MASTER THESIS

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THE EFFECT OF LONG-TERM STRESS ON BASAL LEVELS OF PLASMA CORTISOL AND HYPOTHALAMIC–PITUITARY–INTERRENAL (HPI) AXIS IN LUMPSUCKER (*Cyclopterus lumpus*)

Faculty of Biosciences and Aquaculture

SPRING 2016

NORD University

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PREFACE

First of all, I would like to thank my supervisor, Martin Haugmo Iversen, that has been great support and excellent teacher and helpful guide in the complicated world of experiments and physiology knowledge. I cannot enough express my gratitude for your encouragement and willingness to help me all over the time with the research, writing of this thesis and making it happen despite hundreds of kilometers. Thank both of you for your understanding, patience and kindness.

I would like to thank Bente Sunde for teaching and help with analyses in the laboratory, Roald Jakobsen and Steinar Johnsen for help during sampling. A great thank also to Hilde Ribe and all the other people from Mørkvedbukta that were helpful and enthusiastic. You are a great team! Very special, warm thank to Wanja Braseth - thank you for being supportive, professional, enthusiastic, precise and hard working in all the difficult moments. You are a wonderful person; without your assistance it would probably not have been possible.

Finally, I send a great gratitude to my good friends that I met on my way. I am eternally grateful.

My special gratitude is for my family, my wonderful husband in particular. Morten, you have been most supportive, carrying person I have ever met. I would never be able to fulfill this project without your help, trust and believing in me when I was in the deepest doubts. Thank you!

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SUMMARY

This project investigated the effect of a long-term crowding stressor on HPI axis and basal levels of cortisol in Atlantic lumpfish (*Cyclopterus lumpus*), which is a relatively new subject of research, especially in the light of growing interest from aquaculture companies as a new method for controlling the sea lice population in salmon aquaculture industry. Unfortunately, early reports from fish farms shows high mortality during the early production phase, most likely due to low long-term stress tolerance. Thus, the purpose of this study was to compare the response of the hypothalamic–pituitary–interrenal (HPI) axis subjected to a long-term crowding stressor in lumpsucker (*Cyclopterus lumpus*).

The experiment consisted of two groups. Group 1: Daily stress in the form of crowding for one month and group 2: Control (no stress). Daily routines were permitted in both of the tanks. It included flushing excess feed, faeces, and monitoring of lumpsuckers behaviour.

Changes in resting levels of plasma cortisol, secondary and tertiary stress responses were tested. Blood samples were obtained prior to start of the experiment (pre-stress), and after 7, 14, 21, 28 days of stress (n=6). Stimulation (ACTH) and dexamethasone (DEX) suppression test was conducted at the same days to test HPI axis response.

The results indicated that stress group showed signs of allostatic overload type 2 due to oversensitivity to ACTH, and a reduced negative feedback system with increased baseline levels of cortisol as result. Those primary changes were followed by secondary stress responses as osmolality changes, chloride and magnesium ions concentration imbalances. Negative tertiary stress responses were detected such as reduced growth in terms of overall weight and specific growth rate changes.

If plasma cortisol becomes elevated and organism does not have enough time to stabilise homeostasis, lumpsucker will end up in an allostatic overload type 2. This could put animal welfare of the lumpsucker at risk, and can result in vast mortality during the process of producing and utilising lumpfish in Norwegian aquaculture industry.

SAMMENDRAG

Dette prosjektet undersøkte effekten av en langsiktig stressor - sammentrenging på HPI-aksen og kortisolnivåer hos Atlantisk rognkjeks (*Cyclopterus lumpus*). Rognkjeks er en relativt ny art i oppdrettssammenheng, og har fått økende interesse fra oppdrettsselskaper som en mulig ny metode for å få kontroll på problemet med lakselus. Dessverre, som tidligere rapporter fra havbruksnæringen viser, er det observert økt dødelighet i løpet av tidlig produksjonsfase som trolig skyldes langvarig kronisk stressbelastning. Med dette som bakgrunn ønsket en å gjennomføre et forsøk for å studere effekten av langtidsstress på HPI-aksen, og hvilenivåene av plasmakortisol hos Atlantisk rognkjeks.

Forsøket besto av to grupper. Gruppe 1: Daglig stress i form av sammentrenging for en måned og gruppe 2: Kontroll (ikke stress). Daglige rutiner ble tillatt i begge tanker, og bestod av spyling av overflødig fôr, avføring, og overvåking av fiskens atferd.

Endringer i hvilenivået av plasmakortisol, og ulike sekundære og tertiære stressresponser ble undersøkt. Blodprøver ble tatt før starten av eksperimentet (pre-stress), og etter 7, 14, 21, 28 dager med stress (n = 6). Stimulering (ACTH) og deksametason (DEX) test ble gjennomført ved tilsvarende dager for å teste HPI akseresponsen.

Resultatene viste at stressgruppen viste tegn på allostatisk overbelastning type 2 med en oversensitivitet til ACTH, og en redusert tilbakekoblingsmekanisme med forhøyede nivåer av kortisol. Disse endringene ble fulgt av forandring i sekundær stressresponser som: endringer i osmolalitet, og ubalanse i klorid-og magnesium-ionekonsentrasjonen. Tertiære stressresponser ble påvist i form av kraftig redusert vekst i stressgruppen.

Hvis plasma kortisol blir forhøyet og organismen ikke har nok tid til å stabilisere homeostase, vil rognkjeks fort ende opp i en allostatisk overbelastning type 2. Det kan kompromittere dyrevelferden, og kan gi stor dødelighet i produksjon av rognkjeks.

1. INTRODUCTION

1.1 Aquaculture and salmon lice

Norway is well suited for fish farming, with its long coastline with fjords and islands, which provides calm environment and, by that, perfect surrounding for aquaculture. In aquaculture, the Atlantic salmon (*Salmo salar*) represents 90% of production (Burridge *et al.*, 2010), and it is considered high quality fish meat, which is available around the globe. Worldwide aquaculture production of Atlantic salmon reached 2 million tons in 2013 (Fao, 2014), with Norway being the biggest producer in the world (Torrissen *et al.*, 2011), followed by United Kingdom, Chile, and Canada. Every year, Norwegian fish farmers produce more than one million tonnes of salmon with a combined value of more than NOK 30 billion (EUR 3.7 billion, (Andreassen & Robertsen, 2014)). Some arguments that salmon is the most sustainable farmed animal (Mozaffarian & Rimm, 2006), a "super chicken", with a feed ratio of 100 to 65 (dry feed to meat, (Torrissen *et al.*, 2011), whereas others points out that there are also many problems hiding in the production process. Escapees and sea lice are the biggest environmental concern both from biological and environmental point of view, but also from the consumers' point of view.

The sea lice are marine parasitic copepods that have been a large problem for both wild and farmed fish from the early start of commercial aquaculture (Brandal & Egidius, 1979). The cost of the sea lice control is likely to reach 0.1-0.2 € for a kg of fish or around 6% of the production costs (Costello, 2009). The most common species in Norwegian aquaculture is the salmon lice (*Lepeophtheirus salmonis*, Krøyer, 1838); however, there is a wide variety of sea lice parasites on various fish species. Some of them are specific for a small number of species or families, like salmon lice or cod lice (*Caligus curtus*), which are preliminary attacking different Gadiformes. Other species, such as *Caligus elongates*, have wide range of hosts and can live on almost all available fish species. All three of lice species can be found on farmed salmonids in Norway, and cause changes in physiological homeostasis, as increased cortisol levels, osmotic imbalance, modulated immune system and high mortality (Bjorn *et al.*, 2001; Grimnes & Jakobsen, 1996; Heuch *et al.*, 2005; Holst, 1993; Tully & Nolan, 2002). Even though, aquaculture have struggled with sea lice for a long time, the development of different methods trying to normalise the problem has not been successful, and the problem has been increasing in the last years (Taranger *et al.*, 2014). The sea lice difficulty has manifested itself in increased use of chemotherapeutants, which again has led to escalation of the resistance

problem. Due to this, re-infections of sea lice, effective strategies to control the problem are hard to come by (Mcvicar, 2004).

The chemotherapauts can be classified into two groups depending on the way it is given to fish: in-feed additives and bath treatments (Burridge *et al.*, 2010). All treatments are with avermectins: added to feed (SLICE ®), hydrogen peroxide, pyrethroids and organophosphates (used in the bath treatment) could eventually lead to resistance against these substances in sea lice (Denholm *et al.*, 2002). Resistance towards organophospates (Fallang *et al.*, 2004) and pyretorids (Sevatdal & Horsberg, 2003) has also been reported in Norway, Scotland, and Ireland (Torrissen *et al.*, 2011). In 2005, resistance to emamectin benzoate was reported in Norway, Ireland and Canada in years 2006 and 2007, accordingly. Additionally, to develop resistance in sea lice, these medical treatments are often stressful to fish (Burka *et al.*, 1997), expensive (Costello, 2009), and harmful to the environment (Burridge *et al.*, 2010). Luckily, there are other non-chemical trials to keep sea lice levels under control. The most common measure is usage of cleaner fish.

1.2 **Cleaner fish in farming of salmonids**

Due to the fact that chemical de-lousing is harmful both for fish and environment, aquaculture companies tries to discover better ways to get rid of the parasite, but a perfect method is still yet to be found. The aquaculture industry, environmental organizations and the governments are deeply concerned, and development of non-pharmacological interventions are in constant motion, and have brought various preventive trials such as shields against lice larvae (skirts, submerged cages, power driven), health feed, breeding, laser and a water filters. Unfortunately, none of these is 100% functional by its own, and the use of additional biological factor is needed in all mentioned above cases. As problems with lice and its resistance to chemical agents has increased, the interest for cleaner fish has increased accordingly (Treasurer, 2002). Already 40% of all Norwegian salmon localities use cleaner fish as part of their louse control strategy (Furuset, 2016). The use of cleaner fish is effective, environmentally friendly, sustainable and do not harm the salmon (Waatevik, 2015). The main groups of cleaner fish exploit nowadays are wrasse (*Labridae*) with four main species, and a lumpsucker (*Cyclopterus lumpus*).

Of six species from the order wrasse that are found in Norway, four have been in used in delousing of salmonids: corkwing wrasse (*Symphodus melops*; Linnaeus, 1758), goldsinny

wrasse (*Ctenolabrus rupestris*; Linnaeus, 1758), rock cook (*Centrolabrus exoletus*; Linnaeus, 1758) and ballan wrasse (*Labrus bergylta*; Ascanius, 1767) (Figure 1).

Figure 1 Adult male Ballan wrasse (*Labrus bergylta*, A). Photo Proff. Oddvar Ottesen, Nord University (NO).

The goldshinny wrasse was first tested in laboratory already in 1988 followed by experiments in net cages in following years (Bjordal, 1992). Commercial fishery of goldsinny wrasse started in 1988 (Darwall *et al.*, 1992). Since the need for wrasse in fish farms has been covered by the wild catch, there has emerged a need for regulations and restrictions to avoid overfishing of the population (Skiftesvik *et al.*, 2014). Up to 2010, Norwegian aquaculture used approximately 1.3 million wrasse annually (wild caught), and this has since 2010 increased to about 20 million (Skiftesvik & Nedreaas, 2016). Due to this demand, the supply of wrasse to the aquaculture industry cannot be covered by a natural resource (Waatevik, 2015). Regrettably, wrasses are not common in the northern parts of Norway, partly because of low sea temperatures in the winter (Lein, 2013; Skiftesvik *et al.*, 2014). Some studies showed that efficiency of wrasses (especially ballan wrasse) to eat lice decreases with reduced water temperature (Lein, 2013). As far as wrasses are challenging species both to produce and to keep in the cages with salmon, and lumpsucker appears to be the perfect cure to rescue salmon farms against lice/ answer for the growing need of aquaculture in northern part of Norway.

1.3 The lumpfish and its role in aquaculture

Lumpsucker (*Cyclopterus lumpus*) is a marine cottoid teleost fish from the family Cyclopteridae (lumpsuckers) (Davenport, 1985). The whole body is covered in scale-less skin, nearly spherical with generally plain coloration. Characteristic ridged back have three bony tubercles on each flank. Colour varies between individuals, reaching colours from bluish, olive

through greyish as well as yellowish or brownish (Figure 2). Mature males turn reddish during the breeding season (Davenport, 1985). As the majority of the benthic fish, lumpsuckers does not have swim bladder, compensated by low body density. Pelvic fins have evolved into adhesive disc that are located ventrally, behind the pectoral fins (Bigelow & Schroeder, 1953). Males can grow up to 40 cm in length and become almost 5 kg in weight, while females can become even bigger and reach 60 cm in length and approximately 9.5 kg in weight. They have a lifespan of approximately 12 years (Davenport, 1985).

Figure 2. Juvenile lumpsucker (*Cyclopterus lumpus*). Photo Dr. Martin H. Iversen, Nord University

It is a common species along the coastlines of North Atlantic (both East and West coast), from Svalbard to Portugal, and up to 70°N in the Western North Atlantic (Davenport, 1985), as well as parts of the Arctic Ocean (Holst, 1993), indicating a broad temperature tolerance range. Lumpfish are breeding in shallow waters during spring and summer, and brood fish are easy to obtain along coastlines during that time (Holst, 1993). Lumpfish lay their eggs in a nest site, which is guarded by the male (Kennedy *et al.*, 2014). Female leaves just after the eggs are laid and do not take part in guarding the stocks, whereas males can guard more than one batch of eggs. After hatching, larvae attach itself to the nearest substratum (Pampoulie *et al.*, 2014), after few months will they migrate (up too 100km) to the open waters for feeding purpose (Blacker, 1983; Kennedy *et al.*, 2014).

Lumpfish has significant economic importance, and is exploited in the North Atlantic regions for its high-valued caviar (Hedeholm *et al.*, 2014; Pampoulie *et al.*, 2014). Nevertheless, lumpsucker is starting to have even greater economical meaning, after it was introduced to the Norwegian aquaculture as a potential delouser of salmon. Recent studies have shown that lumpfish is an effective cleaner fish (Norethberg *et al.*, 2015), grazing on pre-adult and adult stages of sea lice (Imsland *et al.*, 2014; Willumsen, 2001). Field studies seem to support these investigations, and suggest that not only the most common salmon lice, *L. salmonis* but also *C. elegans* is a part of the lumpsucker diet (personal observation). It has a natural northern distribution, with broad tolerance of temperature and supposedly sturdiness to stress and suboptimal water conditions, which makes it perfect species for use with Atlantic salmon in northern part of Norway (Imsland *et al.*, 2014). Early pilot experiments confirmed that adult lumpfish could be reared and would spawn in captivity (Klokseth & Øiestad, 1999), which have started a boost in commercial production of lumpsucker juveniles both in Norway and Faroe Islands (Schaer & Vestvik, 2012; Waatevik, 2015; Willumsen, 2001). Waatevik (2015) estimates as many as 20 million of lumpfish will be produced in 2017.

1.4 Stress in fishes

In the last years, there has been a significant interest in studying stress consequences on fish. As most research have proved, there is correlation between increased stress and decreased fish welfare, decreased quality and increased mortality, which will result in reduced income for the commercial aquaculture industry (Iversen et al, 2015).

The word "stress" has its roots in the physiological definition proposed by Selye (1950, 1973). This theory stated that ''*stress is the nonspecific response of the body to any demand placed upon it*". More specific and precise definition has been evolving over the latter years focusing on nervous and/or endocrine system, but as a broad definition, it still gives us the best description of the phenomenon "stress". One can talk about stress as a flow of physiological actions, when an organism tries to rebuild homeostasis by using allostasis or fight against death (Schreck; 2009). The general idea is that stress itself is a positive thing, promoting survival under adverse challenges. Stress activates several physiological mechanisms that are helping fish to return to physiological equilibrium (homeostasis). Therefore, stress, increases the individuals chance of surviving danger (Chrousos & Gold, 1998). Short-term effects of stress are in most cases a positive and important for existence of the organism and the species. On the other hand, long term stress, can have negative consequences for animals' possibilities to

maintain normal life functions over time. Chronic stress reduces the ability of fish to grow, reproduce and survive (Barton, 2002; Iversen, 2008; Iversen & Eliassen, 2014; Wendelaar Bonga, 1997). Animals in farming conditions, opposite to free-living fish, cannot avoid stressors, or escape from them. Farmed fish can be exposed to unwanted environmental changes, poor water quality, repeated handling, transport, delousing, sampling and crowding.

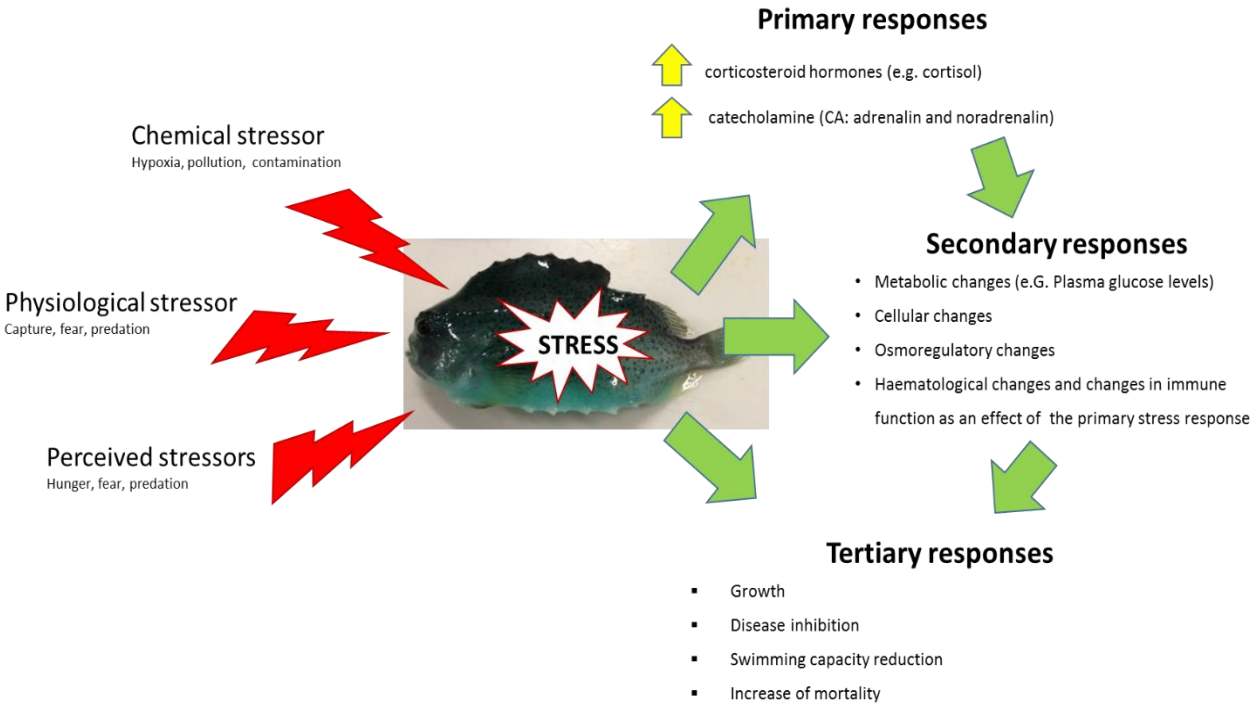

Figure 3. The concept of stress and its possible effect at fish (Iversen, 2013)

Even though, fish lives in a stable environment, they still can be affected by stressors from environment, as chemical stressors (hypoxia, contamination and pollutant exposure), physiological stressors (capture, handling, transport, crowding) and perceived stressors (hunger, fear, predation pressure) (Iversen & Eliassen, 2012a). For the integrated stress response in fish, one can distinguish reaction on few different levels: primary, secondary and tertiary response (Mazeaud *et al.*, 1977; Wendelaar Bonga, 1997; Wendelaar Bonga, 2011). Primary responses happen when the brain centres are activated, resulting in stimulation of hypothalamic-pituratory-interrenal (HPI) axis (Iversen, 2013; Mommsen *et al.*, 1999) and with that, rising levels of corticosteroid hormones (e.g. cortisol, (Balm, 1999)) and catecholamine (CA: adrenalin and noradrenalin) levels (Iversen & Eliassen, 2012a). Secondary responses are characterised by metabolic changes (e.g. plasma glucose levels, cellular changes,

osmoregulatory changes, haematological changes and changes in immune function as an effect of the primary stress response (Wendelaar Bonga, 1997). If changes affect the organism and population level in form of growth, reduced disease resistance, swimming capacity reduction and increase of mortality, one often means a tertiary response (Wendelaar Bonga, 1997). In this study, the focus is on the primary response line in form of corticotropic axis and its influence at secondary and tertiary responses.

1.5 HPI Axis in teleost

Hypothalamic – Pituitary – Interrenal axis (HPI) is one of the tree different endocrine axis, including HPG axis (Hypothalamic–pituitary–gonadal axis) and HPT (Hypothalamic–pituitary–thyroid) which are playing an important role in teleost reactions during stress, and it is characterised by different control layers.

Hypothalamus links the nervous system to the endocrine system via the pituitary gland (hypophysis, (Bernier *et al.*, 2009b). When a fish responds to a stressor, neuronal signals (visual, auditory and sensory) activates the hypothalamus to produce corticosteroid-releasing factor (CRF) in the hypothalamic preoptic area (POA) and induce an activation of sympathetic fibres in the anterior pituitary (Madaro *et al.*, 2015). CRF is 41-residue peptide, similar in the structure to urotesin is found in the urophysis of teleost fish (Hadley, 1992). CRF, via its receptor CRF-R1, induces the synthesis of pro-opiomelanocortin (POMC), which is processed into opioid β-endorphin (β-END) and peptides adrenocorticotropic hormone (ACTH; (Bernier *et al.*, 2009b; Huising *et al.*, 2004; Zhang *et al.*, 2015). Melanin-concentrating hormone (MCH) and dopamine (DA), which both are excreted from hypothalamus, can also have negative influence on the ACTH release (Bird *et al.*, 1990). ACTH is the smallest peptide hormone in the anterior pituitary and consists of a single linear chain of 39 amino acids. It has a significant role in cortisol production, but also in adaptation to hypoosmotic environments (Iversen *et al.*, 2013). ACTH is released into the general circulation and binds to the melanocortin 2 receptor (MC2R), which is expressed completely on cortisol-producing cells on the teroidogenic interrenal cells of the head kidney (Madaro *et al.*, 2015; Wendelaar Bonga, 1997; Zhang *et al.*, 2015). MC2R activation effects the initiation of steroidogenic acute regulatory protein (STAR), which is in control of the transport of cholesterol into the mitochondrial membrane, where it will be transformed to corticosteroids including cortisol (Aluru & Vijayan, 2008; Iversen, 2013; Madaro *et al.*, 2015; Zhang *et al.*, 2015). Cortisol, which is released to general circulation, acts as glucocorticoid and mineralocorticoid in teleosts as these animals do not produce aldosterone

synthase (Wendelaar Bonga, 1997). It is the receptor specificity that effects actions of cortisol: the mineralocorticoid (MR) and glucocorticoid receptor (GR) profile of the target cell. MR and GR are transcription factors, mediating activation or inhibition of target gene expression (Madaro *et al.*, 2015). Cortisol, which work as glucocorticoid, will stimulate protein catabolism in muscles and convert them to glucose in the liver (Iversen, 2013). Cortisol will also prevent glucose uptake by other cells, which will effect in raised glucose levels in the blood, so that organism can utilize it during later functional reaction on the stressor. Failure to suppress activation of the HPI axis during stress will also increase a lactate levels in plasma (Small, 2004). Induced elevations in plasma cortisol are known to suppress immunological capacity in fish (Maule *et al.*, 1989; Pickering & Pottinger, 1989) and suggest glycogen mobilization and breakdown (Small, 2004).

Circulating cortisol levels are also tightly regulated by a negative response loop, including glucocorticoid receptor (GR) preventing the release of trophic hormones (CRF) in response to elevated steroid levels (Flik *et al.*, 2006; Mommsen *et al.*, 1999; Wendelaar Bonga, 1997), and in that way reducing ACTH secretion (Figure 4). Another way to control HPI axis is through CRF binding protein (CRF-BP). CRF-BP modulates the effect of CRF and CRF-related peptides by binding these peptides and reducing their bioavailability (Geven *et al.*, 2006; Huising *et al.*, 2004) which results in decreased release of ACTH (Madaro *et al.*, 2015). There is also a smaller negative response loop, in which, cortisol suppresses ACTH secretion in adenohypophysis (Iversen *et al.*, 2013).

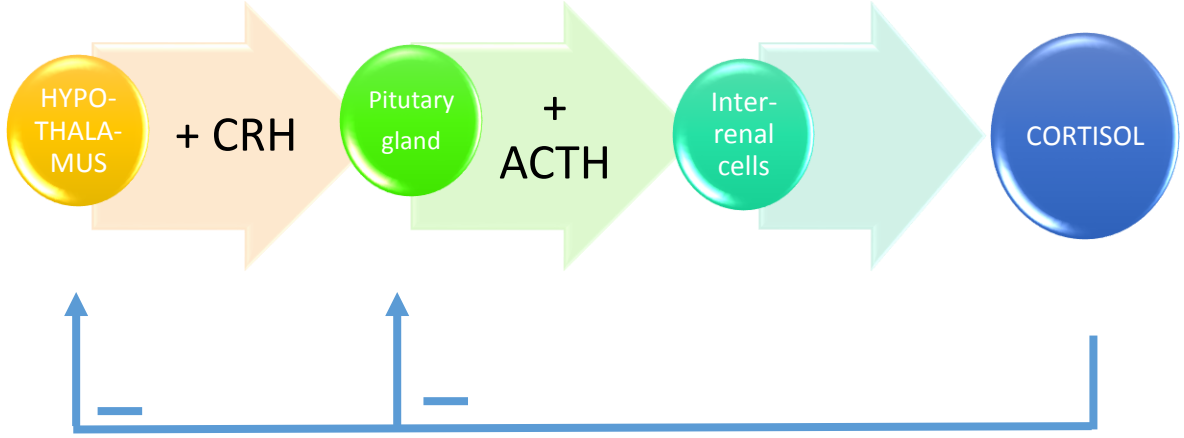

Figure 4. Simplified illustration of the HPI axis in teleosts (after Bernier *et al.* (2009a))

The stress response was created during evolution to help organisms face the dangerous challenges, and to be able escape and survive adverse challenges from their environment. In the short term, putting functions not connected to fight-escape response on hold, have a positive influence. Nevertheless, long term influence of stress on immune system, growth and reproduction cause imbalance (Barton, 2002).

1.6 Main objectives

Lumpsucker **(***Cyclopterus lumpus*) has recently been introduced to the Norwegian aquaculture as a potential efficient delouser of Atlantic salmon. However, early reports from the aquaculture industry indicate that lumpsucker suffers from higher mortality rate during the early production phase and seems to tolerate long-term stress poorly (Pers. Communication, (Hansen)). However as far as one knows no scientific study has been done on the allostatic load on hypothalamic–pituitary–Interrenal (HPI) Axis in lumpsucker subjected to a long-term crowding stressor.

The purpose of this study was to compare the response of the hypothalamic–pituitary–Interrenal (HPI) Axis subjected to a long-term crowding stressor in lumpsucker (*Cyclopterus lumpus*). The following three hypotheses was proposed to be tested:

1. There are no differences in adrenocorticotropic hormone (ACTH) sensitivity in lumpsucker subjected to long-term crowding stressor or not.
2. There are no differences in the negative feedback system of the HPI axis in lumpsucker subjected to long-term crowding stressor or not.
3. There are no differences in the primary (plasma cortisol), secondary (osmolality, chloride and magnesium) and tertiary (growth disturbance) stress responses in lumpsucker subjected to long-term crowding stressor or not.

2 MATERIAL AND METHOD

2.1 Fish stock

Lumpfish roe and milt was collected from Arctic Cleaner Fish AS (Stamsund) from a wild, locally caught brood fish, and delivered to Mørkvedbukta research station (Bodø). It arrived as newly hatched fry before the start of feeding in the end of May 2014. Start feeding was based on Gemma Micro feed delivered by a commercial producer Skretting (Norway) 50-400 µm dry feed pellets. Gemma Micro is a unique larvae feed and has proven to be successful in use from day 10 to 15 in large-scale production. After start, feeding fish were fed Amber Neptun ST (Skretting AS), and pellet size was increased to 1 mm and continued to increase it up to 4mm depending on the size of lumpfish. Until start of the experiment there, were not recorded any diseases, and fish were not vaccinated. The feeding was conducted under 24h light after the first 60 days.

The experimental tanks set up consisted of two quadratic $1m^3$ gray fiber-glass units, each with a rearing volume of 500 L. Automatic feeders (Arvo-Tec T Drum 2000 feeder, Arvo-Tec Oy, Finland) were installed on each tank, and a computer program estimated the daily feeding rates.

The water quality in the tanks was manipulated by controling parameters such as water temperature, oxygen levels and salinity to ensure consistent conditions. Filtered seawater taken from 250m depth was used in the experiment, and the water was also treated with different filters and UV, to provide the best water quality. Water temperature during the experiment was $8 \pm 0.4^{o}C$ and likewise the oxygen level was kept at 85 ± 3 % O_2 saturation. Salinity was stable at 34 ppt throughout the experiment. The experiment was done under continuous feeding and light conditions.

The lumpfish were acclimated, and rested period last one-month prior the start of experiment.

2.2 Experimental design

Experiment took place at Mørkvedbukta research station (Bodø) between 19.01.2015 and 16.02.2015. Approximately 300 lumpsuckers were divided in two tanks (described in chap. 2.1). The experiment consisted of two groups (150 fish per tank). Group 1: Daily stress for 1 month (lowering water to 0m, and reattach plug) and Group 2: Control (no stress). Daily

routines were enabled in both of the tanks. It included flushing excess feed, faeces, and monitoring of fish behaviour.

2.2.1 *Group 1. "Stress"*

Group 1 was exposed to a crowding every day (between 08:00-14:00 every day) for a month, as the stressor. This was done by lowering of the water level so that dorsal fins were drained. The water level was controlled physically with "Munk" (opening of drains) and was kept at an average density of 265 kg/m^3. Average draining time was 6.45 minutes, and normal water level was restored after about 14-15 minutes. Total duration of the stressor was approximately 21 minutes.

2.2.2 *Group 2: "Control"*

Group 2 was not exposed to any stressor during the period of experiment.

2.3 **Blood samples**

To document changes in resting levels of plasma cortisol, lactate, glucose, osmolality, chloride and magnesium during long-term stress responses, blood samples was obtained prior to start of the experiment (pre-stress), and after 7, 14, 21, 28 days of stress (n=6). The blood sample was taken Monday morning every week at 8 am to ensure that the fish had at least 18 hours' rest after last applied stressor. The fish was rapidly transferred to a bucket containing a metomidate solution of 5 mg/L. This concentration has shown to be sufficient in inducing rapid anaesthesia and preventing an increase in blood plasma cortisol (Iversen *et al.*, 2003; Olsen *et al.*, 1995). After being killed with a blow to the head, blood from six fish (per group) at each sampling time was obtained from the caudal vein complex using size 0.50-x16-mm heparinised syringes and lactate and glucose was measured just after taking blood samples. The blood was then centrifuged at 5000 rpm for 5 min and plasma was removed and stored in cryo tubes at -36 °C until analyses were performed.

2.4 **Analytical procedures.**

All samples were analysed by the laboratory of the Univeristy of Nordland research station in Mørkvedbukta, Bodø. The weight and length of lumpfish was measured at day 0 (pre-stress), 7, 14, 21 and 28 days after the start of experiment (n=162).

2.4.1 *Plasma cortisol*

Radioimmunoassay (RIA) method was used to measure plasma cortisol concentrations as described in Iversen et al. (1998). As a tracer, from ^{3}H Energy Technology (Kjeller) was used. Standard ranged from 0.0 to 137.5 nmol/L (nM) were made of hydrocortisone (H 4001, Sigma). The antibody was received from Endocrine Science, Tarzana USA. The samples were centrifuged and incubated at 4-5° C for 24 hours. Antibody-antigen complex was counted in a scintillation counter type Packard Tri Carb 1900 TR. Previous tests at our laboratory gave the following assay specifications: sensitivity of 1,68 nmol/L (nM, samples with hormone levels below detection limit were assigned the value of assay sensitivity) nonspecific binding (NSB) of 2.1–3.7% of total activity; intra-assay coefficient of variation less than 7.0% and inter-assay coefficients of variation of 5.1% at 50 nmol $^{-1}$. Measurements of 4, 17, 34 and 69 nmol $^{-1}$ radiolabelled cortisol added to plasma showed a recovery of 90%, 94%, 96% and 95%, respectively.

2.4.2 *Lactate and glucose*

Lactate and glucose were tested from the whole blood immediately after taking the sample using Lactate Pro^{TM} (Arkray KDK, Kyoto, Japan) and Freestyle Freedom Lite (Abbott Diabetes Care Ltd., Oxon, UK), respectively. Levels of lactate and glucose below detection limit were assigned a value corresponding to the sensor's minimal sensitivity, which was respectively 0.8 mM (lactate) and 1.1 mM (glucose). Use of transportable instruments, for measuring of glucose and lactate, was validated in previous research and proven that they give equivalent results to established laboratory techniques (Wells & Pankhurst, 1999).

2.4.3 *Osmolality and chloride levels.*

Plasma was also analysed for osmolality, and chloride levels using a Wescor 5500 osmometer (Wescor Inc. USA) and a Sherwood Chloride Analyser 926 (Sherwood Scientific Inc. USA), respectively. Magnesium (Mg^{2+}) was analysed by a fluidtest® Mg-XB (Biocon®, Germany) adapted for plate count reader.

2.4.4 *Stimulation and suppression test of HPI-axis*

Design of stimulation (ACTH) and dexamethasone (DEX) suppression test was based on previous study conducted by Pottinger&Carrick (2001a), with some minor modifications. Sampling and testing was conducted at pre-stress day (0) and after 7, 14, 21, 28 days of

experiment. Each of the sampling days 12 fish per group was used and from which six lumpfish was injected with ACTH and six with DEX solution. At pre-stress, 7, 14, 21, 28 days after start of the experiment 12 fish per group were netted from their separate tanks, anaesthetised (as described above), and injected into the peritoneal cavity with 1 mg kg^{-1} dexamethasone (Sigma-Aldrich) in ethanol: phosphate-buffered saline (PBS; 1:3; 1µg μL^{-1}). Afterwards they were moved to two tanks (0.5 m^3). After 24h the lumpfish were netted, anaesthetised, and six fish from each group were given either an intraperitoneal injection of 0.5 mL kg^{-1} adrenocorticotropic hormone (ACTH, fragment 1-24; Sigma-Aldrich in PBS at 45 µg mL^{-1}) or 0.5 mL kg^{-1} PBS, to measure the function of the negative feedback system. The ACTH and PBS groups were kept separate in four different holding tanks. Two hours after the ACTH/PBS injection the fish were netted, terminated and taken blood samples. Lactate and glucose was measured just after taking blood samples. After that, the blood was centrifuged at 5000 rpm for 5 min and plasma was removed and stored in cryo-tubes at -36°C until plasma cortisol analyses can be performed.

2.4.5 *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. In addition, an average SGR was calculated for the completely experimental period (28 days) for all experimental groups.

2.5 **Statistical analysis**

Program SPSS for Windows (ver. 18.00) was used to complete statistical analyses. Kolmogorov–Smirnov test for normality and Levene's test for homogeneity was performed on all the data. Afterward, change in each parameter in time in comparison to pre-stress situation was testes with one-way ANOVA test as well as differences in the same time between control group and stress group (Sokal & Rohlf, 1987). Depending on the result was another tested performed: if the F-values were significant, Bonferroni post hoc test was done to verify if there were differences between groups and between times of experiment. Significant differences were established at level 0.05. Results are presented as mean ± standard deviation (SD). Sign # on

the graphs shows significant change between control and stress group at the same sampling time. Sign * at the figures indicates significant difference at a given sampling compared to pre-stress levels within same experimental group.

3 RESULTS

3.1 Primary response

Figure 5 show the changes in resting levels of plasma cortisol during daily stress and no stress (control) group. The average resting levels of plasma cortisol increased during the experiment in the daily stress group, and become significantly elevated both from pre-stress levels and from control group at the same sampling time at 21 (25.3 ± 10.1 nM) and 28 (22.5 ± 14.1 nM) days after start of the experiment.

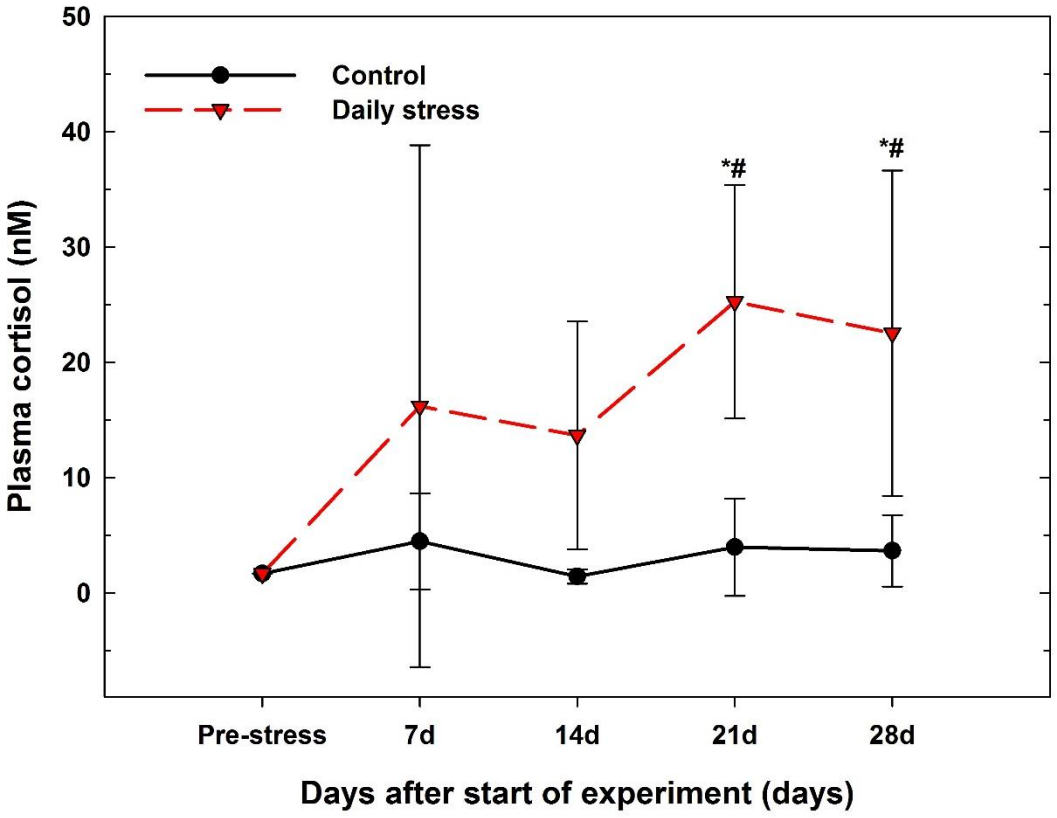

Figure 5. The average values of resting levels of plasma cortisol (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from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

3.2 Secondary response

3.2.1 *Glucose*

The initial mean glucose levels in pre-stressed group ranged between 0.7 to 2.1 mM with average 1.60 (± 0.59 mM) at both groups. The highest glucose levels were detected in the control group at day 14 and ranged 2.97 mM (± 1.78). There was not detected any significant differences between the sampling groups, neither at the same sampling time or compared to pre-stress levels (Figure 6).

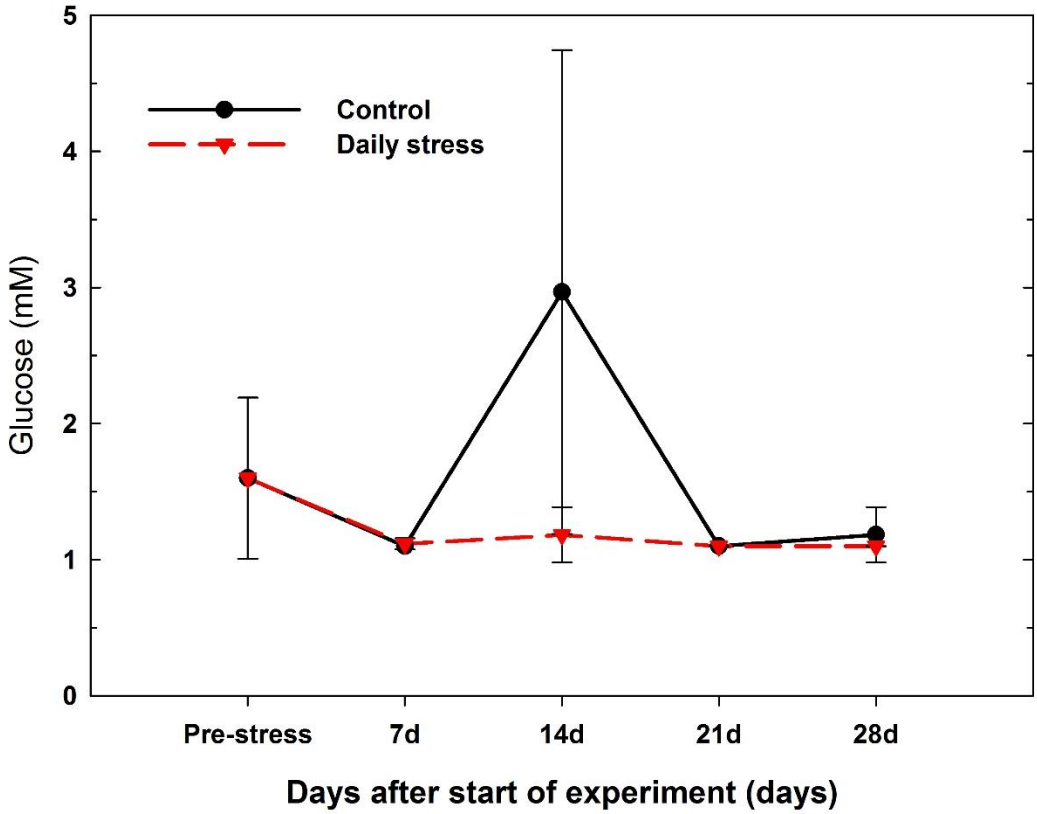

Figure 6. The average values of blood glucose (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12). **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress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

3.2.2 *Lactate*

Average concentration of lactate in blood of fish from control group was 0.77 mM (± 0.08) before the start of experiment. However, plasma lactate was not measured above detection limit of the Lactate Pro™ instrument in any experimental groups or sampling time.

3.2.3 *Osmolality*

Average values of the plasma osmolality pre-stress were 354.83 mOsm/L (± 23.79). There were no significant differences between experimental groups and pre-stress values at 7, 14 and 21 days after the start of the experiment. At 28th day of the experiment in the stress group, the plasma osmolality was measured to 391.67 mOsm/L (± 37.59). This was significantly higher than plasma osmolality levels from pre-stress and was significantly higher than the plasma osmolality level from control group at day 28 (Figure 7).

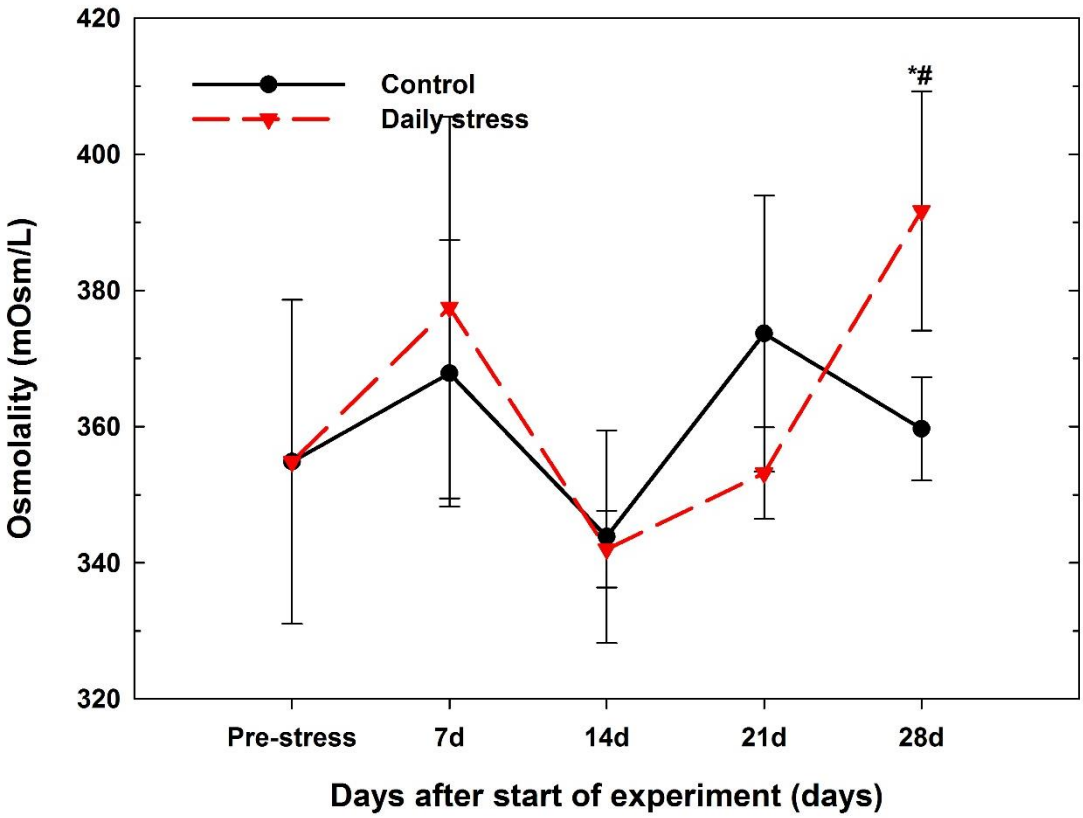

Figure 7. The average values of plasma osmolality (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12). **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress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

3.2.4 *Chloride*

Average concentration of plasma chloride at pre-stress levels were 145 mM (± 9.38). There were no significant differences between experimental groups and pre-stress values at 7, 14 and 21 days after the start of the experiment. At 28th day of experiment stress group had significantly higher plasma chloride values compared to control group and pre-stress levels (171. 33 ± 22.44 mM) (Figure 8).

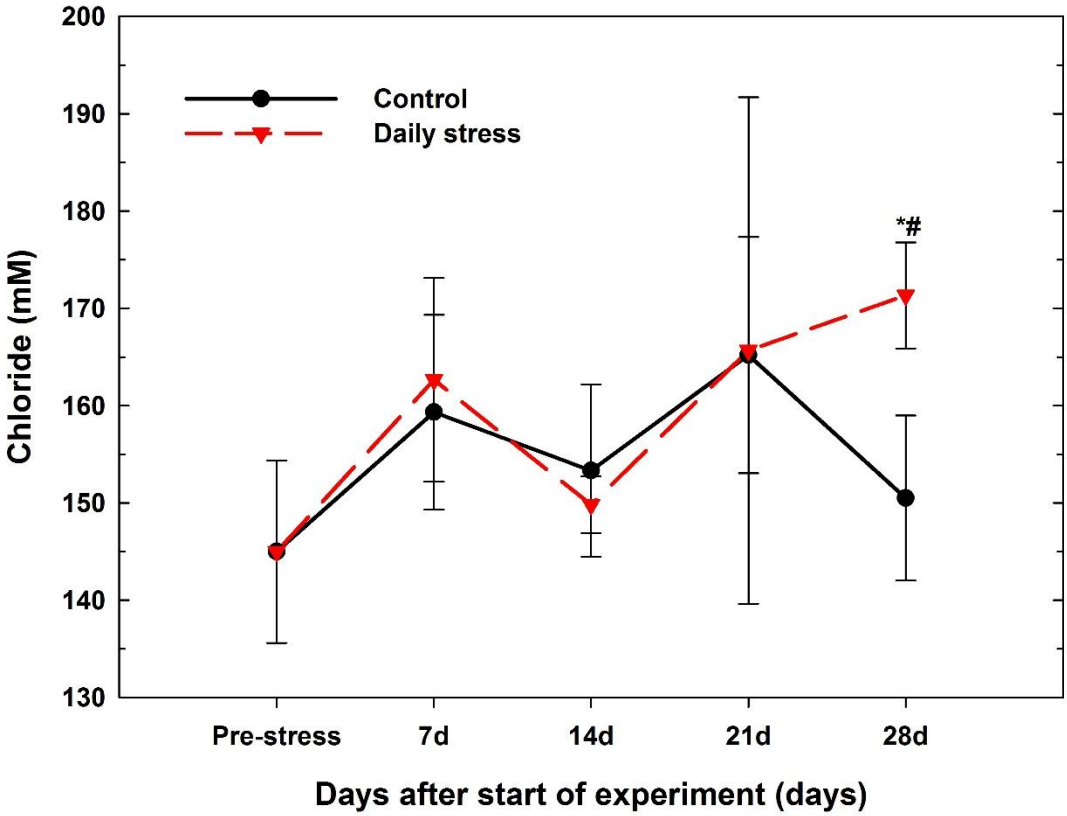

Figure 8. The average values of plasma chloride (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12). **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress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

3.2.5 *Magnesium*

There were no significant differences between experimental groups and pre-stress values at 7, 14 and 21 days after the start of the experiment. At 28th day of experiment stress group had significantly higher plasma magnesium values compared to control group and pre-stress levels (4.57 ± 1.62 mM) (Figure. 9)

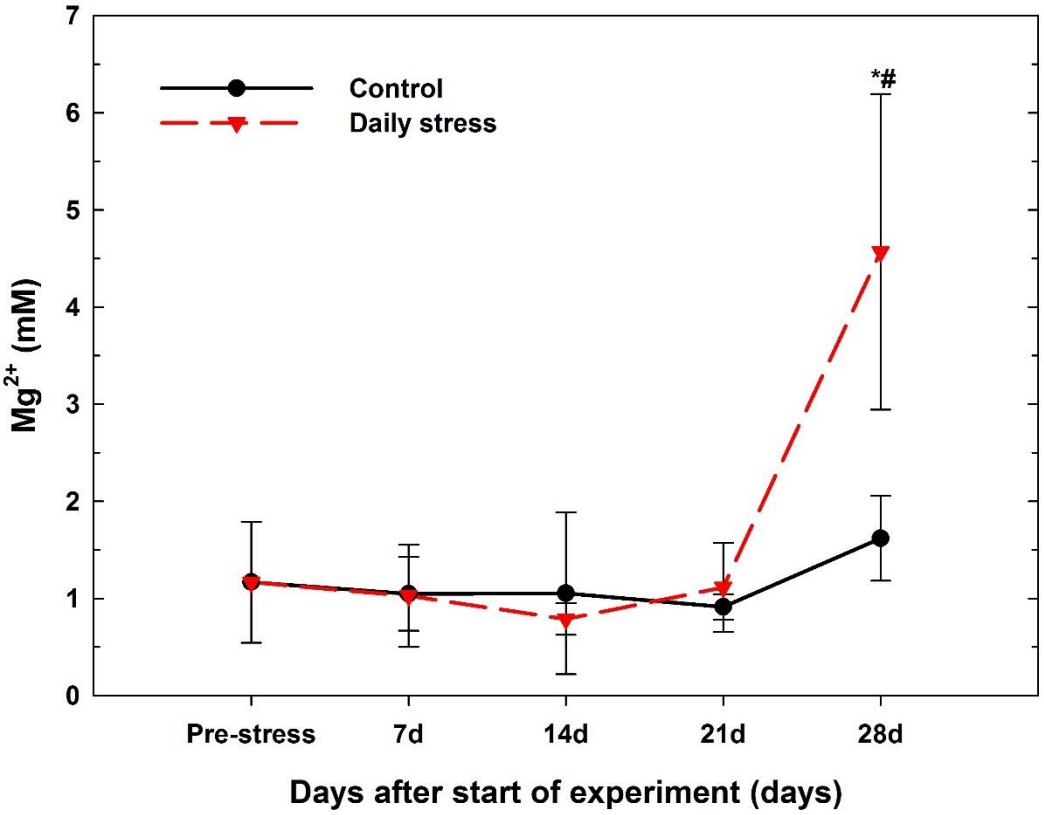

Figure 9. The average values of plasma magnesium (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12). **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress level within the same group at 95% confidence level.

3.3 Tertiary responses

3.3.1 Weight

The average weight of lumpfish from before the start of experiment was 52.7 g (SD ± 15.1). The average weight in the control group was almost even at day 7 and 14 and was 76.8 g (± 47.5) and 74.1 g (± 35.6), respectively. Average weight of fish from control group increased significantly compared to pre-stress and daily stress group at 21 and 28 days after the start of the experiment, and was 152.3 g (± 96.6) and 150.1 g (± 93.9), respectively (Figure 10).

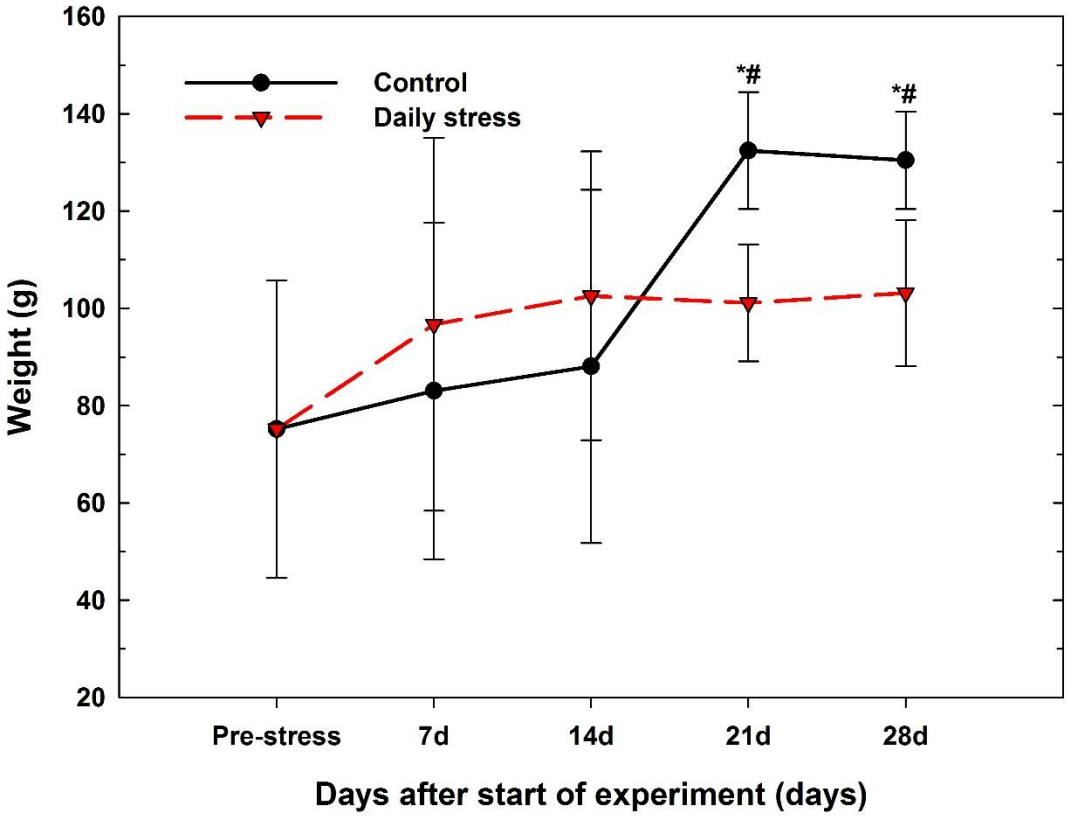

Figure 10. The average values of weight (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12). **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress level within the same group at 95% confidence level.

3.3.2 Specific growth rate

The general increase in specific growth rate was observed from 14th to 21st day of experiment (1.13% and 2.70 % respectively) and significant differences were found in comparison to the pre-stress measurement as well as in comparison to stress group. Similar significance was found also at 28th day of experiment (SGR 1.97%). Statistically significant decrease in specific growth rate was detected at stress group at all the days both in comparison to the pre-stress measurement and to control group at all the days – 7th, 14th, 21st and 28th (3.60%, 2.22%, 1.41% and 1.13% respectively).

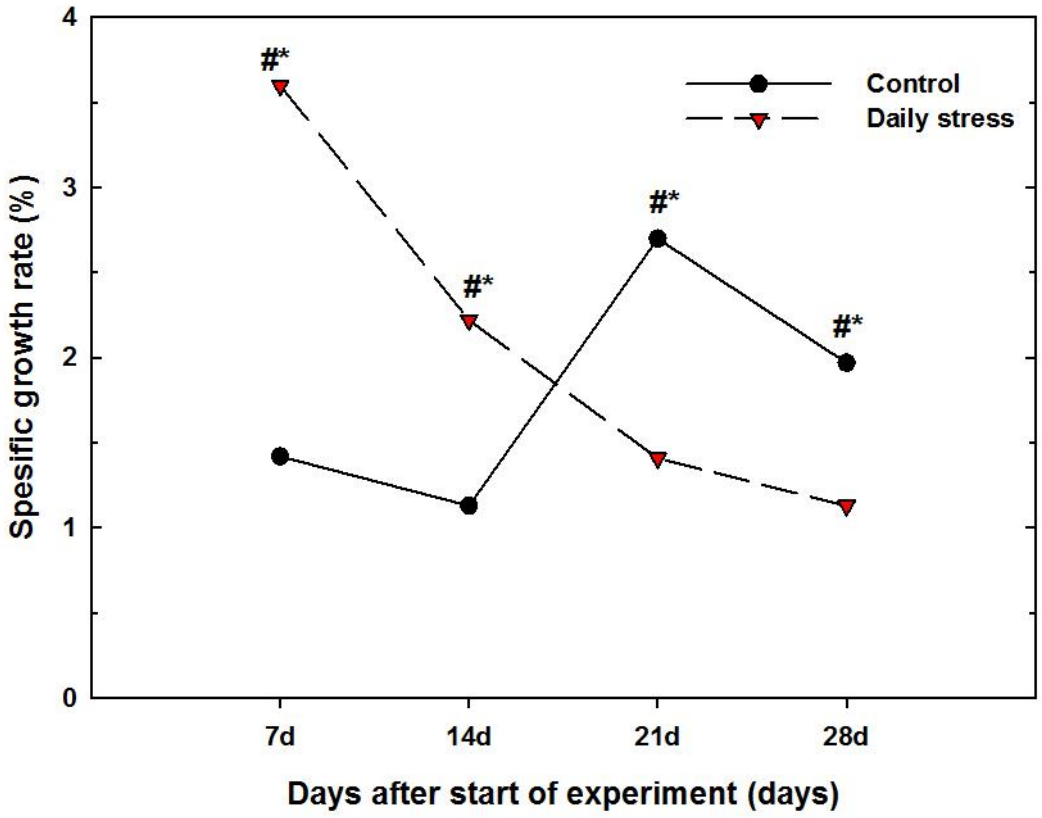

Figure 11. The average values of specific growth rate (%) in the control group and daily stress group at lumpsucker (n=12). # indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress level within the same group at 95% confidence level

3.3.3 Average specific growth rate

Figure 12 shows the difference in average specific growth rate between control group and stress group at the end of the experiment (day 28). The overall SGR was in control group (2.33% ± 0.01%) significantly higher at the end of the experiment compared to the stress group (1.27% ± 0.02%) (Figure 12).

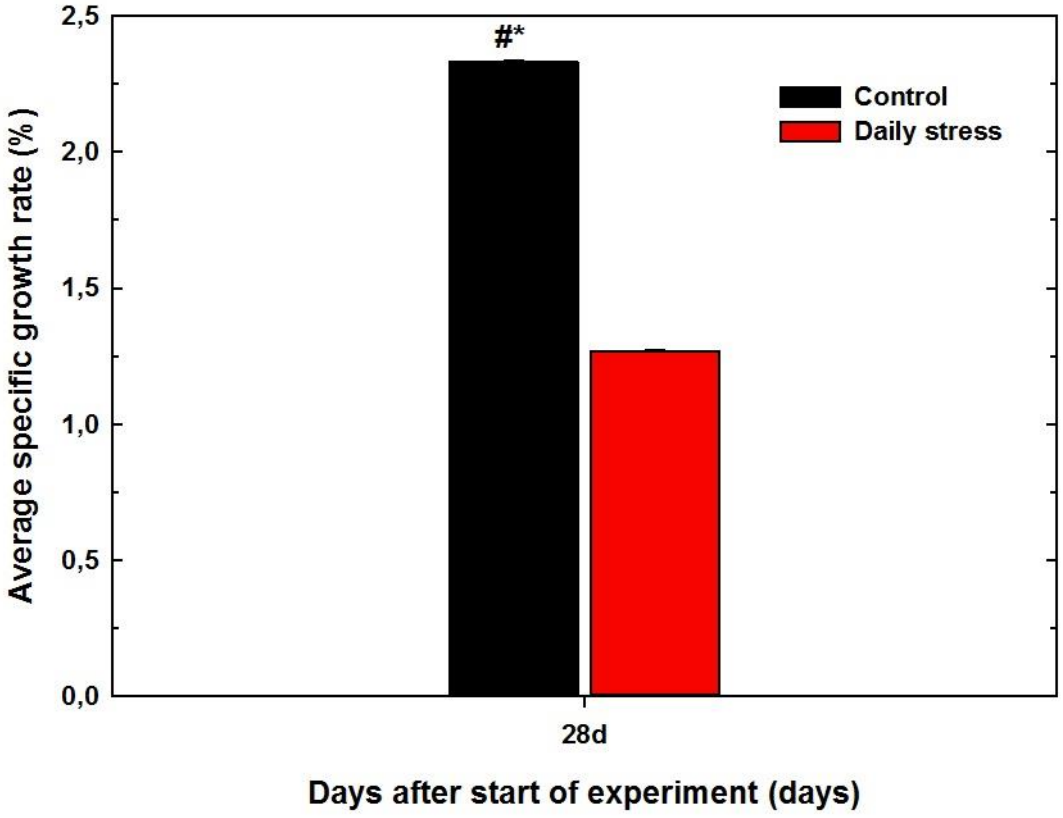

Figure 12. The average values of specific growth rate at 28th day of experiment (%) in the control group and daily stress group at lumpsucker (n=12). **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress level within the same group at 95% confidence level.

3.4 HPI-axis

3.4.1 ACTH sensitivity

Average plasma cortisol levels in fish injected with ACTH solution shows significant increase in comparison to pre-stress levels and control group after 28 days of experiment at stressed group of lumpsuckers (85.11 nM (± 16.05) (Figure 13).

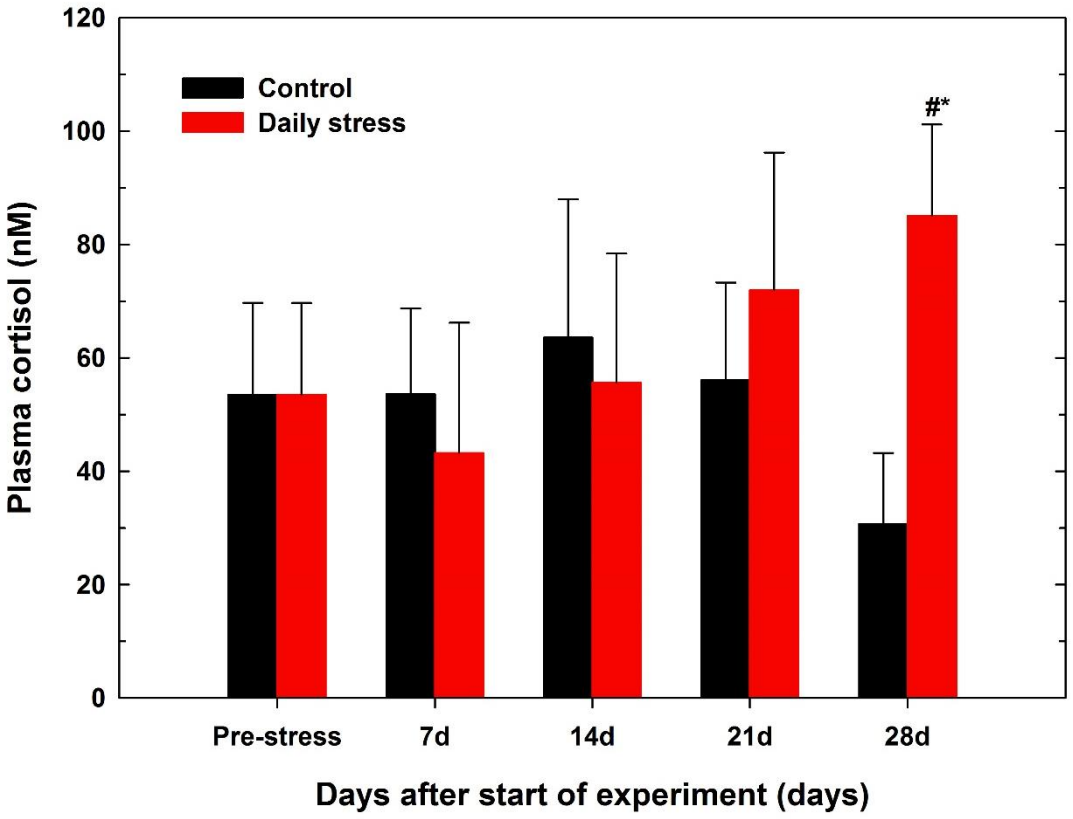

Figure 13. The average values of plasma cortisol (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12) under stymulasjon with ACTH test. **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress level within the same group at 95% confidence level

3.4.2 Negative feedback response

Figure 14 shows the average plasma cortisol levels in lumpsucker injected with PBS solution and a significant increase in the daily stressed group compared to pre-stress levels and control group after 21 and after 28 days (28.98 nM (± 8.6) and 35.27 (±8.95) respectively).

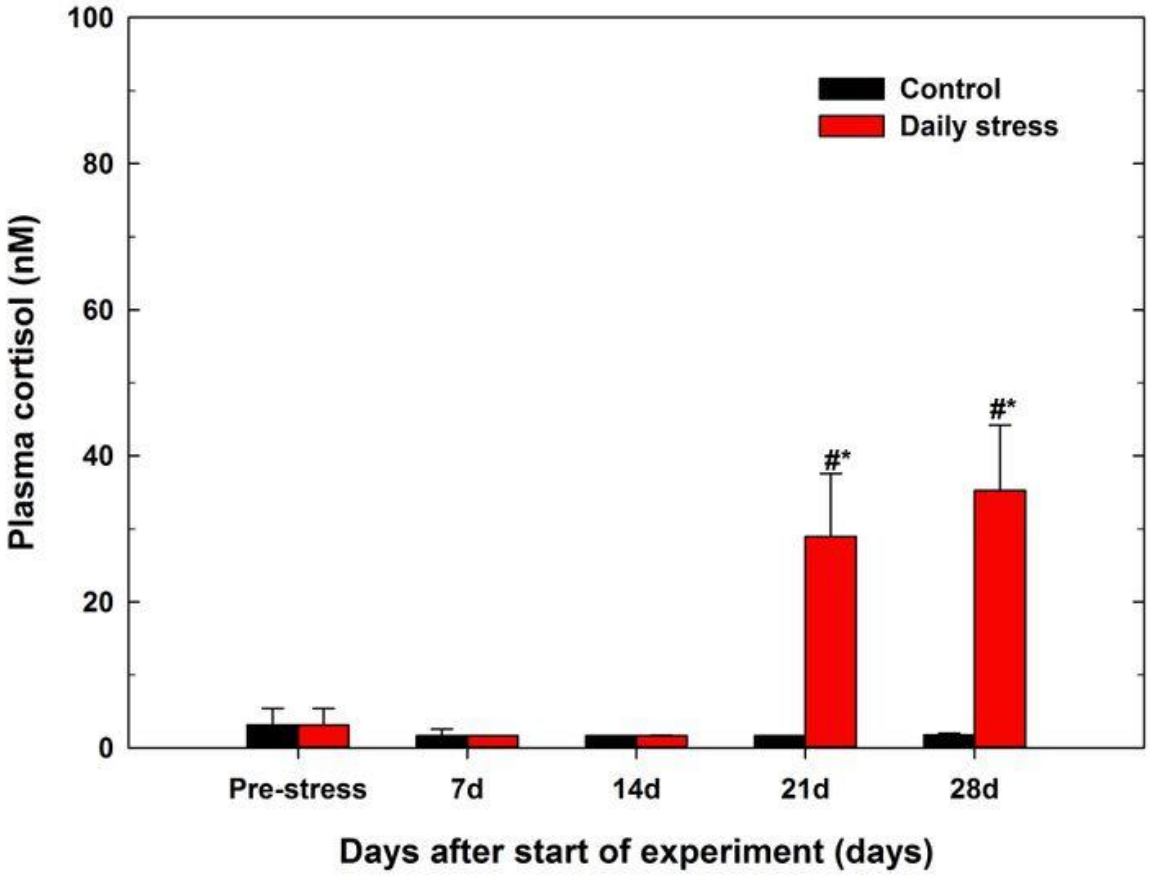

Figure 14. The average values of plasma cortisol (n ± SD) pre-stress (n=6), in the control group and daily stress group at lumpsucker (n=12) under negative feedback response test. **#** indicates significant difference between groups at the same sampling day at 95% confidence level, ***** indicates significant difference from the pre-stress level within the same group at 95% confidence level

4. DISCUSSION

Sea lice is a growing problem in world fish farming of salmonids. Sea lice, when attach to fish, it removes mucus and damage the skin, which then cause biological and enzymatic stress, and eventually could lead to increased suffering and mortality (Costello, 2009; Whelan, 2010). In the nature, where there is a separation of the age classes, and lower density in the wild population, it is also lower possibility of lice infection. Aquaculture of salmon have changed this situation and, salmon hold in captivity under higher density increase the risk of infection by sea lice (Costello, 2009; Liu & Bjelland, 2014; Price *et al.*, 2011). Production of fish farmed salmon and trout has increased from 360 000 tons in 1998 to 1.1 million tons in 2011 (Fiskeridirektoratet). Both the size of the production, the amount of farms of salmon and distance in between them can intensify problem of parasites, like lice (Kristoffersen *et al.*, 2014). The use of pesticides on farmed salmon increases for instants, in Scotland, 188 kg was used in 2008 in comparison to 395 kg used in 2011. To compare, in Norway, 13 000 kg was used only in 2015 (Fhf, 2016).

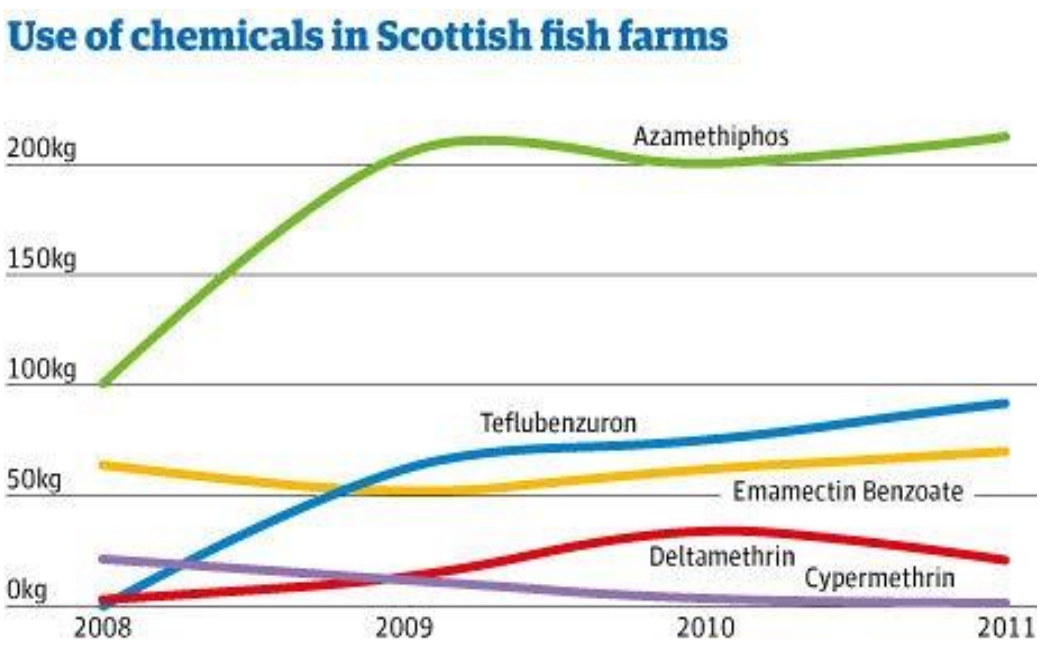

Figure 15. Different pesticides against lice in Scotland in years 2008-2011 (Carell, 2012)

Pressure from customers for production of food without use of chemical treatments is greater than before, additionally, restrictions from Norwegian Food Safety Authority about the amount of salmon lice per fish has become stricter in the later years. Taking these factors into consideration, the pressure on fish farm industry for finding new ways for delousing is high. Only in 2010 fish farming companies used almost one billion Norwegian kroners (NOK) for the delousing, and Research Council of Norway invested 40- mill NOK on the projects connected to sea lice problem (Rykhus, 2010). Additionally, in 2016, The Norwegian Seafood Research Fund allocated 80 mill NOK for projects connected to cleaner fish (Fhf, 2016). One of the non-chemical methods of fighting sea lice and preventing lice infection is to use of cleaner fish. An important species widely used in Norwegian salmon farming, especially in the north of Norway, is lumpsucker (Imsland *et al.*, 2014; Nytrø *et al.*, 2014). Lumpsucker is a robust fish with fast grow rate of 5-6 cm in 4-5 months (Chilvers, 2013), and it tolerates low temperatures (Nytrø *et al.*, 2014) with wide variety of feeding preferences. *C. lumpus* can be used in bigger scale and with bigger meddling percentage compared to other cleaner fish species (Chilvers, 2013).

4.3 Stress and primary response

The knowledge of stress in farmed fish is not a new subject. Already in 1950 (Selye) described stress as a situation where homeostasis of an organism (dynamic equilibrium) is disturbed as the effect of external or internal stressors (Selye, 1950, 1973). However, Selyes definition was considered unsatisfactory, and there for the theory of allostasis was presented to fulfil the understanding of the stress response (Iversen & Eliassen, 2014; Mcewen & Wingfield, 2003; Wingfield, 2005). Allostasis is defined as the ability to achieve stability through changes and can be described as a situation where the activity of the primary mediator is changed and continuous for a substantial time. Mediators can be cytokines, catecholamine and corticosteroids. However, allostasis can only be supported in limited amount of time, and when it last too long, it will cost the organism too much energy, and the animal will enter type of allostatic load, and finally if load continues into type of allostatic overload (Goymann & Wingfield, 2004; Schreck, 2010). Allostasis is a current issue not only for mammals but also for fish (Schreck, 2010), and that concept has been part of some stress studies on fish (Iversen & Eliassen, 2014; Leong *et al.*, 2009; Prunet *et al.*, 2012). Changes in response for the stressor of both physiological and behavioural nature way are called emergency life history stage (ELHS). Wingfield&Sapolsky (2003) discovered the important role of glucocorticosteroids in

the process of EHLS, especially cortisol (Backstrøm *et al.*, 2011; Wingfield, 2005). Increase in the cortisol levels are connected to primary stress response in fish (Barton, 2002; Wendelaar Bonga, 1997). One can talk about primary responses if brain centres are activated, resulting in stimulation of hypothalamic-pituratory-interrenal (HPI) axis (Iversen, 2013; Mommsen *et al.*, 1999) and with that, rising levels of corticosteroid hormones (e.g. cortisol, (Balm *et al.*, 1994)) and catecholamine (CA: adrenalin and noradrenalin) levels (Iversen & Eliassen, 2012b). Pre-stress levels of cortisol at lumpsucker from before the experiment were very low within the level that is generally considered representative for unstressed fish (Barton & Iwama, 1991). Similar resting levels of cortisol at lumpfish were found both by Iversen&Eliassen (2014) and Haatuft (2015). The group that was influenced by daily stress had higher levels of plasma cortisol compared to the control group already after seven days of the beginning of the experiment. The release of the stress hormone suggests that a general stress response was induced, and most likely entering a stage allostatic overload type 1 (Barton & Iwama, 1991; Hatløy, 2015; Iversen, 2013; Wendelaar Bonga, 1997). Resting levels of plasma cortisol in daily stressed lumpsucker continued to increase while experiment lasted and reached over 20 nM at 21st and 28th day. These increased levels of plasma cortisol do not seem to be high in comparison to experiment done on salmon or other cleaning fish - ballan wrasse. However, Iversen *et al.* (2015) showed lumpfish had a different stress response to a short-term stressor compared to several other species as ballan wrasse, cod (*Gadus morhua*), and Atlantic salmon. Actually, the lumpsucker stress reaction was very similar to the reaction of Atlantic halibut (*Hippoglossus hippoglossus*) (Iversen *et al.*, 2015). Halibut, as lumpfish, secretes moderate amounts of cortisol and tries to hide from danger. Elevated levels of plasma cortisol indicate allostatic load on HPI line and primary stress response. Apart from fact that stress response is natural reaction at fish and after short time fish should have possibility to regulate levels of stress hormones down to levels from pre-stress, long time stress can be maladaptive and malicious for fish (Pickering & Pottinger, 1989; Wendelaar Bonga, 2011). In this experiment, one can see that stressed group do not adapt and the resting levels cortisol remain elevated as time passes by (Figure 5).

4.4 Secondary stress response

Primary stress response is often followed by a secondary stress response. Secondary responses are characterised by metabolic changes as shown on the presented above (e.g. plasma glucose levels, cellular changes, osmoregulatory changes, haematological changes and changes in immune (Barton, 2002; Wendelaar Bonga, 1997).

Usually stress influences levels of glucose and lactate (Iversen, 2013), that is why glucose is commonly used indicator of secondary stress response (Cook *et al.*, 2012; Mommsen *et al.*, 1999). When the reaction for the stressor rises the catecholamine levels (mainly adrenaline) the process of glycolysis starts. The second step is process of gluconeogenesis initiated by already increased levels of cortisol. Cortisol intensifies the processes in liver and kidneys, and with that secretion of glucose into the blood stream (Aluru & Vijayan, 2007). The most common reaction on stress, the fight or flight response, is driven by glucose (Barton, 2002). The gills and brain are the two main organs involved in the stress reaction, and it is those organ that beneficent most on the increased glucose levels (Iversen, 2013; Moon, 2004). The several research conducted on salmonids and other species shows that stress response often brings increase in the glucose levels (Cook *et al.*, 2012; Iversen & Eliassen, 2009). Results of this experiment do not show any significant trend – glucose levels were very stable in time at the stressed group and unstable at control group. During the study the observation of sudden growth of glucose levels in control group at 14^{th} day of experiment (2.97 mM (± 1.78)) but the change was not statistically significant (Fig 6). Similar results in the lack of correlation between plasma cortisol and glucose levels are found in several other studies (Cook *et al.*, 2012; Mommsen *et al.*, 1999; Wendelaar Bonga, 1997). Van Heeswijk *et al.* (2006) mentioned that fasting of the animal before the experiment influence metabolic status of the body, and with that it can change glucose levels and stress response of that organism. Fasting have shown to effect ability to introduce a glucose response and increase hepatic sensitivity to adrenergic stimulation (Mommsen *et al.*, 1999). Salmonids are fasted for before transport or delousing to increase their possibility to cope with stress. It brings difficulties to connect plasma cortisol levels and plasma glucose levels without knowing if the stress situation causes acute or chronic overload. One has also to take into consideration that plasma glucose at carnivorous fish can fluctuate a lot more than at mammals, so it cannot be used as the only indicator of metabolic status or stress (Mommsen *et al.*, 1999)(Mommsen et al, 1999).

On the other hand, lack of clear change in glucose level could plausible be explained in the way lumpsuckers react to stress. One does not need high amounts of glucose in the blood when one tries to hide and get himself attached to the surface – especially taking into consideration the way sucking plate is build. The peak at day 14 could possibly be explained by biological characteristics and behaviour of the tested animals.

The elevation of the lactate concentrations immediately after stress is likely due to muscle glycolysis (Moon & Foster, 1995). It is produced from glucose in an anaerobe glycolysis (Olsen *et al.*, 1995). Average concentration of lactate in blood of fish from control group was 0.77 mM (± 0.08) before the start of experiment. However, plasma lactate was not measured above detection limit of the Lactate Pro™ instrument in any experimental groups or sampling time. This could mean that levels of lactate were undetectably low during the experiment, and of no physiological significance. Salmon, cod and ballan wrasse, all had elevated levels of lactate after crowding stress. Those fishes have active fight/escape reaction for stressors with high muscle activity. In contrast to the above mentioned species Atlantic halibut and lumpsucker seek shelter or hide during sever stress, and thus, do not seem to produce any muscle lactate (Iversen *et al.*, 2015).

The release of cortisol into the blood is a primary stress response in fish, and its role is regulation of energy metabolism and the hydromineral balance, oxygen intake and stability of immune system in fish (Bernier *et al.*, 2009a; Mommsen *et al.*, 1999). Release of cortisol has a direct influence on osmolality, chloride and magnesium, and one can use them as an indication of secondary stress response (Veiseth *et al.*, 2006). Cortisol have a major impact in changes in osmolality in salmonids under parr-smolt transformation and during adaptation to freshwater (Wendelaar Bonga, 1997). Different studies have shown correlation between plasma cortisol and uptake by chloride cells in freshwater and size of surface area of gill chloride cells (Mccormick *et al.*, 2008; Perry *et al.*, 1992). Unfortunately, most of the experiment that included measurement of osmolality was conducted on salmonids. In this experiment, changes in plasma osmolality in first seven and fourteen days showed the same pattern both in control and stress group (Figure. 7). The only significant difference in plasma osmolality levels from pre-stress was detected at day 28 (Figure. 7). There was no positive correlation between plasma cortisol and elevated plasma osmolality. One can notice similar trend in chloride levels (Figure 8), also here one observed a delayed effect of stress on plasma chloride similar to osmolality with a significant increase at the end of the experiment. Similar reaction to a stressor on the hydromineral balance has been discussed by Trischitta *et al.* (2005) and Iversen&Eliassen (2014). It is most likely a compensatory response on cell level. When fish cells are exposed to hypotonic environment the cells rapidly swell but will return to its original volume, by eliminating cellular osmolytes and hence water. This regulatory mechanism is termed regulatory volume decrease (RVD). Fish in seawater, however, will work against shrinking of cells (as an effect of elevated plasma osmolality).

To prevent dehydration and because of osmotic water loss, marine teleost must intake seawater. In the same time, they eliminate ions of magnesium and sulphate. As Redding&Schreck (1983) underlines, that uptake of magnesium (Mg^{2+}) and excretion happens in guts and kidneys. In the most tested species of teleost total magnesium concentration in plasma will not be higher than 2nM (Bijvelds *et al.*, 2001). Experiments on Atlantic cod (Staurnes *et al.*, 1994), Coho salmon (Redding & Schreck, 1983), Atlantic salmon (Iversen *et al.*, 2009; Iversen & Eliassen, 2014) and Gilt-head bream (Arends *et al.*, 1999) shows strong correlation between stress and increase in plasma magnesium concentration. Concentration of Mg^{2+} during our experiment was significantly higher after 4 weeks in stress group compared to control group and pre-stress levels (Figure. 9). With values at 4.57 ± 1.62 mM at the end of the experiment in the daily stressed group it, could indicate the start of an allostatic overload type 2 response with dire consequences for the fish, as reported by Iversen&Eliassen (2014). Results from short time experiment with lumpsucker under RENSVEL project experiment, indicates that *C. lumpus* could handle short time stress on magnesium, and osmolality without any problem (Iversen pers. comment). In contrast to salmon, magnesium concentration in the lumpfish plasma started to increase later (at 0h and 1h, respectively) but reached its maximum quicker in comparison to salmon (after 1 hour at lumpsucker and 2 hours at salmon and ballan wrasse) (Iversen *et al.*, 2015). This study shows that even though, lumpfish have big possibilities to cope with some secondary effects of stress in the short time perspective, each day stress and accumulation of negative effects stops the coping mechanism. This gives a reasonable explanation why one did not see any changes before the last measurement. In many cases a significantly elevated magnesium concentrations are followed by increased mortality, and as a result negative impact on the fish welfare (Iversen & Eliassen, 2009; Iversen *et al.*, 2009; Iversen & Eliassen, 2014).

4.5 Tertiary stress response

If changes affect the organism and population in form of growth, disease inhibition, and increase of mortality, one often denotes as a tertiary stress response (Wendelaar Bonga, 1997). To establish homeostasis in the body during and after a stress reaction the organism have to direct all energy to important task as locomotion, and respiration. As long as the danger exists other physiological tasks as growth or reproduction will not be prioritised (Wendelaar Bonga, 1997). The situation is even more critical during chronic stress, as in this experiment. Goede&Barton (1990) implied the reduced growth and condition factor could be used as

trustworthy indicators of fish state. It can give an indication if an organism lives under the "stressful" conditions. Growth rate (specific or average growth rate) reflects appetite and food intake, intestinal uptake, and metabolic rate, and these factors can all be influenced by stressors (Wendelaar Bonga, 1997). Elevated levels of cortisol are an immediate reaction to stress. There are most likely three possible ways, in which cortisol can influence growth (Sorensen *et al.*, 2011). Cortisol reduce directly growth of cells through binding to glucocorticoid receptors (Lee & Bols, 1989). Elevated cortisol levels can also increase the basal metabolic rate in fish, which means a significant reduction in amount of energy that can be used for growth. Additionally, cortisol can also change gut morphology and reduce uptake of nutrients from the food (Barton *et al.*, 1987). Peterson&Small (2004) showed that cortisol reduces concentration of growth hormone and insulin-like growth factors (IGFs) in the blood, which influence feed conversion rate but also food intake itself. In this study one could observe decreased appetite under the experiment, as there were more unconsumed pellets on the bottom of the stressed group tank than in the control group with kept at the same feeding rate (personal observation). Decreased food intake was also followed by decrease in weight and growth rate (Figure 10, 11 and 12). The average weight of lumpfish in the stressed group did not increase after 14 days and remained unchanged until the end of the experiment. The control group, on the other hand, showed rise in average weight, which was higher after 21 and 28 days of experiment. Similar effect of plasma cortisol on growth has been shown on gold fish (Bernier *et al.*, 2004), Atlantic cod (Bernier, 2006) and Atlantic salmon (Bernier *et al.*, 2004; Bernier & Peter, 2001; Mommsen *et al.*, 1999). Average specific growth rate (SGR) shows similar negative trend (Figure 11). In addition, (Bernier *et al.*, 2004) noticed that, the specific growth rate was lower in the stressed group both in comparison to the control group and to pre-stress situation. The average specific growth rate (Figure 12) was significantly higher in the control group compared to the stress group, which most likely confirms significant influence of stress for growth at lumpsuckers, but this subject still needs more research.

4.6 HPI axis

It has been shown that during chronical stress, changes in activity and sensitivity in HPI-axis can be recorded (Iversen & Eliassen, 2014). It can be marked as weight loss, vasculation of corticotrophical cells in the pituitary gland, blocking of the ACTH synthase, reduced feedback effect of GR agonists on the secretion of ACTH, increased size of the adrenal gland, and increased sensitivity to ACTH (Hatløy, 2015). Role of the hypothalamus and pituitary gland

in the control of corticosteroid secretion has been well established for many vertebrates. Corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) plays main role and cortisol, or corticosterone, are important as the end products of the brain-pituitary-adrenal axis. This general pattern discovered in vertebrates applies also to some teleost fish (Wendelaar Bonga, 1997).

There are numerous methods of detecting chronical stress (allostatic overload type 2). Stimulation tests (by CRH, CRH/vasopressin, ACTH) that measure the relative sensitivity of the pituitary, and inhibition test using dexamethasone (DEX) to prove the reduced possibilities of the negative feedback (Mormede *et al.*, 2007). The design of stimulation test was based on previous study conducted by Pottinger&Carrick (2001b) and was used in this experiment. After 28 days of experiment, the daily stressed group showed oversensitivity to the weight-adjusted dose of ACTH compared to the control group and to the status from pre-stress (Figure 13). Even though studies on ACTH sensitivity in fish are not very numerous, the one that were conducted supports theory about chronic stress development (Iversen & Eliassen, 2014). Studies on ACTH sensitivity on mammals showed increased plasma cortisol levels at objects under chronic stress (like raised in bad conditions, kept in restricted space) (Friend *et al.*, 1985). Some studies connect also predisposition for oversensitivity for ACTH to individual predispositions of each separate organism (Pottinger & Carrick, 2001b).

Inhibition test using dexamethasone (DEX) showed significant changed in negative feedback mechanism at the lumpsuckers stressed group in comparison to pre-stress and to control group. Historically, DEX test was used to track different reaction on stress at humans that where depressed (Kumar *et al.*, 1986). Depressed people reacted in the smaller amount to DEX injections, compared to healthy individual whom experienced a significant reduction in the cortisol's morning peak (Kumar *et al.*, 1986). Similarly, to depressed people, chronically stressed animals (with overload type 2), shows the same reaction do DEX injections, and similar regularities were found in chronical stressed Atlantic salmon (Iversen & Eliassen, 2014). The response to DEX in chronic stress situation (Figure 14) seems to be similar to the one discovered for salmon (Iversen & Eliassen, 2014) and for depressed humans (Kumar *et al.*, 1986). Already after three weeks of experiment, one could notice significant difference between stress group and control group and the difference increased with time. Similar results were shown in Iversen&Eliassen (2014) where salmon exposed for daily stress in 4 weeks become oversensitive concerning ACTH stimulation, and had a reduced negative feedback system, and as a consequence elevated resting levels of plasma cortisol. In accordance to the authors, this

group represented most likely an allostatic overload type 2 response with dire consequences for the health of the individual fish. One can conclude that overload type 2 can be connected to chronic stress with severe effect on HPI axis and welfare of the lumpfish. However, there is very few studies (Iversen & Eliassen, 2014) on chronic stress at fish, combining baseline levels of plasma cortisol, sensitivity of the interregnal cells (ACTH), and efficiency of the negative feedback by corticosteroids (DEX). Lack of similar test and unclear definitions of acute/chronic stress makes it difficult to draw precise conclusions, and generalize results for all the fish species. However, in lumpsucker (this study), Atlantic salmon (Iversen & Eliassen, 2014) and rainbow trout (Pottinger & Carrick, 2001b) an oversensitive HPI-axis with reduced negative feedback system and elevated baseline levels of plasma cortisol seems to indicate a chronic stressed fish (allostatic overload type 2) with high potential to compromise the animal welfare of the fish.

5. CONCLUSION

This study investigated the effect of a long-term crowding stressor on basal levels of plasma cortisol and hypothalamic–pituitary–interrenal (HPI) axis in lumpsucker (*Cyclopterus lumpus*). Lumpsucker is considered "new weapon" in the fight with increasing salmon lice problem in fish farming of salmonids. Unfortunately, early reports from the aquaculture industry shows increased mortality during the early production phase most likely due to low long-term stress tolerance.

The results of this survey indicates that *C. lumpus* exposed to daily stress shows allostatic overload type 2nd reaction with a temporary oversensitivity to ACTH, a reduced negative feedback system with elevated baseline levels of plasma cortisol as result. During the study, one could notice a primary stress response in the form of elevated levels of plasma cortisol. Those changes were followed by secondary stress response expressed as osmolality changes, and chloride ions concentration imbalance. Additionally, there was recorded high levels of plasma magnesium ($Mg^{2+)}$ at the end of the experiment which can result in magnesium poisoning. Magnesium poisoning is also a sign of critical imbalance in the body. These changes in the HPI-axis induced a distinct negative tertiary stress response such as reduced overall growth and appetite.

Daily stressed lumpsucker can quickly end up in an allostatic overload type 2 (chronic stress) with subsequent compromised animal welfare. There were significant differences in ACTH sensitivity as well as in negative feedback system of the HPI axis between lumpsuckers subjected to long-term crowding stress, and the unstressed control group.

All rearing and handling of lumpsucker should, therefore, be taken with care and special focus on possible prolonged stressful situations, which could jeopardize the production result and the fish welfare of the species. There is a clear need for further investigation, longer experiments and environmental trials.

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