Burin Region
Protecting Atlantic Salmon Aquaculture Production from Climate-Related Challenges Through Diet Manipulation

Eric Ignatz
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Research on the Burin Region

Protecting Atlantic Salmon Aquaculture Production from Climate-Related Challenges Through Diet Manipulation

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Executive Summary

The objective of this research was to determine: (1) if additional dietary cholesterol could increase the thermal tolerance of female triploid Atlantic salmon (*Salmo salar*), thereby reducing the likelihood of mortality when exposed to rising ocean temperatures; and furthermore (2) if this change in diet could improve fillet quality and fish health. This research built upon the Burin Peninsula's tradition of working on the sea, and was conducted in the hopes of ensuring that the region has future seafood-related employment and economic opportunities regardless of what environmental changes lie ahead. Thus, it fit well within the theme targeted by this funding opportunity 'Ocean Health & Seafood Opportunities'. With the threat of global climate change, and given that a mass mortality event has already occurred in this province, this study examined whether diet manipulation (increasing dietary cholesterol concentration) could be an effective tool that local salmon farmers could use to help mitigate these risks. Our results show that supplemental dietary cholesterol did not improve the upper thermal tolerance of Atlantic salmon as expected, and may actually negatively impact survival at high inclusion levels. Cholesterol was also not effective at preventing fillet 'bleaching', as pigmentation was lost at elevated temperatures regardless of dietary treatment. However, AquaBounty's triploid salmon did not experience large numbers of mortalities until after 22°C, and this suggests that their production may be suitable in Placentia Bay and that ploidy might not influence their survival at high temperatures. The results of this study provide valuable information for companies like Grieg NL, and help ensure that salmon aquaculture in Newfoundland is sustainable.

Introduction

With the stagnation of the global fisheries sector, the aquaculture industry now provides more than half of the world's seafood (Cai and Leung, 2017; FAO, 2018). However, with global seafood demand expected to increase by 47 million tonnes by the early 2020s, expansion of the aquaculture sector is projected to meet only 40% of this requirement (Cai and Leung, 2017). Anthropogenic climate change will only exacerbate the situation, as changes in water temperature, acidification and low oxygen levels (hypoxia) are all anticipated to have long-term effects on aquaculture production (Barange et al., 2018). Further, warmer waters are expected to increase the risk of disease, parasitic infection and harmful algal blooms that can drastically impact the success of aquaculture operations (Barange et al., 2018). Therefore, it is of critical importance that new ways to sustainably expand the aquaculture industry are developed, including those that will offset/mitigate the predicted consequences of climate change.

Recently, Newfoundland's south coast experienced the impacts of an unprecedented summer heat wave on Atlantic salmon (*Salmo salar*) aquaculture production, with the loss of over 2.6 million fish in the summer/fall of 2019 (Burke et al., 2020). With the expectation that Grieg NL will increase provincial production by 33,000 metric tonnes within the next five years (Ignatz, 2019), the industry must be proactive if it is to avoid similar future events. There are several mitigation strategies available, such as selective breeding, using new genetic strains, changing farm management / fish husbandry practices (i.e., improving site selection and/or using deeper net pens) and altering the nutrition of the salmon.

This project addressed the latter of these options, by examining if increased inclusion of dietary cholesterol could improve salmon thermal tolerance and fillet pigmentation (colour), and enhance immune function. Irvine et al. (1957) showed that increased dietary cholesterol can

improve survival at high temperatures in another fish species. However, no research has been published on its effect(s) on Atlantic salmon or other salmonids. Cholesterol is an important component for maintaining cell membrane rigidity, which is relevant as membrane structure becomes more fluid as environmental temperatures increase and this change can ultimately lead to cell death (Crockett, 1998; Farkas et al., 2001; Fodor et al., 1995; Hazel, 1979; Liu et al., 2019). Furthermore, in Tasmania, high temperatures at Atlantic salmon sea-cage sites have led to a decrease in fillet colouration (Grünenwald et al., 2020, 2019; Wade et al., 2019). Rich / darker pigmentation is important as it is desired by consumers (Lerfall et al., 2017). Astaxanthin, a carotenoid typically added to salmon diets for fillet pigmentation, is the most expensive ingredient in salmon feed (Solymosi et al., 2015), and therefore, producers need to maximize its deposition and retention in the fish's muscle (fillet). Increasing dietary levels of cholesterol may assist with this, as cholesterol can increase astaxanthin blood transport and retention in salmon (Chimsung et al., 2013). In addition, there are some studies which show other positive effects of increased dietary cholesterol levels. For example, dietary cholesterol supplementation has been shown to improve the rainbow trout's immune response and disease resistance to the bacterial pathogen *Aeromonas hydrophila* (Deng et al., 2013). Finally, enriching the cholesterol content of bovine cardiac microsomes improves the conformational stability of proteins, and increases their resistance to inactivation at elevated temperature (Ortega et al., 1996).

While a 'high temperature' diet is available in Tasmania and other parts of the world, no peer-reviewed literature is available on its composition or efficacy, and this diet is not currently approved for use in Canada (Ruff, 2015). Therefore, the research we conducted was both novel and practical in its design, and provides information that can benefit the Atlantic salmon aquaculture industry in the Burin region. Given the importance of the salmon aquaculture

industry to the province's economy and coastal communities, especially on the Burin Peninsula where significant expansion is on-going, it is vital to ensure this industry's continued success and sustainability. As discussed, increases in ocean temperature can lead to massive losses of salmon and have sub-lethal effects, that not only adversely affect company profits and employment, but also have consequences for the environment and public trust (the industry's social licence to operate). This project aimed to find ways to avoid such future events through the manipulation of dietary cholesterol, and provide the industry with vital information regarding the production of female triploid Atlantic salmon at elevated temperatures.

Description of the Project

This study was approved by the Animal Care Committee of Memorial University (protocol #20-02-KG), and salmon husbandry and experimental procedures were performed in accordance with the Canadian Council on Animal Care Guidelines on the 'Care and Use of Fish in Research, Teaching, and Testing' (Canadian Council on Animal Care, 2005).

Experimental animals

PIT (Passive Integrated Transponder)-tagged female triploid Atlantic salmon of St. John River origin from AquaBounty Canada (PE, Canada) were received at the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR; Ocean Sciences Centre, Memorial University) where the salmon were smolted. These fish were produced from a single reversed-sex neomale (i.e., functionally masculinized genetic female) that had been crossed with 24 females, producing all female offspring. Fertilized eggs were pooled, and then shocked using hydrostatic pressure to induce triploidy. Ploidy status of the fish was verified by AquaBounty before transfer using flow cytometry (Allen Jr, 1983; AquaBounty Technologies, Inc., 2010).

Based on a subset of 200 eyed eggs, it was estimated that pressure shocking was ≥ 98% effective in inducing triploidy. The fish were initially distributed among eight 2.2 m^3 tanks at 16.5 kg m^{-3} in a flow-through seawater system with temperature set to 10°C for the first week before rising slowly (i.e., +0.3°C day^{-1}) to 12°C over the course of another week. Water oxygen levels were maintained at ≥ 100% air saturation throughout the experiment. The salmon were initially fed a commercial diet (EWOS Dynamic S, 5.0 mm; minimum 46% crude protein, 27% crude fat; EWOS Canada Ltd, BC, Canada) to satiation twice daily by hand. Once the fish reached 12°C, they were re-distributed amongst nine 2.2 m^3 tanks with 50 fish $tank^{-1}$; initial stocking density ~9 kg m^{-3}. Only fish weighing between 300-500 g were included in the study to limit the effect(s) of size variation. Salmon were then given another 18 days before the initial assessment was performed and the trial started.

Overall experimental design and fish sampling

An overview of the experimental design used in this study can be seen in Figure 1. Initial fish weight and fork length were measured after anesthesia (0.2 g L^{-1} AquaLife TMS; Syndel Laboratories Ltd, Nanaimo, BC, Canada). Fish were then allowed 2 days of recovery before their commercial diet was switched to this trial's control diet. Fish were hand fed to satiation twice daily (~9:00 & 15:00), with feeding stopped when a few pellets accumulated on the bottom of each tank. All diets in this study were formulated by Dr. Sean Tibbetts and designed to meet all of the nutritional requirements for Atlantic salmon using conventional ingredients (National Research Council, 2011). The feed was extruded at the Chute Animal Nutrition Lab, Faculty of Agriculture, Dalhousie University (Truro, NS, Canada). In addition to the control diet, 2 experimental diets containing supplemental cholesterol (+1.30% and 1.76% dietary cholesterol,

respectively) were also manufactured. The proximate composition of all 3 diets can be found in Table 1. The amount of supplemental dietary cholesterol included in each experimental diet was selected based on previous research (Deng et al., 2013). All diets contained approximately equal levels of protein (51%), lipid (20%), carbohydrate (18%), digestible energy (21 MJ kg^{-1}), phosphorus (1.6%), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (2%) and astaxanthin (80 mg kg^{-1}), with all essential amino acids at a minimum of 150% of the dietary requirement for Atlantic salmon.

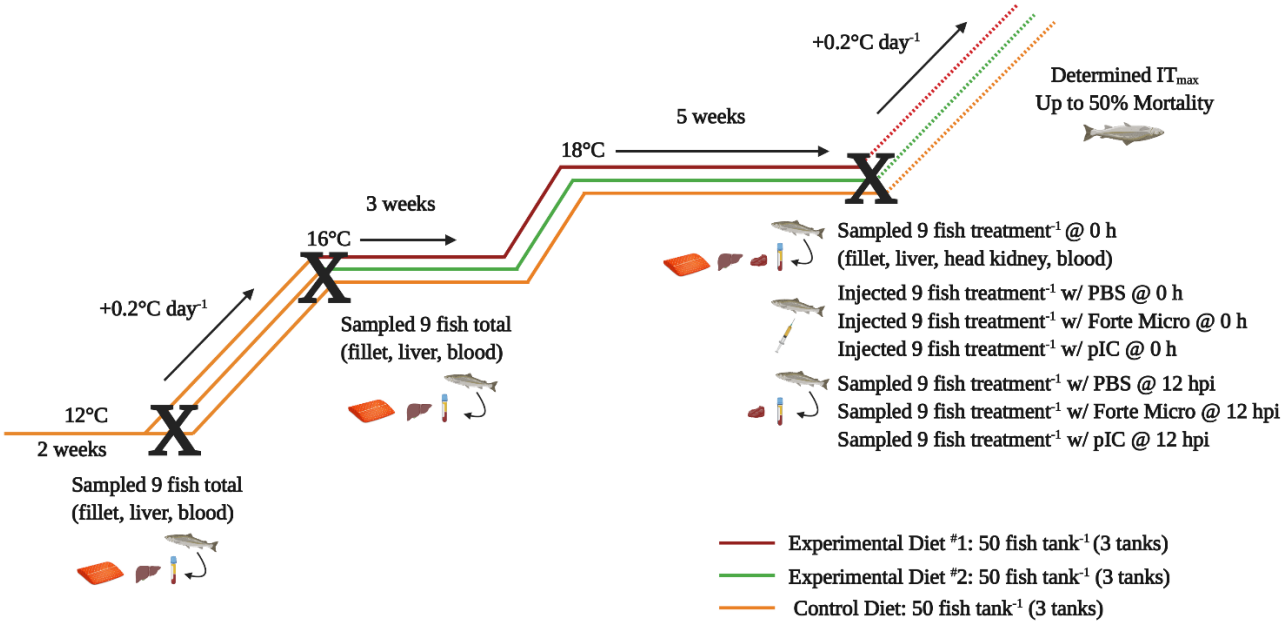

Figure 1. Overview of the protocol used to assess whether supplementary dietary cholesterol affects the thermal tolerance and/or innate immune response of female triploid Atlantic salmon. Figure created using BioRender.com.

Table 1. Proximate composition[1] of the experimental diets (*as fed* basis).

	Control Diet No added cholesterol	Experimental Diet #1 1.30% added cholesterol	Experimental Diet #2 1.76% added cholesterol
Moisture (%)	5.6	6.3	6.1
Ash (%)	10.1	10.4	10.6
Crude protein (%)	51.4	51.7	51.2
Crude lipid (%)	20.5	19.1	19.3
Carbohydrate[2] (%)	18.0	18.8	18.9
Gross energy (MJ kg^{-1})	21.8	21.5	21.4

[1] Analysis performed at the National Research Council of Canada, Marine Research Station, NS. (Sean Tibbetts, Shane Patelakis, Cheryl Wall)

[2] Estimated as: (100% - [Ash + Crude protein + Crude lipid])

Salmon were fed the control diet for a period of 16 days at 12°C, then the first sampling was performed. One fish was randomly netted from each tank (n = 9), euthanized (0.4 g L^{-1} TMS), measured for weight and fork length, and sampled. Blood (1 mL) was collected from each fish via the caudal vein using 1 mL syringes with 23 gauge 1" needles within 2-3 min following euthanasia and placed in heparinized (1000 units mL^{-1}) tubes on ice. Blood was then centrifuged at 1100 x*g* for 1 min at room temperature, whereafter 3 aliquots (100 µL each) of plasma were collected before being flash-frozen in liquid nitrogen. The remaining plasma and white blood cells were carefully pipetted off the top and the leftover red blood cells were then also flash-frozen. The remaining blood from the fish was collected (via multiple collections using a 3 mL syringe and needle), then the viscera was collected, and the liver weighed. Next, duplicate pieces of liver from the most distal portion of the posterior lobe were sampled using standard aseptic

techniques, and quickly flash-frozen. The liver tissue were stored at -80°C until samples could be shipped to the Center for Aquaculture Technologies Canada (CATC; Souris, PE, Canada) for processing. The fish's gonads were examined to confirm that the fish were sterile females. The right side of the fish was then filleted, with the bones removed but the skin remaining. The fillet was weighed before being placed on plain white paper inside a white Styrofoam box with a 100 W halogen light bulb hanging 87.5 cm directly above (Ignatz et al., 2020b). The fillets were then scored using the DSM SalmoFan™ colour chart (DSM, 2021); the industry-recognized method for the visual assessment of the degree of pigmentation in salmon flesh. Two trained technical staff independently scored each fillet to determine its level of colouration, with separate scores assigned to describe the colouration along the lateral line and at the peripheral edges of the fillet. An average of these two values was taken to come up with a final pigmentation score for each fillet. Samples were weighed, then wrapped in aluminum foil and stored in plastic Whirl-Pak® bags (Sigma-Aldrich, Oakville, ON, Canada) that were moved to -80°C until they could be processed.

The following day, the remaining fish were exposed to an incremental temperature increase from 12°C, where temperature was raised by 0.2°C day^{-1} to mimic conditions that these salmon would experience in sea-cages during the summer in Newfoundland (Burt et al., 2012; Johansson et al., 2007, 2006). Once the tanks reached 16°C, another sampling was performed following the same procedure as described above (n = 9; 1 fish sampled $tank^{-1}$) with fish taken off feed 24 h prior to sampling. All remaining fish were also anesthetized (0.2 g L^{-1} TMS), weighed and measured for fork length. A total of 25 fish were removed from the experiment at this point, as these salmon had developed ulcers (e.g., 3 cm x 3 cm) on their right side. Samples of these fish were taken to be analyzed by the Microbial Pathogenesis and Vaccinology

Laboratory (Memorial University), but the results were ultimately inconclusive (data not shown). The next day, 3 tanks were switched onto Experimental Diet #1 (ED1; +1.30% cholesterol) and another 3 tanks were provided with Experimental Diet #2 (ED2; +1.76% cholesterol). The remaining 3 tanks were kept on the control diet. Temperature was then maintained at 16°C for 3 weeks before gradually being increased (+0.2°C day^{-1}) to 18°C, where temperature was held again for 5 weeks. No fish developed ulcers during the remainder of the experiment.

After exposure to elevated temperatures (i.e., ≥16°C) for a total of 65 days, another subset of fish was sampled after being fasted for at least 24 h. Nine fish per dietary treatment (3 fish $tank^{-1}$) were sampled in the same manner as previously described at 12 and 16°C, with the addition that the viscera was weighed, and head kidney tissue was also sampled. Duplicate pieces of head kidney were collected from the most cranial portion of the organ before tissues were quickly flash-frozen in liquid nitrogen. Furthermore, only fish that had gained weight were sampled, with 24 out of 27 fish having gained ≥ 10% weight since their assessment at 16°C and the remaining sampled fish having gained between 5 and 10% weight. This was done to ensure that these fish were actively eating the diets provided.

Immune challenge

Once sampling was completed in a tank, 3 fish per tank were anesthetized (in 0.2 g L^{-1} TMS), measured for weight and fork length, and then injected intraperitoneally (IP) with sterile phosphate buffered saline (PBS; Thermo Fisher Scientific, Mississauga, ON, CA), Forte Micro® (Elanco Limited, Charlottetown, PE, CA) or polyriboinosinic polyribocytidylic acid (pIC; Sigma-Aldrich, Oakville, ON, CA; Catalog #42424-50-0) (9 fish per tank, 27 fish total per dietary treatment). Salmon were injected with a volume of PBS (i.e., to serve as a sham control)

relative to their individual weight (e.g., a 650 g fish was injected with 650 µL of PBS). Forte Micro® is a commercial multi-valent vaccine that contains formalin-inactivated cultures of *Aeromonas salmonicida*, *Vibrio anguillarum* serotypes I & II, *V. ordalii* and *V. salmonicida* serotypes I & II in liquid emulsion with an oil based adjuvant. This vaccine was allowed to warm to room temperature (from 4°C) before fish were IP injected with 50 µL of this vaccine independent of fish weight (as per the manufacturer's instructions). These injections served to stimulate the innate antibacterial immune response of these salmon (similar to Forte V II vaccine stimulation; Zanuzzo et al., 2020). pIC is a synthetic double-stranded RNA (dsRNA) analog that elicits a strong innate antiviral response in fish, and 10 µg of pIC per g of fish (dissolved in PBS) was given to each fish by IP injection (Ignatz et al., 2020a). Solutions of PBS and pIC were kept on ice prior to injection. Of the 80 injected fish (1 fish was not injected with pIC in the ED2 treatment as there was not enough stock solution left), 66 had gained >10% weight, 6 gained between 5 and 10% weight, 4 gained between 0 and 5% weight and 3 maintained or lost < 2% weight from the assessment performed at 16°C. Once the fish were injected, they were moved into one of eight 0.5 m^3 holding tanks (10 fish $tank^{-1}$) supplied with 18°C seawater at 5 L min^{-1}.

At 12 h post-injection (hpi), a time chosen to best capture peak innate anti-bacterial and anti-viral immune responses (Zanuzzo et al., 2020), the fish were euthanized (0.4 g L^{-1} TMS followed by cranial concussion) and sampled. Plasma and red blood cells were collected as previously described, and similarly, the viscera and liver were weighed. Duplicate pieces of head kidney were also collected and flash-frozen. No liver or fillet were sampled. In between injection and sampling times, all remaining fish were anesthetized (0.2 g L^{-1} TMS), and their weights and fork lengths were measured.

Remaining experimental protocol

Once all sampling was completed, temperature was again raised by 0.2°C day^{-1} in all tanks until fish began to reach their incremental thermal maximum (IT_{Max}). When each fish lost equilibrium/succumbed, their weight, fork length, liver weight, viscera weight, ventricle weight, and state of sexual maturity (or lack thereof) were recorded, in addition to the time and temperature at which they were removed from the experiment. The study lasted until 50% of the fish within each dietary treatment reached their IT_{Max}. After the first dietary treatment reached this endpoint, 9 fish per dietary treatment (3 fish $tank^{-1}$) were euthanized (0.4 g L^{-1} TMS), measured for weight and fork length, and sampled. Viscera, liver and ventricle weight were also measured. Fillet weight and colouration were assessed as previously described, and a small piece of dorsal muscle (~1 cm x 1 cm taken below the dorsal fin on the right side) was sampled for possible astaxanthin quantification. This sample was then stored at -80°C. Liver and head kidney were not taken. In addition, after 50% of the fish within each dietary treatment reached their IT_{Max}, the remaining fish within that particular dietary group were euthanized (0.4 g L^{-1} TMS) before weight and fork length measurements were recorded.

Measurements of growth performance

Weight gain was assessed at 16°C, at the end of 5 weeks spent at 18°C and as fish reached their IT_{Max}. Thermal growth coefficient (TGC) was used to assess growth rate using the following equation (Cho, 1992; Iwama and Tautz, 1981):

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

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

Specific growth rate (SGR) was calculated using:

$$\text{SGR} = \left(\frac{\ln(W_f) - \ln(W_i)}{n}\right) \times 100$$

Fulton's condition factor (K) was calculated as:

$$\text{K} = \frac{\text{Fish weight (g)}}{(\text{Fish fork length [cm]})^3} \times 100$$

Feed intake was measured daily by dividing the feed provided to each tank by the number of fish in the tank. Average feed intake was also calculated on a % body weight basis at the assessment points. Liver, viscera, ventricle and fillet weights were used to calculate each fish's hepatosomatic index (HSI), viscerosomatic index (VSI), relative ventricular mass (RVM) and fillet yield, respectively, using the following equations:

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

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

$$\text{RVM} = \left(\frac{\text{Ventricle weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

$$\text{Fillet Yield} = \left(\frac{\text{Fillet weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

Statistical analyses

To confirm the absence of tank effects, replicate tank means were compared first, with dietary treatment as a fixed factor and tank as a random factor, after which the data from replicate tanks were pooled for further analyses. Data were first assessed via Shapiro-Wilk's normality tests and $\log_{10}$-transformed if necessary to meet testing assumptions. Levene's tests to measure homoscedasticity were also performed to confirm the assumptions of the statistical tests. One-way ANOVAs followed by Tukey's HSD post-hoc tests were used to examine differences ($p < 0.05$) in parameters between the dietary treatments. T-tests were used when comparisons were only made between two variables (e.g., assessments performed at 12°C vs. 16°C). Results throughout this article are reported as mean ± standard error (SE).

Ongoing & Future Analyses

One replicate sample each of all liver and head kidney tissues were shipped to CATC on dry ice in June of 2021. CATC will extract RNA from these tissues, including performing DNase treatment and column purification, and will be assessing RNA quality via spectrophotometry and gel electrophoresis. Once complete, they will perform real time quantitative polymerase chain reaction (qPCR) analyses on the synthesized cDNA. Target genes of interest have been chosen (see Tables 2-5) to assess innate antibacterial and antiviral immune responses in the head kidney, and heat stress and lipid/cholesterol metabolism in the liver. Table 6 shows the normalizer genes that will also be tested.

Table 2. qPCR primers for assessing the innate anti-bacterial immune response in Atlantic salmon head kidney.

Gene Name (GenBank Accession Number)	Function	Nucleotide sequence (5'-3')	Amplicon size (bp)	Source
Cholesterol 25-hydroxylase-like protein a (*ch25ha*) (BT046542)	B cell chemotaxis; defense response to virus	F: TAGAGCTGTGATGCTAGTTTAC R: ACCCAGTAGCACTGAGAAGTC	106	Eslamloo et al. (2020)
Cyclooxygenase-2 (*cox2*) (AY848944)	regulation of inflammatory response; cellular response to heat; response to oxidative stress	F: ACCTTTGTGCGAAACGCTAT R: GAGTAGGCCTCCCAGCTCTT	113	Caballero-Solares et al. (2017)
Hepcidin a (*hamp-a*) (BT125319)	acute-phase response; defense response to bacterium; antimicrobial humoral immune response	F: ATGAATCTGCCGATGCATTTC R: AATGGCTTTAGTGCTGGCAG	134	Eslamloo et al. (2020)
Interleukin 1 beta (*il1b*) (AY617117)	cytokine-mediated signaling pathway	F: GTATCCCATCACCCCATCAC R: TTGAGCAGGTCCTTGTCCTT	119	Soto-Dávila et al. (2020)
Interleukin 8a (*il8-a*) (BT046706)	antimicrobial humoral immune response; chemokine-mediated signaling pathway	F: GAAAGCAGACGAATTGGTAGAC R: GCTGTTGCTCAGAGTTGCAAT	99	Soto-Dávila et al. (2020)
Interferon gamma (*ifng*)	apoptotic process; defense response to virus	F: CCGTACACCGATTGAGGACT R: GCGGCATTACTCCATCCTAA	133	Caballero-Solares et al. (2017)
Interferon regulatory factor 7b (*irf7b*) (FJ517644)	defense response to virus; interferon-gamma-mediated signaling pathway	F: GTCAGTGGTAAAATCAACACGC R: CACCATCATGAAACGCTTGGT	91	Caballero-Solares et al. (2017)
Signal transducer and activator of transcription 1 b (*stat1b*) (BT048927)	cellular response to interferon-gamma; cytokine-mediated signaling pathway	F: GTTCAGGATGCAGAGCATGA R: CCATCCCATTCACCTCTTGT	99	Caballero-Solares et al. (2017)
Toll-like receptor 5a soluble (*stlr5-a*) (AY628755)	pathogen recognition	F: ATCGCCCTGCAGATTTTATG R: GAGCCCTCAGCGAGTTAAAG	103	Smith et al. (2018)

Note: Functional annotation of genes came from UniProt.org

Table 3. qPCR primers for assessing the innate anti-viral immune response in Atlantic salmon head kidney.

Gene Name (GenBank Accession Number)	Function	Nucleotide sequence (5'-3')	Amplicon size (bp)	Source
Interferon stimulated gene 15a (*isg15-a*) (BT049918)	defense response to virus; interferon-gamma production	F: AAAGTGGCCACAACAAAGCAG R: ATAGGAGCGGGCTCCGTAATC	140	Caballero-Solares et al. (2017)
Interferon gamma (*ifng*)	apoptotic process; defense response to virus	F: CCGTACACCGATTGAGGACT R: GCGGCATTACTCCATCCTAA	133	Caballero-Solares et al. (2017)
Interferon regulatory factor 7b (*irf7b*) (FJ517644)	defense response to virus; interferon-gamma-mediated signaling pathway	F: GTCAGTGGTAAAATCAACACGC R: CACCATCATGAAACGCTTGGT	91	Caballero-Solares et al. (2017)
RNase helicase lgp2 (*lgp2*, alias *dhx58*) (BT045378)	defense response to virus; positive regulation of type I interferon production	F: TCCAAGACCCGTAAAAGCAC R: GGTGGAGATCAGGAGGTTGA	189	Caballero-Solares et al. (2017)
Interferon-induced GTP-binding protein b (*mx-b*) (BT044881)	defense response to virus; signal transduction	F: ACGCACCACTCTGGAGAAAT R: CTTCCATTTCCCGAACTCTG	184	Caballero-Solares et al. (2017)
Radical S-adenosyl methionine domain containing protein 2b (*rsad2-b*, alias *viperin-b*) (DY728694)	defense response to virus; type I interferon signaling pathway	F: TTCCTGGCATGGATAGGTGT R: CTTGGAGTTGTCGCTGGTTT	113	Caballero-Solares et al. (in prep)
Signal transducer and activator of transcription 1b (*stat1-b*) (BT048927)	defense response to virus; cellular response to interferon-gamma	F: GTTCAGGATGCAGAGCATGA R: CCATCCCATTCACCTCTTGT	109	Caballero-Solares et al. (2017)

Note: Functional annotation of genes came from UniProt.org. The primers for *rsad2-b* were designed by Dr. Albert Caballero-Solares.

Table 4. qPCR primers for assessing the heat stress response in Atlantic salmon liver.

Gene Name (GenBank Accession Number)	Function	Nucleotide sequence (5'-3')	Amplicon size (bp)	Source
Cold-inducible RNA-binding protein (*cirbp*) (BT059171)	response to cold	F: TTGAGTACACAGCGGTGAATT R: ACCAATCTGATGCTATGACGAGA	132	Beemelmanns et al. (2021)
Heat shock protein 70 (*hsp70*) (BT045715)	cellular response to heat	F: AGTGATCAACGACTCGACACG R: CACTGCATTGGTTATAGTCTTG	151	Beemelmanns et al. (2021)
Heat shock protein 90-alpha (*hsp90a1*) (KC150878)	cellular response to heat	F: CGAGGACATGAAGAAGAGGCAT R: ACACTGTCACCTTCTCCACTTT	104	Beemelmanns et al. (2021)
Heat shock protein 90 alpha family class B member 1 (*hsp90ab1*) (NM_001123532)	cellular response to heat	F: AGCCTCACGTTTTTCCAATCG R: TGCGTTGCCCACCATTAACT	150	Ignatz et al. (in prep.)
NADH dehydrogenase 1 alpha subcomplex subunit 1 (*ndufa1*) (BT046880)	mitochondrial electron transport, NADH to ubiquinone	F: TGATGGAGAGAGACAGACGAGT R: AGGTGAGATCTGGGATTAGTGGA	89	Beemelmanns et al. (2021)
Serpin H1 (*serph1*) (XM_014214963)	collagen biosynthetic process; response to unfolded protein	F: GACCATTCAAAAATCAACCTCA R: CATGGCTCCATCAGCATTCT	129	Beemelmanns et al. (2021)
Mitochondrial uncoupling protein 2 (*ucp2*) (XM_014196911)	adaptive thermogenesis; response to cold	F: CTGATCTCTGCCGTCACCAT R: AGAAGACTGATGAGGTGAAGACA	89	Beemelmanns et al. (2021)

Note: Functional annotation of genes came from UniProt.org

Table 5. qPCR primers for assessing lipid and cholesterol metabolism in Atlantic salmon liver.

Gene Name (GenBank Accession Number)	Function	Nucleotide sequence (5'-3')	Amplicon size (bp)	Source
Apolipoprotein A-Ia (*apoAIa*) (BT049771)	cholesterol biosynthetic process	F:AGGTGAAGTTGACTGCACAGAG	117	Xu et al. (2013)
		R:AGGTGGAATCAGCAAACTGC		
Bile acid receptor (*bar*) (GO063627)	bile acid metabolic process; cholesterol homeostasis	F:GCCAAGAGGTAAGCATCTCG	120	Xue et al. (2015)
		R:TCAGGAGGTTCTGTGCAATG		
Cholesterol 7 alpha-hydroxylase b (*cyp7a1b*) (CA042205)	cellular response to cholesterol; cholesterol catabolic process	F:CTGGCCGAGAACTTAAGCAA	94	Hixson et al. (2017)
		R:TCAGGTCATTGAAGGTGGAC		
Elongation of very long chain fatty acids protein 2 (*elovl2*) (NM_001136553)	fatty acid elongation, polyunsaturated fatty acid	F: CGGGTACAAAATGTGCTGGT	145	Morais et al. (2009)
		R: TCTGTTTGCCGATAGCCATT		
Delta-5 fatty acyl desaturase (*fadsd5*) (AF478472)	unsaturated fatty acid biosynthetic process	F:GTCTGGTTGTCCGTTCGTTT	135	Hixson et al. (2017)
		R:GAGGCGATCAGCTTGAGAAA		
Delta-6 fatty acyl desaturase a (*fadsd6a*) (AY458652)	unsaturated fatty acid biosynthetic process	F:CCCCAGACGTTTGTGTCAG	181	Hixson et al. (2017)
		R:CCTGGATTGTTGCTTTGGAT		
3-hydroxy-3-methyl-glutaryl-CoA reductase (*hmgcr*) (NM_001173919)	cholesterol biosynthetic process; bile acid signaling pathway	F:CTCCTATTGGATGGGAAGCA	194	Hixson et al. (2017)
		R:CAGGCCTTGACCTCTACAGC		
3-hydroxy-3-methyl-glutaryl-CoA synthase (*hmgcs*) (AM402497)	cholesterol biosynthetic process	F:CTGTCATCGCACAACACACA	135	Kortner et al. (2014)
		R:TATCCAATCCAGAGCCAGGC		
Prostaglandin-D synthase (*pgds*) (BT048787)	cyclooxygenase pathway	F:GGTGCTCAACAAGCTCTACA	114	Caballero-Solares et al. (2017)
		R:GCAGGAAAGCGATGTTGTCA		
Peroxisome proliferator-activated receptor beta (*pparb1*) (AJ416953)	negative regulation of cholesterol storage	F:AAGGAGGTCAACAACGCCTA	97	Hixson et al. (2017)
		R:ACTCTACTGGGCTGGAGCTG		
Sterol regulatory element-binding protein 1 (*srebp1*) (HM561860)	positive regulation of cholesterol biosynthetic process	F:TCAACAAGTCGGCAATTCTG	100	Hixson et al. (2017)
		R:GACATCTTCAGGGCCATGTT		
Sterol regulatory element-binding protein 2 (*srepb2*) (HM561861)	positive regulation of cholesterol biosynthetic process	F:GAGTGCTGAGGAAAGCCATC	129	Hixson et al. (2017)
		R:TCTCCACATCGTCAGACAGC		

Note: Functional annotation of genes came from UniProt.org

Table 6. qPCR primers for the normalizers.

Gene Name (GenBank Accession Number)	Nucleotide sequence (5'-3')	Amplicon size (bp)	Source
Elongation factor 1 alpha (*ef1a*) (NM_001141909)	F: GTGGAGACTGGAACCCTGAA	155	Jones et al. (2007)
	R: CTTGACGGACACGTTCTTGA		
Eukaryotic translation initiation factor 3 subunit D (*eif3d*) (GE777139)	F: CTCCTCCTCCTCGTCCTCTT	105	Caballero-Solares et al. (2017)
	R: GACCCCAACAAGCAAGTGAT		
Polyadenylate-binding protein 1 (*pabpc1*) (EG908498)	F: TGACCGTCTCGGGTTTTTAG	108	Caballero-Solares et al. (2017)
	R: CCAAGGTGGATGAAGCTGTT		
RNA polymerase II (*polr2*) (CA049789)	F: TTCTGAAAGACCCCCAAGTG	145	Hixson et al. (2017)
	R: AGCTCGCTGATGAGGTCAGT		
60S ribosomal protein 32 (*rpl32*) (BT043656)	F: AGGCGGTTTAAGGGTCAGAT	119	Xue et al. (2015)
	R: TCGAGCTCCTTGATGTTGTG		

Furthermore, fillet samples collected at 12, 16 and 18°C will be analyzed for lipid class composition and undergo fatty acid methyl ester (FAME) derivatization. This will be conducted in association with the Core Research Equipment and Instrument Training (CREAIT) Network at Memorial University. Additional liver and head kidney tissues may also be analyzed in the same manner. However, a final decision on whether this work will be conducted has not been made. Similarly, astaxanthin concentrations in the salmon's fillet tissue may also be quantified if budgetary/time constraints allow. Finally, there are plans to measure plasma cortisol using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY, USA).

Results

Performance Metrics Following an Incremental Temperature Increase to 18°C

Performance metrics of the fish sampled at 12 and 16°C can be found in Table 6. The fish gained almost 100 g, on average, during the 37 day initial period where all fish were fed the control diet as temperature was gradually raised to 16°C from the initial acclimation temperature of 12°C. Although Fulton's condition factor values did significantly decrease slightly ($p < 0.001$) in salmon at 16°C as compared to those assessed at 12°C, there were no differences in feed intake, HSI or fillet yield between the two assessment points. SalmoFan™ colouration in the fillet increased ($p < 0.001$) from 12 to 16°C, indicating that the pigmentation of the fillet increased as the fish grew.

For comparison, performance metrics for the fish as they continued onward and were exposed to temperatures ≥ 16°C can be found in Table 7. Fish in all dietary treatments, did on average, gain weight (~50 g; $p < 0.01$) from when they were assessed at 16°C. However, it is noteworthy that 41.3% of all fish lost weight since the previous assessment (-49 g on average) whereas the remaining 58.7% of the fish gained weight (+199 g on average). Feed intake

decreased significantly ($p < 0.001$) compared to both previous assessments in all three dietary treatments (by 50.8% and 43.8% on average as compared to those measured at 12 and 16°C, respectively). Similarly, growth rate (i.e., as assessed by TGC and SGR) and condition factor declined significantly ($p < 0.001$) in salmon reared up to 18°C as compared to previous measurements. HSI was also lower ($p < 0.05$) at 18°C as compared to at 12°C regardless of dietary treatment, but was only lower ($p < 0.05$) at 18°C in the control dietary group when compared to measurements at 16°C. In contrast, fillet colouration increased significantly ($p < 0.05$) from when it was assessed at 16°C in salmon that were sampled at 18°C. No differences were found in measurement of fillet yield between any of the three temperature sampling points, nor were there any differences for any variable between the three dietary treatments at 18°C.

Table 6. Morphological/production metrics of Atlantic salmon first reared at 12°C and fed the control diet for a period of 16 days, and then after they were exposed to an incremental thermal increase up to 16°C (i.e., after an additional 21 days on the control diet).

	12°C		**16°C**	
	Mean ± SE	**n**	**Mean ± SE**	**n**
Weight (g)	$463.8 \pm 3.5^{a*}$	450	557.3 ± 5.6^{b}	400
Length (cm)	$32.6 \pm 0.1^{a*}$	450	35.1 ± 0.1^{b}	400
K	$1.33 \pm 0.01^{b*}$	450	1.28 ± 0.01^{a}	400
TGC [$g^{1/3}$ (°C d)$^{-1}$]	-	-	0.85 ± 0.03	400
SGR (% body weight day^{-1})	-	-	0.41 ± 0.02	400
Feed Intake (% body weight day^{-1})	$0.80 \pm 0.02^{*}$	9	0.70 ± 0.03	9
HSI (%)	1.33 ± 0.08	9	1.19 ± 0.06	9
Fillet Yield (%)	50.0 ± 1.3	9	52.3 ± 0.9	9
SalmoFan™ Score	21.2 ± 0.1^{a}	9	22.2 ± 0.2^{b}	9

For each parameter, values without a letter in common are significantly different ($p < 0.05$). K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate; HSI, hepatosomatic index
*Weight, length, K and feed intake at 12°C were measured at the start of the experiment.

Table 7. Morphological/production metrics of Atlantic salmon after 65 days on either the control diet, experimental diet 1 (ED1), or experimental diet 2 (ED2). Fish were fed these diets as temperature was maintained at 16°C for 3 weeks, then raised to 18°C where temperature was held for 5 weeks.

	Control		**ED1**		**ED2**	
	Mean ± SE	**n**	**Mean ± SE**	**n**	**Mean ± SE**	**n**
Weight (g)	604.3 ± 14.5	126	609.9 ± 15.5	129	605.7 ± 13.3	130
Length (cm)	37.3 ± 0.2	126	37.4 ± 0.2	129	37.2 ± 0.2	130
K	1.13 ± 0.01	126	1.13 ± 0.01	129	1.15 ± 0.01	130
TGC [$g^{1/3}$ (°C d)$^{-1}$]	0.18 ± 0.04	126	0.19 ± 0.04	129	0.19 ± 0.03	130
SGR (% body weight day^{-1})	0.10 ± 0.02	126	0.11 ± 0.03	129	0.11 ± 0.02	130
Feed Intake (% body weight day^{-1})	0.40 ± 0.03	3	0.43 ± 0.07	3	0.35 ± 0.04	3
HSI (%)	0.89 ± 0.03	27	1.04 ± 0.07	27	0.99 ± 0.04	26
VSI (%)	8.41 ± 0.16	26	8.47 ± 0.22	26	8.08 ± 0.21	26
Fillet Yield (%)	52.8 ± 0.9	9	51.2 ± 1.3	9	50.5 ± 0.9	9
SalmoFan™ Score	23.3 ± 0.4	9	23.8 ± 0.3	9	23.3 ± 0.2	9

No significant differences ($p > 0.05$) were found for any parameter between dietary groups. K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate; HSI, hepatosomatic index; VSI, viscerosomatic index

Performance Metrics & Survival Following an Incremental Temperature Increase Until Mortality Reached 50%

Feed intake declined steeply after temperature passed 18°C in all tanks, to a point where hardly any fish were feeding after 19°C. The ED2 treatment was the first to have 50% of fish reach their IT_{Max} (i.e., at 24.0°C), followed by the control treatment at 24.2°C and the ED1 treatment at 24.4°C. Morphological/production metrics of the first 50% of fish within each dietary treatment that reached their IT_{Max} can be found in Table 8. Fish within this group weighed significantly less ($p < 0.001$) than when they were assessed at 18°C, and their condition factor scores were lower ($p < 0.001$). HSI was higher ($p < 0.05$) in fish that reached their IT_{Max} in comparison to fish assessed at 18°C in the control group. In contrast, although no difference was detected within the ED2 dietary group, VSI scores were lower ($p < 0.05$) in both the control and ED1 treatments at 50% mortality (IT_{Max}) compared to at 18°C. SalmoFan™ colour also decreased significantly ($p < 0.001$) among all dietary treatments compared to when they were assessed at 18°C.

A Kaplan-Meier curve with survival probabilities for each dietary group can be seen in Figure 2. Furthermore, a histogram with the IT_{Max} results can be viewed in Figure 3. In comparison to the control and ED2 treatments, the average IT_{Max} for the first 50% of ED2 fish that succumbed to elevated temperatures was slightly higher, and trended toward significance ($p = 0.068$). Thus, this suggests that fish fed ED2 had reduced survival as compared to those fed the control diet.

Fish that survived to the endpoint of the experiment (i.e., after 50% of each dietary treatment reached their IT_{Max}), weighed more ($p < 0.01$), lost less weight ($p < 0.01$), were longer ($p < 0.01$) and had higher condition factor values ($p < 0.05$) as compared to fish within their

respective dietary treatment that succumbed to the high temperatures (Table 9). No differences were found when comparing survivors between dietary treatments.

Table 8. Morphological/production metrics and incremental thermal maximum (IT_{Max}) of the first 50% of Atlantic salmon to succumb to the increasing temperature protocol. Fish were fed either the control diet, the first experimental diet (ED1) or the second experimental (ED2) diet.

	Control		**ED1**		**ED2**	
	Mean ± SE	**n**	**Mean ± SE**	**n**	**Mean ± SE**	**n**
IT_{Max} (°C)	23.4 ± 0.1	45	23.3 ± 0.2	47	22.9 ± 0.2	48
Weight (g)	416.2 ± 16.9	45	436.4 ± 17.7	47	422.9 ± 17.0	48
Weight Gain (g)	-76.0 ± 7.7	45	-71.2 ± 8.9	47	-78.9 ± 4.3	48
Length (cm)	35.3 ± 0.4	45	35.7 ± 0.4	47	35.4 ± 0.4	48
K	0.93 ± 0.02	45	0.93 ± 0.02	47	0.93 ± 0.02	48
HSI (%)	1.07 ± 0.03	45	1.05 ± 0.04	47	1.05 ± 0.04	48
VSI (%)	7.44 ± 0.30	45	7.31 ± 0.23	47	7.35 ± 0.24	48
RVM (%)	0.073 ± 0.002	45	0.070 ± 0.002	47	0.079 ± 0.004	48
Fillet Yield (%)	47.2 ± 0.5	9	44.9 ± 1.3	9	44.8 ± 1.7	9
SalmoFan™ Score	21.4 ± 0.4	9	22.4 ± 0.7	9	21.9 ± 0.5	9

No significant differences between dietary treatments ($p > 0.05$) were found for any parameter. Weight gain is in comparison to the assessment that was performed at 18°C. K, Fulton's condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; RVM, relative ventricular mass.

Table 9. Morphological parameters for Atlantic salmon that survived to the endpoint of the experiment (i.e., when 50% of each population reached their incremental thermal maximum [IT_{Max}]) ; control (n = 44), first experimental diet (ED1; n = 46), second experimental diet (ED2; n = 47).

	Control	ED1	ED2
	Mean ± SE	**Mean ± SE**	**Mean ± SE**
Weight (g)	541.7 ± 19.1	535.1 ± 23.5	555.6 ± 18.9
Weight Gain (g)	-38.8 ± 7.6	-35.0 ± 5.2	-56.2 ± 5.7
Length (cm)	37.6 ± 0.3	37.5 ± 0.4	37.6 ± 0.4
K	1.00 ± 0.02	0.98 ± 0.02	1.02 ± 0.02

No significant ($p > 0.05$) differences between dietary treatments were found. Weight gain is in comparison to the assessment that was performed at 18°C. K, Fulton's condition factor.

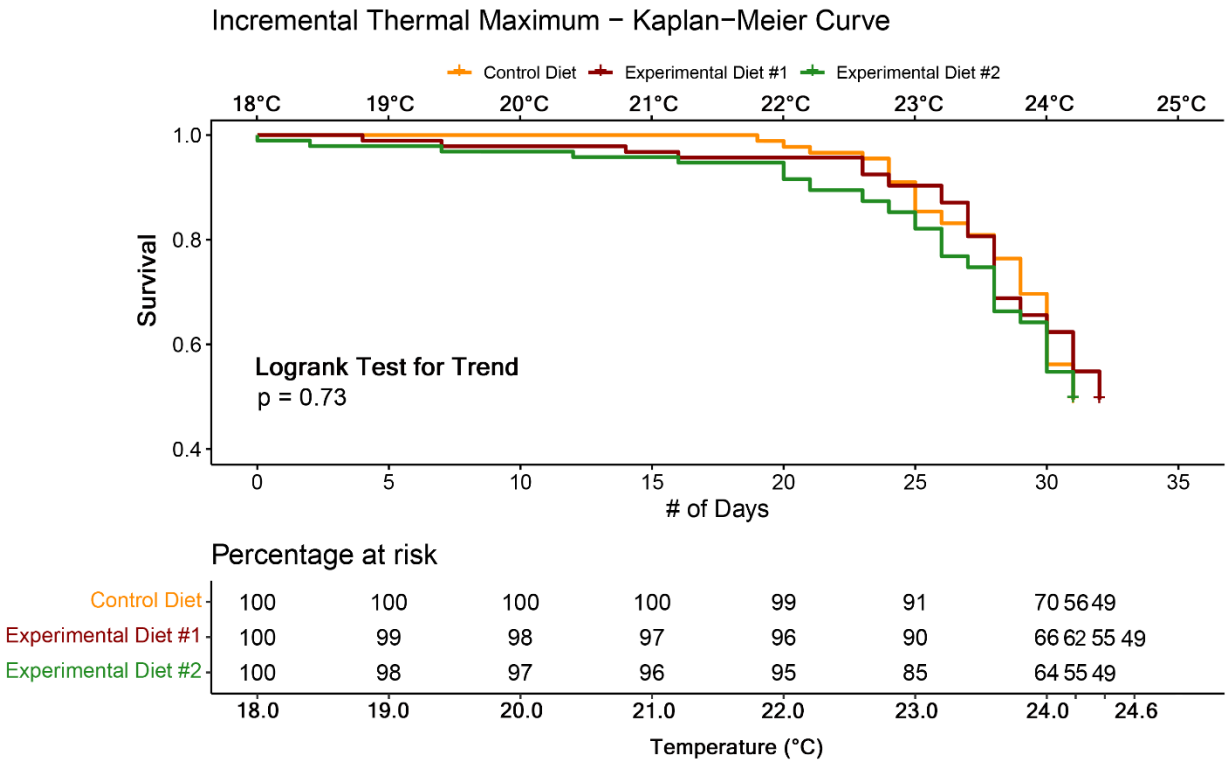

Figure 2. The incremental thermal maximum (IT_{Max}) of female triploid Atlantic salmon exposed to a thermal increase of 0.2°C day^{-1} from 18°C after prolonged exposure to elevated temperatures (≥ 16°C) for 65 days. Kaplan-Meier survival curves are shown with the log-rank test for trend to determine significance.

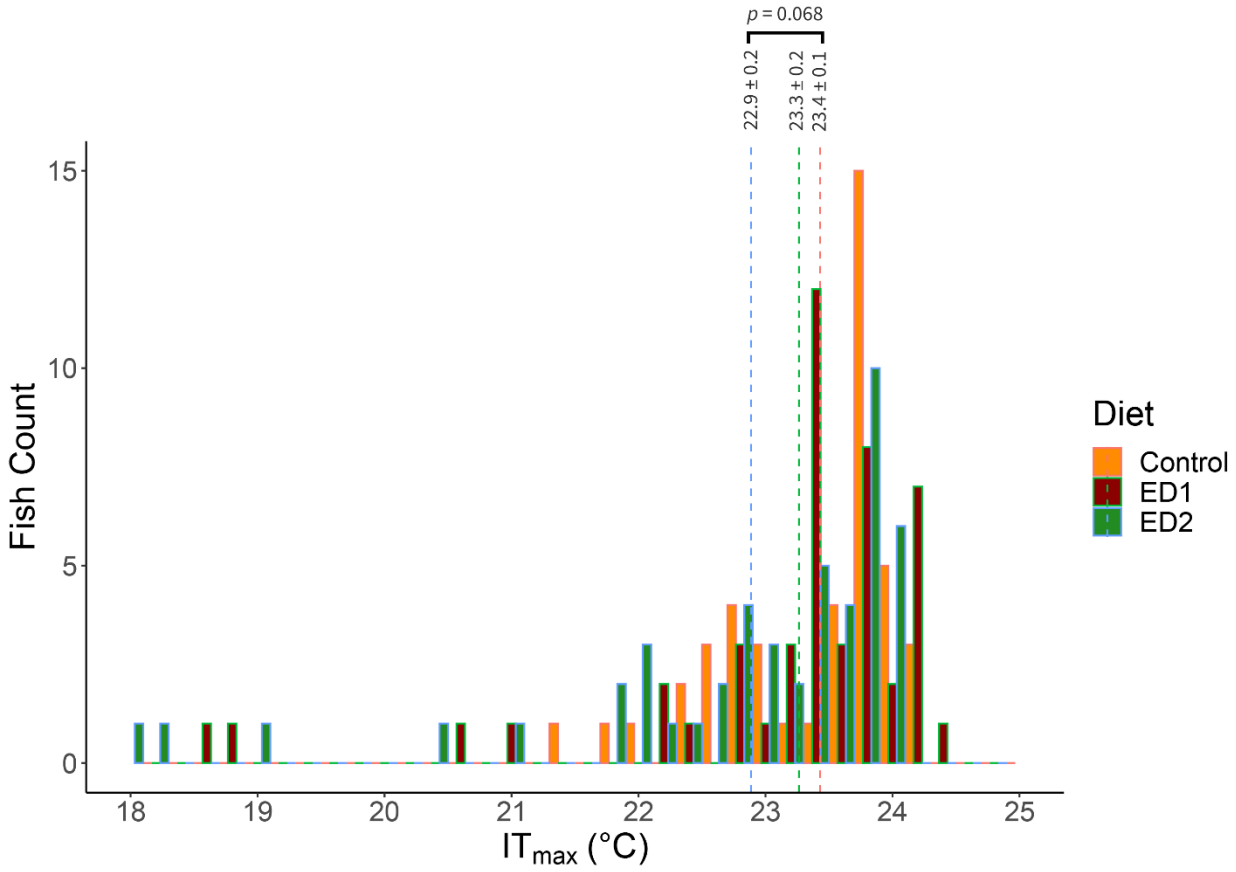

Figure 3. Histogram of the incremental thermal maximum (IT_{Max}) data for female triploid Atlantic salmon exposed to a thermal increase of 0.2°C day^{-1} from 18°C after prolonged exposure to elevated temperatures (≥ 16°C) for 65 days; fish were fed control, first experimental (ED1) or second experimental (ED2) diets. The dashed lines indicate the average IT_{Max} value for the first 50% of fish that succumbed to high temperature for each dietary treatment.

Discussion & Potential Implications

The objective of this research was to determine if supplementary dietary cholesterol could improve the upper thermal tolerance and growth performance of female triploid Atlantic salmon, a commercially relevant farmed fish to the Burin region. Further, this study examined if adding cholesterol to salmon diets could benefit fillet pigmentation and assist the fish in

mounting a more robust immune response against both bacterial and viral antigens (i.e., pathogen-like immune stimuli). While several analyses are still ongoing, the current results indicate that supplemental dietary cholesterol was not successful in improving survival or enhancing growth at elevated temperatures. In fact, the results indicate that the highest inclusion level (+1.13% cholesterol) might even negatively influence upper thermal tolerance in these fish. In addition, adding cholesterol to the diet did not prevent fillet bleaching (i.e., loss of pigmentation) from occurring, as all dietary treatments saw a decrease in SalmoFan™ scores after 50% of fish had reached their IT_{Max}.

Interestingly, a high proportion (i.e., >40%) of salmon lost weight when reared at temperatures between 16 and 18°C. It is unknown whether this was directly related to triploidy, as it has previously been reported that triploids exhibit reduced feed intake at elevated temperatures (i.e., 18°C) as compared to diploids (Sambraus et al., 2018, 2017), or if it may be related to other potential confounding variables. For example, a small proportion of fish in this study developed dermal sores before the assessment at 16°C, so it is possible that these fish were combatting an unknown infection but did not present clinical signs during the remainder of the experiment. Also, feed intake in this experiment regardless of rearing temperature was lower than anticipated (i.e., the diets' palatability or some other aspect related to diet formulation could be associated with reduced feed intake).

However, this is the first study to expose triploid Atlantic salmon to an incremental thermal increase that mimics the natural conditions that these fish are expected to encounter in Placentia Bay as ocean temperatures rise. While it has been suggested that triploid salmonids have reduced survivorship at elevated temperatures as compared to diploids (Hyndman et al., 2003; Ojolick et al., 1995), this finding is not always consistent (Benfey et al., 1997; Bowden et

al., 2018; Ellis et al., 2013). In the current study, our triploid fish did not experience large numbers of mortalities until after 22°C, which is similar to what has been reported for diploid Atlantic salmon exposed to a comparable gradual increase in temperature (Gamperl et al., 2020). Furthermore, the fish in the present study spent an extended period of time (i.e., 65 days) at temperatures ≥ 16°C before their IT_{Max} was determined (a feature not found in Gamperl et al., 2020). Therefore, it could be hypothesized that these triploid salmon might have been able to tolerate even higher temperatures if they spent less time at suboptimal temperatures where their feed intake and weight were reduced beforehand. While it is unfortunate that a high proportion of fish in this trial lost weight at 16°C or above, it is possible that selective breeding could be used to enhance growth performance at elevated temperatures in the future (Yoshida and Yáñez, 2021). Advances in genotyping methods could also lead to the improved selection of Atlantic salmon (stocks) with improved upper thermal tolerance, as this trait has been shown to be heritable in other salmonids (Gallardo-Hidalgo et al., 2021; Ihssen, 1986; Perry et al., 2005).

Further research is also recommended into using dietary manipulation to enhance the production of farmed female triploid Atlantic salmon. While the current results suggest that supplemental cholesterol may not be beneficial to Atlantic salmon reared at elevated temperatures, other ingredients (e.g., prebiotics, vitamins C and E) show promise for improving thermal tolerance and/or offsetting the negative impacts of heat stress in other fish species (Dong et al., 2020; Gupta et al., 2010; Islam et al., 2021; Khosravi-Katuli et al., 2021). It also remains to be determined what effect additional dietary cholesterol had on the innate anti-bacterial and anti-viral immune responses of the salmon in the current study. Therefore, it is still possible that cholesterol could have benefited these salmon in other ways.

Conclusion

The results of this study will be highly valuable to industry members, such as Grieg NL, with particular relevance to the production of female triploid Atlantic salmon. While supplemental dietary cholesterol was not effective at improving survival or growth performance in this experiment, several analyses are yet to be completed, and thus, it is still possible that cholesterol benefited the fish in other ways. It is also beneficial for the industry to now be able to rule out supplemental cholesterol as a dietary ingredient to improve the fish's thermal tolerance. As this is the first study to measure upper thermal tolerance in triploid salmon exposed to an environmentally realistic rate of temperature increase, the current results are invaluable in predicting the impact of current and future climate-change driven changes in the ocean surrounding Newfoundland on salmon production. As the triploid salmon used in this study did not experience high mortalities until after 22°C, this suggests that their production is suitable for Placentia Bay. However, this should be specifically tested on the triploid salmon that Grieg NL plan to put into Placentia Bay. It is hoped that these results will promote the sustainable expansion of the salmon aquaculture industry within the province.

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