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Dietary vs. light stimulated smoltification: effects on smolt development and appetite regulation in pre- and post- smolts

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Abbreviations

ACTH	Adrenocorticotrophic hormones
AgRP	Agouti related protein
ANOVA	Analysis of variance
ARC	Arcuate nucleus
BM	Body mass
CART	Cocaine- amphetamine- related transcript
CC	Mitochondria- rich cells, chloride cells
CCK	Cholesystokinin
cDNA	Complimentary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CRH	Corticotropin- releasing hormone
DNA	Deoxy-ribonucleid acid
FL	Fork length
FW	Freshwater
gDNA	Genomic DNA
GH	Growth hormone
GI- tract	Gastrointestinal tract
IGF- I	Insulin like growth factor I
K	Fulton`s condition factor
LepR	Leptin receptor
LL	Continuous light
mRNA	Messenger ribonucleid acid
MSH	Melanocyte- stimulating hormone
NKA	Na+/K+- ATPase
NKCC	Na+/K+/2Cl- co- transporter
NOK	Norwegian kroners
NPY	Neuropeptide Y
POMC	Pro- opiomelanocortin
PST	Parr- smolt transformation
PYY	Peptide YY
qPCR	Quantitiative polymerase chain reaction
SGR	Specific growth rate
SP	Short photoperiod
SW	Seawater
SWCT	Seawater challenge test
WGD	Whole genom duplication

Abstract

The transfer of smolt from freshwater (FW) to seawater (SW) is a critical stage in the farming of Atlantic salmon (*Salmo salar*) with mortalities of up to 15 % after transfer. One of the reasons for this loss is suboptimal smolt quality. It is therefore important to ensure that the fish are fully SW adapted before transfer to SW. Traditionally, smoltification is stimulated by exposing the fish to a light regime mimicking a natural summer- winter- spring daylength signal (light treatment). In recent years, it has become more and more common to keep the fish at continuous light throughout the FW phase and feeding them a specialized feed supplemented with a salt mixture and in some cases, also with a free tryptophan supplement, during the last weeks prior to SW transfer (dietary stimulation). However, it has not been shown whether the dietary approach triggers a true smoltification and stimulate all the mechanisms needed for optimal growth and welfare in SW. In the present study, we compared smolt development and SW growth performance between the traditional light treatment and a group that received a commercial smoltification feed containing an ion mix and free tryptophan. In addition, we investigated changes in appetite regulators in response to smoltification and treatment given. With regard to smoltification during the FW phase, we found that both treatment groups were changing skin color, but only the light treated group (those subjected to a short photoperiod (SP) 9L:15D) had a reduction in condition factor (*K*) indicating that a true smoltification took place only in this group. In contrast, the dietary treated group showed a higher gain in body mass and better SW tolerance throughout the FW phase. Both treatment groups had similar specific growth rate (SGR) during the SW phase, indicating that feed supplemented with a salt mixture and free tryptophan is a good alternative to the traditional light treatment. Some changes were seen in appetite regulators that could be a response to smoltification and treatment given. The light treated group had a significant higher brain expression of the putative orexigenic neuropeptide Y (NPY) at first sampling (first day on SP) that could indicate an acute response to SP. This group also had a significantly higher brain expression of the putative anorexigenic pro-opiomelanocortin (POMC) on the last sampling in the FW phase, which could be due to endocrine and metabolic changes taking place during a true smoltification. The intestine expression of the putative anorexigenic peptide YY (PYY) was significantly lower in the light treated group before they were transferred to SW and may be related to preparation for a life in SW. The dietary treated group had a significantly lower intestine expression of PYY after one week in SW, most likely indicating a response to SW transfer. However, the measurement of central

and peripheral appetite regulators were inconsistent with feed intake measurements and hence, did not provide a clear evidence for their putative anorexigenic or orexigenic roles. A marked increase in the stomach expression of the putative orexigenic ghrelin -1 and -2 one week after SW transfer may point towards a role of ghrelin in growth stimulation after SW transfer.

1 Introduction

1.1 Atlantic salmon (*Salmo salar*)

The Atlantic salmon (*Salmo salar*) belongs to the subfamily Salmoninae within the family Salmonidae. Salmonids are native to the Northern Hemisphere, but have been distributed to cool waters throughout the world. Although they are predominately freshwater fish, many of the species are anadromous, including the Atlantic salmon (Foote 2018). Anadromy is a migratory strategy where the fish are born and begin their life in freshwater (FW), before they undergo a downstream migration to the nutrient rich ocean to grow and mature, then returns as adults to their natal freshwater rivers to spawn (Cooke et al. 2011). Atlantic salmon usually spend one to six years in FW before they migrate to the ocean, depending on latitude. The migration is a critical part of the anadromous life history, where the fish encounter many natural challenges such as predators, parasites, diseases, dynamic river flows and ocean currents, changing temperatures and variable salinities. In addition anthropogenic challenges, fisheries exploitation, habitat alteration and physical barriers such as dams and other ecosystem alterations pose a threat to these fish (Cooke et al. 2011). The salmon juveniles undergo several adaptive changes when they transform from a stream- dwelling parr to a seaward migrating smolt, through a transformation termed parr- smolt transformation (PST) or smoltification (recently reviewed by McCormick 2013).

1.2 Smoltification

Smoltification includes a series of physiological, morphological and behavioural changes, which are preparatory for a successful life in the marine environment. The process has been a topic of intense research and has been described in several excellent reviews (Hoar 1988, Stefansson et al. 2008, McCormick 2013). Multiple interactions between the environment and the salmon leads to smoltification, the stimulation is under internal control by the neuroendocrine system, and the timing is controlled by the increase in daylength during spring (McCormick et al. 1998), so that seawater (SW) entry occurs when they are fully adapted (physiological smoltwindow) and conditions in the sea are optimal, i.e. the ecological smoltwindow (Boeuf 1993). A size/growth- threshold exists and the smoltification process is

initiated the following spring when the salmon has reached a critical size in late summer (>10 cm; Thorpe et al. 1998). Little is known about the processes taking place during winter, but the increasing daylength during spring leads to a stimulation of the light- brain- pituitary axis (Ebbesson et al. 2003), which in turn leads to the release of hormones that provoke PST.

1.2.1 Morphology

There are clear morphological differences between a parr and a smolt. Parr usually have strong vertical bands (parr marks, "finger marks") and spots along their lateral line (McCormick 2013). During smoltification the visibility of the parr marks are reduced and there is development of increased silvering, caused by the deposition of purines (guanine and hypoxanthine) in the scales and skin. The silvering contributes to counter- shading camouflage that are common in pelagic schooling fish species (Stefansson et al. 2008). Concurrent with the increased silvering the salmon develops darkened fin margins, especially on the pectoral, caudal and dorsal fins, caused by expansion of melanophores (McCormick 2013). Lipolytic growth hormone (GH) and cortisol (Sheridan 1986) together stimulate length growth relative to mass growth during PST, which results in a lower condition factor (*K*; weight to length ratio). Smolt development implies a high energetic demand, which includes an increase in basal metabolic rate- and increased locomotor activity. A slimmer body shape gives a better swimming ability and predator avoidance, pivotal for survival during migration and ocean entry (McCormick 2013).

1.2.2 Ion- and osmoregulation

Like other teleosts, the Atlantic salmon maintain a nearly constant internal osmotic and ionic concentration irrespective of their external environment. In FW the salmon is hyperosmotic towards the external medium and will lose ions and gain water by passive diffusion and osmosis respectively, and to counteract this, the fish actively take up ions and get rid of the excess water by producing a large quantity of diluted urine. In SW the salmon is hypoosmotic towards its surrounding medium and will therefore lose water and gain ions. A parr has only a limited ability to secrete salts if exposed to SW, but during smolt development the capacity for SW hypoosmoregulation increases dramatically. Hence, the developed ability to drink and absorb water and excrete excess ions is critical to the survival of ocean migrating smolts (McCormick 2013). The major osmoregulatory organs are the gills, kidney, intestine and skin

(Rankin and Jensen 1993), of which the gill are the most studied (McCormick 2013). In the gills, the ion transport is carried out by specialized cells termed ionocytes, mitochondria- rich cells or, more commonly, chloride cells (CCs). The number, size and shape of CCs increases during smoltification. The SW type of CCs have an extensive tubular system continuous with the basolateral membrane, giving a large surface area for transport proteins (Stefansson et al. 2008). Three major transport proteins in chloride cells are involved in ion secretion (Fig.1). Two of them are localized in the basolateral membrane; Na^+/K^+- ATPase (NKA) and $Na^+/ K^+/ 2Cl^-$ co- transporter (NKCC), while the third; cystic fibrosis transmembrane conductance regulator (CFTR), is located in the apical membrane. NKA provides low Na^+ concentrations in the CC by active secretion into the extracellular fluid. The NKCC uses the low Na^+ concentration inside the CC to transport Cl^- ions into the CC, which then leaves the CC on a "downhill" electrical gradient through CFTR. Na^+ leaves the CC through NKA and exits the gill through a paracellular pathway between the CC and the accessory cell (McCormick 2013).

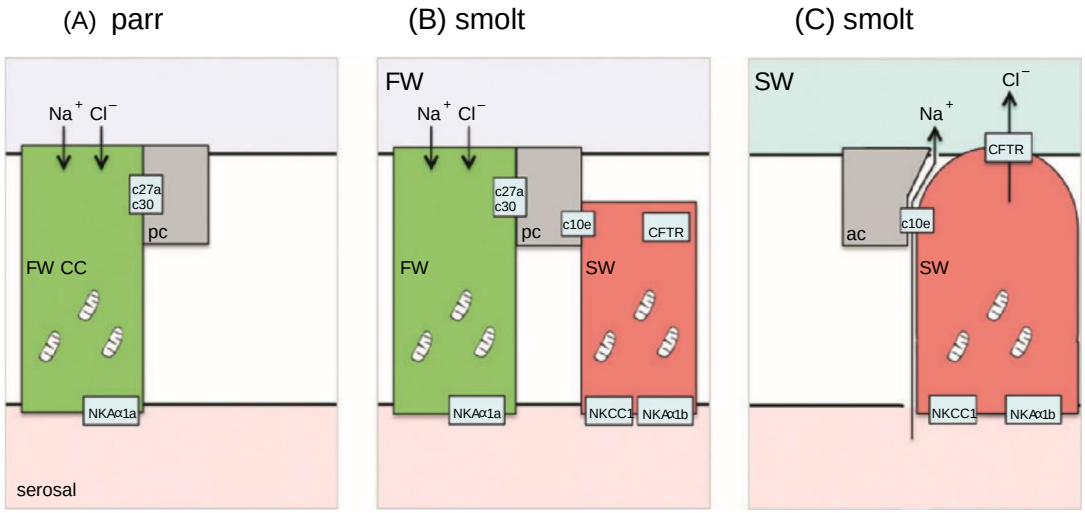

Figure 1: Osmo- regulatory processes in the gills of FW and SW teleosts. The gill NKAα1a (FW type) and NKAα1b ionocyte (SW type) isoforms changes during smoltification in FW and after SW exposure. The NKAα1a predominates in FW parr (A). During smoltification the number of NKAα1b ionocyte isoform increases (B). These cells stay beneath pavement cells (pc) and are most likely inactive at this stage. After SW exposure (C), these cells will increase in size and have transport contact with the external environment through CFTR and the opening between CC and an accessory cell (ac). There will be no NKAα1a cells. NKCC and CFTR are also upregulated during smoltification and continue to increase after SW exposure (McCormick 2013).

In contrast to in FW the Atlantic salmon in SW needs to drink SW to absorb the water lost to its surrounding medium through osmosis. To take up water, the gut first absorbs Na^+ and Cl^- to reduce the osmotic pressure in the intestinal fluid, which is conducted by the absorptive NKCC2 in the apical membrane and NKA in the basolateral membrane (McCormick 2013).

The osmotic pressure in the gut is further reduced by active alkalinisation to precipitate Mg^{2+}, Ca^{2+} and SO_4^{2-}, which is then excreted with the faeces. The fluid uptake in the posterior intestine increases during smolting and after SW exposure, and there is a shift in the intestinal water uptake from a paracellular to a transcellular pathway (Stefansson et al. 2008).

The kidney of FW teleost creates a highly dilute urine to excrete excess water, the glomerular filtration rate is high and tubular reabsorption is low. In SW the urine is sparse and more concentrated, there is a reduction in the number of functional glomeruli (Rankin and Jensen 1993).

1.2.3 Endocrine control

In Atlantic salmon increased photoperiod is the major factor stimulating the increase in GH (McCormick et al. 1995), and this hormone seems to be causally related to osmoregulatory indicators of smolt development, such as the increase in gill NKA activity and SW tolerance (Boeuf et al. 1994). GH stimulates secretion of Insulin like growth factor I (IGF-I) from the liver, leading to stimulation of linear growth and metabolism during PST (Björnsson et al. 2002).

A large number of studies have confirmed an increase in circulating cortisol levels during smolting, which remain low in Atlantic salmon parr of insufficient size for PST in spring, but increase up to 10- fold in those that have exceeded the size threshold when held under the same conditions (McCormick 2013). Cortisol treatment has been shown to stimulate gill NKA activity by increasing CC numbers (Rankin and Jensen 1993). Indeed, it seems that GH and cortisol works in synergy to stimulate NKA activity and SW tolerance in Atlantic salmon (McCormick 1996).

In addition to the hormones already mentioned, thyroid hormones have shown to be essential in many smoltification related processes, including silvering, olfactory imprinting, behaviour and changes in visual sensitivity (Stefansson et al. 2008). Conversely, prolactin promotes ion uptake, is inhibitory to salt secretion and hence is regarded as a FW hormone. The highest gill NKA activity occurs when prolactin levels are low (Rankin and Jensen 1993). Cortisol appears to inhibit prolactin synthesis and release and consequently, prolactin levels decrease with the concomitant surge in cortisol during PST (McCormick 2013).

1.3 Norwegian salmon farming and the control of smoltification

Norway is the dominating producer of Atlantic salmon in the world, from the beginning of the 1970`s up until today the industry has undergone a significant development with a multiplied production. The fish farming industry is a big and important industry for the Norwegian economy. In 2017, Norway produced 52 % of the world total production of Atlantic salmon. Salmon exports in 2019 was a total of 1.1 million tonnes, representing an export value of 72.5 billion Norwegian kroners (NOK). Smolt production has strongly increased over the years, a total of 349 million Atlantic salmon smolt were produced in Norway in 2018, accounting to a total value of 4.5 billion NOK (Fiskeridirektoratet 2018). Transfer of smolt from FW to SW is a critical stage in the farming of Atlantic salmon. On the average, 15 % of the salmon transferred to sea cages died after transfer the last years, and suboptimal smolt quality is considered an important reason for this loss (Hjeltnes et al. 2019). It is therefore important to ensure that the smolts produced are of good quality. Triggering and timing of smoltification in the salmon farming industry has traditionally been based on the natural summer- winter-spring daylength (photoperiod) treatment similar to that triggering smoltification in wild salmon (Saunders et al. 1985); a continuous light (LL) regime that is used in the juvenile rearing is interrupted by a period of short day (12 h of light or less per day) followed by continuous light (hereafter termed "light stimulation"). The establishment of this regime was based on numerous experimental studies in the 80/90ties which showed that the duration of natural change in daylength could be compressed due to high water temperature and continuous excess to feed in farms (e.g. Saunders et al. 1985). At the same time several other studies tested whether addition of salt (NaCl) or more complex salt mixtures in the feed and/or in the FW could be used to adapt salmonid fish to SW. It was found that the use of dietary salts increased gill NKA activity and improved SW tolerance in brook charr (*S fontinalis;* Pellertier and Besner 1992), rainbow trout (*O. mykiss;* Salman and Eddy 1987, Perry et al. 2006), chinook salmon (*O. tshawytscha*; Zaugg et al. 1983) and Atlantic salmon (Basulto 1976, Duston and Knox 1992, Duston 1993). Possibly motivated by these results it has become more common to prepare Atlantic salmon for SW by using specially formulated commercial feeds to induce smoltification characteristics without the use of light stimulation (hereafter termed "dietary stimulation"). However, to the best of my knowledge, there are no scientific reports on the quality of these dietary stimulated smolts in today's salmon farming.

1.4 Appetite regulation

In fish, as in mammals, feeding behaviour is regulated within feeding centers of the brain, that receive, process and respond to information from neuroendocrine signals both from brain and peripheral tissues (Volkoff 2016). These signals consist of hunger (orexigenic) hormones, or satiety (anorexigenic) hormones that stimulate and inhibit food intake, respectively. Neuropeptide Y (NPY) and agouti related protein (AgRP) are examples of central orexigenic neuropeptides, whereas pro- opiomelanocortin (POMC) and cocaine- amphetamine- related transcript (CART) are considered anorexigenic. There are also metabolic and neural peripheral signals, which provide information regarding nutritional status and meal ingestion (Volkoff 2016). In mammals, the arcuate nucleus (ARC) in the hypothalamus is the key area where short- term food intake (meal- to- meal) and long- term (energy homeostasis) are regulated by synthesis of anorexigenic/orexigenic neuropeptides. In the circulation, there are hormones that act acutely initiating (e.g. ghrelin) or terminating (e.g. cholecystokinin; CCK) a meal, and there are hormones that reflect body adiposity and energy status, such as leptin (Wynne et al. 2005).

Most fish species have indeterminate growth (continue to grow during their entire life span), in contrast to the determinate growth found in birds and mammals (Rønnestad et al. 2017). Fish are the most diversified group of vertebrates, and relatively few fish species have been examined with regards to the neuroendocrine regulation of feeding. There are many factors that are considered to affect feeding behavior in fish; the large number of fish species, huge variations in habitats and- feeding strategies, and differences in digestive tract anatomy and physiology (Volkoff 2016). Feeding behavior is also affected by environmental factors such as photoperiod and temperature that may change seasonally. Large variations in appetite exists between species and within a species (individual variation), and a species- specific approach is necessary when studying appetite (Valen et al. 2011a). However, the central regulators mentioned above appear to be present in most of the fish species investigated so far and their presence and putative roles in appetite regulation has recently been comprehensively reviewed by Volkoff (2016) and Rønnestad et al. (2017).

Several neuropeptides and peptides including; AgRP, NPY, POMC, CART, CCK, peptide YY (PYY), arginine vasotocin, corticotropin- releasing hormone (CRH) family (includes; CRH or corticotropin- releasing factor and urocortin 1, 2 and 3), leptin, nesfatin- 1, orexin and

ghrelin have been identified in Atlantic salmon and other salmonids (Volkoff 2016, Rønnestad et al. 2017). The effect of these actors on appetite regulation is, however, still somewhat unclear. Generally, present knowledge indicates that NPY has an orexigenic role, while CART and CCK seems to be anorexigenic in Atlantic salmon (Volkoff 2016). Less clear are the roles of POMC, AgRP, ghrelin and PYY. For the most newly discovered appetite regulating peptides in salmonids, very few studies have been conducted and their role is mostly unknown. Hence, the focus in this thesis will be on six appetite regulators; POMC, NPY, AgRP, CCK, PYY and ghrelin. This selection has been based on their key roles in mammalian appetite regulation and the fact that they all have been described in salmonid fish. All teleost fish share three rounds of whole genome duplication (WGD), 1R, 2R, Ts3R, whereas a fourth WGD occurred for certain teleost linages, including salmonids (Ss4R) (Lien et al. 2016). This make salmonids potentially a more complex model to study with increased gene copy numbers and multiple protein isoforms, which can have various physiological functions (Volkoff 2016).

AgRP is a strong orexigenic peptide released from the hypothalamus and influences food intake mainly by acting as an antagonist to central melanocortin receptors. Two isoforms of AgRP (AgRP1 and AgRP2) have been identified in Atlantic salmon (Murashita et al. 2009a). POMC is a precursor peptide that is post- transcriptionally processed into melanocortins, which include melanocyte- stimulating hormones (α, ß and γ- MSH, teleosts lack γ- MSH) and adrenocorticotrophic hormones (ACTH). These peptides interact with five G- protein-coupled melanocortin receptors MC1R- MC5R (Valen et al. 2011a). The melanocortin system has two endogenous antagonists; agouti and AgRP that modulate the activity of melanocortin peptides. In mammals, POMC and α- MSH are involved in appetite regulation and energy homeostasis. Leptin is a major signaling molecule that activate POMC neurons. As fat stores increase, leptin is secreted from adipose tissue and reaches the ARC via the circulatory system where it binds its receptors (LepR) located in the POMC neurons and activates the transcription of POMC and CART (Wynne et al. 2005). In salmonids three copies of *pomc* gene and one splice variant have been described (*pomc- a1,- a2,- a2s, and- b*) (Rønnestad et al. 2017). In mammals, CCK has been shown to induce satiety as a neurotransmitter in the brain, and CCK is also secreted from the proximal intestine and acts as a short- term satiety peptide and induces the release of digestive enzymes from the pancreas and the gallbladder (Volkoff 2016). Two different *cck* genes have been identified in Atlantic salmon and three distinct *cck* genes exists in rainbow trout (Rønnestad et al. 2017). Peptide YY (PYY) is a

member of the NPY family, but in contrast to NPY it act as an anorexigenic peptide in mammals. Two isoforms of the gene *pyy* have been identified in teleost species including Atlantic salmon, *pyya* and *pyyb*. *Pyy* is predominately expressed in the brain and gastrointestinal (GI)- tract (Rønnestad et al. 2017). Ghrelin is a well described orexigenic peptide in mammals, but its role in teleosts is inconclusive. It is mainly produced in the stomach of fish and mammals, except for some stomach- lacking species. In Atlantic salmon there are two isoforms, ghrelin -1 and -2, that are mainly expressed in the stomach, but also weakly in the pyloric caeca and adipose tissue (Murashita et al. 2009b). When conducting experiments, the role of ghrelin on food intake may also vary with route of administration, source of hormone, species, time of treatment and dose (Jönsson et al. 2007, 2010).

In summary, there is still a lack of knowledge on the neuroendocrine mechanisms that regulate appetite in fish. Such knowledge is strongly needed, not least to better understand feeding regulation in important aquaculture species such as the Atlantic salmon. In this species, a reduction in daylength has been shown to result in an abrupt reduction of appetite (Strand et al. 2018) and, as such, represents an interesting model to study appetite regulating mechanisms. Further, it is a common believe that smolts have a higher scope for growth than parr (McCormick et al. 1998), which may be accompanied with adaptive changes in appetite regulating mechanisms during smoltification. However, no reports on such adaptive changes can be found.

1.5 Aims of the study

-Compare smolt development and SW performance in light- vs. dietary stimulated Atlantic salmon.

-Reveal possible adaptive changes in appetite regulating mechanisms during smoltification and if these also are evoked with a dietary stimulated smolting.

-Investigate if the reduction in appetite upon SP exposure is related to a downregulation of orexigenic peptides and/or upregulation of anorexigenic appetite regulators.

-Compare responses in appetite regulators in the early (stressful) and later (confident) phase of SW residence between light- and dietary stimulated smolts.

2 Material and methods

2.1 Experimental set- up

The experiments were carried out at the Aquaculture Research station in Kårvika outside of Tromsø, Northern Norway (69°N). The fish used in the experiment were Atlantic salmon of the Aqua- gen strain, generation 2018. Eyed eggs were incubated and hatched at the station and start feeding commenced 22.05.2018. Until the start of the acclimation period (four weeks before start of the experiment) they were kept under continuous light (24L:0D) in FW at natural temperatures during summer and 4°C from November onwards and fed a commercial salmon feed (Skretting, Stavanger, Norway) continuously by automatic feeders according to established tables at the station.

On March 25, 2019, 1080 salmon parr (≈ 30 g, ≥ 14 cm fork length; FL) were randomly distributed amongst six, 300 l circular tanks (180 fish/tank). Of these, a total of 90 fish (15 fish/tank) were anesthetised in a low dose of benzocaine (ca. 30 ppm) and then marked with external Floy tags (fingerling tags; www.floytag.com) in order to monitor individual growth during the whole experiment. Temperature was increased to 10°C within the first week of the acclimation period and maintained at 10°C until the end of the FW phase of the experiment. The fish were held in FW until July 16, 2019, during which three of the tanks were exposed to a short photoperiod (SP) of 9 h of light and 15 h of darkness (9L:15D) for six weeks (light on at 10 am and off at 7 pm) followed by LL for another 6 weeks (Fig. 2). These groups were fed with a control feed produced specially for this experiment by Skretting. The fish in the other three tanks were kept on LL during the whole period and fed with the control feed that mimicked a commercial feed supplemented with a salt mix and free tryptophan during the last 6 weeks in FW (Table 1). These two treatment groups are hereafter termed the “light”- and the “dietary” treated groups, respectively. Feed was provided daily and in excess between 11 am and 6.30 pm using automatic feeders. After these 12 weeks, on July 16, 2019, all fish were transferred to SW. The density of fish was reduced by reducing the number of fish in each tank (70 fish/tank, including all Floy- tagged fish; 49.5 kg/m^3 light treated group and 61.8 kg/m^3 dietary treated group). After one week in SW the feeding regime extended and the fish were fed daily and in excess between 11 am and 3 am using automatic feeders.

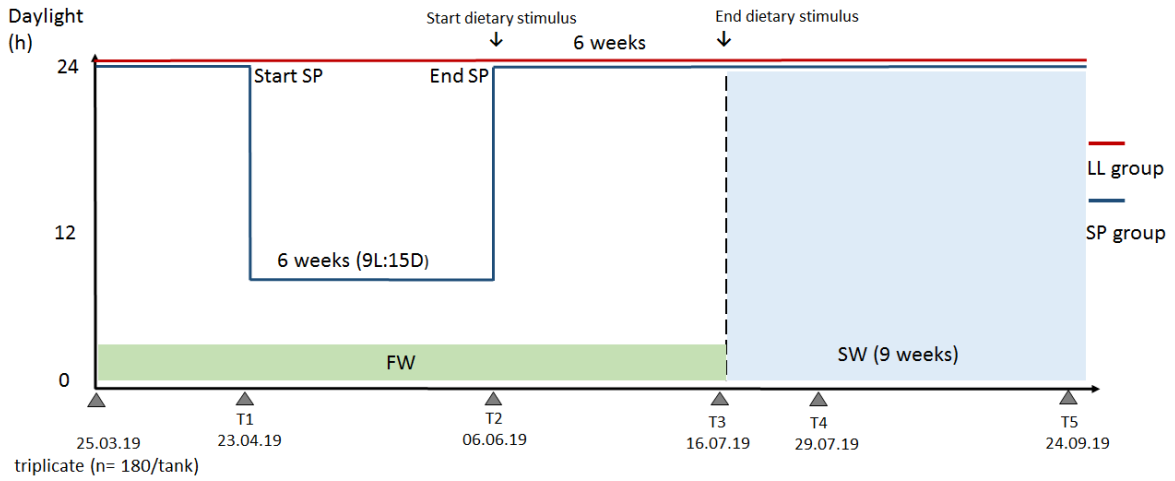

Figure 2: Experimental set- up. The red line represents the dietary treatment group that was kept on LL throughout the experiment and given a dietary treatment for six weeks before transfer to SW. The blue line represents the light treatment group, starting on LL, then given a short winter stimuli (SP; 9L:15D) for six weeks, before returning to LL for another six weeks before transfer to SW. During the SW phase both groups was kept on LL and given normal feed. T1 represents first sampling, the light treated group had been on SP for one day. At T2 the light treated group had been on SP for 6 weeks and was put on LL after sampling, where the dietary stimulus started for the dietary treatment group after sampling at T2. At T3 the dietary stimulus ended and both groups were transferred to SW after sampling. During the SW phase samples were taken after one week (T4) and after nine weeks (T5).

Table 1: The composition in percentage (%) in the control feed and the smoltification (salt) feed.

Diet/composition (%)	Control	Salt
Wheat	15.00	9.90
Wheat gluten	10.00	12.00
Sunflower meal	5.00	2.00
Soy protein concentrate	15.50	15.00
Fababean dehulled	4.80	2.00
Fish meal	31.30	32.3
Rapeseed oil	8.50	8.60
Fish oil	8.50	8.60
Water	0.30	1.00
Vitamin and mineral premixes	1.10	1.10
Sodium chloride	0.00	6.00
Calcium chloride	0.00	0.75
L-Tryptophan	0.00	0.40
Magnesium chloride	0.00	0.25
Total	100.00	100.00
Moisture	8.30	8.30
Protein	43.55	43.24
Fat	21.99	21.99
Ash	6.98	13.36
Gross energy (MJ)	22.17	21.21

2.2 Sampling

During the 12 week long FW phase, untagged fish were sampled on three occasions, at the start of SP period (1 day on SP; T1), at the end of SP period (week 6 last day on SP; T2) and at the end of the FW phase (week 12; T3). During the 9 week long SW phase, untagged fish were sampled on two occasions, after one week (T4) and at the end of the SW phase (week 9; T5). Sampling for each treatment group was done on two consecutive days in order to sample both groups at exactly the same time of the day. On each sampling day a total of 6 fish (2 fish per tank) were sampled at 10 am, one hour before the feeding started. The fish were anesthetized in an overdose of benzocaine (120 ppm). Body mass and FL were measured and photos were taken to assess silvering status. The fish were then decapitated before brain (whole brain without pituitary), stomach and anterior intestine (posterior of pylori caeca, no

posterior intestine) were dissected out and stored in 1 ml RNAlater (Thermo Fisher Scientific; www.thermofisher.com) for later gene expression analyses. The RNAlater fixed tissues were stored at 4°C for 24 hours, then transferred to - 20°C until further analyses.

During the FW phase a 24 h SW challenge test (SWCT) was conducted on each sampling day. Nine fish per treatment (3 fish/tank) were randomly netted and transferred to a test tank with SW (salinity 33 ppt; temperature 7°C). After 24 h the fish was killed with a lethal overdose of benzocaine, body mass and length (FL) were measured and blood was withdrawn from the caudal vein using 1 ml Li- heparinized vacutainers. Blood samples were centrifuged at 3000 x rpm at 4°C, plasma removed and stored at - 80°C until further analyses.

Food intake measurements were done at T1, T2, T3 and T4. A sieve system at the water outlet tube developed at the aquaculture station which sample uneaten food was used. This system is validated and is routinely in use at the station.

2.3 Measurement of plasma osmolality and chloride levels

Plasma osmolality was measured using a Fiske One- Ten Osmometer (Advanced® Osmometer Model 3250). The osmometer determines the concentration of solutions by measuring the freezing point. It utilizes high- precision thermistors to sense the sample temperature, to control the degree of supercooling and freeze induction and to measure freezing point of the sample. The precision of the instrument was first tested by using a reference solution, Clinitrol™ with a known value of 290 mOsm/kg. Then 15 µl plasma were added to specific tubes and measurements were recorded, the sample probe was cleaned with a sponge between each test. Each sample was measured twice, a third measurement was conducted in case of a deviation > 3 mOsm/kg between the two first measurements. A control test was done after every tenth sample taken according to protocol (Manual 2003).

A chloride analyser (Sherwood M926S) was used to measure chloride levels in the plasma. This method is based on titration of chloride ions by passing a known constant current between two silver electrodes, which provides a constant generation of silver ions. Together with chloride in the sample, these silver ions form silver chloride. The number of silver ions introduced into the sample combine with one unit measurement of chloride. When all the

chloride has bound to the silver ions and formed silver chloride, the free silver ions begin to appear and the solution conductivity changes. The electrodes sense this change, the reading stops, and results are displayed in millimoles per litre (mmol/l). Before running the samples, a standard (Manual 2007) test was conducted with a solution containing 100 mmol/l Cl^- as sodium chloride. Then 20 µl sample was added to the acid buffer and titrated. All samples were measured twice, a third measurement was conducted with a deviation > 3 mmol/l between the measurements. Control tests were taken after every tenth sample according to protocol (Manual 2007).

2.4 RNA extraction

The RNA extraction process took place under a fume hood. First step for the RNA extraction was to disrupt and homogenize the tissue samples. This was done by using a TissueLyser (Qiagen TissueLyser II, Model 85300) system. Each sample was placed in a 2 ml Eppendorf tube containing one steel bead for the brain and intestine, and two steel beads for the stomach. Then 900 µl QIAzol Lysis Reagent was added to each tube. Tubes were placed in a TissueLyser Adapter Set, brain samples were treated at 30 Hz for 1 min. Intestine samples were treated at 30 Hz for 2 x 1 min and stomach samples at 30 Hz for 2 x 2min as the tissue was harder to break down. The tubes containing the homogenate were then incubated at room temperature (15-21°C) for 5 min. The next step was phase separation (extraction of the RNA), where 100 µl genomic DNA (gDNA) removal and 180 µl chloroform were added to each tube and shaken vigorously, using a vortex, for 15 sec. The tubes were then incubated at room temperature for 3 min, and then centrifuged at 12000 x g for 15 min at 4°C. Samples had now separated into three different fractions. The upper fraction, containing the RNA, was transferred to a new Eppendorf tube and an equal volume of ethanol was added. Then, half of the sample was transferred to RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 x g for 30 sec at room temperature. The supernatant was removed and the remainder of the sample was added to the spin column and centrifuged as previous. In order to wash the membrane, different buffers were used. First, 700 µl RWT buffer was added to the spin column, centrifuged at 8000 x g for 30 sec at room temperature. Then 500 µl RPE buffer was added in two rounds, first with centrifugation as previous and the last round with centrifugation at 8000 x g for 2 min at room temperature. Between each centrifugation step, the supernatant was removed. In order to eliminate any carryover of RPE buffer the spin columns were placed in new 2 ml collection tubes, and centrifuged at full speed for 1 min. For

the final step the spin column was placed in a new 1.5 ml collection tube and 45 µl RNase-free water was added directly on the membrane in order to elute the RNA. The samples were centrifuged at 8000 x g for 1 min at room temperature. The spin column was then removed, and the tubes with the RNA were stored at -80°C until further treatment.

2.5 RNA purification

RNA was purified using a two- step process according to normal protocols.

2.5.1 Ethanol precipitation

All samples was cleaned by ethanol (EtOH) precipitation, a buffer was made with 1/10 the volume of RNA sample (45 µl; 4.5 µl) 3 M Sodium acetate (final concentration 0.3 M) and 3 x the volume of RNA sample (135 µl) 100 % ethanol. To each sample 140 µl of the buffer was added and stored at - 20°C overnight. Then all samples were centrifuged at 13000 x g for 20 min at 4°C. The supernatant was removed without disturbing the pellet and 500 µl ice cold 80 % EtOH was added, centrifuged at 13000 x g for 5 min at 4°C, removed the supernatant and air-dried the tubes for 5 min. Then 30 µl (20 µl for brain samples) RNase free water was added to each sample, vortex for 10 sec and centrifuged at 10000 x g for 1 min. RNA concentration was measured using a NanoDrop and samples were stored at - 80°C until further treatment.

2.5.2 DNase treatment

The samples were treated with TURBO DNA-*free*™ Kit in order to remove eventual gDNA from the RNA preparations. The TURBO DNase enzyme breaks down the DNA, so that the amount of contaminating DNA left would be too low to be detected by routine polymerase chain reaction (PCR).

1 µl TURBO DNase enzyme and 2.5 µl 10 x TURBO buffer (0.1 x sample volume) were added to 1.5 ml Eppendorf tubes. Then the appropriate volume of RNA (depending on the concentration level for each sample) was added to each tube, followed by x µl RNase free water to fit a maximum volume of 25 µl. All samples were incubated on a heat block at 37°C for 30 min. Then 3 µl DNase inactivation solution was added to each tube and incubated for 5 min in room temperature. The samples were mixed by flicking the tube during incubation

time in order to disperse the DNase inactivation reagent. Then the samples were centrifuged at 10000 x g for 2 min and the supernatant containing the RNA was transferred to a new tube without disturbing the pellet. RNA concentration and quality was measured using a NanoDrop, and samples were stored at - 80°C.

2.6 First strand cDNA synthesis

The RevertAid First Strand cDNA Synthesis kit was used to reverse transcribe the RNA to complimentary DNA (cDNA). A master mix was made according to protocol; reagents included are listed in table 2 and the exact amount of the different components was calculated up to the number of reactions that was run each time. To each 200 µl PCR tubes (MicroAMP) 9 µl master mix was added. Complimentary DNA was made out of 1 µg RNA for the intestine and stomach and 500 ng for the brain samples, the appropriate volume (µl) was calculated based on concentration level for each sample. Then RNase free water was added to a total volume of 20 µl. There were made seven negative control samples (no reverse transcriptase control, no- RT) for each tissue (total 21), in order to ensure that there was no contamination with gDNA. The Applied Biosystems 2720 Thermal Cycler was used to perform first strand cDNA synthesis, program set is shown in table 3 below.

Table 2: List of the reagents used in the master mix for first strand cDNA synthesis.

Reagent	x 1 reaction
Random hexamer primers	1 µl
5 x reaction buffer	4 µl
RiboLock RNAse inhibitor	1 µl
10 mM dNTP Mix	2 µl
RevertAid Reverse transcriptase	1 µl

Table 3: The program used for first strand cDNA synthesis; the different stages, temperature in °C and time in minutes for each stage.

Stage	Temperature (°C)	Time (min)
Pre- PCR hold	25	5
PCR segment	42	60
Post- PCR hold (termination)	70	5
Final hold	4	∞

After the cDNA synthesis all samples were diluted (1:20), aliquoted into four tubes and stored at - 20°C until further analysis.

A dilution series was made for each tissue in order to define standard curves for quantitative PCR (qPCR). For the brain, five samples, with high RNA concentration, were synthesized and then pooled (giving a total volume of 100 µl cDNA). Then 1:3 dilution was made using 80 µl cDNA and 160 µl H_2O. Thereafter a two- fold dilution series were made. For the intestine three samples were synthesized and pooled, giving a total volume of 60 µl cDNA. A 1:3 dilution was made using 50 µl of the cDNA and 100 µl H_2O. Followed by a two- fold dilution series. For the stomach, two samples were used; giving 40 µl cDNA, a 1:3 dilution was made by 30 µl cDNA and 60 µl H_2O, then a two- fold dilution series were made.

2.7 Real- time PCR (qPCR)

All samples were run in duplicates using Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR Plates (Bio-Rad, CA, USA). Quantitative PCR was run in 20 µl reactions, consisting of 10 µl SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA), 5.5 µl distilled H_2O, 4 µl cDNA (final concentration 5 ng; Table 4) and 0.5 µl primer mix (final concentration 250 nM; primers listed in Table 5). No- reverse transcriptase controls and no- template control (NTC) were added to each plate. A dilution series was included in each run as a quality control and to verify primer efficiency.

Table 4: List over the components used during qPCR and the master mix composition. The volume of the different components in the master mix was calculated according to the appropriate number of reactions on each plate which differed depending on tissue and sample numbers.

qPCR components		**Mastermix**
	x1 reaction (µl)	94x reactions (µl)
SYBR Green Mastermix	10	940
250 nM primer mix (forward and reverse)	0.5	47
H_2O	5.5	517
cDNA (5 ng)	4	

Table 5: Gene name, forward and reverse primer sequence for each gene and the amplification efficiency (%) from qPCR run.

Gene	Sequence (5`- 3`)	Efficiency (%)
Ghrelin- 1	Forward: CCAGAAACCACAGGTAAGACAGGGTA Reverse: CTCCTGAAACTCCTCCTCACTCATGG	99
Ghrelin- 2	Forward: GCCCCTCCCAGAAACCACAGGGTAAA Reverse: CTCCTGAAACTCCTCCTCACTCATGG	101
Peptide YY (PYY)	Forward: ACTACACCGCGCTCAGACACTACATC Reverse: TCTCTGGTCTCTCTCTGCATTGTTGCCG	102
Cholecystokinin (CKK-L)	Forward: GCGCGAACTACTGGCAAGATTGATA Reverse: TGTCCTTTATCTTGTGGCTGGGACCCG	104
Neuropeptide Y (NPY)	Forward: ACTGGCCAAGTATTACTCCGCTCTCA Reverse:CTGTGGGAGCGTGTCTGTGCTCTCCTTCAG	103
Pro- opiomelanocortin A1 (POMCA1)	Forward: AAGACAACTCCTCGGAAGAGAAA Reverse: CAGCCACAGCCAATAACCAC	102
Pro- opiomelanocortin A2 (POMCA2)	Forward: TTTGGCGACAGGCGAAGA Reverse: ATGGGAGATTTGGCGGTGAG	100
Agouti related protein 2 (AgRP2)	Forward: GCGGTGTGGTCGTCTGATGG Reverse: GGGCCCAGTCTCCAGCAGTG	115
ß- Actin	Forward: CCAAAGCCAACAGGGAGAA Reverse: AGGGACAACACTGCCTGGAT	106

2.8 Data treatment and statistics

RT- qPCR data

Data from RT-qPCR was analysed by using the $2^{-\Delta\Delta C_T}$ (Livak) method. All target genes were normalized to a reference (ref) gene, ß- actin (I). The dietary treatment group at T1 was chosen to be the calibrator sample. Then the test sample was normalized to the calibrator sample (II) and the expression ratio of the target gene in all other samples are expressed as an increase/decrease relative to the calibrator sample (III).

I. ΔCt (test) = Ct (target, test) – Ct (ref, test)
   ΔCt (control) = Ct (target, control) – Ct (ref, control)
II. $\Delta\Delta Ct = \Delta Ct$ (test) – ΔCt (control)
III. $2^{-\Delta\Delta C_T}$ = normalized gene expression ratio

Normalized gene expression data were log transformed prior to statistical analyses. All figures were made in Excel (2013) and statistical analyses were conducted in SigmaPlot version 14.0. A Shapiro- Wilk normality test was used, and with an overall normal distribution, an independent samples t- test was performed to test for differences between

treatments within time points. If the overall assumption of a normal distribution failed, a non-parametric Mann- Whitney Rank Sum test was used. One- way analysis of variance (ANOVA) was performed in order to look for differences over time within each treatment group. Followed by a Holm- Sidak test for pairwise comparisons. A p- value < 0.05 was considered significant.

Calculations for growth estimates

Fulton's condition factor, *K* was calculated with the following formula (Body mass /Fork length^3) x 100, in which g is body mass and cm is fork length.

The specific growth rate, SGR, % BM increase day^{-1} was calculated with the following formula $[(\ln W_2 - \ln W_1)/T] \times 100$, where BM is body mass, W_2 is final weight and W_1 is initial weight in gram, t= time elapsed in days.

Feed intake

Feed intake was calculated using the following steps:

I- Biomass/tank (g): Body mass (Floy tagged fish; g) x number of fish
II- Dry feed eaten (g): Total dry feed given (g) – dried leftover dry feed (g)
III- Feed intake pr. 100 g fish: (Dry feed eaten (g)/ (biomass/tank (g)/100))

3 Results

3.1 Smoltification development

Silvering

Both treatment groups developed the typical silvering smolt characteristic in the course of this experiment. At T1, there was no silvering in the dietary treated group nor in the light treated group (i.e. parr marks visible; Fig. 3). A difference between the two treatment groups was seen at T2; the dietary treatment group was fully silvery, had no visible parr marks and darkened fin margins, while the light treated group had some silvering but still visible parr marks (Fig. 4). By T3, fish in both groups were fully silvery (Fig. 5).

Figure 3: A photo of a typical fish from both treatment groups at T1.

Figure 4: Morphological differences between the dietary treated fish (A) and the light treated fish (B) at T2.

Figure 5: Morphological differences between the dietary treated fish (A) and the light treated fish (B) at T3.

Body mass and length

There was a significant increase in body mass (g) over time in the dietary treated group and the light treated group ($p < 0.001$, Fig. 6). The dietary treated group had a significantly higher body mass than the light treated group at T2, T3 and T5 ($p < 0.001$).

There was also a significant increase in fork length (cm) over time in both treatment groups ($p < 0.001$, Fig. 7), and this increase was significantly higher in the dietary treated group than in the light treated group at T2, T3 and T5 ($p < 0.001$, $p < 0.001$ and $p = 0.008$ respectively).

Condition factor

Fish from both treatment groups showed a significant increase in *K* from T1 to T2 ($p = 0.002$ and $p < 0.001$ respectively; Fig. 8). There was no significant difference between the two treatment groups at T1 and T2. Between T2 and T3, there was a significant decrease in *K* in the light treated group ($p < 0.001$), while there was no decrease in the dietary treated group ($p = 0.196$), resulting in a significantly lower *K* in the light treated than the dietary treated fish at T3 ($p < 0.001$). From T3 to T5 there was a significant increase in *K* in the dietary treated fish ($p = 0.019$), but not in the light treated group, and hence, *K* was significantly higher in the dietary group than in the light treated ones at T5 ($p < 0.001$). From T1 to T5, the dietary treated group had a significant increase in *K* ($p < 0.001$).

Feed intake

Both treatment groups had a decreasing feed intake throughout the experiment (Fig. 9). There was a significantly lower feed intake in the dietary treated group at T2, T3 and T4 ($p = 0.003$, $p = 0.004$ and $p < 0.001$ respectively) than at T1. The light treated group had a significantly lower feed intake at T4 than at T1 ($p = 0.014$). At T2 and T3 there was a significantly lower feed intake in the light treated group than in the dietary treated group ($p = 0.010$ and $p = 0.035$ respectively).

Specific growth rate

There was a significant higher SGR in the dietary than in the light treated groups from T1- T2 ($p < 0.001$; Fig. 10). Both the dietary treatment group and the light treatment group had a significant decrease in SGR from T1- T2 to T2- T3 (both $p < 0.001$). From T2 to T3, there was no difference in SGR between treatment groups. After SW transfer, both the dietary

treatment group and the light treatment group had a minor, albeit significant increase in SGR in the period between T2-T3 and T3-T5 ($p = 0.012$ and $p < 0.001$ respectively). During T3-T5, there was tendency, albeit not significant ($p = 0.069$), towards a higher SGR in the dietary treated group than in the light treated one. There was a significant decrease in SGR in the dietary treated group in the period from T1-T2 to T3- T5 ($p < 0.001$), but not in the light treated one.

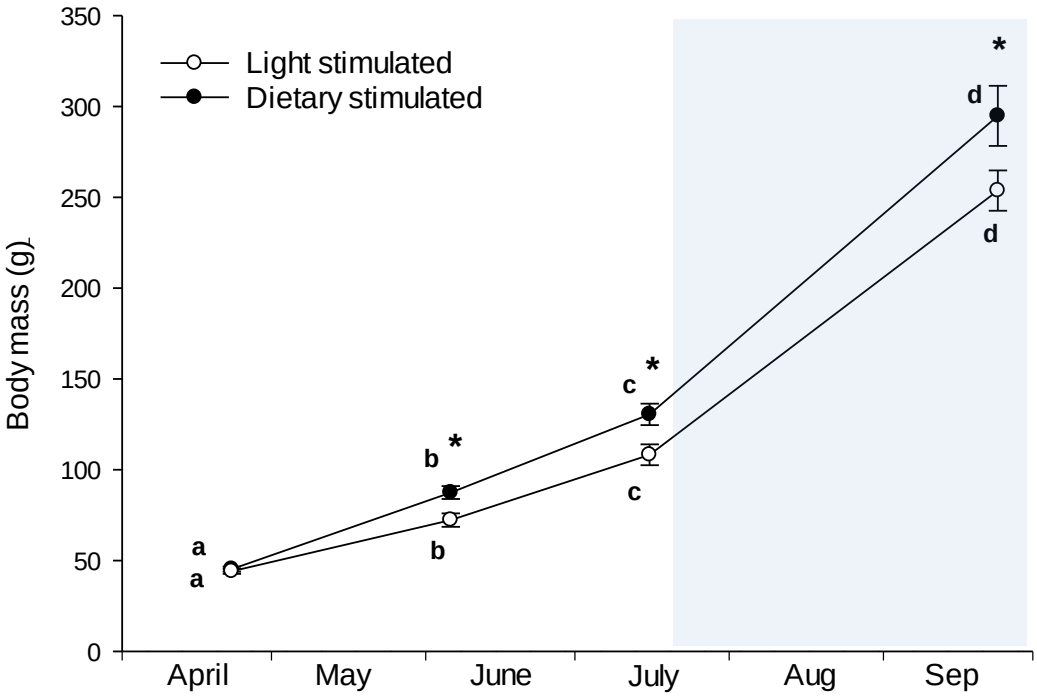

Figure 6: Body mass of Floy tagged fish (g, ± SEM) for the light treatment group and the dietary treatment group at the different sampling points (T1, T2, T3 and T5). Blue shadow illustrates the SW phase. Asterisk indicate significant differences between treatment groups within sampling time points; different lower-case letters indicate significant differences over time within the treatment group ($p < 0.05$).

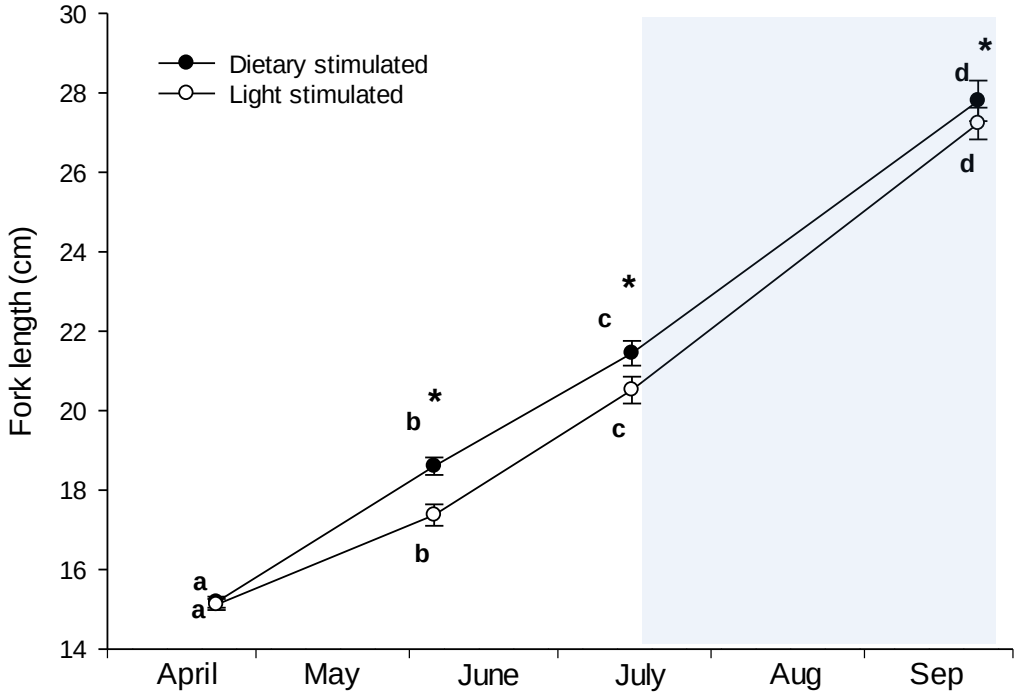

Figure 7: Fork length of Floy tagged fish (cm, ± SEM) for the light treatment group and the dietary treatment group at the different sampling points (T1, T2, T3 and T5). Blue shadow illustrates the SW phase. Asterisk indicate significant differences between treatment groups within sampling time points; different lower-case letters indicate significant differences over time within the treatment group ($p < 0.05$).

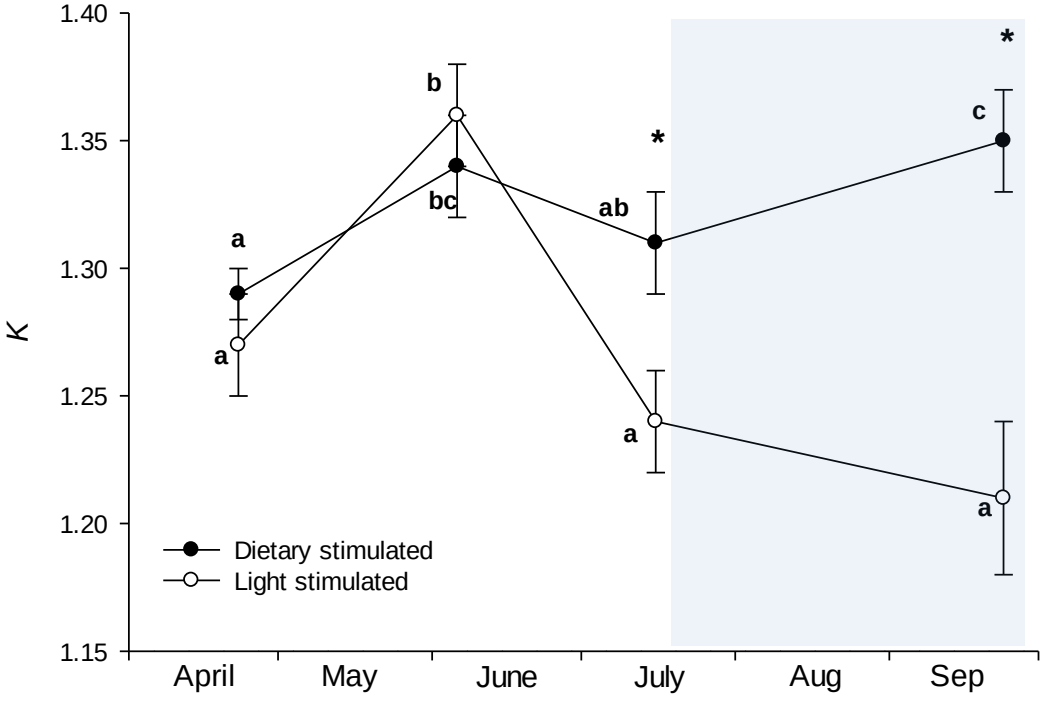

Figure 8: Average Condition factor (*K*, ± SEM) for the light treatment group and the dietary treatment group at the different sampling points (T1, T2, T3 and T5). Blue shadow illustrates the SW phase. Asterisk indicate significant differences between treatment groups within sampling time points; different lower-case letters indicate significant differences over time within the treatment group ($p < 0.05$).

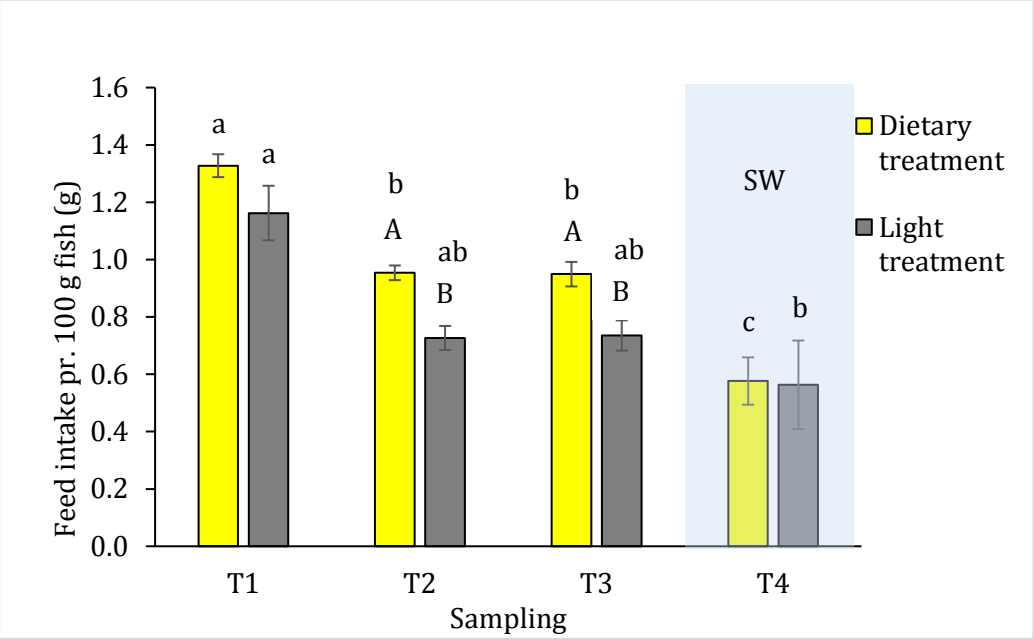

Figure 9: Feed intake pr. 100 g fish (g, ± SEM) for the light treatment group and the dietary treatment group at the different sampling points (T1, T2, T3 and T4). Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

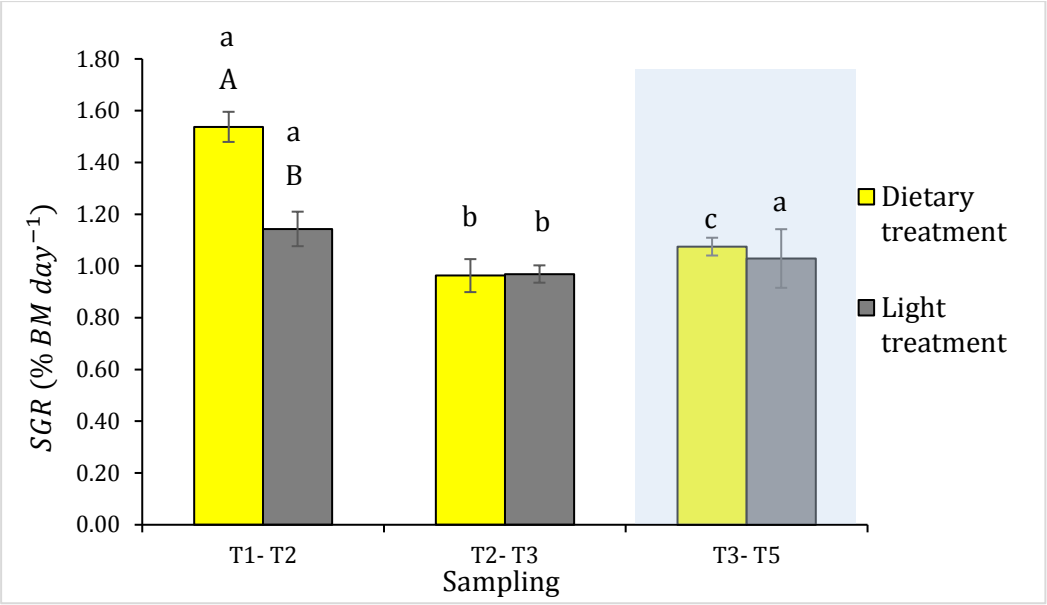

Figure 10: Specific growth rate (± SEM) for the light treatment group and the dietary treatment group between sampling points T1- T2, T2- T3 and T3-T5. Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

Post -SWCT plasma osmolality

At T1, no significant differences were found in plasma osmolality between treatment groups (Fig. 11). Between T1 and T2, there was no difference in plasma osmolality in the dietary treatment group, but in the light treated group, plasma osmolality was significantly higher at T2 than at T1 ($p = 0.002$). At T3, the light treated group had a significantly lower plasma osmolality than at T2 ($p = 0.028$), while no difference was seen between these time points in the dietary treated group. Consequently, plasma osmolality in the light treated group was higher than in the dietary stimulated one at T2 ($p < 0.001$) and T3 ($p = 0.020$). There were no differences in plasma osmolality between T1 and T3 in any of the two treatment groups.

Post -SWCT plasma chloride concentration

At T1, plasma chloride concentrations did not differ between treatment groups (Fig. 12). At T2, plasma chloride levels tended to be higher, albeit not significant, than at T1 in the light treated group. In the dietary treated group there was no difference in plasma chloride levels between T1 and T2, but at T3 chloride levels were significantly lower than at T1 ($p = 0.029$) and T2 ($p = 0.004$). At T2 and T3 there were significantly higher plasma chloride levels in the light treated than in the dietary treated group ($p = 0.006$ and $p < 0.001$ respectively).

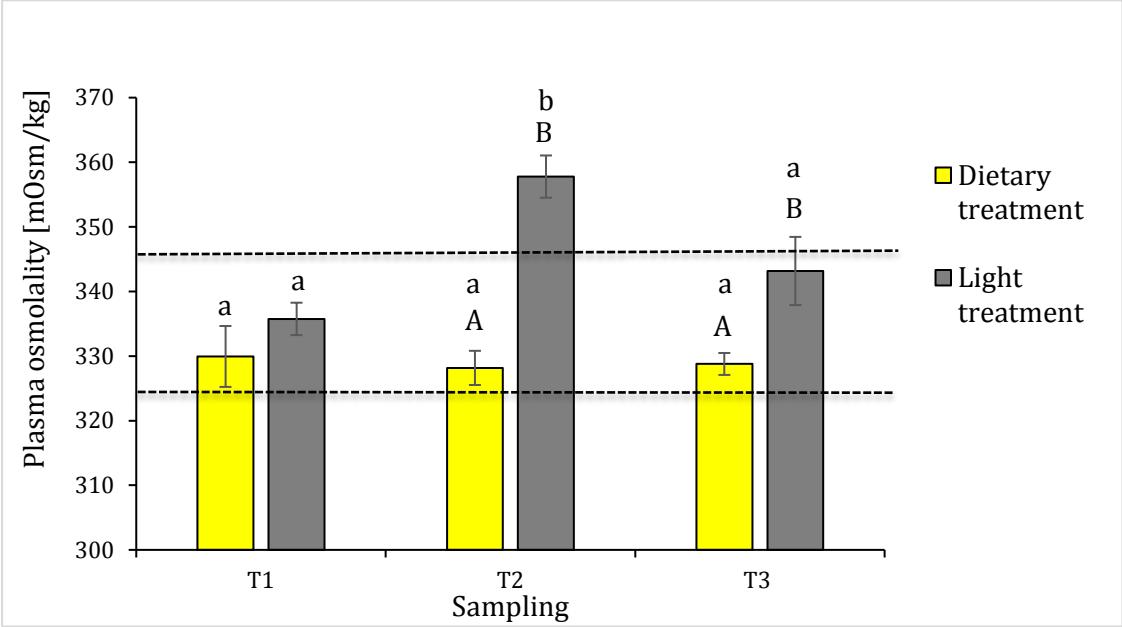

Figure 11: Plasma osmolality (± SEM) measured after SWCT of fish from the dietary treatment and the light treatment group. The two dotted lines represent the normal osmolality range for a SW adapted fish. Different capital letters indicate significant differences between treatment groups within time points; lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

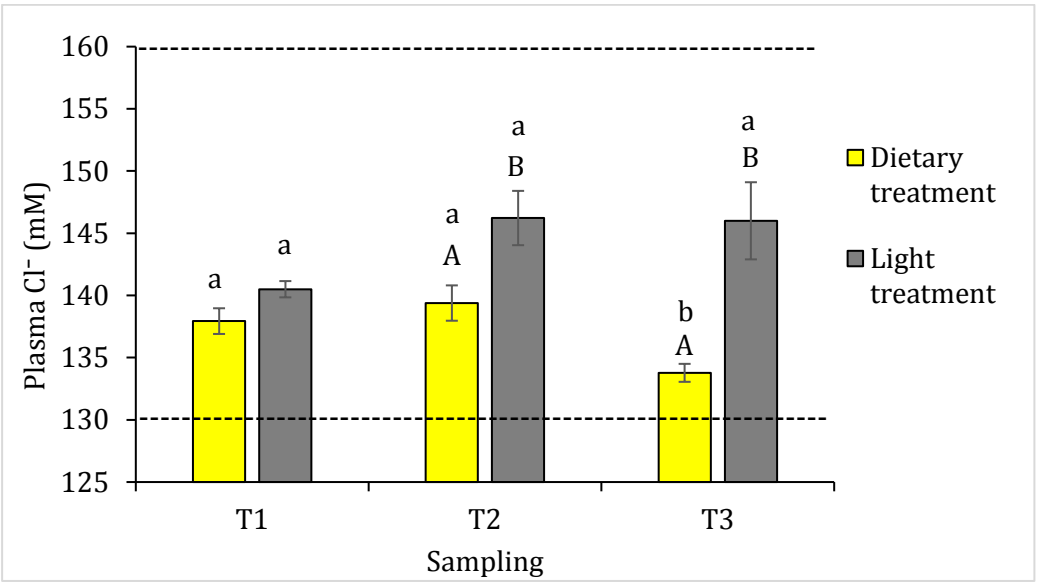

Figure 12: Plasma chloride concentrations (± SEM) measured after SWCT of fish from the dietary treatment and the light treatment group. The two dotted lines represent the normal plasma chloride range for a SW adapted fish. Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

3.2 Gene expression

3.2.1 Gene expression of central orexigenic neuropeptides

NPY

During the FW phase, neither the dietary treated group, nor the light treated groups had significant differences in brain NPY expression between T1 and any of the time points (Fig. 13). However, there was a significant decrease in NPY expression comparing the fish sampled after the first week in SW (T4) and those sampled after 9 weeks in SW, T5 ($p = 0.043$) in the dietary treated group. The light treated group had a significantly lower NPY expression at T5 compared to T1 ($p = 0.002$). At T4 NPY expression in this group tended to be lower than that at T1, albeit not significantly ($p = 0.065$). The light treated group had a significantly higher NPY expression than the dietary treated group at T1 and T5 ($p = 0.002$ and $P < 0.001$ respectively).

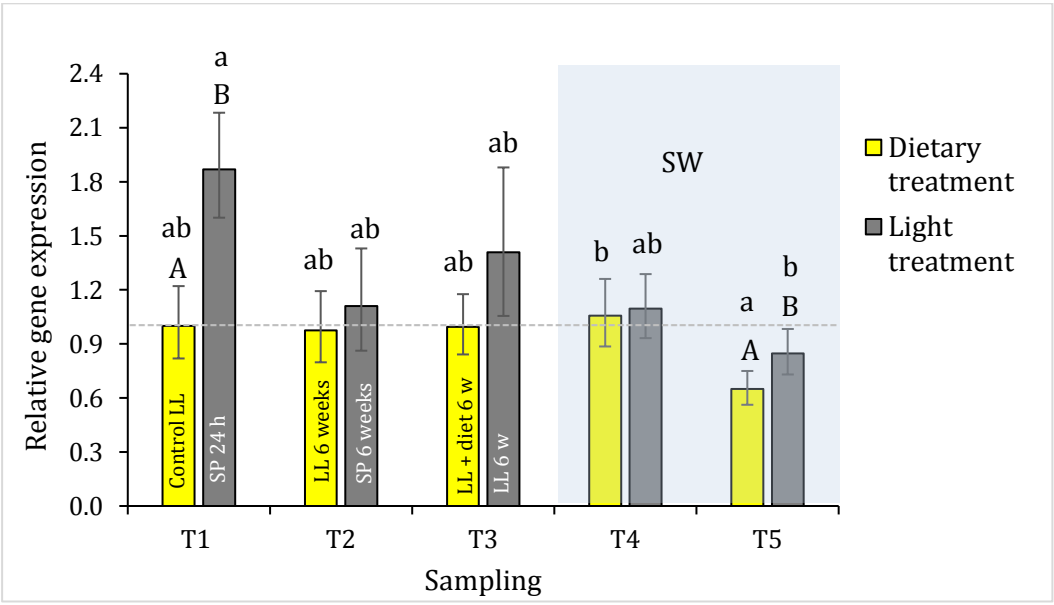

Figure 13: Brain NPY gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

AgRP2

The dietary treated group tended to have a higher AgRP2 expression at T3 compared to T1, albeit not significantly different (p = 0.055, Fig 14). At T4 there was a significantly higher AgRP2 expression relative to T1 (p = 0.012) in the dietary treated group. In the light treated group there were no significant differences in AgRP2 expression over time. No significant differences in AgRP2 expression were found between the two treatment groups at any time point.

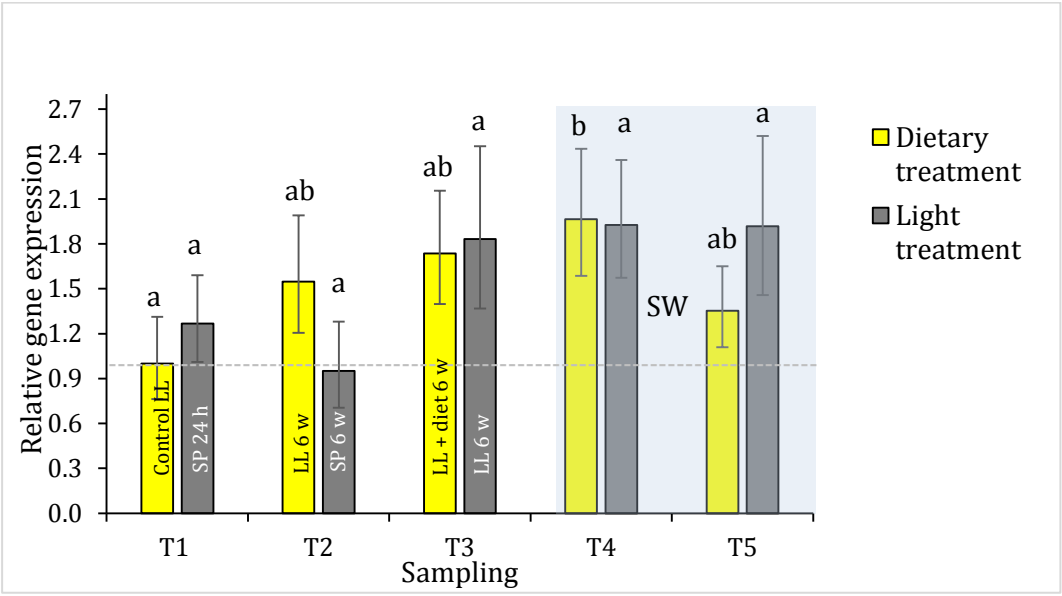

Figure 14: Brain AgRP2 gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). No significant differences were found between treatment groups. Different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

3.2.2 Gene expression of central anorexigenic neuropeptides

POMCA1 and 2

Except for a tendency to a lower POMCA1 expression in the dietary treated group at T5 than at T1 (p = 0.051), there was no differences in POMCA1 expression between time points in neither the dietary nor the light treated groups (Fig. 15). There was a significantly higher POMCA1 expression in the light treated group than in the dietary treated group at T3 (p =

0.032). POMCA1 expression tended to be higher in the light treated group than in the dietary treated group at T5.

POMCA2 expression did not differ significantly over time in any treatment group (Fig. 16), there was a tendency to a lower POMCA2 expression in the dietary treated group at T5 than at T1 ($p = 0.071$). There was a markedly higher POMCA2 expression in the light treated group than in the dietary treated group at T3, albeit no significant differences was found between treatment groups at that time point or any other time points.

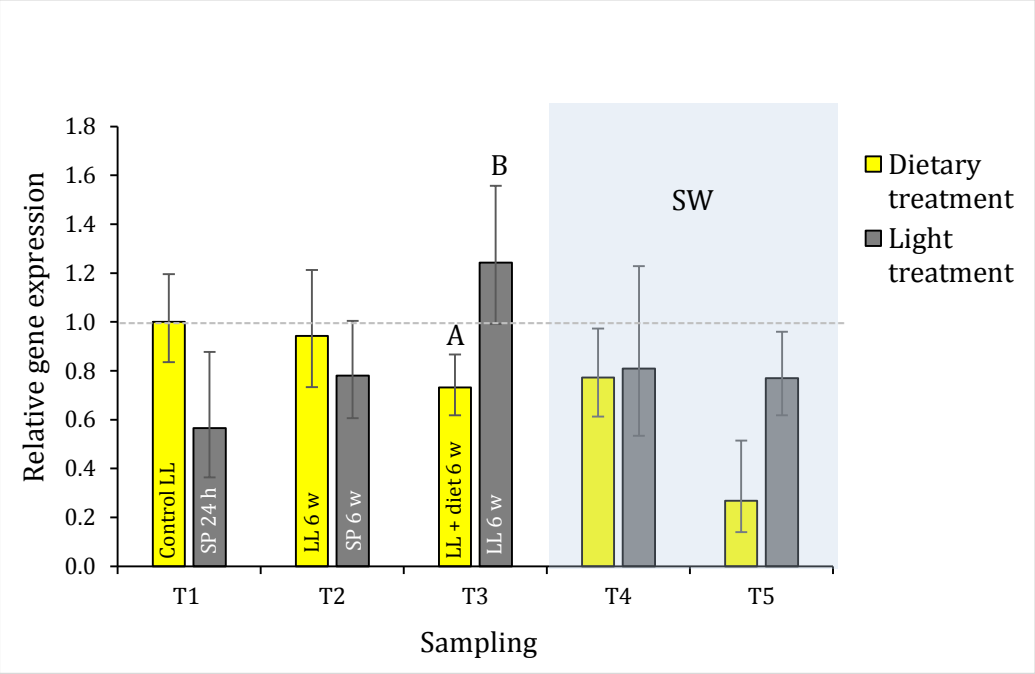

Figure 15: Brain POMCA1 gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). Different capital letters indicate significant differences between treatment groups within time points ($p < 0.05$).

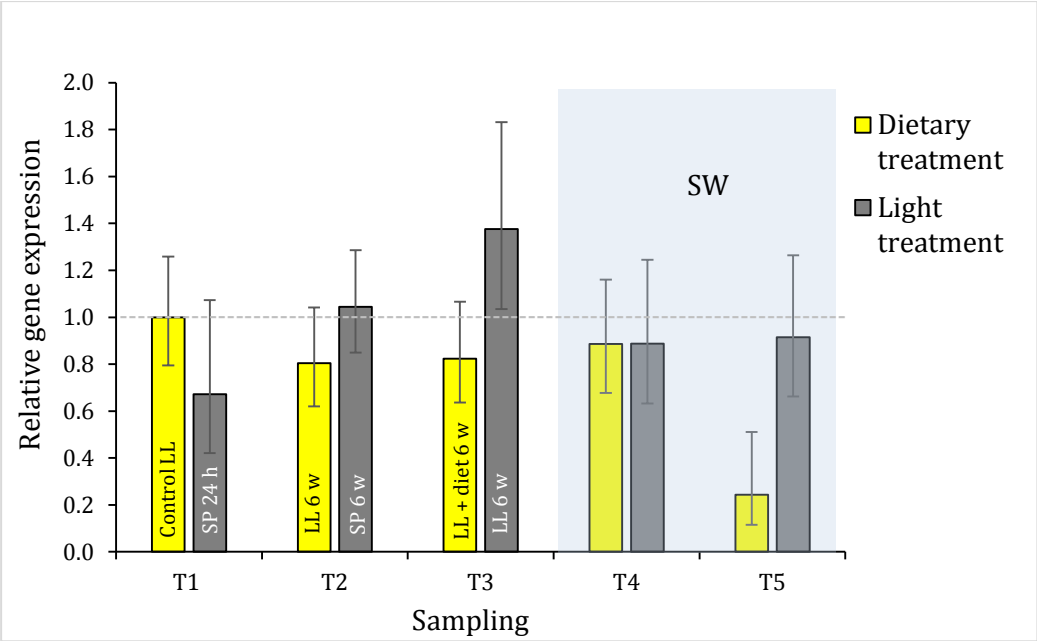

Figure 16: Brain POMCA2 gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line).

CCK- L

There were no significant differences in CCK-L expression in the dietary or the light treated group between time points (Fig. 17). At T5, there was a significantly higher expression of CCK-L in the light treated group than in the dietary group ($p = 0.044$).

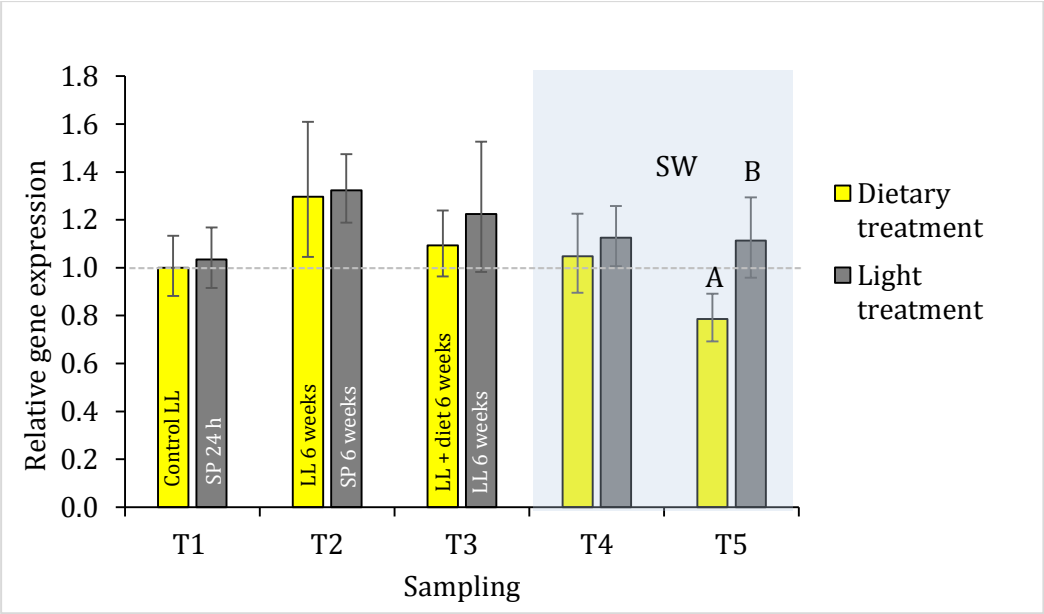

Figure 17: Brain CCK-L gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). Different capital letters indicate significant differences between treatment groups within time points ($p < 0.05$).

3.2.3 Gene expression of peripheral appetite regulators in the intestine and stomach

PYY

The dietary treated group had a significantly lower PYY expression in the SW phase (T4 and T5) compared to the levels at T1 ($p = 0.032$ and $p = 0.009$ respectively) and T3 ($p = 0.033$ and $p = 0.010$ respectively, Fig. 18). The light treated group had a significant lower PYY expression at T3 and T5 compared to T1 ($p = 0.011$ and $p = 0.016$ respectively) and T2 ($p = 0.010$ and $p = 0.014$ respectively), and expression at T4 tended to be lower than at T1 and T2. At T2, the light treated group had a significantly higher expression than the dietary treated group ($p = 0.041$) while PYY expression was higher in the dietary treated group than the light treated one at T3 ($p = 0.015$).

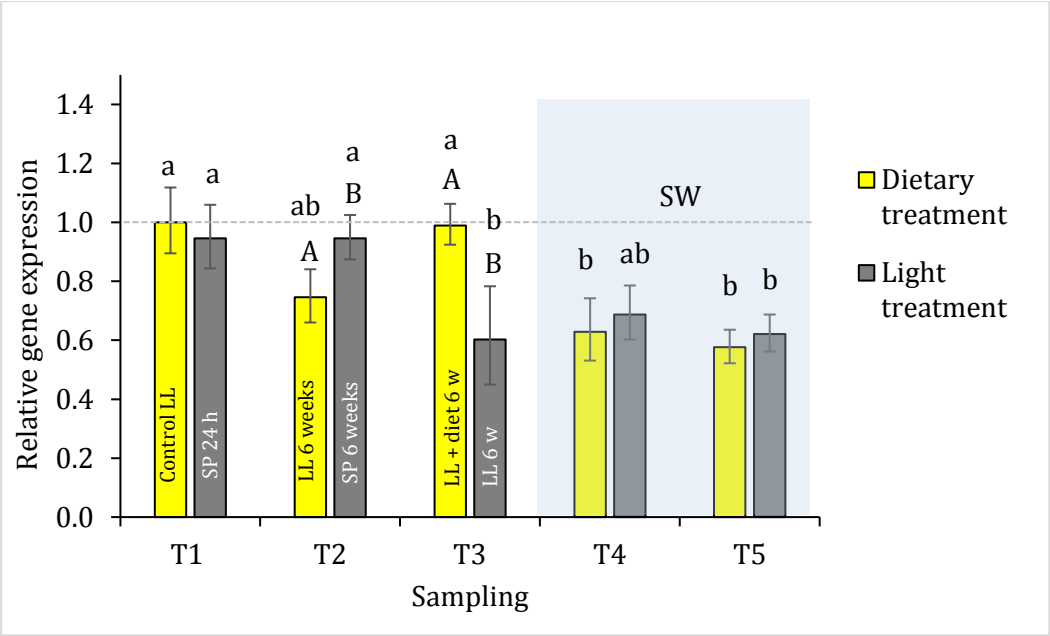

Figure 18: Intestinal PYY gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

Ghrelin- 1 and -2

The dietary treated group showed no significant differences in ghrelin- 1 expression over time (Fig. 19). The light treated group had a significantly higher ghrelin- 1 expression at T4 compared to all other time points (T1: $p < 0.001$, T2: $p = 0.002$, T3: $p = 0.008$ and T5: $p < 0.001$). There was a significantly higher ghrelin- 1 expression in the dietary treated group than in the light treated group at T1 ($p = 0.036$). Ghrelin- 1 expression decreased somewhat in the dietary treated group after T1 and there was a small increase in the light treated group, giving a significant difference between the two treatment groups at T3 ($p = 0.008$).

There were no differences in ghrelin- 2 expression between time points during the FW phase in neither the light nor the dietary treated groups (Fig. 20). A tendency to a decrease in the dietary treated group and a tendency to an increase in the light treated groups during the FW phase resulted in a higher expression in the light treated group than in the dietary treated group at T3 ($p = 0.019$). In the dietary treated group, ghrelin- 2 was higher expressed after transfer to SW at T4 than at T2 and T3 ($p = 0.060$ and $p = 0.032$ respectively). Also the light treated group had a significantly higher ghrelin- 2 expression after transfer to SW, giving a

significantly higher expression at T4 than all other time points during the FW phase (T1: $p < 0.001$, T2: $p = 0.007$, T3: $p = 0.029$). However, at T5 ghrelin- 2 expression was significantly lower in the light treated group than at T4 ($p < 0.006$) and in the same range as during the FW phase.

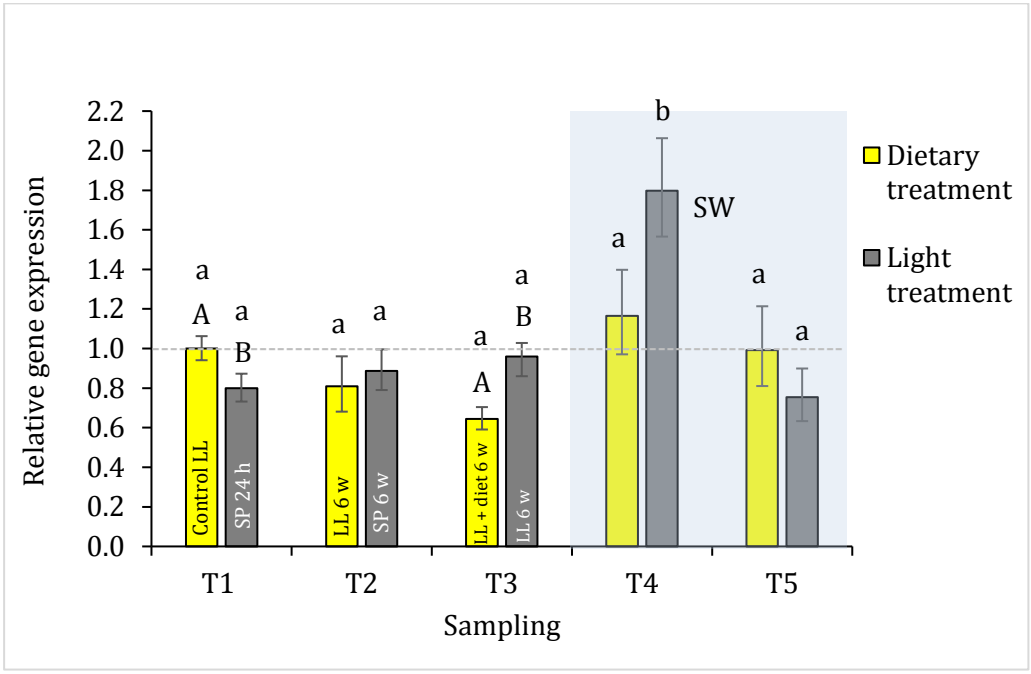

Figure 19: Stomach ghrelin- 1 gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

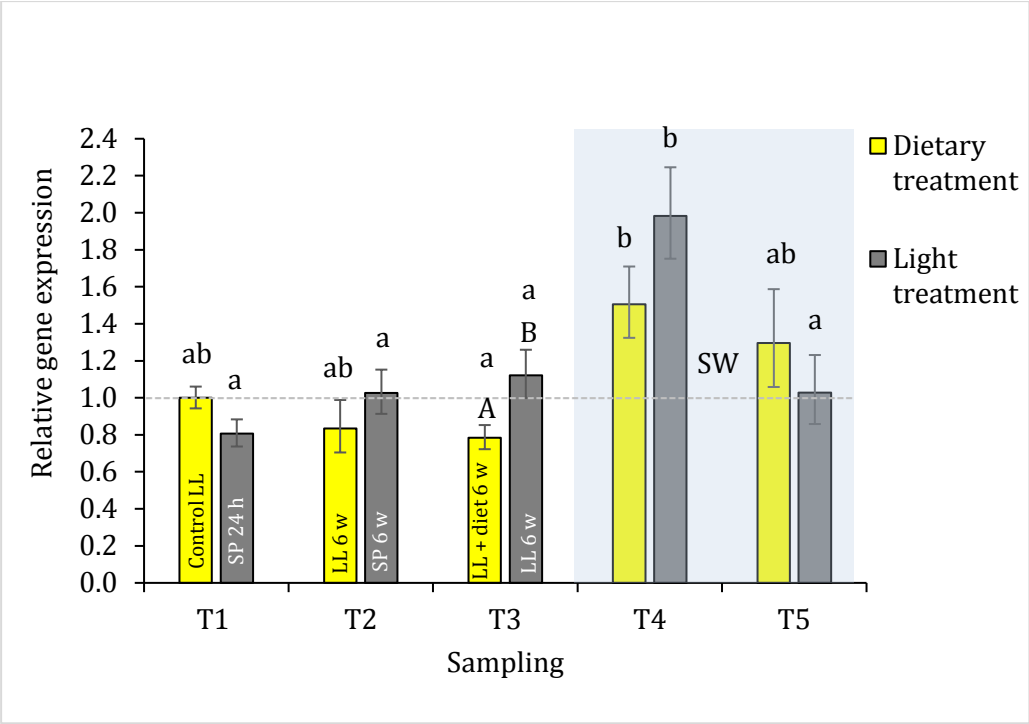

Figure 20: Stomach ghrelin- 2 gene expression (± SEM) in the dietary treatment group and the light treatment group at each sampling point (T1- T5). Gene expression data was normalised to the reference gene ß- actin and is presented relative to control LL T1 (indicated by the dashed line). Different capital letters indicate significant differences between treatment groups within time points; different lower-case letters indicate significant differences over time within the treatment groups ($p < 0.05$).

4 Discussion

The present study investigated the effects of light vs. dietary stimulated smoltification with a particular focus on possible changes in appetite regulators. In the first part of the discussion, differences between the two treatments on smoltification and growth will be discussed, followed by a paragraph on SW performance of the fish, and treatment dependent effects on feed intake and appetite regulation.

4.1 Effect of treatment on smoltification

Skin silvering is the mostly used external characteristic to assess PST status. Fish in both treatment groups were silvery at the end of the FW period (Fig. 3- 5). In the dietary treated group, silvering occurred already from T1 to T2, prior to dietary treatment, and is hence

considered to be spontaneous and not a result of the salt and free amino acid supplementation of the feed. Such a spontaneous silvering (i.e. without any apparent external cues) has been shown before in pre -smolts kept on continuous light when they have passed a critical size (Handeland et al. 2013). Also, the increase in water temperature two weeks before the experiment started may have stimulated silvering, which has been demonstrated before (Johnston and Eales 1970). On the other hand, short -day treatment seemed to reverse silvering in the light treated group from T1- T2, even though these fish had reached a similar size as the dietary treated fish at T1 and had been subjected to the same temperature regime as the fish in the dietary treated group.

An overall good growth of fish within both treatment groups, both regarding body mass and length, throughout the FW- and SW phase (Fig. 6 and 7) indicates that the fish had good growth conditions in this experiment. The lower SGR in the light treated group than in the dietary treated group (Fig. 10) from T1- T2 was expected, since exposure to short daylength (between T1 and T2) has been shown to reduce growth in Atlantic salmon (Saunders and Henderson 1970, Stefansson et al. 1989, Boeuf and Le Bail 1999, Strand et al. 2018). This was not an effect of the duration of the feeding time, since both treatment groups were fed only in the period where the light treated group had daylight. The lower feed intake in the light treated fish at T1 and T2 (Fig. 9; when they were exposed to a short photoperiod) than the dietary treated group (constant light), further implies that the daylength, *per se*, affects appetite (Jørgensen and Jobling 1992). The reduction in SGR from T1-T2 to T2-T3 in the dietary treated group, and the lower SGR in this group than in the light treated group between T2 and T3 when the dietary treated group had a higher feed intake, could be due to a reduction in feed utilisation when fed the salt enriched feed, as shown before (Salman and Eddy 1988, Duston 1993).

An important indicator for true smolt development is the reduction in condition factor after transfer from short day to long day in the smoltifying fish (McCormick 2013). The reduction in condition factor seen in the light treated group and not in the dietary treated group (Fig. 8) indicates that the light treated group went through a true smoltification while the dietary treated group did not. Several studies have shown that an increase in daylength has a strong influence on plasma growth hormone levels, and an increase in GH is expected (Saunders and Henderson 1970, Björnsson et al. 1989, McCormick et al. 1995). The reduction in condition factor is consistent with the growth- regulating effect of GH in salmonids, which together with cortisol stimulates length growth over mass growth (Stefansson et al. 2008). The results

in this experiment therefore imply that these hormonal responses were only seen in the light treated group.

A critical element of smoltification is the development of hypoosmoregulatory ability. A fully SW adapted salmon has a plasma osmolality concentration between 325- 345 mOsm/kg in SW (Noble et al. 2018). At T1, both treatment groups were within this level (Fig. 11) giving further support that the fish had started developing smolt characteristics before any treatment was given (except for the increase in temperature). It has previously been shown that development of hypoosmoregulatory ability without being given a photoperiodic winter stimulus, could take place in fish kept on LL (Duston and Saunders 1990, Sigholt et al. 1995, Handeland et al. 2013). They grew faster, hence reached the size- threshold for smoltification earlier, and developed a good SW tolerance much earlier than the fish kept on a natural photoperiod. In addition, previous studies conducted on Atlantic salmon have shown that body size alone might have an influence on SW tolerance, most likely due to a more favourable surface: volume ratio in larger fish (Handeland et al. 2013). The high plasma osmolality in the light treated group at T2 could be due to the short day stimuli interrupting smoltification processes that had started before the experiment, as noted for the silvering. The decrease in plasma osmolality from T2 to T3 in the light stimulated group confirms that the exposure to 24 h light from T2 to T3 had initiated PST including development of hypoosmoregulatory ability in this group, consistent with what is expected in a smoltifying fish. In contrast to the light stimulated group, the dietary treated group showed an equally good hypoosmoregulatory ability throughout the FW phase, despite the fact that special diet was given to the fish only in the period between T2 and T3. It has been observed previously that the spontaneous development of hypoosmoregulatory ability is lost after a period of 660 day degrees when maintained in FW (Fjelldal et al. 2018). The SWCT at T3 was 840 day degrees after the beginning of the experiment, indicating that the dietary treatment partly prevented the spontaneous loss of hypoosmoregulatory ability and partly stimulated ion extrusion mechanisms, as shown previously in salmon juveniles (Zaugg et al. 1983, Pellertier and Besner 1992, Perry et al. 2006).

Normal plasma chloride concentrations for a SW adapted fish lie between 130 - 160 mmol/l (Noble et al. 2018), and both treatment groups were within these levels throughout the whole FW phase. Plasma chloride concentrations were lower in the dietary treated group at T3 than at T2 as compared to the light stimulated group, indicating a beneficial effect of the diet on

chloride regulation. Why plasma chloride concentration, in contrast to plasma osmolality, did not decrease between T2 and T3 in the light stimulated group remains unknown.

4.2 Growth performance in the SW phase

It is well known that the first week in SW is a very stressful period and a reduction in appetite and growth was expected. Both treatment groups performed well during the SW phase (Fig. 10) with no differences observed between treatments. The SGRs of approximately 1% of body mass in both groups during this period were similar to those during the last 6 weeks in FW and higher or similar to that measured in other comparable experiments with salmon post -smolts in indoor tanks at the same salinity and temperature (Sacobie et al. 2012, Calabrese et al. 2017). Further, the relatively high feed intake of the fish in both treatment groups seen one week after SW transfer (Fig. 9) indicate a fast acclimation to SW since other studies have shown a very low feed intake (Arnesen et al. 2003) and growth rate (Handeland et al. 2000) shortly (3 to 14 days) after SW transfer. The good growth seen in the dietary treated group during the SW phase may be related to good SW tolerance at SW transfer, which reduce the degree of osmotic stress that follows transfer to SW in a less prepared fish (Duston 1993). Previous studies have shown that salt supplemented feed has a positive effect on SW adaptation (Zaugg et al. 1983, Pellertier and Besner 1992). The light treated fish had only a slightly lower SGR than the dietary treated fish during the SW phase. This was surprising taking into account the lower hypoosmoregulative ability of the light treated than the dietary treated fish at T3 (Fig. 11). However, it has been noted in a previous study on light and dietary treated fish that the light treated had lower hypoosmoregulatory ability than the dietary treated group at the end of the FW phase, but had developed full SW tolerance within a week after SW transfer, indicating that they nevertheless were fully ready for SW transfer (Jørgensen, E.H. and Striberny, A., personal communication). On the other hand, the dietary treated fish were 40 g bigger than the light stimulated ones (295 vs. 255 g respectively), and taking into account that growth rate in fish decreases with size (Jobling 1983), the dietary treated fish may have performed somewhat better in SW than the light stimulated ones.

Fish that undergo a true smoltification develop a high scope for growth, likely due to the increase in GH upon SW transfer (Handeland et al. 2000). The dietary treated group might not have developed this, but compensated for it by having a more energy efficient

hypoosmoregulation. Unfortunately, it was not possible to follow growth in these two groups for a longer time in SW, which would have provided more solid data on the long -term performance in SW in these treatment groups. However, both light and dietary stimulated smolts seemed to have a high performance after transfer to SW.

4.3 Feed intake and appetite regulators

All samplings were conducted at 10 am, one hour before start of feeding and excess feed was collected shortly after the end of feeding at 6.30 pm in order to avoid possible differences in gene expression between treatment groups as an effect of sampling in different circadian phases. The reduction in feed intake seen in both treatment groups at T2 compared to T1 (Fig. 9), could possibly be due to stress and elevated cortisol levels, which generally are known to reduce appetite (Madison et al. 2015). The reason for this was a 24 h sampling process taking place at T2, where fish from both treatment groups were sampled every 4 hours. Hence, the fish had been disturbed three times during the feeding period before the leftover feed was collected at 6.30 pm. At all other sampling points, only the pre-feeding sampling at 10 am was conducted. The significantly lower feed intake in the light treated group compared to the dietary treated group at T2, was most likely due to a reduced appetite caused by the SP exposure of the light treated group (Strand et al. 2018). After the light treated group had been taken back on LL, it was expected that the appetite would increase, however, this was not the case. This might have been due to the expected endocrine changes associated with the smoltification and increased cortisol and GH levels which stimulate mobilization of endogenous energy sources from lipid mobilization and protein catabolism (Björnsson et al. 1989, Stefansson et al. 2008). If so, the result indicate that the fish rely more on endogenous energy and substrate resources than feed in this period, which is consistent with the decrease in *K* from T2 to T3. The reduction in feed intake from T3 to T4 in the dietary treated group could, at least partly, be due to the fact that this group experienced a shift from the salt enriched feed to control feed at transference to SW. However, a reduction in feed intake shortly after SW transfer is a common phenomenon (Usher et al. 1991, Arnesen et al. 2003).

Concerning appetite regulation, one might expect that orexigenic neuropeptides/ peptides are upregulated, and anorexigenic regulators downregulated, when feed intake is high, and vice versa. In the following, the gene expression of central and peripheral appetite regulators will

be discussed in relation to the differences seen in feeding and growth between treatment groups, and within treatment groups over time.

4.3.1 Central neuropeptides

NPY is one of the strongest orexigenic signaling molecules in mammals, in teleosts however, data on NPY is elusive. In e.g. zebrafish (*Danio rerio*), rainbow trout and channel catfish (*Ictalurus punctatus*), intracerebroventricular (icv) NPY injections resulted in an increase in feeding (Rønnestad et al. 2017). Furthermore, food deprivation increased brain NPY expression in zebrafish, goldfish (*Carassius auratus*) and many other species (Rønnestad et al. 2017). A higher hypothalamic NPY expression was also found in fasting chinook and coho salmon (Silverstein et al. 1998). However, in Atlantic cod (*Gadhus morhua*), it was found that fasting did not affect NPY brain expression (Kehoe and Volkoff 2007). In the present study, the higher NPY expression in the light treated group compared to the dietary treated group at T1 (Fig. 13), despite the same feed intake between groups (Fig. 9), indicates an acute response to the abrupt change from continuous light to a short daylength in the light treated group. This increase did not comply with an orexigenic role of NPY, since feed intake went down, and may be related to functions other than appetite regulation. NPY is widely expressed throughout the brain and has a wide range of functions, for example a study conducted on rainbow trout showed that stress induced brain NPY expression levels (Doyon et al. 2003). Changes in brain NPY expression in the dietary treated group were only seen from T4 to T5. The decrease, which also tended to happen in the light treated group, could be due to the fact that at T5, brain sampling was conducted 7 h after the feeding ended and hence, that both groups were still in the absorptive state at this time while in the post-absorptive state on the other sampling time points (15 h after feeding ended). The timing of tissue sampling is important and it has been shown in Atlantic salmon that brain NPY expression increased concurrently with the onset of feeding and not prior to (Valen et al. 2011a), and remained high at 1.5 and 9 hours post- feeding.

In salmonids, previous studies have shown conflicting results on the role of AgRP and also differences between salmonid species. In the strongly seasonal Arctic charr it has been shown that voluntary fasting individuals had higher brain AgRP expression levels than feeding fish (Striberny et al. 2015). In Atlantic salmon, AgRP1 and AgRP2 brain mRNA levels increased

after feeding (Valen et al. 2011b), suggesting that AgRP works as an appetite inhibitor. On the contrary a recent study conducted on Atlantic salmon showed that 3 days of fasting increased hypothalamic AgRP1 expression, suggesting that AgRP1 acts as an orexigenic stimulator (Kalananthan et al. 2020). Another study on Atlantic salmon showed that 6 days fasting decreased AgRP1 expression, while AgRP2 expression remained unchanged between fasting and fed fish, suggesting that AgRP1 and AgRP2 have different roles in appetite regulation (Murashita et al. 2009a). In the present study, the increase in brain AgRP2 expression in the dietary treated group and tendency for a higher expression in the light treated group from T1 to T5 (Fig. 14), coincided with a decrease in feed intake in both groups (Fig. 9). These findings may point towards an anorexigenic role of AgRP2 in salmon, however, if this was the case one would have expected to see differences in AgRP2 expression between treatment groups at T2 and no such differences were found. Therefore, it is more likely that the observed change was a response to smoltification or smolt like developments by light and diet, respectively.

No significant differences over time were found in POMCA1 and 2 expression in any of the treatment groups (Fig. 15 and Fig. 16) and hence, there was no indication for a role of these in appetite regulation in the present study. Previous studies have concluded that POMC plays an anorexigenic role in fish; an increase in brain POMCA1 was seen in Atlantic salmon after feeding (Valen et al. 2011a), a decreased hypothalamic expression of POMCA1 was seen in fasted rainbow trout (Leder and Silverstein 2006) and a decreased food intake was seen in coho salmon after intraperitoneal injection with α- MSH, a POMC intermediate (White et al. 2016). The significantly higher POMCA1 expression in the light treated group than in the dietary treated group at T3 coincided with a lower feed intake in this group than in the dietary treated group. However, this high POMCA1 expression was not seen at T2 where the feed intake was the same as it was at T3 in the light treated group, hence this does not necessarily point to a difference related to appetite regulation. Rather, it is more likely that this difference was related to the treatment, since only the light treated groups seemed to go through a true smoltification. The decrease in *K* in this group between T2 and T3 indicates an increase in plasma GH and cortisol and may suggest that the metabolic changes upon return to continuous light may have affected POMCA1 expression. The reason for the tendency to lower POMCA1 and 2 expressions in the dietary treated group than in the light treated group at T5 is unknown, partly due to the missing data on feed intake at T5.

In goldfish, it has been demonstrated that brain CCK expression increased shortly after feeding (Peyon et al. 1999) and in rainbow trout appetite increased when peripheral CCK antagonists were used (Gélineau and Boujard 2001). A study conducted on Atlantic salmon showed that brain CCK-L expression increased during the hours after feeding, indicating a role during the initial phase of feed ingestion (Valen et al. 2011a).

To get a better picture of how possible anorexigenic appetite regulators change in relation to a meal, it would have been interesting and probably more visible to look at the effect on gene expression in the two treatment groups at both onset and after feeding. By measuring brain CCK-L expression one hour before feeding started, we found no significant changes in brain CCK-L expression over time or between treatment groups during the FW phase or first week in SW. Reasons for the significantly lower CCK- L expression in the dietary treated group compared to the light treated group at T5 are unknown.

4.3.2 Peripheral peptides in the intestine and stomach

The expression of the putative anorexigenic PYY as a response to feeding/fasting varies a lot across species and experimental design. Fasting decreased intestine PYY expression in piranha (*Pygocentrus nattereri*), increased intestine PYY expression in yellowtail (*Seriola quinqueradiata*) and had no effect on intestine PYY expression in Atlantic salmon (Murashita et al. 2009b, Rønnestad et al. 2017). Another study on Atlantic salmon showed that there were no postprandial changes in PYY gene expression during the first 12 h post feeding (Valen et al. 2011a). In the present study we looked at intestine PYY expression before feeding and found some significant changes and differences in expression over time within treatment groups and between treatment groups, respectively. The significantly higher PYY expression at T2 and significantly lower expression at T3 seen in the light treated group than in the dietary treated fish (Fig. 18) does not seem to be related to feed intake, which was lower in the light treated group than in the dietary treated group at both time points (Fig. 9). Intestinal PYY expression in the SW phase was generally lower than during the FW phase in the dietary treated group, possibly indicating a response to SW exposure. In the light treated group a reduction was seen from T2 to T3; thereafter it remained low through the SW phase, indicating that the differences in PYY expression were related to smolting in this group.

In some fish species, i.e. brown trout (*Salmo trutta*), grass carp (*Ctenopharyngodon idellus*) and tilapia (*Oreochromis mossambicus*), studies on ghrelin have shown responses indicating the same orexigenic function in fish as in mammals (Rønnestad et al. 2017). In rainbow trout the results in previous studies are conflicting; one study showed that central injections of ghrelin increased feeding after 24 h (Velasco et al. 2016), whereas another study showed that short- term central (1 h) and long- term (weeks) peripheral administration of ghrelin suppressed appetite (Jönsson et al. 2010). Fasting in rainbow trout has shown to decrease plasma ghrelin levels and a single intraperitoneal injection with ghrelin did not affect food intake 12 h post -injection (Jönsson et al. 2007). A study conducted on Atlantic salmon showed that 2 days fasted fish had lower ghrelin- 1 mRNA levels in the stomach compared to fed fish, however no significant differences was found between fasted and fed fish after 14 days of fasting (Hevrøy et al. 2011). Another study on Atlantic salmon found increased ghrelin- 1 mRNA levels in the stomach after 6 days of fasting (Murashita et al. 2009b). It seems like the different sampling times in these experiments are an important factor to take into consideration when looking into the role of ghrelin and explain the contradictory results that have been found. The results in the present study revealed very similar responses to ghrelin-1 and ghrelin-2 to treatment and time, albeit the significances of changes and differences varied somewhat between ghrelin-1 and -2 (Fig. 19 and Fig. 20). The significantly lower ghrelin-1 expression seen in the light treated group compared to the dietary treated group at T1 could be an acute response to the abrupt short day exposure of the light treated group (1 day on SP). There was a trend for a decrease in ghrelin-1 and -2 expression during the FW phase in the dietary treated group, and an opposite trend in the light treated group. This resulted in a significantly higher ghrelin- 1 and -2 expression in the light treated than in the dietary treated group at T3, which again opposes an orexigenic role of ghrelin in Atlantic salmon since feed intake at T3 was lower in the light- than in the dietary treated group. The marked increase in ghrelin- 1 and -2 expression in the light treated group from T3 to T4, could indicate a response to SW transfer *per se*, since feed intake did not differ between T3 and T4. Ghrelin is a ligand of growth hormone secretagogue receptor (Chanoine et al. 2009) and it is possible that the increase seen was related to the transient increase in plasma GH levels seen after SW transfer of smolts. For example, it was shown that plasma GH levels of smolts increased and remained elevated for a week in Atlantic salmon smolts transferred to SW with a comparable temperature as in the present study (Handeland et al. 2000). The decrease in ghrelin expression between T4 and T5 is also in line with the decrease in plasma GH levels seen after one week in SW (Handeland et al. 2000). A role of ghrelin during

smoltification in Atlantic salmon remains to be investigated in future experiments. But given that ghrelin is involved in stimulation of the somatotrophic axis during smoltification, the attenuated increase in ghrelin expression in the dietary treated group as compared to the light treated group between T3 and T5 would provide further support for the conclusion that only the light treated group went through a complete smoltification.

Some notes of caution must be added regarding studies on appetite regulation mechanisms. The whole brain was sampled in the present study. Hence, the samples included RNA mix from several brain compartments and may therefore encompass changes that may be associated with other roles than appetite regulation. Further, changes and differences in mRNA abundance do not always reflect similar changes and differences at the protein levels, which may result in wrong conclusions.

5 Conclusions

The following conclusion can be derived from the present study:

1: The light treatment resulted in a reduced growth and smaller smolt compared to the dietary treatment. However, there was a reduction in SGR in the dietary treated group when the dietary treatment was given, likely due to a reduction in feed utilization.

2: A significant reduction in *K* was only seen in the light treated group, indicating that this group went through a true smoltification and the dietary treated group did not.

3: The dietary treated group had the best SW tolerance throughout the FW phase, which seemed to have a positive effect on SW performance and a fast adaptation to SW. The light treated group did not have as good SW tolerance as the dietary treated group throughout the FW phase. However, the decrease in plasma osmolality seen when the light treated group was back on LL, confirmed that the increase in daylength stimulated hypoosmoregulatory ability.

4: SGRs of both treatment groups in SW were similar, leading to the conclusion that feed supplemented with a salt mix and the free tryptophan is a good alternative to the traditional light treatment.

5: Changes in NPY, POMCA1 and 2, PYY and ghrelin over time are interesting and could be related to preparation for a life in SW. The marked increase in ghrelin-1 and -2 in the light

treated group at T4 and decrease at T5, could be seen in relation to changes in plasma GH levels seen after SW transfer, as ghrelin is a GH secreatagogue receptor agonist, but also this needs further investigation.

6: The measurement of central and peripheral appetite regulators did not bring clear evidences for their putative anorexigenic or orexigenic roles, despite changes in appetite over time within treatment groups and differences between groups.

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Appendix I – Supplementary tables

Supplementary table 1: Body mass and *K* of fish (n= 6-8/ treatment group) sampled for qPCR on each sampling point (T1-T5). Data presented as mean ± SEM.

Time		**Treatment**	**Body mass (g)**	***K***
T1	23.04.19	LL	42.8 ± 1.3	1.30 ± 0.01
	24.04.19	SP (1 day)	43.5 ± 2.0	1.30 ± 0.02
T2	04.06.19	LL	90.9 ± 3.6	1.39 ± 0.03
	05.06.19	SP	70.7 ± 4.9	1.34 ± 0.02
T3	17.07.19	LL+ diet	151.5 ± 6.4	1.52 ± 0.03
	16.07.19	SP-LL	109.4 ± 7.3	1.29 ± 0.02
T4	29.07.19	LL	128.4 ± 6.8	1.26 ± 0.03
	29.07.19	SP-LL	116.3 ± 11.2	1.17 ± 0.04
T5	24.09.19	LL	297.0 ± 12.2	1.34 ± 0.04
	25.09.19	SP-LL	251.8 ± 13.8	1.23 ± 0.03

Supplementary table 2: Body mass and *K* of fish (n= 9/treatment group) that survived the 24 h SWCT at T1, T2 and T3. Data presented as mean ± SEM.

Time	**Treatment**	**Body mass (g)**	***K***	**Mortality (n)**
T1				
23.04.19	LL	40.7 ± 2.4	1.20 ± 0.02	0
24.04.19	SP (1 day)	43.6 ± 1.6	1.18 ± 0.02	0
T2				
04.06.19	LL	90.1 ± 4.9	1.30 ± 0.02	0
05.06.19	SP	63.6 ± 3.0	1.31 ± 0.02	0
T3				
17.07.19	LL+ diet	135.0 ± 7.2	1.23 ± 0.03	0
16.07.19	SP-LL	110.8 ± 9.0	1.21 ± 0.02	0