the current trial was ~200 bp, about the same length as the low molecular weight pIC used in the aforementioned study (Poynter and DeWitte-Orr, 2015). Response in Mx protein expression lasts for at least 4 days after injection with pIC in Atlantic cod spleen, kidney, liver and gill (Das et al., 2008). In contrast, the results of the current study show pIC versus PBS fold-changes of *mxb* decreasing by 48 hpi compared to 24 hpi in salmon reared at 10.5°C and 13.5°C, and levels returning to baseline by 48 hpi at 16.5°C. Furthermore, expression of *mx* in liver and kidney tissue increases during female sexual maturation in Atlantic salmon (Fourrier et al., 2017), but this is unlikely to have affected results in this study as the fish were triploid females, avoiding the development of ova. IP injection of 500 µg pIC/fish in Atlantic salmon parr, post-smolts and adults reared at 11°C showed that expression of *mxb* in the liver significantly increased by 24 hpi, peaked at 72 hpi and decreased to undetectable levels a week after injection (Lockhart et al., 2004). The results of that study suggest that *mx* response may be similar regardless of growth stage or dosage of pIC (Lockhart et al., 2004). Temperature significantly influences *mx* expression in juvenile sevenband grouper (*Epinephelus septemfasciatus*) after intramuscular injection of 100 µg pIC, where peak transcript levels were detected at 3 hpi in fish reared at 25°C and 30°C, but not until 24 hpi in fish at 15°C and 20°C (Thanasaksiri et al., 2014). In the current study, no differences were detected amongst temperature groups in terms of transcript abundance of *mxb* at any time point.

In fish, IFNG induces ISGs (Gan et al., 2019). Structure of exon and intron regions of *ifng* in teleosts is similar to that of mammals, and functional characterization in a rainbow trout homologue suggests *ifng* plays a similar role in innate immune cell responses (Zou et al., 2005; Robertsen, 2006). This is evidenced in a study exposing an Atlantic salmon cell line (TO) to

ISAV and SAV, where upregulation of *ifng* induced several interferon regulatory transcription factors (IRFs) and cytokines, as well as other immune effectors (Sun et al., 2011). There was a significant correlation between responses in *ifng* and *isg15a* in the current study, as similar differences between expression at 10.5°C and 16.5°C are evident in both genes.

The effect on pIC on the antiviral immune response of finfish aquaculture species is well documented (Rise et al., 2008; Feng and Rise, 2011; Fierro-Castro et al., 2013). In a study where farmed diploid Atlantic salmon were reared at ~12°C and fed diets containing different protein and oil sources, 24 hpi pIC-injected fish demonstrated a response in *tlr3* head kidney expression, with similar low fold-change values reported to those found at 10.5°C in the current study (Caballero-Solares et al., 2017). Higher fold-changes were consistently found at 10.5°C in the present study than those reported in *lgp2*, *stat1b*, *mxb*, *rsad2*, *isg15a* and *ifng* amongst any of the dietary treatments from Caballero-Solares et al. (2017). Fish reared at 13.5°C and 16.5°C in the current study exhibited higher fold-changes than those previously reported across most of the target transcripts as well, however pIC was injected at a 9.36 μg/g concentration in the current trial compared to only 2 μg/g in the dietary experiment (Caballero-Solares et al., 2017) which might explain these differences. In addition, temperature impacts the spleen transcriptome in response to pIC in Atlantic cod held at 10°C and 16°C (Hori et al., 2012a). Unlike in the current study which examined gene expression in head kidney tissue, no response was found in *tlr3* in Atlantic cod at either 10°C or 16°C at 6 or 24 hpi (Hori et al., 2012a). A significantly higher response was found at 10°C compared to the higher temperature for *lgp2* (Hori et al., 2012a), which was also identified in the present experiment. In Atlantic cod, a difference was found between the two temperatures in response to *stat1* (Hori et al., 2012a), but no difference was found between the 10.5°C and 16.5°C temperatures in the present study. Differences between

PBS- and pIC-injected treatments at 6 hpi were reported as significant in *stat1*, *isg15*, and *rsad2* (Hori et al., 2012a), which were effects not seen in the current study. At 24 hpi, response to pIC differed between cod reared at 10°C and 16°C in *rsad2*, but not *isg15* (Hori et al., 2012a), but the opposite was found in the current 10.5°C and 16.5°C treatments. Microarray results showed that a much smaller number of differentially expressed genes were found between 10°C and 16°C rearing temperatures when Atlantic cod were injected with formalin-killed *Aeromonas salmonicida* compared with pIC (Hori et al., 2012a, 2013). This highlights that temperature's effect on immune response may be pathogen-dependent, with a larger effect seen in viral infection than in bacterial infection (Hori et al., 2013).

Further, studies have shown that Atlantic cod head kidney macrophages are responsive to pIC, as indicated by time-dependent qPCR assays to validate microarray and deep sequencing results, demonstrating that the same antiviral biomarker genes tested in the current study and specific microRNAs are relevant at the immune cell level (Eslamloo et al., 2016, 2018). In olive flounder, higher temperature (25°C) induces an earlier response to intramuscular injection of pIC than in fish reared at 15°C with evidence of higher expression of type I interferon-related genes in the spleen at 3 hpi compared to 24 hpi (Thanasaksiri et al., 2015). A similar trend was not seen in the present trial, but this pattern does coincide with other research (Hori et al., 2012a; Inkpen et al., 2015). As the results of the PERMANOVA analyses for the current trail demonstrate, temperature plays a role in mediating aspects of AAS immune and stress response to a viral mimic. More research is required to better characterize the impact temperature has on these fish, as this study is limited in scope to examining only seven antiviral biomarker genes. It is not possible to say with certainty that the higher fold-change values seen at 10.5°C compared to 16.5°C in *isg15a*, *rsad2* and *ifng* are truly indicative of a higher overall immune response in

those genes, or whether transcript levels peaked earlier at the elevated temperature. The fact that expression levels returned to baseline across all target genes in the 16.5°C group at 48 hpi, where some differences were still detected between pIC- and PBS-injected treatments at the lower two temperatures, might suggest fish reared at 16.5°C experienced an accelerated immune response. Further investigation is necessary to confirm this hypothesis.

In viral pathogen challenges, similar impacts on the IFN signalling cascade are noted and these effects are generally conserved across vertebrates (LeBlanc et al., 2010; Verrier et al., 2011; Gong et al., 2019). During ISAV infection in Atlantic salmon, upregulation of several protein degradation genes has been observed alongside the increased prevalence of antiviral gene expression, suggesting reallocation of amino acid reserves and disruption of regular metabolic pathways (Heidari et al., 2015). A total of 117 viral responsive genes have previously been identified in Atlantic salmon, as assessed by their rapid induction, low tissue specificity and positive correlation with viral load in response to a variety of viral challenges (Krasnov et al., 2011). In addition, as several studies have shown that temperature's impact on virulence and host immunity is virus specific (Xu et al., 2011; Binesh, 2014; Souto et al., 2015; Vo et al., 2015), future research could focus on measuring the immune and stress response of AAS during a live pathogen challenge to gain more information on whether rearing temperature affects survival and the pathogenesis of targeted viruses of interest.

Cortisol plays an important role in the innate immune response by modulating complement and lysozyme activity in plasma and helping regulate immune-related gene expression in the liver of rainbow trout (Cortés et al., 2013). Similar to earlier work in Atlantic cod (Hori et al., 2012a), neither rearing temperature nor injection treatment influenced plasma cortisol concentrations in the current study. However, the mean cortisol levels observed in this

present study were all considerably lower than what was reported in diploid Atlantic cod exposed to similar conditions (Hori et al., 2012a), but that may be attributable to their different phylogeny when comparing a marine pelagic species such as Atlantic cod with an anadromous species such as Atlantic salmon. In contrast, Pérez-Casanova et al. (2008) found a 2.9-fold increase in plasma cortisol at 16°C compared to the control group held at 10°C with diploid Atlantic cod, but no differences were seen in comparison of the control and treatments above or below 16°C. Further research with diploid Atlantic cod has found significant negative correlations between high cortisol responsiveness to handling and heat stress and growth rate (Hori et al., 2012b). Triploids and diploids demonstrated a similar plasma cortisol response following acute handling and confinement stress in brook charr (*Salvelinus fontinalis*) and rainbow trout, showing the same initial rapid increase within minutes, followed by a gradual decrease toward resting levels after ~2 hours post-stress (Biron and Benfey, 1994; Benfey and Biron, 2000). In Atlantic salmon, no differences in plasma cortisol were detected between triploids and diploids after confinement stress in either freshwater parr or saltwater smolts (Sadler et al., 2000). These results suggest that triploidy does not affect cortisol responsiveness in salmonids (Biron and Benfey, 1994; Benfey and Biron, 2000; Sadler et al., 2000). In a study involving diploid Atlantic salmon exposed to either short- or long-term handling stress, total plasma cortisol only differed from controls at one and three hours after short-term handling (Fast et al., 2008). The results of that experiment also demonstrate that unstressed salmon typically have total plasma cortisol concentrations below 10 ng mL^{-1} (Fast et al., 2008), a threshold at which most fish in the current study were below. No differences in plasma cortisol levels were found among injection treatments or rearing temperatures in the present trial; therefore, it remains unknown what role plasma cortisol plays in relation to the antiviral response of AAS. Future studies could include

more time point assessments for cortisol, as the current study is limited to only non-injected fish at 0 h and injected fish at 24 hpi.

3.6 Conclusions

The results of this study demonstrate that temperature influences the immune response of AAS after IP injection with pIC. Aside from *tlr3* (only activated at 24 hpi in the 10.5°C group) and *lgp2* (activated by 6 hpi at 10.5°C, and by 24 hpi in the 13.5°C and 16.5°C groups), all other antiviral biomarker genes targeted in this study were first upregulated at 24 hpi, followed by a decline in transcript expression at 48 hpi amongst all temperature groups. At 24 hpi, rearing temperature influenced transcript abundance and/or fold-change comparing time-matched pIC- and PBS-injected fish in *tlr3*, *lgp2*, *stat1b*, *isg15a*, *rsad2* and *ifng*. However, there was no effect of temperature or injection treatment on cortisol response. Collectively, these results suggest that AAS at 10.5°C exhibit a more robust antiviral response compared to the other two temperature groups. However, it is possible that higher rearing temperature could have accelerated AAS responses that were not captured by the sampling time points used in this study. This is the first report on the immune and stress response of AAS following IP injection of a viral mimic involving temperature and provides a new foundation for future studies to expand upon.

3.7 References

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CHAPTER 4: Summary

4.1 Summary of research findings

Research is currently limited regarding nutrition and growth performance of AquaBounty's salmon, but past studies highlight their unique characteristics. For example, mixed-sex juvenile growth hormone (GH) transgenic (TG) triploid Atlantic salmon reach target weight in approximately 40% of the time it takes their non-transgenic (NTG) siblings, exhibit lower feed conversion ratios (FCRs) and they are more efficient in retaining dietary nitrogen when reared at 10°C in a flow-through system (Tibbetts et al., 2013). Additionally, these fish can maintain their enhanced growth performance when fed a diet with high amounts of plant protein (substituting fish meal) in a recirculating aquaculture system (RAS) (Ganga et al., 2015). Family-specific differences in GH TG triploid salmon growth rate and hepatic transcriptomes have also been described, highlighting the need to carefully manage breeding programs for their production (Xu et al., 2013). Interestingly, previous research has not investigated rearing temperature's effect on AAS growth performance or ability to utilize dietary nutrients. The current research is the first to specifically study AquAdvantage Salmon (AAS), as all fish from the current experiments were GH TG triploid females as opposed to mixed-sex populations in the aforementioned trials. This is relevant because only AAS are currently approved for commercial production and sale in the United States and Canada.

The objective of this research was to investigate the impact of rearing temperature on the growth performance, nutrient utilization and antiviral immune response of AAS. Over the course of a 17-month grow-out trial where AAS were reared from first-feeding fry up to 1500 g in a freshwater RAS at 10.5°C, 13.5°C or 16.5°C, frequent assessments and samplings aided in the determination of differences in growth rate and nutrient retention among treatments. As expected, based on past research in conventional Atlantic salmon (Johnston and Saunders, 1981;

Koskela et al., 1997; Imsland et al., 2014), AAS reared at 16.5°C reached 1500 g in a significantly shorter amount of time compared to the other two temperature groups. However, comparing thermal-unit growth coefficients (TGCs), salmon at 10.5°C exhibited greater growth rates than those reared at 16.5°C for the majority of the experiment. Fish reared at 16.5°C also directed more fat deposition towards their viscera instead of fillet tissue, as evidenced by their higher viscerosomatic index (VSI) and condition factor (k) scores, compared to AAS reared at 10.5°C or 13.5°C. Nutrient deposition rates and/or retention efficiencies were significantly lower at 16.5°C for protein, lipid and omega-3 (ω3) fatty acids, among other nutrients, especially compared to the 10.5°C treatment. Additionally, FCRs were higher at 16.5°C in comparison to fish reared at 10.5°C, further emphasizing that elevated temperature negatively affects nutrient utilization as fish at 16.5°C required more feed to gain the same amount of body weight when compared to fish reared at 10.5°C. Lastly, among the three temperature treatments, fillet yields were lowest among AAS reared at 16.5°C, and fillet colour was also lighter at this elevated temperature compared with fish reared at 13.5°C. The results of this research suggest that AAS should be reared at less than 16.5°C, as either 13.5°C or 10.5°C would be preferable during their full commercial production cycle.

Furthermore, the current research examined the antiviral immune and stress responses of AAS reared at 10.5°C, 13.5°C and 16.5°C. The use of pIC to study the antiviral immune response of finfish aquaculture species is well documented (Rise et al., 2008; Feng and Rise, 2011; Fierro-Castro et al., 2013; Caballero-Solares et al., 2017) and has previously been used in conjunction with differing rearing temperatures in Atlantic cod (*Gadus morhua*) (Hori et al., 2012). In the current study, real-time quantitative polymerase chain reaction (qPCR) analysis of RNA derived from head kidney samples collected at various time points post-injection revealed

that rearing temperature significantly influenced targeted gene expression. Target genes were chosen to capture different aspects of the interferon (IFN) pathway to provide an overview of antiviral response: pattern recognition (*tlr3, lgp2*), signal transduction and transcription (*stat1b*), immune effectors (*isg15a, rsad2, mxb*) and a cytokine (*ifng*). Aside from *tlr3* [only activated at 24 h post-injection (hpi) in the 10.5°C group] and *lgp2* (activated by 6 hpi at 10.5°C, and by 24 hpi in the 13.5°C and 16.5°C groups), all other antiviral biomarker genes targeted in this study were first upregulated at 24 hpi, followed by a decline in transcript expression at 48 hpi amongst all temperature groups. At 24 hpi, rearing temperature affected transcript abundance and/or fold-change comparing time-matched pIC- and PBS-injected fish in *tlr3, lgp2, stat1b, isg15a, rsad2* and *ifng*. There was significantly higher induction of *isg15a*, *rsad2* and *ifng* in fish reared at 10.5°C compared to 16.5°C. Higher fold-changes at 10.5°C were also found in *lgp2*, *stat1b*, *isg15a*, *rsad2* and *ifng* compared to fish reared at 13.5°C at 24 hpi. Collectively these results suggest that AAS reared at 10.5°C exhibit a more robust antiviral response compared to the other two temperature groups. However, it is possible that higher rearing temperature could have accelerated AAS responses, but they were not captured at the sampling time points used in this study.

Plasma cortisol was also quantified in non-injected fish and PBS- and pIC-injected fish at 24 hpi by enzyme-linked immunosorbent assay (ELISA) analysis, however, no effect of rearing temperature or injection treatment was detected. Overall, the results of this study are comparable to previously reported research where temperature also influenced similar antiviral gene expression in aquaculture species (Avunje et al., 2012; Hori et al., 2012; Hwang et al., 2018; Jeong et al., 2018) and where gradual, incremental temperature increase (10°C to 16°C over ~ 5

weeks) did not significantly affect plasma cortisol concentrations in Atlantic cod (Hori et al. 2012).

In summary, this thesis reports novel findings on AAS growth performance, nutrient utilization, and antiviral gene expression. As AAS reared at 16.5°C used more feed per unit of biomass gain and were less efficient at retaining commercially relevant feed ingredients, such as ω3 fatty acids and astaxanthin pigment, than their counterparts at 10.5 and 13.5°C, this temperature is not recommended for the duration of their production. Furthermore, it appeared that AAS reared at 16.5°C exhibited an attenuated antiviral immune response compared to the 10.5°C group. The information gathered from this thesis will be useful for the ongoing development and commercial production of AAS.

4.2 Future research

Much remains unknown regarding AAS and the differences between them and conventional diploid Atlantic salmon. This thesis did not include any NTG counterparts for comparison, but it would be interesting to investigate if GH transgenesis impacts response to pIC in Atlantic salmon. In GH TG diploid coho salmon (*Oncorhynchus kisutch*), a reduced response to pIC was noted in muscle tissue when compared to wild-type coho (Alzaid et al., 2018). However, NTG triploid Atlantic salmon have exhibited signs of enhanced performance during viral infection compared to diploids (Herath et al., 2017; Moore et al., 2017). Therefore, it is unknown what the individual versus combined effects of both triploidy and GH transgenesis would have on AAS antiviral response.

Similarly, the immune response during a live pathogen challenge in AAS is currently unknown. For example, infecting fish reared at 10.5°C, 13.5°C and 16.5°C with infectious

salmon anaemia virus (ISAV), an industrially relevant pathogen, would be an interesting comparison to the pIC data collected in this thesis to determine if responses are similar between studies. Temperature would play an interesting role as it is known to influence both host responses as well as virulence in other viruses (Xu et al., 2011; Binesh, 2014; Souto et al., 2015). In addition, the research described in this thesis could be repeated and improved upon in order to gain more knowledge on AAS antiviral response. For example, additional sampling time points (e.g. 12 and 18 hpi) would aid in the determination of whether maximum transcript expression following pIC stimulation was reduced at 16.5°C, or whether it was accelerated and peaked earlier than at 10.5°C. Analysis of plasma cortisol at additional time points would be useful shortly after injection to ascertain whether differences exist among rearing temperatures.

Additionally, future research focusing on tailoring a diet towards AAS would likely improve their growth and health. Previous research has suggested that feeding diploid Atlantic salmon a high-protein diet will increase condition factor, feed efficiency and slaughter yield, while decreasing muscle fat and VSI (Dessen et al., 2017; Weihe et al., 2019). Testing a high protein diet with AAS at elevated rearing temperatures could aid in the reduction of the amount of lipid deposited in the viscera, subsequently improving their use of dietary lipid through greater deposition in the fillet. As the dietary protein-to-lipid ratio was a significant predictor of body lipid content in the current research, further investigation of this factor is warranted with cost-benefit analysis conducted to ensure the cost of a higher protein diet does not exceed production benefits. Furthermore, methionine deposition rates decreased during the final stage of the trial (i.e., dropping below zero) suggesting that fish reared at 13.5°C and 16.5°C mobilized dietary methionine faster than it could be assimilated, as it was either being poorly digested and/or was deficient in the diet. Previous research has shown that increasing crystalline DL-methionine by

approximately 17% in the diet can improve methionine intake and apparent energy digestibility in post-smolt diploid Atlantic salmon (Espe et al., 2011). Collectively, these results suggest that AAS reared at temperatures above 10.5°C may require additional methionine in their diet when they reach 800 g. Increasing the amount of methionine by 17%, from 0.51% to 0.60% (as-fed), in 6.0 mm feed (Appendix I) as fish reach 500 g is therefore recommended. Furthermore, current results suggest that diets developed for AAS should be tailored based on both weight stage and rearing temperature. As well, the potential to reduce astaxanthin levels could be beneficial when developing a diet for AAS. As reported in Chapter 2, average DSM SalmoFan™ scores of fillets of 1500 g fish, regardless of rearing temperature, were higher than Norwegian farmed salmon (Alfnes et al., 2006). This could equate to substantial savings for the producer as astaxanthin is an expensive and necessary feed ingredient (Solymosi et al., 2015).

Overall, there are numerous avenues for future research on AAS. The research summarized in this thesis provides a foundation upon which new studies could expand. Research into maximizing growth performance and understanding immune response will continue to benefit the production of AAS.

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APPENDICES

Appendix I. Description and nutritional composition of all commercial diets fed to each experimental group throughout the trial.

	Commercial Diet	Nutra ST 0.3 mm	Nutra XP 0.5 mm	Nutra XP 0.7 mm	Nutra XP 1.0 mm	Nutra XP 1.2 mm	Nutra Fry NP 1.8 mm	Nutra Fry NP 2.3 mm	Nutra RC NP 3.0 mm	Nutra RC 200 SP 4.0 mm	Optiline Summer 500 EP 6.0 mm
	Fish Weight Range (g)	First Feeding Fry - 0.5	0.5 - 2	2 - 3	3 - 5	5 - 10	10 - 25	25 - 50	50 - 200	200 - 500	500 - 1500
	% Dry Matter	94.78	93.52	93.25	92.00	92.25	93.28	93.75	96.22	96.41	95.58
Macronutrients (% As-Fed)	Crude Protein	58.58	54.84	56.40	54.89	53.84	54.92	53.90	55.12	55.12	47.67
	Ash	10.39	9.54	9.38	7.41	7.89	8.57	8.31	7.71	7.35	6.91
	Crude Lipid	17.20	15.32	15.90	14.95	15.64	16.57	16.85	19.61	19.02	25.82
Fatty Acids (% of Total Fatty Acids)	12:0	0.07	0.09	0.11	0.09	0.10	0.10	0.10	0.09	0.08	0.07
	14:0	4.65	6.04	6.20	5.78	6.25	4.21	4.08	3.75	2.82	2.45
	15:0	0.38	0.39	0.43	0.39	0.39	0.33	0.32	0.35	0.19	0.18
	16:0	18.51	18.90	18.54	19.30	17.94	19.09	18.84	18.01	16.07	16.25
	17:0	0.41	0.41	0.42	0.40	0.40	0.39	0.36	0.41	0.26	0.23
	18:0	3.81	3.75	3.55	3.72	3.53	4.44	4.45	4.14	4.53	4.63
	20:0	0.35	0.37	0.37	0.35	0.34	0.28	0.28	0.32	0.42	0.43
	21:0	0.09	0.09	0.08	0.09	0.08	0.09	0.09	0.09	0.06	0.05
	22:0	0.18	0.14	0.14	0.13	0.10	0.16	0.17	0.20	0.74	1.00
	24:0	0.13	0.11	0.11	0.12	0.10	0.12	0.11	0.17	0.13	0.13
	14:1n-7	0.05	0.05	0.06	0.04	0.03	0.09	0.10	0.08	0.09	0.12
	14:1n-5	0.13	0.14	0.15	0.14	0.13	0.14	0.13	0.12	0.07	0.06
	15:1n-5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Fatty Acids (% of Total Fatty Acids)	16:1n-9	0.24	0.27	0.27	0.27	0.24	0.26	0.26	0.25	0.26	0.29
	16:1n-7	5.42	6.70	6.84	6.44	7.11	6.26	6.42	6.60	4.71	5.89
	16:1n-5	0.15	0.17	0.18	0.15	0.20	0.14	0.13	0.12	0.09	0.09
	17:1	0.05	0.05	0.05	0.05	0.06	0.04	0.04	0.04	0.05	0.04
	18:1n-9	13.52	12.23	11.38	12.75	10.36	17.57	18.20	17.37	30.19	32.41
	18:1n-7	2.94	3.33	3.16	3.14	2.94	2.51	2.48	2.28	2.36	2.44
	18:1n-5	0.16	0.17	0.18	0.12	0.19	0.21	0.22	0.20	0.12	0.12
	20:1n-11	0.31	0.34	0.40	0.20	0.28	0.44	0.56	0.50	0.36	0.34
	20:1n-9	1.62	1.95	2.27	1.13	2.85	3.58	4.38	4.50	2.89	2.69
	20:1n-7	0.20	0.23	0.23	0.19	0.18	0.31	0.35	0.43	0.11	0.20
	20:1n-5	0.03	0.03	0.03	0.03	0.03	0.00	0.03	0.04	0.04	0.03
	22:1n-11+13	1.79	2.19	2.96	1.19	3.59	5.52	6.80	6.63	4.89	3.73
	22:1n-9	0.24	0.30	0.31	0.18	0.29	0.61	0.74	0.83	0.29	0.48
	22:1n-7	0.06	0.10	0.11	0.08	0.09	0.14	0.17	0.18	0.06	0.09
	24:1n-9	0.51	0.52	0.54	0.43	0.47	0.46	0.49	0.42	0.31	0.20
	24:1n-7	0.06	0.07	0.07	0.06	0.04	0.05	0.06	0.06	0.01	0.01
	16:2n-6	0.34	0.38	0.39	0.38	0.36	0.25	0.22	0.23	0.11	0.09
	16:2n-4	0.66	0.90	0.87	0.89	0.95	0.49	0.50	0.48	0.31	0.32
	18:2n-9	0.28	0.38	0.32	0.36	0.29	0.08	0.08	0.07	0.05	0.04
	18:2n-6 cis	12.11	6.03	4.83	7.68	7.06	9.21	7.78	10.09	14.10	14.25
	18:2n-6 trans	0.04	0.04	0.03	0.03	0.03	0.03	0.04	0.03	0.06	0.03
	18:2n-4	0.27	0.34	0.31	0.34	0.30	0.19	0.19	0.16	0.11	0.08
	20:2 NMIi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20:2 NMIj	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	20:2n-9	0.45	0.66	0.53	0.61	0.47	0.05	0.05	0.05	0.03	0.05
	20:2n-6	0.18	0.15	0.17	0.15	0.12	0.14	0.14	0.15	0.15	0.11
	22:2 NMIi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	22:2 NMIj	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	22:2n-6	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.02	0.05	0.04
	16:3n-4	0.64	0.90	0.87	0.92	0.95	0.52	0.52	0.51	0.33	0.35

Fatty Acids (% of Total Fatty Acids)	16:3n-3	0.12	0.10	0.14	0.10	0.11	0.13	0.11	0.11	0.06	0.03
	18:3n-6	0.16	0.20	0.20	0.19	0.17	0.15	0.15	0.15	0.11	0.13
	18:3n-4	0.17	0.17	0.18	0.17	0.18	0.12	0.12	0.13	0.10	0.12
	18:3n-3	1.79	0.99	0.86	1.31	1.27	0.84	0.80	0.84	2.80	2.68
	20:3n-6	0.10	0.14	0.13	0.13	0.13	0.14	0.13	0.12	0.12	0.12
	20:3n-3	0.10	0.10	0.09	0.11	0.07	0.06	0.07	0.06	0.06	0.03
	16:4n-3	0.07	0.06	0.07	0.07	0.14	0.07	0.07	0.10	0.05	0.04
	16:4n-1	1.12	1.63	1.45	1.67	1.62	0.80	0.89	0.65	0.44	0.28
	18:4n-3	1.54	1.71	1.90	1.82	2.26	1.22	1.20	1.04	1.01	0.57
	18:4n-1	0.16	0.23	0.21	0.23	0.23	0.11	0.12	0.11	0.08	0.08
	20:4n-6	0.91	1.06	1.05	1.00	0.96	0.91	0.81	0.82	0.58	0.52
	20:4n-3	0.53	0.58	0.61	0.61	0.56	0.36	0.32	0.33	0.21	0.22
	22:4n-6	0.13	0.11	0.11	0.07	0.10	0.12	0.08	0.13	0.09	0.09
	22:4n-3	0.05	0.04	0.05	0.05	0.05	0.04	0.05	0.04	0.03	0.02
	20:5n-3	10.84	13.41	13.31	13.44	13.49	7.42	6.98	6.58	3.50	2.77
	21:5n-3	0.43	0.55	0.55	0.55	0.57	0.28	0.27	0.23	0.17	0.13
	22:5n-6	0.26	0.22	0.25	0.21	0.19	0.18	0.17	0.24	0.08	0.09
	22:5n-3	1.20	1.44	1.49	1.41	1.41	0.97	0.94	1.14	0.50	0.48
	22:6n-3	9.35	8.42	9.98	8.59	7.71	7.65	6.90	7.28	2.60	1.67
	Omega-3	26.02	27.41	29.06	28.06	27.64	19.03	17.70	17.75	11.00	8.63
	Omega-6	14.19	8.29	7.13	9.82	9.10	11.13	9.53	11.95	15.40	15.44
	Omega-3 / Omega-6	1.83	3.31	4.08	2.86	3.04	1.71	1.86	1.49	0.71	0.56
	SFA	28.51	30.21	29.84	30.29	29.13	29.11	28.70	27.44	25.20	25.35
	MUFA	27.49	28.84	29.20	26.60	29.09	38.34	41.57	40.66	46.89	49.23
	PUFA	44.00	40.95	40.96	43.11	41.78	32.56	29.73	31.90	27.91	25.42
Amino Acids (% As-Fed)	Asparagine + Aspartic Acid	3.65	3.04	2.73	3.79	3.15	3.43	2.80	2.53	2.66	2.28
	Glutamine + Glutamic Acid	7.64	7.24	6.84	8.65	7.11	7.18	5.07	6.02	5.23	4.82

Amino Acids (% As-Fed)	Hydroxy-proline	0.28	0.29	0.25	0.21	0.15	0.70	0.61	0.46	0.67	0.53
	Serine	1.99	1.75	1.64	2.09	1.80	2.43	1.86	1.93	2.15	2.03
	Glycine	2.50	2.27	2.15	2.41	1.98	3.27	2.70	2.47	2.78	2.38
	Histidine	1.28	1.06	1.07	1.44	1.18	1.26	1.00	1.14	1.12	0.91
	Arginine	3.24	2.77	2.63	3.42	2.78	3.70	2.94	2.53	2.74	2.37
	Taurine	0.29	0.31	0.34	0.32	0.27	0.36	0.28	0.29	0.19	0.12
	Threonine	2.00	1.70	1.69	2.12	1.83	2.11	1.71	1.70	1.61	1.40
	Alanine	2.34	2.09	2.02	2.41	1.98	2.81	2.21	2.49	2.28	1.99
	Proline	2.45	2.35	2.29	2.80	2.35	3.27	2.39	2.76	2.87	2.67
	α-Amino-butyric Acid	0.07	0.06	0.04	0.06	0.07	0.06	0.06	0.04	0.04	0.03
	Tyrosine	1.76	1.51	1.47	1.92	1.68	1.72	1.41	1.63	1.42	1.30
	Valine	2.51	2.11	2.00	2.70	2.31	2.57	2.26	2.14	2.39	2.14
	Methionine	0.74	0.66	0.67	0.76	0.66	0.61	0.61	0.59	0.53	0.51
	Isoleucine	1.99	1.69	1.62	2.08	1.85	1.69	1.64	1.50	1.48	1.32
	Leucine	3.24	2.83	2.72	3.42	2.99	3.21	2.89	3.37	3.24	3.04
	Phenyl-alanine	2.00	1.77	1.84	1.99	1.93	1.70	1.81	1.81	1.89	1.77
	Ornithine	0.08	0.08	0.10	0.08	0.08	0.12	0.15	0.10	0.13	0.14
	Lysine	2.95	2.43	2.70	2.59	2.59	2.01	2.45	1.75	2.07	1.98
	Cysteine A	0.43	0.41	0.43	0.41	0.43	0.58	0.56	0.47	0.77	0.77
	Tryptophan	0.03	0.02	0.03	0.03	0.03	0.02	0.07	0.00	0.02	0.02
Minerals (% As-Fed)	Aluminum	0.0026	0.0012	0.0016	0.0016	0.0024	0.0011	0.0034	0.0038	0.0055	0.0061
	Antimony	0	0	0	0	0	0	0	0	0	0
	Arsenic	0.00053	0.00062	0.00059	0.00026	0.0003	0.00011	0.00014	0.00016	0.000089	0.00012
	Barium	0.00013	0.000086	0.000085	0.000069	0.000096	0.00015	0.00025	0.00014	0.00033	0.00023
	Beryllium	0	0	0	0	0	0	0	0	0	0
	Bismuth	0	0	0	0	0	0	0	0	0	0
	Cadmium	0.00002	0.00003	0.00003	0.00002	0.00002	0.00001	0.00002	0.00002	0.000007	0.00001

Minerals (% As-Fed)	Calcium	2.50	2.20	2.20	1.70	1.70	1.90	2.30	2.30	1.70	1.90
	Chromium	0.0002	0.0001	0.0002	0.0002	0.0002	0.0000	0.0001	0.0001	0.0002	0.0002
	Cobalt	0.00002	0	0	0.00001	0.00002	0	0	0	0	0.00002
	Copper	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	Iron	0.019	0.017	0.017	0.014	0.02	0.014	0.025	0.021	0.031	0.024
	Lead	0	0	0	0	0	0	0	0	0	0
	Lithium	0.00021	0.000059	0.000077	0.000058	0.00013	0.00004	0.00004	0.00005	0.00003	0.00003
	Magnesium	0.17	0.14	0.13	0.10	0.15	0.11	0.13	0.14	0.09	0.09
	Manganese	0.004	0.003	0.003	0.003	0.005	0.003	0.003	0.002	0.003	0.003
	Molybdenum	0.00003	0.00002	0.00002	0.00003	0.00004	0.00003	0.00002	0.00003	0.00004	0.00004
	Nickel	0.00009	0.00006	0.00008	0.00007	0.00009	0.00003	0.00005	0.00004	0.00009	0.00009
	Phosphorus	2.00	1.80	1.70	1.50	1.60	1.70	1.60	1.60	1.30	1.30
	Potassium	0.84	0.66	0.80	0.54	0.68	0.56	0.64	0.55	0.51	0.36
	Selenium	0.0002	0.0002	0.0002	0.0001	0.0002	0.0002	0.0001	0.0001	0.0001	0.0001
	Silver	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Sodium	0.84	0.83	0.70	0.34	0.44	0.34	0.44	0.57	0.32	0.22
	Strontium	0.0081	0.0075	0.006	0.0023	0.0056	0.0025	0.0028	0.0035	0.0018	0.002
	Thallium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Titanium	0.0003	0.0002	0.00024	0.00022	0.00028	0.00016	0.00024	0.00016	0.00029	0.00027
	Vanadium	0.00061	0.00064	0.00052	0.00035	0.00054	0.0003	0.00037	0.00039	0.00036	0.00039
	Zinc	0.02	0.02	0.02	0.03	0.03	0.02	0.02	0.01	0.01	0.03
	Zirconium	0.00003	0.00002	0.00002	0.00001	0.00003	0.00000	0.00003	0.00000	0.00002	0.00003

Appendix II. Fillet composition (% total fatty acid basis) for total omega-3 fatty acid (Σ ω3), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of AquAdvantage Salmon held at three rearing temperatures and assessed at 500, 800 and 1500 g. Mean values are given with their standard errors (±SEM) (n=3). Upper and lower-case letters denote significant differences among weights at the same temperature or among temperatures at the same weight, respectively ($p < 0.05$).

Variables	Treatments					
	10.5°C		13.5°C		16.5°C	
	Mean	(±SEM)	Mean	(±SEM)	Mean	(±SEM)
500 g						
% Σ ω3	13.54^{Ca}	0.11	14.57^{Cb}	0.12	14.48^{Cb}	0.05
% DHA	6.60^{Ca}	0.07	7.78^{Cb}	0.10	7.79^{Cb}	0.07
% EPA	2.56^{Ca}	0.04	2.71^{Cb}	0.02	2.58^{Cab}	0.03
800 g						
% Σ ω3	11.40^{Ba}	0.17	12.94^{Bb}	0.08	13.2^{Bb}	0.03
% DHA	5.19^{Ba}	0.11	6.43^{Bb}	0.07	6.68^{Bb}	0.03
% EPA	2.11^{Ba}	0.04	2.39^{Bb}	0.01	2.38^{Bb}	0.01
1500 g						
% Σ ω3	10.04^{Aa}	0.08	10.54^{Aa}	0.14	11.26^{Ab}	0.16
% DHA	4.34^{Aa}	0.06	4.91^{Ab}	0.09	5.51^{Ac}	0.12
% EPA	1.84^{A}	0.02	1.86^{A}	0.04	1.95^{A}	0.02

Appendix III. Permutational multivariate ANOVA (PERMANOVA) and similarity percentages analysis (SIMPER) for genes of interest alongside plasma cortisol results and phenotypic measurement data amongst all fish injected with pIC and sampled 24 hpi. The p (perm) value is the statistical significance determined by PERMANOVA after conducting 9999 permutations. SIMPER provided average dissimilarities (%) and the top 70% contributing variables.

	16.5°C vs 10.5°C	13.5°C vs 10.5°C	16.5°C vs 13.5°C
p (perm)	0.0499	0.0299	0.0468
Average dissimilarity (%)	35.83	39.10	42.93
Contributing variables (top 70%)	*ifng*	*tlr3*	*stat1b*
	isg15a	*lgp2*	k
	VSI	*rsad2*	*tlr3*
	rsad2	*stat1b*	VSI
	lgp2	VSI	*ifng*
	tlr3	*isg15a*	*mxb*
	k	HSI	HSI