MASTER THESIS

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Course code: AK306F Master thesis in Aquaculture

The Effect of Chronic Stress on the Triploid Atlantic Salmon HPI-axis

Faculty of Biosciences and Aquaculture

SPRING 2016

NORD University

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TABLE OF CONTENTS

FOREWORD

This thesis was composed in accordance with the Faculty for Biosciences and Aquaculture, Nord University Bodø as part of criteria for Master of Science (MSc) in aquaculture.

To Martin: over the past years I owe my prospects and undying gratitude, both in every opportunity offered and step accomplished regarding this project, all written manifestations, and academic guidance. In my undergraduate career we designed a plan that was followed despite obstacles on both sides, through to today at the height of my academic learning. I could not be more proud of myself nor happier to have asked you for a point in the right direction what seems now so long ago. Thank you for the support and the vote of confidence, I never forget my true believers.

Working alongside me were Bente, Roald, and Steinar, each with their own unique contributions to practical assistance and guidance. Even more importantly, their wisdom and humor made every day in the lab a joyful and positive experience.

Tone deserves a special note for her acceptance of my coattail-riding and for raising a new son into the world throughout the process: congratulations on your professional advancement and life developments, I have appreciated every moment of knowing another young parent is sitting up late doing something other than studying for an exam or polishing a chapter.

The foundation and purpose I have found in my family, an entity so rare I hardly accept its existence, exhausts and energizes me every single day. Our clan grew over the course of this journey as well, establishing roots in grand swaths and making leaps into beyond—Kari, half of me and most of my good sense, I can never repay you for the hours we somehow scraped together to make all of this possible. I hope everyone above recognizes the value of my gratitude, but you, my wife will have the rest of this future to build from here by my side.

April 18th, 2016

Kyle David Rogers

Summary

The physiology of triploid fish in comparison with diploids has been extensively investigated, more recently including welfare differences in aquaculture settings. Atlantic salmon (*Salmo salar*) have garnered considerable attention in this respect due in part to high individual product value and environmental conservation concerns, especially prevention of genetic dissemination by escaped fish. Notable advancements have been made in ensuring successful implementation of triploid Atlantic salmon with welfare debate in tow. The review and experimentation incorporated here further elucidate the implications of triploid salmon use in Norwegian aquaculture with a focus on endocrine stress response and welfare.

Achieving greater understanding of long-term hypothalamic-pituitary-interrenal/adrenal (HPI/A) feedback response was the primary goal of this study, directly associating hormones with their systemic response elements. Four groups of salmon were formed based on ploidy and treatment: control diploid, stressed diploid, control triploid, and stressed triploid, each consisting of 480 0+ saltwater adjusted smolt. Daily, irregularly timed crowding was the experimental treatment in which the two stressed groups had their water drained then immediately refilled. Adrenocorticotropic hormone (ACTH) was used to differentiate a basal plasma cortisol group from an ACTH-injected and a phosphate-buffered saline (PBS) group in each of the four tanks, producing significantly increased plasma cortisol concentrations in all stressed triploids (basal, ACTH, PBS) from the 14 day sampling interval onwards. Osmoregulatory evidence and previous studies suggest however that this increase occurred sometime after the 7 day interval. Plasma magnesium was the osmoregulatory factor and described a loss of glomerular secretory electrolyte balance in both stressed triploids and diploids, though beginning in the former by the 7 day interval and the latter at the 28 day interval. Growth and specific growth rate (SGR) were both significantly differential over the course of the study, with stressed triploid exhibiting less than 1% average SGR both between sample groups and pre-stress values and an average weight loss at early sampling intervals.

Review of relevant studies presents concern as to triploid stress coping, most especially throughout regular aquaculture production which includes potentially lethal requisite stressors at developmental intervals. Investigation into economic and ethical components surrounding triploid implementation is necessary and must consider many factors, including those presented here and beyond.

Sammendrag

Fysiologien til triploid fisk sammenlignet med diploider har blitt grundig undersøkt, mer nylig også i forhold til velferdsforskjellene i akvakultursammenheng. Atlantisk laks (*Salmo salar*) har fått stor oppmerksomhet, noe som delvis skyldes høy verdi av enkeltprodukt og miljøvernhensyn, da spesielt forebygging av genetisk spredning av rømt fisk. Det har også blitt gjort fremskritt for å sikre en vellykket innføring av triploid laks med velferdsdebatten på slep. Gjennomgangen og eksperimenteringen beskrevet i denne oppgaven vil videre belyse konsekvensene av triploid laks brukt i norsk akvakultur med fokus på endokrin stressrespons og velferd.

Det primære målet med denne oppgaven er å oppnå større forståelse av langsiktig hypothalamus-hypofyse-interrenal/adrenal (HPI/A) akse “negative feedback” respons, og direkte knytte HPI-hormoner med sine systemiske responselementer. Fire grupper laks ble kategorisert basert på ploiditet og behandling: kontroll-diploid, stressbehandlede diploid, kontroll-triploide, og stressbehandlede triploide. Disse gruppene bestod av 480 0+ saltvann-justerte smolt. Daglig, uregelmessige trenginger er en eksperimentell behandling der de to stressede gruppene fikk vannet sitt uttappet, for å rett etter få det fylt opp igjen. Adrenokortikotropt hormon (ACTH) ble brukt for å skille en basal plasmakortisol gruppe fra de ACTH-injiserte og en fosfat-bufret saltvann (PBS)-injisert gruppe fra hver av de fire karene. Etter fjorten dager hadde ACTH produsert signifikant økning i plasmakortisolkonsentrasjoner i de tre stressede triploide gruppene (basal, ACTH, PBS). Osmoregulatoriske funn og tidligere studier foreslår at denne økningen skjedde en gang etter syv dagers intervall. Plasma-magnesium var den osmoregulatoriske faktoren og beskrev et tap av glomerulær sekretoriske elektrolyttbalanse i både belastede triploider og diploider, men vises tidligere ved 7 dagers interval for triploider og etter 28 dager for diploider. Både vekst og spesifikk veksthastighet (SGR) viste betydelig forskjell i løpet av studiet, med stressbehandlede triploider som viste mindre enn 1% gjennomsnittlig SGR både mellom prøvegrupper og “pre-stress” og en gjennomsnittlig vekttap i disse fiskene på tidlige prøvetakingsintervaller.

Gjennomgang av relevante studier presenterer bekymring med hensyn til triploid stressmestring, spesielt når man ser på regelmessig akvakulturproduksjon som inkluderer nødvendige belastninger som er potensielt dødelige for disse fiskene. Både økonomisk og etisk gransking innenfor dette feltet er nødvendig, og mange faktorer må vurderes, både de beskrevet i denne oppgaven og videre.

1. Introduction

Selective breeding found its roots in ancient practices as domestication for both practical and aesthetic purposes, and technology has progressed to selective gene and ploidy alteration to allow transcendence from many diseases and ecological harm. Such power rests only with humanity and so does accountability for their application on animals whose welfare must be monitored and maintained. In Atlantic salmon, triploid stocks are an interesting commodity which were induced artificially to prevent genetic dissemination from farms to wild stocks—value and practicality studies are ongoing, and the survivability of triploids versus diploids remains in question due to stressors along production chains, e.g. grading, vaccination, smoltification, transport and transfer to sea. Stress has been observed to affect triploid salmon more severely than diploid, so a method for improving efficiency and welfare in both groups is to limit these stressors (Benfey and Biron, 2000, Cnaani *et al.*, 2014, Fraser *et al.*, 2012, Fraser *et al.*, 2015, Hatløy, 2015, Iversen, 2013, Maxime, 2008). Given the triploid salmon's potential on great industrial scales, studies are carried out weighing costs against benefits, e.g. "*Salmotrip*" in which production was field tested, monitored, and consumer perception was assessed for 3 years through the University of Stirling (Taylor *et al.*, 2007).

The particularly crucial issue of farmed fish genes reproducing on wild spawning grounds is the primary reason for these studies, though ecological impacts of escaped, selectively bred fish into spawning and feeding grounds stem only partly from this fact; competition for food with wild salmon can edge out wild stocks in genetic representation through more than the reproductive route (Naylor *et al.*, 2005). More than 90% of all farmed salmon production is attributed to Atlantic salmon, making the prevention of escape important. Those caught from natural sites in Norway, home to many of those few remaining, are often (11-35% mean in 2001) farmed bloodlines (Fiske *et al.*, 2001, Naylor *et al.*, 2005). These statistics are troubling for select Norwegian wildernesses, especially considering some stocks are upward of 80% farmed ancestry, and this distinction is key when deciding whether to produce triploids on a given site (Naylor *et al.*, 2005). Genetic manipulation beyond selective breeding certainly provides a potential avenue for wild stock recovery capability, but at what cost?

1.1 Animal welfare and fish welfare

Instated by the Farm Animal Welfare Council (FAWC) as early as December 1979, the Five Freedoms extended eventually to all corners of UK animal welfare management and further influenced many aspects of European animal welfare in general (McCulloch, 2013). The modern and ubiquitously referenced form of the Five Freedoms is available online, though its origins lie in the 1965 Brambell Report which declared animals should have the abilities to stand, lie, groom, turn, and stretch (Brambell, 1965). Primitive in today's perspective, and nearly unrelated to fish, adaptation of The Five Freedoms is currently:

1. Freedom from Hunger and Thirst - by ready access to fresh water and a diet to maintain full health and vigor.
2. Freedom from Discomfort - by providing an appropriate environment including shelter and a comfortable resting area.
3. Freedom from Pain, Injury or Disease - by prevention or rapid diagnosis and treatment.
4. Freedom to Express Normal Behavior - by providing sufficient space, proper facilities and company of the animal's own kind.
5. Freedom from Fear and Distress - by ensuring conditions and treatment which avoid mental suffering.

Although its application has found no end, only recently has its accuracy regarding animal welfare itself been sufficiently questioned (McCulloch, 2013). Definition without the Five Freedoms proves difficult, given their indispensable wording, but efforts have been made from different perspectives, some specifically including fish. Singling out fish welfare for its objective factors provides an avenue to increase understanding of distress and pain, whereas subjective discussions of ethics often result in arguments of anthropomorphism (Arlinghaus *et al.*, 2009). The mental faculties which set cerebrates apart from other animals are often debated in comparison with fish, a lack of evidence satisfying objectivity requirements to disprove piscine perception of pain beyond nociception (Rose *et al.*, 2014). As more information becomes available regarding what pain, fear, and distress mean to animals, our management practices adapt and have improved for animals, the environment, and consumers on every level. In this respect, arguments against consciousness become increasingly irrelevant (Arlinghaus *et al.*, 2009, Ellis *et al.*, 2012, Fraser and Duncan, 1998, Huntingford *et al.*, 2006, McCulloch, 2013, Rose *et al.*, 2014, Selye, 1950, 1973).

1.2 Concept of stress

The culminated knowledge in the fields of biosciences, medicine, and philosophy appeared as “the general adaptation syndrome” or G.A.S. before becoming its modern equivalents (Selye, 1950). This lifelong balance of “alarm reaction” with stages of “resistance” and “exhaustion” eventually proved too primitive, as Selye (1950, 1973) ascertained would occur once stress had become “satisfactorily elucidated”. Essentially an amalgam of presumptuous conclusions, G.A.S. has been respectfully altered as necessary to bring the stress definition into its current form (Ellis *et al.*, 2012, Iversen, 2013, Rose *et al.*, 2014, Selye, 1950). The data collectively referring to the HPA axis certainly applies today, also within piscine stress studies (Ellis *et al.*, 2012, Iversen 2013). The specific physiological mechanisms for stress in fish have essentially been uncovered and understood for their contribution to the organism, though the sum of their parts is still largely unknown: neural triggers for endocrine signals to central nervous and renal secretory tissues lead to whole-animal effects, each consisting of well-documented components but imparting effects unseen both immediately and over time as either chronic or permanent effects (Barton, 2002, Ellis *et al.*, 2012, Fraser *et al.*, 2012, Hatløy, 2015, Iversen, 2013, Iversen and Eliassen, 2014, Mommsen *et al.*, 1999).

Given historical ignorance of stress in any fish, both technological application and policy debate has been necessary. The characterization of pain and fear perception in fish has been, until the past few decades, strafed, with some of the earliest, basic studies on the topic non-existent before 1970 (Ellis *et al.*, 2012, Iversen 2013). Resultant controversy surfaced once consciousness became part of the discussion, since delimiting pain to nociception simplifies any issue of handling or treatment to anthropomorphism. Fear is unnecessary as a component of pain, but knowledge of pain implies fear and this awareness inconveniences production protocol (Ellis *et al.*, 2012, Rose *et al.*, 2014). The opposite argument is made still based on anthropomorphism, identifying the search for piscine consciousness as unsupported and only self-serving (Rose *et al.*, 2014). Either way, technology and policy are under constant criticism and techniques are thus constantly developing. Current metrics depend heavily on largely inaccurate corticosteroid responses, though combination approaches integrating neurochemistry, physiology, and behavior allow ever-increasing elucidation, e.g. study revolving around endocrine feedback using allostasis (Burghardt and Rivas, 2002, Edwards, 2009, Ellis *et al.*, 2012, Fraser and Duncan, 1998, Hatløy, 2015, Jensen, 1996, Paul *et al.*, 2005, Rose *et al.*, 2014, Veissier and Boissy, 2007).

The distinction between stress and distress is a fine one, respectively referring to adaptive and maladaptive responses (Barton, 2002). Stress in itself provides the animal with situationally appropriate change and under optimal circumstances results in adaptation. Helpful responses are not inherently negative, barring transient discomfort, as opposed to maladaptive situations (Barton, 2002, Ellis *et al*., 2012, Hatløy, 2015, Iversen, 2013). Distressed animals do not exhibit healthy negative feedback responses and instead counteract them by suppression of required endocrine signals, exhibiting sub-optimal adaptation or no adaptation at all (Barton, 2002, Iversen, 2013).

1.2.1 Hypothalamus – pituitary -interrenal axis in fish

Cortisol, a glucocorticoid, is prominent across the animal kingdom with similar influences: an immunosuppressive capacity, a promoter of gluconeogenesis, and gamete quality effects (Breuner, 2008, Iversen, 2013, Pankhurst, 2011, Schreck, 2010, Schreck *et al.*, 2001). Teleostean physiological mediation is broadly influenced by cortisol, including these areas but also osmoregulation, a crucial element in aquatic survival and fitness (Ellis *et al*., 2012, Fraser *et al*., 2012, Iversen, 2013). As cultured animals' welfare hinges upon human regulation, cortisol is often used as a gauge to determine stress perceived from negative experiences such as handling and crowding (Ellis *et al*., 2012).

The promotion and regulation of plasma cortisol circulation is dictated by the HPA/HPI axis of cascading hormone activity, where Atlantic salmon do not possess adrenal glands and instead utilize the interrenal tissue of the head kidney (Iversen, 2013). Stressors which impact the central nervous system (CNS) are perceived internally or from outside the body by the peripheral nervous system (PNS), and thus induce a hormonal cascade effect (Ellis *et al*., 2012). The HPI axis is influenced by any stimulus able to initiate the secretion of corticotropin release hormone (CRH) from hypothalamic neurons (See Figure 1, p11) (Aluru and Vijayan, 2007). CRH receptors on corticotropic pituitary cells transduce CRH signalling to ACTH, which enters the general circulation before entering the head kidney to cells located near chromaffin cells and the posterior cardinal vein. Indirect from this juxtaposition, cortisol works in tandem with endocrine factors such as catecholamines to promote rapid release via paracrine signalling (Ellis *et al*., 2012, Iversen, 2013).

ACTH is a linear peptide chain of 39 amino acids derived from pro-opiomelanocortin and secreted from the adenohypophysis, specifically the rostral pars distalis (RPD, frontal hypothalamus), and is the primary actuator behind interrenal steroidogenesis in the piscine head kidney (Bernier *et al.*, 2009, Wendelaar Bonga, 2011). ACTH circulates to interrenal melanocortin-2 receptors (MC2Rs), a form of G-protein-coupled receptors, triggering an ATP→cAMP reaction. ACTH is essential to this reaction, as MC2R only responds to ACTH (Bernier et al. 2009). Although the primary promoting factor in ACTH secretion is CRH, some species variation causes urotensin-I to be more effective than CRH, e.g. goldfish (Fryer *et al.*, 1985). Cumulative effect in tandem with CRH and angiotensin I and II causes amplified ACTH secretion, whereas paracrine signals from the neurohypophysis can act alone as a secretory factor, e.g. arginine vasotocin, an osmoregulator and isotocin, a piscine vasopressin analog. Angiotensin-I, II and thyrotropin have also been related to corticotropic release (Bernier *et al.*, 2009).

The primary negative regulatory hormones of ACTH are dopamine (DA) and melanin-concentrating hormone (MCH), both of hypothalamic origin. Regulation of ACTH can occur physiologically by these endocrine factors at pituitary binding sites, making them neurotransmitters within the CNS which locally actuate negative feedback from the PNS. Although MCH and DA regulate ACTH secretion, their presence *in situ* has also been documented as necessary to normal promotion of ACTH in the primary stress response (Bernier *et al*., 2009). ACTH binds on the interrenal surface to MC2Rs, preceding corticosteroid-genesis and cortisol circulation (Aluru and Vijayan, 2009).

Cortisol circulates within blood plasma through assistance of yet unidentified carrier–proteins to target tissues exhibiting one mineralocorticoid and two glucocorticoid receptors (MR, GRs) (Aluru and Vijayan, 2009, Iversen, 2013). The genetic mechanism behind this adaptive activation or suppression is the cortisol-GR complex, binding to nuclear DNA of effector cells at the active site, the glucocorticoid response element (GRE), allowing a cascading promotion or regulation of subsequent expression. Local activity at the tissue site may also be activated by signaling of surface membrane proteins. The cascading effect of cortisol seems governed by heat shock protein-90 presence as with mammals, with further elucidation on extracellular signaling largely lacking; cortisol does however act as a GR regulator, with increased plasma circulation increasing proteasome GR catabolism (Iwama *et al.*, 2004). Primarily, this affects the gills, liver, and gastrointestinal tract, made evident by chloride retention and increased plasma osmolality, rapidly increasing gluconeogenesis (endogenous glucose synthesis), and a halt on hunger and digestion. Cortisol thus allows fish access to quickly catabolized energy reserves, hyperventilation without ionic imbalance (providing necessary respiration to facilitate rapid gluconeogenesis), and the ability to excrete any waste and flee, hide, or fight as long as possible (Aluru and Vijayan, 2009, Ellis *et al.*, 2012, Iversen, 2013). Other important effects have been documented, such as exogenous cortisol injection suppressing testosterone and 17β-estradiol in vitellogenic rainbow trout (sexually mature females developing yolk, preovulatory), 1 and 3 hours post injection (hpi), and 3 and 6 hpi respectively, though gonadotropin was unaffected, possibly linking it to cortisol transduction (Pankhurst, 2011).

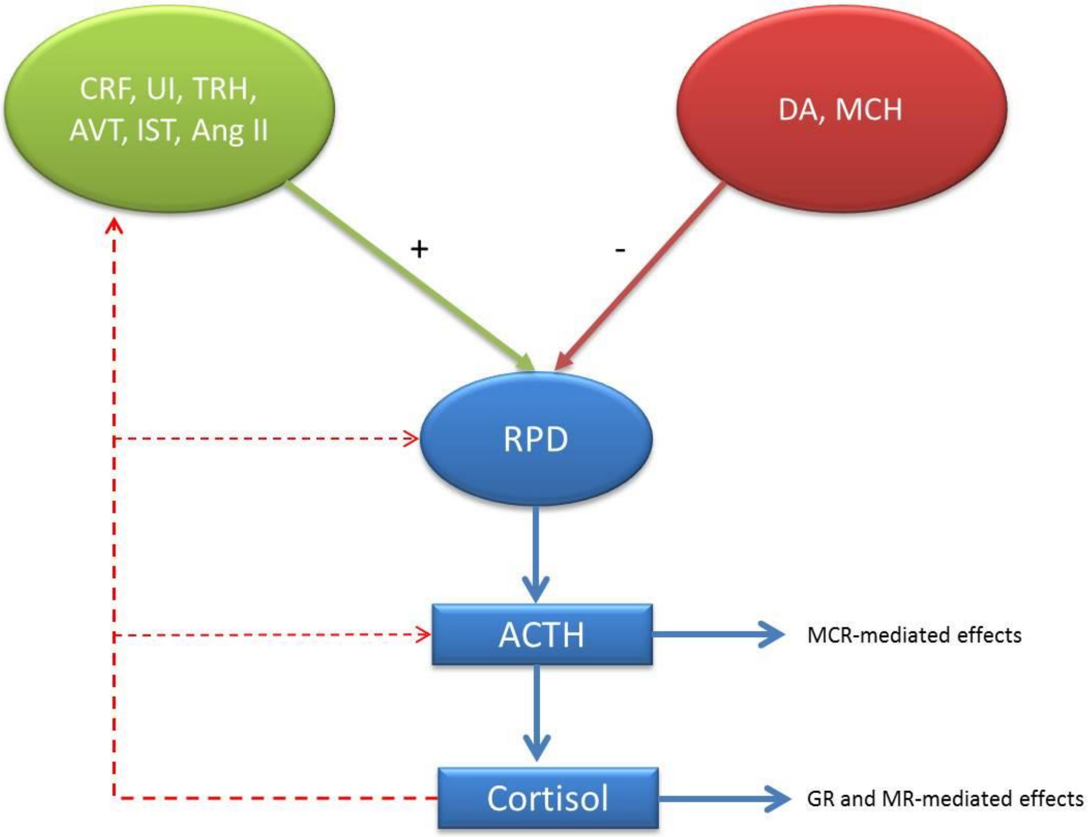

Figure 1. *Overview of major factors that affect the activity of the HPI axis. Green arrows=stimulatory effect, Red arrows=inhibition, dashed red arrows=negative feedback. CRF=corticotropin-releasing factor (CRH), UI=urotensin I, TRH=thyrotropin-releasing hormone, AVT=arginine vasotocin, IST=isotocin, Ang II=angiotensin II, DA=dopamine, MCH=melanin-concentrating hormone, MCR=melanocortin receptors, GR=glucocorticoid receptor, MR=mineralcorticoid receptor, and RPD=rostral pars distalis. Iversen (2013).*

The relationship of ACTH and cortisol is of specific relevance, as the cascading effect of CRH→ACTH→cortisol has a negative feedback based on cortisol MR/GR saturation, leading to MC2Rs feedback, then pituitary neurons. It is therefore understood wherever MR/GR, MC2R, or pituitary saturation is left unchecked, this feedback system no longer has vertical synergy, and negative feedback is needed to maintain balance (Ellis *et al.*, 2012, Iversen, 2013, McEwen and Wingfield, 2003, Sterling, 2012). A fat-soluble hormone will either be found in bound form with various transport proteins (biologically inactive) or in unbound form free in the plasma (biologically active), and in addition one will find the hormone in metabolized and inactive form inside of cells. Cortisol levels are reduced by metabolic change, deactivating and making it unavailable to GR and MR, as well as increasing its water solubility. Hormone and receptor will finally detach from each other, with cortisol sent to the liver through the hepatobiliary tract as the main cortisol secretion route (Davis, 2006, Greenspan and Gardner, 2004, Mommsen *et al.*, 1999).

The greatest reduction in cortisol is realized through reduction of the 4/5 double-bond in the A-ring and the ketone-group-3 (Greenspan and Gardner, 2004). Conjugation of the resulting hydroxyl group on carbon 3 together with sulphate and glucuronic acid makes the steroid more water-soluble, such that it may secrete through glomerular capillaries for urinary excretion (Davis, 2006, Greenspan and Gardner, 2004, Mommsen *et al.*, 1999).

1.2.2 Allostasis

ACTH presented internally over natural levels per HPI primary stimulation therefore overloads corticosteroid-genesis, inducing relatively immediate cortisol concentration spiking and a long term sensitivity loss to endogenous ACTH circulation due to allostatic overload (Iversen and Eliassen, 2014, McEwen and Wingfield, 2003). Allostasis is described as maintenance of homeostasis through adaptation and changes, where change to the organism is evident but the internal stability must be maintained (Figure 2). This term was coined first in reference to humans, but has since been adapted to animals and fish (Barton, 2002, McEwen and Wingfield, 2003, Sterling, 2011). Where homeostasis implies consistent stability within rigid parameters, allostasis conceptualizes the interaction of the homeostatic organism with its environment and inherent endogenous and exogenous stressors; stress is thus observed adaptive response to stimuli towards homeostasis (Figure 2). Divided into three degrees of effect, stress has primary (1°), secondary (2°), and tertiary (3°) tiers. Primary stress encompasses neural stimulation, signal transduction, promotion and regulation of endocrine factors, and tissue response; secondary stress includes resulting behavioral and organism-level adaptive change; tertiary stress reaches to post-event time, affecting the organism beyond the stress itself in the greater realm of fitness (Barton, 2002, Ellis *et al*., 2012, Iversen and Eliassen, 2014, McEwen and Wingfield, 2003).

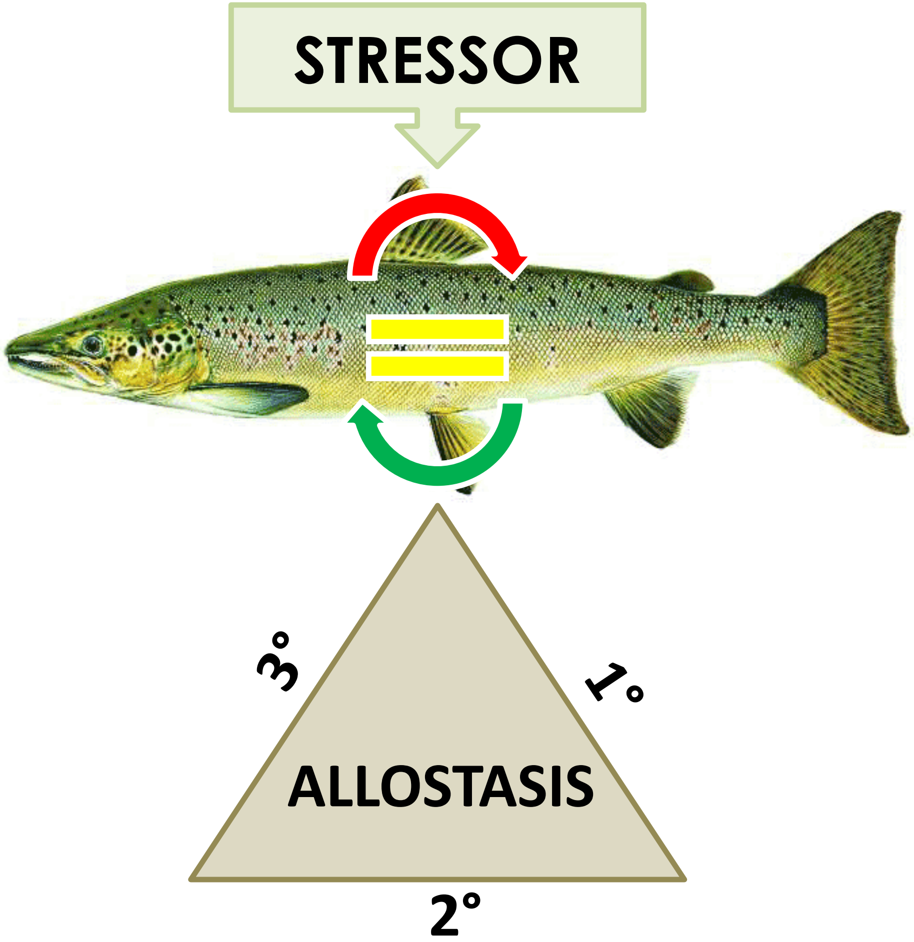

Figure 2. *Physical, chemical and other perceived stressors act on fish to evoke physiological and related effects, which are commonly grouped as primary, secondary and tertiary responses. These responses are collectively regulated to promote homeostasis, as indicated by red (downregulation), green (secretion), and equal (the balanced release and control of primary response factors).*

The adaptive response to stress is the principle of the theory of allostasis and neural action potential is conceptually similar to the functional adaptive mechanism, allostatic overload, which is experienced as Type I or Type II. Type I allostatic overload describes acute stress in which 1° and 2° effectively provide a behavioral or otherwise whole-animal response to stressors, adaptation to and relief from stress, and finally a return to a pre-stress steroidogenic profile as cortisol is catabolized and ACTH is regulated (McEwen and Wingfield, 2003). This regulation of ACTH and cortisol occurs per 1° event, and therefore provides an adaptive 3° where homeostasis is maintained. Type II allostatic overload results wherever consistent hyperstimulation of the HPI axis occurs over time, leading 1° and 2° to 3° where typical adaptive response is not realized, i.e. homeostasis is affected and resulting maladaptive, atypical recovery is apparent. Practically, Type I allostatic overload can be described as typically adaptive acute stress, while Type II is a chronic condition with variable recovery potential (Barton 2002, Ellis *et al.*, 2012, Iversen, 2013, Iversen *et al.*, 2003, Iversen and Eliassen, 2014, McEwen and Wingfield, 2003). Specifically referencing Type II overload, allostasis provides the animal with adaptation, be it conducive to fitness or not; 2° occurs in predictable ways, but physiology is multi-tiered, intertwined, and fluid. Therefore, 3° is a dependent variable, with 1° and 2° independent: as the HPI feedback reaches apex to regulate ACTH secretion and promote uptake by cortisol concentration and tissue saturation, everything returns to normal, however this can be theoretically hindered with consistently sub-potential interrenal exposure to ACTH and/or GR/MR saturation with cortisol (Figure 3) (Iversen, 2013).

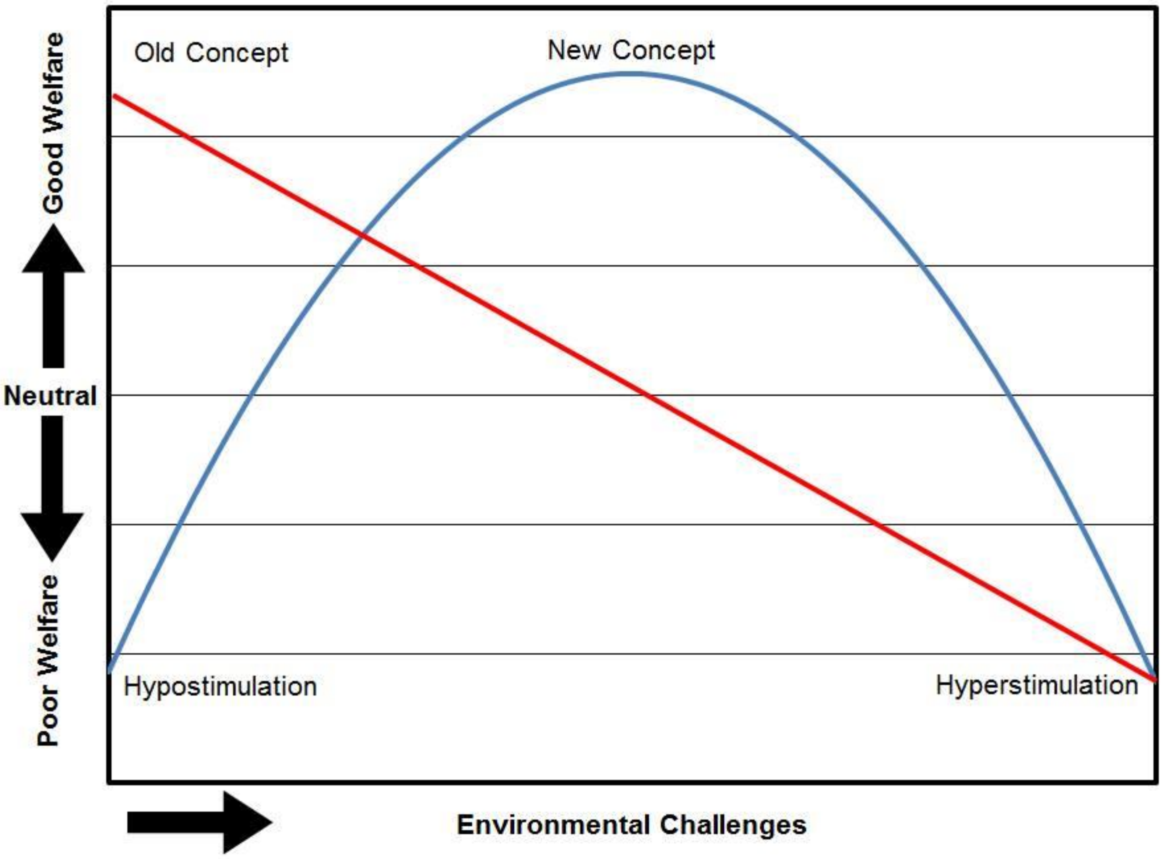

Figure 3. A recreation of the bell-curve model representing the perspective change with respect to animal welfare in relation to hypostimulation and hyperstimulation. Redrawn from Korte *et al.*,(2005).

1.2.3 Plasma cortisol and animal welfare

Plasma cortisol is the best available and most commonly used metric in piscine stress studies, based on its increased concentration following controlled stressor exposure (Barton, 2002, Ellis *et al.*, 2012, Mommsen *et al.*, 1999, Ramsay *et al.*, 2009). The consistent and predictable nature of cortisol circulation, metabolism, excretion and relatively low resting concentrations (>13.8nM or 5ng/mL in salmon, 50-75nM during smoltification) make cortisol objectively functional (Barton, 2002, Iversen, 2013, Wendelaar Bonga, 2011). Use of cortisol thus imparts evidence on otherwise unfounded phenomena, i.e. poor growth performance or mortality increases without obvious disease, and often provides fish welfare with a supporting argument (Ellis *et al*., 2012, Iversen, 2013).

Functional changes in fish welfare are often represented by cellular activity as they are impacted by plasma cortisol increase or decrease (Iversen, 2013). References to growth, reproduction, locomotion, and osmoregulation exist connected with plasma cortisol, relating it intimately to essential physiological functions in fish. Definition of functional changes in fish is established and description of the feelings fish have, though mostly negative, is achieved with accuracy and careful interpretation via plasma cortisol (Ellis *et al*., 2012).

Investigation into alternative welfare objectification methods seems necessary in the face of such lacking evidence supporting positive affective indicators in fish with respect to feelings. Although function is explained clearly and behaviors are studied thoroughly in cultivated fish, the capabilities these animals have in understanding of such processes are left to ethical questions. Research has proven fish can perceive threats and danger without physical altercation of any kind, namely visual stimuli followed by increase in plasma cortisol (Davis, 2006, Ellis *et al.*, 2012, Ramsay *et al.*, 2009, Schreck, 1981, Schreck *et al.*, 2001). This could imply fear, an emotional response associated with feeling or memory, since these stimuli range from watching the handling of other individuals and simply seeing a capture net after observing its function, to only visual predator contact (Ellis *et al*., 2012). These responses of feeling have been represented by plasma cortisol increases, linking stress hormones to conceptual understanding via behavioral and functional expression, however these are no better than proxies for interpretation of a more complex concept (Ellis *et al*., 2012). So although there is some association between feelings and cortisol, there is no proven causation, and thus is no more than a *de facto* representation. There is an interest in expanding the definition of welfare to include more than just plasma cortisol indications, but records of alternative methods are lacking (Ellis *et al*., 2012).

1.3 Triploids, triploid salmon, and welfare implications

Polyploid animals feature more genome copies than are naturally selected for in a species, usually diploids with two (2n), while triploids exhibit exactly three (3n) complete sets of nuclear chromosomes in each cell. These are naturally occurring phenomena throughout Animalia with polyploidy induction used in livestock. Induction can result from different forms of shock during meiosis II to fertilized ova, though selective breeding is a possible route resulting in sex-reversal (Maxime, 2008, Otto and Whitton, 2000).

Induction processes do vary and within salmonid applications, thermal and hydrostatic shock tend be the most common procedures, with success most consistent in hydrostatic pressure application during the second meiotic division (Maxime, 2008). Additional benefits are afforded by genetic manipulation and selective breeding, including potential sex-reversal for all-female egg stocks and prevention of precocious spawning, which directly benefit growth production. Refinement of induction technique specifics over more than 30 years has thus far improved induction success in survival and triploidization (Levandusky *et al.*, 1990, Salimian *et al.*, 2016). No matter the case, the process involves a shock to fertilized, meiosis II eggs before expulsion of the second polar body; this extra set of chromosomes is retained and reproduced during mitosis, producing triploids with an infertile set (Figure 4) (Benfey and Sutterlin, 1984, Quillet and Gaignon, 1990, Teskeredžić *et al.*, 1993, Utter *et al.*, 1983).

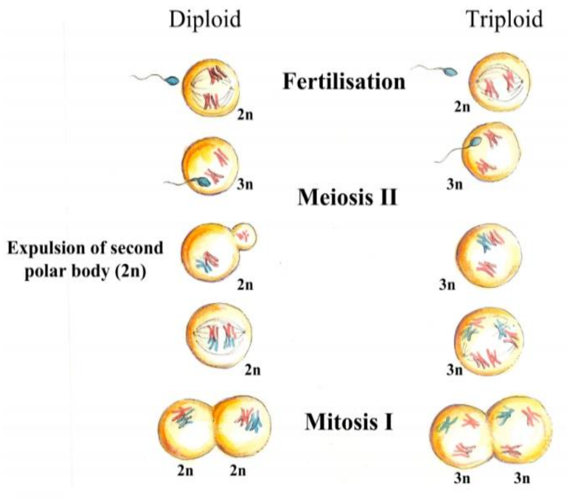

Figure 4. *Induction of triploidy, as occurs in fish. Pressure shock is applied along the triploid development column post-fertilization, before the second polar body is expelled. Adapted from T. Hansen, IMR (Salmotrip project, October 2008).*

Morphology and behavior of both diploid and triploid Atlantic salmon are indistinguishable despite hypertrophic triploid nuclei with a maintained cytoplasmic ratio, resulting in similarly sized organs and tissues but with fewer cells (Fraser *et al.*, 2012, Maxime, 2008). Vital to whole animal stress is cellular physiology and signal transduction, which are strongly influenced by stressors due to heat shock proteins and many receptor types, although this potential for dysfunction is well-studied, it does not include knowledge of (Aluru and Vijayan, 2007, Iwama *et al.*, 2004, Osborne *et al.*, 2007). Reproductive implications arise, given inherent incompatibility between polyploids and diploids, even though exceptions exist within teleost species (Otto and Whitton, 2000, Piferrer *et al.*, 2009). Sex-reversed, induced Atlantic salmon triploidy produces all-female populations incapable of gonadal maturation, achieved commonly by thermal shock treatment and results in increased early growth (Piferrer *et al.*, 2009). Immunity seems to function similarly between triploids and diploids, and externally regarding sea lice. Some variation is documented in intestinal microbiota communities and with regard to antibacterial resistance, as well as an apparently slight disadvantage of triploids to exogenous bacterial infection (LPS injection) (Cantas *et al.*, 2011, Frenzl *et al.*, 2014, Jhingan *et al.*, 2003, Maxime, 2008). Myriad accounts of triploid salmonids exhibiting various inferiorities compared to diploids have been presented and continue to be, including (in Atlantic salmon) decreased marine growth performance, jawbone deformity, gill filament deformity, acute stress response and complete cataracts (100% ocular coverage) in one eye or the other (Cotter *et al.*, 2002, Hatløy, 2015, Sadler and King, 2001). Some physiological differences are remediated with nutritional or environmental management, though baseline deformity and morbidity is typically higher in triploids (Taylor *et al.*, 2014).

A host of inferiorities implies greater stress for similar fitness, allowed for by principles of allostasis: individual difficulty in achieving growth, reproductive, and survival success impacts 1° response more or less profoundly, producing varying 2° response and most likely 3° responses. Understanding and quantifying this difference between individual experiences is key to stress evaluation, given its subjective nature; separate evaluations of physiological stress factors and co-factors, while eliminating confounders, as of yet provides the most accurate information to this effect (Barton, 2002, Cotter *et al.*, 2002, Davis, 2006).

1.4 Hypothesis

Use of triploid Atlantic salmon has merits in the face of ecological destruction, though unknown factors in their welfare raise questions as to ethics of their implementation. A challenge study of ACTH sensitivity could demystify some uncertainty both in direct relation to physiological welfare standards and indirectly through possible effects of chronic stress.

- H_0: ACTH sensitivity, as well as 2° and 3° stress response, is not differentially affected by chronic crowding stress between diploid and induced triploid Atlantic salmon (*Salmo salar*).
- H_1: ACTH sensitivity, as well as 2° and 3° stress response, is differentially affected by chronic crowding stress between diploid and induced triploid Atlantic salmon.

2 Materials and methods

2.1 Experimental animals

480 0+ Atlantic salmon (*Salmo salar*) smolt were halved as diploid and triploid individuals (AquaGen DIPLOID R*E QTL IPN/PD+ILA IPN/PD and AquaGen TRIPLOID R*E QTL, respectively) and randomly distributed within the groups between four 450 L open, white cylindrical plastic tanks in Hall 5, Mørkvedbukta. Flow cytometry of erythrocyte DNA confirmed 100% triploidy induction within the triploid group (AquaGen AS). Smolts were delivered to Mørkvedbukta research station in Bodø from Cermaq facilities in Hopen 05/09/2014 via enclosed 600 L transport tanks aboard approved transport with oxygen injection during loading and unloading. A 30 day acclimitization period followed transfer into the 4 tanks, during which and throughout the study salinity (33-34%), temperature (9.2±0.2 °C), and oxygen (76.1±8.5% saturation) of continuously flowing water were maintained. Dry feed was automatically dispensed to the fish, light period was 24:0 unless specimens were in separate holding tanks post-injection awaiting further study, and human interaction was limited to those directly handling the fish at variable times on set days by closed doors, opaque tank shrouds, and appropriate notification to those potentially entering Hall 5.

2.2 Experimental approval

Norwegian Animal Research Authority (NARA) registered this experiment with approval number ID 6933 by the approval of the locally responsible laboratory animal science specialist.

2.3 Pre-stress

Maintaining 1° and 2° background values requires pre-stress evaluation of morphological and hematological factors, providing control figures comparing potential physiological change *in vivo*. This was performed by first measuring weight, length, glucose, and lactate of 3 fish per tank as control values (n=12), followed by injecting 6 individuals from each of the 4 tanks (n=24) with 0.1mL/100g dexamethasone (DEX) under surgical anesthesia (Stage IV; Schoettger and Julin, 1967) which were divided by ploidy and treatment into four groups of 6 and held in 4 separate tanks (9 fish sampled per tank, 3 of these per tank sampled for pre-stress evaluation, 6 of these per tank injected with DEX and isolated for 24 hours in four separate 40 L tanks). ACTH and PBS challenges followed as described below for these 24 pre-stress fish.

2.3.1 Experimental design

Values were obtained randomly and separately between diploid and triploid groups, allowing 18 individuals of both groups' (diploid and triploid) total weight and length measurement, followed by glucose and lactate readings of blood samples of control fish. Those fish held for 24 h after DEX injection were again anesthetized (directly in 40 L holding cells, water flow stopped prior to dissolution) to Stage IV in 5mg/L metomidate before being injected with either 0.1 mL/100 g crystalline ACTH in ethanol solution or 0.1 mL/100 g PBS solution, in order to determine a control for healthy function of a negative feedback system in cortisol concentration regulation versus exogenous ACTH introduction.

2.3.2 Sampling procedures

Prior to blood sampling each fish was euthanized with blunt head trauma during Stage IV anesthesia (Schoettger and Julin, 1967). Blood was sampled with a heparinized 1mL syringe via caudal vasculature of the fish, and thereafter deposited into previously labeled Eppendorf® Micro-lock centrifuge tubes, sorted by test group. Evaluation of blood glucose and lactate concentrations was performed using Freestyle Freedom Lite™ (0.3 µL) and Lactate Scout™ (0.2 µL), respectively prior to plasma extraction. Tubes were centrifuged at 5-6000 rpm until plasma was separated from blood and extracted into new, sealed Eppendorf® tubes and labeled for storage in a commercial freezer (-20 °C, long-term storage at -40 °C).

2.3.3 Stressor

Each of two 450L tanks, stressed diploid (SD) and stressed triploid (ST), were drained of their water each day by removing a vertically adjacent and external drain plug, without halting water influx. The tanks were drained simultaneously until crowding prevented dorsal submersion and subsequently refilled. Randomization of this process was ensured by recording their intervals weekly and selecting times no less than an hour apart, without sequential repeats.

2.3.4 Sensitivity and negative feedback response

The ACTH sensitivity test was carried out weekly, applied to 12 fish per group (48 fish weekly, 24 weekly basal samples, 360 total samples, and additionally 24 pre-stress subjects) for the duration of the study. This negative feedback response test was dependent upon background values and blocking of ACTH secretion by DEX peritoneal injection (0.1mL/100g), performed each week at 08:00 Monday prior to daily stressors, and metomidate (5mg/L) was used to ensure minimal confounding influences by unwanted cortisol response. The test transferred 18 fish per tank (6 control and 12 challenge) into two 10L buckets of anesthetic, followed by sampling of control fish and DEX injection of challenge test fish and their transfer to isolated 40L tanks (separated by ploidy, stress, and ACTH exposure into 8 separate tanks) for 24 hours. After 24 hours, 6 of these fish were anesthetized and sampled for basal cortisol readings, with 6 peritoneal injections of ACTH and 6 injections of PBS, then replaced into 40L isolation tanks for two hours. These 12 fish per group were then transferred to anesthetic once more, euthanized, then sampled as previously described to determine by challenge the sensitivity to ACTH comparatively.

2.4 Analytic procedures

2.4.1 Plasma cortisol

The concentration of plasma cortisol was analyzed via radio immunoassay (RIA) methods, as described by Iversen *et al.*, (1998). [3H]-cortisol (TRK 407, Institutt for Energiteknikk, Kjeller) was used as the tracer in the analytical procedure. Hydrocortisone (H 4001, Sigma) comprised the standard analytical series (0-137.5nM (nmol/L)). Anti-cortisol rabbit serum (F3-314, Endocrine Science, Tarzana, USA) was the chosen antibody. Samples were initially centrifuged (Haraeus sepatech Omnifuge 2 ORS, 154mm radius, rotor 3360) and subsequently incubated (24h at 4-5°C). The antibody-antigen complex was analyzed via liquid scintillation counter to determine radioactivity (Packard Tri Carb 1900 TR). RIA sensitivity was at 1.68nM, with any sub-minimum readings attributed to minimum sensitivity values. Inter assay Coefficient of Variability (CV) was 12.5% and intra assay CV was below 10%. Non-specific binding (NSB) varied from 2.1to 4.8%.

2.4.2 Glucose

A handheld device designed to register blood glucose concentrations at or above 1.1mM in diabetics, Freestyle Freedom Lite™ (Abbott Diabetes Care, ltd, Oxon, United Kingdom). Efficacy of such handheld instruments in non-human animal glucose evaluation has been addressed and found sufficient (Wells and Pankhurst, 1999).

2.4.3 Lactate

A handheld device, Lactate Scout+ (Arkray KDK, Kyoto, Japan) registers lactate values of 0.8mM. Efficacy of such handheld instruments in non-human animal glucose evaluation has been addressed and found sufficient (Wells and Pankhurst, 1999).

2.4.4 Plasma magnesium

Magnesium (Mg^{2+}) analysis was performed using a Fluitest Mg-XB analysis kit (Biocon Diagnosemittel GmbH and Co., Germany). The kit utilizes colorimetrics by photometric absorbance analysis of xylidyl blue from a Mg-Xylidyl blue complex, which is purple in color. 10µL plasma for each sample is diluted individually according to kit metrics by 1mL xylidyl blue in Eppendorf® centrifuge containers and incubated (to 26°C) prior to deposition and analysis in 520nm plates, controlled by Fluitest xylidyl blue (null) and Mg standard (control, total standard) and produced in mM (mmol/L).

2.4.5 Fin erosion

Subjective observation of fin erosion was personally performed at each sampling interval, scored according to minor alterations on Hoyle et al. (2007). These intervals were pre-stress, 7, 14, 21, and 28 days of daily crowding and were scored from 0-3, wherein erosion could be non-existent (0%), mild (1-24%), moderate (25-49%) or severe (>49%), respectively.

2.4.6 Specific growth rate

Specific growth rate (SGR) was calculated using the following equation:

$$\frac{(\ln(final\ weight\ (g)) - \ln(start\ weight\ (g))}{\#\ days} \times 100$$

SGR was calculated during the experiment for all experimental groups from 0-7d, 7-14d, 14-21d and 21-28 days with daily stressor. An average SGR was calculated for the experimental period (28 days) for all groups.

2.5 Statistical analysis

Statistical tests were performed using the statistical program SPSS 18.0 for Windows. Homogeneity and normality were determined for each dataset with Levene’s and the Kolmogorov-Smirnov tests, respectively. As described by Sokal and Rohlf (1987), one-way ANOVA testing was implemented to investigate differences both within and between experimental groups based on physiological parameters per sampling event. If significant F-values resulted, a Bonferroni post-hoc test was used to determine which groups differed. Wherein parametric evaluation did not correctly apply, Kruskal–Wallis ANOVA (non-parametric) and Mann–Whitney-U tests with a Bonferroni-adjusted significance level were used. Statistical significance was set at 0.05 to determine differences within two STD. Results provided are expressed in mean ± standard deviation (SD) format. Those figures noted with # indicate significant difference between experimental groups at the same sampling time and * indicates significant difference at a given sampling compared to pre-stress levels within the same experimental group. Sample group acronyms were applied as follows: diploid control (DC), diploid stress (DS), triploid control (TC), triploid stress (TS).

3 Results

3.1 Primary stress responses

3.1.1 Plasma cortisol

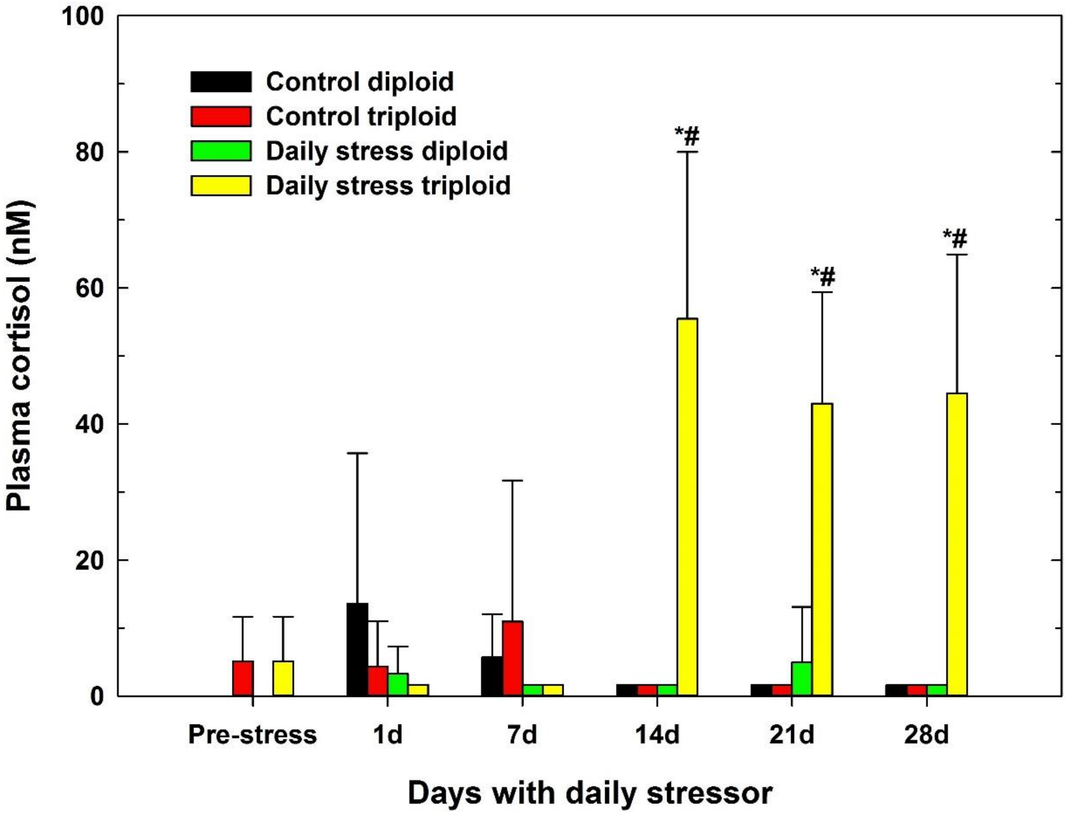

Figure 5. The average values of plasma cortisol (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The resting levels of plasma cortisol concentrations for the four sample groups is shown in Figure 5. At the beginning of the experiment the average baseline levels of plasma cortisol were 5,14 ± 6,56 nmo/L (nM) and 5,26 ± 6,51 nM for control diploid (CD) and control triploid groups (CT), respectively. The daily stress triploid group experienced a significant increase in resting levels of plasma cortisol compared to pre- stress and the other groups at same sampling time at 14 (55.47 ±24.53 nM), 21 (43.02 ±16.39 nM) and 28 (44.52 ± 20.39 nM) days after the start of the experiment.

3.1.2 Sensitivity of the HPI-axis

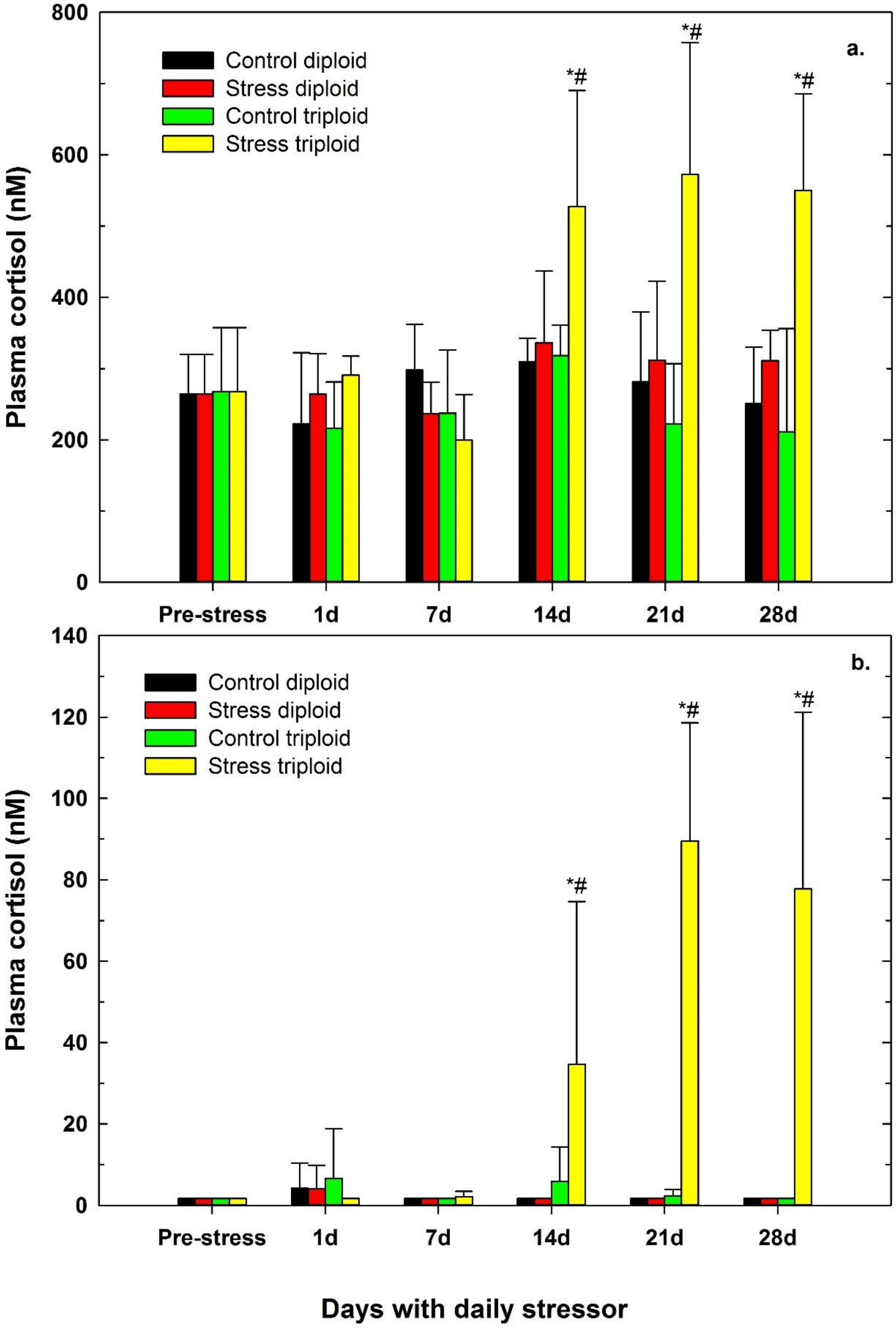

Figure 6. The average values of plasma cortisol (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average levels of plasma cortisol concentrations subjected to an ACTH (Figure 6a) and a negative feedback test (Figure 6b) are shown in Figure 6. At the beginning of the experiment (pre-stress) the average levels of plasma cortisol exposed to a ACTH injection were 264.53 ± 55.62 nM (control diploid (CD) and stress diploid (SD)), and 267.72 ± 89.50 nM (control triploid (CT) and stress triploid (ST)). The ST group experienced during ACTH test a significant increase in plasma cortisol compared to pre- stress and the other groups at same sampling time at 14 (527.37 ±162.72 nM), 21 (572.40 ±184.75 nM) and 28 (550.30 ± 135.47 nM) days after the start of the experiment.

Pre-stress levels of plasma cortisol subjected to a negative feedback test were 1.68 ± 0.01 nM (control diploid (CD) and stress diploid (SD)), and 1.68 ± 0.01 nM (control triploid (CT) and stress triploid (ST)). The ST group experienced during the negative feedback test a significant increase in plasma cortisol compared to pre- stress and the other groups at same sampling time at 14 (34.62 ± 40.02 nM), 21 (89.51 ± 29.06 nM) and 28 (77.74 ± 43.43 nM) days after the start of the experiment..

3.2 Secondary stress responses

3.2.1 Glucose

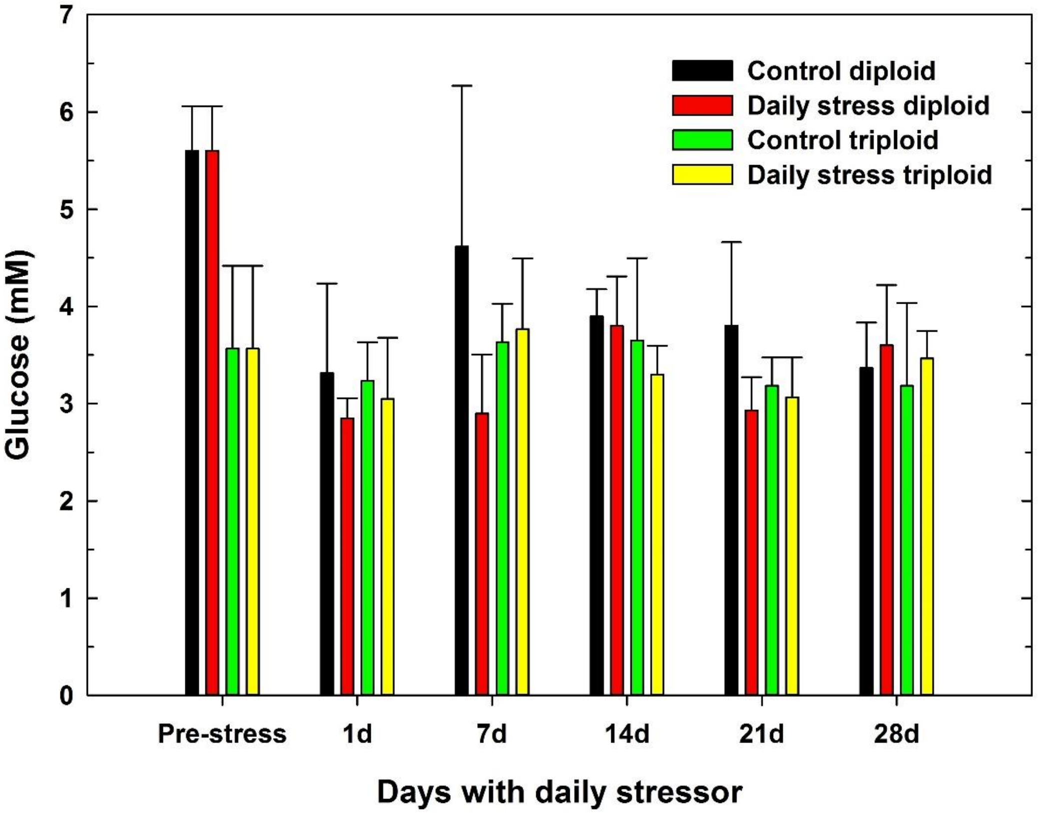

Figure 7. The average values of blood glucose (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average levels of blood glucose concentrations for the four sample groups are shown in Figure 7. At the beginning of the experiment (pre-stress) the average levels of blood glucose were 5.6 ± 0.46 mM (control diploid (CD) and stress diploid (SD)), and 3.27 ± 1.12 mM (control triploid (CT) and stress triploid (ST)).

3.2.2 Lactate

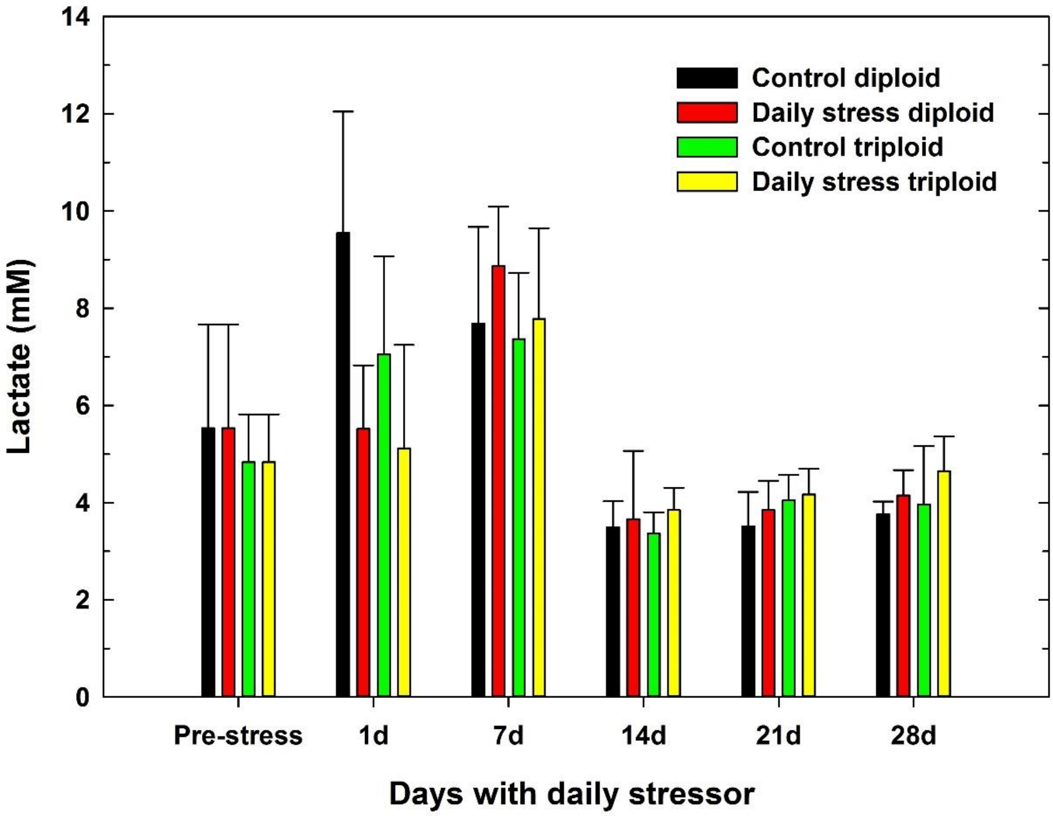

Figure 8. The average values of blood lactate (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average levels of blood lactate concentrations for the four sample groups are shown in Figure 8. At the beginning of the experiment (pre-stress) the average levels of blood lactate were 5.53 ± 2.14 mM (control diploid (CD) and stress diploid (SD)), and 4.77 ± 1.55 mM (control triploid (CT) and stress triploid (ST)).

3.2.3 Plasma magnesium

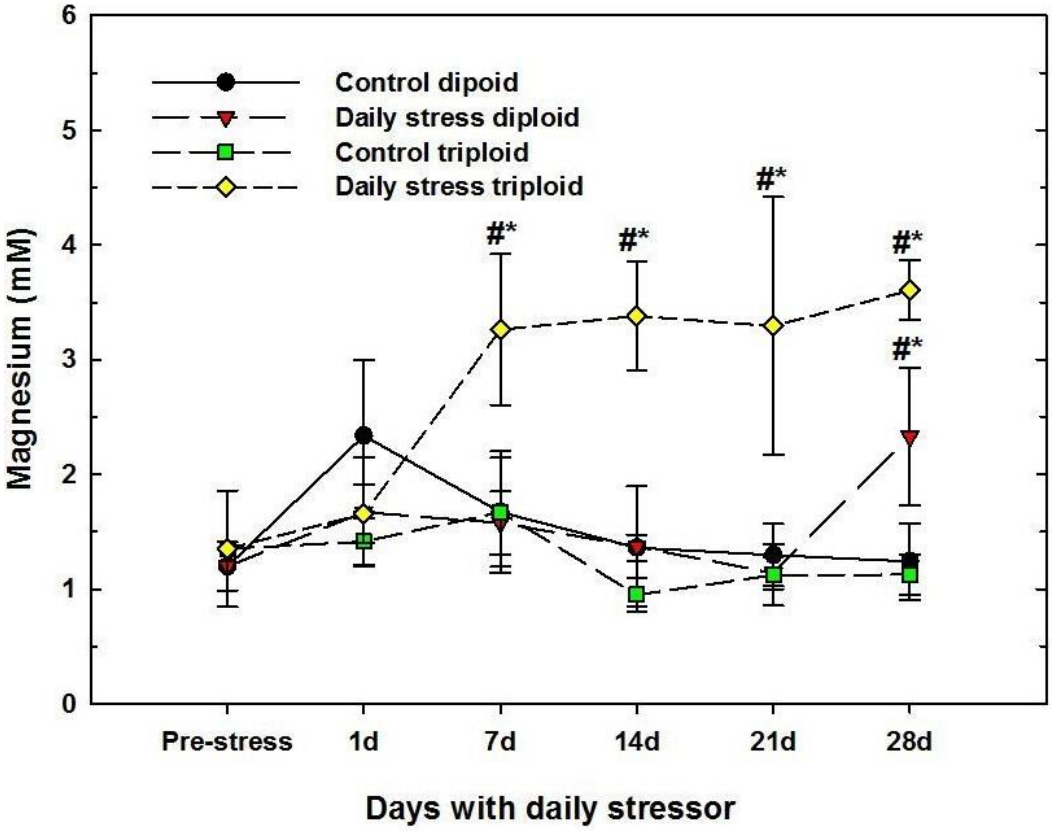

Figure 9. The average values of plasma magnesium (Mg^{2+}) (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average levels of plasma magnesium (Mg^{2+}) concentrations for the four sample groups are shown in Figure 9. At the beginning of the experiment (pre-stress) the average levels of plasma magnesium were 1.20 ± 0.21 mM (control diploid (CD) and stress diploid (SD)), and 1.35 ± 0.51 mM (control triploid (CT) and stress triploid (ST)). Significant differences exist both between ST and other 7d (3.26 ± 0.66 mM), 14d (3.38 ± 0.48 mM), and 21d (3.29 ± 1.12 mM) groups and their pre-stress magnesium levels. At 28 days of daily stress, SD (2.33 ± 0.60 mM) and ST (3.60 ± 0.90 mM) both exhibited significant differences between 28d controls and pre-stressvalues.

3.3 Tertiary stress responses

3.3.1 Fin erosion

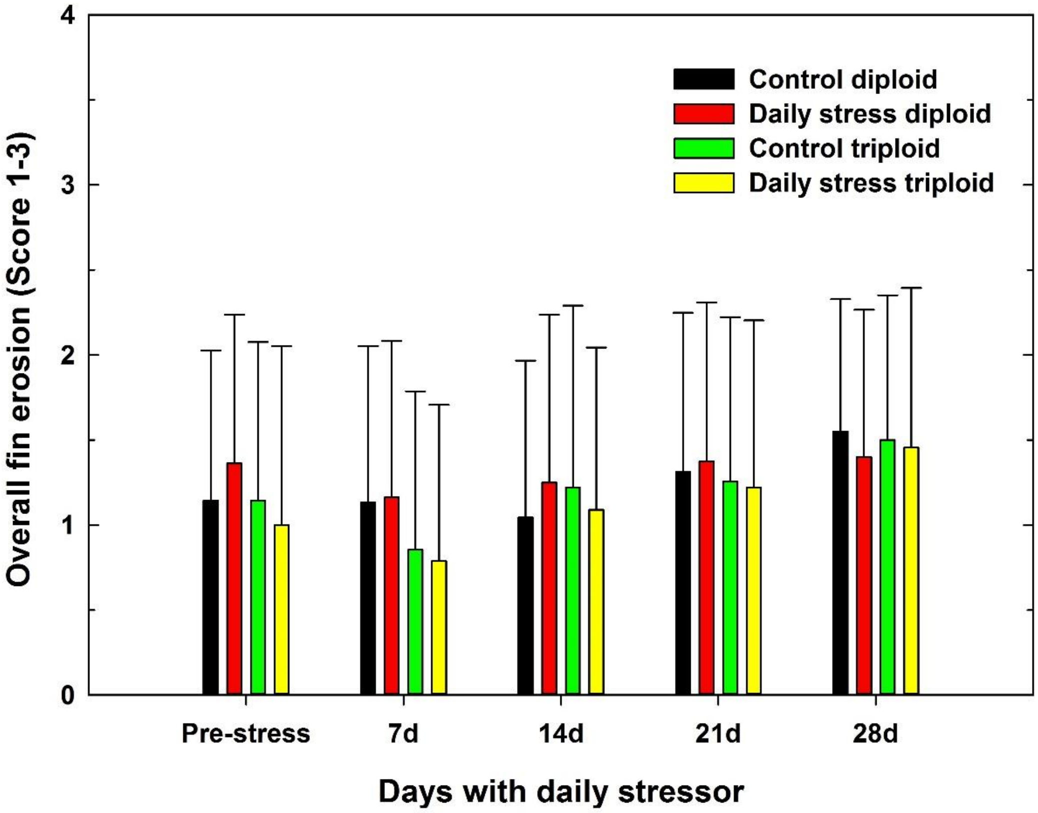

Figure 10. The average scores of fin erosion (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average fin erosion scores for the four sample groups are shown in Figure 10. At the beginning of the experiment (pre-stress) the average scores were 1.25 ± 1.33 (control diploid (CD) and stress diploid (SD)), and 1.07 ± 0.99 (control triploid (CT) and stress triploid (ST)).

3.3.2 Growth

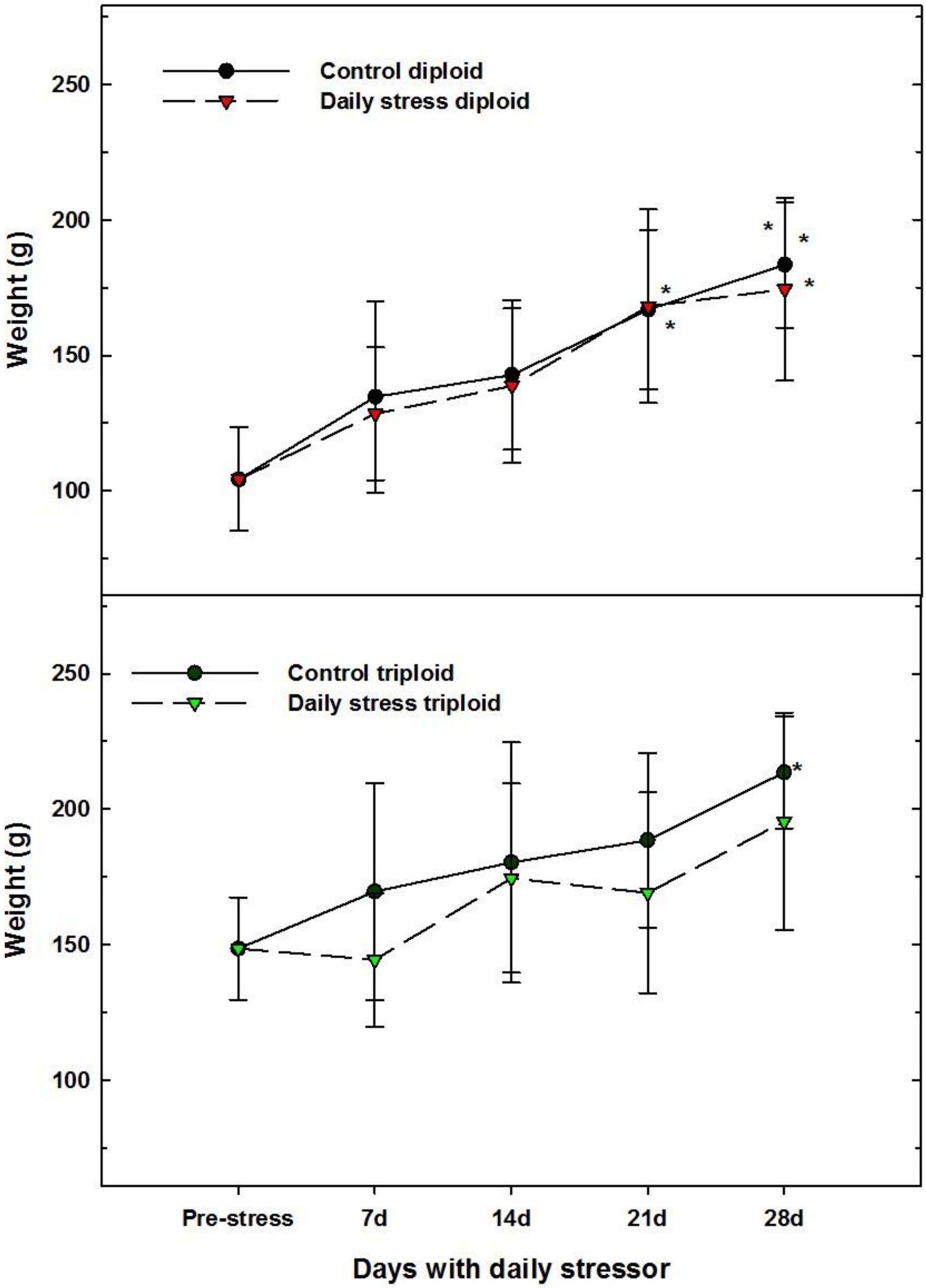

Figure 11. The average wet weight (n ± SD) pre-stress (n=6), in the diploid control, daily stress diploid (12a) and triploid control, daily stress triploid (12b) group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average wet weight of the four sample groups is shown in Figure 11. At the beginning of the experiment (pre-stress) the average wet weights were 104.31 ± 19.08g (control diploid (CD) and stress diploid (SD)), and 148.47 ± 18.89g (control triploid (CT) and stress triploid (ST)). Both diploid groups (CD, SD) experienced a significant increase in wet weight compared to pre-stress 21 (166.94 ± 29.38g, 168.17 ± 35.69g) and 28 (183.44 ± 23.18g, 174.44 ± 33.82g) days after the start of the experiment, and CT experienced a significant increase in wet weight compared to pre-stress 28 (213.56 ± 50.63g) days after the start of the experiment.

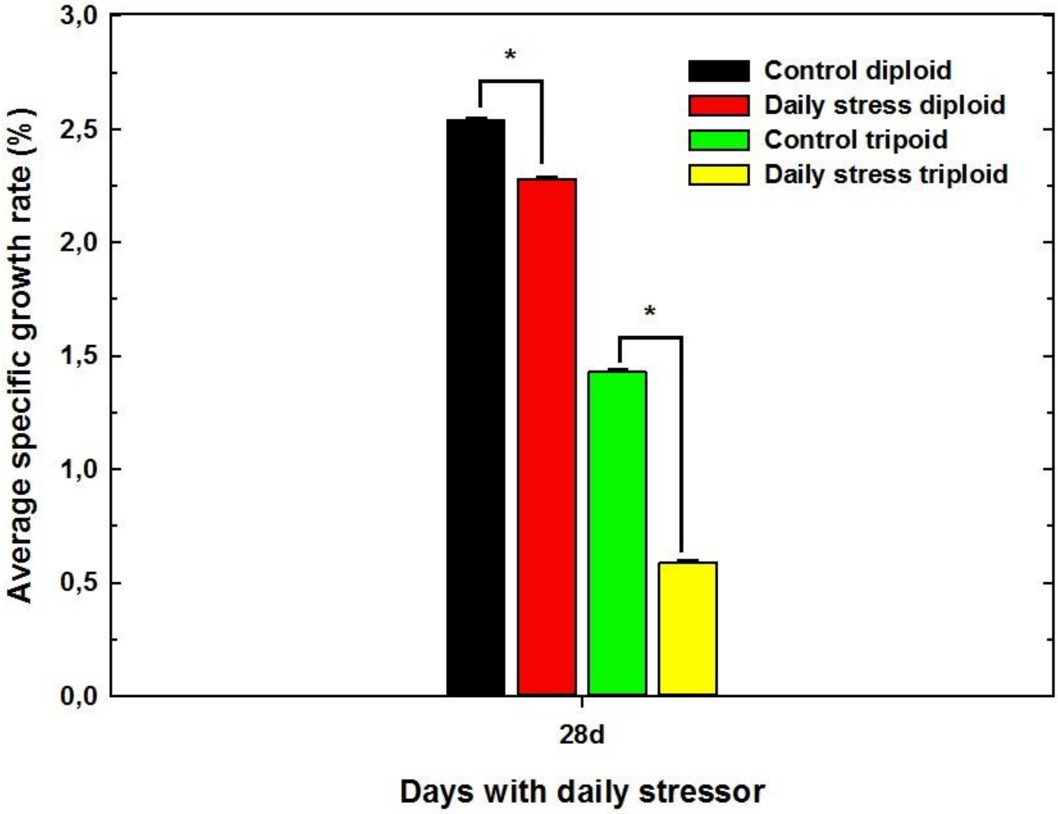

Figure 12. The average SGR (n ± SD) pre-stress (n=6), in the diploid control, triploid control, daily stress diploid and daily stress triploid group of Atlantic salmon smolts (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, * indicates significant difference from the pre-stress level within the same group at 95% confidence level.

The average specific growth rate (SGR) of the four sample groups 28 days after the start of the experiment are shown in Figure 12. Both diploid groups (CD, SD) experienced a significant difference in SGR compared with pre-stress weight (2.54% ± 0.01% and 2.28% ± 0.00%) and triploid groups (CT, ST) experienced a significant difference in SGR compared to pre-stress weight (1.43% ± 0.00% and 0.59% ± 0.01%).

3.3.3 Mortality

No mortality occurred during the experiment.

4 Discussion

4.1 The benefit of triploid salmon

The relative fitness of farmed versus wild genotype Atlantic salmon, ploidy notwithstanding, has been illustrated as negative based on consistent recapture study results over ≥20 years and more recently experimentally characterized *in vivo* under semi-natural conditions behaviorally (Naylor *et al.*, 2005, Skilbrei *et al.*, 2015, Solberg *et al.*, 2015). The importance of feed competition of escaped individuals thus seems much higher than that of spawning for transgenic rainbow trout, which may relate to triploid Atlantic salmon (which also grow at a faster rate post-developmental bottleneck) especially under threat of predation. If the same proves true for Atlantic salmon, then induced triploidy reaches an even more narrow potential range of Norwegian farms especially, though all Atlantic salmon farms must still be considered (Naylor *et al*., 2005).

Given the hazards of such farmed individuals on spawning grounds, studies must illuminate emergence behavior of wild Atlantic salmon fry and have begun to do so (Larsen *et al.*, 2015). Contrasting wild and farm type individuals may possibly lead to some preliminary remediation of wild-spawned farm salmon through understanding of natural environment requirements. Increased susceptibility to predation is an oversimplification largely unfit for illustration of fry fitness in nature, since predation is not consistently observed, no studies exist to accurately propose such a hypothesis, and other potential mortality or disappearance factors exist (Naylor *et al*., 2005, Solberg *et al*., 2015). Therefore, learning more about survivability and coping behavior of fry hatched in the wild (farm vs. wild) could eliminate more confounding factors from this assumption. Evaluating the escape risks inherent could describe the potential effect of replacing a model farm population with triploids by weighing the impacts these potentially spawning diploids could have versus how greatly induced triploid populations could decrease these impacts.

Logically, triploid induction removes farm-borne eggs from consideration by effectively preventing their conception; despite this, spawning and feeding sites are still at risk from escapees (Naylor *et al*., 2005). Evidence describing the out-of-pen survival at known intervals is non-existent for Atlantic salmon triploid escapees, and barely beginning with diploids under experimentally controlled conditions; use of data describing other salmonid species is therefore apparently justified, i.e. rainbow trout (*Oncorhynchus mykiss*) (Solberg *et al*., 2015). A direct feed competition under semi-controlled natural conditions variable in predator presence, genetic source, and age group (cutthroat trout present or not, non-transgenic (W) or transgenic-wild (TW) or transgenic-domesticated/wild (TD/W), first-feeding (FF) fry or 60 days post-first-feeding) of rainbow trout demonstrated the utter susceptibility of TW FF-fry to predation and relative weakness even without them (8 and 30% survival, respectively) compared to wild-type FF-fry (31 and 81%, respectively). Survival was however similar between TW and W trout 60 days post-FF (overwinter), with a growth disadvantage to wild spawn (Crossin *et al.*, 2015). Essentially, these data illustrate a life stage key to survival in the wild which farm-type trout poorly traverse compared to wild-type somewhere within the 60 days after first feeding. This is not necessarily evidence of which factors are responsible for this limited survival of wild-hatched farm-related fry, though predator presence did significantly change survival rates in those youngest (transgenic or not). Survival of salmonid fry in nature is dependent on many things, and whether or not the transgenic offspring live to propagate themselves is vital to their genetic dissemination. Further research should in the author's opinion be conducted on viability of those offspring before deciding if a given marine grow out facility should preferentially raise triploid salmon.

Escaped aquaculture fish can affect wild populations in many ways, but permanent alteration of the wild salmon stock gene pools is a major concern. Total escaped aquaculture salmon in Norway, the world's greatest producer, was in 2001 at 272 000, in 2006 at 921 000, in 2014 at 287 000, with preliminary values for 2015 at 160 000 (FDIR, 2016). Many causative factors in salmon escape exist on land and at sea, though investigations indicate human failure, technical failure, improper equipment use, net pen damage by passing vessels, as well as extreme weather all contribute substantially (Hatløy 2015). Fish still escape from the pens despite preventative measures and production of triploid salmon has begun with the intention to reduce the genetic risk escapees present to the wild Norwegian Atlantic salmon stocks (Hatløy 2015, FDIR, 2016). The implementation of commercially produced triploids is only as viable as their necessity in the production cycle, so if escape is further hindered and better knowledge of spawn success is known in target regions then the viability of their implementation may decrease.

4.2 Primary stress responses (tier 1°)

HPI-axis negative feedback function is explored by this study in its cumulative capacity as opposed to the immediate response to acute stressors, both of which exhibit significant changes to the HPI-axis systemic response element, plasma cortisol, in triploid Atlantic salmon

(Hatløy, 2015). ACTH is directly responsible for these changes, as illustrated by these methods, with results highlighting the importance of hypothalamus-pars distalis endocrine signalling. Similarly, to this cascading stimulation of cortisol secretion must ACTH be naturally downregulated, another endocrine cascade suppressing the secretion of cortisol again at the hypothalamic-pituitary interface (Iwama *et al*., 2005). Dopamine (DA) is a negative feedback response element secreted from the hypothalamus which has been posited as a behavioral regulator following stress, helping salmonids recover and maintain homeostasis, though its exact functions are not clear (Fraser *et al*., 2015). Recent evidence has shown triploid Atlantic salmon exhibit lacking DA catabolism following stress, with respect to DA:DOPAC (a catabolite of telencephalon DA activity) and could reflect a depressed hypothalamic negative feedback control either because it does not respond similarly to diploids or that it is simply lacking (Fraser *et al*., 2015). If triploids are biochemically inferior in ACTH regulation from the hypothalamic tier resultant of lacking DA reactivity to stress, comparative diploid study could elucidate a primary endocrine factor in this respect, though no conclusion can be drawn from this correlation alone. An interesting aspect would however include this DA:DOPAC comparison in relation to behavioral stress response, given the possible link between social hierarchy and DA/5-HT concentrations versus their respective metabolites under distress.

As the ACTH-cortisol relationship affects so many physiological processes, any significantly detrimental variation between ploidies must be thoroughly explored before intensive aquaculture production can be justified from a welfare perspective. Described below in the following chapters are these individual effects on the 2° and 3° tiers, representing the visible reactions which produce those changes qualifiable into categories, e.g. lost production, reduced welfare; the 1° must be extrapolated into these areas to explain beyond endocrine hormone plasma concentrations and cellular responses.

4.2.1 HPI -axis response to cumulative stress response

Vulnerability to distress accumulation is exacerbated in triploid salmon, which express the chronic stress response significantly higher than diploid seawater transfers (Fraser *et al.*, 2015, Langston *et al*., 2001). The importance of plasma cortisol at this point is greater to triploid salmon than diploids, since osmoregulatory ion regulation is poorer in the former under cortisol saturation of regulatory tissues than the latter (Cotter *et al*., 2002, Fraser *et al*., 2012). Prior to seawater release, a crucial resting period provides stress delimiting and negative feedback recovery—this recovery period has been shown to greatly increase in duration requirements for triploid salmon by these results, assuming a return to pre-stress values is possible and HPI-axis response normalizes.

4.2.2 Plasma cortisol and stress-related losses

Susceptibility of fish to cumulative stress-related loss is shown in this study, as increasing resting plasma cortisol concentrations correlated to negative SGR response and lost BW. It seems therefore that triploid Atlantic salmon do handle daily crowding stress in a profoundly negative and significant way compared to diploids, both under immediate stress and not. This accumulation of stress has been characterized in Atlantic salmon as indicative of a chronic stress condition beginning at different life stages individually and to various degrees, with significantly high mortality rates (of 50,970,000 lost Norwegian smolts post-transfer in 2011, 77% were of recorded mortality), and unknown production loss to reduced growth (Iversen, 2013). Mortality is not always directly associated with stress coping, however, since it affects all aspects of the juvenile to grow out salmon. During the especially stress-vulnerable life stage from parr to smolt, a list of stressors encumbers healthy HPI negative feedback response: this is ensconced within smoltification itself, since plasma cortisol concentrations naturally rest higher during the process (>13.8nM or 5ng/mL in salmon, 50-75nM during smoltification) (Alne *et al.*, 2011, Barton, 2002, Iversen, 2013, Iversen and Eliassen, 2009, Iversen *et al.*, 2009, Iversen *et al.*, 2005). The factors themselves are essentially composed of transfer to marine water and vaccinations, actually involving multiple loading and unloading events (especially potent stressors), anesthesia, vaccination (often oil-adjuvanted, commonly causing peritoneal cavity morbidity, e.g. adhesion), transport by land (tank truck) and/or by sea (well boat), and exposure to open seawater (osmoregulatory stress and natural environment stressors, e.g. pathogens). Profound variation in morbidity and mortality can occur between production chains, i.e. choice and application of anesthetics and vaccine(s), transport route and duration, and weather qualities (Iversen, 2013).

From these results and a variable host of commercial stressors imparting substantial productivity losses in presumably otherwise healthy diploid salmon, subjecting induced triploid individuals to similar treatment would cause even greater losses. Losses experienced are resultant both in welfare and productivity, though the latter is characterized numerically both by individuals and monetary cost, factors carefully calculated and monitored by financial forces at large including Marine Harvest in the NYSE (Asche and Sikveland, 2015). In short, stress experienced by triploid Atlantic salmon is significantly higher than in diploids, both statistically and physiologically. This illustrates both reduced welfare and production, translating to quickly realized losses with a profound impact during smoltification. Plasma cortisol is used to designate these facts, as its whole-organism nature describes stress at all levels of organisation in these fish.

4.3 Secondary stress responses (tier 2°)

These impacts are those quantifiable stress responses which determine the adaptive nature of allostatic overload type I (Barton, 2002, Ellis *et al.*, 2012, Iversen and Eliassen, 2014, McEwen and Wingfield, 2003). Describing 2° in this study are glucose and lactate, best representative of immediate reactions and not chronic stress, and plasma magnesium which produces significant changes over extended stressor exposure in cumulative fashion but also in triploids 48 hours after exposure to a 20-minute acute crowding stressor (Hatløy, 2015).

4.3.1 Blood glucose

2° in fish has a directly regulatory effect on blood glucose concentrations, namely by way of cortisol saturation in those hepatic cells responsible for glycogenolysis and gluconeogenesis. The resultant blood glucose spike from catabolizing glycogen and rapidly releasing glucose provides substantial energy to carbohydrate-obligate tissues, allowing the gills, brain, and muscles an opportunity to react under distress (Barton, 2000, Barton *et al.*, 1988). Experimental triploids have now shown a profound cortisol response under repeated stress, though no correlative evidence links this fact to blood glucose concentrations as the results are largely inconclusive and not significantly variable. Therefore, the use of a single factor in determination of stress, its presence or its degree, is inaccurate and must be avoided (Barton, 2000, 2002; Barton *et al.*, 1988). Some studies have also suggested correlative activity between plasma cortisol and blood glucose, since a long-term coexistence in circulation may indicate a direct regulation of glucose by cortisol (Vijayan and Moon, 1992, Vijayan *et al.*, 1997). If there is a lingering glucose-cortisol regulatory action, it may reflect in the results in that they are inconclusive: with elevated plasma cortisol and empirically similar blood glucose concentrations in all individuals, readings possibly provide neither evidence of the gradual process of glucose metabolism nor the immediate gluconeogenesis-glycogenolysis which follows 1° (see Fig. 7). This is in opposition to sampling intervals, which always took place before crowding stress in a cortisol reduction effort and from fish which had been separated from the crowding tanks for over 24 hours—this effectively prevented stressor exposure in sample fish for two full crowding intervals and could allow removal of excess glucose from the bloodstream. Ample time to regulate blood glucose would logically affect these readings and simply reflect homeostatic function in those tissues presumably affected by significantly changed plasma cortisol concentrations, suggesting the possibility of lost homeostasis at more immediate intervals. This has been reflected in a study performed parallel to this, with acutely stressed triploids exhibiting significantly increased blood glucose at 24 and 48h post-crowding within their respective interval groups (not relative to pre-stress values). This crowding was of similar method, though instead maintained for 20 minutes (Hatløy, 2015).

4.3.2 Blood lactate

A resultant metabolite of glucose oxidation to pyruvate, an exercise-dependent process in piscine muscle, lactate forms from lactic acid both aerobically as such and anaerobically by fermentation (Pankhurst and Dedual, 1994, Wells and Pankhurst, 1999). Quantification of lactate concentrations in salmon blood clarifies a portion of its behavioral stress in theory by describing a ratio of lactate to muscular activity, though directly linking this to quantifiable stress response is a haphazard approach (Iversen, 2013, Wells and Pankhurst, 1999). Peaks experienced in blood lactate values relate to activity such as deep anesthesia and vaccination, including their recovery periods, as well as crowding and air exposure; these activities are in addition to strenuous exercise, which produces the highest values in salmon and other species (>20mM) (Iversen, 2013).

Given the immediate nature of lactate secondary response in allostasis, it would suffice to say its representation in a chronic stress study would be at most a correlative trend and not significant, as is apparent by the results (see Fig. 8).

4.3.3 Osmoregulation

Renal maintenance of magnesium (Mg^{2+}) concentrations is directly correlated by plasma secretion from peritubular capillaries to associated proximal tubules, especially with respect to the early proximal tubule. This, whether the fish is glomerular or aglomerular (Atlantic salmon are glomerular), is part of the nephritic osmoregulatory process for secreting, excreting, and retaining ions and organic metabolites, e.g. glucose and creatinine. In addition to Mg^{2+}, chloride (Cl^-) and sulfate (SO_4^{2-}) are key electrolytes in marine teleost osmoregulation via glomerular secretion, and do so in tandem (McCormick, 2013). Along channels according to its electrochemical gradient, Mg^{2+} is secreted into the tubule lumen from blood plasma and passively excreted to the urinary bladder via collection tubules by tonicity after active hydrogen (H^+) excretion passively diffuses H^+ back into the lumen. This renal excretion of Mg^{2+} accounts for the amount taken up intestinally, about 10-20% absorption of Mg^{2+} and Ca^{2+}. These processes are made stable by Na^+, K^+-ATPase, H^+-pump, and carbonic anhydrase (CA) functions, as the osmoregulatory balance act is not so simple (Marshall and Grosell, 2006).

Given the results, Mg^{2+} retention in the plasma in ST from between pre-stress and 7d onwards illustrates a loss of stability in these osmoregulatory functions to a significant degree after a relatively short period of mild stress (see Fig. 9). Even before significant plasma cortisol concentration changes occur (from 14d) in ST, Mg^{2+} had already responded and may indicate some osmoregulatory action independent of corticosteroid stress response. Triploid osmoregulatory fitness during smoltification is at best comparable with diploids and often depressed, exhibiting decreased rates of maturation and greater apparent sensitivity to gill parasite infection (Maxime, 2008). These results reflect one aspect of this by way of a key electrolyte both in triploids and diploids exposed to daily stressors, additionally Mg^{2+} is significantly affected by acute crowding stress in triploids highest 1 (2.5 ± 0.8 mM), but also 2, 24, and 48 hours post-stressor compared to pre-stress concentrations (1.1 ± 0.2 mM) (Hatløy, 2015). Commercial salmon cultivation in Norway depends currently in part upon salmon lice (*Lepeoptheirus salmonis*) management, a constant process over the grow-out period to varying degrees across the country often implementing hydrogen peroxide (H_2O_2) baths shown in sea bass (*Dicentrarchus labrax*) to significantly increase plasma Mg^{2+} (Helgesen *et al.*, 2015, Roque *et al.*, 2010). Given the frequent exposure of commercial Atlantic salmon to lice and treatment baths, the sensitivity of triploids to osmoregulatory stress may pose a problem. Osmoregulatory stress is apparently correlated strongly with both cortisol and secondary stressors, namely vaccination: a similar stress evaluation over a 28 day period in diploid Atlantic salmon produced similar Mg^{2+} significant fluctuation, and a severe cortisol spike following vaccination at the end of the 28 day stress trial (Iversen and Eliassen, 2014). The connection between Mg^{2+} and cortisol could be related to the apparently paracrine effect in head kidney stress response, an adrenergic process tying catecholamines, Mg^{2+}, and corticosteroidogenesis together in 1° (Iversen, 2013, Iversen and Eliassen, 2014). If similar correlation exists in triploids, in which cortisol and Mg^{2+} appear significantly affected in a positively correlative manner sometime between 7d and 14d of chronic stress trials, such a vaccination at 28d could result in widespread mortality given their profound response compared to diploids (see Figs. 4, 5, 9). The Mg^{2+} increase in the SD 28d interval points to a correlation both between other work on diploids but also the ST group, and illustrates the apparent effect chronic crowding stress has on osmoregulation since CT and CD had similar basal concentrations over the whole study. Aforementioned similar results are also depicted on the short term osmoregulatory changes in triploids over a 48 hour timespan (Hatløy, 2015).

Vaccination and H_2O_2 salmon lice treatment are thus avenues to critical stress response both in renal osmoregulation and directly on HPI-axis negative feedback, setting triploids and diploids apart because crowding stress and vaccination are not only commonplace but requisite aquaculture production protocol.

4.4 Tertiary stress responses (3^o tier)

Beyond the immediate events following stress response, the affected animal ideally enters an adaptive state and maintains homeostasis without maladaptive changes to fitness (Barton, 2002, Ellis et al., 2012, Iversen, 2013). Tertiary stress responses are those which will reflect such changes, in this study represented by growth performance and fin erosion scoring, and describe the welfare impacts 1° and 2° have had on the individual.

4.4.1 Growth performance and fitness

Aquaculture production revolves around flesh biosynthesis in all species, aesthetic and food livestock alike. The significant losses to specific growth rate (SGR) both on average and by body weight (BW) at intervals in chronically stressed triploid salmon have demonstrated potential losses in welfare, flesh amount and quality (given impact of stress on quality), and finance (from valuation of triploid eggs, through vaccination and feeding, to extended smoltification periods and finally to apparent mortality) (Iversen, 2013, Iwama *et al.*, 2005). Controlled aquaculture production delimits the definition of fitness in relatively isolated populations, though disease, feed availability, and social standing all exist in marine net pens which contain many competing individuals (Clements and Raubenheimer, 2005). Disease is of particular difference between wild and farmed stocks, since high densities and exposure to different environments increase the probability for pathogen proliferation (Mennerat *et al.*, 2010). Allostasis physiologically calculates these variable stressors and produces homeostasis, however if the negative feedback mechanisms governing it lose control of hormonal regulation, then fitness will be reduced and these results have shown triploids will also lose growth potential (see Figs. 12, 13).

Significant growth and SGR loss experienced by ST is characterized by this and other plasma cortisol studies, reflecting a collapsed HPI-axis resultant of increased basal plasma cortisol concentrations. Studies using cortisol-enriched feed have clearly described this suppressive relationship between plasma cortisol and growth, i.e. goldfish (*Carassius auratus*) in a 21 day feeding trial comparing feed containing 0, 50, or 500µg cortisol/g feed (Bernier, 2006). Other examples of this cortisol-influenced growth suppression are documented for rainbow trout (*Oncorhynchus mykiss*), matrinxã (*Brycon cephalus*), Atlantic cod (*Gadus morhua*), channel catfish (*Ictalurus punctatus*), and gilthead seabream (*Sparus auratus*) *vis à vis* direct effect on growth as well as appetite changes (Bernier, 2006, Bernier *et al.*, 2004, Bernier and Peter, 2001, Bjornsson *et al.*, 2002, Mommsen *et al.*, 1999, Olsen *et al.*, 2008, Pankhurst, 2011, Pankhurst *et al.*, 2008, Pickering, 1990, Pickering, 1992, 1993, Small, 2004, Sørensen *et al.*, 2011, Tsigos and Chrousos, 2002, Weil *et al.*, 2001). The cascading effect of plasma cortisol circulation has multiple affective tiers from reduced cellular growth described in cultures treated with cortisol, a GR-mediated response by way of GRE in nuclear DNA, to increased oxidative activity in stressed fish observed as increased BMR resulting in reduced energy availability, and suggested to influence intestinal morphology, which would directly alter nutrient acquisition from gut lumen surface activity reduction. Cortisol also has an endocrine influence on feed consumption and FCR, given its documented effect on growth hormone and hepatic insulin-like growth factors (Bernier, 2006, Bernier *et al.*, 2004, Bernier and Peter, 2001, Bjornsson *et al.*, 2002, Mommsen *et al.*, 1999, Olsen *et al.*, 2008, Pankhurst, 2011, Pankhurst *et al.*, 2008, Pickering, 1990, Pickering, 1992, 1993, Small, 2004, Sørensen *et al.*, 2011, Tsigos and Chrousos, 2002, Weil *et al.*, 2001).

4.4.2 Communal rearing

Performance of triploids (3N) versus diploids (2N), both in ploidy-isolated (ISO) and ploidy-mixed (MIX) rearing cohorts, is reduced significantly. This restriction on the use of induced triploid Atlantic salmon is further specified by negative performance between families of similar groups (thus, within and between 3N-ISO, 3N-MIX) (Taylor *et al*., 2014). The ratings of performance tend to be based on comparison of body weight (BW, a wet weight metric) and condition factor (CF or K), commonly associated with other metrics, i.e. specific feeding rate, thermal growth coefficient, basal metabolic rate, and feed conversion ratio, though variability between these occurs as a result of differing sample collection methods and standards (Alne *et al*., 2011, Taylor *et al*., 2014). Specific condition factor was not identified through review of several growth performance studies for *Salmo salar* and was instead reported as

K=(L/W3)*100, with the value of 3 being a common stand-in for freshwater fish, as K is often used in their weight characterization (Anderson and Neumann, 1996).

Relevance for K is expressed through triploid stock growth efficiency and possible welfare implications, since inferior growth efficiency should by commercial logic be avoided especially given the sensitive nature and careful handling required to raise such stocks. Reduced BW and K in both isolated and communal pens demonstrates triploid susceptibility to any number of environmental stressors; their exceptionally poor (6.8 and 26.4% lower than 2N-ISO/MIX, respectively) growth performance in communal pens makes them even less attractive (Taylor *et al*., 2014). Morphological deformity vis à vis ocular (cataracts), mandibular (reduced jaw mobility), and opercular (exposed gill tissue) are also more prevalent in triploid stocks. These malformations are also significantly affected by heredity and accelerated growth, with 2N/3N-ISO both exhibiting higher rates of cataracts than communal families with variable frequency (familial similarities) and especially higher deformity in 3N-ISO than 3N-MIX (30.4 vs 5.8%) (Taylor *et al*., 2014). Prevention of cataracts via dietary histidine supplementation has however been experimentally demonstrated as a possible remedy to cataract formation tendency in triploid salmon (Taylor *et al*., 2014). A decreased growth performance is thus illustrated in comparison of communal pen stress with isolated families, as well as performance between ploidies and a differential deformity rate; BW and SGR results from this study also illustrate their significant decrease both within and between ploidies based on crowding stress in isolated familial tanks with no determination of deformities in 0+ smolt. The results are especially significant between diploid groups (both stressed and unstressed) and stressed triploids, for which the 28d SGR was almost -0.5% and BW intervals (rather than averages) resulted in significantly negative values at 3 of 5 samplings. Average SGR also implies a highly depressive effect for stressed triploid salmon at just over 0.5%, concurrent with those in Taylor *et al*. (2014) which presumably experienced social stress under communal conditions.

The importance of evaluating communal pen interactions between ploidies is to demonstrate the necessary separation of the two groups, thus rearing them indiscriminately is effectively advised against. If they cannot be reared in the same group, then more facilities must either displace diploid production or construct entirely new facilities for triploids, if these results are to be applied to that of Taylor *et al*. (2014).

4.5 Animal welfare of triploid aquaculture salmon

The increasing interest in aquaculture sustainability is a key driving factor in introducing triploids into commercial Norwegian production cycles both from animal and energetic perspectives, given the requirements of alternative methods of precocious maturation prevention (Fraser *et al*., 2012). Considering every caveat to their stress coping capability compared with diploids, these fish exhibit a weakened state after relatively minimal stressor periods with disproportionate response both in the endocrine system and numerous production quality affecters. Specifically discussing welfare parameters also illustrates a troubling dichotomy within the ploidy topic, clearly translating to studies of their differences based on indices, i.e. survival, metabolism, stress response (Fraser *et al*., 2015).

Debate on the conscious experience of fish is relevant here, in that inherent biochemical and physiological differences exist between ploidies of salmon, often to the detriment of triploid individuals and leading to additional care requirements (Cotter *et al*., 2002, Davis 2006, Fraser *et al*., 2012, Hatløy, 2015, Iversen, 2013, Langston *et al*., 2001, Maxime, 2008, Sadler *et al*., 2001, Salimian *et al*., 2016, Taylor *et al*. 2014). Included previously in this paper have been myriad publications describing many aspects of environmental conservation made possible by successful and reliable triploid salmon induction, though none are published regarding the economic burden such an overhaul would require; given the apparent welfare management requirements of triploid salmon, implementing them on a commercial scale entails reconsideration of many production protocols, e.g. communal rearing, feed choice, and the logistics of transport and handling. Animal welfare is therefore a gauge for not only the wellbeing of triploid salmon, but also their additional costs if ethical treatment is carried out.

Conclusion

Introduction of induced triploids could significantly reduce genetic dissemination and is researched for viability as a solution to an endemic issue in Atlantic salmon aquaculture. The results from this study raise concerns for chronic allostatic overload type 2 in triploid salmon subjected to a daily crowding stressor. Study has shown that additional stress following such overload has significantly detrimental results in diploids, which are better equipped to cope with stress than triploids.

HPI-axis response is significantly affected in triploid Atlantic salmon compared with diploids, chronic and acute, as expressed by blood glucose changes over days and by plasma cortisol and magnesium concentrations, as well as growth performance, over weeks. These results indicate that if triploid salmon are to be used in commercial production, their more fragile nature compared to diploid salmon must be considered. Specifically, negative feedback response in the triploid salmon HPI-axis could be more receptive to environmental changes in production, thus increasing the probability of disease, heightening morbidity and mortality, and decreasing overall welfare and production.

Avoiding systematic animal suffering must constantly be weighed against human benefit and environmental conservation. These results present evidence depicting difficult division between these principles: triploid salmon could significantly improve wild stock bloodlines genetically, though their sensitivity may prove too great for commercial aquaculture production in meeting projected quotas. Balance must then be realized between yet undefined economic, environmental, and ethical guidelines for triploid salmon aquaculture.

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