Effects of temperature regimes on proportion of early maturation and the activation of the BPG axis in male Atlantic salmon (*salmo salar L.*) postsmolts

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Abstract

Early maturation of Atlantic salmon (*salmo salar L.*) male postsmolts is a growing concern for the Norwegian aquaculture industry due to an increasing occurrence in intensive rearing facilities, negatively impacting growth, welfare, and survival after transfer to sea. The neuroendocrine pathway to maturation is not well known in fish, and finding key hormones involved in the regulation could give insight to how, and when, temperature exerts influence on the decision to mature early. The experiment consisted of rearing four groups in duplicates under four different temperature profiles (15, 15-8L, 15-8E, and 8) from May 24th to November 2nd, 2022. Atlantic salmon in the four treatments (900 parr, initial weight ~50g) was reared in flow-through under continuous light (LD24:0, LL) with a 5-week winter signal (LD12:12) introduced on the 28th of July 2022 to promote developmental events. Two groups were kept at constant temperature: group 15 at 15°C and group 8 at 8°C. Two groups experienced a decrease in temperature from 15°C to 8°C simultaneously to change in photoperiod: group 15-8E at the start of WS and 15-8L at the end of WS. Body weight, condition factor (K), gonadosomatic index (GSI) and transcript levels from the diencephalon brain region of gonadotropin releasing hormones (*gnrh2, gnrh3*), deiodinase type 2 (*dio2b*), kisspeptin receptor (*gpr54*), and gonadotropin inhibitory hormone (*gniha*) were assessed. Results showed that rearing individuals at 15°C allowed for higher energy accumulation, leading to 100% of male maturity in mid-September after WS, while there was no maturation seen in the group reared at 8°C. There were little physiological differences between the two temperature regimes (15-8E, 15-8L). Results suggest that reduction in temperature decreases physiological development, leading to a lower percentage of maturing individuals than the constant 15°C treatment, however, the timing of reduction in temperature from 15°C to 8°C did not make a significant difference in biometry. Results from the gene transcripts revealed temperature to influence expression, showing a trend between genes in the lower temperature treatment (8°C) following increase in photoperiod after WS, while elevated temperature appeared to have a disruptive effect on the BPG axis causing early maturation. The results of this thesis further supports the use of less intensive water temperatures in industry production in order to reduce occurrences of early maturation. The clear effect of temperature on sexual maturation remains a mystery at the neuroendocrine level. Results suggest a disruption of the BPG pathway at elevated temperatures, but the picture of regulatory factors remains unclear.

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And to all those whom I have tried explaining my thesis to, I apologise, and I promise, this will be the last time.

Happy reading,

Marte Karlsen, May 2023

"I want to thank me for believing in me. I want to thank me for doing all this hard work."
– The lord and saviour, Snoop Dogg.

Glossary of species (alphabetic order)

Carassius auratus	Goldfish
Coturnix japonica	Japanese quail
Cyprinodon variegatus	Sheepshead minnow
Danio reio	Zebrafish
Dicentrarchus labrax	European sea bass
Oncorhynchus masou	Masu salmon
Oncorhynchus mykiss	Rainbow trout
Oncorhynchus nerka	Sockeye salmon
Oreochromis niloticus	Nile tilapia
Oryzias latipes	Medaka
Salmo salar	Atlantic salmon

Abbreviations (alphabetic order)

1R	First round of whole genome duplication
2R	Second round of whole genome duplication
3R	Third round of whole genome duplication
4R	Fourth round of whole genome duplication
ANOVA	Analysis of variance
BPG	Brain-pituitary-gonad
cDNA	Complementary DNA
Cq	Quantification cycle
DIO2/3	Deiodinase type 2/3 enzyme
DNA	Deoxyribonucleic acid
Fsh	Follicle-stimulating hormone
FW	Freshwater
FRW	Forward primer
GnIH	Gonadotropin-inhibitor hormone

GnRH	Gonadotropin-releasing hormone
GPR54	G protein-coupled receptor / Kisspeptin receptor
GSI	Gonadosomatic index
K	condition factor
KISS	Kisspeptin
LD	Light Dark photoperiod
Lh	Luteinizing hormone
LL	Continuous light photoperiod
mRNA	Messenger RNA
PT	Pars tuberalis
qPCR	Real-time Quantitative Polymerase Chain Reaction
RAS	Recirculating Aquaculture System
RNA	Ribonucleic acid
RV	Reverse primer
SDG	Sustainable Development Goals
SEM	Standard mean error
SSWDG	Salmonid specific whole genome duplication
SW	Seawater
T3	Triiodothyronine
T4	Thyroxine
TSH	Thyroid-stimulating hormone
TSWDG	Teleost specific whole genome duplication
UN	United Nations
VA	Vertebrate ancient
WGD	Whole genome duplication
WS	Winter signal

Nomenclature:

This thesis applies the nomenclature recommendations of ZFIN

(https://zfin.atlassian.net/wiki/spaces/general/pages/1818394635/ZFIN+Zebrafish+Nomenclature+Conventions):

- Gene/protein fish – Gene transcript: *fsh* (italicized, small letters). Protein: Fsh (first letter uppercase)
- Gene/protein human – Gene transcript: *FSH* (italicized, uppercase). Protein: FSH (uppercase)
- Gene/protein rodent – Gene transcript: *Fsh* (italicized, first letter uppercase). Protein: FSH (uppercase)
- GnRH**2**/GnRH**3** – paralogs indicated by numbers = 3R
- GnIH**a**/GnIH**b** – paralogs indicated by lower case letters = 4R

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1 Introduction

1.1 Challenge of early maturation in Norwegian Salmon Aquaculture

Norwegian production of Atlantic salmon (*salmo salar L.*) has become highly intensive since its inception in the 1970s and is now Norway´s fourth biggest export commodity, bringing in a yearly revenue of over NOK 70 billion (Yajie Liu et al., 2011; Olaussen, 2018; StatistiskSentralbyrå, 2020). According to BarentsWatch (2022) there were 1065 registered farming sites in Norway in 2021, and in line with the United Nation´s (UN) Sustainable Developmental Goals (SDG) along with the ambition to provide the growing population with a sustainable protein source, the industry is set to grow 5-fold by 2050. However, though the industry increases its revenue each passing year, the exponential growth has in recent years stagnated in large part due to clashes of development and environmental challenges (FAO, 2020). These challenges are unacceptable according to the United Nations (UN) Sustainable Development Goals (SDGs) (United Nation, 2015), and in an attempt to overcome this challenges and meet the UN goals, the industry has shifted from the traditional farming system towards land-based farming.

Traditional salmon farming comprises of two main production systems, early on-land freshwater (FW) flow-through systems for hatching and rearing of smolt (8 – 12 months), followed by the large-scale grow-out stage in seawater (SW) in net-pens or cages (12 – 24 months) until slaughter (4.5 – 6kg) (Taranger et al., 2010). However, this traditional production has raised question and concerns from the government, the public, and stakeholder on the sustainability of this strategy long-term. The concerns are mainly related to the SW production, its impact on wild populations, the pollution from production, as well as the welfare of the farmed fish after SW transfer (Dalsgaard et al., 2013; Hynes et al., 2018). The biological challenges faced today include the infestation of sea lice and diseases in cultured salmon that could cause mortality and potentially spread to wild strains, the risk of genetic loss due to escapee introgression, welfare and mortality rates in sea-launched smolt, and organic and chemical waste pollution (Brauner et al., 2012; Yajie Liu et al., 2011; Torrissen et al., 2013). To combat the sustainability issues, reduce the SW production period (by producing bigger smolt), improve public opinion and fish welfare, several innovative production systems have been invented, such as semi-closed or closed-containment systems,

such as recirculating aquaculture system (RAS), and the production of postsmolts up to larger size (500g). The exact definition of "postsmolts" is not agreed upon, but in terms of its use in Norway it refers to salmon that has gone through smoltification and have been transferred to SW (Bjørndal & Tusvik, 2017). In this document the term "postsmolts" will refer to the Norwegian approach. The aim of the rearing of postsmolts, an extended land-based phase in RAS, is to reduce time spent at sea, thus shortening the SW production and decreasing the risk of escapees, sea lice infestation, diseases, and mortality.

While land-based rearing facilities solve a number of sustainability issues and boosts the growth potential of individuals, the intensive rearing conditions used in such land-based facilities bring along some production challenges such as an increased occurrence of early male maturation (Fjelldal et al., 2011; Good & Davidson, 2016; Imsland et al., 2014). Land-based rearing allows for the artificial control of environmental parameter, such as photoperiod and water temperature, creating the optimal conditions for quick growth but also for maturation (Dalsgaard et al., 2013). Maturation causes stagnant growth, decreased filet quality, and osmoregulatory changes which could cause mortality, representing a serious loss in production for the aquaculture industry (Yajie Liu et al., 2011; Mobley et al., 2021; Taranger et al., 2010). Better understanding on how early maturation can be mitigated in these intensive rearing facilities requires more knowledge on the neuroendocrine pathway that leads to the onset of maturation and how internal and external cues effect it. These pathways in seasonal breeding mammals and birds are well studied and highly conserved and used for the basis for this research into the neuroendocrine regulation of early maturation in Atlantic salmon.

1.2 Atlantic salmon life cycle

Atlantic salmon is an anadromous fish in the Salmonidae family (Thorpe, 1986). Like other species in the family, salmon typically spend part of its life in freshwater (FW) and parts in seawater (SW) (McCormick, 2012). The life cycle of wild Atlantic salmon (Figure 1) begins with mature adult salmon spawning in their natal river, during the autumn and winter months. Adult spawning females bury their eggs in the river gravel, creating nests (redds). The eggs are fertilized by one or more milt-releasing males. The roe develops over winter and hatch in early spring into alevins. Alevins hatch with a part of the yolk-sac still attached and uses this

for nutrients for several weeks post-hatching. When the nutrient-sac is depleted, the fish emerge from the gravel and swim up to the surface and commence first feeding. Now referred to as fry, the fish starts predating on microscopic invertebrates. During late summer and early autumn, the fish develops into parr, distinctly characterized by dark vertical bands along its body length (known as parr marks), territorial behaviour, and feeding on bigger invertebrates. Parr remain in the river until a certain size threshold (10-15cm) is reached. When the growth threshold is reached, they undergo a crucial life history transformation called smoltification. Smoltification is a series of physiological, morphological, and behavioural changes the salmon undergoes to transition from a life in FW to SW (Adams & Thorpe, 1989; McCormick, 2012; McCormick & Saunders, 1987; Mobley et al., 2021). The juvenile fish, now smolts, begins swimming downriver towards the sea where they will spend most of their adult life. Adult salmon spend anywhere between 1-5 years feeding in the ocean until their energy reserves are full before they return migration to their natal river for spawning. Sexual maturation happens while migration up the river and when the reach the top, spawning occurs.
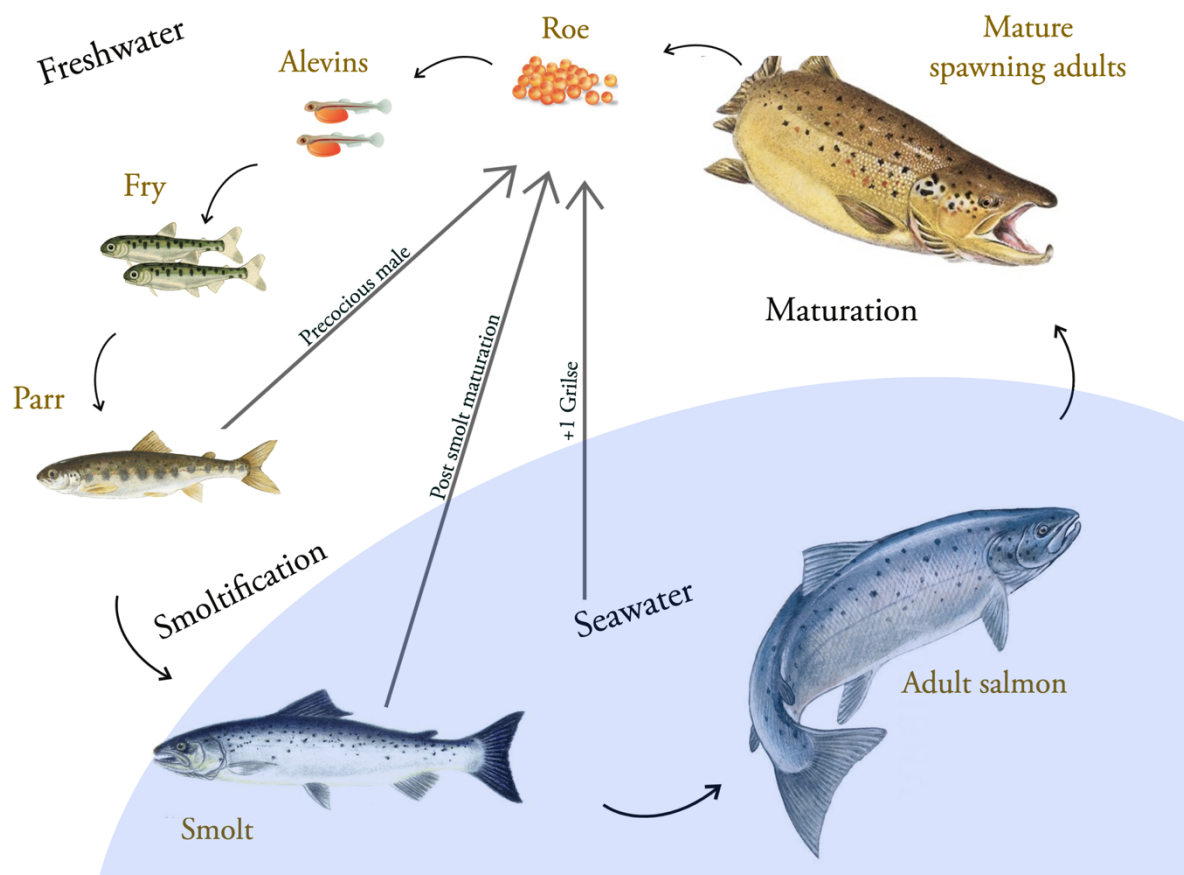

Figure 1: Schematic overview of the life cycle of Atlantic salmon. *Figure shows the different life stages of Atlantic salmon. Eggs from adult spawning females are fertilized by adult spawning males in*

autumn. The roe hatches into Alevins during spring and develops into fry. The juvenile fish develops into parr over summer and from here the fish either goes through smoltification, becoming smolts, or mature (precocious male) and partake in spawning. The juvenile fish can spend between 1-7 years in the river before smoltifying. Upon migrating to the sea, the salmon experiences exponential growth. Usually, an individual spends 2-4 years at sea before returning to freshwater to spawn, but there are incidences where individuals, called grilse, only spend 1 year at sea before returning. A phenomenon called post smolt maturation ("jacking") has been observed in smolt rearing facilities where smolts mature after transfer to seawater. This is the thought to be the result of intense rearing, with high temperatures and unnatural photoperiods.

Smoltification, or parr-smolt transformation, is a series of physiological, morphological, and behavioural changes, induced by the increase in photoperiod and the warmer temperatures during spring (McCormick & Saunders, 1987). During this transition to a marine environment the juvenile salmon take on a silvery colouration and a decrease in conditions factor (K), while their fins also take on darker colouration. Organs like the gills, kidneys, and gut goes through osmoregulatory adaptions to SW and behaviourally the fish start to showcase a negative rheotaxis, less aggression, and begin to school. Smoltification depends on photoperiod and temperature and developmental thresholds. Thorpe et al. have argued that there are certain developmental thresholds and growth parameters that are determining of the timing of smoltification, and if the overwinter lipid storage of the parr is sufficient, it will smoltify the following spring (Thorpe, 2007; Thorpe et al., 1998; Thorpe & Metcalfe, 1998).

For Atlantic salmon, an alternate life history strategy may occur when male parr, instead of smoltifying, become sexually mature. Referred to as precocious parr, these males have the shape of a parr but have fully running milt and can take part in spawning (Good & Davidson, 2016; Saunders et al., 1983). This is believed to be in part due to genetic and favourable environmental conditions, allowing the precocious male to reach an energy threshold that allows for spawning (Adams & Thorpe, 1989; Saunders et al., 1983). A different phenomenon, called post-smolt maturation ("jacking" or "jacks"), is the maturation of post-smolts shortly after smoltification (Fjelldal et al., 2011; Imsland et al., 2014). This phenotype is rarely observed in the wild and is thought to be a response to the intense farming conditions (Fraser et al., 2019).

1.3 Sexual maturation in Atlantic salmon

The life cycle of Atlantic salmon shows considerable plasticity in life history variation, the timing and routing of maturation varying greatly both within and between strains (Good & Davidson, 2016; Thorpe et al., 1998). The onset of puberty in teleost is linked to genetic factors, metabolic signals e.g. related to energy stores, and environmental conditions and although the precise pathway that leads to maturation in fish is generally not well known, puberty implies the functional competence of the brain-pituitary-gonad (BPG) axis (Schulz et al., 2010; Taranger et al., 2010). Maturation commences based on certain biological thresholds such as size, growth rate, genetics, energy status, and environmental conditions (Thorpe, 1994). During the maturation process appetite and feed intake decreases with hormonal changes, such as increased production of leptin which inhibits hunger (Kadri et al., 1996; Taranger et al., 2010). This stunts the growth and leads to weight loss which is a direct consequence of the reallocation of energy to the development of the gonads, gametogenesis, and secondary sex characteristics (Kadri et al., 1996; Thorpe, 1986). The muscle tissue of sexually mature salmon experiences a marked depletion in lipids, protein, and astaxanthin (red pigment) due to the allocation of investment of energy (Taranger et al., 2010). Reproduction related behaviour also drains heavily on the energy reserves of salmonids, and it has been calculated that as much as 59% of the energy reserve is spent during maturation (I. A. Fleming, 1998). Maturation can also have an impact on the immune system of the fish, in part due immunomodulatory role of hormones that change in association with reproduction, and in part due to the agonistic behaviour associated with maturation can lead to skin damage and thus also impact the susceptibility for secondary infections and parasite infections (Taranger et al., 2010).

Early sexual maturation is a natural occurrence the wild, where some parr mature before smoltification and par-take in spawning. Precocious males will mature if conditions allow for fulfilment of required energy thresholds by key period (Thorpe, 1994, 2007; Thorpe et al., 1998). Due to the artificial conditions to promote growth in smolt producing facilities, this threshold may be reached earlier and for far more individuals than in the wild and the issue of early maturation becomes a bigger problem (Dalsgaard et al., 2013; Imsland et al., 2014). The high energy availability in rearing facilities could be the reason for the issue of post-smolt maturation, individuals who mature right after smoltification (Fjelldal et al., 2011; Good & Davidson, 2016). This phenomenon has only been observed in aquaculture-conditions and

only in male individuals. The reason for the male-only early maturation phenomenon is most likely due to the fact that it requires a greater amount of energy to develop ovaries that testis (Mobley et al., 2021; Taranger et al., 2010; Thorpe, 1994). The deleterious nature of sexual maturation is considered a huge welfare issue, and the hypo-osmoregulatory ability of farmed Atlantic salmon can also be compromised if kept in SW throughout the reproductive process, resulting in dehydration and eventually mortality (McCormick & Saunders, 1987; Taranger et al., 2010).

1.4 Regulation of sexual maturation

1.4.1 Genetic background

The influence of genetics on maturation is often linked to genetic factors determining growth and availability of energy, which could be the reason for the variability of age at maturation in Atlantic salmon (Thorpe, 2007). The discovery of the existence of the vestigial-like protein 3 (*vgll3*) locus reported that the gene controls 35-38% of the variation of age at maturity in wild and domesticated Atlantic salmon (Ayllon et al., 2015). In farmed salmon, the homozygous males with the early maturing genotype of the *vgll3* locus were more likely to mature earlier than the homozygous males with the late maturing genotype and the heterozygous males, demonstrating the genetic control of early maturation (Ayllon et al., 2019; Fjelldal et al., 2020). The recent discovery of a genetic factor that plays a role in determining age-at-maturation time exemplifies the lack of knowledge surrounding this topic. With high resolution genomic sequencing techniques, understanding the genetic influence on age-at-maturation may be greatly advanced in the coming decades.

1.4.2 Whole Genome Duplication event

Whole genome duplication (WGD) events are mutational events in evolutionary history where the whole genome is duplicated. Ohno (1970) suggested that WGDs could be the main mechanism behind the increased complexity during evolution. He hypothesized that the original function of the genome would retain its roles, while the duplicated genes could take on a new functions which could allow for large-scale adaptations (Ohno, 1970). Teleosts share at least three rounds of WGDs (Lien et al., 2016). 1R and 2R happened before the divergence of lamprey (Petromyzontomorphi) from jawed vertebrates (Gnathostomata) and a third teleost-specific WGD (Ts3R or 3R) occurred at the base of teleosts around 320 million

years ago (Meyer & Van De Peer, 2005; Smith et al., 2013). Salmonids have gone through a fourth WGD (Lien et al., 2016). The salmonid-specific WGD (Ss4R or 4R) is thought to have happened roughly 80 million years ago in a common salmonid ancestor.

For each WGD the genome is duplicated, meaning that salmonids having gone through 4R have 16 duplications (1:2:4:8:16) (Meyer & Van De Peer, 2005). Mutations in the new duplicated gene can lead to new genetic information, information that codes for new proteins with new functions. Predominantly after a duplication event, the two paralogue daughter genes are identical and serve the same function (redundancy), however, in rare cases, the duplicated genes may adopt new functions in three potential fates; one being silenced by degenerative mutations (non-functionality); one retaining the original function while one attaining a new and beneficial function preserved by natural selection (neo-functionality); or both copies are partially compromised and then together serving the original function of the single-copy ancestral gene (sub-functionality) (Lynch & Conery, 2000). Lien et al. (2016) found that there was a duplicate loss in Atlantic salmon following 4R likely caused by a higher frequency of neo-functional than sub-functional cases. They also found that 20% of the duplicates from 3R and 55% from 4R have been retained in a functional manner (Lien et al., 2016).

1.4.3 Anatomy of the teleost brain

The teleost brain is composed of three main regions as in other vertebrates. In fish the three brain regions are called the *prosencephalon*, *mesencephalon*, and *metencephalon* (Kryvi & Poppe, 2021, pp. 147-153; Yamamoto, 2009). Both the pro- and metencephalon is subdivided further into two regions making five regions: *telencephalon*, *diencephalon*, *mesencephalon*, *metencephalon*, and *medulla oblongata*. The diencephalon is a protruding region in the teleost brain, containing the hypothalamus, thalamus, and chiasma opticus. The hypothalamus is the regulatory hub of the brain, controlling homeostasis, while thalamus (grey substance) is important for the coordination of sensorics and motoric. Uniquely among vertebrates, teleost fish does not have a hypothalamo-pituitary portal system, the pars tuberalis (PT) (Whitlock et al., 2019), instead nerve fibres from the brain are directly innervated to the dorsal end of the pituitary (pars nervosa). Therefore, the activity of the

different cell types are controlled by neurohormones released directly by nerve endings located close to their target cells (Zohar et al., 2010).

1.4.4 Neuroendocrine control

For vertebrates in general, the BPG axis plays a crucial role in regulating reproduction (Nakane & Yoshimura, 2014). The BPG axis is a complex system of hormonal regulation, and this neuroendocrine junction is activated in response to internal (e.g. size, energy status, growth factor, and genetics) and external cues (e.g. photoperiod, temperature, and diet) (Schulz et al., 2010; Taranger et al., 2010). The endocrine response to activation of the BPG axis leads to the onset of maturation. In mammals and birds, exposure to long day results in the stimulation of thyroid-stimulating hormone (TSH) in the PT of the pituitary gland, which in turn stimulates increased levels of type 2 iodothyronine deiodinase (DIO2) in the hypothalamus (Dardente et al., 2016; Nakane & Yoshimura, 2014). DIO2 converts thyroxine (T4) to the more active triiodothyronine (T3). T3 further stimulates the neuropeptide kisspeptin (KISS1), which in turn stimulates the production of gonadotropin-releasing hormone (GnRH) 1. Long-day stimuli, subsequentially, decrease expression of type 3 deiodinase (DIO3), which converts both T4 and T3 to inactive metabolites. The production of PT-TSH is stimulated by the rise and fall of melatonin in mammals (Bronson, 2009; Dardente et al., 2016), while in birds it is stimulated by light information in deep-brain opsin photoreceptors (Hang et al., 2016; Nakane & Yoshimura, 2014; Philp et al., 2000). However, in fish, the PT of the pituitary is not anatomically distinct. Despite similarities in the signalling pathway of the BPG axis there are fundamental differences between how light is perceived. Mammals have melatonin, birds have deep-brain photoreceptors, salmon have both but neither solely regulate the BPG axis.

Melatonin plays a key role in regulating photoperiodic responses in both mammals and teleosts, as it provides a physiological signal of the length of night (for review: (Ciani et al., 2021). Despite a number of teleosts showing relationship between melatonin and gonadotropin release (though inconsistent and contradictory); no similar relationship has been established in salmonids to date (Kuz'mina, 2020). Recently, Horne et al (2022) mapped the localization of GnRH to the same neurons as the deep brain photoreceptor

vertebrate ancient (VA) opsin, suggesting a direct photoreceptive regulation of the onset of maturation, much like what happens in birds.

TSH is a pituitary glycoprotein composed of two subunits, $tsh\alpha$ and $tsh\beta$. Maugars et al. (2014) discovered several $tsh\beta$ paralogs in the genome of a number of teleost species that arose from the 3R WGD. Furthermore, two paralogs of the beta-subunit was recently found in Atlantic salmon, Tshßa and Tshßb, where transcription of $tsh\beta b$, located in the dorsal region of the proximal pars distalis, is greatly stimulated during spring (M. S. Fleming et al., 2019, 2020). An increase in Tsh expression in mammals and birds due to an increase in daylength is stimulated by $Dio2$ expression in the PT (Nakane & Yoshimura, 2014). Lorgen et al. (2015) identified two Dio2 paralogs, $dio2a$ and $dio2b$ in Atlantic salmon thought to have originated from 4R. The study found that $dio2b$ expression is dependent on light regulation related to the freshwater period and smoltification, while $dio2a$ was induced in the gills during transfer to sea water (Lorgen et al., 2015). This could indicate a system for photoperiodic signalling ($tsh\beta b$ – $dio2b$) exists in fish, similar to what occurs in mammals and birds (Figure 2). Irachi et al. (2021) found no significant effect of photoperiod on Dio3 paralog $dio3a$ in Atlantic salmon, indicating that $dio2b$ may be the main pathway for photoperiodic signalling, and a system similar to that in birds and mammals exists in fish as well.
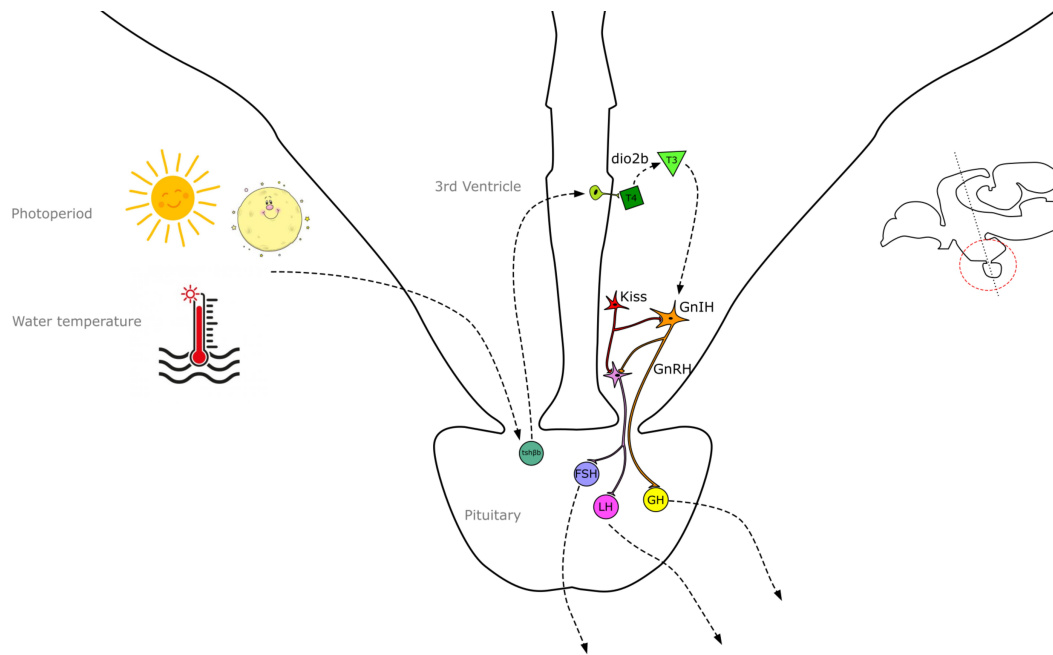
Figure 2: *Schematic representation of regulatory signalling pathways in the BPG axis during sexual maturation in Atlantic salmon. Schematic overview of the proposed BPG axis in Atlantic salmon with the roles of Dio2b, kisspeptin/Gpr54, Gnrh, and Gnih. External cues and internal factors activate Tshßb, which in turn stimulates the Dio2b. Dio2b stimulate the kisspeptin system which activates Gnrh neurons leading to the onset of spermatogenesis. Gnih is believed to either have a stimulatory of inhibitory effect on Gnrh-release.*

1.4.5 Gonadotropin-releasing hormone (GnRH)

It is well established that gonadotropin-releasing hormone (GnRH), a highly conserved neuroendocrine decapeptide, is the major upstream regulator of the pituitary gonadotropins, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), in vertebrates (Whitlock et al., 2019; Zohar et al., 2010). GnRH released from the hypothalamus stimulates the synthesis and release of Fsh and Lh from the anterior pituitary, which in turn activates somatic cells in the gonads to induce the synthesis and release of gonadal steroids and peptide hormones, ultimately promoting gonad development and maturation (Nóbrega et al., 2009; Schulz et al., 2010; Taranger et al., 2010). GnRH is expressed in the brains of all vertebrate species in at least two paralogs, with some teleost species expressing three forms (Ciani et al., 2020; Zohar et al., 2010).

GnRH is usually split into three main branches; GnRH1 is found in the hypothalamus of amphibians, mammals, and a number of fish species, GnRH2 (chicken GnRH or cGnRH2) describes the forms found in the mesencephalon of vertebrates, and GnRH3 (salmonGnRH3 or sGnRH3) includes only the salmon (teleost) isoforms. (Amano, Urano, et al., 1997; Kuo et al., 2005; Okubo & Nagahama, 2008; Whitlock et al., 2019; Zohar et al., 2010). It is suggested that GnRH branches 1 and 2 are found in both terrestrial vertebrates and fish indicates that these branches are ancient and emerged before the divergence of these groups and duplication (1R and 2R) of an ancestral GnRH isoform, while GnRH3 is from the teleost specific 3R duplication (Okubo & Nagahama, 2008; Sefideh et al., 2014). The *gnrh1* gene, responsible for stimulating the release of the gonadotrophs in most vertebrates, is lacking in certain teleosts like Cyprinidae and Salmonidae, while other teleostean species, like Siluriformes or Anguilliformes, lack the *gnrh3* gene (Kuo et al., 2005; Muñoz-Cueto et al., 2020; Okubo & Nagahama, 2008; Whitlock et al., 2019). The lack of the *gnrh1* in Salmonidae poses the obvious question, which gene exerts the Gnrh1 function. Kuo et al. (2005) summarized that the location of the zebrafish (*Danio reio*) *gnrh3* promoter in the telencephalon and its sequence, which is also conserved in the *gnrh1* gene, suggested that teleost *gnrh3* could possess a similar regulatory domain to mammalian GnRH1. Ciani et al. (2020) recently identified a total of 6 GnRHr (GnRH-receptors) paralogs in Atlantic salmon, all, except for one, expressed in male parr pituitary during gonadal development.

1.4.6   Kisspeptin and the gpr54 system

Kisspeptins, the peptide products of the *kiss1* gene, and its G protein-coupled receptor GPR54 (Kiss1r) have been identified as key factors in the regulation of GnRH secretion and controlling onset of maturation in mammals (Roa et al., 2008; Seminara et al., 2003; Taranger et al., 2010). Research into the system and whether a similar system exists in teleost is still in its early stages, however, *kiss1* expressing neurons have been identified in the brains of several species of teleost, suggesting that the Kisspeptin/Gpr54 system is conserved in teleost evolution (Chi et al., 2017). Different from the mammalian Kisspeptin/GPR54 system, non-mammalian vertebrates have multiple kisspeptin/Gpr54 paralogs. Teleosts have two paralogous kisspeptin genes (*kiss1* and *kiss2*) and four genes encoding the kisspeptin receptors, although most teleost species only have two (*kiss2r/gpr54-2b* and *kiss3r/gpr54-1b*) (Ohga et al., 2018). The first evidence of a kisspeptin system in teleost fishes came from the Nile tilapia (*Oreochromis niloticus*), where Gnrh1 neurons express *kiss2r* mRNA (Parhar et

al., 2004). However, in zebrafish normal gonadal development and maturation was displayed with *kiss* or *kissr*-knockout mutants (Tang et al., 2015). This could indicate that the kisspeptin system is dispensable for maturation in some teleosts species. Studies into medaka (*Oryzias latipes*) (Kanda et al., 2013) and European sea bass (*Dicentrarchus labrax*) (Escobar et al., 2013) have both indicated that their Gnrh1 neurons does not express kisspeptin receptors. As mentioned earlier, GnRH1 is lost in salmonids, however, Chi et al. (2017) found co-expression of *kiss2r* (*gpr54 or salmon gpr54(sgpr54)*) and the Gnrh paralog *gnrh3* in the hypothalamus of Atlantic salmon, indicating that kisspeptin may directly influence GnRH secretion primarily during the onset of maturation and the later stages of gonad development.

1.4.7 Gonadotropin-inhibitory hormone (GnIH)

Gonadotropin-inhibitory hormone (GnIH) is hypothalamic neuropeptide involved in the regulation of the BPG axis first discovered in Japanese quail (*Coturnix japonica*) by Tsutsui et al. (2000). In mammals and birds, GnIH acts directly on the pituitary to inhibit the release of gonadotropins (Di Yorio et al., 2019; Kriegsfeld et al., 2015; Tsutsui et al., 2000, 2012). Gnih has also been identified in several species of teleosts, although research into its location and function is scarce and seems to vary drastically between species (Di Yorio et al., 2019; Sawada et al., 2002; Zhang et al., 2010). Gnih has been found to have both stimulating and inhibitory effect on the gonadotropin secretion and reproduction in fish (Muñoz-Cueto et al., 2017). In goldfish (*Carassius auratus*), intraperitoneal administration of zebrafish GnIH has been shown to inhibit gonadotropin levels (Zhang et al., 2010), while goldfish GnIH has been shown to stimulate gonadotropin release from cultured sockeye salmon (*Oncorhynchus nerka*) pituitary cells (Amano et al., 2006). In some analysed species, immunohistochemistry revealed the presence of Gnih-immunoreactive cells in several brain regions (e.g. telencephalon, diencephalon, mesencephalon), unlike birds and mammals where it is restricted to the posterior preoptic area/hypothalamus (Di Yorio et al., 2019; Muñoz-Cueto et al., 2017). However, mapping of the expression pattern of *gnih* in Atlantic salmon revealed a distinct cluster of cells in the diencephalon, in the ventral thalamus close to the third ventricle (Horne et al., 2022). In mammals and birds there are evidence supporting the fact that GnIH is mediated through the effects of photoperiod and temperature, as other systems in the BPG axis. Recently, Maugars et al. (2020) found two *gnih* genes in salmonids, such as Atlantic salmon and rainbow trout (*Oncorhynchus mykiss)*, thought to have derived from the 4R.

1.5 Intensive postsmolt production

Traditionally, production of smolts in Norwegian aquaculture has been carried out in flow-through systems until they are ready to be transferred to sea pens for the rest of production. In an attempt to combat the mentioned sustainability issues related to increased production, the industry is shifting towards postsmolts production (Hagspiel et al., 2018). Postsmolts production involves growing the salmon larger in a protected environment before the sea-transfer, shortening the SW production time and reducing the operational risk and cost associated with it. One of the production technologies of postsmolts production is Recirculating Aquaculture Systems (RAS). RAS is a closed-looped land-based farming solution with considerable potential for growth. RAS decreases water consumption and reduces waste loss to the environment significantly, there is also low risk of parasitic infestation and escapees, potentially solving many of the challenges stunting the growth of the industry today (Dalsgaard et al., 2013). With RAS there can be close to full recirculation of water used in production (99%), and the artificial control over production parameters (such as food availability, water temperature, and photoperiod) allows optimal growing condition of a given species and the opportunity for diversification of species in the industry. However, RAS is expensive to establish and requires high operation cost and increased technical competence on system management (Yajie Liu et al., 2016). It is therefore necessary to identify and minimize risk factors that could compromise its economic feasibility and reduce fish welfare. One of the most concerning risk factors is early maturation and the welfare and mortality issues derived from it (Good & Davidson, 2016).

1.5.1   Early maturation as a result of intensive postsmolt production

Although early postsmolt maturation has rarely been observed in the wild, this is an increasing happening in aquaculture settings that use intensive rearing conditions, such as RAS (Good & Davidson, 2016). The complex interaction of environmental and internal factor influencing sexual maturation is all artificially controlled under rearing conditions allowing Atlantic salmon to quickly meet the required physiological threshold related to energy (Taranger et al., 2010; Thorpe, 2007). To induce smoltification before transfer to SW, a photoperiod regime, called winter signal (WS), is commenced, however, if the theorized energy threshold is reached beforehand, this photoperiod change can act as a *zeitgeber* that synchronizes initiation of sexual maturation (Bromage et al., 2001; Fjelldal et al., 2011; Handeland et al., 2008; Taranger et al., 2010). Consequently, intensive rearing contributes to

early maturation in postsmolts in two manners; promoting rapid growth and energy gain, and by acting as entraining cues that activates the BPG axis. The elevated temperature, constant light condition and feed availability strongly influence Atlantic salmon growth and development (Kadri et al., 1996; Rowe & Thorpe, 1990; Taranger et al., 2010; Thorpe, 2007). All the while the external factors, temperature and photoperiod, acts as the entraining cue that synchronizes the onset of maturation (Bromage et al., 2001; Good & Davidson, 2016).

1.6 External factors affecting maturation

1.6.1 Photoperiod

Atlantic salmon are called short-day breeders, spawning in fall-winter, and shows distinct photoperiodic responses such as smoltification, migration, and maturation (Nakane & Yoshimura, 2014; Taranger et al., 2010; Thorpe, 1994). The absolute consistency of annual changes in photoperiod acts as a *zeitgeber* or synchronizing cue for onset of such responses, signalling the change to suitable environmental conditions and ensures that hatching will occur at the right time to maximize survival of the offspring. (Björnsson et al., 1989; Bromage et al., 2001; Handeland et al., 2008; McCormick et al., 1995).

Several studies have been performed to understand the effects of photoperiod manipulation on early maturation in aquaculture environments during the FW phase. Saunders & Henderson (1988) found that Atlantic salmon subjected to stimulated natural photoperiod (LDN), the only photoperiod regime they tested with a stimulated switch from winter to spring light conditions, showed the highest indication of maturation. Adams & Thorpe (1989) also found a higher yield of maturing individuals in Atlantic salmon subjected to LDN and elevated temperatures than those not exposed to the "maturation window" unrelated to temperature. When looking at the interaction between early maturation and smoltification, Fjelldal et al. (2011) subjected Atlantic salmon previously reared under LD12:12 to LD18:6 or LD24:0 (continuous light, LL) at three different temperatures (5, 10, and 16°C). Results showed 47% of male maturity in the LL16°C groups compared to no maturation in the other groups, suggesting a stronger activation of the BPG axis in response to greater light stimuli from the shift LD12:12 to LL, compared to the shift LD12:12 to LD16:8. When looking at Atlantic salmon postsmolts kept in FW at two temperatures (1.2 and 15°C) and two photoperiods (one kept in LL, and one subjected to 5-week WS), Pino Martinez et al. (2023a)

reported 100% male maturation in the 15°C-WS group and 75% in 15°C-LL. At the lower temperature, 12.5°C the percentage of maturation was still high and smoltification poor. The WS groups revealed a high synchronization of maturation, while the groups reared a LL displayed high variability of maturation and the proportion of male maturation was highly dependent on temperature.

1.6.2 Temperature

Elevated temperature is often used in land-based rearing facilities to promote the growth rate of farmed fish, which also can affect the rate of early maturation in male Atlantic salmon post-smolt (Dalsgaard et al., 2013; Good & Davidson, 2016; Imsland et al., 2014). Temperature is an important factor for all physiological and endocrine processes in teleost fish. The rate of all biological processes is regulated by the surrounding temperature in nearly all fish due to the nature of being ectotherm poikilotherms (Schmidt-Nielsen, 1997, p. 232-239). It has been suggested that warmer waters likely offer a conjunction of higher quality and more abundant food as well as better conditions for growth, as well as a potential early activation of the BPG axis (Good & Davidson, 2016; Handeland et al., 2008).

Several studies have investigated the influence of water temperature on the early maturation in Atlantic salmon, often in combination with other factors such as photoperiod. Adams & Thorpe (1989) reported a higher yield of maturing individuals at elevated temperatures (ambient +5°C) in Atlantic salmon exposed to a synchronizing photoperiod cue. The results suggested that the elevated temperatures were not enough to trigger the onset of maturation in the absence of light stimuli. Imsland et al. (2014) found a high percentage of maturing males (82%) in postsmolts reared at elevated temperatures and subjected to LL. In this study they also found the group reared at lower temperatures subjected to LL had reduced growth in comparison and low yield of maturing individuals. Similarly, Pino Martinez et al. (2023b) reared Atlantic salmon parr at three different temperatures (8, 12.5, and 18°C) under LL except for a 5-week WS (LD12:12). Results showed that WS triggered the onset of maturation in the elevated temperature groups, while individuals reared at lower temperatures showed best signs of smoltification. Studies such as Fjelldal et al. (2011, 2018) and Melo et al. (2014) reported a high percentage of early maturation in Atlantic salmonusing "maturation regimes" that consisted of a simultaneous increase in temperature and photoperiod. The

simultaneous increase of the environmental conditions in these studies makes it unclear when the elevated temperature exerted influence on the decision to mature early, and how temperature changes affected this.

1.6.3 Food availability, growth rate, and energy budget

Growth, size, and availability of energy are directly influenced by diet and food availability or, in aquaculture, feed regime (Handeland et al., 2008; Kadri et al., 1996). Physiological changes such as smoltification and maturation is extremely energy costly, and to undergo such changes a certain energy threshold must be reach (Thorpe, 1994, 2007; Thorpe et al., 1998). If the required energy is assessed as sufficient, photoperiod will act as the zeitgeber, initiating the maturation process, meaning that the external factors regulating the onset of maturation is directly influenced by the internal factors and status. Thorpe (2007) stated that there is a genetically determined energy (lipid) threshold that must be exceeded for completion of sexual maturation. If the lipid threshold is not exceeded, maturation is inhibited. The relationship between energy and maturation can be studied by limiting the availability of energy through different feeding regimes. Rowe & Thorpe (1990) found a suppression of maturation through limiting food availability during spring month in juvenile Atlantic salmon. However, Pino Martinez et al. (2023b) reported decreased growth in experimental groups with restricted diets (67%), but the effect on maturation was temperature dependent. At 18°C, the proportion of maturation was not affected by the restricted diet, at 12.5°C the impact on proportion was greater, but not for all individuals, reinforcing the idea that temperature and photoperiod are the main driving external factors regulation the BPG axis.

1.7 Objective

As the demand for Atlantic salmon grows it is essential to solve the issue of early sexual maturation in postsmolts, and vital to reach a broader understanding of the neuroendocrine control and its signalling. Water temperature is shown to be the most important factor contributing to early postsmolt maturation. However, high proportion of early maturation has been reported under both constant temperatures, or when using maturation regimes that involves simultaneous increase in temperature and daylength. As such, times during production at which the use of high temperature can have a larger influence on early

maturation is not clear. Thus, with the present study the aim is to identify periods during postsmolt production where an exposure to high temperature can exert a larger influence on the incidence of early maturation, and to assess if a large reduction in temperature at critical time point (i.e., before or after WS) could delay or arrest early maturation. In addition, the way in which high water temperature stimulate the early activation of the neuroendocrine regulation of the BPG axis leading to early maturation is not well understood. For that purpose, analysis of the transcription of several major/important genes involved in the neuroendocrine control of sexual maturation in response to different temperature profiles introduced was performed.

Atlantic salmon genes selected for analysis was chosen based on their assumed involvement in sexual maturation through the existing knowledge of the reproduction axis in mammals and birds (*dio2b, gnrh2*, *gnrh3*, *gpr54*, *gniha)*. Recently, Horne et al. (Horne et al., 2022) mapped the cellular location of the central neuroendocrine regulatory factors in the BPG axis in Atlantic salmon which made the basis for the genes transcribed in the present study.

2 Methodological approach

2.1 Experimental setup

The experiment took place from May 24th to November 2nd, 2022, at the flow-through facilities of the Institute of Biological Sciences (BIO) of the University of Bergen (UiB). A total of 900 Atlantic salmon parr (50±SD g) were transferred from a commercial RAS facility (Bremnes Seashore AS, Trovåg, Rogaland, Norway) and randomly distributed among 8 tanks of 500L (112 per tank). The experiment consisted of rearing four groups in duplicates under four different temperature profiles (Figure 3). Group 15 was kept at 15°C throughout the entire experiment, and group 8 was kept at 8°C. Groups 15-8E and 15-8L was kept at 15°C at the beginning of the experiment, and at 8°C by the end. Temperature was reduced over the course of two days simultaneously to the change in photoperiod: group 15-8E at the start of WS, and group 15-8L at the end. Temperature was measured and logged every 10 minutes throughout the entire experiment (Figure 4). The photoperiod regime was the same for all four groups; continuous light (LL or LD 24:0) with a 5 week long WS (LD12:12) lasting from 28th of July to 1st of September. The fish were fed daily with BioMar AS commercial feed provided by Bremnes Seashore AS over a 12-hour cycle.
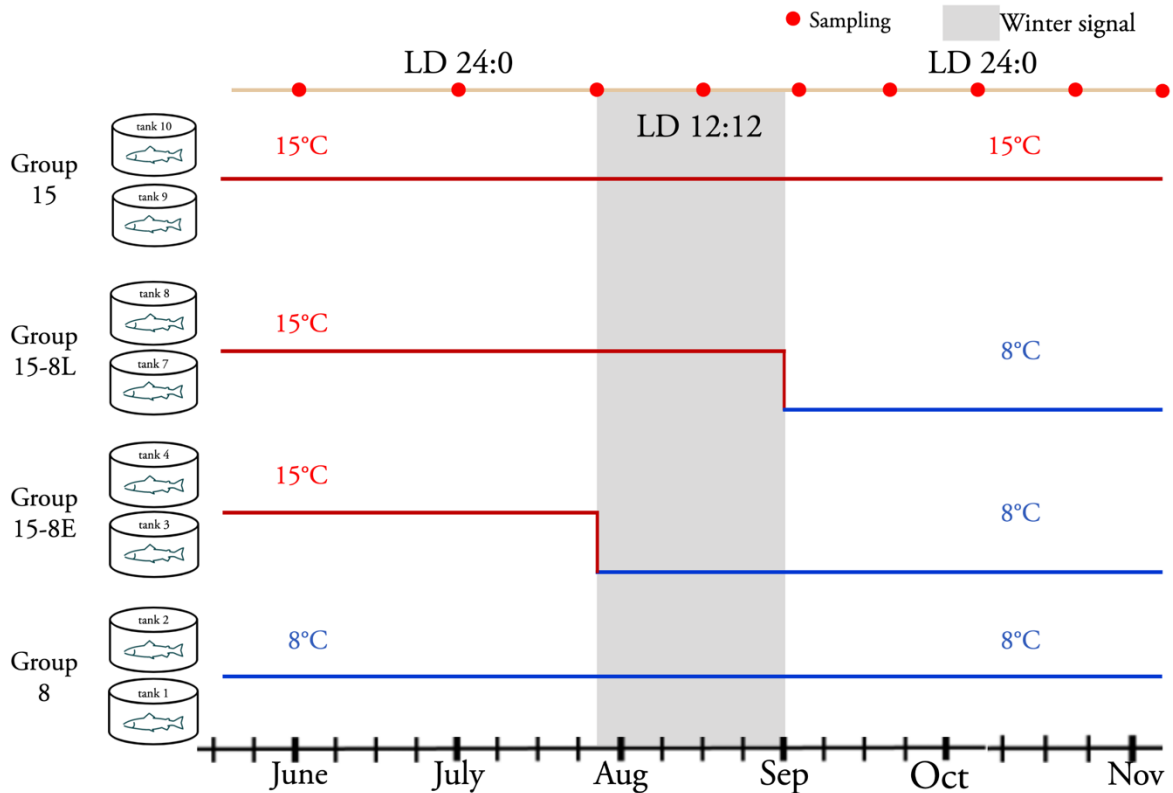
Figure 3: Experimental design. *The experimental setup consisted of four experimental treatment groups in duplicates reared under four different temperature profiles. Every group followed the same light-regime, switching from LL (continuous light) to a five week long LD12:12 winter signal (WS) marked in the design as the grey area. WS lasted from 28th of July to 1st of September. Two groups were kept at constant temperature: group 15 at 15°C and group 8 at 8°C. Two groups experienced a decrease in temperature from 15°C to 8°C simultaneously to change in photoperiod: group 15-8E at the start of WS and 15-8L at the end of WS.*

Figure 4: *Temperature profiles. Measured temperature for group 15, 8, 15-8E, and 15-8L throughout the experiment. Temperature was measured and logged every 10 minutes. Grey area in every plot indicates the WS period when temperatures were regulated.*

2.2 Sampling protocol

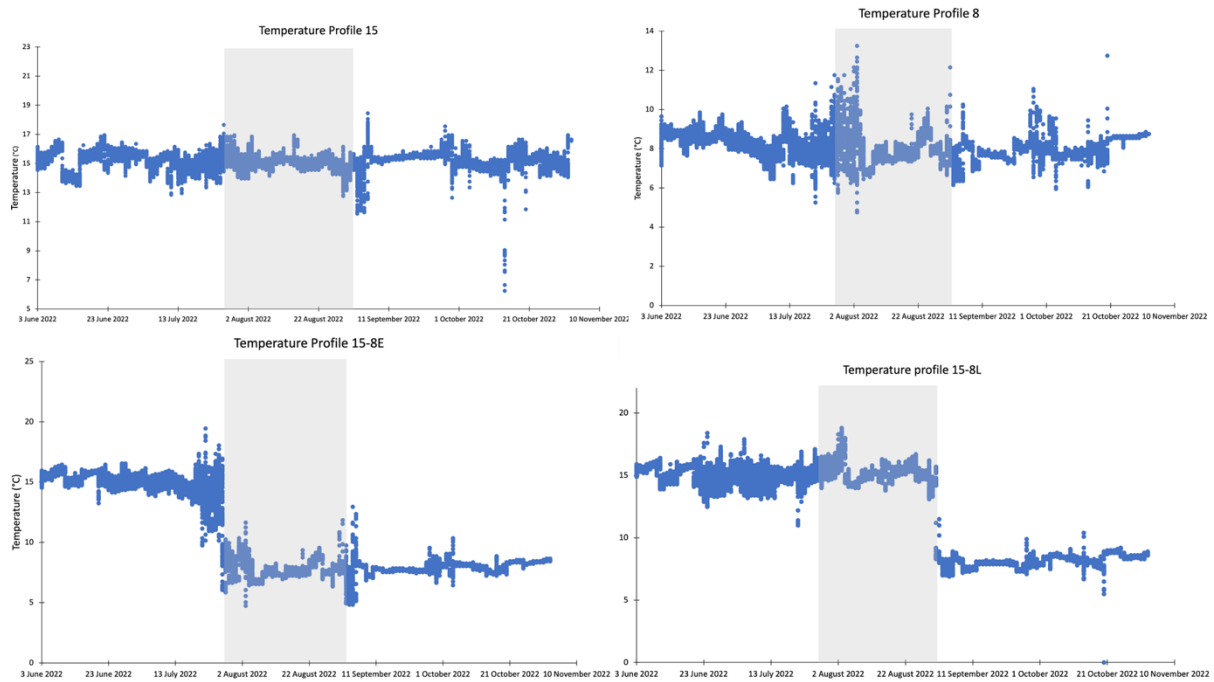Throughout the experiment, eight samplings were conducted (Figure 3). Samplings were held on the 2nd of June, 30th of June, 28th of July, 18th of July, 9th of September, 22nd of September, 6th of October, 20th of October, and 2nd of November. For each sampling, 12 male fish from each group was sampled, six from each tank, resulting in 48 fish per sampling. All fish were netted out and immediately euthanized with an overdose of benzocaine by bath (>50mg/L Benzoak vet.® 20%, ACD Pharma AS, Norway). Fish was measured and weighed (fork length in cm, body weight in g). Fish were dissected and gonads were examined to determine gender and degree of maturation, keeping only males. Testes were dissected and weighed to subsequently calculate gonadosomatic index (GSI), which us used as a reliable index to estimate maturation status.

To sample brains, the heads of the fish were cut of laterally behind the neurocranium whilst making sure to include both pectoral fins. By putting a finger through the mouth and two fingers behind the pectoral fins, the head was placed down, mouth facing up (Figure 5). To open the cranium a vertical incision was made dorsally of the eyes. Cutting through the

nostril bone down to the eyes and then pulling the scalpel away from the head while continuing to cut downwards revealed the entirety of the brain. Using a pair of thinly pointed tweezers to pull the notochord out, the fish head was then tilted so that the brain fell naturally out of its cavity. To remove the brain entirely, the nervus opticus was lightly tugged until the brain was separated from the cranium. The brains were each placed in 1ml RNALater™ (Sigma-Aldrich, Burlington, Massachusetts, United States) and then stored at -80°C.
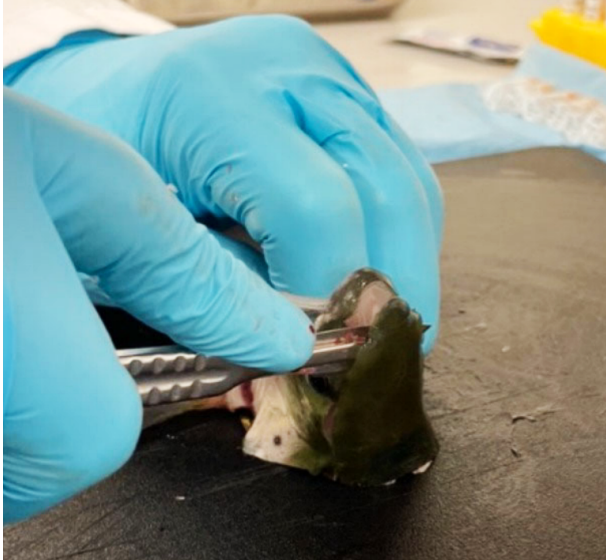
Figure 5: Brain extraction. *Photograph taken of the extraction of the brain and pituitary during sampling. Shown here is the vertical cut made through the nostril bone dorsally of the eyes to open the cranium without damaging the brain.*

2.3 Post dissection

The brains were taken out of the -80°C freezer and needed about 30 minutes to thaw on ice before dissection could start. Each brain was carefully removed from the RNALater™ and placed on parafilm under a Olympus SZ51 light microscope (Olympus copr., Tokyo, Japan) (Figure 6). Dissection was performed using a pair of thinly pointed tweezers, a pair of forceps and a 17mm scalpel. To begin the saccus vasculosus was located at the ventral side of the brain and carefully removed by pulling it posterior (Figure 7). Next the membrane was removed. As each brain was slightly morphologically different, the membrane was either easier to remove immediately or continuously during dissecting. Between the hypothalamus and the medulla oblongata was usually a good place to start removing the membrane. Next the brain was turned on its ventral side and the telencephalon, along with the lobus

olfactorius, was dissected (Figure 8). Tectum opticum was then carefully separated by pulling it slightly away from cerebellum and then separating it down the middle (Figure 9, Figure 10). Careful not to tear the third ventricle or to cut the hypothalamus bulbs on the ventral side, tectum opticum was then dissected (Figure 11). Laying the brain on its side and pushing gently down on the diencephalon and the medulla oblongata made room for lifting the cerebellum and the habenula away (Figure 12). A straight line was made, dissecting the habenula and the cerebellum from the diencephalon. Lastly the medulla oblongata was dissected from the diencephalon (Figure 13). The diencephalon was placed in RNA-free 1,5 ml tubes, weighed using the Sartorius Entris 64-1S (Sartorius AG, Goettingen, Germany), and then placed in the -80°C freezer.
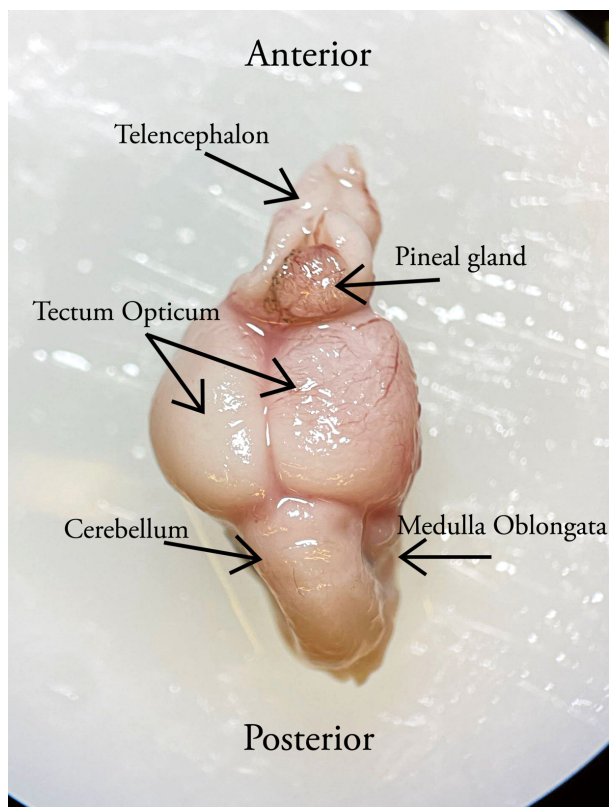
Figure 6: Dorsal view of the brain of Atlantic salmon juvenile. *Photo taken through a light microscope of the dorsal view of the brain of a juvenile Atlantic salmon male pre-dissection. Visible anatomical structures are included.*

Figure 7: Ventral view. *Photo taken through a light microscope of the ventral view of the brain of a juvenile Atlantic salmon male pre-dissection. Visible anatomical structures are included.*
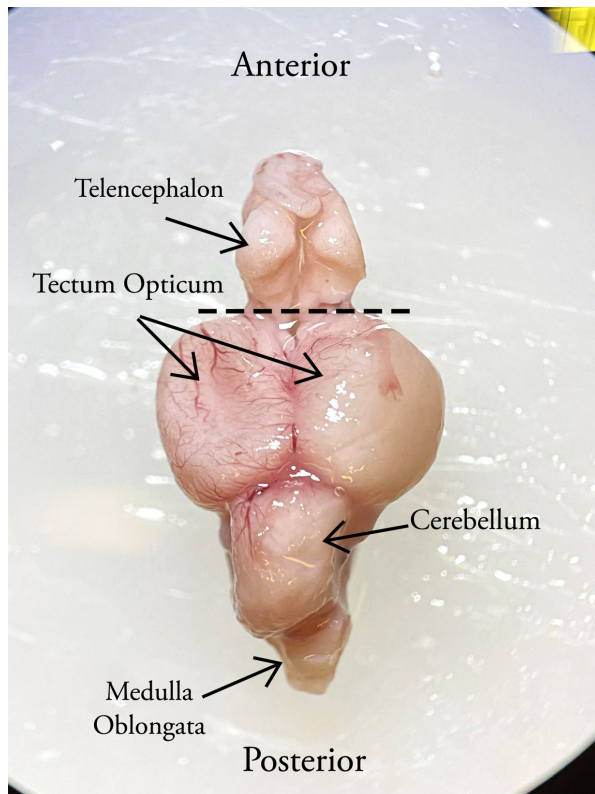
Figure 8: Dorsal view. *Photo taken through the lens of a light microscope. The photo shows the dorsal view of the brain of a juvenile Atlantic salmon male. Here the pineal gland has been removed and the dotted line indicates where the cut to remove the telencephalon region will be made. Visible anatomical structures are included.*
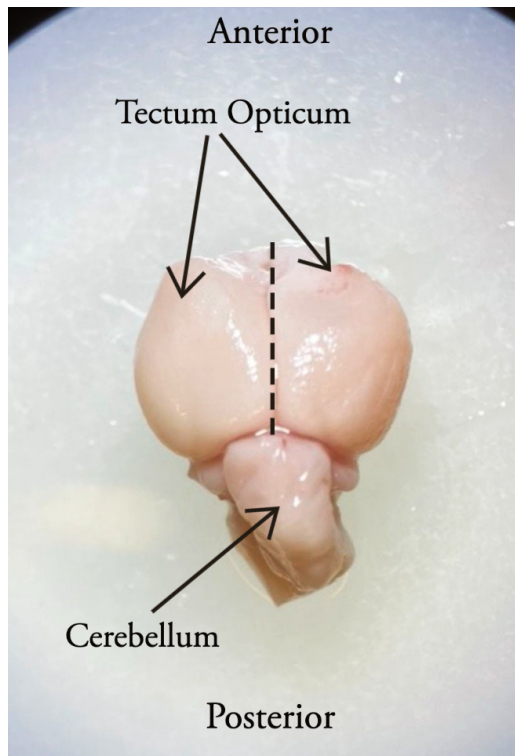
Figure 9: Dorsal view. *Photo taken through the lens of a light microscope showing the dorsal view of a juvenile Atlantic salmon male. Here the telencephalon and the brain membrane has been removed. Visible anatomical structures are shown. The dotted line indicated where tectum opticum will be separated before dissected.*
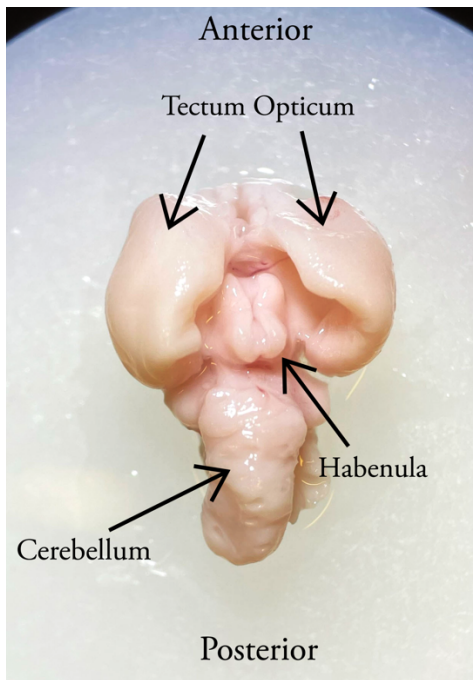
Figure 10: Dorsal view. *Photo taken through the lens of a light microscope showing the dorsal view of a juvenile Atlantic salmon male. Here the telencephalon and the brain membrane has been removed. The tectum opticum has also been separated. Visible anatomical structures are included*
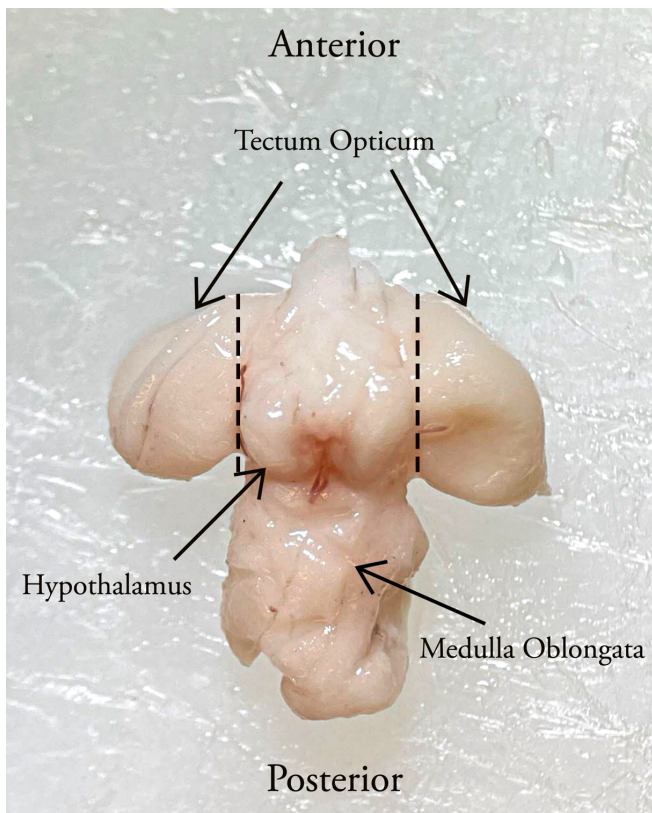
Figure 11: Ventral view mid-dissection. *Photo taken through the lens of a light microscope mid-dissection of the brain of a juvenile Atlantic salmon male. Here the saccus vasculosus has been*

removed along with the brain membrane. The tectum opticum has been separated and the dotted line indicates where the cut will be made to dissect them. Visible anatomical structures have been included

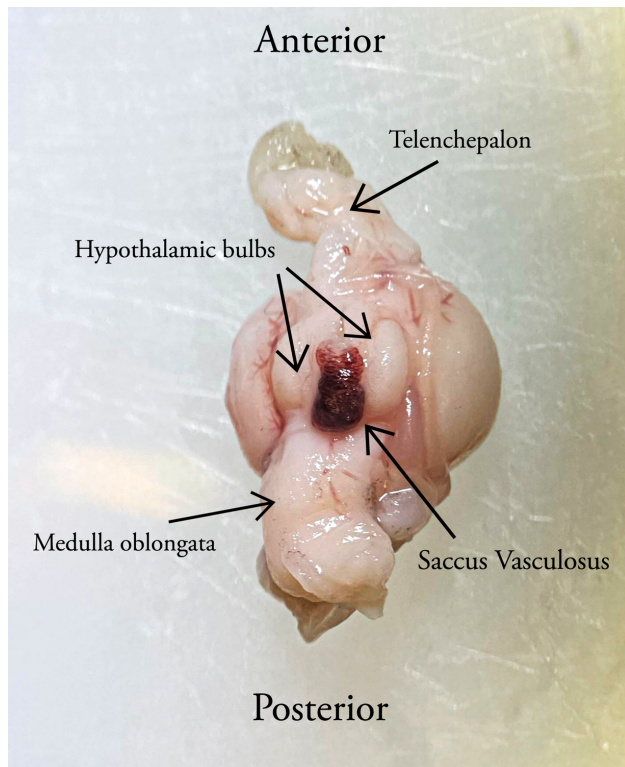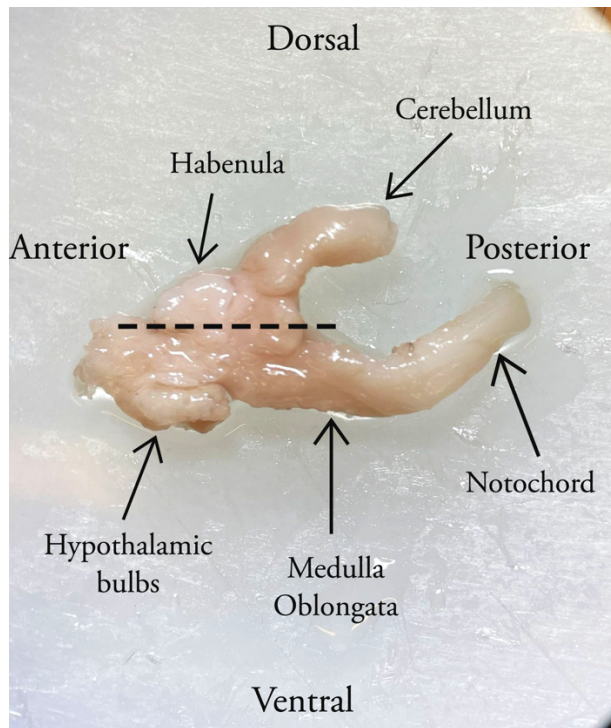**Figure 12: Sagittal view.** *Photo taken through light microscope of the sagittal view of the brain of a juvenile Atlantic salmon male. Here the tectum opticum has been dissected. Anatomical structures visible are included. Dotted line indicates where the cut to remove the habenula and cerebellum from the diencephalon will be made.*
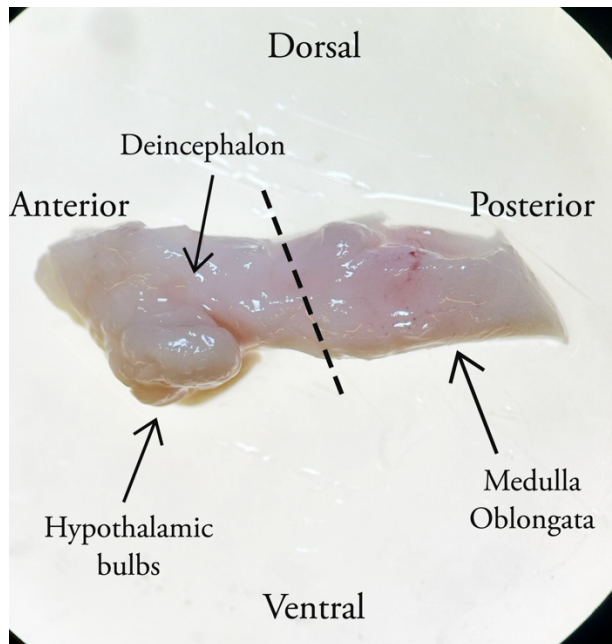
Figure 13: ***Diencephalon region***. *Photo taken through light microscope of the sagittal view of the brain of a juvenile Atlantic salmon male. Here the cerebellum and habenula has been dissected. Anatomical structures visible are included. Dotted line indicates where the cut to remove the medulla oblongata from the diencephalon will be made.*

2.4 Lab analysis

2.4.1 RNA extraction/isolation

Raw RNA was manually isolated from 349 diencephalon tissue samples using the TRI-reagent (TRIzol) method following a modified standard protocol based on the single-step report by Chomczynski (1993). The tissue was homogenized in 1mL TRI Reagent® (Thermo Fisher Scientific, Waltham, Massachusetts, United States) using 0.6g Precellys® ceramic bulk beads (Bertin Technologies, Montigny-le-Bretonneux, France) in a Percellys Tissue Homogenizer (Bertin Technologies) on program 2 (5000-1.15-005) x 2. 200µl of chloroform (Sigma-Aldrich) was added to the samples and vortexed for 20-30 seconds. The samples were centrifuged at max speed for 15 min at 4°C in an Eppendorf 5415R Centrifuge (Eppendorf AG, Hamburg, Germany). Supernatant was transferred to a new tube, making sure to only transfer the aqueous phase. 500µl of isopropanol (Sigma-Aldrich) was added to each sample and mixed thoroughly. Then the samples were left to rest for a 20 min incubation time, mixing every 10 min. Precipitated RNA was collected by centrifugation at max speed for 10 min at 4°C, the liquid was removed, and remaining pellet was rinsed with

1ml ethanol (80%). After removing the ethanol, the pellet was left to dry for up to 20 min, once the RNA-pellet turned opaque it was resuspended in 50µl nuclease free water (Sigma-Aldrich).

The total RNA purity and concentration (ng/µl) of each sample was measured and quantified using a NanoDrop One microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) using 1µl total RNA. The ratio of absorbance for samples was ~2 for 260/280 nm ($A_{260/280}$) and < 2 for 260/230 nm ($A_{260/230}$), which indicated sufficient purity of contaminants. The raw RNA was stored in a -80°C freezer until further analysis was performed.

When the tissue (diencephalon region) reached approximately 0.050 grams, the volumes in the protocol was increased by 1/3. TRI Reagent® (Sigma-Aldrich) was increased to 1500µl, Chloroform (Sigma-Aldrich) was increased to 300µl, and Isopropanol (Sigma-Aldrich) was increased to 750µl. 1,5ml RNA-free tubes were also switched out for 2ml RNA-free tubes. Volume of nuclease free water to resuspend the RNA-pellet was increased to 70 µl.

2.4.2 cDNA synthesis

First-strand complementary DNA (cDNA) synthesis was performed using qScript® cDNA Synthesis kit (Quantabio, Beverly, Massachusetts, United States). 750 ng/µL of RNA was reversed transcribed according to manufactures protocol. This step was performed by my supervisor Mitchell S. Fleming.

2.4.3 Quantitative polymerase chain reaction (qPCR)

Before mRNA expression analysis, primers were validated for efficiency and optimal dilution by running two-fold dilution in series of triplicates using the cDNA pool covering the whole experimental period and the different experimental groups. A serial dilution was made in a 1/5 dilution in a ½ series (1/5, 1/10, 1/20, 1/40, 1/80, 1/160). Primer amplification efficiency was determined by the slope of the regression line (Equation 1) generated from standard curves using the values from the serial dilution of each gene (

Equation 2). A scatterplot was then made with the quantification cycle (Cq) values, using the mean of the triplicate. According to the Cq values of the standard curve, dilutions were

determined for each gene of interest. Real-time Quantitative Polymerase Chain Reaction (qPCR or RT-qPCR) was performed to quantify the relative abundance of *actin, gnrh2, gnrh3, gpr54, dio2b,* and *gniha* using the C1000 Touch Thermal Cycler, CFX96™ Real-Time Detection System (Bio-Rad Laboratories, CA, US) and CFX Manager software (version 3.1) (Bio-Rad Laboratories). The qPCR reactions were done in duplicates in Hard-Shell PCR 96-wells plates (Bio-Rad Laboratories) with a total volume of 14µl in each well, using 6.5 µl iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.25 µl of each forward (FW) and reverse (RV) primer (0.2 µM), 3 µl rna-free water (Sigma-Aldrich) (Table 1, Table 2), and 4 µl cDNA. Two plates were needed when testing one gene on one condition and for simplicity a master-mixture was made to fit all plates (Table 1). All plates were sealed with microseal® 'B' seals (Bio-Rad Laboratories) and contained a 'non-template control' (NTC) for contamination-check and a common pool positive control (POS) (Table 3). The RT-PCR protocol consisted of 3 min at 95 °C followed by 38 cycles at 95 °C for 15 s and 61 °C for 1 min.

Table 1: *Calculations for SYBR-mixture.* *Calculating the amount of each substance in SYBR-mixture for qPCR. Two plates were made when testing one gene on one condition. Each plate has 96 wells, 96*2= 192. To account for error, an extra 10% was added, 192+10% ≈ 210.*

Content	Mix x1	Mix x210
SYBR	6.5µl	1365µl
Primer FW	0.25µl	52.5µl
Primer RV	0.25µl	52.5µl
RNA-free H_2O	3µl	630µl

Table 2: *Primers used in diencephalon qPCR.* *Overview of the specific primers and in their respective nucleic acid sequences used for each qPCR assay to measure mRNA abundance in diencephalon tissue in the present study.*

Gene	Primer sequence (5′ → 3′)		Ref
dio2b	FRW	ATGGAAGATGAAATCGCCGC	Lorgen et al., 2015
	RV	TGCCAGATCTTCTCCATGTCG	

gnrh2	FRW	ACCTGAGACCACAGCGAAGG	M. S. Fleming et al., unpublished
	RV	AGGGTAAAGAAGGGATGCGACA	
actin	FRW	ATGGAAGATGAAATCGCCGC	Lorgen et al., 2015
	RV	TGCCAGATCTTCTCCATGTCG	
gpr54	FRW	ACCCTTTAAAGTCCCTACGCC	M. S. Fleming et al., unpublished
	RV	GGTGGATAGAATGAAGGAACCGAT	
gnrh3	FRW	CAGGTGCCCATGTCCCAGAG	M. S. Fleming et al., unpublished
	RV	TGAATGCTCCATCATCGTTGTCT	
gniha	FRW	CCATGACCAACGACAACGACGG	Maugars & M. S. Fleming, unpublished
	RV	TTGACAGGTGGCGGGTAGAGT	

Table 3: *Plate-setup for qPCR. Example of well setup for qPCR. Shown here is the plate-setup for plate A1 for treatment 8°C.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	2	2	3	3	4	4	5	5	6	6
B	13	13	14	14	15	15	16	16	17	17	18	18
C	60	60	61	61	62	62	63	63	64	64	65	65
D	108	108	109	109	110	110	111	111	112	112	113	113
E	157	157	158	158	159	159	160	160	161	161	162	162
F	202	202	203	203	204	204	205	205	206	206	207	207
G	250	250	251	251	252	252	253	253	254	254	255	255
H	300	300	301	301	POS	POS	302	302	303	303	NTC	NTC

Quantification of mRNA abundance was completed using the mean of the duplicate target Cq values and the corresponding mean of the duplicate reference Cq values used for normalization (Equation 3). *actin* was used as the reference gene.

Equation 1: Slope of regression line

$$slope = \frac{(y - b)}{x}$$

Equation 2: Primer Amplification Efficiency (E)

$$E = 10^{\left(\frac{-1}{slope}\right)}$$

Equation 3: Relative mRNA quantification of genes

$$ratio = \frac{(E_{target})^{\Delta Cq_{mean\,target}}}{(E_{ref})^{\Delta Cq_{mean\,ref}}}$$

b = The intercept

E = Primer amplification efficiency

E$_{target}$ = qPCR efficiency of the target gene

E$_{ref}$ = qPCR efficiency of the reference gene

x = Explanatory variable

y = Dependent variable

ΔCq$_{mean\,target}$ = Quantification cycle (Cq) of the target gene in replicates

ΔCq$_{mean\,ref}$ = Quantification cycle (Cq) of the reference gene in replicates

2.5 Data analysis

2.5.1 Condition factor and gonadosomatic index

Fulton´s conditions factor (K) (Ricker, 1975) of each fish was calculated to assess the condition of each fish according to energetic reserves as a predictor of developmental changes, using the Fulton formula:

$$K = \left(\frac{W}{L^3}\right) * 100 = \left(\frac{Body\ weight\ (g)}{Body\ length\ (cm)^3}\right) * 100$$

Gonadosomatic index (GSI) for each fish was calculated to determine the degree of sexual maturation, expressing gonad compared to the total body weight, by the following equation (Fjelldal et al., 2011):

$$GSI\ (\%) = \frac{Gonad\ weight\ (g) * 100}{Body\ weight\ (g)}$$

To distinguish between maturing and immature individuals GSI (%) thresholds were chosen (Melo et al., 2014; Peterson & Harmon, 2005; Taranger et al., 2010; Thorpe, 1994). The thresholds defining onset of maturation where chosen based on recent work and published literature: "immature" (GSI < 0.06%), "maturing" (GSI > 0.06%) (Pino Martinez et al., 2023b).

2.5.2 Statistical analysis

All plotting and statistical analysis was performed in RStudio 2022.12.0+353 (RStudio Inc, Boston, Massachusetts, United States), using the packages "tidyverse" (Wickham et al., 2019), "car" (Fox & Weisberg, 2019), "emmeans" (Lenth et al., 2023), "lme4" (Fox & Weisberg, 2019), "nlme" (Pinheiro & Bates, 2000). Fisher´s Exact Test for count data was used to compare the incidence of male maturation between treatments and each sampling point. Shapiro-Wilk´s test was initially used to check normality of response variables (body weight, K, GSI, and relative mRNA abundance). Generalized Linear Mixed Models (GLMM) or Linear Mixed Effects Model (LME) were fitted between response variables and the predictors "treatment", "time" and their two-way interactions as fixed effects, and "tank" as random effect. When necessary, model residuals were checked graphically to assess model assumptions such as normality of residuals (q-q plots), non-linear patterns (residuals vs fitted), homogeneity of variance (scale-location plots), or influential outliers (residuals vs leverage plots with Cook´s distance). Levene´s test was also used to check for homogeneity of variance. Significant models were followed by Tukey HSD post-hoc test to find significant differences in the response variable between treatment at each sampling and within treatments over time. All plots of response variables over time for the different treatments were presented as mean ± standard error of mean. Significance level was α = 0.05 unless specified.

3 Results

3.1 Proportion of maturation

Percentage of maturing individuals (GSI > 0.06%) at sampling points displayed an increase towards the end of the experiment for all groups expect group 8 (Figure 14). Group 15 had a significantly higher percentage of maturing individuals than the other groups from mid-September until the end of the experiment ($p < 0.05$), displaying 100% maturing individuals at sampling point 7 and 8, mid-September. There was no significant difference in percentage of maturing individuals between group 15-8L and 15-8E at the end of the experiment. Group 8 experienced very low percentage of maturation.
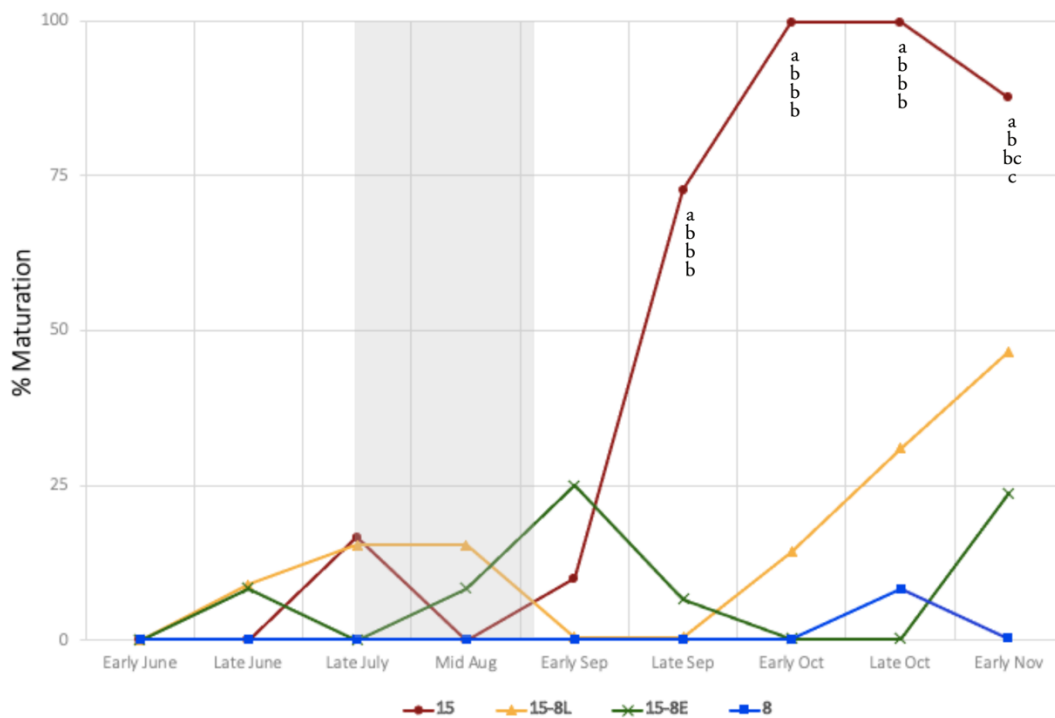
Figure 14: ***Proportion of maturation (%) over time.*** *Percentage of maturing individuals in juvenile Atlantic salmon reared in different temperatures regimes (8, 15-8E, 15-8L, and 15) during the period between May 24th to November 2nd, 2022. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance (p < 0.05) between groups at sampling points. Datapoints are marked with corresponding shape to treatment at each sampling point and presented as mean ± SEM.*

3.2 Morphometrics and GSI

3.2.1   Body weight development

Body weight was significantly dependent on treatment, time, and their interaction (all $p <$
0.001). All groups experienced a gradual increase in body weight throughout the experiment
(Figure 15). The experimental groups reared at elevated temperatures displayed a higher
growth rate and mean body weight than the group reared at 8°C.
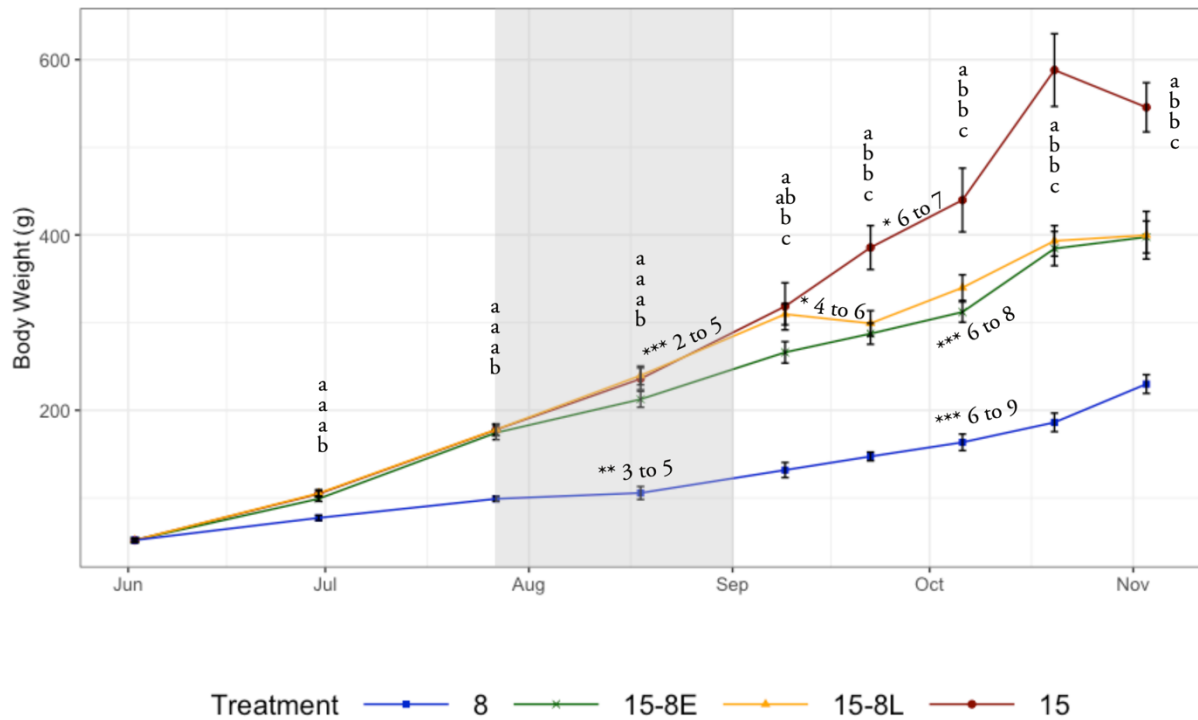
Figure 15: *Mean body weight (g) over time. Body weight (g) development in juvenile Atlantic salmon
reared in different temperature regimes (8, 15-8E, 15-8L, and 15) during the period between May 24th
to November 2nd, 2022. The winter signal period (LD 12:12) is marked by the grey area. Different
lower-case letters showcase significance (p < 0.05) between groups at each sampling point. Asterisks
(* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling
points. Datapoints are marked with corresponding shape to treatment at each sampling point and
presented as mean ± SEM.*

Body weight increased faster at 15°C than in the rest of the groups, reaching a significantly
larger weight in the final sampling than the rest of the treatments (545.6±28.1g). In contrast,
group 8 showed the slowest increase in weight, ending at 229.9±10.6g. The two groups
experiencing a reduction in temperature, 15-8E and 15-8L, reduced their growth

simultaneously to the drop in temperature, and ended with a similar final weight (397.6±18.2g and 399.7±27.2g respectively).

3.2.2 Condition factor (K) development

K was significantly influenced by treatment ($p < 0.001$), time ($p < 0.001$), and the interaction between treatment and time ($p < 0.01$). All treatment groups displayed a variety of K values (mean ± SEM) adjustments (Figure 16). All groups experienced a decline in condition factor from July to Sep-Oct. Then, K in all groups expect 15 continued to decrease until the end of the experiment. In contrast, group 15 displayed an increase, ending with a significantly higher condition factor than the rest.
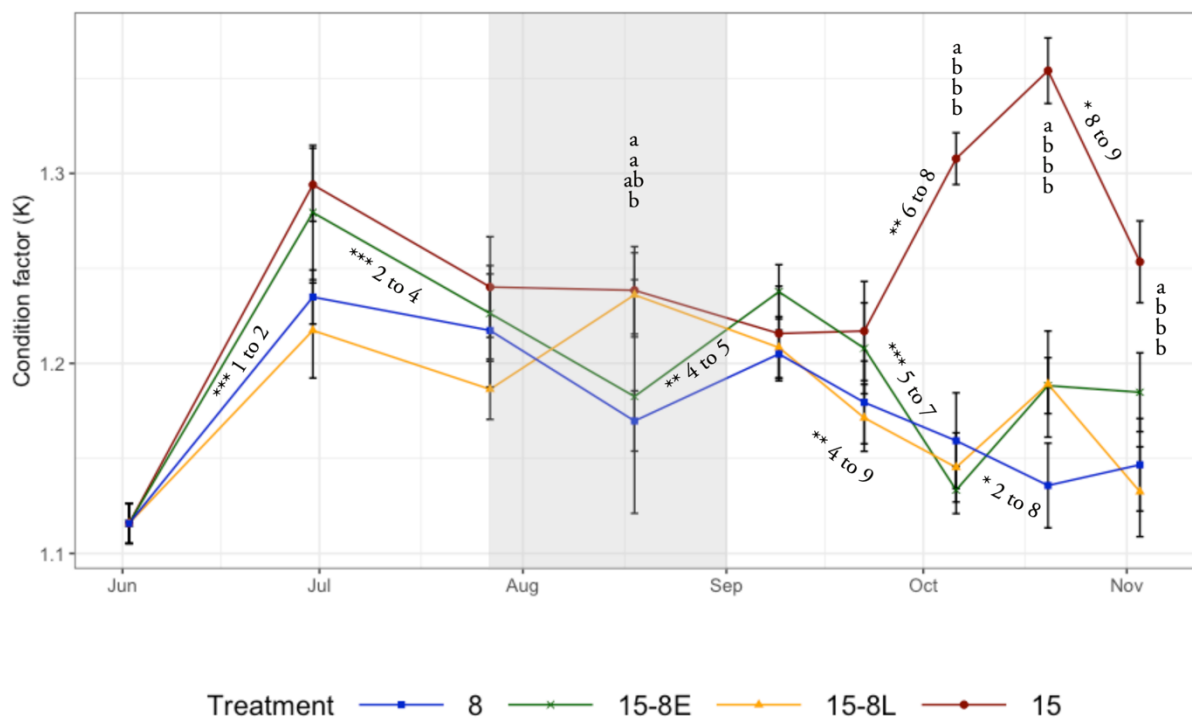
Figure 16: *Mean condition factor over time. Condition factor (K) development in juvenile Atlantic salmon reared in different temperature regimes (8, 15-8E, 15-8L, and 15) during the period between May 24th to November 2nd, 2022. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance (p < 0.05) between groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points. Datapoint are marked with corresponding shape to treatment at each sampling and presented as mean ± SEM.*

3.2.3 Changes in mean gonadosomatic index (GSI) over time.

GSI was significantly dependent on treatment, time, and their interaction (all $p < 0.001$). Up until the WS period, GSI remained low for all groups (Figure 17). After WS, GSI increased dramatically in group 15 ($p < 0.001$), while it remained low for the other groups. Group 15-8L experienced a significant increase in GSI between sampling point 8-9 ($p < 0.05$). In the last sampling, GSI in group 8 was significantly different from 15-8L ($p < 0.05$), but not 15-8E. Group 15 was significantly different from all the other groups ($p < 0.05$) with a GSI of 5.2±0.668% at the last sampling point.
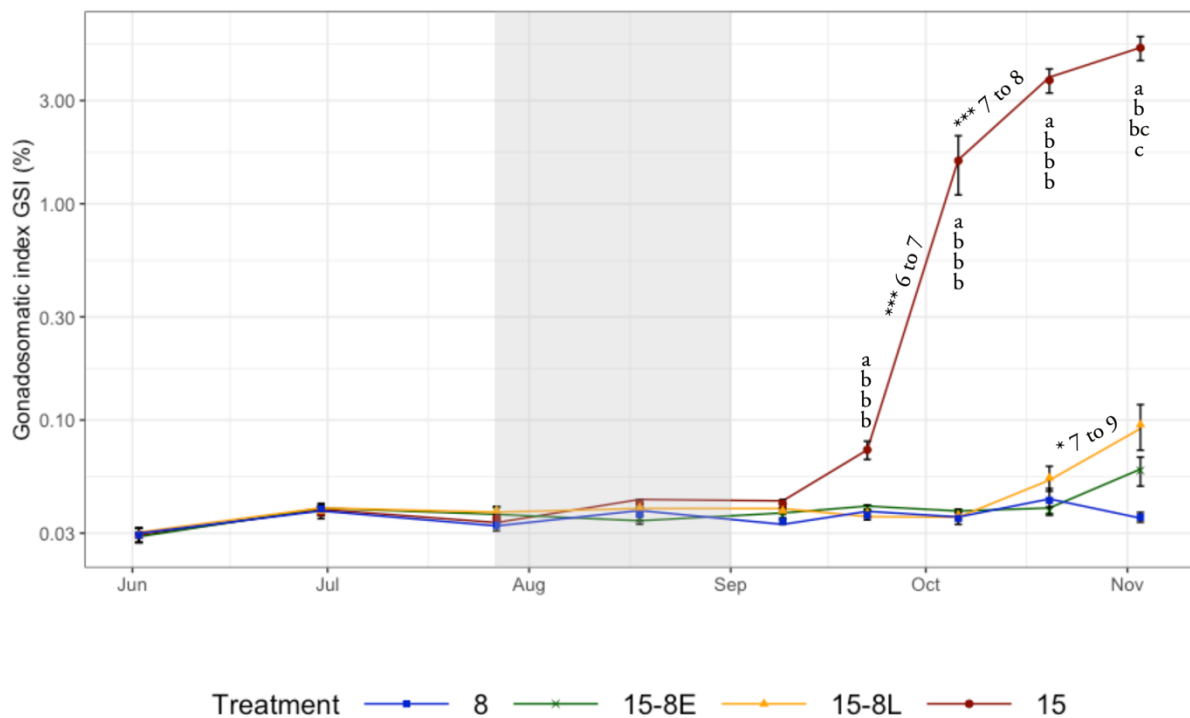
Figure 17: *Mean GSI (%) over time.* *Changes in GSI % in juvenile Atlantic salmon reared in different temperature regimes (8, 15-8E, 15-8L, and 15) during the period between May 24th to November 2nd, 2022. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance ($p < 0.05$) between groups at each sampling point. Asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant changes between consecutive sampling points. Each datapoint is marked with corresponding shape to treatment at each sampling and presented as mean ± SEM.*

3.3 Relative mRNA abundance in diencephalon

3.3.1 Changes in relative mRNA abundance of *dio2b*

Relative *dio2b* mRNA abundance was significantly dependent on time ($p < 0.001$) and temperature ($p < 0.01$), and on interactions between time and treatment ($p < 0.001$). All treatment groups displayed a variety of relative *dio2b* mRNA abundance (mean ± SEM) adjustments, with treatment 15-8E displaying the highest expressed abundance (Figure 18).

Figure 18: Relative mRNA abundance of *dio2b*. *Changes in mean relative abundance mRNA of dio2b in juvenile Atlantic salmon reared in different temperature regimes (8, 15-8E, 15) during the experimental period. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance ($p < 0.05$) between treatment groups at each sampling point. Asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant changes between consecutive sampling points. Each datapoint is marked with corresponding shape to treatment at each sampling and presented as mean ± SEM. Datapoints have been jittered to reduce overlap.*

Group 8 had a significant increase in relative *dio2b* mRNA abundance from sampling point 2 to 4 ($p < 0.001$), and then again after WS between sampling point 5 and 7 ($p < 0.001$). Group 15 experienced a significant increase in relative mRNA abundance from sampling point 4 to

6 (p < 0.001). Group 15-8E displayed a significant increase from sampling point 2 to 3, and again from sampling point 5 to 7 (p < 0.001). At sampling point 2 the transcript of group 8 was significantly lower than group 15 and 15-8E (p < 0.05). Group 15-8E displayed a significantly higher transcript than the other groups at sampling point 3 and 4. At sampling point 5 there was no difference in the transcript between groups 15 and 15-8E, however, both were significantly higher than group 8 (p < 0.05). Group 15 displayed its highest transcript at sampling point 5 with 1.16±0.15. Sampling point 7 was the highest recorded transcription for both 15-8E and 8 (1.51±0.13 and 1.33±0.12) where they displayed a significantly higher transcription than group 15 (p < 0.05). At sampling point 8, group 15-8E displayed a significant higher transcript than group 15 (p < 0.05), while group 8 displayed no significant difference to either group.

Overall, increases over time can be observed in all groups, but differently dependent on temperature treatment. In group 15, an increase in expression can only be seen after the change from WS to LL, remaining lower than the other two groups. In groups 15-8E and 8, major increases after WS can be observed, displaying significantly higher *dio2b* transcript levels than 15.

3.3.2 Changes in relative mRNA abundance of *gnrh2*

Relative *gnrh2* mRNA abundance was significantly dependent on time and the interactions between time and treatment (all p < 0.001). *gnrh2* transcript did not display significant changes in expression over time in group 15 and 15-8E (Figure 19). In contrast, group 8 display a varied *gnrh2* transcript over time, ending with a remarkable increase from September to October that resulted in a significantly higher increase than groups 15 and 15-8E.
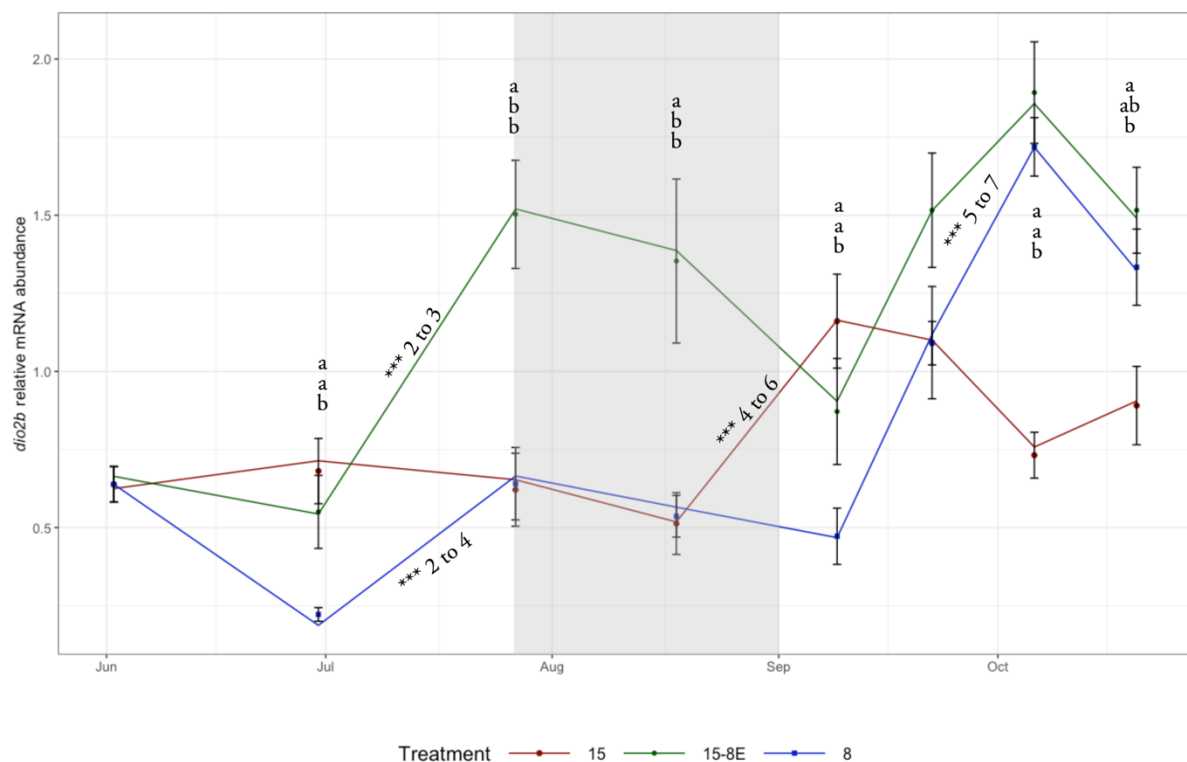
Figure 19: Relative mRNA abundance of *gnrh2*. *Changes in mean relative abundance mRNA of gnrh2 in juvenile Atlantic salmon reared in different temperature regimes (8, 15-8E, 15) during the experimental period. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance (p < 0.05) between treatment groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points. Each datapoint is marked with corresponding shape to treatment at each sampling and presented as mean ± SEM. Datapoints have been jittered to reduce overlap.*

Group 8 had a significant increase in relative *gnrh2* mRNA abundance from sampling point 2 to 4 (p < 0.001), remaining stable until a significant decrease after WS between sampling point 5 and 6 (p < 0.01). From sampling point 6 to 8 there was another significant increase in relative *gnrh2* mRNA abundance for group 8. 15-8E experienced a significant increase in relative mRNA abundance from sampling point 2 to 4 (p < 0.001). At sampling point 2, group 15 displayed a significant higher transcript than group 15-8E (p < 0.05), while group 8 displayed no significant difference to either group. There was no significant difference in transcription until sampling point 8, where group 8 displayed a significantly higher transcription than the other groups (p < 0.05).

3.3.3   Changes in relative mRNA abundance of *gnrh3*

Relative *gnrh3* mRNA abundance was significantly dependent on time and temperature, and on interactions between time and treatment (all p < 0.001). All treatment groups displayed a general increasing trend of *gnrh3* mRNA abundance (mean ± SEM), with treatment 15-8E displaying the highest expressed abundance (Figure 20).
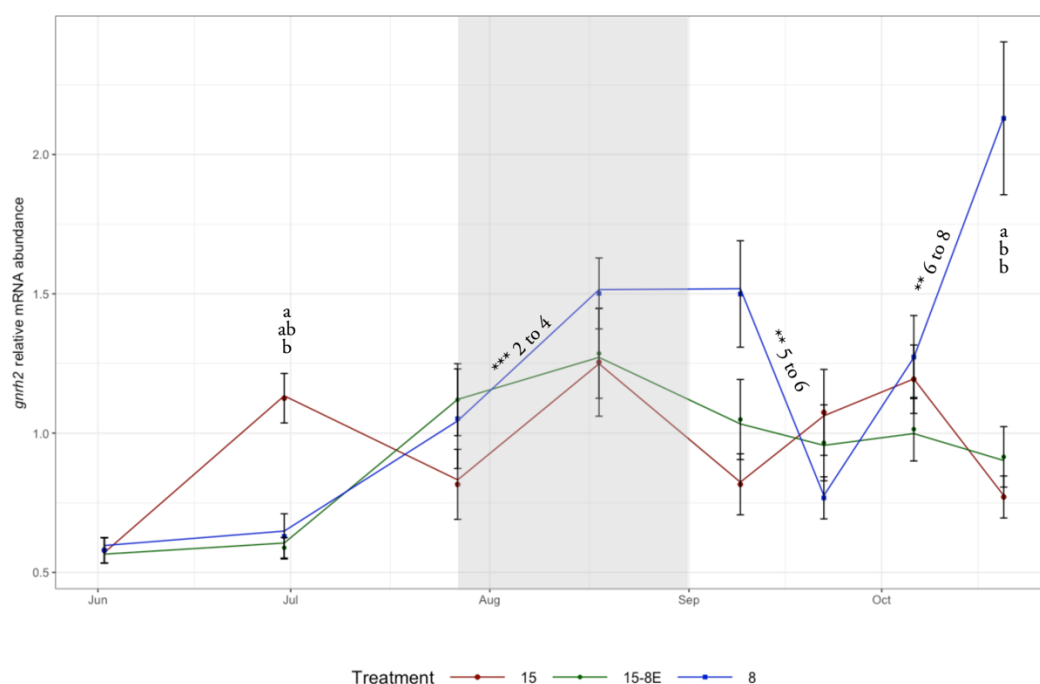

Figure 20: Relative mRNA abundance of *gnrh3*. *Changes in mean relative abundance mRNA of gnrh3 in juvenile Atlantic salmon reared in different temperature regimes (8, 15-8E, 15) during the experimental period. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance (p < 0.05) between treatment groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points. Each datapoint is marked with corresponding shape to treatment at each sampling and presented as mean ± SEM. Datapoints have been jittered to reduce overlap.*

Relative *gnrh3* mRNA abundance at the start of the experiment was 0.76±0.06 for all groups and increased throughout the experiment for all groups. Group 8 had a significant increase in relative *gnrh3* mRNA abundance from sampling point 2 to 5 (p < 0.001), and again from sampling point 6 to 8. Group 15 had a significant increase in relative *gnrh3* mRNA

abundance from sampling point 2 to 7 ($p < 0.001$), while 15-8E experienced a significant increase from sampling point 2 to 4 ($p < 0.001$). At sampling point 4, there was a significant difference between all treatment groups of which group15-8E displayed a considerably higher transcription than group 8, which was again higher than group 15 ($p < 0.05$).

Overall, there was a general gradual increase in expression for all treatment groups, but different depending on the treatment. In group 15 there was a gradual increase over time, generally showing lower transcript levels. Group 15-8E displayed an intense upregulation in expression of *gnrh3* during WS, followed by stable levels after WS. In group 8 there was a sustained gradual increase over time, followed by an intense increase after WS.

3.3.4   Changes in relative mRNA abundance of *gpr54*

Relative *gpr54* mRNA abundance was significantly dependent on time ($p < 0.001$) and on interactions between time and treatment ($p < 0.05$). All groups experienced significant increases in *gpr54* expression over time, that was generally similar in all treatment groups, but with some variability in group 8 (Figure 21).
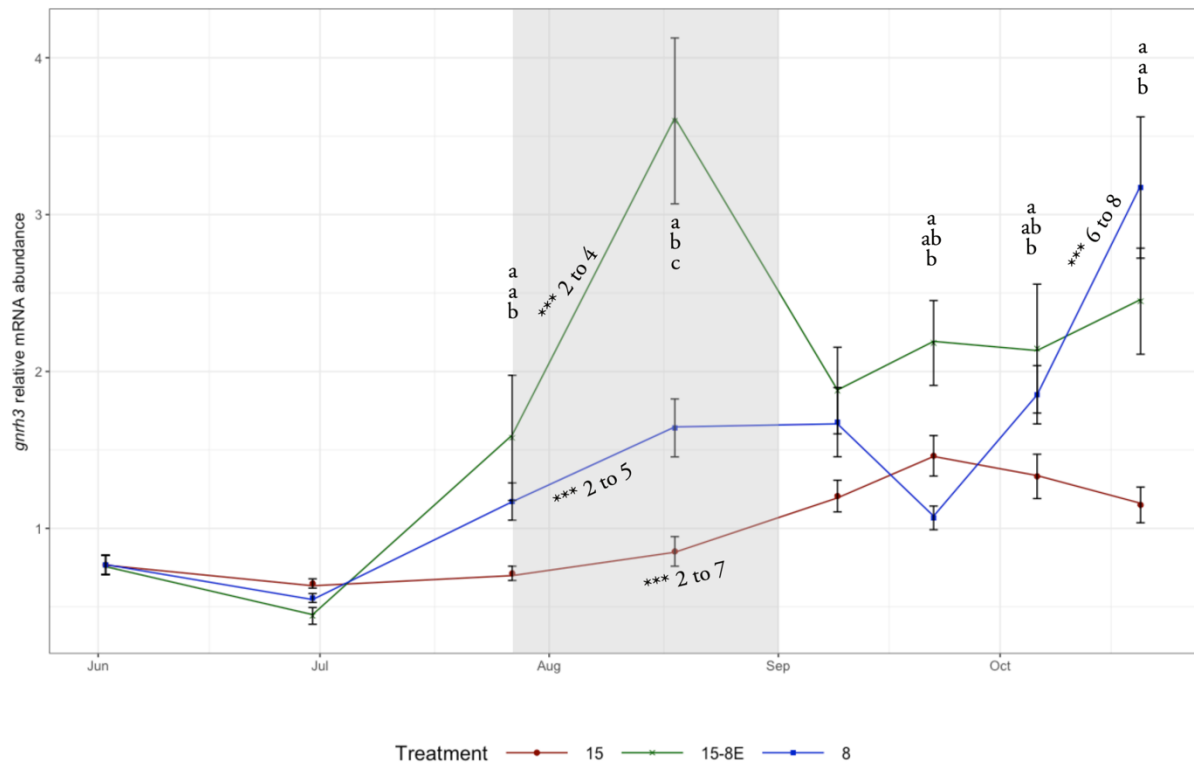
Figure 21: Relative mRNA abundance of _gpr54_. _Changes in mean relative abundance mRNA of gpr54 in juvenile Atlantic salmon reared in different temperature regimes (8, 15-8E, 15) during the experimental period. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance (p < 0.05) between treatment groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points. Each datapoint is marked with corresponding shape to treatment at each sampling and presented as mean ± SEM. Datapoints have been jittered to reduce overlap._

Relative _gpr54_ mRNA abundance at the start of the experiment was 1.13±0.06 for all groups and increased throughout the experiment for all groups. For group 15, relative mRNA abundance increased significantly between sampling point 2 to 5 (p < 0.05). Group 15-E increased significantly between sampling point 2 and 8 (p < 0.001). Group 8 experience a significant increase between sampling point 2 and 5 (p < 0.001). After WS, between sampling point 5 and 6 (p < 0.001), it had a significant decrease in relative mRNA abundance, before significantly increasing between sampling point 6 and 8 (p < 0.05). All groups followed the same trend throughout the experiment. The only showcased significant difference in transcription between groups was at sampling 6 between group 8 and 15-8E.

3.3.5 Changes in relative mRNA abundance of *gniha*

Relative *gniha* mRNA abundance was significantly dependent on time and on interactions between time and treatment (all $p < 0.001$). Transcription of *gniha* remained stable in groups 8 and 15 for most of the experiment (Figure 22). Group 8, did however, experience a remarkable upregulation in *gniha* at the end of the experiment, ending with significantly higher transcript levels than group 15.
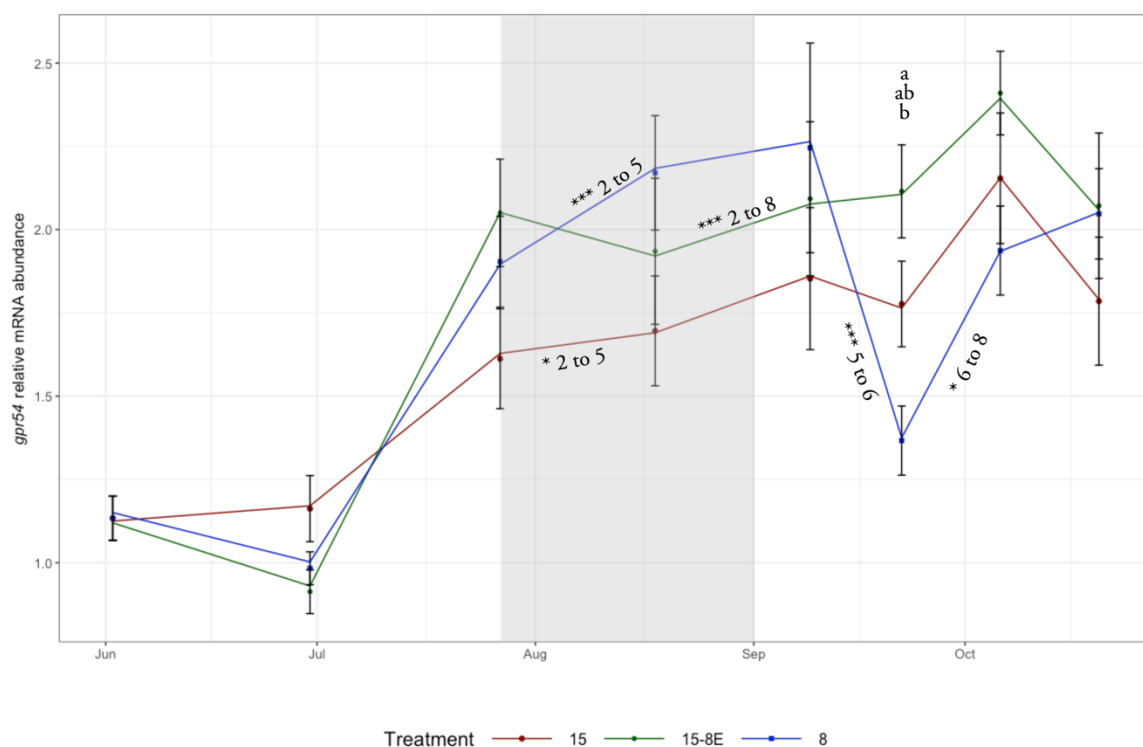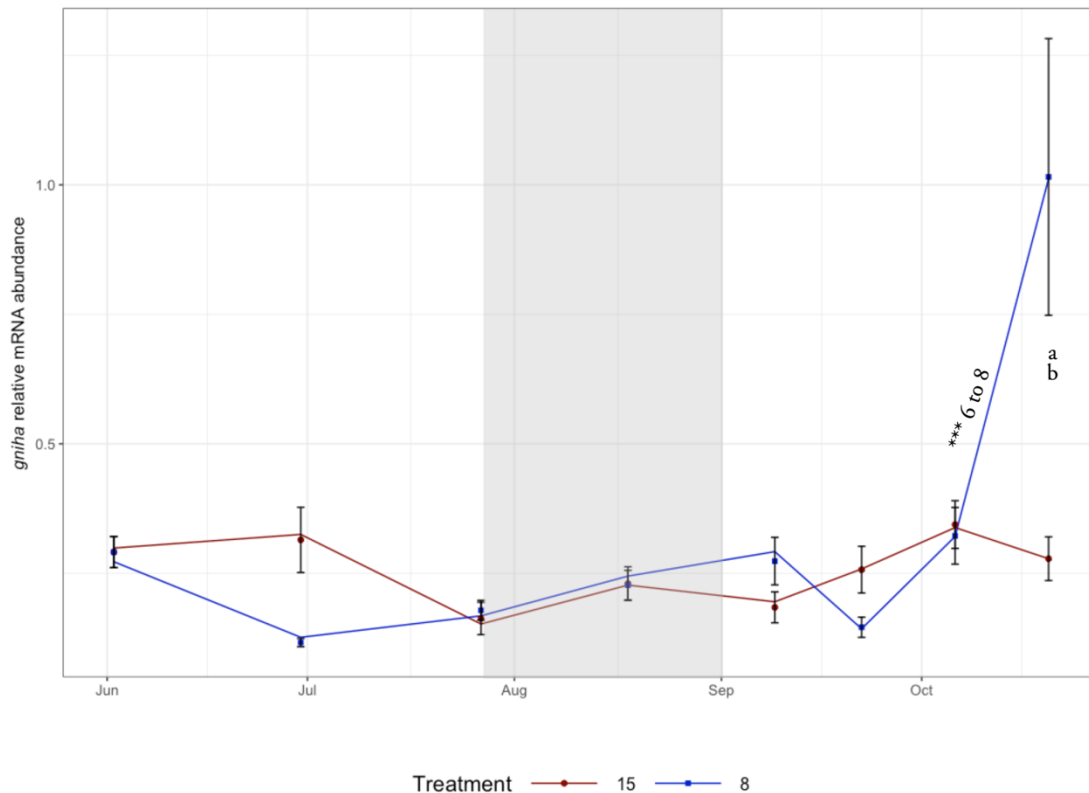

Figure 22: Relative mRNA abundance of *gniha*. *Changes in mean relative abundance mRNA of gniha in juvenile Atlantic salmon reared different temperature regimes (8, 15-8E, 15) during the experimental period. The winter signal period (LD 12:12) is marked by the grey area. Different lower-case letters showcase significance (p < 0.05) between treatment groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points. Each datapoint is marked with corresponding shape to treatment at each sampling point and presented as mean ± SEM. Datapints have been jittered to reduce overlap.*

Relative *gniha* mRNA abundance at the start of the experiment was 0.29 ± 0.03 for both groups. Only group 8 displayed an increase in expression throughout the experiment. The expression in group 15 remained relatively unchanged for the entirety of the experiment.

From sampling point 6, group 8 experienced a significant increase in transcription ($p < 0.001$). The only significant difference in transcription between the two groups was displayed at sampling point 8 ($p < 0.05$).

3.3.6 mRNA abundance in each group

Mean relative mRNA abundance of the tested genes expressed in treatment group 15. The transcript of the different genes displayed no similar trend for this group (Figure 23). Overall there seems to be a relative increase in most of the genes mRNA abundance.

Figure 23: *mRNA expression in group 15. Relative mRNA expression for every gene tested in the diencephalon of male Atlantic salmon juveniles. Shown here are the transcripts of genes* dio2b, gpr54, gnrh2, gnrh3, *and* gniha *expressed in treatment group 15. Each sampling point is presented as mean ± SEM. The grey area indicates WS period.*

Mean relative mRNA abundance of the tested genes expressed in group 15-8E displayed no strong trend between genes (Figure 24). There seems to be a general increase in relative mRNA abundance for all genes throughout the experiment.

Figure 24: ***mRNA expression in group 15-8E.*** *Relative mRNA expression for every gene tested in the diencephalon of male Atlantic salmon juveniles. Shown here is the transcripts of genes* dio2b, gpr54, gnrh2, *and* gnrh3 *expressed in treatment group 15-8E. Each sampling point is presented as mean ± SEM. The grey area indicates WS period.*

Mean relative mRNA abundance of the tested genes mRNA abundance in group 8 (Figure 25). From the transcript in group 8 there seems to be a general trend for most the genes tested. Overall the trend follows an increase pre-WS followed by a sudden drop after switch to LL, and rapid increase again towards the end of the experiment.
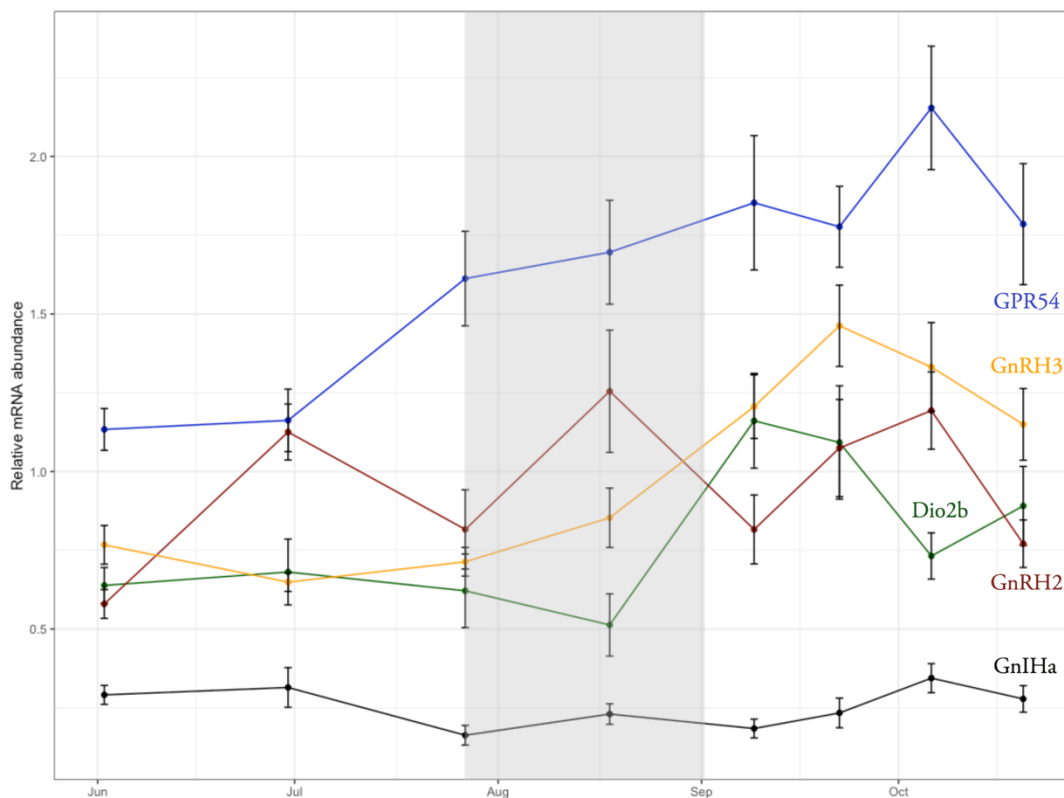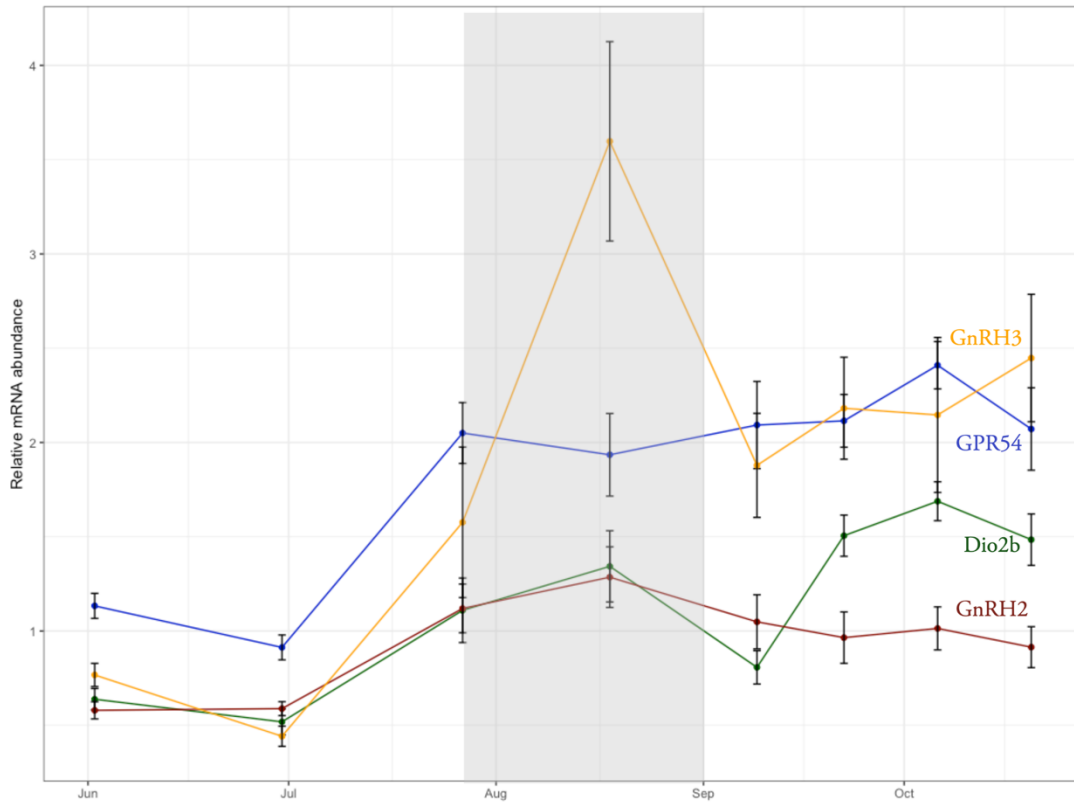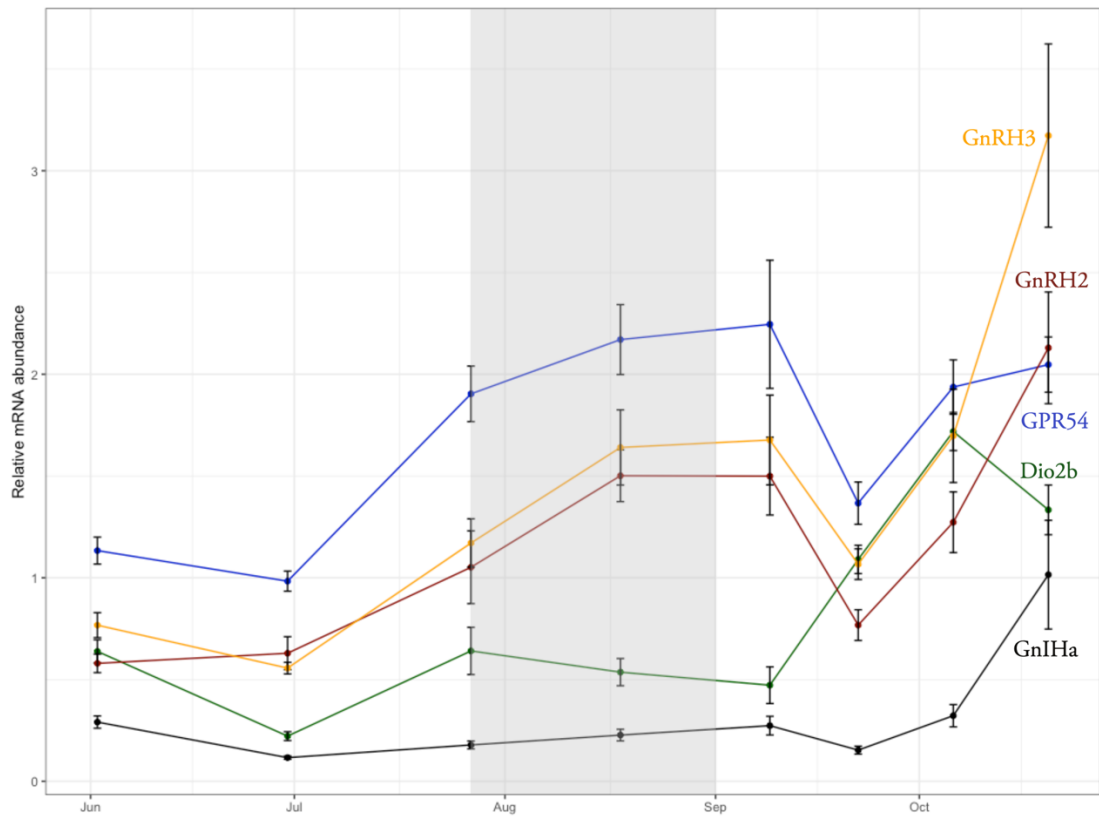
Figure 25: ***mRNA expression in group 8.*** *Relative mRNA expression for every gene tested in the diencephalon of male Atlantic salmon juveniles. Shown here is the transcript of genes* dio2b, gpr54, gnrh2, gnrh3, *and* gniha *expressed in treatment group 8. Each sampling point is presented as mean ± SEM. The grey area indicates WS period.*

4 Discussion

4.1 Methodological considerations

The project experienced difficulties with keeping stable water temperatures in the experimental tanks, due to the temperature being found through mixing warm and cold water in the lab. The setup for this could be improved through better methods of heating and cooling the water, for example with a heat pump.

Due to lack of funding, and the little physiological differences between group 15-8E and 15-8L, only one of the groups were chosen for gene expression. Group 15-8E was chosen as the early period seemed more interesting to this experiment. The original idea was to do both GnIH paralogs, *gniha* and *gnihb*, however, funding for qPCR was limited and *gniha* was chosen for gene expression. For *gniha*, only group 8 and 15 was transcribed, also due to funding.

It should also be noted that primers can only be designed up to a certain point. If two paralogs have similar nucleotide sequences, discerning the difference between to two can be difficult and the current molecular biology methodological constraints makes it difficult to look at this in detail.

4.2 Discussion of results

4.2.1 Morphometric development

Body weight and K are valuable biometric indicators for smoltification and development used in aquaculture research and industry. In the present study, growth was significantly higher in the elevated temperature (15°C) than in the lower (8°C) (Figure 15). Both groups had consistent body weight development, with group 8 ending at a body weight around 300g lower than group 15 (final weight 8 vs final weight 15). Supporting the importance of temperature as one of the main factors influencing growth and development (Schmidt-Nielsen, 1997), consistent with multiple previous studies (Björnsson et al., 1989; Handeland et al., 2004, 2008; Sigholt et al., 1998). Handeland et al. (2008) showed that optimal growth for Atlantic salmon during the postsmolt phase occurs at 13-14°C, therefore, close to optimal

growth at 15°C was expected. Handeland et al. (2008) also showed diminished growth rate at 18°C indicating an upper thermal limit, which is consistent with the temperature-size rule in ectotherms (for review: Angilletta & Dunham, 2003). Interestingly, despite the differences in the temperature during WS, 15-8E and 15-8L did not display any significant differences at any point during the experiment. There seems to be a reduction in growth rate directly after temperature change for both 15-8E and 15-8L, once again supporting the importance of temperature in growth (Figure 15). The timing of this reduction did not, however, affect final mean weight with both groups ending at similar weights. It should be noted that the short amount of time with differentiated temperatures was likely not sufficient to cause a larger diversion in growth. The switch from WS to LL normally induces stress, which increases cortisol, suggesting that the group 15-8L experienced more stress during this period than group 15-8E, which at this point would have been acclimated to 8°C.

K gives us an indication of the chosen life history path of an individual as a direct effect of the reallocation of energy. A decrease of condition factor (K) is a characteristic feature of growth during smoltification in salmon, derived from the weight loss and changes in body composition (Björnsson et al., 1989; McCormick & Saunders, 1987). On the other hand, an increase in K after photoperiodic stimuli indicates potential early maturation or disruption of the smoltification process in postsmolts. In the present study, after WS there was a clear decrease in K for groups 8, 15-8E, and 15-8L (Figure 16). From this trend in K, it can be assumed that the majority of individuals, especially in group 8, prepared for smoltification. However, large variance in both 15-8E and 15-8L indicates potential disruption in smoltification for some individuals. Furthermore, a larger disruption in smoltification was observed in group 15, where there was a significant increase in K, suggesting early maturation. Fjelldal et al. (2011) found that the mature individuals had a K near 1.4, and Pino Martinez et al. (2023b) had consistently a K near 1.4 in the group that later matured at 100%, which is consistent with the present study, where the group 15 experienced a K of 1.35±0.01.

4.2.2 Proportion of maturation

In group 15, all males matured in a synchronized manner with low variability in GSI% (Figure 14, Figure 17) in response to increase in daylight after WS. In contrast, group 8 experienced no early maturation and likely, according to the K value, smoltified. The main

aim of the experiment was to observe if a reduction in temperature (from 15°C where 100% maturation is likely to occur, to 8°C where 100% smoltification is likely to occur) simultaneously to change in photoperiod would arrest maturation in individuals where maturation had most likely been induced by early rearing at 15°C. From the proportion of maturing individuals in group 15-8E and 15-8L there appears to be a delay in maturation (Figure 14), likely caused by the reduction in temperature, however, the timing of the decrease did not make any significant difference on the proportion. It should be noted that, like with body weight development, the lack of significant difference could be because the time spent in different temperatures was not long enough to cause any difference. The proportion of maturing individuals was remarkably lower when reducing temperature in both treatment groups, which initially could suggest that reducing temperature can be an alternative to decrease proportion of maturation. However, after WS there was an increase in percentage of maturing males over time, indicating that early onset of maturation had been synchronized in a number of individuals and that the maturation was an ongoing process that the reduction in temperature delayed. This is also seen in the increase in GSI experienced towards to the end of the experiment for both 15-8E and 15-8L (Figure 17). The difference in maturation status between the groups in the present study is line with previous studies that temperature influences the timing and rate of early maturation (Björnsson et al., 1989; Imsland et al., 2014; McCormick et al., 2002; Pino Martinez et al., 2023b).

Results from the present study indicates that the early period before a photoperiodic change can be determinant for Atlantic salmon pre-smolt to decide which life history pathway they follow, and that any changes in the rearing condition afterwards may not change that decision. In the case of the present study, the reduction in temperature did not change the decision to mature, only delaying it. This is likely tied to the ectotherm nature of the fish, at which the rate of developmental changes is determined by temperature. As such, the drop in temperature probably slowed the intensity and speed of the process causing early maturation in males but did not alter the decision. Further research should focus on what has been stimulated or what processes have been induced during this early period at high temperatures so that the decision is made irrespective to some extent of the rearing conditions experienced afterwards.

4.2.3 Effects of temperature regimes on transcriptional changes of neuroendocrine factors

4.2.3.1 *dio2b* transcription

DIO2 is an enzyme that converts T4 to T3 causing thyroid-driven mechanisms. Lorgen et al. (2015) discovered the expression of Dio2 paralogs in Atlantic salmon regulated under different environmental conditions; *dio2a* is induced in the gills during transfer to SW, while *dio2b* expression is dependent on light regulation related to the FW period and smoltification. Since the present study was exclusively carried out in FW, only expression of *dio2b* was investigated. In mammals and birds, increased levels of DIO2 in the hypothalamus is stimulated by long-day stimulated TSH (Nakane & Yoshimura, 2014). DIO2 stimulates kisspeptin release, which in turn stimulates GnRH leading to the onset of maturation. In Atlantic salmon, Irachi et al. (2021) found long day photoperiodic stimuli to increase *tshßb* mRNA levels in the pituitary and *dio2b* mRNA levels in the hypothalamus. If the signalling pathway in birds and mammals is preserved in salmonids, *tshßb* subsequentially stimulates *dio2b*, which in turn could activate the GnRH-system (Figure 2) .

In the present study the mean diencephalon expression of relative *dio2b* mRNA increased in all groups after the end of the WS period and exposure to LL (Figure 18), consistent with increased daylight activation of *dio2b* (Irachi et al., 2021; Lorgen et al., 2015; Strand et al., 2018). Interestingly, there seems to be a difference in timing of the increase of *dio2b* between groups 15-8E and 8 and group 15, suggesting that the increased expression due to increase in daylight could be influenced by temperature. Group 15 seems to experience the significant increase after WS earlier than 8 and 15-8E, however, due to the timing of the switch to LL and the next sampling point it is difficult to say if the increase was instant or gradual. For group 8 and 15-8E the increase begins after the first sampling point after switching back to LL. The increase for both group is greater than for group 15, group 8 even greater than 15-8E. These findings suggest the change in photoperiod from WS to LL stimulates production of *dio2b* in male Atlantic salmon juveniles.

Confusingly, before WS, group 15-8E experienced a high increase in expression between sampling point 2 to 3. After sampling point 3 is when WS was commenced and temperature was lowered from 15°C to 8°C, therefore there is a potential role of temperature where

sudden changes in temperature could affect the expression of *dio2b*. This is the first report of a potential effect of temperature on Atlantic salmon *dio2b* expression, which warrants further research into the relationship between the effects of temperature and the early activation of the BPG axis. It should also be noted that Dio2 has a wide range of physiological roles (for review: Orozco & Valverde-R, 2005), thus making an assumption on its role in maturation difficult.

4.2.3.2 Gnrh transcriptions

GnRH was first discovered in the early 1970s and is the main regulator of pituitary gonadotropin hormones Fsh and Lh. While GnRH´s stimulatory role holds true for mammals and birds, a similar role has not been defined in teleosts (Zohar et al., 2010). Taranger et al. (2010) stated that puberty implies a functional BPG axis, and as Fsh stimulates early spermatogenesis and its progression (Nóbrega et al., 2009; Schulz et al., 2010); in the present study, we assume elevated Fsh correlates higher GSI levels (Pino Martinez et al., 2023b). In vertebrates, GnRH1 is the main promoter of gonadotropin secretion. GnRH2, however, which has been conserved for ~500 million years of evolution, is absent or inactivated in some mammalian species, such as rodents (for review: Desaulniers et al., 2017). The conservation of the gene supports its importance and highly critical biological role, yet the absent of the gene in some species signalizes a divergent function to that of GnRH1. Gnrh1 neurons are found in the hypothalamic region for the brain and most teleost species have three forms of Gnrh. In teleost fish with the three Gnrh forms there appears to be overlap of the function of the three paralogs and species-specific differences in the overlap (Muñoz-Cueto et al., 2020; Zohar et al., 2010). In some species who lack Gnrh1, such as goldfish, either Gnrh2 or Gnrh3 has adopted the hypothalamic releasing form (Kobayashi et al., 1997).

As Gnrh1 lacks in salmonids, the question then remains which of Gnrh2 and Gnrh3 has adopted the gonadotropin releasing function. Amano, Kitamura, et al. (1997) noted that only Gnrh3 had been found in the pituitary by immunocytochemistry or radioimmunoassay in the rainbow trout and masu salmon (*Oncorhynchus masou*), and therefore Gnrh3 was the most likely to be the gonadotropin secretor in salmonids. In their study they found a correlation between increase ventral telencephalon and preoptic area *gnrh3* mRNA levels with higher

GSI levels and concluded that activated Gnrh3 synthesis is related to the appearance of precocious males (Amano, Kitamura, et al., 1997).

In the current study, temperature within the optimal range, like treatment group 8, show potential consistency between the hypothesized signal relationships in which *gnrh2*, *gnrh3*, and *gpr54* (which will be discussed later) have similar expression trends (Figure 25). The transcripts from treatment group 8 suggests that the signalling pathway is preserved in Atlantic salmon. Group 8 had a significant and rapid increase in expression of both *gnrh2* and *gnrh3* towards the end of the experiment, with no occurrence of maturation in this group. This could be due to a potential inhibitory role of increased *gniha* expression, which will be discussed later.

In group 15 there seems to be a disassociation amongst this particular signalling pathway, as the trend seen in group 8 were not observed (Figure 23). Instead, there was a high variance with little change in abundance throughout the entire experiment. These results are hard to interpret due to the conserved role of Gnrh as there was a high yield of maturing individuals in this groups. When looking at this system in zebrafish, Marvel et al., (2018) generated a double Gnrh knockout zebrafish line and found that the loss of both GnRH isoforms resulted in no major impact on reproduction. Furthermore, Yun Liu et al. (2017) found no impairment on puberty or reproductive defects with the inactivation of *kiss* genes in a *gnrh-/-* mutant background. They did, however, find an increase in neuropeptides reported to stimulate gonadotropin release. This could indicate that a compensatory response outside of the Gnrh system was activated in zebrafish.

The disruption in signalling in group 15 in the present study could be due to the Gnrh-system being affected by temperatures outside of the optimal thermal range. This has been supported by studies done in mammals (for review: Bronson, 2009) and in teleosts (Shahjahan et al., 2017). Through the current study and previous studies, it is apparent that the GnRH-system is highly affected by temperatures outside of salmon's optimal range, creating disruption of this particular signalling pathway. In saying that, it is important to note the difficulties experienced in this experiment with temperature fluctuations especially in and around the time of changing conditions, which could have potentially affected gene expression. In a

similar manner to zebrafish (Yun Liu et al., 2017), the elevated temperatures could have initiated a compensatory mechanism for the onset of maturation. In salmonids, the potential for undiscovered compensatory mechanism is high due to the generous amount of paralogs present after the 4R. With increasing molecular biology techniques our ability to investigate these paralogs in greater detail will surely help our understanding of the Gnrh-system in teleosts.

4.2.3.3 *gpr54* transcription

GPR54 is G-coupled protein receptor activated by kisspeptin, best known for its role in activating GnRH in mammals and birds (Roa et al., 2008). Pioneering studies of the kisspeptin system in mammalian species revealed that inactivating mutations in GPR54 resulted in that affected individuals did not undergo puberty (Seminara et al., 2003). The research on the effect of kisspeptin gonad development and maturation is limited and contradictory in teleosts. In goldfish, Li et al. (2009) found that kisspeptin cannot elicit Lh secretion from the pituitary, while Yang et al. (2010) found that kisspeptin can affect pituitary Lh secretion. In salmonids, even fewer studies have been done on the kisspeptin-system with inconclusive results on its role in the BPG axis (Chi et al., 2017), further complicated by the number of paralogs.

Despite no concrete evidence of kisspeptin-system on the activation of Gnrh, hypothesises on its role and the effect of temperature can be discussed. If maturation is induced through an activation of the kisspeptin receptors, the trends from the present study indicate that groups 15 and 15-8E have a receptor concentration which could allow for the activation threshold to be met (Figure 21). In group 8 there was a significant decrease in *gpr54* expression right after WS. If maturation is induced through an activation of the kisspeptin receptors, the decrease in receptor abundance prevents the possibility of early maturation, supported by the GSI and K levels of this groups suggesting smoltification. If the signalling pathway of early activation of the BPG axis is preserved in Atlantic salmon, the sudden increase in *gpr54* expression seen in group 8 after WS could have been stimulated by daylength increased *dio2b* mRNA levels. This could also explain the upregulation seen in *gnrh2* and *gnrh3* at this time, stimulated by increased kisspeptin.

4.2.3.4 *gniha* transcription

The role of GnIH in the BPG axis and maturation is generally considered inhibitory on gonadotropin secretion from the pituitary in birds and mammals (for review: Kriegsfeld et al., 2015), however, there are some contradictory reports in teleosts (Di Yorio et al., 2019). Zhang et al. (2010) reported that intaperiotoneal administration of zebrafish Gnih to goldfish inhibited gonadotropin levels, Bock et al. (2021) reported that sheepshead minnow (*Cyprinodon variegatus*) with a higher expression of hypothalamic *gnih* had reduced GSI values, and Spicer et al. (2017) found Gnih to downregulate gene expression of *gnrh3* in the pituitary of male zebrafish. In sockeye salmon, however, Amano et al. (2006) found that goldfish Gnih stimulated pituitary gonadotropin release *in vitro*. Although Maugars et al. (2020) found two *gnih* paralogs in salmonids and Horne et al. (2022) mapped *gnih* expression in Atlantic salmon to the diencephalon region, there is little research on the involvement of GnIH on the BPG axis in salmonids.

In the present study group 8 experienced a rapid increase in expression came between the two last sampling point (Figure 22). Evidence of stimulated gonadotropin relase in salmonids (Amano et al., 2006) could suggest that the increase is connected to the increase in *gpr54* expression seen in this group at the same time. However, there was no sign of early maturation in this group, where both low GSI and K suggest smoltification. The role of GnIH on the gonadotropin release is disputed, but if looking at, as the name suggest, a inhibitory role, a potential role could be explained. The photoperiod switch to LL stimulated *dio2b* expression, likely stimulated *tshßb* mRNA levels. Once again, comparing the regulatory pathway of birds and mammals, Dio2b could have stimulated kisspeptin, which stimulated Gnrh. The increase in both Gnrh paralogs could cause an early activation of the BPG axis, however, as the individuals in group 8 decides to smoltify instead, *gniha* expression was increased, inhibiting gonadotropin release. The non-active role of *gniha* in group 15, who reached 100% male maturation, supports the suggestion of a inhibitory role on gonadotropin release, however, the missing transcript from group 15-8E makes this hard to discuss.

4.2.4 Factors that may determine the decision to initiate early maturation

Although some parts of the early activation of the BPG axis relates to factors influencing growth, like temperature, size, and genetics, the initiation of maturation is still largely unclear.

While there are some parts of the BPG axis in mammals and birds that translates to salmonids, it is apparent that there are some stark differences in the early activation of maturation between these vertebrates. The photoperiodic WS effect on *dio2b* expression in the FW-phase gives convincing evidence of its involvement in the early activation of maturation, yet how it could potentially influence the Gnrh-system, or which Gnrh neuron it effects, is still unclear. In this study the trend between gene expression was investigated and showed a baffling difference between temperature regimes. It could appear that normal BPG function is expressed within the optimal thermal range, however, in temperatures outside of the range, the function is changed. While no studies on a secondary regulatory system for reproduction has been conducted in salmonids, there are evidence of this in zebrafish (Yun Liu et al., 2017; Marvel et al., 2018; Tang et al., 2015). In elevated temperatures outside the optimal thermal range, it could be possible that a secondary regulatory system for maturation is activated, which would explain the lack of responsiveness seen in the present study from group 15. Another possibility could be that, as elevated temperatures increase the rate of biological processes, samplings in the pre-WS phase were not performed early enough and perhaps too spread apart, missing a possible increase in expression for the maturing individuals.

Thorpe (2007) states that a certain energy threshold must be reach for an individual to mature. If this threshold is reached before they migrate, they will remain in FW to sexually mature and partake in spawning as precocious males. If the threshold is not met, they either remain in the river or undergo smoltification and migrate. In aquaculture rearing settings, where environmental conditions are artificially controlled, the opportunity for quick growth are much higher than in nature as observed with a higher yield of early maturation. In the present study there was a clear link between body size and maturation, as group 15 had the highest growth rate and the highest yield of maturing individuals, which is consistent with the energy threshold theory. Another prominent theory for early maturation and the occurrence of precocious males is based in the temperature size rule. Temperature controls the rate of

biological processes where warmer temperatures elevates the rate. Warmer water also holds less oxygen, and this conjunction makes being big in warmer water more difficult for an individual than being small. Thomas W. K. Fraser (personal communication, May, 2023) from the Institute of Marine Research (Bergen, Norway) proposes, in relation to maturation, that this is a balance. Males mature at a smaller size than females, who require more energy to produce eggs, however, there are reports about females maturing at <500g in >15°C (Fjelldal et al., 2018). These females had very few eggs, meaning their reproductive success was probably limited. Maturing at this stage, if reproductive success is limited, does not make sense unless the individual believes this is their only chance at reproducing. This suggest that the elevated temperatures cause such a high amount of stress for the individual that they believe they must rush to mature before they die, which could be the point in which a secondary regulatory system comes in, overriding the GnRH-system. Stress-induced cortisol release (including high temperature-induced effects) has been shown in several teleost species to promote the synthesis of 11-ketotestosterone (hormone produced in the testis) in response to gonadotropin stimulation and several salmonids species have displayed higher levels of plasma cortisol during maturation (for review: Rousseau et al., 2021). In Atlantic salmon, cortisol levels have been shown to increase during photoperiod change to long-day (Mobley et al., 2021; Pino Martinez et al., 2021).

It's important to consider the role genetics play in developmental variance amongst a cohort. When looking at two different strains of Atlantic salmon, Handeland et al. (2004) found that overall, the individuals reared at 12°C had a higher growth rate than at 8.9°C. On the other hand, they found that one of the strains had a higher growth potential in the lower temperature, hinting to the involvement genetics could have on the suggested lipid threshold and the occurrences of postsmolt maturation in aquaculture setting, and precocious males and grilse in the wild. Kallio-Nyberg et al. (2020) showed that there is an increase in the proportion of grilse, individuals that return to spawn after only 1 year at sea (L'Abée-Lund et al., 2004), in the wild likely caused by climate change increasing the temperatures in both the sea and in rivers. This is an intriguing find as it is possible that the intense rearing conditions in aquaculture produce post-smolt who would have been grilse in the wild, again pointing to the role of genetics in maturation.

5 Conclusion

Results from the present study further supports the idea that temperature is the main driving factor for early maturation. Physiological development was significantly higher in group 15 (kept at constant 15°C) than the other treatment groups, reaching 100% maturation towards the end of the experiment, and the percentage of maturation decrease with temperatures. It is likely that the elevated temperatures exert influence on early maturation in two manners: promoting physiological development and inducing energy accumulation, and through earlier activation of the BPG axis than at lower temperatures. The photoperiodic switch from LD12:12 WS to continuous light (LL) likely acted as the environmental cue to trigger maturation in the groups with higher energy accumulation. The role of photoperiodic activation is supported by the weaker, yet still significant, physiological response related to sexual maturation development seen in the groups with lower proportion of maturing individuals. The timing of reduction in temperature simultaneously to switch in photoperiod did not cause any significant physiological differences in groups 15-8E and 15-8L but the reduction did appear to have a delaying effect on the onset of maturation for both groups. Photoperiodic effect on the activation of the BPG axis could be explained in the expression trend seen in group 8, where there is a response in several of the genes to the increase in light after WS. However, this is not seen in the other treatment groups, hinting to a possible earlier activation of the axis due to early elevated temperatures. In the groups with higher accumulation of energy, there could be an earlier activation of the BPG axis, or a secondary regulatory system to the Gnrh-system. Furthermore, the simultaneously increase in expression of several genes after WS seen in group 8 makes it hard to conclude if a gene, and which, exerts stimulatory function on another. The present study suggests the need for more research in this topic, especially that of the effects of Dio2b and temperature, Gnih´s role during smoltification/early maturation, and deciphering the different roles of Gnrh paralogs plays in teleost maturation.

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Appendix
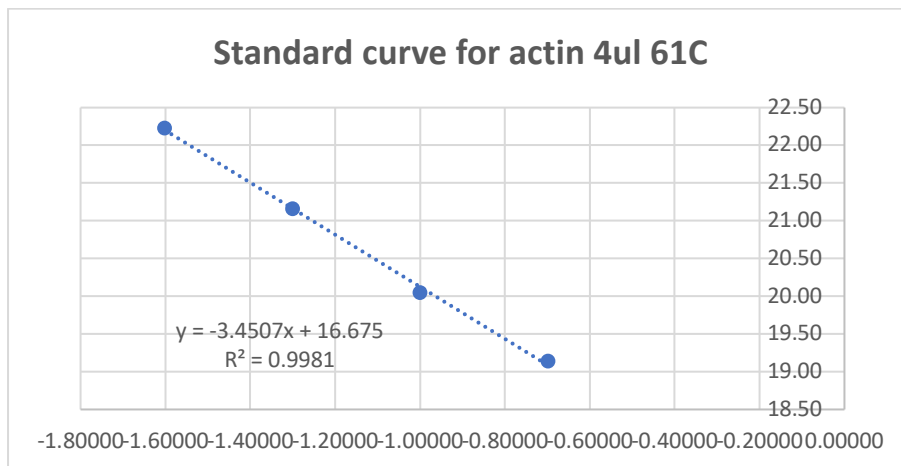
Supplementary figure I: *Example of standard curve from dilution series. Dilution series were made for every primer to validate for efficiency and optimal dilution. Shown here is the standard curve for reference gene* actin.
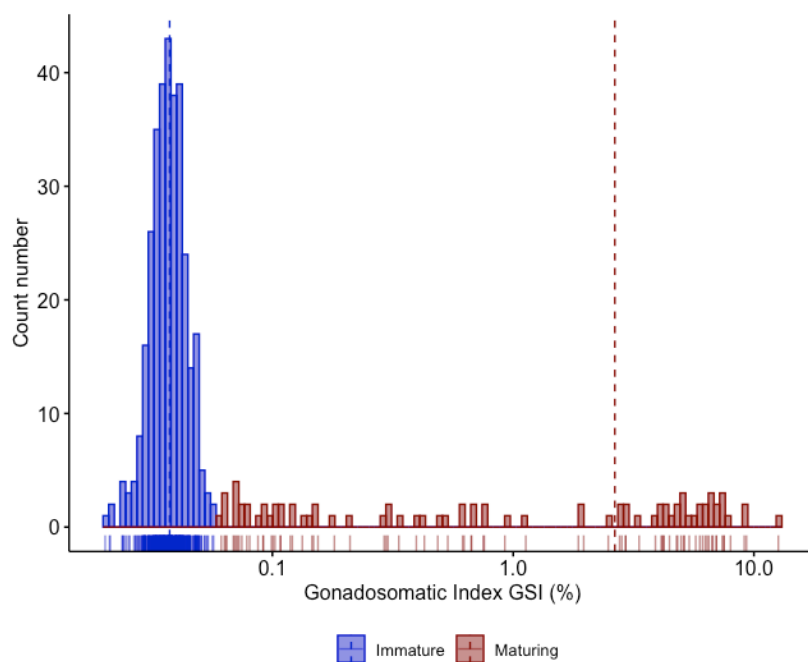
Supplementary figure II: *Proportion of the variable GSI% from the experiment differentiating between immature (GSI < 0.06) and maturing (GSI > 0.06). The dashed lines represent the mean GSI value of the maturity categories. X-axis is displayed in logarithmic scale. In immature males, GSI is normally distributed and as maturation proceeds, GSI starts increasing, thus changing the distribution from normal to highly right-skewed (). GSI variability showed that values of GSI up to 0.06% followed a normal distribution with mean $0.036 \pm 0.007\%$.*

Supplementary table I: *Sample of working data from RNA extraction and qPCR. Overview of morphometrics, GSI, data from RNA extraction used for cDNA synthesis, and the relative mRNA abundance of each gene tested. Shown here is the data collected from sampling 3, on the 28th of July. Red marks outliers based on GSI%.*

fish_id	SAMPLING	treatment	Weight (g)	K	GSI%	tank_id	Nucleic Acid	A260/A280	A260/A230	A260	A280	[dio2b/actin]	[gnrh2/actin]	[gpr54/actin]	[gnrh3/actin]	[gniha/actin]
60	3	8	86.0	1.29427	0.030	1	928.839	1.996	2.456	23.221	11.636	0.3893	0.3094	2.3469	1.9026	0.2969
61	3	8	110.0	1.12241	0.029	1	802.719	2.003	2.444	20.068	10.021	1.5439	0.6105	2.2684	1.2265	0.0830
62	3	8	111.0	1.30747	0.031	1	1208.469	2.026	2.42	30.212	14.913	0.5563	0.6745	1.2616	0.5948	0.1143
63	3	8	107.0	1.07663	0.041	1	841.258	2.013	2.439	21.031	10.448	1.0505	0.6485	1.8251	0.7334	0.1131
64	3	8	95.0	1.36340	0.042	1	880.973	1.962	2.41	22.024	11.226	0.2294	0.6723	1.7409	1.3335	0.1288
65	3	8	99.0	1.23750	0.031	2	736.749	2.003	2.462	18.419	9.194	0.4681	0.6367	1.9708	0.9529	0.1909
66	3	8	95.0	1.26170	0.021	2	826.935	2.001	2.454	20.673	10.331	0.3968	1.0782	2.5292	0.9815	0.2067
67	3	8	104.0	1.15570	0.038	2	940.442	1.991	2.398	23.511	11.809	0.4003	1.1767	1.6656	1.1956	0.1814
68	3	8	102.0	1.35467	0.029	2	718.045	1.974	2.414	17.951	9.096	0.2830	1.0281	2.2692	0.8124	0.1949
69	3	8	108.0	1.05693	0.031	2	1033.157	2.001	2.411	25.829	12.909	0.9867	2.5141	2.4372	1.9131	0.1583
70	3	8	89.0	1.18201	0.027	2	864.543	2	2.394	21.614	10.805	0.9680	1.5617	1.2845	1.2556	0.1846
71	3	8	82.0	1.19551	0.040	2	877.635	1.966	2.417	21.941	11.158	0.4158	1.7101	1.2457	1.1500	0.2914
72	3	15-8E	119.0	1.13296	0.028	3	865.796	1.997	2.38	21.645	10.839	1.4151	0.8249	2.3652	1.1934	-
73	3	15-8E	202.0	1.12318	0.035	3	1064.726	1.991	2.406	26.618	13.368	1.6872	1.3066	2.6485	1.8845	-
74	3	15-8E	184.0	1.17760	0.048	3	1052.197	1.991	2.383	26.305	13.163	0.8830	1.2258	2.0161	1.4950	-
75	3	15-8E	177.0	1.16043	0.032	3	880.24	1.954	2.274	22.006	11.264	2.0035	0.6802	2.8001	4.8969	-
76	3	15-8E	188.0	1.31020	0.045	3	967.044	1.97	2.297	24.176	12.274	1.8471	0.5626	2.1452	3.1053	-
77	3	15-8E	170.0	1.21450	0.035	4	1019.218	1.981	2.426	25.48	12.86	1.1809	1.3299	2.7361	1.1248	-
78	3	15-8E	152.0	1.31680	0.039	4	1175.598	1.999	2.411	29.39	14.7	0.4048	0.7784	1.2588	0.4650	-
79	3	15-8E	197.0	1.26080	0.038	4	1036.635	1.998	2.379	25.916	12.971	0.3959	1.7306	1.8304	0.7470	-
80	3	15-8E	154.0	1.26572	0.036	4	852.091	1.977	2.402	21.302	10.773	0.9318	0.8072	1.6422	0.8770	-
81	3	15-8E	209.0	1.37022	0.045	4	955.068	1.989	2.363	23.877	12.005	0.5470	1.8671	1.6793	0.8377	-
82	3	15-8E	166.0	1.15688	0.036	4	1004.736	1.981	2.4	25.118	12.679	0.9134	1.2067	1.4312	0.7167	-
83	3	15-8L	197.0	1.18808	0.037	7	954.97	1.979	2.395	23.874	12.064	-	-	-	-	-
84	3	15-8L	214.0	1.21757	0.036	7	901.29	1.965	2.379	22.532	11.467	-	-	-	-	-
85	3	15-8L	133.0	1.26625	0.063	7	895.972	1.977	2.479	22.399	11.33	-	-	-	-	-
86	3	15-8L	199.0	1.15876	0.045	7	929.326	1.992	2.438	23.233	11.664	-	-	-	-	-
87	3	15-8L	187.0	1.28728	0.048	7	1241.334	2.002	2.452	31.033	15.501	-	-	-	-	-
88	3	15-8L	163.0	1.12207	0.034	8	1189.074	2.001	2.457	29.727	14.855	-	-	-	-	-
89	3	15-8L	169.0	1.14918	0.044	8	1288.745	2.005	2.406	32.219	16.067	-	-	-	-	-
90	3	15-8L	155.0	1.17922	0.032	8	1121.271	1.974	2.417	28.032	14.2	-	-	-	-	-
91	3	15-8L	179.0	1.14560	0.039	8	1291.951	2.009	2.435	32.299	16.077	-	-	-	-	-
92	3	15-8L	162.0	1.18665	0.037	8	1067.459	1.997	2.414	26.686	13.365	-	-	-	-	-
93	3	15-8L	189.0	1.26957	0.037	8	1183.179	2.001	2.426	29.579	14.783	-	-	-	-	-
94	3	15-8L	145.0	1.14631	0.031	8	986.973	1.99	2.382	24.674	12.401	-	-	-	-	-
95	3	15-8L	95.0	1.50040	12.632	8	766.157	1.948	2.477	19.154	9.834	-	-	-	-	-
96	3	15	183.0	1.21440	0.033	9	1346.844	2.017	2.385	33.671	16.693	0.5885	0.7661	1.3534	0.6417	0.4167
97	3	15	188.0	1.20320	0.033	9	1281.654	2.011	2.421	32.041	15.933	0.7503	0.9772	2.2092	0.7520	0.2176
98	3	15	200.0	1.19209	0.039	9	1183.967	1.987	2.452	29.599	14.899	1.3303	0.4896	1.3500	1.0111	0.0807
99	3	15	187.0	1.30324	6.495	9	985.093	1.997	2.344	24.627	12.329	0.6107	0.7157	1.7537	0.7440	0.1027
100	3	15	187.0	1.27158	0.024	9	1438.011	2.018	2.419	35.95	17.815	0.5412	0.7592	2.4893	0.6160	0.1683
101	3	15	212.0	1.34065	0.038	10	1451.409	2.016	2.415	36.285	18	1.2065	0.6452	1.7860	0.8200	0.0975
102	3	15	120.0	1.27742	0.302	10	1383.904	1.926	2.327	34.598	17.966	0.1425	0.8561	1.1311	0.7050	0.1237
103	3	15	161.0	1.16464	0.035	10	1051.818	1.993	2.217	26.295	13.191	0.4194	0.4200	1.7124	0.6339	0.1781
104	3	15	175.0	1.12000	0.033	10	1126.266	2.001	2.404	28.157	14.073	0.4079	0.7220	1.7924	0.5485	0.1053
105	3	15	162.0	1.21694	0.038	10	1288.259	2.011	2.318	32.206	16.017	0.3764	1.8437	1.0012	0.7381	0.1018
106	3	15	143.0	1.39945	0.043	10	830.766	1.975	2.4	20.769	10.516	0.3068	0.6511	1.2518	0.5562	0.1323
107	3	15	166.0	1.27910	0.036	10	1047.045	2.004	2.148	26.176	13.065	0.2862	0.8870	1.1782	0.8176	0.1304

Supplementary table II: *Proportion of male maturing individuals (GSI > 0.06 %) in the four experimental treatment groups. Lower-case letters indicate significant differences between treatment groups (p < 0.05).*

Treatment	Immature	Maturing	Significance
15	47	50	a
15-8L	83	19	b
15-8E	95	11	b
8	99	1	c

Supplementary table III: *Mixed models used, percentage of total model variance identified as random variance caused by tank replicates, final model fitted, and family or transformation applied, for each response considered. All mixed models used were either General Linear Mixed Models (GLMM) or Linear Mixed Effects Models (LME).*

Response	Mixed model	Family (link)	Transformation	Random tank variance (%)
Body weight (g)	LME	-	Log	0.1
Condition factor K	LME	-	Log	< 1
GSI (%)	GLMM	Gamma (log)	-	0.2
dio2b relative expression	GLMM	Gamma (log)	-	6.8
gnrh2 relative expression	GLMM	Gamma (log)	-	7.3
gnrh3 relative expression	GLMM	Gamma (inverse)	-	1.4
gpr54 relative expression	GLMM	Gamma (inverse)	-	1.7
gniha relative expression	GLMM	Gamma (log)	-	4.2

Supplementary table IV: *P-value from random effects nested ANOVA for the predictors (treatment, date, and the interaction between the two) tested against the response (Body weight, K, GSI, and relative mRNA abundance). Significant effects (p<005) are highlighted in red.*

	Weight	K	GSI	*dio2b*	*gnrh2*	*gnrh3*	*gpr54*	*gniha*
Treatment	<.0001	<.0001	<.0001	.1027	.6155	.1485	.5884	.7579
Date	<.0001	<.0001	<.0001	<.0001	.0175	<.0001	<.0001	<.0001
Treatment:date	<.0001	.0021	<.0001	<.0001	.002	.0399	.3157	.0005