Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Brynjar Muren

A comparative study of the physiological responses of chronically stressed diploid and triploid Atlantic salmon (*Salmo salar*)

Master's thesis in Natural Science with Teacher Education
Supervisor: Rolf Erik Olsen
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NTNU
Kunnskap for en bedre verden

Foreword

This thesis constituting 30 study points was composed to fulfil my Master of Science (MSc) in Marine Biology and Aquaculture to become a teacher in biology and chemistry. The experimental work was conducted at The Norwegian Institute of Marine Research (IMR) in Matre in October and November of 2021. In writing the thesis I was supervised by Professor Rolf Erik Olsen at the Department of Biology, NTNU, and co-supervised by Senior researcher Angelico Madaro at IMR.

Writing the thesis has been a very humbling experience. The five years of previous studies had given me a lot of theoretical and practical knowledge in biology and trained my writing skills. Nonetheless, participating in serious scientific inquiry has been frustrating and turbulent, requiring long hours and many rounds of analyses and rewriting. Knowing now what I have learned in the process "the hard way", I could probably have done it again spending significantly less time, and the process would be much smoother.

Working part time as a natural science teacher for the last year, the knowledge from writing the thesis has already been incredibly valuable and relevant. The basic skills in natural science that I will continue to train pupils in, like reading and writing in science, and computing and using digital tools to present data, I have now been thoroughly trained in myself. The basic biology and the physiological stress response investigated in the experiment is also highly relevant for the curricula in both natural science, biology, and chemistry.

I would like to thank my supervisors Professor Rolf Erik Olsen and Angelico Madaro for great help and solid guidance in the writing process. You have certainly elevated the quality of my thesis, but more importantly enhanced my writing skills and biological understanding which I carry with me into my professional career. A big thank you to all of my great friends in Trondheim for making these years at NTNU such a joy. Especially thank you to my good friend Håvard, who sadly passed away after our undergraduate years. You will always be remembered. Most of all, thank you so much to my amazing wife Fanny for enduring this hectic year with a busy husband, caring for our beautiful child, and in the last few months carrying our second. I will work diligently to repay you for the long hours you have spent to make this thesis possible. I look very much forward to our continuing journey.

May 27th, 2022

Brynjar Muren

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Sammendrag

Rømt oppdrettslaks (*Salmo salar*) regnes for å være en av de største truslene som påføres ville populasjoner av Atlantisk laks grunnet introgressiv hybridisering. Dette kan unngås ved å indusere sterilitet i oppdrettslaksen, noe som er mulig i kommersiell skala gjennom triploidisering. Triploid laksefisk har vist høyere mortalitet og lavere yteevne i kommersiell produksjon, noe som indikerer redusert dyrevelferd. Lite er kjent om triploid laks sin fysiologi. Denne studien tok sikte på å utforske den triploide stressfysiologiden under kroniske stressforhold for å få innsikt i hvordan laksen mulig håndterer suboptimale forhold i kommersiell sammenheng.

I denne studien ble triploid og diploid Atlantisk lakseparr utsatt for en uforutsigbar kronisk stress (UKS)-test over en tre ukers periode. Etter testen ble fisken eksponert for en ny stressor-test sammen med kontrollgrupper, og plasmaprøver ble analysert for HPI-akse aktivering og fysiologisk håndtering av akutt stress. Begge ploidier responderte på UKS-testen med en redusert spesifikk vekstrate (SGR) og en økt forkonverteringsratio (FCR). Det var ingen statistisk forskjell mellom ploidienes UKS respons i SGR og FCR. Kronisk stressede triploider hadde høyere grunnverdier i sirkulerende kortisol sammenlignet med sin kontrollgruppe, mens kronisk stressede diploider hadde lignende kortisolverdier som den diploide kontrollgruppen. Sirkulerende adrenokortikotropt hormon (ACTH) viste inkonsistent effekt av UKS, hvor stressede triploider hadde de høyeste verdiene, mens stressede diploider hadde de laveste verdiene av alle gruppene. Sammenlignet med vekst ble grunnverdier i plasma (ACTH, cortisol, Na^+, Cl^-, laktat, glukose) evaluert som upålitelige indikatorer for opplevelsen av kronisk stress i dette eksperimentet.

Både kronisk stressede og kontrolltriploider responderte på akutt stress-testen med en høyere utskillelse av både ACTH og kortisol sammenlignet med de diploide, noe som ikke var forventet. På tvers av analyser (ACTH, cortisol, Na^+, Cl^-, K^+, laktat, glukose) konkluderer vi at UKS har lignende effekter på den akutte stressresponsen for begge ploidier. Sammenlignet med kronisk stresset fisk har naiv fisk en større akutt stressrespons og erfarer økt allostatisk belastning, spesielt triploider. Dette eksperimentet peker ikke på opplevelsen av kronisk stress som en enkeltstående faktor i å forklare den lavere ytelsen til triploid laks i opprett. Triploid laks virker ikke å erfare lavere velferd en diploid laks under kroniske stressforhold.

Abstract

Farmed Atlantic salmon (*Salmo salar*) escapees is regarded as one the greatest threats imposed on the wild Atlantic salmon due to introgressive hybridization. This can be avoided by inducing sterility in the farmed salmon, which is commercially possible through triploidization. Triploid salmonids have shown to have higher mortalities and in general lower performance in commercial settings, indicating a concerning reduced animal welfare. The knowledge of the triploid salmon physiology is scarce. This study aimed to investigate the triploid stress physiology under chronic stress conditions to gain insights to how the salmon might cope with suboptimal conditions in aquaculture settings.

In this study, triploid and diploid Atlantic salmon parr were exposed to an unpredictable chronic stress (UCS) trial over a three-week period. The fish were then exposed to a novel stressor trial together with respective control groups, and plasma was analysed for HPI-axis activation and physiological coping under acute stress. Both ploidies responded similarly to the UCS trial with a reduced specific growth rate (SGR) and increased feed conversion ratio (FCR). There was no statistical difference between the ploidy UCS response in SGR and FCR. Chronically stressed triploids had higher baseline circulating cortisol than the respective control group, while chronically stressed diploids had similar cortisol values to the diploid control. Circulating (adrenocorticotropin releasing hormone (ACTH) showed inconsistent effects of UCS, with the highest value in stressed triploids, while stressed diploids had the lowest value of all the groups. Compared to growth, baseline plasma values (ACTH, cortisol, Na^+, Cl^-, lactate, glucose) were in this experiment evaluated as inconsistent indicators for experiencing chronic stress.

Both chronically stressed and control triploids responded to the novel stressor trial with a higher release of both ACTH and cortisol compared to the diploids, which was not expected. Across analysis (ACTH, cortisol, Na^+, Cl^-, K^+, lactate, glucose) we conclude that UCS had similar effects on the acute stress response for both ploidies. In comparison to chronically stressed fish, naïve fish had a larger novel stressor response, experiencing a great allostatic load, particularly the triploids. This experiment does not point to the experience of chronic stress as a sole factor explaining lower performance of triploid salmon in aquaculture. Triploid salmon does not seem to experience lower overall welfare than diploid salmon under chronic stress conditions.

Introduction

Norwegian Atlantic salmon aquaculture and the problem of escapees

Fish farming has been practiced by humans for thousands of years, particularly in East Asia (Nash, 2010). Historically, Asian finfish aquaculture has been mostly utilizing freshwater species local to their environment in an extensive or semi-intensive manner. Norway is home to the anadromous Atlantic salmon (*Salmo salar*) with over 400 watercourses with wild Atlantic salmon and approximately 25% of the global stocks (Aas et al., 2010). With a long history of salmon fishing, the Norwegian nation has cultural bonds to the Atlantic salmon (Kvamme, 2019). In the 1960s, experimentation with salmonids for aquaculture was initiated, and over the next few decades, intensive farming in open sea water cages was established. In 2021, Norway harvested a farmed salmon biomass of more than 1,5 million ton, consisting of approximately 300 million fish (Fiskeridirektoratet, 2022). In comparison, the entire wild Norwegian salmon population consists of approximately 500 000 fish, with a biomass of about 400 ton (Thorstad et al., 2021).

As in almost all domestic farming, selective breeding of farmed Atlantic salmon has enhanced commercially important traits (Gjøen & Bentsen, 1997). Since the 1960s, growth, feed consumption and protein and energy retention have been selectively bred for. In 12 generations of breeding, this has led to an improved growth rate and increased feed consumption. The genetic composition of farmed Atlantic salmon is now clearly distinct from the wild salmon and identifiable. Generic genetic differences can be detected trough single nucleotide polymorphisms (SNPs) (Karlsson et al., 2011), while SNPs also reveal clear genetic differences between wild populations (Bourret et al., 2013). Salmon rivers may be very different in ecological structure, with differences in temperature, size, steepness, water flow, organic content, oxygen saturation etc., and local adaptations to the environment have led to clear group differences in genetic structure.

Escapees of farmed salmon is of great concern for the wild salmon population (Diserud, Fiske, et al., 2019). Reported Norwegian annual numbers of escapees has been as high as 920 000 in the record year 2006. For the past decade, the average number of escapees has been around 168 000 annually (Wennevik et al., 2021). Karlsson et al. (2016) showed that breeding between farmed and wild salmon in Norwegian rivers has led to an average farmed genetic introgression of 6,4% in the populations (median = 2,3%), with a range between 0.0% and 42.2%. The genetic changes have been evaluated as “large” in 68 of the 239 populations

evaluated (Diserud, Hindar, et al., 2019). As farmed Atlantic salmon has a lowered fitness in the wild, farmed genetic introgression is believed to lower the fitness and eliminate local adaptations of the wild salmon populations (Fleming et al., 2000; McGinnity et al., 2003; Skaala et al., 2019; Wacker et al., 2021). Forseth et al. (2017) have concluded that escaped farmed salmon is the largest current threat to wild salmon populations, exceeding the problem with salmon lice. These two factors combined may affect certain populations to the extent of critical endangerment. Glover et al. (2017) concludes that only a major reduction in the number of escapees and/or sterility of farmed salmon can eliminate further negative impacts.

The physiology of triploid fish

As of today, the only effective commercially available technique to induce sterility in Atlantic salmon is triploidization (Benfey, 2001; Golpour et al., 2016; Hansen et al., 2012). Triploid organisms have three sets of haploid chromosomes (*3n*), and are both euploid and polyploid as they contain one complete extra set of chromosomes besides the normal two (*2n*) chromosome sets (Sellars, 2013). Triploids are infertile as a consequence of improper gamete formation. During the first meiotic division, the three chromosome homologs segregate in a random manner such that genetically balanced and fertile gametes are extremely unlikely.

Triploidy occurs naturally in some flowering plant populations containing both diploid (*2n*) and tetraploid (*4n*) plants. In cultivation, large, sterile and seedless bananas have been produced for thousands of years by crossing species like the *2n Musa acuminata* and the *4n Musa balbisiana* (Sellars, 2013). The first triploid vertebrates, four newt larvae (*Triturus viridescens*) were discovered in nature in 1938 (Fankhauser, 1941). Techniques for experimental induction of triploidy in *T. viridescens* were then developed, giving newly fertilized eggs a cold or hot shock treatment (Fankhauser & Griffiths, 1939; Fankhauser & Watson, 1942; Griffiths, 1941). In 1984, Benfey and Sutterlin developed a technique to induce 100% triploidy in fertilized Atlantic salmon eggs (Benfey & Sutterlin, 1984b). Heat and pressure shocks are utilized on newly fertilized eggs in the meiosis II stage to prevent the expulsion of the second polar body. The result is triploid fertilized eggs containing two haploid sets of chromosomes from the mother and one set from the father (Figure 1).

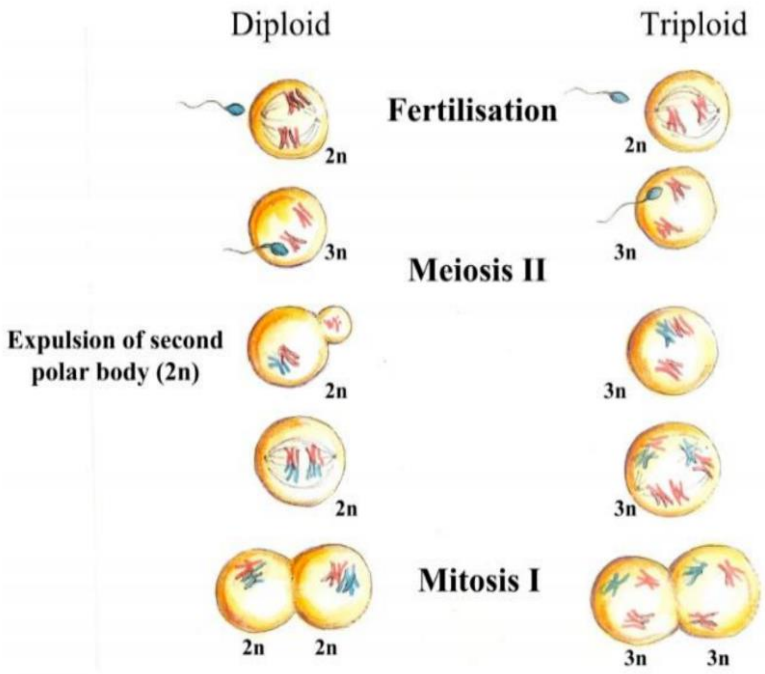

Figure 1: A comparison between normal fertilization of a fish egg resulting in a diploid individual, and fertilization where the expulsion of the second polar body is prevented due to a pressure shock, leading to a triploid individual. Figure by Hansen et al. (2012), adapted by Rogers (2016).

The physiology of triploids is broadly similar to that of diploids (Maxime, 2008), with the largest physiological difference being underdevelopment of the gonads in triploids (Benfey & Sutterlin, 1984a). Differences in growth are not conclusive, as studies differ in results (de Fonseka et al., 2022; Galbreath et al., 1994; McGeachy et al., 1995; Oppedal et al., 2003). One clear tendency of triploids across species is a larger cell volume without an increase in organ and body size, implying a reduction in cell number and density (Benfey, 1999; Fankhauser, 1941; Swarup, 1959). The physiological consequences of this phenomenon is poorly understood (Maxime, 2008).

Though the differences in growth for triploid and diploid Atlantic salmon is not that great, the overall difference in performance is much larger. Triploids have a higher frequency of skeletal deformities like the lower jaw deformity syndrome (Amoroso et al., 2016; Sutterlin et al., 1987), vertebral deformities (Fjelldal & Hansen, 2010), ocular cataracts (de Fonseka et al., 2022; Leclercq et al., 2011), as well as higher mortality, especially under suboptimal rearing conditions (Madaro et al., 2021). Compared to diploids they have higher requirements for dietary phosphorous to prevent vertebral deformities (Fjelldal et al., 2015), and dietary histidine to avoid ocular cataracts (Sambraus et al., 2017). Triploid salmon are less tolerant

toward high water temperatures (Ojolick et al., 1995) and reduced O_2 saturation, especially towards the two factors in combination (Hansen et al., 2015; F. Sambraus et al., 2017; Sambraus et al., 2018). Most studies have found the O_2 carrying capacity, unloading capacity or consumption rates to not differ much between ploidies (Fraser, Fjelldal, Hansen, et al., 2012). Studies have however found lower O_2 carrying capacity in triploid Chinook salmon (*Oncorhynchus tshawytscha*) during sustained swimming exercise (Bernier et al., 2004). This capacity might to a greater degree than diploids be affected by temperature (Altimiras et al., 2002; Fraser, Fjelldal, Hansen, et al., 2012; Riseth et al., 2020).

Animal welfare and triploidy

The Animal Welfare Act (Dyrevelferdsloven, 2009) demands that keepers of animals must ensure adequate food and water (§24), protect against unnecessary discomfort (§3), protect against harm and disease (§24), ensure possibility for natural behaviour (§23) and protect the animals from unnecessary stress (§3). Further, The Norwegian Regulation for Operation of Aquaculture facilities (Akvakulturdriftforskriften, 2008) requires operating the fish farm in a way that promotes welfare and avoids unnecessary strain (§28). The key term that sums up the goal of the act and the regulations is to achieve high *animal welfare*. Many definitions of animal welfare exist. Researchers from The Norwegian Veterinary Institute and The Institute for Marine Research have interpreted the term in summing up the act and regulations to best be defined by the established definition; “The quality of life as perceived by the animal itself” (Gismervik et al., 2020; Noble et al., 2018; Stien et al., 2013).

By alluding to a perception of its own life, the concept of animal welfare therefore presupposes some form of animal awareness or consciousness regarding its own experience. The conscious experience of fish has been debated on scientific and philosophical grounds. Rose (2002) argued that fish lack a neocortex and therefore the neurological basis for consciousness. However, more recent data suggests that the fish brain may be more similar to other mammalian vertebrates than previously thought. Broglio et al. (2005) gave convincing arguments for the existence of areas in the teleost brain being homologous to the mammalian limbic system. For example, the fish lateral pallium and medial pallium were shown to be homologous to the mammalian hippocampus and amygdala respectively. This suggests that fish may have a spatial, relational, and temporal memory system, and an emotional memory system. Furthermore, classical Pavlovian conditioning works on both cod, halibut and salmon, who can learn to anticipate feed based on unconditioned stimuli (Bratland et al., 2010;

Nilsson et al., 2008; Nilsson et al., 2010). Unconditioned stimuli can elicit innate fear responses, which through habituation and conditioning can be extinguished and even learned to anticipate positive events (Bratland et al., 2010). In other cases, omission of expected feed due to conditioning triggered the display of negative emotion as aggressive behaviour in Atlantic salmon (Vindas et al., 2012). Numerous behavioural and neurological findings support criteria for fish to experience pain (Sneddon, 2009). All of this support the notion that fish have a qualitative experience of the world, suggesting some sort of subjective conscious experience.

As mentioned, triploid salmon show higher prevalence of physical disease, with the disease origins being environmental, nutritional, or genetic factors. This suggests a possible lower animal welfare in triploid salmon compared to their diploid counterpart (Fraser, Fjelldal, Hansen, et al., 2012). Therefore, the use of triploid salmon to avoid genetic introgression in wild salmon populations meets ethical challenges. Due to welfare concerns The Norwegian Food Safety Authority is planning to temporarily stop the commercial use of triploid salmon in Norway in 2023 (Berge, 2021; Bøhren, 2021).

Understanding what limits the performance of triploid salmon is beneficial and necessary on both environmental, economic, ethical, and legal grounds. Fish in aquaculture are subjected to several different stressors which may impact their coping capacity and performance. Insight into the physiological stress response in the triploid Atlantic salmon is very limited, particularly when stressors are long lasting (chronic). Understanding the stress response in triploid Atlantic salmon and highlighting possible differences with their analogue diploids might be crucial to understanding the lower performance of triploids when subjected to intensive aquaculture settings.

The Stress concept

The original term “stress” is adopted from physics and describes the interplay between a force and the resistance offered to it, like the resistance of a steel bar towards a bending force (Schreck & Tort, 2016; Selye, 1950). Appropriately, the term “stress” in biology is used to describe an organism’s attempt to resist the stressor. The term refers to the processes that tune physiology and behaviour, and prepare the body to handle challenges in the environment (Sterling, 2011). The stress response can then be defined as “the physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostatic norms in the face of insult” (Schreck & Tort, 2016).

The General Adaptation Syndrome, homeostasis and allostasis

Historically, the stress response has been explained through Hans Selye's comprehensive model of the General Adaptation Syndrome (G.A.S.) (Selye, 1936, 1950). G.A.S. develops through three phases: The Alarm Phase, The Resistance Phase, and Exhaustion. This concept is still valid today, with some modifications. Selye's concept drew on the work of Claude Bernard and Walter Cannon. It was Cannon (1932) who coined the well-known terms *"fight or flight*" and the term *homeostasis* used to explain the ability of the body to maintain internal stability. Many physiological variables like blood pressure, blood glucose concentration and intracellular osmolarity have set points of value, and deviations from these will be counteracted by physiological processes. According to G.A.S. theory, when encountering a stressor, the goal of the adaptation syndrome is to maintain internal stability, i.e., homeostasis. Considering present knowledge of the stress response however, the homeostasis concept is not fully explanatory. The maintenance of a stable internal environment despite change in the external environment does not square well with what happens in the neuroendocrine stress response. This was noted by Selye himself (Selye, 1975). The concept of homeostasis in explaining the stress response is therefore today to a great degree replaced by the concept of *allostasis*.

Allostasis can be described as "the ability to achieve stability through change" (Sterling & Eyer, 1988). The set points for physiological processes essential for life can change due to stress (Schreck, 2010). If the exposure extends over time the organism can enter a changed *allostatic state*, with the changed state having negative heath consequences (Madaro et al., 2020; Sterling & Eyer, 1988). The cumulative cost to the body of allostasis is called the *allostatic load*. We can distinguish two types of allostatic load; *emergency responses* and *coping responses* (McEwen & Wingfield, 2003). If the load leads to serious pathophysiology, we talk about *allostatic overload*, similar to the stage of "exhaustion" in the G.A.S. model, or the state of "distress" as described later. Allostatic overload as a result of emergency responses is called type 1 and is the result of energy demands exceeding supplies short term. Type 2 allostatic overload is the result of coping, where energy consumption is sufficient, but constant stressors like an overcrowded rearing environment for fish leads to chronically stressed animals (Schreck & Tort, 2016). The total energy cost of chronic stress imposes a greater allostatic load than any acute stress, because of the prolonged energy demand.

The physiological stress response

The physiological stress response is explained as three responses: the primary, the secondary and the tertiary responses (Figure 2). These terms are similar to the three phases of the G.A.S, but primarily explain the effect of stress on different sets of physiological processes (Schreck & Tort, 2016).

The stress response is initiated with some sort of detection of a threatening stressor. The threat might be real, or *perceived* as real (Schreck, 1981). The primary response follows, where neuroendocrine cascades lead to the synthesis and secretion of hormones into the blood. Catecholamines (adrenaline and noradrenaline) are released through the hypothalamus-sympathetic-chromaffin cells (HSC) axis, and corticosteroids (cortisol) are released through the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). The HSC-axis releasing catecholamines acts fast with neurological signalling to the chromaffin tissue, while the HPI-axis releasing cortisol acts slower, signalling through hormones released into the circulatory system. The HPI-axis will be described in more detail later.

The hormones released from the axes lead to the secondary stress responses in different tissues and organs. In the gills, catecholamines increase the respiratory ability of the fish, altering the permeability of the gills. Catecholamines also increase heart rate and thereby cardiac output. Together with increased liberation of energy substrates like glucose into the blood, the animal's availability of oxygen and energy to the handling of the stressor is increased (Wendelaar Bonga, 1997). The increased gill permeability to enhance respiration compromises the hydromineral balance. Water then flows down its osmotic gradient, either in or out of the gills depending on the osmolarity of the environment (Madaro et al., 2020; McDonald & Milligan, 1997; Schreck & Tort, 2016). One of the many roles suggested for cortisol is to restore the hydromineral balance to prestress conditions. If the balance is not sufficiently restored, the fish will experience hydromineral dysfunction (Schreck & Tort, 2016).

While the catecholamines released have immediate effects in a matter of seconds to minutes to prepare for fight or flight, cortisol released through the HPI-axis rather works as an *adaptation hormone* (Gorissen & Flik, 2016). Cortisol redirects the energy flow within the body, having suppressive effects on functions less relevant for short term survival, while stimulating functions increasing survival probability (Sapolsky et al., 2000). Cortisol stimulation is for example shown to increase AA catabolism and gluconeogenesis (Mommsen et al., 1999), thereby increasing energy available for coping. Over the span of hours to days,

cortisol elevation seeks to aid the animal in restoring prestress conditions, i.e. achieve stability through change, as explained earlier with the allostasis concept.

If the stressor is not mitigated and the stress response is extended it will have negative consequences for the animal and act maladaptive (Korte et al., 2005). The animal is then said to be in distress (Holden, 2000), and experiences a high allostatic load. The state is characterized by sustained elevated plasma cortisol levels (Wendelaar Bonga, 1997). The tertiary stress responses occur when the fish energy resources are allocated in the attempt to cope with the challenge at the expense of other functions. The consequence might be a decrease in disease resistance, reproduction, growth, learning, and normal functional behaviour. A prolonged condition of chronic stress for fish in aquaculture thereby can lead to low growth and survival (Schreck & Tort, 2016).

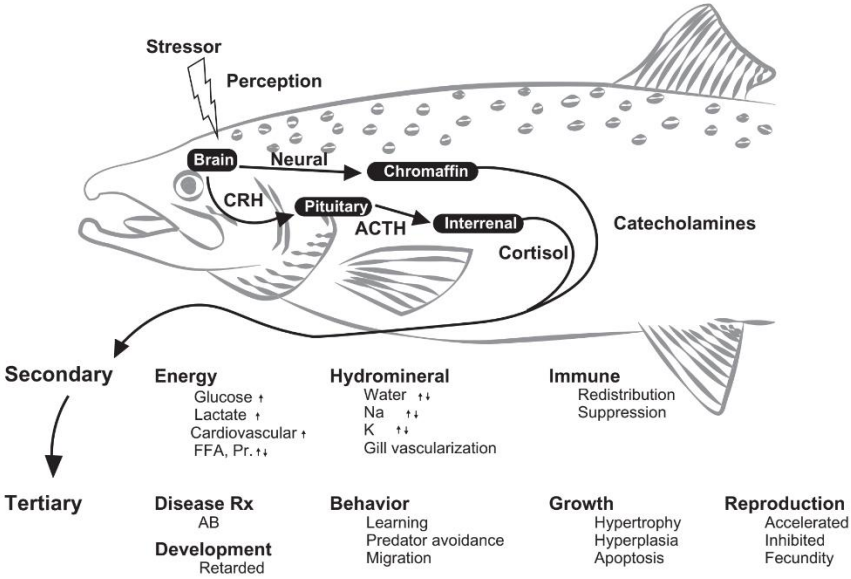

Figure 2: Description of the primary (inside the fish), the secondary and the tertiary stress responses in a fish during distress. Figure taken from Schreck and Tort (2016).

The hypothalamic-pituitary-interrenal axis

The HPI-axis is the endocrinal cascade leading to the secretion of cortisol into the blood in fishes. The name is different from that of the HPA-axis in mammalian and avian species as these utilize the adrenal cortex (A) for the secretion of corticosteroids, compared to fishes who secrete corticosteroids from interrenal cells (I) in the head kidney (Iversen, 2013).

The perception of a stressor leads to the secretion of corticotropin releasing factor (CRF), a short-lived 41-amino acid peptide, from preoptic neurons from the hypothalamus directly in the pituitary near corticotropic cells. CRF triggers the pituitary cells in pars distalis to release adrenocorticotrophic hormone (ACTH) into the general circulation (Gorissen & Flik, 2016).

ACTH is a 39 amino acid polypeptide synthesized from the larger precursor polypeptide pro-opiomelanocortin (POMC). The α-melanocyte-stimulating hormone (α-MSH) is also produced from POMC and released from pars intermedia, having different effects in different fish species, possibly acting as an anorexic compound (Cerdá-Reverter et al., 2003). In fish, the receptor for ACTH, melanocortin receptor 2 (MC2R), is almost exclusively found in the head kidney, presumably the interrenal cells (Metz et al., 2005; Metz et al., 2006). As in humans, the main product of the corticosteroid producing cells in fish is cortisol, which is produced and released into the peripheral circulation following stimulation (Gorissen & Flik, 2016).

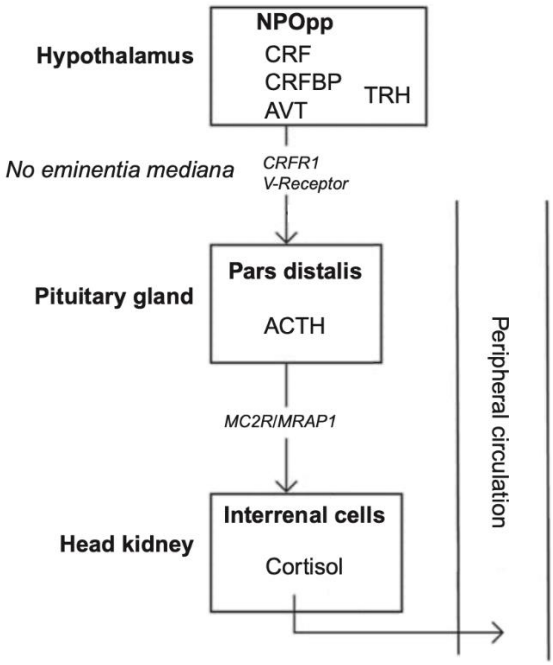

Figure 3: Simplified diagram of the teleostean hypothalamic-pituitary-interrenal (HPI) axis. The most important understandings of the HPI-axis activation are explained in the following: Corticotropin Releasing Factor (CRF) is released through neurons in the *nucleus preopticus* (NPOpp) to pituitary adrenocorticotropic hormone (ACTH) cells in the pars distalis in the pituitary gland. ACTH is released to the blood, primarily binding to the melanocortin receptor 2 (MC2R) in the interrenal cells in the head kidney, leading to the production and release of the corticosteroid cortisol to the peripheral circulation. The diagram is made by simplifying a figure from Gorissen and Flik (2016).

Unlike humans, fish don't produce a specific mineralocorticoid (Wendelaar Bonga, 1997). Instead, cortisol functions as either a glucocorticoid or a mineralocorticoid, depending on binding to a glucocorticoid receptor (GR) or a mineralocorticoid receptor (MR). GR subtypes are derived from a single gene, but alternative splicing of the transcript and posttranslational modifications leads to a variety of receptors having diverse cellular and genomic responses when activated (Oakley & Cidlowski, 2013). The "classic" pathway when binding cortisol following stress leads to a genomic response. Cortisol binds to a GR through intracellular

ligands in the cytoplasm, and the cortisol-GR heterocomplex translocates into the nucleus, forming homodimers that bind to glucocorticoid response elements (GRE) in the promoter region of target genes (Anon, 2014; Dinarello et al., 2020). Through slight differences in GREs and other transcription factors, different GRs and MRs in the same cell can be activated, leading to the regulation of different genes (Dinarello et al., 2020). Thus, the cortisol-GR heterocomplex can modulate either the transactivation or transrepression of different genes inside the same cell or different cells, coding for proteins involved in metabolism, growth, reproduction, and immune function (Aluru & Vijayan, 2009; Mommsen et al., 1999; Prunet et al., 2006).

Variations in stress coping style and cortisol release

Observations in different fish species like rainbow trout (*Oncorhynchus mykiss*) (Pickering et al., 1991), zebrafish (*Danio rerio*) (Pavlidis et al., 2015), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*) and more (Fanouraki et al., 2011) show that peak cortisol release takes place between 30 and 60 minutes post stress (reviewed in Madaro et al. 2020). Following stress activation, when the fish have coped with the challenge, cortisol is involved in a negative feedback loop that downregulates the HPI-axis and shuts down the stress response. Cortisol downregulates the transcription of the CRF gene in the hypothalamus (Bernier et al., 1999), the expression of POMC (Palermo et al., 2008) and the release of ACTH from the pituitary (Fryer et al., 1984). The effects of cortisol are terminated by the enzyme 11β-hydroxysteroid dehydrogenase 2 as it converts cortisol into its inactive form cortisone (Funder et al., 1988; Mommsen et al., 1999).

Stress coping capacity varies greatly between species, and also between individuals of the same species and same population. Differences in coping strategy between species greatly affect the nature of the stress response, leading to different amounts of cortisol being released to the blood. Facing a stressor, species like Atlantic salmon, European sea bass and ballan wrasse *(Labrus bergylta)* usually respond with a quite traditional “fight or flight” response, while species like the lumpfish usually “freeze” in order to cope, which demands less cortisol release (Fanouraki et al., 2011; Jørgensen et al., 2017; Leclercq et al., 2014). In the same species, life experiences like earlier stress episodes can affect the stress response (De Kloet et al., 2005; Vindas, Madaro, et al., 2016; Winberg et al., 2016). Environmental conditions like temperature also affect the stress response with the profile and magnitude of cortisol release (Madaro et al., 2018). Ontogeny and life stage also influence the magnitude of the stress

response, as critical periods like transitional stages leaves the fish more vulnerable (Feist & Schreck, 2001). Smoltification in salmon is an example of this.

In salmonid populations, established dominance hierarchies lead to differences in serotonergic profiles. Subordinated fish with lower serotonin levels also sustain elevated levels of cortisol (Gilmour et al., 2005). Subordination is perceived like a form of chronic stress. Inside a population, differences in serotonin levels, phenotype, and baseline cortisol levels can lead to different coping mechanisms when stressed (Gilmour et al., 2005; Vindas, Johansen, et al., 2016). This suggests variation in cortisol release under acute stress between individuals in the same population.

The unpredictable chronic stress paradigm

In salmon aquaculture in open cages, the fish are potentially exposed to a number of different stressors like poor water quality, repeated handling, transport and crowding (Madaro et al., 2015). The effect of individual stressors on fish have been studied, like netting (Gorissen et al., 2012), transportation (Barton & Peter, 1982), stocking density (Di Marco et al., 2008) and more. To simulate and understand the possible impact of multiple stressors placed on Atlantic salmon in aquaculture, Madaro et al. (2015) developed an unpredictable chronic stress (UCS) paradigm for Atlantic salmon. A similar paradigm has also been tried on zebrafish (Pavlidis et al., 2015). The Atlantic salmon UCS paradigm involves exposing the fish to multiple instances of stress daily, each time utilizing one of many possible stressors simulating what the fish might experience in an aquaculture setting. After weeks of experiencing chronic stress, subsequent trials and sampling can reveal how the chronic stress condition affects the stress response, growth, and other measures. In diploid Atlantic salmon, the exposure to UCS clearly reduces the growth rate (Madaro et al., 2015). It also reduces pituitary capacity to mount a proper acute stress response, reducing the cortisol response to the stressors, downregulating the stress response under chronic stress conditions (Madaro et al., 2015). The reduced cortisol response can also be observed in fish stressed with only one stressor, though the growth might not be affected to the same degree (Barton, 2002; Barton et al., 1987). In explaining the lessened cortisol response and sustained growth in one-stressor trials it is suggested that *habituation* takes place. In that case the fish is no longer stressed by the exposure (Barton, 2002; Barton et al., 1987).

In triploid salmonids, acute stress tests have been studied earlier (Benfey & Biron, 2000; Biron & Benfey, 1994). Overall, triploids have been evaluated as having a similar and typical

acute stress response compared to diploids, with similar levels of cortisol and glucose measured in the plasma. Almost nothing however is known about triploid Atlantic salmon performance under chronic stress conditions. A chronic stress trial has been conducted on triploids utilizing crowding as the stressor (Rogers, 2016), but this trial only utilized one repeating stressor, possibly allowing for habituation. Subjecting triploid Atlantic salmon to a UCS regime could allow a better understanding of the physiological coping capacity of triploids to long lasting stress conditions. In understanding so one could attempt to draw a link to the lower performance of triploids in commercial conditions when compared to the homologue diploid fish.

Working hypotheses

Triploids have a larger cell volume without having a larger body size, suggesting a lower cell density. The endocrinal stress response relies on preoptic neurons, corticotropic pituitary cells, and interrenal cells for secretion of CRF, ACTH and cortisol, respectively. Due to a lower cell density, during chronically stressed conditions, this could possibly mean that an exhausted triploid *nucleus preopticus*, pituitary and/or interrenal may secrete an insufficient amount of stress hormones. This could entail an impaired adaptive response due to lower cortisol release. The triploid Atlantic salmon could then possibly not be able to handle as high an allostatic load as diploids. By comparing the stress response of chronically stressed triploids and diploids with naive control groups, this phenomenon and other possible weaknesses in triploid salmon physiology can be detected. The following hypotheses will be tested in this experiment:

- Three weeks of chronic stress will have reduced growth for both ploidies, and more so for triploids.
- Three weeks of chronic stress will have increased feed conversion ratio for both ploidies, and more so for triploids.
- Both ploidies experiencing chronic stress will have increased baseline concentrations of circulating cortisol compared to unstressed fish.
- Experiencing a novel stressor, triploids will secrete less ACTH and cortisol into the plasma compared to diploids.
- Experiencing a novel stressor, chronically stressed triploids will secrete less ACTH and cortisol into the plasma compared to triploids not experiencing chronic stress.

Materials and methods

Experimental approval

The experiment was approved by the Norwegian Animal Research Authority (NARA), FOTS ID 28400.

Experimental animals and facilities

Atlantic salmon (*S. salar*, Aquagen/NLA strain, male, diploid and triploid) were hatched and reared at the Institute of Marine Research (IMR), Matre. The fish were produced by crossbreeding one homozygous female clonal line (line 113) with one doubled haploid male (frozen sperm). One batch of these eggs were pressurized in the second meiotic division to generate triploids. Triploidy was confirmed comparing erythrocyte diameter on blood smears from 30 diploid and 30 triploid fish with a technique established by Benfey et al. (1984) (Figure 4). The rearing conditions resembled natural winter conditions (12 h light:12 h dark, 8 °C, tank size 1m^3 400 L).

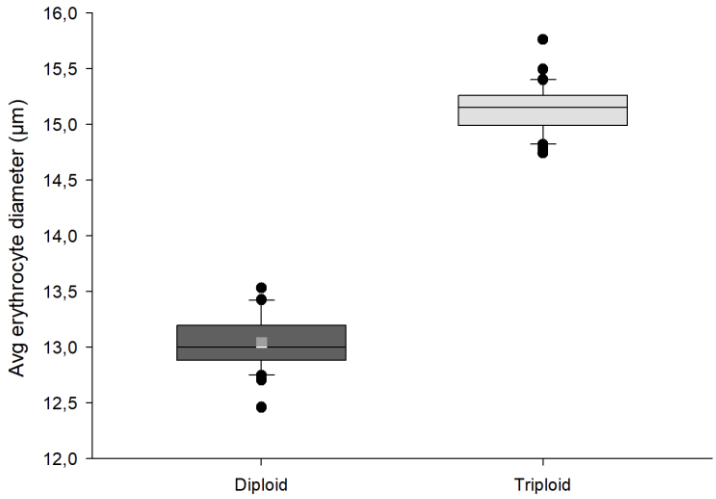

Figure 4: The average erythrocyte diameter (µm) in 30 fish from the diploid experimental tanks and 30 fish from the triploid experimental tanks. This comparison was used to confirm the ploidy of the fish used in the experiment, using a method developed by Benfey et al. (1984).

360 diploid fish (154.6 ± 18.6 g) and 360 triploid fish (187.8 ± 21 g) were moved to the indoor experimental cell and divided between 12 square 400 L tanks (60 fish each) filled with fresh water at 12°C, with a water flow of 15 L/min, which ensured a O_2 saturation of 90% in the tanks. The diploid and triploid fish were randomly divided on their respective 6 tanks,

making up 4 experimental groups each consisting of 3 tanks each (Control 2N, UCS 2N, Control 3N, and UCS 3N). Each experimental group had its own water inlet, allowing for independent control of water quality between groups. A sketch overviewing the tanks in the facility is shown in Figure 5. While handling the fish they were mildly sedated with 25 mg/L Finquel ®vet (ScanAqua AS, Årnes, Norway), buffered with 25 mg/L sodium bicarbonate. 30 fish for each tank were individually measured for weight and length, while the remaining 30 fish for each tank were weighed in bulk.

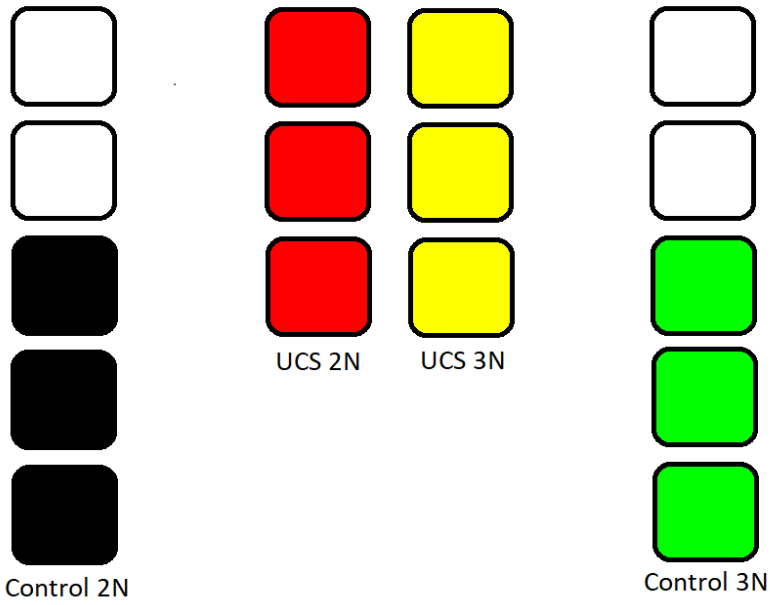

Figure 5: Sketch overviewing the tanks in the experimental facility. Each experimental group consisted of three tanks containing 60 fish each. The tanks making up the groups are coloured in the same respective colours that are used indicating the groups in the graphs in the results. Each group had the same water inlet, allowing to adjust water flow and water temperature independently between groups, which was utilized in some of the stressors applied. The white tanks contained no fish for the UCS trial but were utilized for containing fish during the novel stressor trial at the end of the experiment.

The tanks were equipped with lids containing fluorescent light tubes and automatic feeders (Arvo-tec feeding units, Arvo-Tec T drum 2000, Huutokoski, Finland). After the transfer a 9 day acclimation period followed. During the acclimation period and the experimental period the fish were fed using commercial feed (Skretting Nutra Olympic, 2 mm in the acclimation period, 3mm in the UCS trial period), giving 60% of the daily feed from 9:00-11:00 a.m. and the remaining 40% from 1:00-3:00 p.m. Each tank received 150 g/day through the acclimation period + the first day of the UCS trial. The following 7 days they were fed 200 g/day, and 250 g/day the remaining period of the trial.

Unpredictable chronic stress trial

After the acclimation period, a three-week unpredictable chronic stress (UCS) trial began for the diploid and triploid UCS groups. The Control groups were left undisturbed with as little interference as possible. The UCS fish were stressed two times a day. The first stressor was given at 8:00 a.m. and the second at 12:00 noon, with the exemption of when the stressor was a temperature shock, which started ca. 30 min earlier, as the required temperature took ca. 45 minutes to reach, giving a total time of about 90 minutes to arrive at the original 12 °C again, before the start of feeding. The stressor regime was a version of the UCS trial put forward by Madaro (2015), slightly modified with the aim to minimize physical damage to larger experimental fish and solely induce a stress response (Table 1).

Table 1: Description of the stressors given to the UCS groups during the 3 weeks UCS trial. The stressors given were a slight modification of the stressors put forward by Madaro et. al. (2015) to minimise direct physical damage and solely induce a stress response. The stressors "netting", "chasing" and "noise + darkness + flashlight" were given by opening the tank lids and continuously move between all the tanks for a 5-minute period, giving each tank a 5-10 second stressor exposure each time.

Stressor	Time (min)	Description
Chasing	5	Stirring in the tank with a blue tank cleaning brush
Netting	5	Stirring in the tank with a black hand net
Temperature shock 12-6-12°C	90	Reduction of the water temperature from 12°C to 6°C and back up again.
Temperature shock 12-19-12°C	90	Increasing the water temperature from 12°C to 19°C and back down again.
Noise + darkness + flashlight	5	Turning off tank light and knocking on the tank while flashing with a flashlight.
Emptying the tank	5	Removing the tank plug while leaving the water flow on resulting in a 3-5 cm deep layer of water.
Brief hypoxia	5	Closing off the water inflow and emptying half of the tank until oxygen saturation reached 60%.

The seven different stressors used in the trial were delivered in a semi-random manner in such a way that each stressor was given two times a week. This weekly schedule was repeated for the three weeks (Table 2).

Table 2: The stressor regime for the 3-week UCS trial. The weekly regime was repeated for all three weeks. Stressor 1 was given at 8:00 a.m., and stressor 2 at 12 noon, except for the temperature stressors which started 30 minutes earlier. The stressors are further explained in Table 1.

Daily stressor no.	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1	Chasing	Netting	T 12-6-12°C	Empty tank	Hypoxia	Netting	Empty tank
2	T 12-19-12°C	Hypoxia	L/D + noise	T 12-19-12°C	T 12-6-12°C	L/D + noise	Chasing

Feeding continued as during the acclimation period dispensing 60% of the daily feed from 9:00-11:00 a.m. and the remaining 40% from 1:00-3:00 p.m. In this way, the UCS fish got ca. 55 minutes from the stressor exposure until the start of feeding, with the exemption when given the temperature shock stressors. Feed collection was done by putting out sieves under the water outlets of the tanks just before the start of feeding, and removing them for weighing of content 30 min after stop of feeding, i.e. feed collection was done from 9:00-11:30 a.m. and from 1:00-3:30 p.m.

Novel stress trial and sampling

Around 21:00 p.m at the last day of the 21 day UCS trial, the UCS 3N group which was to be sampled the next day was weighed in bulk in order to record the end total biomass of fish in each tank. The same procedure was completed the next night for the UCS 2N group and the Control 3N group, and the following night the Control 2N group was weighed. In this way, the UCS groups were weighed the night before their sampling day, while the Control groups got one more day between weighing and sampling. During weighing, the fish were sedated as earlier with 25 mg/L Finquel ®vet (ScanAqua AS, Årnes, Norway), buffered with 25 mg/L sodium bicarbonate. After weighing a group, the feed collection for this group stopped.

After the UCS trial sampling started, with one of the four experimental groups each day. The sampling started with the UCS 3N group and continued the following days with the UCS 2N group, the Control 3N group, and lastly the Control 2N group. Feeders in all groups were stopped at 11:00 a.m. the day before sampling, and the fish were therefore starved for 21 h before sampling occurred. Both UCS groups ended their UCS trial with equal stressors the last day before sampling. This was accomplished by replicating the last day of the trial for the UCS 3N fish one more day for the UCS 2N fish (Table 2). All four experimental groups went through the same novel stressor trial and sampling procedure.

Sampling started at 9:00 a.m. each day. Ten fish in total were then immediately randomly sampled from the three tanks in the experimental group and anesthetized with an overdose of 1 g/L Finquel ®vet (ScanAqua AS, Årnes, Norway), buffered with 1 g/L sodium bicarbonate. The rest of the fish from all three tanks were quickly collected into the same 100 L mobile tank on wheels, resulting in a crowded environment. When all fish were collected in the small tank, a clock was started giving a one-minute countdown. Oxygen saturation in the crowded tank was continually measured, ensuring acceptable O_2 levels, which never fell below 60%. After the one minute, the fish were randomly divided into 8 different tanks, with at least 10 fish in each tank. The lids for the tanks were then closed, ensuring no additional accidental stressor exposure for the fish. The tanks were then sampled one at the time after 15, 30, 45, 60, 90, 120, 240 and 300 minutes post stress. 10 fish per tank were sampled and anesthetized with 1g/L Finquel ®vet. Blood was collected by 1 mL heparinized syringes fitted with a 23 G needle. Blood plasma was separated immediately by centrifugation at 13,000 rpm for 3 min. Plasma was immediately put on ice and then analysed for ion and metabolite content before being stored at -80 °C for further hormonal analysis.

Analysis

Specific growth rate

Specific growth rate from first to last weighing was calculated for each tank according to the following formula:

$$Spesific\ growth\ rate\ (\%) = \frac{ln\ (weight\ finish) - ln\ (weight\ start)}{\#days} * 100 \quad (1)$$

As the end weight was registered on different days, the #days varies between the SGR calculations, being 30 for the UCS 3N group, 31 for the UCS 2N and Control 2N groups, and 32 for the Control 2N group.

Feed collection and feed conversion ratio

Collected uneaten pellet was used to determine daily feed as in Helland et al. (1996):

$$Air - dry\ feed\ eaten\ (g) = \frac{\left(A * \frac{Adm}{100}\right) - \left(W * \frac{Wdm}{R}\right)}{\frac{Adm}{100}} \quad (II)$$

$$R = 100\% * \frac{W * Wdm}{A * Adm} \quad (III)$$

A is weight of air-dry feed, Adm is dry matter content of air-dry feed (%), W is weight of waste feed collected (g), Wdm is dry matter content of waste feed (%), and R is recovery of dry matter content of waste feed (%).

The model for the calculations was made as follows: 3*50 pellets were weighed and thrown into three empty fish tanks. The waste feed was collected with sieves in the same manner as for the experimental tanks. The three collected pellet samples were weighed before they were put in an oven at 60°C together with triplicate pre-weighed samples of 50 *dry* pellets. 24h later the samples were weighed. The values calculated based on these results are presented in Table 3.

Table 3: The values obtained and used in the calculations of the air-dry feed eaten during the experimental period. *Wdm* is dry matter content of waste feed (%), *Adm* is dry matter content of air-dry feed (%), while *R* is the recovery of dry matter of waste feed.

***Wdm* (%)**	***Adm* (%)**	***R* (%)**
29.47	91.60	73.66

The feed conversion ratio (FCR) for each tank was then calculated with the following equation:

$$FCR = \frac{Total\ feed\ eaten\ (g)}{Biomass\ increase\ (g)} \quad (IV)$$

At the end of the experiment, some feed was still left in the automatic feeders. The weight of the leftover feed was registered after the sampling and subtracted from the total weight of dry feed given.

As feed collection started at the first day of the UCS trial, no waste feed was collected during the 9 day acclimation period. In calculating the FCR, the following assumptions were made about feed eaten in this period to get reasonable values. Feeding started at day 3 after transfer. It is assumed that the feed eaten by the fish increased exponentially the first four days of feeding, so that normal feeding behaviour is reached at day 6 after transfer to the facility. This gives four days of normal feeding behaviour before the start of the UCS trial. It is assumed that the Control tanks ate the same amount these four days as at the first day of the UCS trial (first day of feed collection), while the UCS tanks ate the average of the Control tanks of their respective ploidies. The average of the controls is used here as the UCS groups the first day of feed collection were stressed and had a lower appetite.

Blood analysis

Ions (Na^+, Cl^-, K^+), pH, lactate and glucose concentrations in the plasma were analysed using an ABL90 FLEX blood gas analyser (radiometer) of a 65 µL subsample of the blood plasma minutes after the centrifugation. The samples were then stored at -80 °C until further hormonal analysis.

Plasma ACTH and cortisol concentrations were analysed utilizing enzyme-linked immunosorbent assays (ELISA). The frozen samples were thawed over ice before the ACTH levels were measured utilizing 50 µL plasma subsamples with a Fish ACTH kit (Cat.no.CSB-E15926Fh, CusaBIO). The cortisol ELISA was carried out utilising 20 µL subsamples (standard range: 20 to 800 ng/mL, RE52061 IBL-International, Hamburg, Germany).

No ACTH levels were analysed for the 300 min sampling point.

Statistical analysis and figures

All statistical tests are performed using the statistical program SPSS 27.0 for MacOS. One-way ANOVA tests were performed on the SGR and FCR data, and the pre-stress plasma data (Time = 0). The total plasma data was analysed by making generalized linear models (GLM). The factors tested for were Time (Time post stressor (min)), Ploidy (2N, 3N) and Treatment (Control, UCS). The models generated do not include the data at Time = 45, as no data for the UCS 3N group was collected at this time point.

Figures are made using SigmaPlot 14.0 for Windows. For the blood data, the data at Time = 45 is shown in the figures even though these data are not included in the statistical tests. This is done in order to give an impression of the general developmental trends.

Results

Growth and feed conversion

Average weight increase

Table 4: Average individual start and finish weight, and biomass increase for each group across the experimental period. Weight measures represent the average fish weight based on the total biomass measurements at transfer to the experimental tanks, and the bulk measurements at the end of the experiment. Standard deviation between average weight in the three experimental tanks.

Group	Weight start (g)	Weight finish (g)	Biomass gain (g)
Control 2N	154.0 ± 2.2	269.4 ± 10.6	115.4 ± 10
UCS 2N	155.2 ± 1.6	196.0 ± 3.0	40.8 ± 2.6
Control 3N	181.8 ± 10.3	281.9 ± 13.8	100 ± 5.8
UCS 3N	193.8 ± 5.4	238.0 ± 9.9	44.2 ± 6.8

Table 4 shows the average individual weight per fish for each group at the start and end of the experiment, with the average biomass gain. The Control 2N fish had an average biomass gain of 115.4 ± 10g while the UCS 2N fish gained 40.8 ± 2.6g. The Control 3N fish gained 100 ± 5.8g on average, while the UCS 3N fish gained an average mass of 44.2 ± 6.8g during the experimental period.

Two fish, one UCS 3N fish and one Control 3N fish, at day 5 and day 16 of the UCS trial respectively, were observed with abnormal behaviour, laying on the tank bottom and having some fin erosion. The fish were evaluated as moribund and were immediately removed from their tanks and euthanized with an overdose of 1g/L Finquel ®vet buffered with 1g/L sodium bicarbonate, implementing early humane endpoints as described in the FOTS application. The weight and fork length of the two fish were recorded and considered in the growth and feed intake data analyses.

Specific growth rate

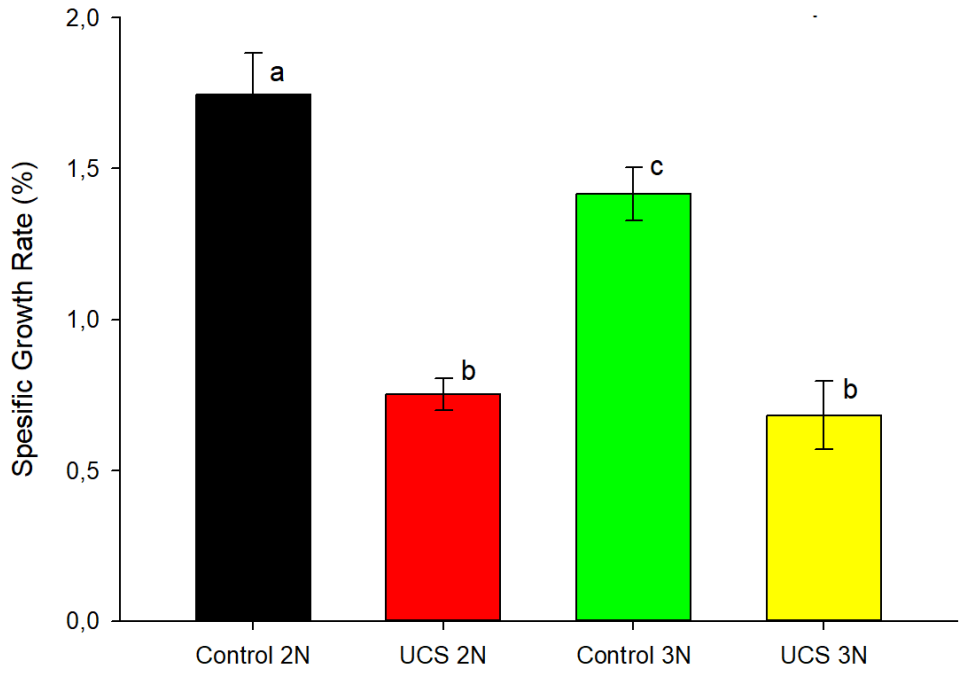

Figure 6: Average SGR (%) values for the four experimental groups based on the total biomass measurements at transfer to the tanks, and the total biomass at the end of the experiment (n=3). Different letters mark significant differences at the 95% confidence level. Error bars show standard deviation.

Figure 6 shows the average specific growth rate (SGR) for the four experimental groups based on the total biomass at transfer and the total biomass at the end of the experiment. The Control 3N group (1.42 ± 0.05%) had a significantly lower SGR than the Control 2N group (p = 0.019). The Control 2N group (1.74 ± 0.08%) had a significantly larger SGR than all the other groups. The UCS 2N group (0.75 ± 0.03%) and the UCS 3N group (0.68 ± 0.07%) had the lowest SGR values and were not significantly different from each other (p = 0.844).

The weight of the two removed fish were subtracted from the start weight of their respective tanks.

Feed conversion ratio

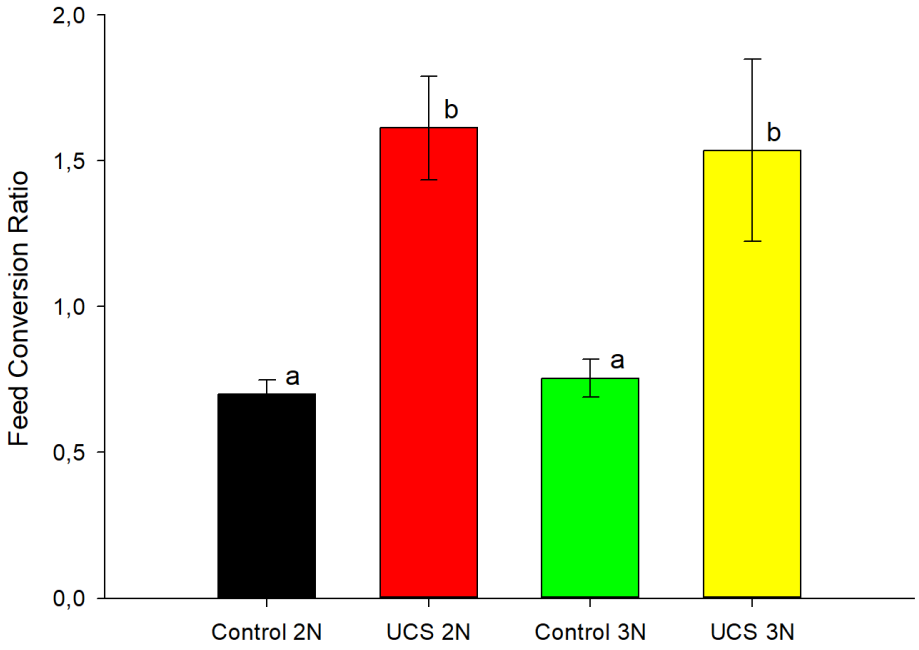

Figure 7: Average FCR values for the four experimental groups based on the total biomass measurements at transfer to the tanks, the total biomass at the end of the experiment (n=3), and the collected feed during the experimental period. Different letters mark significant differences at the 95% confidence level. Error bars show standard deviation.

The calculated feed conversion ratio (FCR) values are shown in Figure 7. The values are calculated from the total biomass at the start and the end of the experiment and collected feed during the experimental period. Throughout the UCS trial, the UCS groups consistently left more feed uneaten to be collected compared to the Control groups. Values used for the calculation of total feed eaten are presented in Table 3. The Control 2N (0.70 ± 0.05) and Control 3N (0.75 ± 0.06) groups had significantly lower ratios than the UCS groups. The Control groups were not significantly different form each other (p = 0.983), and the UCS 2N (1.61 ± 0.18) and UCS 3N (1.54 ± 0.31) groups were not significantly different from each other either (p = 0.954).

The two fish that were removed from the tanks during the UCS trial both had K-factors above 1. This was interpreted as that the fish had eaten while staying in the tanks. In calculating the FCR for the two tanks missing one fish each, the biomass of the two fish was subtracted from the start weight (as done when calculating the SGR), and the assumed 1/60 of the total feed eaten by the fish was removed from the daily feed eaten on the days that the two fish were in their tanks.

Blood plasma levels

The average measured levels of plasma parameters at each time point post stressor exposure are presented in Figure 8 to 15. For each graph, GLM Between-Subject effects are shown, calculated without the Time = 45 data due to the lack of data at this time point for the UCS 3N group. The data at Time = 45 are shown in the figures to give a greater visual impression of the general trends. In describing the blood parameters, trends are prioritized over describing specific time point values. Particular emphasis is placed on the primary stress response hormones.

ACTH

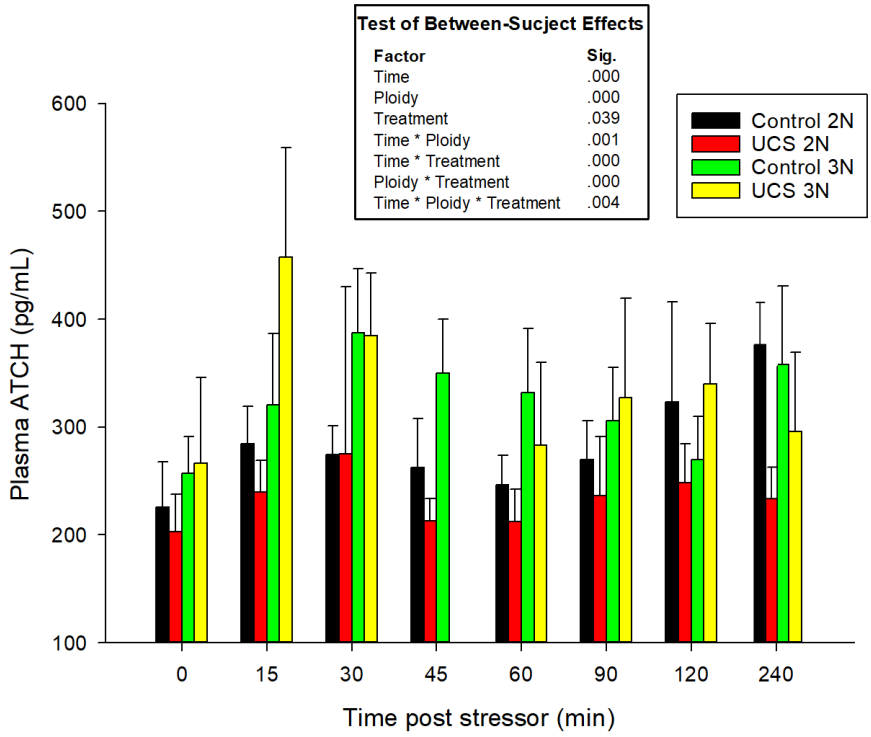

Figure 8: The average values of plasma ACTH (pg/mL) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 8 shows the average plasma ACTH levels (pg/mL). Pre-stress values were significantly different between the UCS groups (p = 0.041), with the UCS 3N group having the largest value. Otherwise, no pre-stress significant differences were found. In the novel response, all factors and interactions showed significant effects. Clear trends though are not so easy to identify. The triploid fish seem to mount a larger ATCH release after the stress (Ploidy $p<0.001$), and the highest average value recorded is for the UCS 3N group at 15 min post stressor (457.4 ± 101.5 pg/mL). For all groups the ACTH level comes down from the

initial response between 45 and 120 minutes, but all groups also experience an increase in ACTH plasma levels towards 240 minutes post stress, particularly the Control 2N group, which ended at 240 min with the highest recorded value for this group (378.0 ± 39.2 pg/mL)

Cortisol

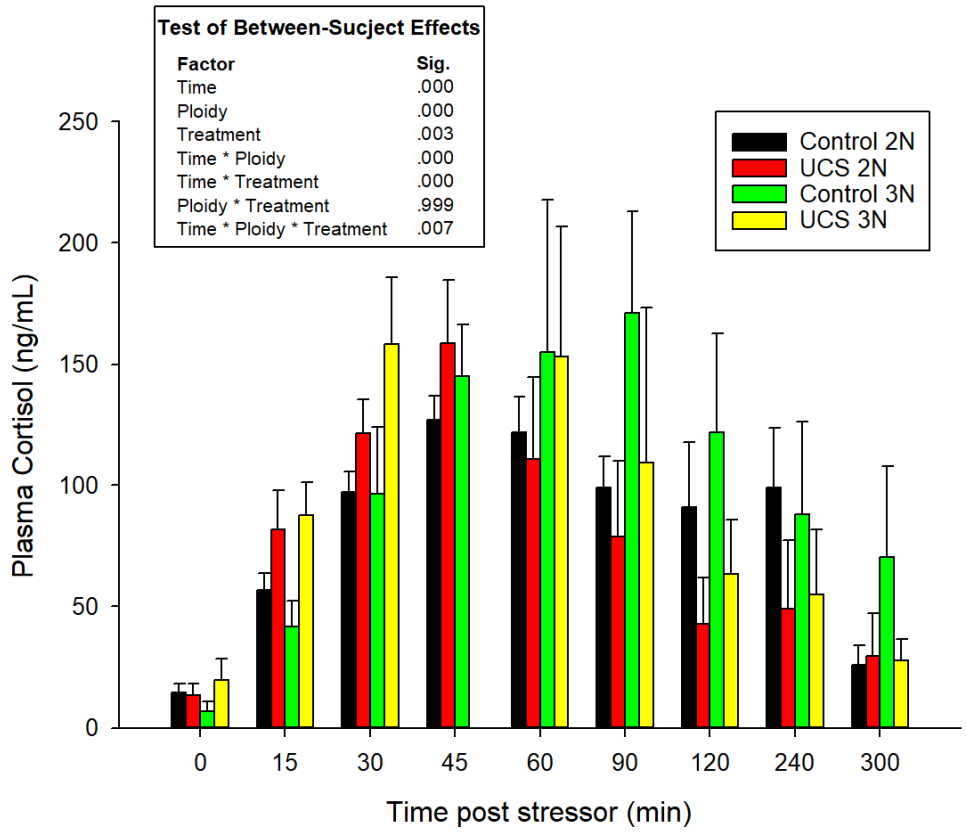

Figure 9: The average values of plasma cortisol (ng/mL) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

The development of cortisol for all groups from pre-stress to 300 minutes after stress is given in Figure 9. Pre-stress, the recorded values of plasma cortisol for the Control 2N group were 14.6 ± 3.4 ng/mL, for the UCS 2N group 13.3 ± 4.9 ng/mL, for the Control 3N group 6.8 ± 4.1 ng/mL, and for the UCS 3N group 19.7 ± 8.8 ng/mL. The 3N UCS group had significantly larger pre-stress levels than the Control 3N group ($p < 0.001$), and the Control 3N group was significantly lower than the Control 2N group ($p = 0.020$).

The cortisol curve followed a Gauss curve pattern (Time $p < 0.001$) increasing from around 20 ng/ml in all groups before stress to around 150 ng/ml at 30-90 minutes after stress. The level was then reduced throughout until the end of trial (300 min) but without reaching original levels. There was a significant effect of Treatment on the cortisol curves (Treatment $p = 0.003$). In particular, the UCS groups had a faster increase of plasma cortisol at 15 and 30 minutes following stress than the Control groups. The UCS groups did on the other hand appear to clear the cortisol faster from the plasma than the Control groups. This was particularly evident at 60, 120 and 240 minutes after stress. At 240 min the plasma cortisol of the Control groups was around 100 mg/mL while the UCS groups approached 50 ng/mL. There was also a significant ploidy effect (Ploidy, $p < 0.001$, Ploidy x Time $p < 0.001$). This was to a large extent due to a very high cortisol response seen in the Control 3N fish compared to the Control 2N. In the former group, the cortisol level peaked at 90 minutes (171.3 ng/mL) and remained higher than all other groups until the end of sampling. There did not appear to be any significant interactional effect between Ploidy and Treatment ($p = 0.999$), while all other effects were significant.

Glucose

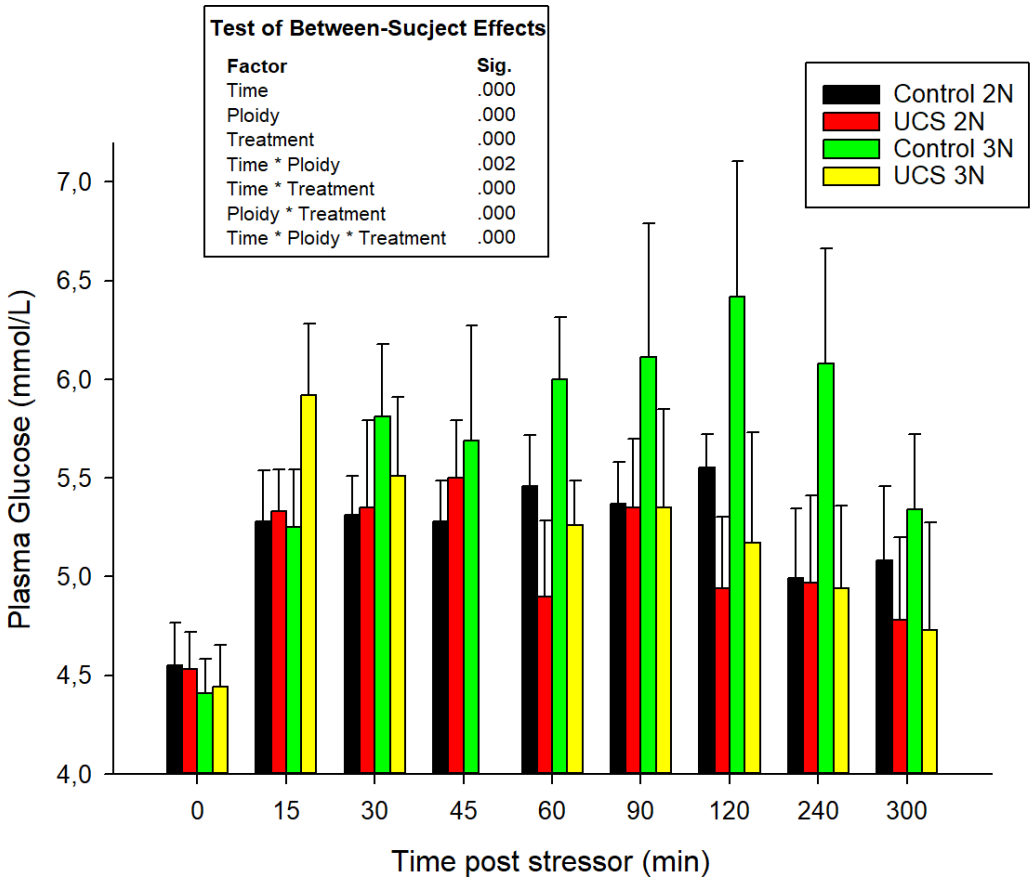

Figure 10: The average values of plasma glucose (mmol/L) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 10 shows the average plasma glucose levels. There were no significant differences in pre-stress values. For the novel stressor, all factors and interactions showed significant effects. From 0 to 15 min a markable increase in plasma glucose took place for all groups, with the UCS 3N response being higher than the rest. For the rest of the sampling, the groups were pretty similar in their glucose levels, except for the Control 3N group which had a remarkably higher and prolonged response, particularly from 60 to 240 min.

Lactate

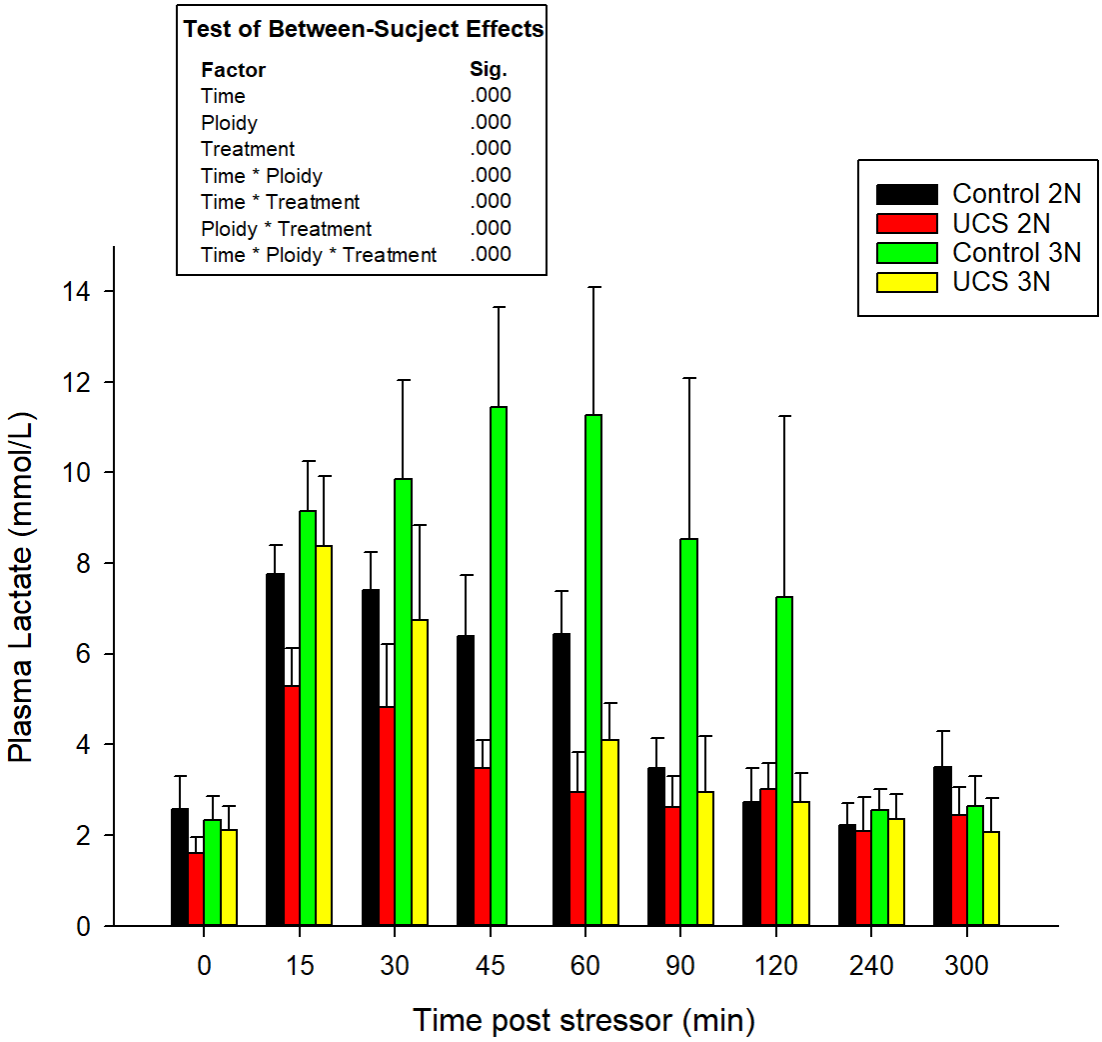

Figure 11: The average values of plasma lactate (mmol/L) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 11 shows the average plasma lactate levels. The UCS 2N group had significantly lower pre-stress values than the Control 2N group (p = 0.002) and the Control 3N group (p = 0.024). In the novel response, all factors and interactions showed significant effects. All groups show an increase in plasma lactate from 0 to 15 min, with the lowest response in the UCS 2N group. Both Control groups show a higher concentration and prolonged increase compared to their respective UCS groups, and the Control 3N group shows substantially higher concentrations with recorded values almost twice the values of the Control 2N group at Time 60 and 90 min. All groups approached pre-stress values at the end of sampling.

pH

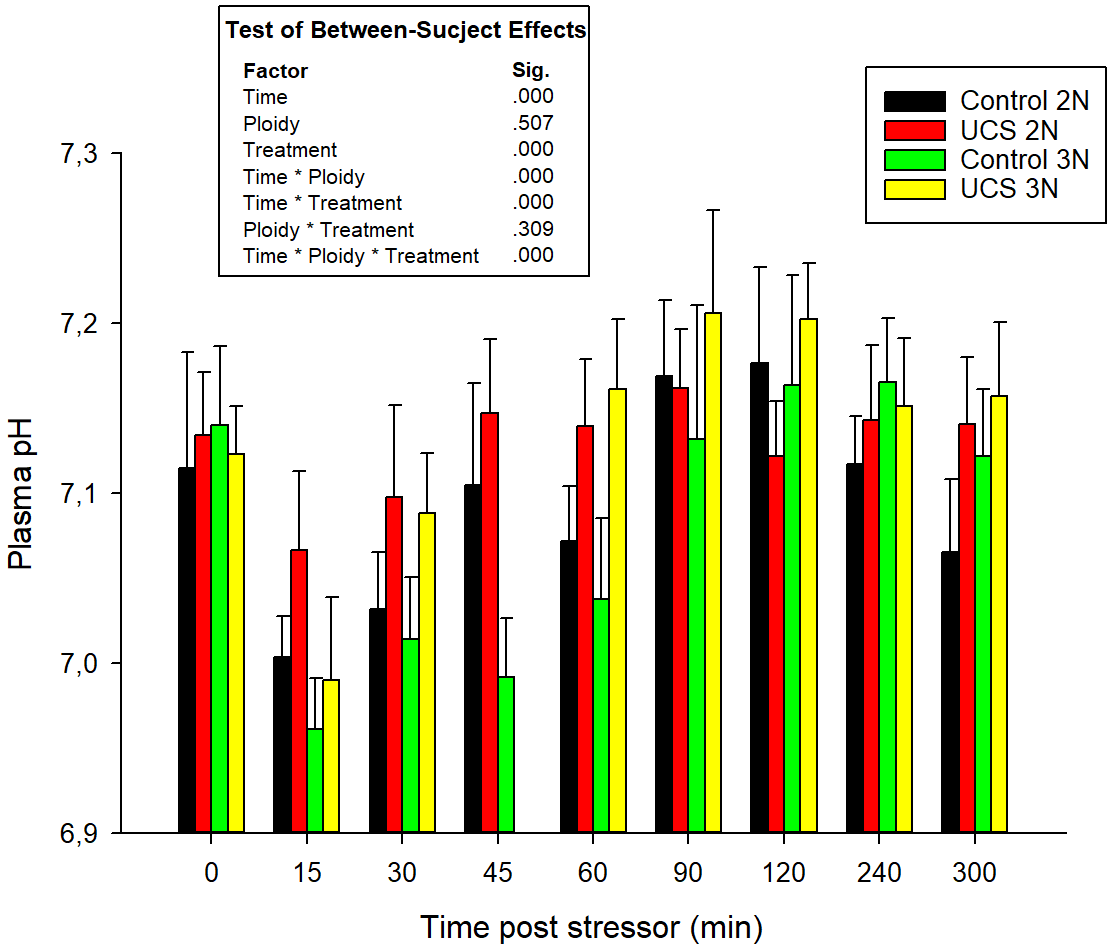

Figure 12: The average values of plasma pH measured for the fish (n = 10) collected at each point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 12 shows the average plasma pH across the samples. No significant pre-stress differences were observed. In the novel response there was no significant effect of Ploidy (p = 0.507) and the interaction between Ploidy and Treatment (p = 0.309), while all other effects were significant. From 0 to 15 min a drop in pH occurred for all groups, with the largest decrease recorded for the Control 3N group and the smallest decrease for the UCS 2N group. The Control 3N group had prolonged lower pH than the rest of the groups until the 90 min sample, where all groups were quite similar again.

Sodium (Na^+)

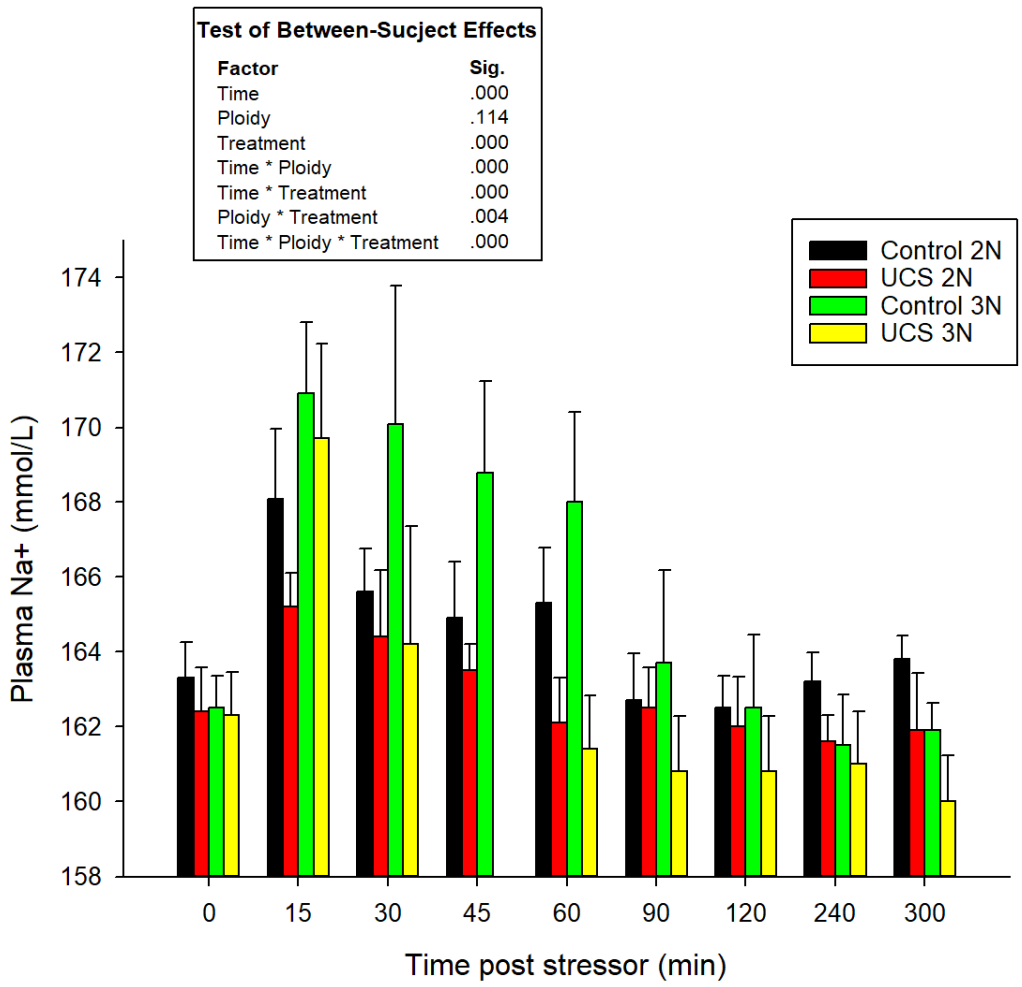

Figure 13: The average values of plasma sodium (Na^+) (mmol/L) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 13 shows the average plasma sodium (Na^+) levels. There were no significant differences in pre-stress levels. For the novel response, there was no significant effect of Ploidy ($p = 0.114$), while all other effects were significant. From 0 to 15 min, a marked increase in Na^+ can be observed for all groups, with the UCS 2N group having a lower response than the other groups. Comparing the triploids, the recovery of the UCS 3N group was fast, with a marked decrease from 15 to 30 min post stressor. The Control 3N group had a remarkably prolonged high response before it came down at 90 min. Both UCS groups performed very similarly compared to their respective Control groups (Treatment $p < 0.001$), which might account for the insignificant effect of Ploidy ($p = 0.114$)

Chloride (Cl^-)

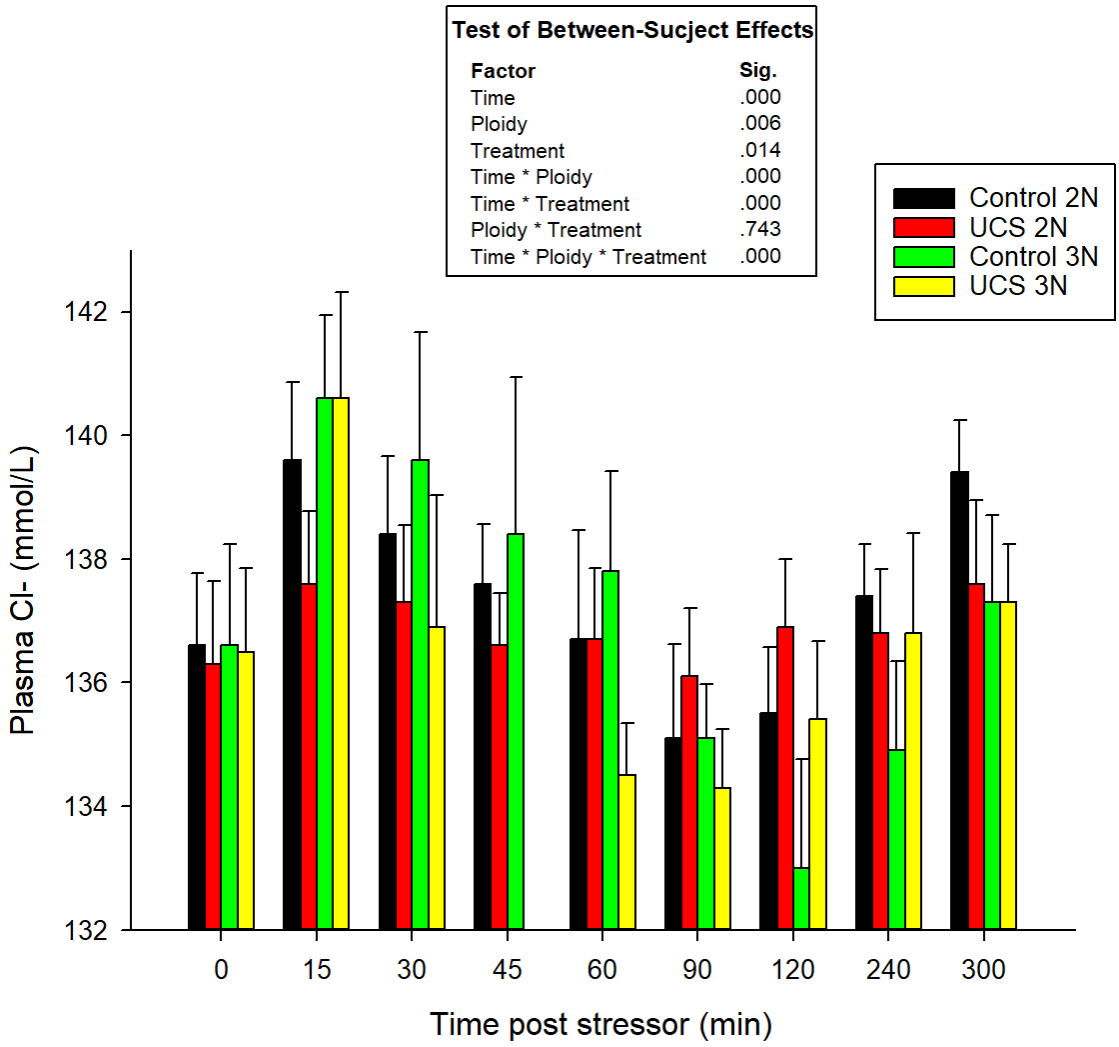

Figure 14: The average values of plasma chloride (Cl^-) (mmol/L) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 14 shows the average plasma chloride (Cl^-) levels. No significant pre-stress values were found. In the novel response, there was no significant effect of the interaction between Ploidy and Treatment (p = 0.743), while all other effects were significant. As with the Na^+, all groups respond to the stressor with an increase in plasma Cl^- concentration at 15 minutes, with the smallest response in the UCS 2N (137.6 mmol/L) group and the largest response in both 3N groups (140.6 mmol/L). All groups experienced a drop in Cl^- towards 90 min (120 min for the Control 3N group), followed by an increase. Especially the Control 2N group increased a lot from 90 min, ending at 300 min with almost the same concentration as at 15 min post stressor (139.4 mmol/L).

Potassium (K^+)

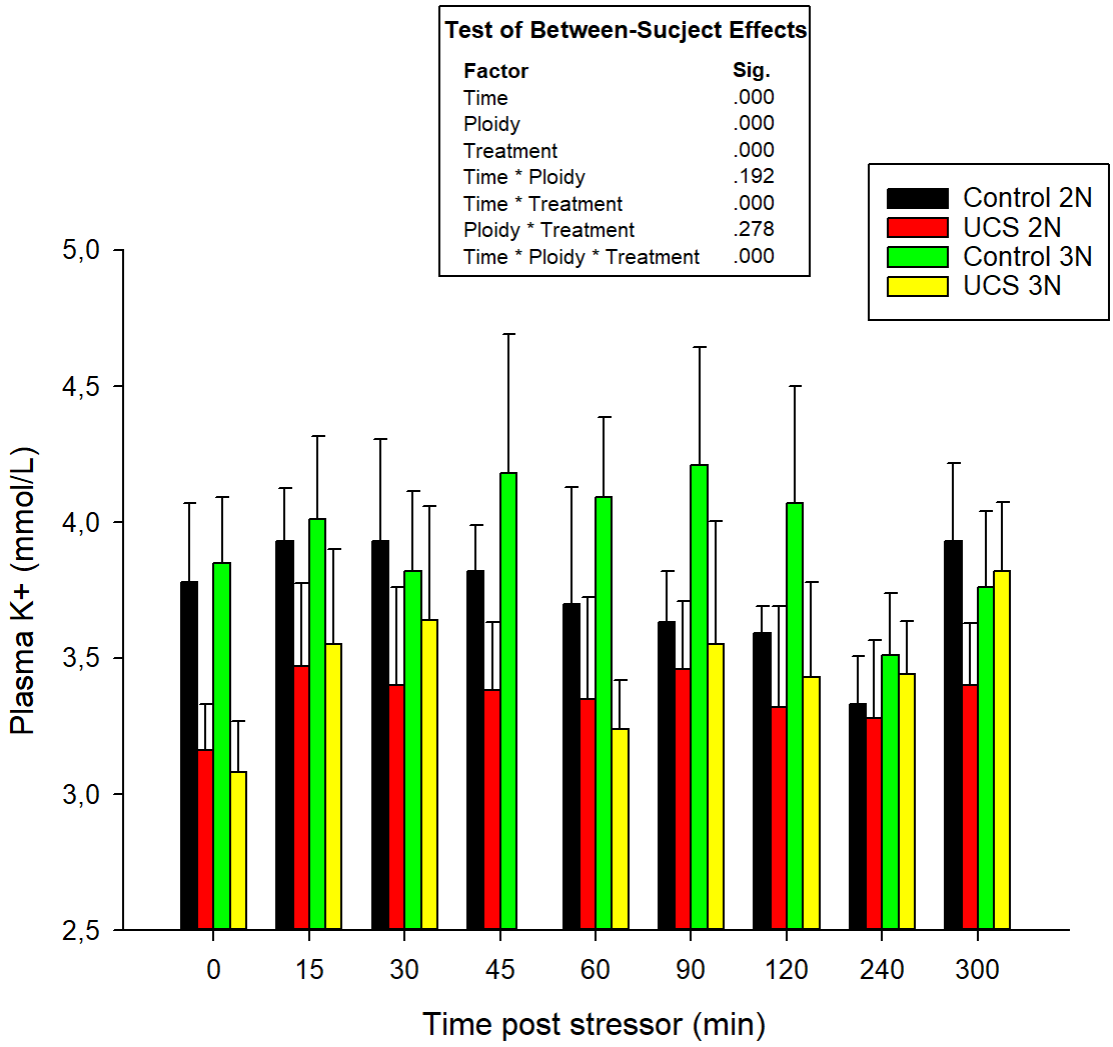

Figure 10: The average values of plasma potassium (K^+) (mmol/L) measured for the fish (n = 10) collected at each time point before and after the exposure to stress for the four experimental groups. Error bars show standard deviation. GLM Between-Subject effects calculated without Time = 45 measurements.

Figure 15 shows the average plasma potassium (K^+) levels. Pre-stress, the Control and UCS groups were significantly different form each other ($p < 0.001$) with the UCS groups having lower K^+ levels. In the novel response, there was no significant effect of the interaction between Time and Ploidy ($p = 0.192$) and the interaction between Ploidy and Treatment ($p = 0.278$), while all other effects were significant. A small K^+ increase was observed from 0 to 15 min, while the Control groups continued to have higher concentrations than the UCS groups. Across the sampling period, the UCS groups and the Control 2N group become more and more similar, while the Control 3N group showed prolonged higher values from Time = 45 to Time = 120.

Discussion

The discussion will start with a comparison of the Control groups, discussing the effects of ploidy on growth and the novel acute stress trial. Following, the impact of chronic stress on salmon physiology will be discussed, and further highlighting the possible interactive effects ploidy can have on the effect of chronic stress. The working hypotheses for the experiment will be highlighted during the discussion and some unexpected results investigated.

Comparison of diploid and triploid physiology and performance

Research on growth performance of diploid and triploid salmonids has earlier given inconsistent results, and generally growth is presumed to be similar between ploidies (reviewed in Fraser et al. (2012)). In our experiment, the SGR indicated higher growth in the Control 2N group compared to the Control 3N group, while the FCR was similar between the Control groups. The higher growth rate can be due to the fact that the Control 3N fish was on average 27.8g heavier than the Control 2N fish at the start of feeding. As growth rates in Atlantic salmon decreases as the weight increases (Austreng et al., 1987), the differences in SGR may be affected by the differences in initial weights. Therefore, one should not conclude from the experimental data that the Control 3N group performed worse in growth than the Control 2N group.

Triploidy alters brain morphology in Atlantic salmon. Though the total brain mass is similar for both ploidies, triploids have a significantly larger olfactory bulb, cerebellum and telencephalon (Fraser, Fjelldal, Skjæraasen, et al., 2012). As in the rest of the body, triploids have larger cells in the central nervous system, having fewer cells in the same volume of nervous tissue (Fraser, Fjelldal, Skjæraasen, et al., 2012). The difference in brain morphology implies possible differences in behaviour, but the relationship between the two is hard to interpret. For example, larger telencephalon correlates with more active behaviour and foraging activity (Wilson & McLaughlin, 2010), but triploid fish having a larger telencephalon generally show less aggressive behaviour and reduced foraging capacity (Carter et al., 1994; Czesny et al., 2002). Almost no studies have been conducted on the learning ability or the memory of triploid salmonids, with the only study published showing no effect of ploidy (Deeley & Benfey, 1995). If the cognitive ability of triploids is impaired, their ability to interpret and handle stressful stimuli might be hindered. This is speculative, and

more research into the behaviour and the neurophysiology of triploids is needed to enlighten this topic.

In the novel stressor trial, the effect of Ploidy was significant in most of the analyses. This seems to a degree to be the result of a larger stress response in the Control 3N group. The Control 3N cortisol curve had a much slower clearing rate and at 300 min post stress, the group had remarkably higher values than the other experimental groups. The physiological parameters glucose, lactate pH and K^+ do to a large extent confirm this. A lower triploid O_2 carrying and unloading capacity (Altimiras et al., 2002; Bernier et al., 2004; Fraser, Fjelldal, Hansen, et al., 2012) and more violent response to stress could lead to earlier oxygen exhaustion and more lactate production (Nielsen et al., 1994). Changes in pH and K^+ are also indicators of severe muscular activity (Nielsen et al., 1994; Thomas et al., 1999). Prolonged elevated cortisol and glucose levels indicate high metabolic needs, with cortisol communicating the need for the allocation of energy resources for coping (Benfey & Biron, 2000; Biron & Benfey, 1994). All this indicates that the naïve triploid Atlantic salmon physiology interprets the stressor as a greater threat compared to the other groups and seems to have a larger physiological challenge in coping with the stress, experiencing a type 1 allostatic overload. The above description of the Control 3N response is also valid in interpreting the Control 2N response, though the diploid group reacted less severely across all analyses.

In addition to the severe response observed in the Control 3N group to the novel stressor, some anecdotal observations during the UCS trial can supplement in the understanding of the triploid stress response. During the UCS trial when implementing the "Chasing" and "Netting" stressors, the UCS 3N group seemed to have a more severe flight reaction than the UCS 2N fish. In implementing the two stressors, the person stressing the fish went back and forth between the UCS tanks for 5 minutes, giving each tank a 5-10 second stir each time. The initial stirring lead to a volatile flight reaction from the fish in all UCS tanks. When returning to the UCS 2N tanks for subsequent stirs, the diploid fish had a lower fear reaction when trying to avoid the brush/net. When returning to the UCS 3N tanks however (having maybe had a 30 second rest), the triploid fish had an *almost identical fear reaction* as at the first encounter. It seemed like the UCS 3N fish could not accommodate the earlier exposures in the 5 minute period, unable to interpret the risk or danger of the returning stressor.

One of the more surprising results from the trial was how plasma concentrations of Na^+ and Cl^- for all four groups *increased* from 0 to 15 min post stress. It was expected that in fresh water, a net influx of water over the gills would result in *lower* plasma ion concentrations. However, gill ion losses will not always be reflected in a reduced plasma osmolarity in freshwater fish. During acute stress, plasma water can move out of the circulation and into the tissues, leading to an increase in plasma osmolarity (Okimoto et al., 1994) (see review in Wendelaar Bonga, 1997). What we observe might be explained well by the effect that the *β*-adrenergic response has on red blood cells (RBCs) in teleosts after stress-related sympathetic activation, reviewed by Jensen (2004). The mechanism aims to protect RBCs and increase the respiratory ability of the fish during stress by increasing internal pH (Nikinmaa, 1983) and diluting haemoglobin (Hb) by cell swelling (Holk & Lykkeboe, 1995), leading to an increased Hb-O_2 affinity, i.e. the Bohr shift. The net consequence for the plasma is a drop in pH due to the expulsion of H^+ from RBCs, and increased osmolarity due to RBC water uptake, which is exactly what we observed in the experiment. The phenomenon is well established, but the large effect was a little surprising for the marine-focused research group. Again, the effect was the largest in the Control 3N group.

The effect of unpredictable chronic stress on diploid and triploid salmon performance

Knowledge about the effect of chronic stress on triploid salmonids is very limited. Ojolick et al. (1995) found increased mortality and lower body weight in triploid rainbow trout reared in high temperatures (21 °C). However these results might be specific to the effect of the high temperature on triploids. Rogers (2016) exposed both ploidies to a stressor regime consisting in daily emptying daily the experimental for 28 days. In Rogers' results, the difference in SGR between the control and daily stressed diploids was not so large (2.54 ± 0.01% and 2.28 ± 0.00%), while the difference between the control and daily stressed triploids was larger (1.43 ± 0.00% and 0.59 ± 0.01%). The triploid SGRs in Rogers' study were similar to those found in the current experiment. In contrast, the stressed diploid values in the two experiments are very different. It seems like Rogers' stressed diploids were very much able to cope with the given stressor and to habituate well, being able to grow. This difference indicates validity of the UCS paradigm in the current experiment, as the UCS 2N fish seems to have experienced chronic stress and have not habituated in a similar manner. It seems like the diploid salmon were able to habituate to single stressors, while triploid salmon were

comparatively lacking this ability, experiencing chronic stress also under a single stressor regime.

It was apparent that the UCS procedure worked on both ploidies, as both UCS groups had significantly lower growth compared to the Control groups. This was expected as loss of appetite is a general consequence of chronic stress, including Atlantic salmon exposed to repeated acute stress (McCormick et al., 1998). The causes are assumed to be related to direct and downstream consequences of CRF activation (Bernier & Craig, 2005), including increased α-MSH synthesis from POMC that will give an anorexigenic signal (Cerdá-Reverter et al., 2003). In addition to being anorexigenic, chronic stress has been reported to reduce diet digestibility in Arctic charr (Olsen et al., 2002), most likely due to reduced transit time of the diet or reduced rate of digestive hydrolysis. Stress increases energy demands, reflected in elevated oxygen consumption in salmonids (Laitinen & Valtonen, 1994; Sloman et al., 2000). Cortisol stimulation is also shown to increase gluconeogenesis and amino acid (AA) catabolism (Mommsen et al., 1999), prioritizing more AAs for energy rather than growth. Following stress there will also be significant repair of damages. For example, stress in salmonids will cause enterocyte and tight junction damages in addition to massive mucus loss (Olsen et al., 2005) that will require extensive energy to repair and replace. Both the reduced digestibility and increased metabolism to cope with stress would lead to massively increased FCR. This was obvious in the current study where the UCS groups had FCR values about twice that of the respective Control groups.

Plasma samples taken pre novel stressor trial were used to investigate the general effect of UCS on blood chemistry. We expected both UCS groups to have elevated levels of cortisol, indicating a state of chronic stress (Wendelaar Bonga, 1997). For ACTH, we were unsure what to expect. Earlier research has shown an increase in *pomc* mRNA paralogue abundance in diploid UCS salmon, indicating overall higher ACTH production (Madaro et al., 2015; Winberg & Lepage, 1998). In an acute stress response however, *pomc* expression decreases in UCS salmon (Madaro et al., 2015). One study also found increased pre-stress ACTH in chronically stressed Brown trout (*Salmo trutta*) (Pickering et al., 1987), while on the other hand, chronically elevated plasma cortisol as a response to social subordination is shown to decrease plasma ACTH in rainbow trout (Jeffrey et al., 2012). Chronic and daily stress has not shown an increase in baseline glucose values in fish (Barton et al., 1987; Santos et al., 2010), while baseline plasma K^+ is reported in increase in chronically stressed juvenile Chinook salmon (Barton et al., 1986). In our analyses, the cortisol levels for the UCS 3N

group were in fact increased, but not for the UCS 2N fish, having similar values to the Control 2N. This was not expected, and our hypothesis was in this regard false regarding the much more investigated diploids. For ACTH, the experimental results were inconsistent, and unexpectedly, the UCS 2N group had the lowest pre-stress ACTH level, while the triploid groups both had higher ACTH values. This difference in ploidy will be further discussed below. Our results were as expected with no differences in pre-stress glucose between the groups, while K^+ levels interestingly was the most consistent plasma indicator of chronic stress, with significantly lower values for both UCS groups. The correlation between reduced growth and increased circulating plasma cortisol in chronically stressed fish is reviewed by Van Weerd and Komen (1998). They conclude that using baseline plasma cortisol for measuring chronic stress as it relates to growth is inconsistent, questioning the reliability of the method in this context. Our experimental results agree with this statement, and across the analysis we made, with the exemption of K^+, baseline plasma values might not be great indicators for the experience of chronic stress, compared to growth.

The effect of ploidy on the UCS response

The novel stressor trial was aimed at clarifying the original hypothesis regarding lower cell density in the triploid pituitary possibly leading to a lower ACTH and cortisol release to the blood. A potentially exhausted HPI-axis for the UCS 3N group was also expected to release less ACTH and cortisol than the Control 3N group. The results in the ACTH graph seem to falsify this hypothesis. Both triploid groups have clearly *higher* plasma concentrations of ACTH 0 to 60 minutes post stressor. Also at 15 minutes, the first measurement post stress exposure, the UCS 3N group had the *highest* ACTH concentration, the opposite of what the hypothesis put forward. In the cortisol data as well, both UCS and Control 3N groups must be said to achieve higher plasma concentrations than their respective 2N fish groups. Sadly, we lack data for the UCS 3N group at 45 min due to human error. One could expect a very high peak cortisol value at this point, but that is only speculation. The cortisol data falsifies the hypothesis regarding lower triploid cortisol release also. One can therefore conclude that our experiment does *not* point towards insufficient ACTH or cortisol release as an explanation for the lower ability of triploid salmon to cope with suboptimal conditions and a stressful environment. With the increased ACTH and cortisol release, the 3N fish groups both seem to physiologically interpret the novel stressor as more severe than their 2N counterpart, and we do not observe any signs of an exhausted HPI-axis in the manner observed in earlier studies in diploids (Madaro et al., 2015). As the hypotheses regarding the primary stress responses for

the experiment were false, a clear physiological explanation for the differences in HPI-axis activation can not be offered.

Triploid salmon perform worse in aquaculture settings under suboptimal rearing conditions (Madaro et al., 2021). We therefore expected the triploids to respond to UCS with a lower SGR and higher FCR. This was not the case as the ploidies did not have significantly different values for the UCS groups, and our hypothesis was false. Overall including the novel stressor response, we interpret the UCS trial to have similar physiological effects for both ploidies. A large standard deviation in the plasma data makes specific sample comparisons hard to interpret, a possible consequence of the methodology used, as individual fish can vary greatly in the stress level induced (R.E. Olsen, personal communication, May 30, 2022). Trends are nonetheless observable, and when investigating the novel stressor response, the Control groups and the UCS groups seem to follow different patterns in plasma concentrations for the compounds across time, indicating similarities in the response to UCS. Though following the same patterns, the UCS 3N fish initially respond more severely at 15 min post stressor with higher levels of Na^+, Cl^-, glucose and lactate, and a lower pH. This seems consistent with the descriptions of the higher Control 3N response discussed earlier, giving further evidence of a more severe stressor appraisal in triploids. At 30 min however, the UCS 3N and 2N values are very similar, continuing throughout the sampling period. The similar responses across ploidies suggest some form of physiological adaptation to an environment where unpredictable stressors may appear. We will still define the UCS fish as stressed though some adaptation might have happened (compared to the habituated salmon with higher growth rates discussed earlier). Regarding cortisol, both UCS groups have a faster and larger response, and a faster clearance rate. The large and fast release can be expected to aid the fish in coping with the stressor (Van Weerd & Komen, 1998). The rapid clearance rate suggest a faster inactivation of cortisol by 11β-hydroxysteroid dehydrogenase 2 (Mommsen et al., 1999), a mechanism possibly adapted to avoid the long lasting negative effects of high levels of active cortisol (Wendelaar Bonga, 1997). In this regard, the chronically stressed fish interestingly seem to better cope with the novel stressor, not experiencing the same type 1 allostatic overload as the Control groups.

Conclusion and implications for animal welfare in aquaculture

The effect of the UCS treatment in this experiment shows the massive negative effect chronic stress can have on the growth efficiency of both diploid and triploid Atlantic salmon. Across analyses, both ploidies seem to react more similarly than differently to the UCS treatment, implying that both may experience chronic stress in a similar manner. As no insufficient HPI-activation was observed in triploids, the experiment does not allude to any clear physiological explanation for the lower performance of triploid Atlantic salmon.

The chronic stress analysis in this experiment does not seem indicate to any lower triploid animal welfare compared to diploids. As triploids in fact do perform worse in aquaculture settings, chronic stress alone is not solely able to explain this phenomenon, though it might be a factor having an effect in combination with other negative health factors. Triploids seemed to have a larger initial acute stress response compared to diploids, a phenomenon lacking a physiological explanation. Further investigation into the cognitive abilities and neurophysiology of triploids might be a way provide relevant information.

The possibly clearest welfare implication observed in this study is already well established, and we can conclude; in on-growing aquaculture, regardless of ploidy, it should be in the greatest interest of fish farmers both for economic and animal welfare reasons to increase the growth and the welfare of their fish by reducing unnecessary stress episodes as much as possible.

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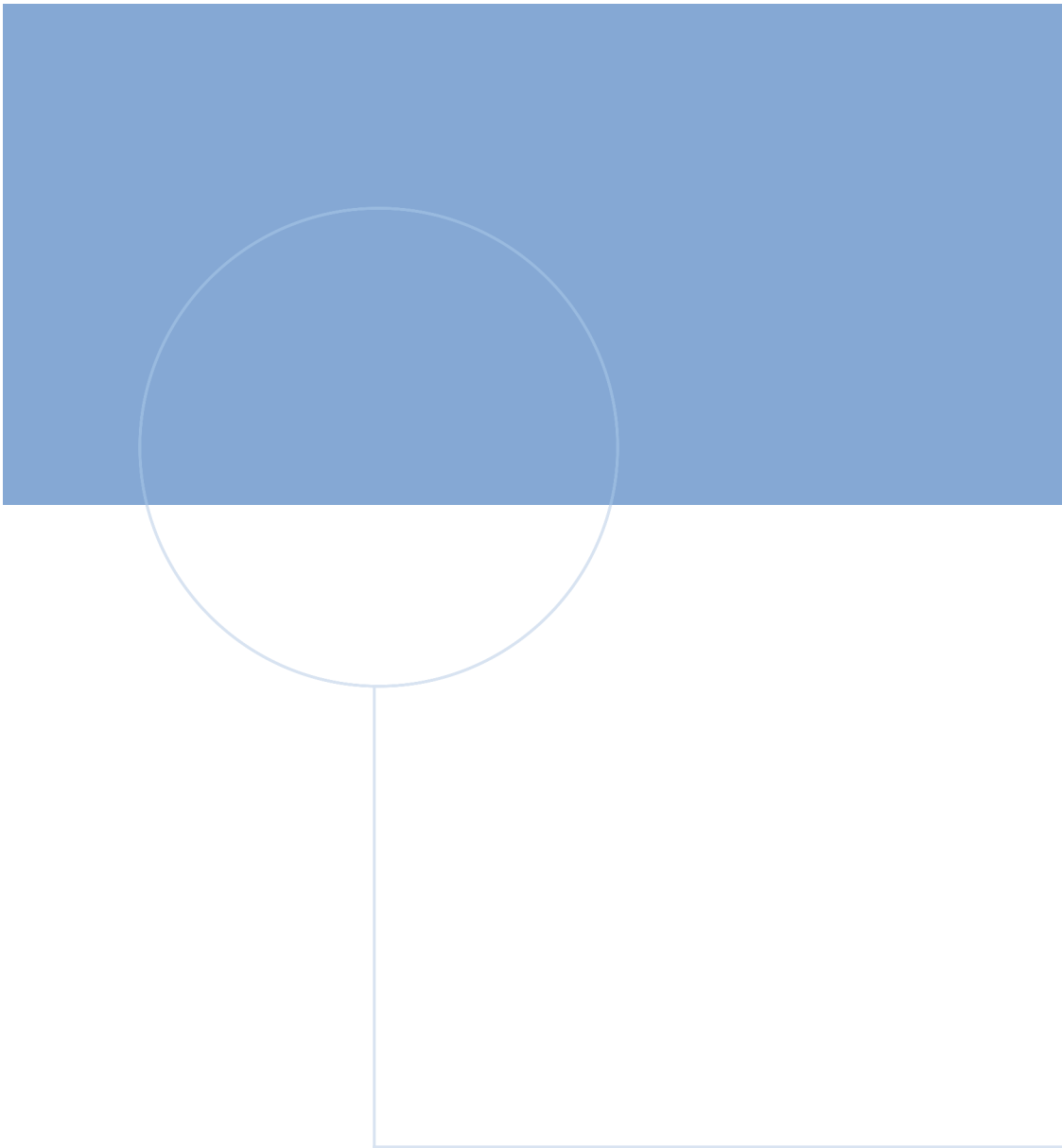

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