THE EFFECT OF DIETARY SUPPLEMENTATION OF ASTAXANTHIN ON ACUTE HYPOXIA AND THERMAL TOLERANCE IN TRIPLOID AND DIPLOID BROOK CHARR, *SALVELINUS FONTINALIS*

by

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ABSTRACT

Triploid fish, by way of their inherent sterility, show promise in averting interbreeding between wild and farmed fish, but they are more vulnerable to environmental stressors than diploids. This study investigated whether supplementing diets of triploid and diploid brook charr (*Salvelinus fontinalis*) with the carotenoid astaxanthin (AX) enhances their tolerance towards hypoxia and elevated temperatures. Both ploidies were fed diets of differing AX supplementation (18, 80, and 190 mg/kg) for a minimum period of eight weeks, and then assessed for their acute hypoxia tolerance and their critical thermal maximum (CTmax). Results showed that triploids were less hypoxia tolerant than diploids, but there was no difference between ploidies in their CTmax. Diet had no effect on either hypoxia tolerance or CTmax. This study suggests ploidy, not diet, is more indicative of stress tolerance in brook charr. Further research is needed to examine other possible benefits AX has on farmed fish health.

DEDICATION

This thesis is dedicated to my mother, whose endless love, support, and encouragement helped me become the person I am today.

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Table of Contents

List of Tables

List of Figures

1. INTRODUCTION

1.1 Canadian and Global Aquaculture

The practice of farming aquatic organisms for food has been employed around the world for millennia, evolving over time to take pressure off natural and cultivated land and water ecosystems and ecosystem services (Costa-Pierce, 2008). As the human population continues to increase exponentially, there is a greater need for a sustainable and cost-effective way to meet the protein requirements of the world. Since 1961, annual global growth in fish consumption has been twice as high as human population growth, cementing the usefulness of aquaculture as an effective strategy for meeting animal protein demand (Food and Agriculture Organization, 2018). In that time, species diversity in aquaculture has increased, with Atlantic salmon (*Salmo salar*) becoming one of the most popularly farmed species globally.

The Canadian aquaculture industry emerged in the 1970s in response to the growing demand for seafood, and it has since become an important revenue source (Government of Canada, 2013). Production value has increased by 63% over the last 20 years, reaching $1.2 billion in 2021 (Government of Canada, 2023). British Columbia dominates Canadian aquaculture, and in 2021, contributed 60% ($712 million) of the total national output (Government of Canada, 2023). New Brunswick accounts for the second largest contribution, which totaled $240 million in 2021 (Government of Canada, 2023). Despite the first harvest of farmed Atlantic salmon in New Brunswick having been as recent as 1978, in 2021 this species was the largest single food commodity produced in the province in terms of sales value (Government of Canada, 2023). Atlantic salmon

represents 90% of New Brunswick's aquacultural production value, with various other species of finfish and shellfish comprising the remainder (Government of New Brunswick, 2021). Roughly 60% of New Brunswick's farmed Atlantic salmon production is exported to the east coast of the United States, 35% is exported to other parts of Canada, and the remainder is sold locally (Government of New Brunswick, 2021).

1.2 Salmonid Aquaculture

During the first half of their captive three-year lifecycle, encompassing the time from egg to smolt, Atlantic salmon are reared in land-based freshwater facilities. Following smoltification (the period where salmonids undergo various behavioural, developmental, and physiological changes to prepare for their transition from fresh water to salt water [McCormick, 2012]), salmon are moved to saltwater floating net pens where they spend roughly 18 months growing until they are harvested. While in these net pens, farmed salmon pose risks to local, and genetically distinct, wild salmon populations (Glover et al., 2017). Significant advancements in containment technologies and measures have been implemented over the short tenure of the aquaculture industry to reduce the interaction between farmed and wild fish stocks, but none are without flaw. Net tearing/ripping due to environmental factors (e.g., severe winds, waves, or currents) and/or human operational and maintenance errors allow for the escape of potentially thousands of fish at a time (Jensen et al., 2010; Jensen et al., 2013). Reports of escape events are common; however, the number of lost farmed fish varies among events (Jensen et al., 2013).

Escaped fish have the potential to significantly impact wild populations genetically, as farmed fish have been shown to breed with wild stocks (Glover et al., 2017; Wringe et al., 2018). Wild and farmed fish differ genetically, with the latter displaying increased phenotypic divergence from the former due to the intentional and unintentional selection inherent in aquaculture (Wringe et al., 2018). Genotypic selection may be passed on through genetic introgression when wild and farmed individuals interbreed, which has caused global concern. A possible solution to this issue is to remove the chance of interbreeding completely through the stocking of sterile, triploid fish.

1.3 Triploidy

Triploidy describes a genetic condition where an organism has three sets of homologous chromosomes in their cells, as opposed to the more typical diploid organism with two sets (Bazaz et al., 2020). Triploidy induction has been achieved in many species of fish, including various species of salmon, trout, catfish, and bass, by using physical treatments such as thermal or pressure shock to disrupt the second meiotic division shortly after fertilization and thereby retain the second polar body with its haploid set of maternal chromosomes (Benfey, 2001; Maxime, 2008; Piferrer et al., 2009; Bazaz et al., 2020). The resulting triploid zygote (3n) has two sets of maternal chromosomes (2n) and one set of paternal chromosomes (1n) (Piferrer et al., 2009; Bazaz et al., 2020). Triploid fish of both sexes can still create gametes, but these are typically reduced in number and aneuploid, and any viable embryos produced normally will not survive early development, meaning triploid fish are functionally sterile (Benfey, 2001; Piferrer et al.,

2009; Li et al., 2016). Naturally occurring triploid individuals have been observed in wild populations of diploid species, but their frequency is very low (Jørgensen et al., 2018).

Triploid sterility is beneficial to the aquaculture industry as it removes the potential for interbreeding between wild and farmed stocks in the event of escapes (Benfey, 2016). It is also useful for reducing production losses associated with pre-harvest sexual maturity, including slower growth and increased susceptibility to disease (Piferrer et al., 2009). In addition to sterility, triploids exhibit several characteristics of interest in physiological studies related to cell size and number because they have larger, but fewer, cells in most tissues and organs relative to diploids of the same species (Benfey, 1999; Maxime, 2008). This is due to the need to accommodate the added set of homologous chromosomes in their cell nuclei. Changes in cell size and number have been suggested as the underlying cause for triploid underperformance when subjected to stressful conditions. Despite these differences in biological parameters, triploids are similar to diploids when studied at the whole-animal level (Benfey, 1999). Since they do not need to devote much energy towards reproductive activity and gonadal development, triploids, especially females, exhibit greater maximum body sizes when compared to similarly aged diploids of the same species (Bazaz et al., 2020). Triploid salmonids have also been shown to have increased muscle pigmentation when compared to sexually mature diploids, resulting in a higher quality fillet (Bjørnevik et al., 2004).

Despite these potential advantages, triploids are not widely used in aquaculture. For instance, in Norway, which is the world's largest producer of farmed Atlantic salmon, triploids are no longer allowed for commercial production due to ethical concerns related to poor performance (Madaro et al., 2022) that is likely associated with

their larger cell size (Maxime, 2008; Benfey, 2016). Triploid individuals show an overall lower tolerance towards both chronic and acute stress, including elevated temperature and hypoxia (low oxygen), when compared to sibling diploids. For example, there is growing evidence that triploids have an innately lower thermal optimum for metabolism and higher mortality when exposed to elevated temperatures (Hyndman et al., 2003a; Hyndman et al., 2003b; Atkins & Benfey, 2008; O'Donnell et al., 2017; Sambraus et al., 2017a; Sambraus et al., 2017b; Daigle et al., 2021). This has been shown to directly impact the heart's ability to deliver oxygenated blood throughout the body, causing onset of cardiac arrhythmia at lower temperature in triploid rainbow trout compared to diploids (Verhille et al., 2013). During chronic thermal stress experiments, triploids have been shown to express lower amounts of ubiquitin (a type of regulatory protein) and heat shock proteins than diploids reared at the same conditions, implying that triploids have greater difficulty coping with protein damage when exposed to thermal stress (Saranyan et al., 2017). Exhaustive exercise experiments conducted at elevated temperatures have also resulted in higher mortality of triploid individuals, leading to the presumption that they also have lower aerobic and anaerobic capacities when compared to diploids (Hyndman et al., 2003a; Hyndman et al., 2003b; Bernier et al., 2004). Research has shown that there are significant interactions between limitations in oxygen uptake and cellular surface area to volume ratio (Verberk et al., 2022). Since triploids have larger, but fewer, cells they tend to require more oxygen during periods of stress to maintain homeostasis, and thus show lower aerobic scope when compared to diploids of the same species (Hansen et al., 2015; Leal et al., 2021; Verberk et al., 2022). It has, however, been shown that acclimating triploids to higher temperatures can reduce the negative

physiological impacts of hypoxia on triploids (Hansen et al., 2015; Jensen & Benfey, 2022).

Several studies have highlighted the usefulness of dietary manipulation to mitigate triploid underperformance and shortcomings. For instance, supplementing feed with phosphorus can improve growth and prevent the formation of vertebral deformities in Atlantic salmon (Burke et al., 2010; Fjelldal et al., 2016; Smedley et al., 2016; Sambraus et al., 2020), and supplementation with histidine prevents the formation of cataracts in adult triploid Atlantic salmon (Taylor et al., 2015). In their study examining dietary supplementation with L-carnitine, an amino acid which aids in lipid metabolism for obtaining energy, Ozório et al. (2012) found that fish fed diets with higher L-carnitine supplementation stockpile more dietary lipids. These findings highlight the usefulness of dietary modification in mitigating the physical problems of triploids; however, there is a lack of research on whether dietary supplementation of carotenoids can improve high temperature and hypoxia tolerance in triploid salmonids.

1.4 Dietary Supplementation of Carotenoids

Carotenoids encompass a diverse group of over 700 organic, lipid-soluble molecules that are produced *de novo* by many species of bacteria, photosynthetic freshwater algae and terrestrial plants, and fungi (Weber & Davoli, 2003; Lim et al., 2018). These molecules have been extensively studied for their numerous health benefits to humans, including reducing the risks of developing certain cancers, cardiovascular diseases, and bone, skin, or eye disorders (Meléndez-Martínez et al., 2022). Their beneficial properties are attributed to their antioxidant mechanisms, which act to protect

biological molecules from oxygen-deprived free radicals (Weber & Davoli, 2003; Meléndez-Martínez et al., 2022). The most well studied carotenoids include ß-carotene, lutein, fucoxanthin, zeaxanthin, canthaxanthin, and astaxanthin (Lim et al., 2018).

Astaxanthin (AX) is a naturally occurring xanthophyll carotenoid utilized in many different fields including food production, cosmetics, pharmaceuticals, and aquaculture for its potent antioxidant properties (Davinelli et al., 2018; Lim et al., 2018). The powerful antioxidant capacity of AX stems from its molecular structure's affinity for donating electrons to reduce endogenously produced free oxygen radicals and convert them into stable products (Lim et al., 2018). Free radicals are commonly produced during normal essential metabolic processes in the body or following periods of high bodily stress, and the number present at any one time can seriously impact an organism's ability to maintain homeostasis during and after such an event (Lim et al., 2018). In their study looking at high temperature–induced alterations to hypothalamic mitochondria of mice, Chen et al. (2021) found that mice fed olive oil mixed with AX were protected from hypothalamic mitochondrial degradation, by way of preserving redox homeostasis through the reduction of free oxygen molecules. In looking at the hypoxia tolerance of the golden pompano (*Trachinotus ovatus*), Niu et al. (2020) similarly found that suitable AX supplementation shows neuroprotective effects due to its ability to upregulate the expression of anti-apoptotic pathways through the reduction of reactive oxygen species.

AX is a natural derivative of ß-carotene, the most abundant carotenoid, and displays antioxidant effects 10 times higher than that of ß-carotene and 100 times stronger than vitamin E, another common and potent carotenoid (Li et al., 2020). In nature, AX is the molecule responsible for the orange/red colouration observed in many

crustaceans, fungi, freshwater red algae, and in the flesh of wild salmonids. Salmonids accumulate AX in their bodies entirely through their diets (Weber & Davoli, 2003). *Haematococcus pluvialis*, a freshwater green algal species of the Chlorophyta, contains the highest levels of AX known and is responsible for most of the global production of AX and its derivative canthaxanthin (Weber & Davoli, 2003; Zhu et al., 2022). It is known to accumulate in many parts of the body, but salmonids accumulate high concentration of AX in the liver, muscle, skin, and gonads (Meléndez-Martínez et al., 2022). The supreme antioxidant properties shown by AX reveal its great potential for supplementation in salmonid feed.

Currently, AX is used in salmonid aquaculture as a pigment-enhancement agent in finishing diets, which leads to the characteristic orange–red colouration of salmonid fillets. Aside from pigment enhancement, AX has been shown to have important health benefits among other farmed aquatic animals, stemming from its antioxidant properties (as described above). Several studies have examined the capability of fish fed diets supplemented with AX to reduce bacterial infections. Zhu et al. (2022) found that when grouper (*Plectropomus leopardus*) were fed diets supplemented with AX, their ability to resist infection by the bacteria *Vibrio harveyi* increased, as well as enhancing their digestive enzyme activity and antioxidant capacity. Comparable results were found by Jagruthi et al. (2014), who examined the ability of the common carp (*Cyprinus carpio*) to fight off infection by the bacteria *Aeromonas hydrophila*. Carp fed diets of highest supplementation showed significantly improved growth rates and immunocompetence. Saleh et al. (2018) found that AX also plays a role as an immunostimulant during periods of sudden osmotic decline by increasing intestinal mucosal phagocytic and lysosome

activities, as well as survival, in sea bass (*Dicentrarchus labrax*) fed highly supplemented diets. In a similar study examining the same species, Goda et al. (2018) found that fish fed diets of highest carotenoid concentration showed a marked improvement in their resistance to thermal and salinity stress. They also found that those same fish showed increased total body carotenoid content. During instances of hypoxic stress, AX has been shown to improve growth performance and antioxidant capacity, while reducing inflammatory response in the golden pompano (Xie et al., 2020).

AX has been examined in the scope of salmonid aquaculture, but not to the extent of other farmed fish, with attention focused on its well-known pigment enhancement properties. In a study focusing on gene expression in Atlantic salmon, Ytrestøyl et al. (2021) found that even low levels of AX supplementation increased the expression of genes related to lipid metabolism, biosynthesis of terpenoids and steroids in the liver, immunosuppression in the intestine, and over 311 genes related to immunity and stress response in skeletal muscle. Choubert et al. (2009) found that rainbow trout (*Oncorhynchus mykiss*) fed diets of high supplementation displayed increased AX concentration in their muscle tissue with corresponding profound colouration. In a similar study looking at triploid rainbow trout, Wei et al. (2019) found that with increasing supplementation of AX enriched diets, growth, survivability, and muscle pigment concentrations increased. In examining rainbow trout response to hyperoxia, Kalinowski et al. (2019) found that trout fed an AX-supplemented diet showed decreased plasma glucose and an increase in hepatic glycogen when stressed, two factors which are known to accompany hyperoxia and increased flesh redness.

1.5 Objective, Hypotheses and Significance

The goal of this study was to determine whether AX supplementation can improve the tolerance of triploids and diploids to hypoxia and high temperature. This was achieved by feeding triploid and diploid brook charr (*Salvelinus fontinalis*) diets of differing AX content and then assessing their acute hypoxia and high temperature tolerances in separate experiments. There were two main hypotheses of this study. Firstly, I hypothesized that if high temperature and hypoxia tolerance are dependent on antioxidant activity, then the ability of any individual fish to tolerate these stressors should show improvement with higher dietary AX supplementation. Secondly, I hypothesized that if the reduced high temperature and hypoxia tolerance of triploids is due to greater oxidative stress, then AX supplementation will decrease the differences between diploids and triploids in their acute stress tolerance.

This study aims to give insight into a possible dietary modification that would be useful in addressing hypoxia and elevated temperature, two common issues faced in aquaculture (Oppedal et al., 2011; Stehfest et al., 2017; Gamperl et al., 2021), and to improve farmed fish health overall. The differences between triploid and diploid fish in terms of stress tolerance is clear, and if a beneficial dietary modification can be found, these differences may be mitigated. This may also lead to the increased incorporation of triploid fish in aquaculture.

Brook charr were chosen as a model salmonid for this study due to their small size, ease of rearing, and resilience under laboratory conditions (e.g., Ellis et al., 2013; Jensen & Benfey, 2022). The fish were tested as juveniles to allow for an increased sample size during testing in the experimental apparatus, and to remove sexual

maturation as a confounding variable. High-temperature tolerance was determined using the critical thermal maximum (CTmax) protocol with loss of equilibrium as the non-lethal endpoint (based on Lutterschmidt & Hutchison. 1997). This research builds upon the work of past students of the Benfey lab, where triploid versus diploid hypoxia and thermal stress tolerance have been examined (e.g., Ellis et al., 2013; Benfey & Devlin, 2018; Jensen & Benfey, 2022), but not through the lens of dietary manipulation.

2. METHODS

2.1 Husbandry Information

This research was approved by the UNB Animal Care Committee (Animal Use Protocol 22004), following the guidelines of the Canadian Council on Animal Care. The fish used for this research were spawned in the University of New Brunswick aquatic facility on November 30th, 2020, from an in-house breeding program of a mixed progeny of three-year-old brook charr (23 females, 18 males). Triploidy was induced in half of the eggs via a hydrostatic pressure shock treatment at 65.5 mPa for 5 minutes, which began at 200°C-min post-fertilization (Model: TRC-APVM, TRC Hydrolics Inc., Dieppe, NB, CA). The remaining half of the eggs were left untreated as diploid controls. The embryos, both triploid and diploid, were left to incubate in the dark until yolk-sac absorption occurred in February of 2021. Fry were then moved into separate 56L circular flow-through tanks, based on assumed ploidy, and supplied with dechlorinated municipal water at ambient temperature and reared according to standard salmonid protocols (Jobling et al., 2010).

The fish used for this research (n = 450) came from two of the previously described stock tanks. They were visually size matched and transferred into a photoperiod-controlled room with eighteen 56L circular tanks (25 fish per tank, with 3 tanks per ploidy–diet combination) on January 18th, 2022 (Figure 1). Tank assignments (ploidy and diet; Figure 2) were predetermined using a random number generator. The tanks were supplied dechlorinated municipal water through a custom-bult PVC flow-through system fed by two connected 416L head tanks, oxygenated by compressed air via

two large air stones in each head tank, with the water passing through de-gassing columns prior to entering each head tank (Figure 1). The system was designed so that each tank had its own valve to control water flow and to evenly supply all the tanks in the system from both head tanks. Water flow into each tank was maintained at approximately 1L/min (checked biweekly) and the photoperiod set to have lights on from 8:00 AM to 6:30 PM. Tank dissolved oxygen and temperature were monitored daily using a handheld oxygen meter (Model: ProODO^{TM}, YSI Inc., Yellow Springs, OH, USA), and ranged between 78 – 99% air saturation, with temperature consistent with seasonal trends (between 9.6°C – 15°C).

Fish were initially fed a maintenance ration (0.5% of tank biomass) of standard 3mm floating salmonid pellets (Corey Feed Mills Ltd., Fredericton, NB, CA) (Table 1) once daily between 8:30 and 9:00 AM. On February 3rd, 2022, fish were anesthetized in a 50mg/L solution of ethyl 4-aminobenzoate (Sigma-Aldrich; Product # 1003344493), measured (body mass [to 0.1g] and fork length [to 0.1cm]), as to compare growth after trials had been completed, and their pelvic fins clipped for later identification (no clip for control diet and right and left fin clips for the intermediate and high diets, respectively) (diet formulation is detailed below). Feeding of the experimental diets began on March 28th, 2022, with the goal of tripling fish body mass prior to starting hypoxia and high-temperature tolerance testing (detailed below). Fish were fed once in the morning, between 8:30 and 9:00 AM, and again in the evening between 5:30 and 6:00 PM. They were initially fed a total of 2% of tank biomass daily, divided equally between the two feedings, but this was later reduced to 1.5% per day on April 30th, 2022, and kept at this

percentage for the remainder of the feeding period, due to most of the fish exhibiting an unwillingness to consume the entire daily ration.

2.2 Experimental Diets

Standard 3mm floating salmonid pellets (Corey Feed Mills Ltd.), the same feed used prior to the March 28th feeding period, were used as the control diet (Table 1), hereafter designated as the "low" AX diet. The "high" diet was created by Corey Feed Mills using their in-house top-coating procedure. Briefly, 200kg of 3mm pellets were placed in an industrial mixer, 2kg of poultry fat (Sanimax Marketing Ltd.; Guelph, ON, CA) was added as a binding agent, and the pellets were thoroughly mixed to ensure equal coating of the fat. Next, 0.38kg of synthetic astaxanthin powder (AF/L AstaFeed 10%; Product Code ANAX10K, Divis Nutraceuticals, Florham Park, NJ, USA) was added and the pellets were once again thoroughly mixed to allow the powder to bind to the pellets. Pellets were then dried, bagged, and stored frozen. The "intermediate" diet was created, as needed, by mixing pellets from the "low" and "high" diets in a ratio of 17:8 (low/high). The actual AX concentration of these diets was determined independently by the Research and Productivity Council (Fredericton, NB, CA) and the Valorēs Research Institute (Shippagan, NB, CA), and are hereafter taken to be 18, 80, and 190 mg/kg for the "low", "intermediate", and "high" diets, respectively (Table 2). A more thorough analysis of the composition of these diets was obtained from the Valorēs Research Institute (Table 2).

2.3 Acute Hypoxia Trials

Hypoxia trials began on May 30th, 2022, with two trials conducted daily (one beginning between 9:20 and 9:30 AM and the other between 12:00 and 12:30 PM) for six consecutive days. Each trial used two fish per ploidy–diet combination (i.e., 12 fish per trial) with tanks randomly assigned to a trial such that each home tank was sampled four times across the six days (n = 144 fish in total, with 24 for each ploidy–diet combination). Feed was withheld from the assigned home tanks for 24 hours prior to fish removal for experimentation on any given day to reduce any effects of feeding on metabolism, which could potentially impact the stress tolerance tests. Test fish for a given trial were randomly selected from the assigned tanks and transferred to a 44L stainless steel water bath (90 cm × 28 cm × 17.5 cm deep) (Figure 3) containing ~40L of dechlorinated municipal water from the same source and left at the same temperature as that of the home tanks. Compressed air was injected into the water using a gas-bubble diffuser to maintain dissolved oxygen as close to 100% of air saturation prior to commencement of trials (maintained between 80 – 87 % of air saturation and between 15.4 – 16.3°C across all trials). Dissolved oxygen and water temperature were monitored using the same handheld YSI meter described above and a calibrated digital thermometer (Model: VWR Total-Range Digital Thermometer, VWR International, Radnor, PA, USA), respectively. The water bath also contained an immersion heater/circulator (Model: D3; Haake, Berlin, DE) (Figure 3) used to ensure uniform mixing throughout the water bath, with the heating element turned off. This circulator was placed behind a mesh-PVC screen to prevent fish from interacting with it. A clear piece of plexiglass was used as a covering for the water bath to help prevent the escape of any fish.

The immersion circulator and compressed air were turned on after the fish were transferred to the water bath, and the fish were habituated to the experimental water bath for 30 minutes, which was modified from previous work on triploid acute hypoxia and thermal tolerance (e.g., Ellis et al., 2013; Benfey & Devlin, 2018; Jensen & Benfey, 2022), and based on past research which shows that triploidy has no effect on acute handling stress (Biron & Benfey, 1994; Benfey & Biron, 2000; Fraser et al., 2012). The compressed air was then turned off and the bubble diffusers removed, and two microbubble gas diffusers (Model: MBD 300; Pentair Aquatic Eco-systems, Apopka, FL, USA), connected to an external tank of compressed nitrogen gas, were then used to inject compressed nitrogen into the water to displace oxygen. Oxygen concentration was assessed continuously via the YSI handheld oxygen meter, and a steady rate of decline was maintained by manually adjusting the valve on the nitrogen cylinder, with the goal of 1.4% dissolved oxygen reduction per minute. Temperature, dissolved oxygen (as % of air saturation), and time elapsed were recorded for each fish as it lost equilibrium (LOE), i.e., where the fish was unable to maintain upright dorsal–ventral positioning for 5 seconds. Each fish was then removed from the water bath via a dipnet and transferred to an individual recovery bucket containing ~6L of dechlorinated, oxygenated water at the ambient water temperature of the home tanks.

Once all 12 fish had sufficiently recovered from the trial, they were anesthetized (as above) in the same order as they had reached LOE, and a ~1mL sample of blood was collected from the caudal vasculature. The time elapsed from LOE to collection of the blood sample was not recorded for individual fish but ranged from approximately five to 10 minutes. A blood smear was made and set aside for ploidy confirmation, as detailed

below. Heparinized capillary tubes were used to determine hematocrit (in duplicate), and single measurements of blood glucose and lactate levels (mmol/L) were collected via a FreeStyle Lite (Abbot Diabetes Care Ltd., Whitney, Oxen, UK) and Lactate Plus meters (Nova Biomedical, Waltham, MA, USA), respectively. Blood samples were diluted (1:200 blood: isotonic saline [0.9% NaCl]), transferred to a Neubauer hemocytometer, covered with a cover slip, and photographed at 10X magnification to determine average red blood cell count per sample. Each fish was then euthanized in 500mg/L solution of ethyl 4-aminobenzoate for measurement and dissection (see below).

2.4 Critical Thermal Maximum (CTmax) Trials

CTmax trials were also completed in six days, beginning on June 6th, 2022, and followed the same protocol as outlined for the hypoxia trials, but adding a second gas-bubble diffuser supplying more dissolved oxygen to the water prior to the commencement of trials, as the starting dissolved oxygen for the hypoxia trials was not adequate. The nitrogen gas injection was replaced by the steady increase of temperature, for a desired 0.4°C/min using the heating element of the Haake immersion heater/circulator described above. Water was maintained at ambient temperature prior to the introduction of thermal stress (maintained between 14.4 – 16.2°C and between 94 – 98% air saturation across all trials).

2.5 Dissections

Fish were measured immediately following euthanasia (body mass [to 0.01g], and fork length [to 0.01cm]), and their dietary treatment group determined from their pelvic fin clip status. Large dissection scissors were used to sever the spinal cord just posterior

to the skull and then a second cut was made along the entire length of the abdominal (ventral) side of the fish from vent to opercula. The gonads were excised and weighed (to 0.01g), and the sex and relative maturity of each fish determined visually. The whole liver and a sample of the left epaxial muscle (from between the dorsal fin and lateral line) were excised, weighed (to 0.01g), and transferred to a separate 10mL scintillation vial on ice (until all fish had been processed) and then frozen for later astaxanthin concentration analysis, as detailed below. The entire right lateral fillet was excised from three of the larger fish per trial (i.e., 1 fish per diet) and frozen at −20°C for later pigment analysis (hypoxia: n = 14, 12, and 12 samples of the low, intermediate, and high diets, respectively; CTmax: n = 12, 12, and 12 sample of the low, intermediate, and high diets, respectively), as detailed below. Larger fish were intentionally selected for fillet analysis due to size limitations of the instrumentation.

The measurements described above were used to calculate Fulton's condition factor (K), gonadosomatic index (GSI), and hepatosomatic index (HSI) using the following formulae:

Formula 1: $$K = \frac{10^2 \times Body\ mass\ (g)}{Fork\ length\ (cm)^3}$$

Formula 2: $$GSI\ (\%) = \frac{Combined\ gonad\ mass\ (g)}{Body\ mass\ (g)} \times 100$$

Formula 3: $$HSI\ (\%) = \frac{Whole\ liver\ mass\ (g)}{Body\ mass\ (g)} \times 100$$

2.6 Ploidy Confirmation and Hematology

Ploidy was confirmed from blood smears as described by Benfey & Devlin (2018) using image analysis software (AmScope MU900 AmLite for macOS version

2.1.18573.20210304) to measure red blood cell (RBC) length. Average RBC count was calculated using the digital images from the Neubauer hemocytometer (Formula 4) and these data were used to calculate mean RBC volume (Formula 5):

Formula 4: $$RBC\ count\ \left(\frac{cells}{mm^3}\right) = \frac{(Sum\ of\ RBCs\ counted \times dilution\ (200)}{Total\ area\ measured\ (0.2mm^2) \times depth\ (0.1mm)}$$

Formula 5: $$RBC\ volume\ (\mu m^3) = \frac{hct\ (\%) \times 10^9}{RBC\ count\ \left(\frac{cells}{mm^2}\right) \times 100\%}$$

2.7 Astaxanthin (AX) Concentration in Tissue

Excised livers and muscle tissue samples were randomly selected for two rounds of analysis, with the first occurring on July 8th, 2022, and the second on September 2nd, 2022 (54 of each tissue per round of sampling, *n* = 108 total samples). The samples were thawed and then homogenized using a tissue homogenizer (Model: Brinkmann PT 10/35, 110 Volts, 6 Amps, 60 Hz; Brinkmann Instruments Co., Switzerland) in liquid chromatography mass spectrometry (LC-MS) grade methanol (OmniSolv; CAS-No: 67-56-1) in ratios of 1:2 for liver and 1:3 for muscle (tissue:methanol [w:v]). Samples were homogenized until little to no solid tissue remained. Once homogenized, a 1mL volume was centrifuged for 10 minutes at 15,000 RPM (Model: 5425, Eppendorf, Hamburg, DE) and the resulting supernatant decanted into separate 1mL microtubes and refrigerated. Prior to analyzing these samples, a 1mL syringe fitted with a 0.2µm membrane filter (VWR North America; Cat. No. 28145-491) was first cleaned by plunging 1mL of LC-MS grade methanol through the syringe/filter two times. A 100µL sample of supernatant and 100µL of LC-MS grade methanol was then added to the cleaned syringe/filter and deposited into a 2mL autosampler vial and capped (VWR North America; Cat. No. 46610-724A).

The samples were analyzed by the Ultivo Triple Quadrupole LC-MS/MS system from Agilent (Agilent Technologies, Inc., Santa Clara, CA, USA) to determine each sample's AX concentration (Department of Chemistry, University of New Brunswick, NB, Canada). It was equipped with a Poroshel 120 EC-C18 column (100 × 4.6mm, particle size: 2.7μm) (Agilent Technologies, Inc., Santa Clara, CA, USA) and photodiode array detector (range: 190 – 640nm). Pre-made astaxanthin standards were injected into the system (1, 2, 4, 8, 16, 28 ng/μL), produced from 97% AX powder (Sigma Aldrich; CAS No: 472-61-7), and were used for both analyses. Two solvents were used: 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The linear elution gradient used for both analyses was 1:3 (A:B) for 0–5.6 minutes, 1:99 for 5.6–6 minutes, and 1:3 for 6.01–8 minutes, with flow kept at 0.6mL/min.

2.8 Muscle Pigment Analysis

The excised fillets were removed from their storage at −20°C and placed on dry ice and transported to the Agricultural Campus of Dalhousie University in Truro, Nova Scotia (CA), on September 15th, 2022, for pigment/colour analyses. The samples were thawed and analyzed using a Hunter Lab MiniScan EZ Spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA, USA). Lightness (L), red–green intensity (a), and blue–yellow intensity (b) coordinate values were collected from each sample. "L" is measured on a scale from 0 to 100, with 0 indicating perfect black and 100 indicating perfect white. "a" and "b" are measured on a positive and negative scale, with positive values corresponding to red/yellow and negative values corresponding to green/blue, respectively, and with "0" acting as neutral for both values. The L*a*b colour values

were then used to calculate the lightness, redness index, hue (colour), and chroma (colour intensity/saturation) of each sample using the following formulae (Ottestad et al., 20l2; Wei et al., 2021):

Formula 6: $Redness\ Index = (\frac{a}{b})$

Formula 7: $Hue = tan^{-1}(\frac{a}{b})$

Formula 8: $Chroma = (a^2 + b^2)^{0.5}$

In addition to the L*a*b colour values, a Roche SalmoFan™ (DSM Nutritional Products Canada Inc., Dartmouth, NS, CA) designed specifically to assess the colour of salmon fillets was used to visually assess the colour of each fillet. Each sample was given a value, chosen by myself, between 20 and 34 (i.e., the SalmoFan™ scale), with 20 indicating the least amount of colouration and 34 indicating the most.

2.9 Statistical Analysis

All statistical analyses were performed in R version 4.1.2 (R Core Team, 2021). Separate linear mixed-effects models (lme4 package, version 1.1-28; Bates et al., 2015) were used to test the effects of ploidy and diet on time to LOE (Model 1) and PO_2 at LOE (Model 2) for the hypoxia experiment, and time to LOE (Model 3) and temperature at LOE (Model 4) for the CTmax experiment:

Model 1:

```
M1.lmer<-lmer(LOE.Time~(Ploidy*Diet)+(1|Trial)+(1|Tank),data=d)
```

Model 2:

```
M2.lmer<-lmer(LOE.PO2~(Ploidy*Diet)+(1|Trial)+(1|Tank),data=d)
```

Model 3:

M3.lmer<-lmer(LOE.Time~(Ploidy*Diet)+(1|Trial)+(1|Tank),data=d)

Model 4:

M4.lmer<-lmer(LOE.Temp~(Ploidy*Diet)+(1|Trial)+(1|Tank),data=d)

The variables evaluated were Ploidy (2 levels) and Diet (3 levels), which were included as fixed effects. Trial (12 levels) and Tank (18 levels) were included as random effects. Sex of the fish was not included in the analysis because all fish had very low GSIs (see Results), and gonadal development could therefore be excluded as a confounding variable. All data met the assumption of homoscedasticity. Linear mixed-effect models were built to determine if ploidy and diet had any effect on the body mass, fork length, and condition factors of the original population of fish (n = 450) prior to the feeding period, to be used as reference for post-experimental growth models, with tank (18 levels) and individual fish (25 levels) added as random effects. Similar to the LOE models, additional linear mixed-effects models were built to determine if ploidy and diet had any effects on post-experimental body mass, fork length, condition factor, blood glucose and blood lactate, hematocrit, RBC count and RBC volume, HSI, retained AX in the muscle and liver, muscle lightness, muscle red–green and blue–yellow intensities, and muscle redness, hue, chroma, and SalmoFan values. The random effects included in the models described above were used for all analyses, only deviating from these models when one produced either a singularity or convergence error. Singularity occurs when the predictor variables used in the model are perfectly correlated with each other, making it impossible to estimate unique coefficients for each of the predictors. Convergence errors

stem from the model's optimization process failing to find any further adjustments which could improve the fit of the data. In the case of a singularity error, model selection using the ranova () function was used to remove the least significant random effect until the model was fitted without a singularity. If both random effects were estimated as zero (i.e., highly insignificant), both variables were removed, and that specific model was re-run to determine which of the least significant variables should be removed. In the case of a convergence error, model selection with the ranova() function occurred in an equivalent manner; however, if both of the random effects were still estimated as zero, the accuracy of the model could not be determined and the model was not fit for that particular variable.

Separate correlation matrices were created to determine if LOE parameters (oxygen concentration and temperature at LOE for the hypoxia and CTmax experiments, respectively, as well as time to LOE for both) were correlated with body mass, fork length, condition factor, or HSI, and if the LOE parameters were correlated with blood glucose, blood lactate, hematocrit, RBC count, or RBC volume. Separate matrices were also created to determine if these LOE parameters were correlated with AX concentration in the muscle or liver. The LOE data for one fish of the last CTmax experimental trial were excluded from all statistical analyses because it had wedged itself behind the net separating the water bath from the heater/circulator. Thus, the hypoxia experiment evaluated 144 fish, whereas the CTmax experiment evaluated 143 fish. Post-hoc Tukey's tests were performed in the instance of any significant findings from these analyses. A significance level of $\alpha = 0.05$ was used for all tests.

3. RESULTS

Average astaxanthin (AX) concentrations of the three experimental diets were approximately 18, 80, and 190 mg/kg for the low, intermediate, and high AX diets, respectively (Table 2). Erythrocyte length measurements of the fish used in the acute hypoxia and CTmax experiments (n = 288; 144 fish per experiment) revealed two distinct populations, i.e., diploids and triploids (Figure 4). One fish was not of the assumed ploidy: a presumed triploid from the fourth CTmax trial was found to be a diploid and was treated as such in all subsequent analyses. All analyses were therefore based on 72 diploids and 72 triploids (n = 144) for the acute hypoxia experiment, and 73 diploids and 70 triploids (n = 143) for the CTmax experiment, with one less fish in the latter case following the removal of all data from a triploid that had wedged itself behind the mesh-PVC net separating the water bath from the heater/circulator (as noted above).

Triploids weighed less (by ~5%) and had lower condition factor (by ~3%) than diploids within the initial populations of fish used to stock the experimental tanks (n = 450) prior to feeding the experimental diets (Tables 3 & 4). The sex ratio of the fish subsequently used in the experiments was 53:47 (male:female), but visual examination of excised gonads and measurement of GSI showed that all fish were immature (GSI < 1%), and sex therefore could be excluded from the analyses as a confounding variable.

It is important to note that during pigment analyses, a number of the fillet samples were too small (n = 7 and 11 samples from the hypoxia and CTmax trials, respectively), despite randomly selecting the largest three fish per diet of any specific trial to avoid this issue and were not completely contained by the Hunter Lab MiniScan EZ Spectrophotometer, possibly allowing for light to enter influence lightness values. Also,

several samples had notable blood clots (n = 3 samples per experiment) which may have influenced red–green intensity, redness index, and hue values.

Statistical analysis of the data using the lmer () function in R produced singularity errors in both experiments, resulting in modifications to the specific base models (see Methods). When included as a random factor, Tank was extremely non-significant and was removed in the following hypoxia lmer models to resolve a singularity error: condition factor of the initial population prior to feeding of the experimental diets, all final body parameters, blood glucose and lactate, RBC count (also resolving an additional convergence error) and RBC volume, muscle AX content, and all muscle pigment values except hue, with chroma being removed from the analyses due to an additional unresolved convergence error (see Methods for statistical approach). When included as a random factor, the variable Tank was extremely non-significant and was removed from the following CTmax lmer models to resolve a singularity error: final body mass and condition factor, all LOE and blood parameters, muscle and liver AX content, and muscle lightness. Trial was also added as a random factor and by removing it, when non-significant, from the following CTmax lmer models fixed singularity errors in the models: final HSI, SalmoFan, blue-green intensity, and chroma (which also resolved an additional convergence error).

3.1 Acute Hypoxia Experiment

Final body mass approximately tripled from the start of the eight-week feeding period to the end of the acute hypoxia experiment, with an average weight gain of ~90g (197%) across all three diets, and with fork length and condition factor increasing by

~6.1cm (42%) and ~0.06 (4%), respectively (Table 5). There was a significant effect of both ploidy and diet on body mass ($p = 0.029$ and 0.019, respectively), and diet on fork length ($p = 0.003$), but there was no significant interaction between ploidy and diet found on any of these parameters following the hypoxia experiment (Table 6). Triploids weighed less than diploids (by ~7%) but did not differ in condition factor; however, triploids were also lighter than diploids at the beginning of the feeding trials. Tukey's post-hoc analyses determined that fish fed the high AX diet were significantly heavier (by ~6%) and longer (by ~3%) than those fed the low AX diet, but the fish fed the intermediate AX diet were neither heavier nor longer than those fed the high or low AX diets. Ploidy, but not diet, had a significant effect on HSI ($p < 0.001$), with triploids having lower values than diploids (by ~10%; Tables 5 & 6).

The average rate of oxygen depletion during the acute hypoxia trials was 1.43 ± 0.10% of air saturation per minute (mean ± SD) (Figure 5). Ploidy, but not diet, affected both time to LOE and the PO_2 at LOE (Table 7), with triploids reaching LOE sooner than diploids (40.77 ± 4.25 min versus 41.57 ± 4.33 min, respectively [mean ± SD]; Figure 6) and at higher PO_2 (4.95 ± 0.55 kPa versus 4.66 ± 0.55 kPa, respectively [mean ± SD]; Figure 7). Triploids had significantly lower post-hypoxia hematocrit and RBC count ($p = 0.040$ and < 0.001, respectively), and larger RBC volume ($p < 0.001$), than diploids, but diet had no effect on any of these parameters, and neither ploidy nor diet affected post-hypoxia blood glucose or lactate levels (Tables 5 & 7). Neither ploidy nor diet affected muscle or liver AX content, and none of the muscle pigment parameters were affected by ploidy (Table 8). Diet did, however, affect SalmoFan, and red–green intensity values ($p < 0.001$ and 0.021, respectively) (Tables 5 & 8), with a Tukey's post-hoc analysis

determining that fish fed the high AX diet showed greater values than those fed the low AX diet. Fish fed the intermediate AX diet also showed greater SalmoFan values than those fed the low AX diet, while the other muscle pigment parameters did not differ between the intermediate and low AX diets.

There was a significant negative correlation ($r = -0.32$) between time to LOE and PO_2 at LOE (Table 9). Time to LOE was positively correlated with RBC count (r = 0.23) and liver AX content (r = 0.30) and negatively correlated with HSI (r = −0.22) and RBC volume (r = −0.24), whereas PO_2 at LOE was negatively correlated with RBC count (r = −0.30) and positively correlated with RBC volume (r = 0.30) (Tables 9-11). There was a significant negative correlation (r = −0.73) between RBC count and RBC volume, and a positive correlation (r = 0.33) between blood glucose and lactate (Table 10).

3.2 Critical Thermal Maximum (CTmax) Experiment

Including an extra week of feeding before the start of the CTmax experiment (i.e., the time during which the hypoxia trials were underway) resulted in an increase in final body mass (by ~91g or 201% from the initial weight) but no further increase in fork length or change in condition factor (Table 12). Neither ploidy nor diet affected the final body mass, fork length, or HSI; however, ploidy, but not diet, had a significant effect on condition factor (p = 0.018), with triploids having lower values (by ~2%) than diploids (Tables 12 & 13). No significant interaction terms were noted (Table 13).

The average rate of temperature increase during the CTmax trials was 0.37 ± 0.01 °C per minute (mean ± SD), with minimal variation among trials (Figure 8). Neither ploidy nor diet affected time to LOE or temperature at LOE (38.59 ± 1.76 min and 29.47

± 0.60 °C, respectively [mean ± SD]; Table 14, Figures 9 & 10). Like the hypoxia experiment, diet had no effect on any hematological parameter, and ploidy had no effect on post-CTmax blood glucose and lactate levels. Ploidy was, however, found to affect both RBC count and RBC volume ($p < 0.001$ for both variables), with triploids having lower and greater values, respectively, than diploids (Tables 12 & 14). Ploidy again had no effect on muscle or liver AX content, or any of the muscle pigment parameters (Table 15); however, unlike the hypoxia experiment, diet affected both muscle and liver AX content (Table 15), with Tukey's post-hoc analysis determining that fish fed the high AX diet had significantly greater values for both tissues than those fed the low diet ($p < 0.001$ for both variables), and those fish fed the intermediate AX diet had greater muscle AX content than those fed the low AX diet (Table 12). Diet had no effect on any of the muscle pigment parameters (Table 15).

There was no correlation between time to LOE and temperature at LOE, but both were negatively correlated with body mass ($r = -0.20$ for both parameters; Table 16) and RBC volume ($r = -0.20$ for both parameters; Table 17). Time to LOE was negatively correlated with both blood glucose and lactate ($r = -0.22$ and -0.20, respectively), while temperature at LOE was positively correlated with blood glucose only ($r = 0.30$) (Table 17). Similar to the hypoxia experiment, there was a significant negative correlation ($r = -0.90$) between RBC count and RBC volume, and a positive correlation ($r = 0.23$) between blood glucose and lactate (Table 17), but unlike the hypoxia experiment, there was a significant positive correlation ($r = 0.40$) between muscle and liver AX content, and a significant positive correlation between both tissues and body mass ($r = 0.41$ and 0.31, respectively; Table 18).

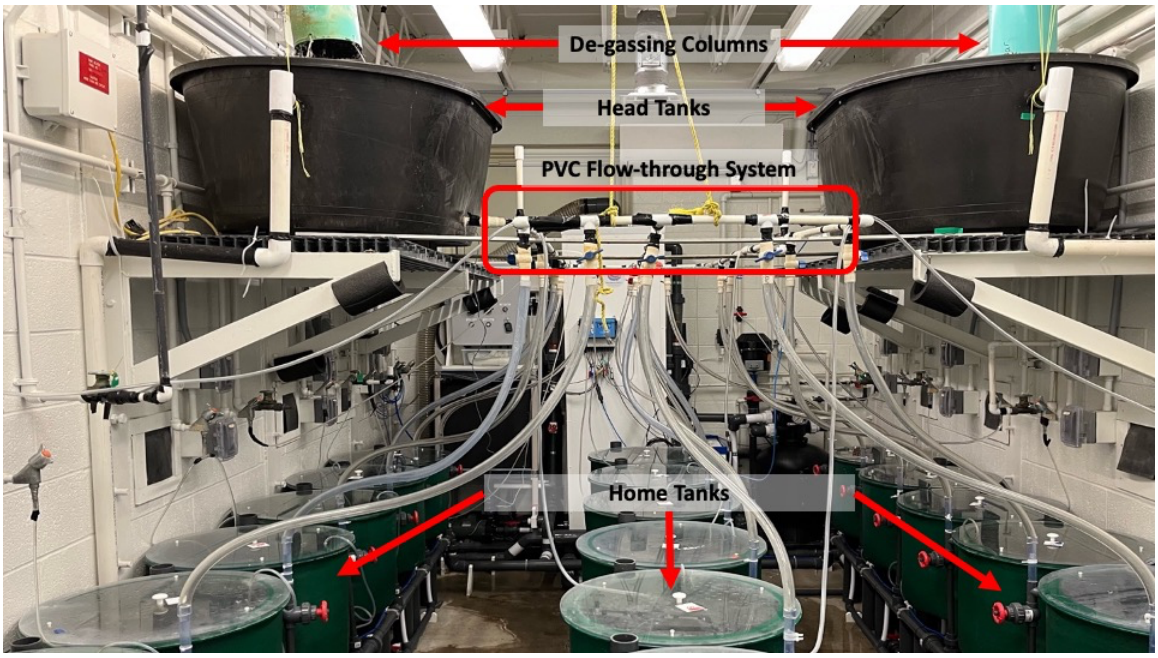

Figure 1: Tank setup for the experimental rearing and feeding period. Municipal water was supplied to the two 416L head tanks (after first passing through de-gassing columns). From the head tanks, water was delivered to each individual home tank using a custom-built PVC flow-through system to allow for equal ambient water distribution and individual flow manipulation to each of the 18 home tanks.

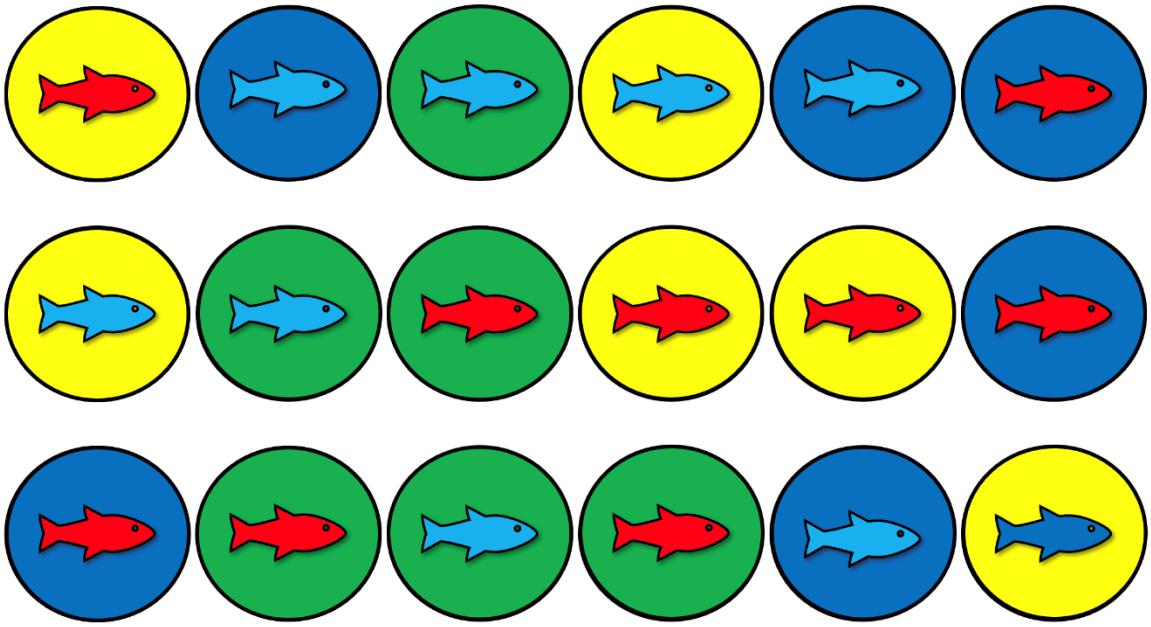

Figure 2: Home tank layout showing specific ploidy and diet designations. The blue and red fish represent tanks containing diploids and triploids, respectively, and the blue, yellow, and green circles represent tanks receiving the low (control), intermediate, and high astaxanthin (AX) content diets, respectively (n = 450 fish in total, with 25 fish per tank, 225 per ploidy, and 150 per diet). Experimental tanks (ploidy and diet designations) were randomly assigned.

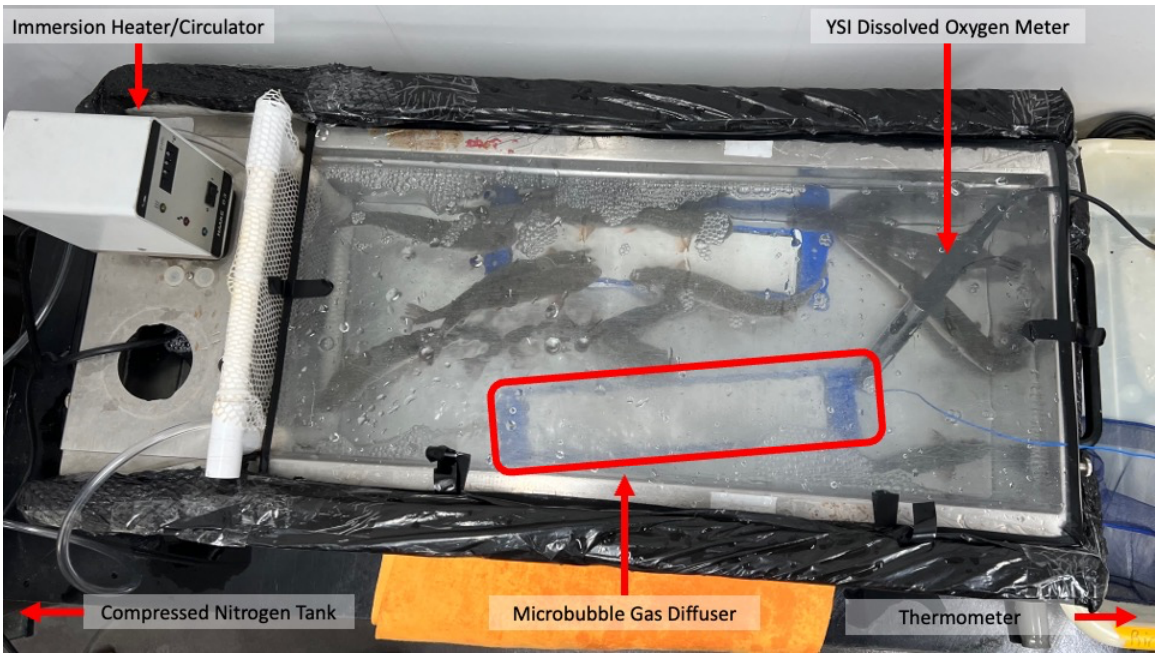

Figure 3: Experimental water bath used for both the acute hypoxia and critical thermal maximum (CTmax) experimental trials. The nitrogen gas microbubble diffuser was removed for the CTmax experiment. Water was circulated and heated using the immersion heater/circulator for the hypoxia and CTmax trials, respectively. Water dissolved oxygen and temperature were monitored continuously during both experiments using a YSI dissolved oxygen meter and thermometer, respectively.

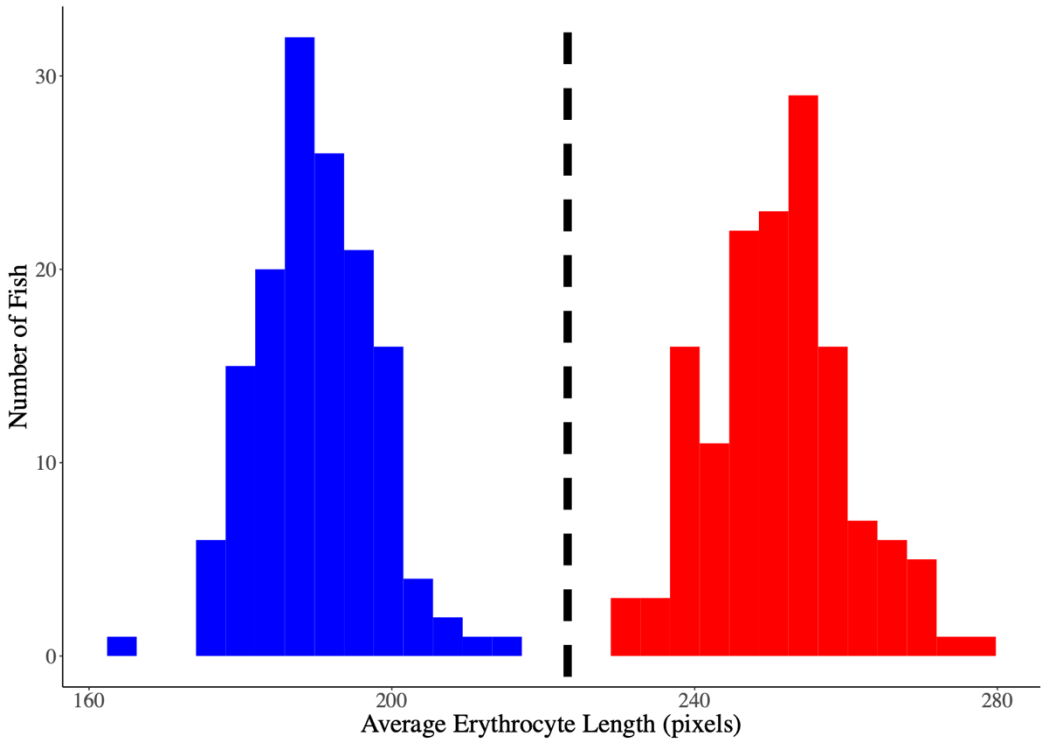

Figure 4: Erythrocyte length distribution for juvenile brook charr (*Salvelinus fontinalis*) used to determine the effect of ploidy and dietary astaxanthin (AX) supplementation on acute hypoxia and critical thermal maximum (CTmax). The dashed line indicates the cutoff used to classify a fish as either diploid (blue) or triploid (red) (n = 288 [144 fish per experiment]).

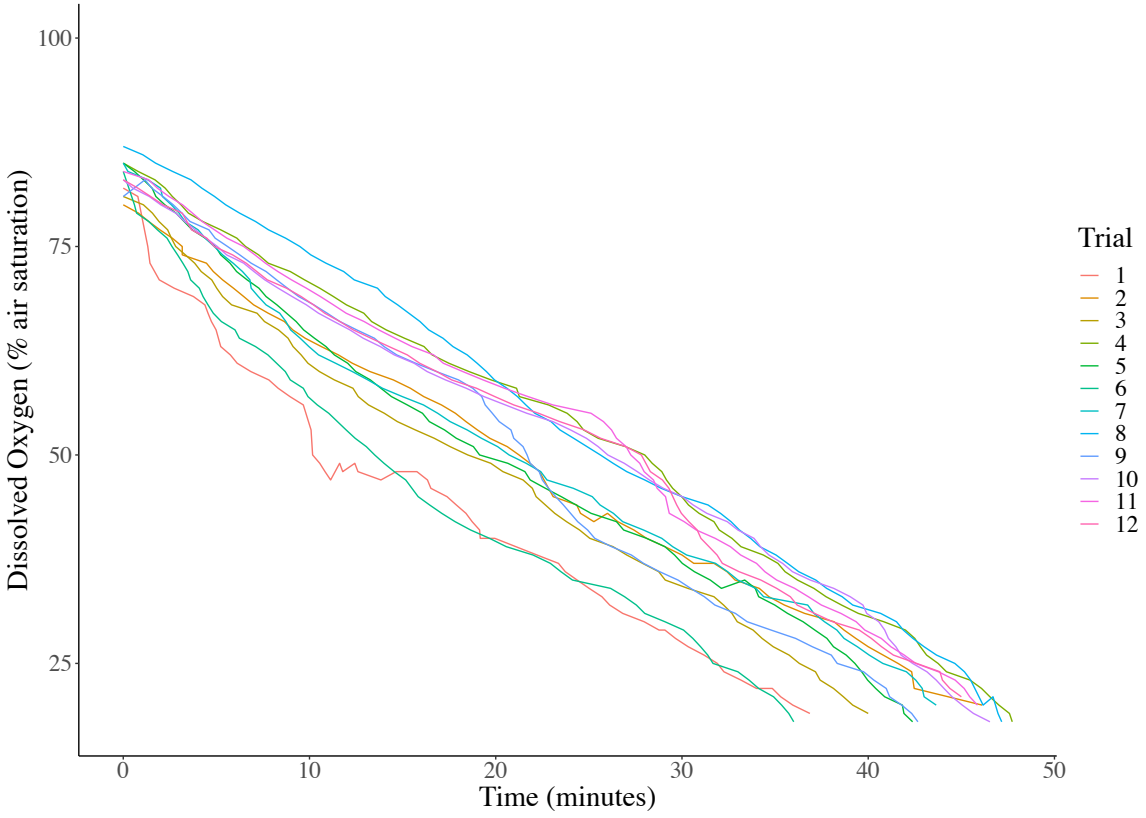

Figure 5: Rate of oxygen depletion during the acute hypoxia trials. Each line represents a single trial with 12 fish (n = 12 replicates). The average rate was 1.43% of air saturation decrease per minute.

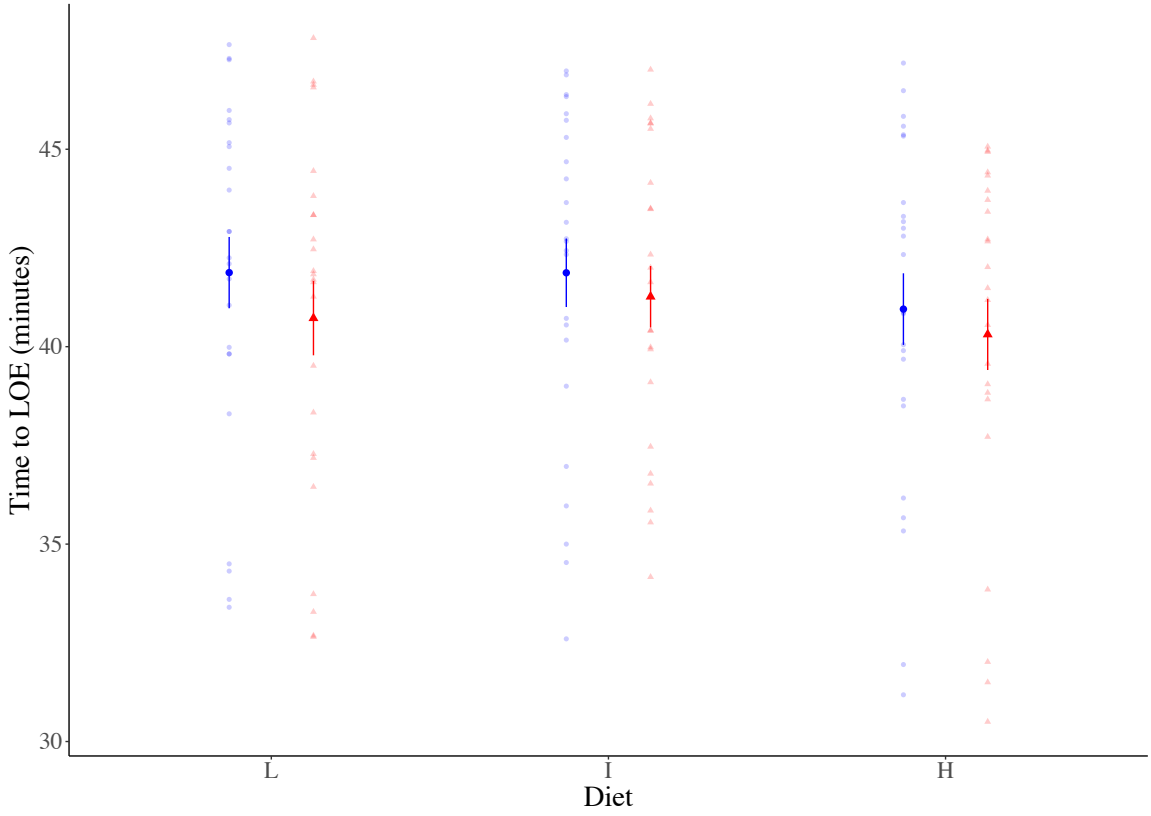

Figure 6: The effect of ploidy and dietary astaxanthin (AX) content on time to loss of equilibrium (LOE) of individual juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia stress. Diploids are represented by the blue circles and triploids by the red triangles. Dietary AX supplementation is represented by L, I, and H for the low, intermediate, and high AX diets, respectively (mean ± SE, n = 24 fish per ploidy-diet combination [144 fish in total]).

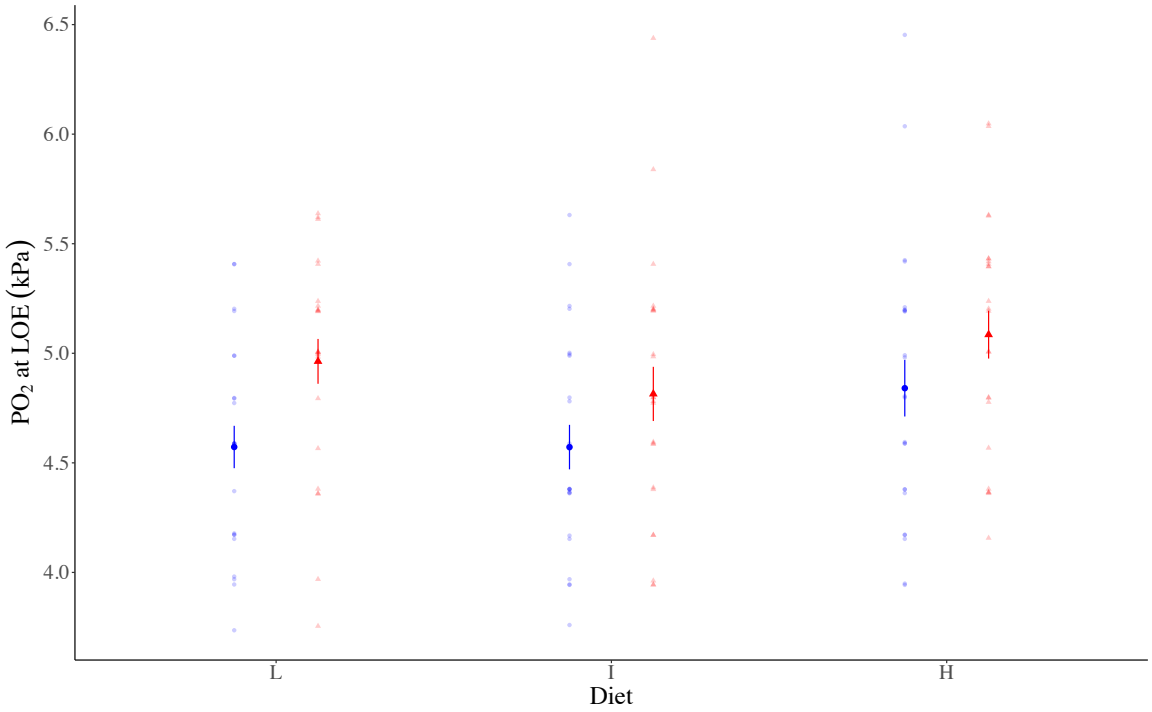

Figure 7: The effect of ploidy and dietary astaxanthin (AX) content on the PO_2 value at loss of equilibrium (LOE) of individual juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia stress. Diploids are represented by the blue circles and triploids by the red triangles. Dietary AX supplementation group is represented by L, I, and H for the low, intermediate, and high AX diets, respectively (mean ± SE, n = 24 fish per ploidy-diet combination [144 fish in total]).

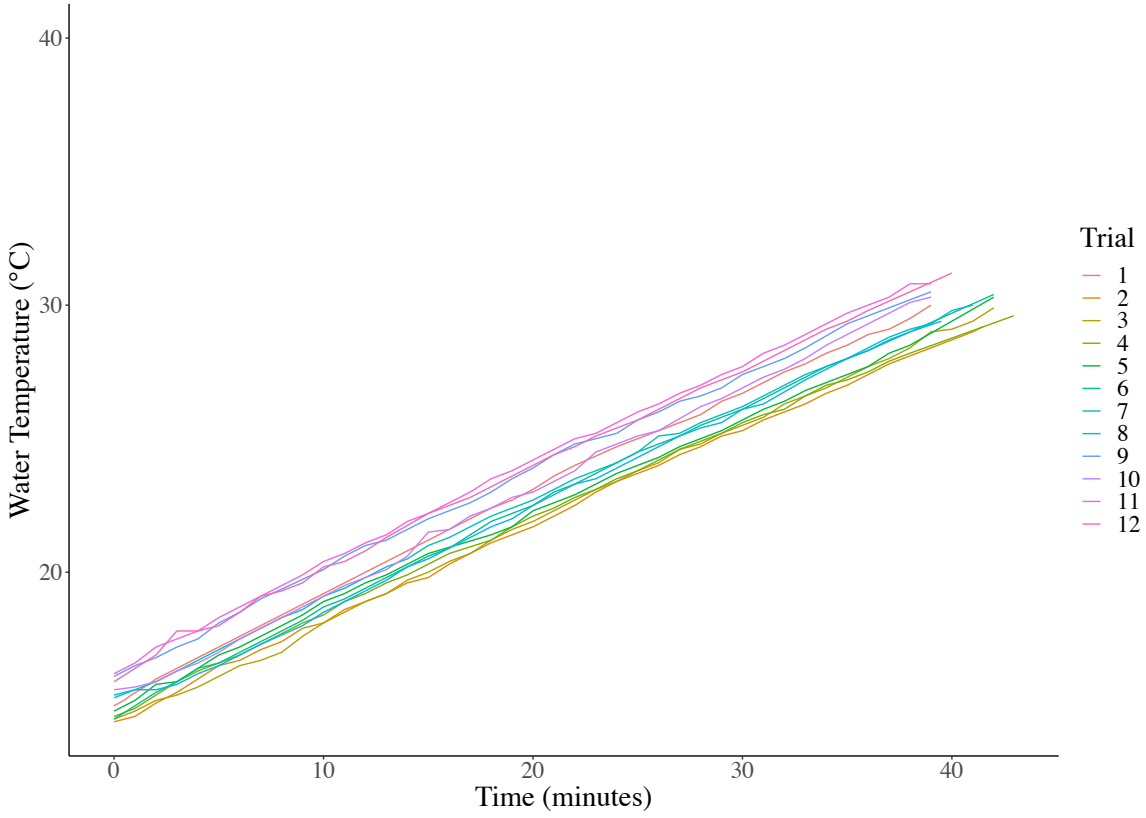

Figure 8: Rate of temperature increase during the critical thermal maximum (CTmax) trials. Each line represents a single trial with 12 fish (n = 12 replicates). The average rate was 0.37°C increase per minute.

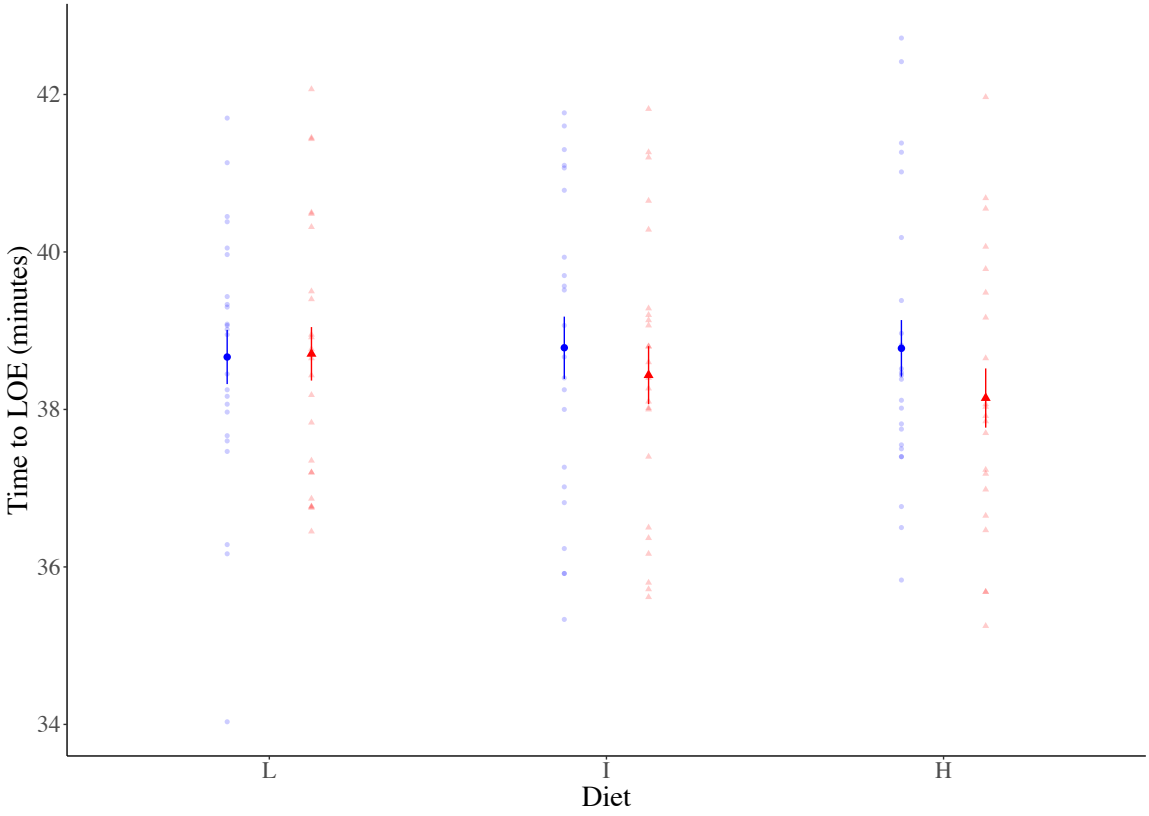

Figure 9: The effect of ploidy and dietary astaxanthin (AX) content on time to loss of equilibrium (LOE) of individual juvenile brook charr (*Salvelinus fontinalis*) at their critical thermal maximum (CTmax). Diploids are represented by the blue circles and triploids by the red triangles. Dietary AX supplementation group is represented by L, I, and H for the low, intermediate, and high AX diets, respectively (mean ± SE, n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]).

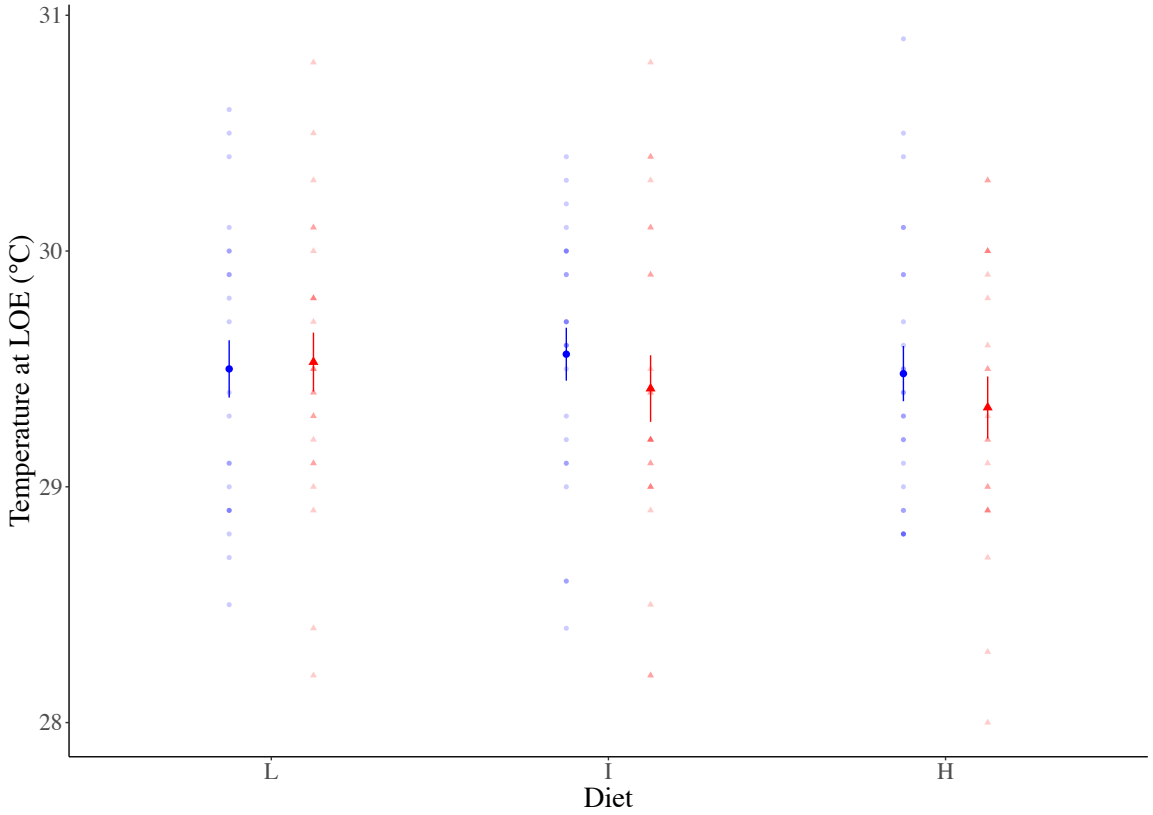

Figure 10: The effect of ploidy and dietary astaxanthin (AX) content on temperature at loss of equilibrium (LOE) of individual juvenile brook charr (*Salvelinus fontinalis*) at their critical thermal maximum (CTmax). Diploids are represented by the blue circles and triploids by the red triangles. Dietary AX supplementation is represented by L, I, and H for the low, intermediate, and high AX diets, respectively (mean ± SE, n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]).

Table 1: Corey Feed Mills salmonid feed analysis (control, low astaxanthin diet, 3mm diameter floating pellets).

Ingredient	Amount Incorporated
Crude Protein	42% (minimum)
Crude Fat	14% (minimum)
Crude Fiber	2.9% (maximum)
Calcium	1.0% (actual)
Phosphorous	1.0% (actual)
Sodium	0.45% (actual)
Vitamin A	2500 IU/kg (minimum)
Vitamin D3	2400 IU/kg (minimum)
Vitamin E	160 IU/kg (minimum)

Table 2: Independent analyses of the control and experimental diets. The values in the top portion of the table represent independently measured astaxanthin (AX) concentrations (mg/kg) of all three diets and their corresponding averages, while the values denoted by an asterisk were analyzed separately by the Valorēs Research Institute.

	Control (Low AX)	Intermediate AX	High AX
Research and Productivity Council	15.6	76.6	171.9
Valorēs Research Institute	21.2	83.1	205.4
Average	18.4	79.85	188.65
Fat (%) *	13.8	15.1	15.5
Total Astaxanthin (mg/kg) *	21.2	83.1	205.4
All-trans Astaxanthin (mg/kg) *	14.8	61.3	146.0
9-cis Astaxanthin (mg/kg) *	3.5	6.2	11.9
13-cis Astaxanthin (mg/kg) *	2.9	15.6	44.1
di-cis Astaxanthin (mg/kg) *	< 2.1	< 2.1	3.4

Table 3: Body size parameters of the initial population of diploid (2N) and triploid (3N) juvenile brook charr (*Salvelinus fontinalis*) prior to the start of the feeding trials. Each group was assigned randomly to an experimental tank (in triplicate for each ploidy–astaxanthin diet [Low, Intermediate, or High] combination), but all groups were fed the same standard salmonid diet prior to the commencement of trials (n = 450; 25 fish per tank; mean ± SD).

	Low		Intermediate		High	
	2N	3N	2N	3N	2N	3N
Body Mass (g)	45.17 ± 9.45	42.49 ± 12.01	48.44 ± 11.55	44.64 ± 10.41	45.86 ± 10.15	44.76 ± 11.94
Fork Length (cm)	14.75 ± 2.34	14.39 ± 1.56	14.89 ± 1.35	14.64 ± 1.22	14.60 ± 1.19	14.61 ± 1.39
Condition Factor	1.45 ± 0.20	1.40 ± 0.12	1.45 ± 0.15	1.40 ± 0.09	1.46 ± 0.10	1.41 ± 0.12

Table 4: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid), and diet (low, intermediate, or high astaxanthin [AX] content) on body mass, fork length, and condition factor of the initial population of juvenile brook charr (*Salvelinus fontinalis*). Variables denoted by an asterisk indicate the models which had Tank removed as a random effect. Bold values indicate a significant effect ($n = 450$; $p<0.05$).

Source of Variation	SS	MS	NumDF	DenDF	F-value	*p*-value
Body Mass:						
Ploidy	**43.428**	**43.428**	**1**	**12**	**6.998**	**0.021**
Diet	33.443	16.722	2	12	2.694	0.107
Ploidy x Diet	8.345	4.173	2	12	0.672	0.528
Fork Length:						
Ploidy	2.169	2.169	1	12	2.085	0.174
Diet	1.632	0.816	2	12	0.784	0.478
Ploidy x Diet	1.345	0.672	2	12	0.646	0.541
Condition Factor: *						
Ploidy	**0.267**	**0.267**	**1**	**444**	**14.584**	**<0.001**
Diet	0.003	0.001	2	444	0.095	0.908
Ploidy x Diet	<0.001	<0.001	2	444	0.003	0.996

Table 5: Body size and blood parameters, muscle and liver astaxanthin (AX) concentrations, and muscle pigment parameters of diploid (2N) and triploid (3N) juvenile brook charr (*Salvelinus fontinalis*) fed diets of low, intermediate, or high levels of dietary AX content. Values were recorded following loss of equilibrium (LOE) when exposed to acute hypoxia (body size and blood parameters: $n = 24$ fish per ploidy-diet combination [144 fish in total]; muscle and liver [AX]: $n = 54$ for each tissue [108 samples in total]; muscle pigment parameters: $n = 37$; mean ± SD). GSI and HSI refer to the gonadosomatic and hepatosomatic indices, respectively.

	Low		Intermediate		High	
	2N	3N	2N	3N	2N	3N
Body Mass (g)	128.68 ± 26.81	122.08 ± 32.41	147.45 ± 30.75	129.23 ± 29.39	143.30 ± 21.60	137.36 ± 24.97
Fork Length (cm)	20.38 ± 1.46	20.00 ± 1.81	21.23 ± 1.59	20.46 ± 1.46	21.25 ± 1.15	21.14 ± 1.13
Condition Factor	1.50 ± 0.08	1.49 ± 0.11	1.52 ± 0.10	1.49 ± 0.09	1.49 ± 0.14	1.44 ± 0.09
GSI (%)	0.47 ± 0.26	0.27 ± 0.39	0.56 ± 0.34	0.16 ± 0.14	0.61 ± 0.35	0.25 ± 0.17
HSI (%)	2.19 ± 0.31	1.86 ± 0.26	2.02 ± 0.31	1.92 ± 0.28	2.07 ± 0.31	1.89 ± 0.30
Glucose (mmol)	7.24 ± 1.39	6.85 ± 1.07	6.94 ± 1.53	7.53 ± 1.01	7.03 ± 1.38	7.30 ± 1.15
Lactate (mmol)	13.02 ± 1.61	13.29 ± 1.22	12.44 ± 2.02	13.30 ± 1.74	12.50 ± 2.30	12.56 ± 1.74
Hematocrit (%)	42.88 ± 6.17	42.04 ± 6.55	44.67 ± 5.19	39.33 ± 4.96	42.21 ± 6.87	42.08 ± 3.81
RBC Count (x 10^5 cells/mm^3)	9.60 ± 1.73	7.00 ± 2.71	10.70 ± 2.11	6.70 ± 2.10	10.03 ± 3.50	6.43 ± 1.60
RBC Volume (μm^3)	462 ± 98	699 ± 355	432 ± 90	686 ± 423	510 ± 385	687 ± 150

Table 5 cont.:

	Low		Intermediate		High	
	2N	3N	2N	3N	2N	3N
Muscle [AX] (mg/kg)	34.51 ± 8.32	34.31 ± 5.12	38.28 ± 5.98	38.30 ± 10.78	38.92 ± 9.52	40.46 ± 8.77
Liver [AX] (mg/kg)	9.28 ± 0.54	9.75 ± 1.32	9.41 ± 0.63	9.58 ± 1.10	10.40 ± 1.86	9.18 ± 0.54
Muscle SalmoFan	25.00 ± 2.19	25.13 ± 2.23	26.50 ± 1.41	27.00 ± 0.82	28.00 ± 2.45	28.33 ± 1.86
Muscle Lightness	50.14 ± 3.69	49.47 ± 3.20	48.12 ± 4.02	49.27 ± 0.29	47.95 ± 4.33	47.54 ± 1.35
Muscle a (R-G Intensity)	24.54 ± 2.80	21.53 ± 4.90	25.53 ± 2.92	24.55 ± 3.15	26.85 ± 3.35	27.53 ± 2.29
Muscle b (B-Y Intensity)	38.47 ± 1.88	35.00 ± 4.69	38.77 ± 4.62	37.73 ± 3.04	40.13 ± 2.69	40.75 ± 3.06
Muscle Redness Index	0.64 ± 0.04	0.61 ± 0.08	0.66 ± 0.03	0.65 ± 0.04	0.67 ± 0.06	0.68 ± 0.02
Muscle Hue	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00
Muscle Chroma (x 10^6)	4.44 ± 1.22	3.24 ± 2.00	5.00 ± 2.24	4.30 ± 1.41	6.00 ± 2.00	6.02 ± 2.00

Table 6: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid), and diet (low, intermediate, or high astaxanthin [AX] content) on body mass, fork length, condition factor, and hepatosomatic index (HSI) of juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia. Variables denoted by an asterisk indicate the models which had Tank removed as a random effect. Bold values indicate a significant effect (n = 24 fish per ploidy–diet combination [144 fish in total]; $p<0.05$).

Source of Variation	SS	MS	NumDF	DenDF	F-value	*p*-value
Body Mass: *						
Ploidy	**3787.1**	**3787.1**	**1**	**138**	**4.867**	**0.029**
Diet	**6325.3**	**3162.6**	**2**	**138**	**4.064**	**0.019**
Ploidy x Diet	1143.3	571.7	2	138	0.734	0.481
Fork Length: *						
Ploidy	6.291	6.291	1	138	2.977	0.086
Diet	**24.991**	**12.495**	**2**	**138**	**5.912**	**0.003**
Ploidy x Diet	2.601	1.300	2	138	0.615	0.541
Condition Factor: *						
Ploidy	0.031	0.031	1	127	3.082	0.081
Diet	0.041	0.020	2	127	2.026	0.136
Ploidy x Diet	0.014	0.007	2	127	0.706	0.495
HSI: *						
Ploidy	**1.444**	**1.444**	**1**	**127**	**19.454**	**<0.001**
Diet	0.075	0.037	2	127	0.506	0.603
Ploidy x Diet	0.318	0.159	2	127	2.147	0.121

Table 7: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid) and diet (low, intermediate, or high astaxanthin [AX] content) on time to loss of equilibrium (LOE), partial pressure of oxygen (PO_2) at LOE, blood glucose, blood lactate, hematocrit, RBC count, and RBC volume of juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia. Variables denoted by an asterisk indicate the models which had tank removed as a random effect. Bold values indicate a significant effect (n = 24 fish per ploidy-diet combination [144 fish in total]; p<0.05).

Source of Variation	SS	MS	NumDF	DenDF	F-value	p-value
Time to LOE:						
Ploidy	**10.652**	**10.652**	**1**	**9.996**	**5.457**	**0.041**
Diet	10.340	5.170	2	9.996	2.648	0.119
Ploidy x Diet	1.043	0.521	2	9.996	0.267	0.770
PO_2 at LOE:						
Ploidy	**1.315**	**1.315**	**1**	**11.042**	**5.940**	**0.032**
Diet	0.794	0.397	2	11.042	1.794	0.211
Ploidy x Diet	0.074	0.037	2	11.042	0.167	0.848
Glucose: *						
Ploidy	0.902	0.902	1	127	0.626	0.430
Diet	0.884	0.442	2	127	0.306	0.736
Ploidy x Diet	5.980	2.990	2	127	2.075	0.129
Lactate: *						
Ploidy	5.640	5.640	1	127	1.866	0.174
Diet	9.466	4.733	2	127	1.566	0.212
Ploidy x Diet	4.100	2.050	2	127	0.678	0.509
Hematocrit:						
Ploidy	**146.598**	**146.598**	**1**	**10.987**	**5.396**	**0.040**
Diet	4.874	2.437	2	10.987	0.089	0.914
Ploidy x Diet	177.311	88.656	2	10.987	3.263	0.077
RBC Count: *						
Ploidy	**$4.154e^{+12}$**	**$4.154e^{+12}$**	**1**	**127**	**82.561**	**<0.001**
Diet	$6.108e^{+10}$	$3.054e^{+10}$	2	127	0.606	0.546
Ploidy x Diet	$1.306e^{+11}$	$6.533e^{+10}$	2	127	1.298	0.276
RBC Volume: *						
Ploidy	**$1.795e^{+6}$**	**$1.795e^{+6}$**	**1**	**11.249**	**23.933**	**<0.001**
Diet	$0.037e^{+6}$	$0.018e^{+6}$	2	11.249	0.253	0.780
Ploidy x Diet	$0.038e^{+6}$	$0.019e^{+6}$	2	11.249	0.261	0.774

Table 8: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid), and diet (low, intermediate, or high astaxanthin [AX] content) on muscle and liver AX concentration and SalmoFan, lightness, red-green intensity, blue-yellow intensity, redness index, hue, and chroma values of juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia. Variables denoted by an asterisk and cross indicate the models which had tank and trial removed as a random effect, respectively, and those missing data were excluded from analysis due to unresolved statistical convergence errors. Variables denoted by an asterisk indicate the models which had tank removed as a random effect. Bold values indicate a significant effect (muscle and liver [AX]: n=54 samples per tissue [108 samples in total]; muscle pigment: n = 14 samples from the low diet, 12 from the intermediate and high diets [38 samples in total]; $p<0.05$).

Source of Variation	SS	MS	Num DF	Den DF	F-value	*p*-value
Muscle [AX]: *						
Ploidy	2.864	2.864	1	44.314	0.049	0.825
Diet	200.377	100.188	2	44.683	1.718	0.190
Ploidy x Diet	7.549	3.774	2	45.397	0.064	0.937
Liver [AX]:						
Ploidy	0.049	0.049	1	9.594	0.061	0.809
Diet	1.076	0.538	2	10.357	0.665	0.534
Ploidy x Diet	4.243	2.121	2	10.650	2.622	0.118
SalmoFan: *						
Ploidy	2.424	2.423	1	27.643	1.088	0.305
Diet	**49.819**	**24.909**	**2**	**21.231**	**11.191**	**<0.001**
Ploidy x Diet	0.008	0.003	2	25.049	0.001	0.998
Lightness: *						
Ploidy	0.571	0.571	1	29.450	0.073	0.787
Diet	16.784	8.392	2	21.866	1.082	0.356
Ploidy x Diet	4.043	2.021	2	26.701	0.260	0.772
a (R-G Intensity): *						
Ploidy	8.059	8.059	1	30.991	0.731	0.398
Diet	**101.098**	**50.549**	**2**	**21.767**	**4.590**	**0.021**
Ploidy x Diet	19.976	9.988	2	29.216	0.906	0.414
b (B-Y Intensity): *						
Ploidy	14.678	14.678	1	31	1.071	0.308
Diet	83.542	41.771	2	31	3.049	0.061
Ploidy x Diet	26.156	13.078	2	31	0.954	0.395
Redness Index: *						
Ploidy	<0.001	<0.001	1	28.203	0.266	0.609
Diet	**0.035**	**0.017**	**2**	**21.480**	**5.536**	**0.011**
Ploidy x Diet	<0.001	<0.001	2	25.549	0.141	0.868
Hue: *						
Ploidy	$1.661e^{-11}$	$1.660e^{-11}$	1	30.241	0.028	0.866
Diet	$2.274e^{-11}$	$1.137e^{-10}$	2	22.121	0.019	0.980
Ploidy x Diet	$5.598e^{-10}$	$2.799e^{-10}$	2	27.590	0.483	0.622
Chroma: *						
Ploidy	----------	----------	----------	----------	----------	----------
Diet	----------	----------	----------	----------	----------	----------
Ploidy x Diet	----------	----------	----------	----------	----------	----------

Table 9: Correlation matrix for paired combinations of loss of equilibrium (LOE) endpoints and body size parameters of juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia. Pearson's correlation coefficients (r) and p-values are below and above the diagonal, respectively (n = 24 fish per ploidy-diet combination [144 fish in total]). PO_2, CF, and HSI represent the partial pressure of oxygen at LOE, condition factor, and hepatosomatic index, respectively. Bold values indicate a significant effect ($p<0.05$).

	Time to LOE	PO_2 at LOE	Body Mass	Fork Length	CF	HSI
Time to LOE		**<0.001**	0.10	0.20	0.70	**0.01**
PO_2 at LOE	**-0.32**		0.90	0.40	0.053	0.20
Body Mass	-0.14	0.02		**<0.001**	**0.01**	0.20
Fork Length	-0.12	0.10	**0.94**		0.20	0.21
CF	0.03	-0.20	**0.22**	-0.11		0.70
HSI	**-0.22**	-0.11	0.11	0.11	-0.03	

Table 10: Correlation matrix for paired combinations of loss of equilibrium (LOE) endpoints and blood parameters of juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia. Pearson's correlation coefficients (r) and p-values are below and above the diagonal, respectively (n = 24 fish per ploidy-diet combination [144 fish in total]). PO_2, and Hct represent the partial pressure of oxygen at LOE, and hematocrit, respectively. Pearson's correlation coefficients and p-values relating to the combination of LOE endpoints are identical to those in Table 9. Bold values indicate a significant effect ($p<0.05$).

	Time to LOE	PO_2 at LOE	Glucose	Lactate	Hct	RBC Count	RBC Volume
Time to LOE		**<0.001**	0.30	0.90	0.93	**0.01**	**0.004**
PO_2 at LOE	**-0.32**		0.80	0.90	0.20	**<0.001**	**<0.001**
Glucose	0.10	0.02		**<0.001**	0.80	0.51	0.90
Lactate	0.01	0.01	**0.33**		0.50	0.43	0.73
Hct	0.01	0.11	-0.03	-0.10		**0.04**	0.10
RBC Count	**0.23**	**-0.30**	-0.10	-0.10	**0.20**		**<0.001**
RBC Volume	**-0.24**	**0.30**	0.01	0.03	0.14	**-0.73**	

Table 11: Correlation matrix for paired combinations of loss of equilibrium (LOE) endpoints, body mass, hepatosomatic index (HSI), and muscle and liver astaxanthin (AX) concentrations of juvenile brook charr (*Salvelinus fontinalis*) subjected to acute hypoxia. Pearson's correlation coefficients (r) and p-values are below and above the diagonal, respectively (n = 24 fish per ploidy-diet combination for the LOE parameters, body mass, and HSI [144 fish in total]; 54 samples per tissue [108 samples in total]). PO_2, and HSI represent the partial pressure of oxygen at LOE, and hepatosomatic index, respectively. Bold values indicate a significant effect ($p<0.05$).

	Time to LOE	PO_2 at LOE	Body Mass	HSI	Muscle [AX]	Liver [AX]
Time to LOE		**0.02**	0.50	0.40	0.30	**0.04**
PO_2 at LOE	**-0.32**		0.83	0.90	0.65	0.90
Body Mass	-0.10	0.03		**0.03**	0.60	0.70
HSI	-0.13	-0.02	**0.31**		0.53	0.63
Muscle [AX]	0.14	-0.10	0.10	-0.10		0.32
Liver [AX]	**0.30**	0.03	-0.10	-0.10	-0.14	

Table 12: Body size and blood parameters, muscle and liver astaxanthin (AX) concentrations, and muscle pigment parameters of diploid (2N) and triploid (3N) juvenile brook charr (*Salvelinus fontinalis*) fed diets of low, intermediate, or high levels of dietary AX content. Values were recorded following loss of equilibrium (LOE) at each fish's critical thermal maximum (CTmax) (body and blood parameters: n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]; muscle and liver [AX]: n = 54 for each tissue [108 samples in total]; muscle pigment parameters: n = 36; mean ± SD). GSI and HSI refer to the gonadosomatic and hepatosomatic indices, respectively.

	Low		Intermediate		High	
	2N	3N	2N	3N	2N	3N
Body Mass (g)	142.62 ± 28.76	122.59 ± 33.75	138.72 ± 22.11	131.98 ± 26.17	139.02 ± 33.66	142.39 ± 25.87
Fork Length (cm)	21.05 ± 1.41	20.13 ± 1.78	20.89 ± 1.25	20.17 ± 1.29	20.97 ± 1.73	21.32 ± 1.44
Condition Factor	1.51 ± 0.10	1.47 ± 0.09	1.52 ± 0.13	1.47 ± 0.10	1.48 ± 0.11	1.46 ± 0.09
GSI (%)	0.56 ± 0.27	0.20 ± 0.20	0.62 ± 0.29	0.24 ± 0.22	0.53 ± 0.22	0.29 ± 0.26
HSI (%)	1.94 ± 0.28	2.02 ± 0.39	2.06 ± 0.24	1.95 ± 0.28	1.93 ± 0.26	1.99 ± 0.19
Glucose (mmol)	7.11 ± 1.28	7.39 ± 1.59	7.12 ± 1.25	7.10 ± 1.31	7.15 ± 1.24	7.18 ± 1.01
Lactate (mmol)	10.59 ± 1.81	11.60 ± 1.77	10.89 ± 1.84	11.10 ± 1.94	11.16 ± 1.64	11.28 ± 1.65
Hematocrit (%)	44.08 ± 6.43	42.17 ± 6.35	42.13 ± 5.70	42.13 ± 4.75	41.92 ± 7.38	40.27 ± 5.30
RBC Count (x 10^5 cells/mm^3)	10.22 ± 0.24	6.60 ± 1.41	10.42 ± 1.90	6.34 ± 1.71	10.70 ± 2.20	5.92 ± 1.50
RBC Volume (μm^3)	452 ± 112	668 ± 175	416 ± 95	708 ± 204	405 ± 93	712 ± 161

Table 12 cont.:

	Low		Intermediate		High	
	2N	3N	2N	3N	2N	3N
Muscle [AX] (mg/kg)	36.50 ± 6.98	33.38 ± 7.22	42.41 ± 5.25	40.41 ± 5.10	49.78 ± 12.61	45.82 ± 10.81
Liver [AX] (mg/kg)	9.69 ± 0.85	9.48 ± 1.00	10.34 ± 1.14	10.35 ± 1.45	11.36 ± 1.90	11.76 ± 1.84
Muscle SalmoFan	25.00 ± 2.00	25.75 ± 1.26	25.75 ± 1.71	25.25 ± 2.49	27.20 ± 2.49	26.57 ± 2.15
Muscle Lightness	49.28 ± 2.66	48.70 ± 2.44	48.96 ± 3.55	49.26 ± 2.28	47.56 ± 3.01	47.14 ± 2.13
Muscle a (R-G Intensity)	24.86 ± 1.90	24.07 ± 2.52	25.57 ± 3.36	24.83 ± 3.38	26.41 ± 5.17	26.12 ± 5.21
Muscle b (B-Y Intensity)	37.78 ± 3.51	37.77 ± 1.24	38.55 ± 2.16	37.04 ± 2.68	38.75 ± 3.79	37.79 ± 2.64
Muscle Redness Index	0.66 ± 0.03	0.64 ± 0.06	0.66 ± 0.06	0.67 ± 0.06	0.68 ± 0.08	0.69 ± 0.10
Muscle Hue	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00	1.57 ± 0.00
Muscle Chroma (x 10^6)	4.40 ± 2.00	4.10 ± 0.70	4.70 ± 1.40	4.13 ± 1.50	5.21 ± 2.31	4.73 ± 1.61

Table 13: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid), and diet (low, intermediate, or high astaxanthin [AX] content) on body mass, fork length, condition factor, and hepatosomatic index (HSI) of juvenile brook charr (*Salvelinus fontinalis*) following loss of equilibrium (LOE) at each fish's critical thermal maximum (CTmax). Variables denoted by an asterisk and cross indicate the models which had tank and trial removed as a random effect, respectively. Bold values indicate a significant effect (n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]; $p<0.05$).

Source of Variation	SS	MS	NumDF	DenDF	F-value	*p*-value
Body mass: *						
Ploidy	1819.4	1819.40	1	12.192	2.274	0.157
Diet	1357.5	678.74	2	12.186	0.848	0.451
Ploidy x Diet	2688.1	1344.04	2	12.191	1.680	0.226
Fork Length:						
Ploidy	1.781	1.781	1	12.348	0.815	0.383
Diet	5.863	2.931	2	12.347	1.341	0.297
Ploidy x Diet	7.377	3.688	2	12.347	1.687	0.225
Condition Factor: *						
Ploidy	**0.059**	**0.059**	**1**	**126.38**	**5.659**	**0.018**
Diet	0.013	0.006	2	126.06	0.651	0.523
Ploidy x Diet	0.002	0.001	2	126.37	0.120	0.886
HSI: †						
Ploidy	0.004	0.004	1	12.221	0.063	0.806
Diet	0.061	0.030	2	12.220	0.390	0.685
Ploidy x Diet	0.249	0.124	2	12.220	1.590	0.243

Table 14: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid), and diet (low, intermediate, or high astaxanthin [AX] content) on time to loss of equilibrium (LOE), temperature at LOE, blood glucose, blood lactate, hematocrit, RBC count, and RBC volume of juvenile brook charr (*Salvelinus fontinalis*) following loss of equilibrium (LOE) at each fish's critical thermal maximum (CTmax). Variables denoted by an asterisk indicate the models which had tank removed as a random effect. Bold values indicate a significant effect (n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]; $p<0.05$).

Source of Variation	SS	MS	NumDF	DenDF	F-value	*p*-value
Time to LOE: *						
Ploidy	2.761	2.761	1	126.04	1.917	0.168
Diet	1.603	0.801	2	126.01	0.556	0.574
Ploidy x Diet	1.999	0.999	2	126.04	0.694	0.501
Temp at LOE: *						
Ploidy	0.285	0.285	1	126.03	1.709	0.193
Diet	0.219	0.109	2	126.00	0.657	0.519
Ploidy x Diet	0.252	0.126	2	126.03	0.755	0.471
Glucose: *						
Ploidy	0.432	0.432	1	126.09	0.422	0.517
Diet	0.482	0.241	2	126.03	0.235	0.790
Ploidy x Diet	0.565	0.282	2	126.09	0.275	0.759
Lactate: *						
Ploidy	5.803	5.803	1	126.00	2.275	0.133
Diet	0.855	0.427	2	125.88	0.167	0.845
Ploidy x Diet	6.676	3.338	2	126.00	1.308	0.273
Hematocrit: *						
Ploidy	52.814	52.814	1	126.41	1.555	0.214
Diet	97.580	48.790	2	126.16	1.436	0.241
Ploidy x Diet	26.772	13.386	2	126.40	0.394	0.675
RBC Count: *						
Ploidy	**$6.160e^{+12}$**	**$6.160e^{+12}$**	**1**	**125.96**	**178.413**	**<0.001**
Diet	$2.528e^{+9}$	$1.264e^{+9}$	2	125.53	0.036	0.964
Ploidy x Diet	$7.587e^{+10}$	$3.793e^{+10}$	2	125.95	1.098	0.336
RBC Volume: *						
Ploidy	**$2.63e^{+6}$**	**$2.63e^{+6}$**	**1**	**126.14**	**125.921**	**<0.001**
Diet	442	221	2	126.71	0.010	0.989
Ploidy x Diet	$0.057e^{+6}$	$0.028e^{+6}$	2	126.14	1.371	0.257

Table 15: Results of linear mixed-effects models (Type III SS) testing the effects of ploidy (diploid or triploid), and diet (low, intermediate, or high astaxanthin [AX] content) on muscle and liver AX concentration and SalmoFan, lightness, red-green intensity, blue-yellow intensity, redness index, hue, and chroma values of juvenile brook charr (*Salvelinus fontinalis*) which had reached loss of equilibrium (LOE) at each fish's critical thermal maximum (CTmax). Variables denoted by an asterisk and cross indicate the models which had tank and trial removed as a random effect, respectively. Bold values indicate a significant effect (muscle and liver [AX]: n=54 samples per tissue [108 samples in total]; muscle pigment: n = 12 fish from each diet [36 samples in total]; $p<0.05$).

Source of Variation	SS	MS	Num DF	Den DF	F-value	*p*-value
Muscle [AX]: *						
Ploidy	76.12	76.12	1	39.778	1.418	0.240
Diet	**1738.03**	**869.01**	**2**	**39.974**	**16.188**	**<0.001**
Ploidy x Diet	10.54	5.27	2	42.357	0.098	0.906
Liver [AX]: *						
Ploidy	0.054	0.054	1	48	0.026	0.870
Diet	**35.203**	**17.601**	**2**	**48**	**8.638**	**<0.001**
Ploidy x Diet	0.858	0.428	2	48	0.210	0.810
SalmoFan: †						
Ploidy	0.092	0.092	1	13.241	0.025	0.874
Diet	10.463	5.231	2	13.231	1.453	0.268
Ploidy x Diet	2.071	1.035	2	13.231	0.287	0.754
Lightness: *						
Ploidy	0.468	0.468	1	30	0.068	0.795
Diet	21.821	10.910	2	30	1.596	0.219
Ploidy x Diet	1.186	0.593	2	30	0.086	0.917
a (R-G Intensity):						
Ploidy	0.867	0.867	1	9.973	0.110	0.746
Diet	8.877	4.438	2	8.902	0.564	0.587
Ploidy x Diet	0.342	0.171	2	11.618	0.021	0.978
b (B-Y Intensity): †						
Ploidy	4.509	4.509	1	12.308	0.574	0.462
Diet	1.904	0.952	2	12.292	0.121	0.886
Ploidy x Diet	2.587	1.293	2	12.292	0.164	0.849
Redness Index:						
Ploidy	$8.582e^{-5}$	$8.582e^{-5}$	1	10.258	0.045	0.836
Diet	$1.872e^{-3}$	$9.361e^{-4}$	2	9.246	0.491	0.626
Ploidy x Diet	$1.133e^{-3}$	$5.666e^{+4}$	2	12.047	0.297	0.747
Hue:						
Ploidy	$1.654e^{-9}$	$1.654e^{-9}$	1	10.360	0.226	0.644
Diet	$1.606e^{-9}$	$8.031e^{-10}$	2	9.095	0.109	0.897
Ploidy x Diet	$5.791e^{-10}$	$2.895e^{-10}$	2	11.629	0.039	0.961
Chroma: †						
Ploidy	$1.114e^{+12}$	$1.114e^{+12}$	1	12.710	0.487	0.497
Diet	$2.664e^{+12}$	$1.332e^{+12}$	2	12.698	0.582	0.573
Ploidy x Diet	$8.027e^{+10}$	$4.013e^{+10}$	2	12.698	0.017	0.982

Table 16: Correlation matrix for paired combinations of loss of equilibrium (LOE) endpoints and body size parameters of juvenile brook charr (*Salvelinus fontinalis*) which had reached loss of equilibrium (LOE) at each fish's critical thermal maximum (CTmax). Pearson's correlation coefficients (r) and p-values are below and above the diagonal, respectively (n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]). Temp, CF, and HSI represent the temperature at LOE, condition factor, and hepatosomatic index, respectively. Bold values indicate a significant effect ($p<0.05$).

	Time to LOE	Temp at LOE	Body Mass	Fork Length	CF	HSI
Time to LOE		0.62	**0.050**	0.03	0.80	0.71
Temp at LOE	-0.04		**0.03**	0.10	0.50	0.98
Body Mass	**-0.20**	**-0.20**		**<0.001**	**0.03**	**0.003**
Fork Length	-0.20	-0.20	**0.94**		0.10	**0.01**
CF	0.03	-0.10	**0.20**	-0.14		0.10
HSI	0.03	-0.002	**-0.30**	**-0.21**	-0.14	

Table 17: Correlation matrix for paired combinations of loss of equilibrium (LOE) endpoints and blood parameters of juvenile brook charr (*Salvelinus fontinalis*) which had reached loss of equilibrium (LOE) at their critical thermal maximum (CTmax). Pearson's correlation coefficients (r) and p-values are below and above the diagonal, respectively (*n* = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total]). Temp, and Hct represent the temperature at LOE, and hematocrit, respectively. Pearson's correlation coefficients and p-values relating to the combination of LOE endpoints are identical to those in Table 16. Bold values indicate a significant effect ($p<0.05$).

	Time to LOE	Temp at LOE	Glucose	Lactate	Hct	RBC Count	RBC Volume
Time to LOE		0.62	**0.01**	**0.02**	0.83	0.10	**0.04**
Temp at LOE	-0.04		**0.002**	0.23	0.82	**0.01**	**0.03**
Glucose	**-0.22**	**0.30**		**0.01**	0.10	0.97	0.50
Lactate	**-0.20**	-0.10	**0.23**		0.52	**0.01**	**0.01**
Hct	-0.02	-0.02	-0.20	-0.10		0.06	**0.004**
RBC Count	0.20	**0.23**	0.003	**-0.23**	0.20		**<0.001**
RBC Volume	**-0.20**	**-0.20**	-0.10	**0.21**	**0.24**	**-0.90**	

Table 18: Correlation matrix for paired combinations of loss of equilibrium (LOE) endpoints, body mass, hepatosomatic index (HSI), and muscle and liver astaxanthin (AX) concentrations of juvenile brook charr (*Salvelinus fontinalis*) which had reached loss of equilibrium (LOE) at their critical thermal maximum (CTmax). Pearson's correlation coefficients (r) and p-values are below and above the diagonal, respectively (n = 24 fish per ploidy-diet combination for the low and intermediate diets, and 25 diploid and 22 triploid fish for the high diet [143 fish in total] for the LOE parameters, body mass, and HSI; 54 samples per tissue [108 samples in total]). Temp, and HSI represent the temperature at LOE, and hepatosomatic index, respectively. Bold values indicate a significant effect ($p<0.05$).

	Time to LOE	Temp at LOE	Body Mass	HSI	Muscle [AX]	Liver [AX]
Time to LOE		0.08	0.06	0.70	0.40	0.30
Temp at LOE	-0.24		0.95	0.60	0.30	0.22
Body Mass	-0.30	0.01		0.90	**0.002**	**0.02**
HSI	0.10	0.10	0.03		0.41	0.32
Muscle [AX]	-0.13	-0.20	**0.41**	0.12		**0.003**
Liver [AX]	-0.15	-0.20	**0.32**	0.14	**0.40**	

4. DISCUSSION

The main objective of this study was to determine if dietary supplementation with astaxanthin (AX) improves acute hypoxia and thermal tolerance in triploid fish. This has practical relevance because the use of triploid stock can limit the harmful environmental impacts of aquaculture escapees by way of their inherent sterility (Benfey, 2001; Piferrer et al., 2009; Benfey, 2016; Li et al., 2016). As climate change continues to increase global water temperatures, thereby possibly reducing dissolved oxygen levels, farmed salmonids are at risk because, as ectotherms, their metabolism is largely dependent on temperature and oxygen availability (Frölicher and Laufkötter, 2018). Instances of hypoxia and increasing temperatures in aquaculture are negatively affecting the fitness of farmed stocks, by reducing survival, growth, and reproductive rates (Oppedal et al., 2011; Stehfest et al., 2017; Smale et al., 2019; Gamperl et al., 2021). It is therefore key to understand salmonid, and specifically triploid salmonid, performance under these stressors when reared in an aquaculture setting.

During a thermal stress event, the overall number of reactive oxygen species (ROS) increases (Fedyaeva et al., 2014). Due to its antioxidant capabilities, AX suppresses ROS accumulation during these events, thereby reducing the harmful oxidation of other cellular molecules. This in turn allows for greater upregulation of heat shock proteins, which have been shown to promote acute thermal tolerance in other species of fish such as the pufferfish (*Takifugu obscurus*: Cheng et al., 2018). In the absence of research on AX's effect on hypoxia and thermal tolerance in triploid fish, I hypothesized that as AX supplementation increases, so too would the tolerance of triploid

fish towards both acute hypoxia and thermal stress. Improved tolerance towards these stressors may make triploids more favourable for use in aquaculture, allowing for their inherent sterility to promote greater sustainability in farmed salmonid stocks.

To promote AX retention in relevant tissues, I prepared feeds of increasing AX supplementation and fed them to sibling populations of juvenile diploid and triploid brook charr until they tripled their body mass. I then assessed the acute hypoxia tolerance of a subset of this population followed by the critical thermal maximum (CTmax) of another subset. Thus, the fish used in the CTmax experiment were fed their experimental diets for an additional week. The two experiments also differed in the initial dissolved oxygen level of the water bath, which was lower in the hypoxia experiment (80–87% of air saturation) than in the CTmax experiment (94–98%) due to the use of a single gas-bubble diffuser. While this resulted in shorter hypoxia trials, it did not affect comparisons between ploidies and diets within the experiment. This was corrected in the CTmax experiment through the addition of a second gas-bubble diffuser, which brought the starting oxygen levels to ~95% of air saturation.

Statistical analysis revealed that no significant interactions between ploidy and diet existed for any of the response variables recorded, and the effects of ploidy and diet are therefore discussed separately.

4.1 Ploidy Effects

Ploidy had a significant effect on body mass in the hypoxia experiment, with triploids being lighter and displaying lower HSI values than diploids. This effect of ploidy on body mass may be due to the pre-existing difference in body mass between

triploids and diploids observed prior to the feeding period. Ploidy also had a significant effect on HSI in the hypoxia experiment, but contrasting results in the literature show that HSI does not differ between triploids and diploids (Leal et al., 2021). Triploid fish also had lower condition factors in the CTmax experiment, and this result is supported by previous research (Ozório et al., 2012; Fjelldal et al., 2016; Sambraus et al., 2017b). The reduced triploid condition factor in the CTmax experiment was surprising, given that there were no effects of ploidy on any other body parameter (e.g., weight, length) measured. Interestingly, ploidy had no effect on condition factor in the hypoxia experiment, which is supported by the work of Leal et al. (2021), who found that condition factor also did not differ between triploid and diploid white sturgeon (*Acipenser transmontanus*). Triploid growth is variable, with some studies showing improved growth (Galbreath et al., 1994; Smedley et al., 2016; Sambraus et al., 2017b; Bazaz et al., 2020) while others show either reduced or variable growth in triploids (Hansen et al., 2015; Taylor et al., 2015; Leal et al., 2021; Madaro et al., 2022). In my study, I found that triploids showed similar growth rates as compared to diploids following the experimental feeding period.

Triploids displayed reduced hypoxia tolerance when compared to their diploid counterparts, reaching LOE sooner and at a higher PO_2. Although only a small difference (<5%), this finding of reduced hypoxia tolerance in triploids is supported by recent research on the hypoxia tolerance of triploid salmonids (Hansen et al., 2015; Sambraus et al., 2017a, b; Benfey & Devlin, 2018; Sambraus et al., 2020), including brook charr (Jensen & Benfey, 2022). The reduced hypoxia tolerance observed in triploids may be due to their reduced cell surface area to volume ratio, brought about by their increased

cell size (Benfey & Sutterlin, 1984; Maxime, 2008; Leal et al., 2020). It has been hypothesized that the rate of oxygen diffusion is correlated with red blood cell size (Lay & Baldwin, 1999), due to an increase in intracellular diffusion distance (Benfey, 1999). Organisms with smaller cells (i.e., diploids) have shorter intracellular diffusion distances and therefore have a greater capacity for oxygen uptake as compared to organisms with larger intracellular distances and larger cells (i.e., triploids) (Miettinen et al., 2017; Hermaniuk et al., 2021).

Triploids have been shown to possess reduced aerobic scopes compared to diploids (Altimiras et al., 2002; Hyndman et al., 2003a; Bernier et al., 2004; Beyea et al., 2005; Mercier et al., 2005; Bowden et al., 2018; Leal et al., 2021). Aerobic scope represents the difference between the maximum metabolic rate (i.e., the maximum amount of oxygen consumption of an organism) and standard metabolic rate (i.e., the baseline oxygen consumption of an organism at rest) (Fry & Hart, 1948; Eliason et al., 2013; Chabot et al., 2016). Triploid brook charr have been shown to have an increased standard metabolic rate (O'Donnell et al., 2017) and a lower maximum metabolic rate (Hyndman et al. 2003b), and triploid white sturgeon, a reduced maximum metabolic rate (Leal et al., 2020), thus supporting the limited overall aerobic performance of triploids during periods of low oxygen or high temperatures. However, contrasting results have been seen, suggesting that the difference in aerobic performance between ploidies is both subtle and variable (Sadler et al., 2000; Bowden et al., 2018; Daigle et al., 2021).

Critical thermal maximum did not differ between triploid and diploid brook charr in the present study, a result similarly noted by Benfey et al. (1997). Other studies, however, have shown variable differences between the thermal tolerance of diploids and

triploids using both chronic (Galbreath et al., 2006) and acute (Ellis et al., 2013; Benfey & Devlin, 2018; Bowden et al., 2018) thermal challenges. One of the current hypotheses to explain the physiological mechanism of thermal tolerance in ectotherms is the oxygen capacity limited thermal tolerance (OCLTT) hypothesis (Pörtner, 2006; Pörtner, 2010; Verberk et al., 2016; Pörtner et al., 2017; Blasco et al., 2020). The core concept of the OCLTT hypothesis is that thermal tolerance is limited by the capacity of the cardiorespiratory system to meet basic tissue oxygen demands (i.e., an organism's standard metabolic rate) (Chabot et al., 2016). The OCLTT hypothesis relates to an organism's aerobic scope and has frequently been applied to fish, as modern technologies allow for more precise tracking of the movement of oxygen through the circulatory system of fishes (Farrell, 2016). During a thermal stress event, standard metabolic rate increases to the point where oxygen demands for bodily maintenance require the entirety of the cardiorespiratory output of oxygen (Blasco et al., 2020; Lefevre et al., 2021; Earhart et al., 2022, Ern et al., 2023). At this point, aerobic scope for any additional activities (e.g., growth, exercise, and reproduction) is zero, and beyond this point, rapid physiological decline, including a decrease in aerobic performance, a reduction in activity, or death may occur (Pörtner and Farrell, 2008; Pörtner, 2010; Schulte, 2015). As noted above, triploids may have lower aerobic scopes than diploids (Verhille et al., 2013), thereby making them especially susceptible to thermal stress based on the OCLTT hypothesis. The universality of the OCLTT hypothesis has become a topic of active debate (Schulte, 2015; Farrell, 2016; Jutfelt et al., 2018; Blasco et al., 2020; Lefevre et al., 2021), and some studies have shown that LOE at CTmax may not be linked to tissue oxygen supply (Ern et al., 2014; Wang et al., 2014; Ern et al., 2016, Ern et al., 2023).

Another reason for this potential reduction in the thermal tolerance of triploid fish (as noted in other studies) may be related to a lower thermal optimum (Atkins & Benfey, 2008; Sambraus et al., 2017b). In their recent study examining the effect of warm-temperature acclimation on the metabolic rate of triploid and diploid zebrafish, Hermaniuk et al. (2021) have shown that standard metabolic rate is highest in triploids at lower temperatures, suggesting that they have a lower thermal optimum. In addition to this, triploids show greater levels of heat shock proteins after chronic temperature acclimation and greater protein breakdown after acute thermal stress, suggesting that triploid thermal tolerance may instead be due to a reduced ability to maintain proteostasis during periods of thermal stress (Saranyan et al., 2017).

A possible solution for improving triploid hypoxia and thermal tolerance may be prior warm-temperature acclimation, as past studies have shown improvement towards acute stress events (Bowden et al., 2018; Jensen & Benfey, 2022), and this has also been shown to improve thermal tolerance in diploid coho salmon (*Oncorhynchus kisutch*) (Becker & Genoway, 1979) and brook charr (Morrison et al., 2020). This suggestion may not be relevant in all cases; for example, Hyndman et al. (2003a) found that high temperature–acclimated triploid brook charr exhibited higher mortality than diploids following exhaustive exercise (with 90% triploid mortality within four hours).

Post-challenge blood glucose and lactate levels were not affected by ploidy in either experiment, with similar results found in previous studies (Biron & Benfey, 1994; Hyndman et al. 2003a; Hyndman et al., 2003b; Beyea et al. 2005; Benfey & Biron, 2000). Post-hypoxia hematocrit was lower in triploids, but there was no effect of ploidy on post-CTmax hematocrit. In their study examining the effects of different water

temperature and oxygen concentrations on triploid Atlantic salmon, Sambraus et al. (2017b) found that post-challenge hematocrit was also similar between ploidies. This suggests that triploids do not differ from diploids in their ability to regulate hematocrit, which is used as a measure of blood oxygen carrying capacity during periods of acute thermal and hypoxic stress. Past research has, however, shown hematocrit to be highly variable between diploids and triploids (Biron & Benfey, 1994; Benfey & Biron, 2000). The contrasting results in the literature may reflect that hematocrit is only a broad measure of oxygen carrying capacity in fish.

Triploidy had no effect on tissue AX content or any of the muscle pigment parameters measured in either experiment. The work of Choubert & Blanc (1989) supports this, as their research on pigment fixation in juvenile rainbow trout showed that triploids and diploids do not differ in their ability to fix dietary carotenoids. Fraser et al. (2022), however, found that triploid Atlantic salmon and brown trout hybrids show improved fillet colouration, through an improvement in their SalmoFan™ values, then their diploid counterparts, which contradict the findings of this study.

4.2 Diet Effects

Dietary AX content had no effect on acute hypoxia tolerance or CTmax in all fish. Regarding hypoxia tolerance, this may be due to several reasons, with the most obvious being that more time was needed to assimilate AX within the body; despite the fish approximately tripling their initial body mass while on their respective diets, and that muscle and liver AX concentration did not differ among dietary supplementation groups. This is highly plausible given the clear relationship between dietary AX content and both

muscle and liver AX content in the CTmax experiment, where these fish were fed an additional week prior to commencement of experimental trials. This is consistent with previous research showing that salmonids retain more AX in their muscle tissue when fed diets supplemented with AX (Choubert & Storebakken, 1989; Torrissen, 1989; Storebakken & Choubert, 1991; Choubert & Heinrich, 1993; Choubert et al., 1995; Choubert et al., 2006; Choubert et al., 2009). The insufficient tissue assimilation of AX observed in the hypoxia experiment may have also been attributed to the use of synthetic AX instead of naturally derived AX, as past research suggests naturally derived AX to be more effectively retained in the muscle (Choubert & Heinrich, 1993; Choubert et al., 1995; Choubert et al., 2006).

As previously stated, higher levels of dietary AX supplementation resulted in higher liver AX concentration in the CTmax experiment, suggesting that the liver plays a role in the uptake and use of AX during acute stress. However, the absence of AX assimilation in the livers of fish exposed to acute hypoxia contradicts the findings of Page & Davies (2006), specifically for AX in rainbow trout and canthaxanthin (CAX; a related xanthophyll carotenoid) in Atlantic salmon, with the liver and kidneys found to be important sites for carotenoid catabolism in both species. This difference between experiments can likely be be attributed to the longer duration of feeding AX-supplemented diets in the CTmax exerpiment. It has also been shown that when carotenoids become degraded, they are stored in the liver and subsequently re-secreted into necessary tissues via circulation, further contradicting the non-significant amount of AX assimilated in the livers of fish exposed to acute hypoxia (Okada et al., 2009; Zuluaga et al., 2018; Elbahnaswy & Elshopakey, 2023). AX has also been suggested to

influence rainbow trout liver physiology by modulating the expression of lipogenic transcription factors and stimulating the oxidative phase of the pentose phosphate pathway of liver glycolysis, which acts to counteract oxidative stress (Kalinowski et al., 2019; Kalinowski et al., 2023).

Fish fed the high AX diet were larger (body mass and fork length) than those fed either the low or intermediate diets in the hypoxia experiment, suggesting a possible positive effect of dietary AX on growth rate. However, the high AX diet also had the highest fat content, so no conclusions can be drawn and, in any case, the lack of an effect of diet on growth in the CTmax experiment suggests that any effects of diets were negligible. Dietary AX supplementation has been shown to promote nutrient utilization, and therefore, growth, in different species of salmonids (Torrissen, 1989; Christiansen et al., 1994; Christiansen et al., 1995; Řehulka, 2000; Bazyar Lakeh et al., 2010). However, this is not supported by the absence of an effect of diet on any body size measurement in the CTmax experiment. Reasons for these differences between the hypoxia and CTmax experiments are not known, and additional research is required to elucidate any mechanisms.

Surprisingly, muscle pigmentation was only influenced by dietary AX supplementation in the hypoxia experiment, with those fish fed the high AX diet having significantly greater SalmoFan, red–green intensity, and redness values than those fed the low AX diet. Similar results have been observed in previous studies (No & Storebakken, 1992; Choubert & Heinrich, 1993; Choubert et al., 2006; Choubert et al., 2008), so the lack of an effect of diet on muscle pigmentation in the CTmax experiment was not expected given that these fish were fed their respective diets for an additional week and

assimilated significantly greater amounts of AX in their muscle and liver. These muscle pigment parameters are central to the marketability of salmonid flesh, as greater values equate to redder (i.e., more desirable) fillets. This observation is supported by past research which also saw increased fillet pigmentation with increased muscle AX retention (Choubert & Storebakken, 1989; No & Storebakken, 1992; Choubert & Heinrich, 1993; Choubert et al., 1995; Storebakken et al., 2004; Choubert et al., 2006; Choubert et al., 2008; Choubert et al., 2009; Kalinowski et al., 2019).

4.3 Correlations

Body mass was positively correlated with AX content in muscle and liver tissue in the CTmax experiment (i.e., larger fish assimilated greater amounts of AX per unit tissue mass than smaller fish). Similar results were observed by Torrissen (1989) in rainbow trout, with fish heavier than 90g assimilating more carotenoids in the flesh than those lighter than 90g when fed combined diets supplemented with 200 mg/kg of AX and CAX.

Time to LOE and temperature at LOE in the CTmax experiment were both found to be negatively correlated with body mass, suggesting that smaller diploids and triploid are better able to manage thermal stress, as previously shown for this species (Benfey et al., 1997; Galbreath et al., 2006). This negative correlation between CTmax and body mass has also been observed in other diploid species such as the shortnose sturgeon (*Acipenser brevirostrum*) (Zhang & Kieffer, 2014).

As expected, there was a negative correlation between RBC count and RBC volume in both experiments, confirming the well-documented increase in cell size and

corresponding decrease in number of circulating RBCs in triploids compared to diploids (Benfey, 1999). Correlations between the RBC parameters and the LOE parameters of both experiments can therefore be attributed to the ploidy effects mentioned above, which support that triploids are less tolerant towards both acute hypoxia and thermal stress than their diploid counterparts (Hansen et al., 2015; Benfey & Devlin, 2018; Jensen & Benfey, 2022). Further supporting the findings of underperformance in triploids towards acute stress, Verberk et al. (2022) found there to be an interaction between triploidy and warm water–induced hypoxia tolerance stemming from their reduced ability to take up oxygen in the blood, as described above.

There was no relationship between hypoxia tolerance (either time to LOE or PO_2 at LOE) and blood glucose and lactate, which was surprising given that there was a negative correlation between post-CTmax blood glucose and lactate with time to LOE, and a positive correlation between post-CTmax blood glucose and temperature at LOE. Hypoxia and thermal stress have been shown to illicit a secondary stress response, specifically changes in blood glucose, lactate, and hematocrit, in fish (Barton, 2002). The work of Ellis et al. (2013) supports the positive correlation observed between post-CTmax blood glucose and temperature at LOE, where they also observed glucose to be significantly higher at, and immediately following, brook charr CTmax in hypoxia conditions. Post-challenge blood glucose and lactate were positively correlated with each other in both experiments, which is expected, as both are documented measures of secondary stress response in fish (Barton, 2002).

There was a positive correlation between liver AX content and time to LOE but no relationship between muscle AX content and LOE in the hypoxia experiment. There

was no relationship between either muscle or liver AX content and LOE in the CTmax experiment. There was also a significant amount of AX assimilated into the liver of the fish exposed to CTmax (the longer of the two experiments), indicating that the liver may play a role in the use of AX in the body. The works of Kalinowski et al. (2019 & 2023) support this, as they found that AX helps to counteract oxidative stress through stimulating hepatic function, which may explain the relationship between liver AX content and time to LOE observed in this study. There were positive correlations between liver AX content and body mass and muscle AX content and body mass, which is supported by past research (Christiansen et al., 1995). Past research has, however, shown there to be little difference in body mass between AX-supplemented and non-supplemented diets in rainbow trout and Atlantic salmon (Choubert et al., 1995; Page & Davies, 2006), which suggests that AX's impact on salmonid body mass is species-specific.

5. CONCLUSIONS AND FUTURE RESEARCH

This study confirmed that (i) triploidy reduces hypoxia tolerance but does not affect CTmax, and (ii) showed that dietary AX supplementation, at least under the present experimental conditions, does not improve acute thermal or hypoxia tolerance in diploid or triploid fish. However, dietary supplementation improved both muscle and liver AX retention and pigmentation, confirming that there is merit for its incorporation in greater amounts in salmonid feed to promote greater colouration. There are, however,

limits in place as to the maximum amount of AX which may be included in salmon feed (100 mg/kg in the European market [Bampidis et al., 2022] and 80mg/kg in the Canadian market [Government of Canada, 2023]), due to uncertainties regarding an ideal dose for human consumption and the effects that any higher level may have on both fish and human health. The work of Choubert & Storebakken (1989) suggests there to be a reduction in the rate at which salmonids retain AX in their muscle tissue between the range of 50 mg/kg to 100 mg/kg, further discouraging the supplementation of AX in greater amounts than already allowed. While this study demonstrated that fish benefit from improved pigmentation when fed the intermediate and high AX diets (80 mg/kg and 190 mg/kg, respectively), and improved growth with the high AX diet, given that these were either at or above the concentration currently allowed in salmonid feed, more research is necessary as to the effect that these levels of AX supplementation may have on both farmed salmonid and human health.

Future studies should focus on increasing the amount of time fish are fed AX supplemented diets prior to exposing them to hypoxia or thermal stress. Insufficient assimilation of dietary AX was one of the main reasons why there was no significant difference between diets in both muscle and liver AX retention in the hypoxia experiment, given that the additional week of feeding by the CTmax fish produced a significant diet-dependent increase in both muscle and liver AX retention.

Another possibility for future studies is to examine the effect of dietary AX on chronic stress tolerance, rather than acute stress tolerance. As described above, triploid fish respond differently to chronic stressors than their diploid counterparts. Chronic stress affects an organism for longer periods, allowing for an organism to adjust itself to its new

environment, and additionally, more time to assimilate AX into their bodies. In relation to this, there has been some debate as to the usefulness of acute stress in relation to hypoxia and temperature changes in aquaculture systems (Schulte, 2015; Farrell, 2016; Lefevre, 2016; Jutfelt et al., 2018; Blasco et al., 2020; Lefevre et al., 2021; Strowbridge et al., 2021; Bartlett et al., 2022; Ignatz et al., 2023). An example of this debate is the CTmax protocol (Lutterschmidt & Hutchison, 1997) used in this study. The acute nature of this protocol has been viewed to not accurately mimic the chronic nature of thermal stress that fish are exposed to in commercial aquaculture systems, since these fish cannot escape to more favourable conditions. This leads to possible compounding effects of acute stress in addition to a pre-existing chronic stress event, leading some to question its relevance as an indicator of thermal tolerance (Galbreath et al., 2006; Bartlett et al., 2022; Ignatz et al., 2023).

In conclusion, this thesis has explored the potential benefits of dietary manipulation of AX to improve acute stress tolerance by triploid brook charr for aquaculture considerations. This study has, unfortunately, found no impact of AX supplementation on either triploid acute hypoxia or thermal tolerance. It did, however, demonstrate the potential for greater AX incorporation in salmonid feeds to promote improved pigmentation and growth. Although triploid fish have the potential to be a beneficial replacement for diploid stocks due to their inherent sterility, their overall lower stress tolerance makes them less appealing for mass stocking. Despite muscle pigmentation and growth benefiting from greater AX supplementation, more research is necessary to determine the role AX may play in the acute stress response of triploids in aquaculture.

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Baker CA*, Benfey TJ, Kieffer JD. April 2023. The Effect of Dietary Supplementation of Astaxanthin on Acute Hypoxia and Thermal Tolerance in Triploid and Diploid Brook Charr, *Salvelinus fontinalis*. Conference of the Biological Sciences (Fredericton, New Brunswick).

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