The effects of water temperature and photoperiod: Investigating the cause of early activation of the BPG axis in male Atlantic salmon (*Salmo salar*) during the freshwater stages of development

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Abstract

Early sexual maturation of Atlantic salmon (*Salmo salar*) post-smolts has been a major issue over the years and has recently become a prevalent issue in land-based production systems like the recirculating aquaculture systems (RAS), where early maturation is more prevalent in males. Atlantic salmon that mature early experience a loss of growth, may have a higher risk of infections and are at risk of dying if they are kept in sea water during maturation, and the aquaculture companies experience a loss of profit and altered production schedules. Male Atlantic salmon were exposed to two different temperatures regimes of 12.5℃ and 15℃ and photoperiod regimes, where all salmon were exposed to long light, LL (LD 24:0) prior half being exposed to a winter signal period, WS for 5 weeks (LD: 12:12) and then LL. Body weight and condition factor (K) was measured and calculated to examine changes in growth and the gonadosomatic index (GSI) was calculated to discern maturation levels in the four experimental groups (12.5℃-WS, 12.5℃-LL, 15℃-LL and 15℃ WS). Gene expression profiles were calculated as relative mRNA abundance, where diencephalon expressions of *gnrh2*, *gpr54*, *gniha*, *gnihb*, and *dio2b* and pituitary *tshβb* expression were analyzed to ascertain their potential influence on maturation. The salmon in all experimental groups grew steadily throughout the experiment, and condition factor mainly increased in all groups suggesting the salmon did not smoltify normally. Early maturation was observed in the 15℃-LL group, indicating that exposure to a higher water temperature and continuous light may trigger early onset maturation in Atlantic salmon. Temperature was the key factor influencing changes in diencephalon expression of *gnrh2*, *gpr54*, *gniha* and *gnihb* over time, where a possible effect of the onset of maturation may be stimulated by exposure to LL and inhibited by exposure to WS. Photoperiod was the key regulator on the pituitary expression levels *tshβb*, which increased in only the 15℃-WS group and 12.5℃-WS group following exposure to LL. Results indicate that there is a TSH-DIO2 signaling system in Atlantic salmon, but its role related to maturation is not as clear as in mammals and birds. The lack of a *tshβb* expression peak in the salmon exposed to continuous lights suggest that this light regime disrupts the physiology during freshwater development and emphasizes the importance of subjecting salmon to a winter signal during freshwater development. Further studies are needed to elucidate the possible role of TSH in smoltification and maturation in Atlantic salmon. Of the tested photoperiod and temperature regimes, exposure to a water temperature of 12.5℃ and a winter signal period presents as the best option to avoid early post-smolt maturation in Atlantic salmon.

Glossary

BPG – Brain-pituitary-gonad

cDNA – Complementary DNA

Cq – Quantification cycles

DIO2 – Iodothyronine Deiodinase 2

DNA – Deoxyribonucleic acid

Ef1a – Elongation factor 1-alpha

FSH – Follicle stimulating hormone

FRW – Forward primer

GnRH – Gonadotropin releasing hormone

GnIH – Gonadotropin-inhibiting hormone

GPR54 – G-protein coupled receptor / kisspeptin peptide receptor

GSI – Gonadosomatic index (%)

K – Condition factor

Kiss – Kisspeptin

LD – Light, Darkness

LH – Luteinizing hormone

LL – Long light

mRNA – Messenger RNA

MS-222 – Tricaine methanesulfonate

Na+-K+-ATPase – Sodium-potassium pump

NTC – No template control

PCR – Polymerase chain reaction

PN – Pars nervosa

PT – Pars tuberalis

qPCR – Quantitative real-time PCR

RAS – Recirculating Aquaculture System

REV – Reverse primer

RNA – Ribonucleic acid

Ss4R – Salmonid-specific fourth WGD

T_3 – Tsriiodothyronine

T_4 – Thyroxine

TRH – Thyrotropin-releasing hormone

TSH – Thyroid-stimulating hormone

WGD – Whole genome duplication

WS – Winter signal

Ethical statement

All ethical requirements were met when conducting this experiment.

1. Introduction

1.1. Atlantic salmon (*Salmo salar*)

Atlantic salmon (*Salmo salar*) have an anadromous life history with both freshwater and seawater life stages, which involves vast differences in physiology, morphology, and behavior (McCormick, 2013; Staurnes and Stefansson, 1998). They are widely distributed in the North Atlantic Ocean and the connected freshwater rivers. Mature salmon naturally spawn in freshwater rivers where the females lay their eggs in gravel (Helfman et al., 2009), and following fertilization the eggs then develop through the winter until hatching in the spring (Jonsson and Jonsson 2011). The eggs hatch into larvae with yolk sacs attached to them for nutrition, which is a life stage called alevins (Helfman et al., 2009). When the yolk sac is gone, the salmon become fry and start developing parr marks (Helfman et al., 2009), which are vertical bands along the sides of the fish (McCormick, 2013). After becoming parrs, they stay in the rivers for at least one year to feed and grow until they are ready to become smolts (Helfman et al., 2009; McCormick, 2013).

The process of becoming smolts is called smoltification, which happens when certain conditions are met (e.g. body size and environmental factors) (Staurnes and Stefansson, 1998). Morphological changes during smoltification include less visible parr marks, silvering of the scales, darkening of the fins and dorsal side of the salmon and an elongation of the body causing the salmon to become slimmer (McCormick, 2013; Staurnes and Stefansson, 1998). Parrs have a positive rheotaxis and swim towards the current in the river and display an aggressive and territorial behavior towards other parrs (Staurnes and Stefansson, 1998). During smoltification, they develop a more negative rheotaxis and form schools which lowers the risk of being attacked by predators in the ocean (Staurnes and Stefansson, 1998). Transitioning from freshwater to seawater involves major physiological changes in the gills, the digestive system and endocrinology, where smolts become hypoosmotic to regulate the new osmotic environment in seawater (McCormic, 2013; Staurnes and Stefansson 1998), and involves an increase in Na^+-K^+-ATPase activity (Saunders et al., 1982). An indicator of smoltification is a decrease in condition factor (K), which is the relationship between weight and length (Stefansson and Hansen, 1998). Condition factor is thought to decrease during smoltification due to the high energetic cost of the process (McCormick, 2013), which involves an increased metabolism (Staurnes and Stefansson), a decreased lipid content (Sheridan, 1989) and a higher growth in length to increase survival and swimming capacity during the following downstream migration and transition to the ocean (McCormick, 2013; Staurnes and Stefansson., 1998).

Following a successful smoltification process, post-smolts then migrate to feeding grounds in the ocean later in the spring and through the summer (Hansen and Quinn, 1998). After spending 1-4 years in the ocean, the salmon then later return to their native rivers for spawning and mature during upstream migration (Hansen and Quinn, 1998; Jonsson and Jonsson 2011).

1.2. Early sexual maturation and relevance for salmon aquaculture

Early sexual maturation happens naturally in the wild at the parr stage in male Atlantic salmon prior to smoltification (called precocious males), where they become sexually mature within the first year after hatching and stay in the river to spawn (Jonsson and Jonsson 2011; Saunders et al., 1982). However, in intense aquaculture settings where they control the temperature and photoperiod to increase growth rate, the salmon has sexually matured early at the smolt stage or post-smolt stage (Good and Davidson, 2016). This is a big problem in the aquaculture industry and involves multiple negative effects such as poor salmon welfare, increased mortality, (Taranger et al., 2010), loss of growth (Taranger et al., 1999), poor fillet quality (Hansen et al., 1992), altered production cycles (Thorpe et al., 1990) and economic loss for the aquaculture companies (McClure et al., 2007). Post-smolt maturation is a serious issue in closed land-based production systems such as recirculating aquaculture systems (RAS), which have negatively affected both production and profit (Good and Davidson, 2016).

Early maturation is more common in males than in females (Taranger et al., 2010). It is thought to be more prevalent in males than in females because ovary and egg development have a higher energetic cost than do the development of testes and sperm, causing females to mature later than males (Thorpe, 1994). The varying timing of age and size within and between populations of Atlantic salmon poses issues in aquaculture (Good and Davidson, 2016), probably because it makes it difficult to predict if and when the salmon will mature in the farms before the end of the production cycle.

Negative effects on salmon welfare are of great concern when it comes to early maturation, where it has been shown to have adverse effects on the health and survival of the fish (Taranger et al., 2010). A study conducted on farmed Atlantic salmon in Canada found that mature individuals had a 13.6 times higher likelihood of getting infected with a parasite known as *Kudoa thyrsites* than the immature individuals (St-Hilaire et al., 1998). It is therefore possible that early maturation can lead to increased infections and cause welfare issues and higher mortality rates. Early maturation also involves growth loss and adverse effects on feed

utilization in Atlantic salmon and can lead to high mortality rates if the mature individuals are kept in sea water during maturation (Taranger et al., 1999; Taranger et al., 2010).

Early maturation causes multiple problems for the aquaculture industry, such as affecting the production schedules in the farms (Thorpe et al., 1990) and having deleterious effects on the fillet quality of the fish (Hansen et al., 1992). When Atlantic salmon become sexually mature, there is a change in content of protein, fat, and water which decreases the fillet quality and taste experience for consumers (Aksnes et al., 1986), which can cause big economic losses for the producers (McClure et al., 2007).

Multiple approaches have been used to prevent early maturation in aquaculture, which have had various degrees of success (Good and Davidson, 2016). One approach is selective breeding, which farmed Norwegian Atlantic salmon have been subject to for nearly 50 years, where individuals shown to be best suited for aquaculture have been selected for each breeding event (Refsti, 1998). Traits that make up the basis for these selections have increased over the years, and include growth in both freshwater and seawater, age at sexual maturation, fillet quality, resistance to various diseases and body weight (Gjerde et al., 2007). Although age at maturation has been a trait included in selective breeding, the heritability of age at maturation is low (Gjedrem, 2000). This implies that selective breeding for this trait can have less success in preventing early maturation than other approaches.

Another approach is photoperiod manipulation, where exposing the salmon to long light regimes can postpone the onset of maturation (Bromage et al., 2001; Hansen et al., 1992). A broader understanding of how different photoperiod and temperature regimes affect early maturation can help prevent it in salmon aquaculture, especially in closed land-based systems where these environmental factors can be altered.

1.3. Whole genome duplication events (WGD)

Four whole genome duplication events (WGD) have taken place in the common ancestor of salmonids (Lien et al., 2016). The first widespread gene duplication event (1R) is thought to have happened adjacent to the origin of vertebrates, which may have been genome-wide (Donoghue and Purnell, 2005; Holland et al., 1994). A second widespread gene duplication (2R) is thought to have taken place around the origin of jawed vertebrates (gnathostomes) (Holland et al., 1994) before lamprey diverged from gnathostomes (Lien et al., 2016). A third and teleost-specific whole genome duplication (WGD) (3R) occurred in the common teleost

ancestor between 320 and 350 million years ago (Glasauer and Neuhauss, 2014). The fourth and most recent WGD (4R) took place in salmonids and cyprinids between 50 and 80 million years ago (Glasauer and Neuhauss, 2014), where the salmonid-specific fourth WGD (Ss4R) is thought to have happened in the common ancestor of salmonids after they diverged from Esociformes (Lien et al., 2016).

Gene duplication results in two paralogue daughter genes that originally are identical and serve the same function but can later acquire different functions following different processes (Glasauer and Neuhauss, 2014). One process is neofunctionalization where one of the daughter genes will serve a new function, which is thought to be the rarest outcome after gene duplication (Glasauer and Neuhauss, 2014). The gene duplicate serving the new function might be preserved if the function is beneficial, and the second duplicate will serve the function of the ancestral gene (Lynch and Conery, 2000). A second process is subfunctionalization where the two duplicates together serve the function of the ancestor gene at a single-copy level (Glasauer and Neuhauss, 2014; Lynch and Conery, 2000). The third process is nonfunctionalization where one of the duplicates is silenced following multiple deleterious mutations and the other duplicate serves the function of the original gene and is considered the most likely outcome of gene duplication (Glasauer and Neuhauss, 2014). Lien et al. found that nonfunctionalization (also called pseudogenization) most likely is the main cause of duplicate loss in Atlantic salmon following Ss4R, and that 20% of the duplicates from the teleost-specific WGD and 55% of the duplicates from Ss4R have been retained in a functional manner (Lien et al., 2016).

This study will investigate multiple genes that might be related to sexual maturation in Atlantic salmon, and it is therefore important to know that Ss4R probably have led to Atlantic salmon having a higher number of duplicates of these genes than does many other teleosts, where an unknown number may have been retained and play stimulating or inhibiting roles in maturation. Additionally, some genes and duplicates from previous WGD events that have been found to stimulate or inhibit sexual maturation in other teleosts may have been lost or silenced in Atlantic salmon, thereby indicating the complexity of the neuroendocrine control of sexual maturation in this species.

1.4. Sexual maturation and the brain-pituitary-gonad axis (BPG axis)

Gonad development during maturation starts in the spring and continues during the summer until spermiation and ovulation near the end of fall and early winter, whereafter spawning

occurs (Pankhurst and King, 2010). The timing of maturation in Atlantic salmon varies considerably both within and between different populations when it comes to the size and age at maturation, which is thought to be due to varying adaptations to each population's native rivers and their environment when migrating to the ocean (Good and Davidson, 2016; Taranger et al., 2010). The process of sexual maturation comes at a high energetic cost for Atlantic salmon (Thorpe, 1994), where they undergo major anatomical and morphological changes (e.g. secondary sexual characteristics), behavioral (e.g. aggression and courting), and physiological changes (e.g. neuroendocrine regulation of gonad development) (Fleming, 1996; Jonsson and Jonsson 2011; Mobley; 2021).

A way to determine whether and in what degree the salmon are maturing is to calculate the gonadosomatic index (GSI), which is a relationship between the gonad weight and total weight of the fish (Duncan et al., 1999). In Atlantic salmon males, the GSI quickly increases from less than 0.1% when the salmon are smolts up to between 5% and 10% when they are mature and start spawning (Taranger et al., 2010). There is some variation in the GSI levels that are used to define the stage of maturation in Atlantic salmon in different studies (Ciani et al., 2021; Fjelldal et al., 2011; Good and Davidson, 2016; Melo et al., 2014). Based on recent work in our lab, the GSI levels used to define the different stages of maturation in this thesis are based on recent research in our lab, which suggested that the salmon were immature at a GSI $\leq$ 0.06%, that the salmon were at an early stage of maturation at a 0.06 $\leq$ GSI < 0.01%, maturing at a 0.1 $\leq$ GSI < 0.1% and mature at a GSI > 1.0%. (Martinez., 2021).

Many factors contribute to a "critical period" for Atlantic salmon to mature, where the thresholds for these are regulated by genetics and must be exceeded for the fish to start maturing (Taranger et al., 1999; Thorpe, 1994). The factors influencing the timing of maturation are both internal and external, where some of the proposed internal factors include body size (Thorpe, 1994), genetics (Ayllon et al., 2015; Thorpe, 1994), endogenous rhythms (Weltzien et al., 2004) and energy storage (Taranger et al., 2010), and external factors include photoperiod (Hansen et al., 1992; Taranger et al., 1999), temperature (Adams and Thorpe, 1989; Jonsson et al., 2014), behaviour (Taranger et al., 2010), pheromones (Moore and Waring, 1996) and salinity (Melo et al., 2014) (Fig. 1.1).

When the thresholds for these factors are exceeded for the salmon to become mature, a series of endocrine activities take place along the brain-pituitary-gonad axis (Taranger et al., 2010), which is stimulated by photoperiod (Jonsson and Jonsson, 2011) (Fig. 1.1.). Gonadotropin-releasing hormones (GnRH) are produced and released in the brain where they stimulate both

the production in and secretion of gonadotropins from the pituitary gland (Taranger et al., 2010; Weltzien et al., 2004). The secreted gonadotropins are follicle-stimulating hormone (FSH) and luteinizing hormone (LH), where production of the two takes place in specialized cell types in teleosts (Ciani et al., 2020; Whitlock et al., 2019). FSH and LH are transported by the circulatory system to their specific receptors in the gonads (Mobley et al., 2021; Weltzien et al., 2004), where they stimulate gonadal sex steroid hormone production (e.g. androgens and estrogens) and germ cell development (Ciani et al., 2020; Schulz et al., 2010; Taranger et al., 2010). Sex steroids will along with growth factors send either positive or negative feedback to the brain or pituitary, which will either stimulate or inhibit the production and secretion of gonadotropins (Taranger et al., 2010). FSH is coupled to the early stages of maturation and gametogenesis, and LH is associated with the later stages of maturation (e.g. spermiation and gamete maturation) and spawning (Ciani et al., 2020; Taranger et al., 2010).

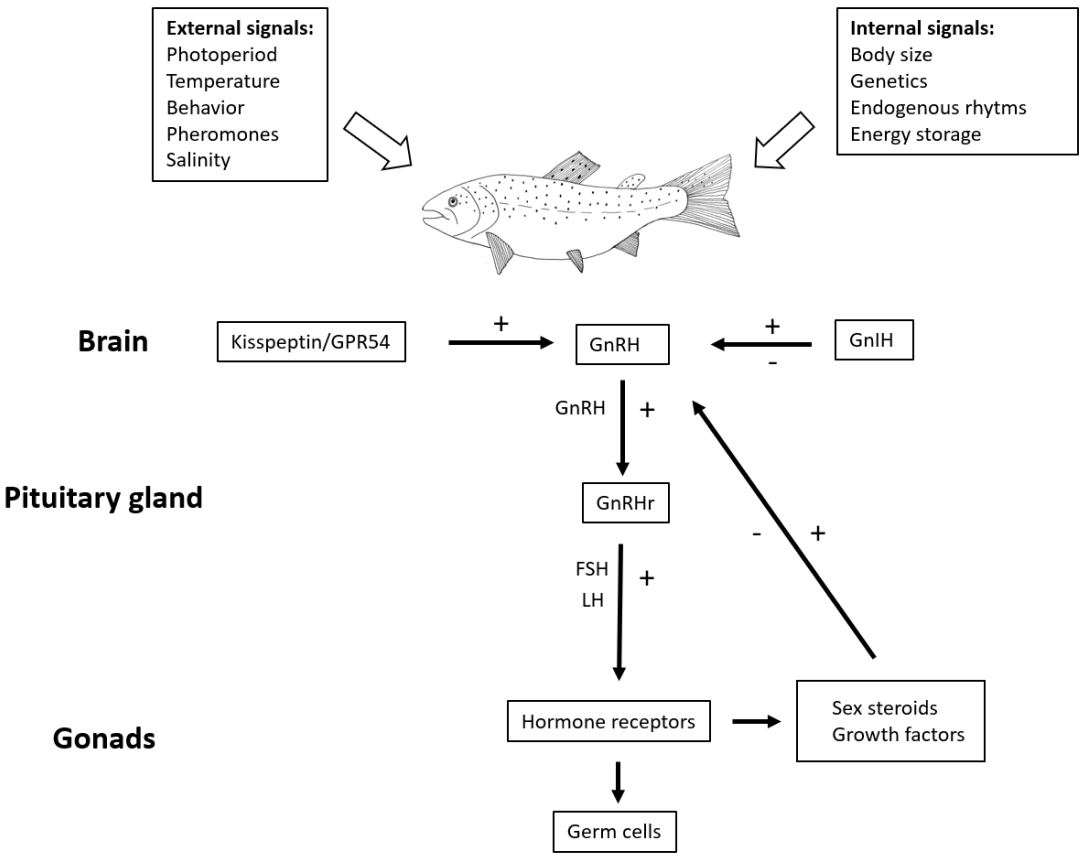

Figure 1.1. A suggested brain-pituitary-gonad axis in Atlantic salmon with the proposed roles of kisspeptin/GPR54, GnRH and GnIH. Inspired by Muñoz-Cueto et al. (2017), Taranger et al. (2010) and Weltzien et al. (2004).

1.4.1. Gonadotropin-releasing hormone (GnRH)

In vertebrates, it is well established that GnRH is the key hormone that regulate maturation and reproduction, and it is also thought to play the same role in teleosts (Whitlock et al., 2019). The structures in the hypothalamus are different from the ones in mammals, but the same cell types have been found in teleosts (Whitlock et al., 2019). GnRH has been proposed to have originated 600 million years ago and 8 forms of GnRH have been identified in teleosts (Lethimonier et al., 2004). Between two and three forms of GnRH are expressed in teleosts (GnRH1, GnRH2 and GnRH3) where GnRH3 is teleost specific (Muñoz-Cueto, 2020; Whitlock et al., 2019). In cyprinids and salmonids however, the *gnrh1* gene has been lost and only GnRH2 (also known as chicken GnRH II or cGnRH) and GnRH3 (also known as salmon GnRH, sGnRH or sGNRH3) are found in these species (Ando and Urano, 2005; Whitlock et al., 2019). Neuroendocrine neurons produce GnRH in multiple regions of the brain in teleosts (Weltzien et al., 2004), but the neuroendocrine pathway of GnRH2 and how it affects maturation in Atlantic salmon or other teleosts is not yet well known (Oka, 2009; Muñoz-Cueto et al., 2020). However, GnRH2 has, along with GnRH3, been shown to stimulate LH secretion in goldfish (*Carassius auratus*) (Pemberton et al., 2013), implying that GnRH2 possibly has a similar role in other teleosts and maybe Atlantic salmon.

1.4.2. Kisspeptin/kisspeptin receptor (*gpr54*) system

In mammals, hypothalamic peptides called kisspeptins are produced by the *kiss-1* gene, which along with the kisspeptin receptor (*GPR54*) are presumed to regulate the release of GnRH and subsequent maturation (Somoza et al., 2020; Taranger et al., 2010). Research on kisspeptin and the kisspeptin receptor *gpr54* (also called *skissr*) in teleosts is still in its early stages (Chi et al., 2017), and whether a similar kisspeptin regulatory system exists in teleosts is not yet determined (Somoza et al., 2020). However, cloning of the relevant sequences in multiple species (Taranger et al., 2010), and research supports the notion that a similar system has been conserved and exist in teleosts species (Parhar et al., 2004; Zmora et al., 2012), including Atlantic salmon (Chi et al., 2017). Two kisspeptins have been shown to be produced in some teleosts (Kiss1 and Kiss2) where different species display various expressions of one or two at different stages of maturation, indicating that a kisspeptin regulatory system in teleosts is complex and distinct between species (Park et al., 2016). Only Kiss2 has been found in some teleost species like the Nile tilapia (*Oreochromis niloticus*) (Ogawa et al., 2013) and Kiss1 is suggested to have been lost in some teleost species such as sticklebacks (Felip et al., 2009) and the Senegalese sole

(*Solea senegalensis*) (Mechaly et al., 2012). Zmora et al. suggested that in striped bass (*Morone saxatilis*) which produce both kisspeptins, Kiss1 may be on the verge of being lost (Zmora et al., 2012). These findings suggest the possibility that Kiss1 may have been lost or can be close to becoming lost in Atlantic salmon.

Recently, Chi et al. found that *gpr54* was expressed in various regions in the brain, where the highest levels of expression were found in the hypothalamus and in the saccus vasculosus in Atlantic salmon, where the expression levels were highest at both the beginning and end of maturation (Chi et al., 2017). Another interesting finding from their study was that there was co-expression of *gpr54* and *gnrh3* at some stages of maturation, which suggests that kisspeptin may regulate the secretion of GnRH in the start and end of maturation in Atlantic salmon (Chi et al., 2017). The researchers also found that *gpr54* expression levels were influenced by photoperiod, where higher expression levels were found in salmon exposed to longer photoperiods (Chi et al., 2017). Their findings indicate that there is a kisspeptin/*gpr54* regulatory system stimulating GnRH release in Atlantic salmon similar to that of mammals.

1.4.3. Gonadotropin-inhibitory hormone (GnIH)

Gonadotropin-inhibitory hormone (GnIH) is a neuropeptide named after its inhibitory role on maturation in birds and mammals, where it is found in the hypothalamus and leads to a reduced synthesis and secretion of gonadotropins (Di Yorio et al., 2019; Ogawa and Parhar, 2014). Research on the location and function of GnIH in teleosts is novel and scarce, where studies have shown that both the location and role of GnIH is more complex in teleosts and varies among species (Muños-Cueto et al., 2017). The function of GnIH seem to vary between different stages of maturation and has been found to serve either or both inhibitory and stimulating functions regarding synthesis and secretion of gonadotropic hormones (Muños-Cueto et al., 2017; Ogawa and Parhar, 2014). In most species, GnIH cells have been found in multiple parts of the brain (e.g. hypothalamus, optic tectum and preoptic area) and GnIH immunoreactive fibers (GnIH-ir) from the ventral hypothalamus have been found to innervate the pituitary gland in multiple species, where they were located near FSH and LH cells in some species (Muños-Cueto et al., 2017).

Most teleosts, including Salmoniformes, have GnIH precursor polypeptides that encode three GnIH peptides (also called LPXRFamide peptides), GnIH1 (also called LPXRFa-1), GnIH2 (also called LPXRFa-2) and GnIH3 (also called LPXRFa-3) (Muños-Cueto et al., 2017; Pinelli

et al., 2022). In goldfish (*Carassius auratus*), three GnIH peptides in the diencephalon and telencephalon have been sequenced (also called gfGnIH1-3 or gfLPXRFa1-3) (Sawada et al., 2002). Research on the role of GnIH in goldfish have found that different GnIH peptides have different inhibitory effects on maturation-related gene expression (*fsh*, *lh* and *gnrh3*) as well as on the production of LH and FSH stimulated by GnRH in females (Qi et al., 2013a) and stimulatory effects on sex steroid production in males (Qi et al., 2013b). These findings suggest a complex regulatory system where GnIH primarily play inhibitory roles within the same species. Amano et al. located gfGnIH1-3 in multiple brain regions in Sockeye salmon (*Oncorhynchus nerka*) and found that all three had a significant stimulating effect on the secretion of LH and FSH as well as growth hormone (GH), indicating that GnIH plays stimulating roles in salmonid maturation in similar manners as GnRH (Amano et al., 2006). GnIH peptides have also been sequenced in Atlantic salmon (Muños-Cueto et al., 2017), but whether they play stimulating, inhibiting or both roles in maturation is not yet known.

1.5. The effect of temperature and photoperiod on sexual maturation

1.5.1. Temperature

Atlantic salmon are poikilotherms and their body temperature changes in relation with the surrounding water temperature (Smith and Smith, 2015). Water temperature is an important external cue that affects multiple processes in Atlantic salmon, such as feeding, growth, smoltification (Hansen, et al., 1998a), and the timing of sexual maturation and spawning (Pankhurst and King, 2010). Poikolitherms have a thermal range in which the individual can survive and perform (Smith and Smith, 2015), where each physiological process has a different optimum temperature (Hansen et al., 1998a). Biological processes happen faster at higher temperatures in the thermal range than at the lower ones (Zuo et al., 2011), where feeding and growth rates in Atlantic salmon has been shown to increase with increasing temperature up to an optimum temperature (Elliott and Hurley, 1997; Handeland et al., 2008).

Water temperature can affect maturation either directly or indirectly (Taranger et al., 2010), and the direct influence of temperature has been coupled with photoperiod in Atlantic salmon where studies have found that exposure to long light regimes along with higher water temperature leads to a higher number of maturing or mature individuals (Fjelldal et al., 2011; Fjelldal et al., 2018; Imsland et al., 2014). Imsland et al. found that exposing Atlantic salmon pre-smolts to different photoperiods (natural photoperiod and continuous light) and temperature regimes

(8.3℃ and 12.7℃) led to a much higher number of males maturing in both photoperiod groups exposed to a higher temperature compared to the ones exposed to a lower one (Imsland et al., 2014). Fjelldal et al. found that exposing Atlantic salmon to three temperature regimes (5℃, 10℃ and 16℃) and two photoperiod regimes (LD 18:6 and LD 24:0) following a winter signal period (LD 12:12) led to smolts maturing only in the group that were exposed to the highest temperature and longest photoperiod regime (Fjelldal et al., 2011). Temperature has also been shown to affect reproductive investment in Atlantic salmon, where one study found that higher embryonic temperatures led to a higher gonad size in females and males, as well as a higher egg mass and larger egg size in females (Jonsson et al., 2014). The findings of these previous studies indicate a coupled effect of temperature and photoperiod regime on the onset of maturation in Atlantic salmon and was therefore studied in the present thesis.

1.5.2. Photoperiod

Photoperiod is an external cue that has a significant impact on multiple biological processes, such as growth rate, smoltification and reproduction (Hansen et al., 1998b), and seasonal changes in photoperiod is thought to be the key regulator of maturation in salmonids (Bromage et al., 2001). Altering the timing of maturation can be done by manipulating photoperiod regimes in farms (Taranger et al., 1998; Taranger et al., 2010), and maturation has been shown to be inhibited by exposing the salmon to a long light regime during the period between winter or the beginning of spring (Hansen et al., 1992; Oppedal et al., 1997; Taranger et al., 1998).

Exactly how the changes in photoperiod are sensed and signaled in Atlantic salmon to impact these processes is not yet well understood. In mammals, the hormone melatonin affects the biological rhythms connected to reproduction, whereof production and secretion take place in the pineal gland during night hours (Campbell et al., 2015). Melatonin stimulates the pars tuberalis (PT) to release thyroid-stimulating hormone (TSH) causing a stimulating pathway to release FSH and LH from the pituitary, causing gonad development (Dardente et al., 2010).

The role of TSH is to recruit the enzyme Iodothyronine Deiodinase 2 (DIO2) to cause local conversion of thyroxine (T_4) into triiodothyronine (T_3) which is more active (Lorgen et al., 2015). Nakao et al. found that exposure to long light caused increased expression of *TSH-β* in the pars tuberalis in the Japanese quail (*Coturnix japonica*) and that TSH generated expression of *DIO2*, suggesting that a similar system exists in birds (Nakao et al., 2008). In birds, conversion of T_4 to T_3 regulates the release of GnRH and the subsequent release of gonadotropis

(Yoshimura, 2013), where increases in *Dio2* have been observed prior to secretion of LH in the Japanese quail (Yasuo et al., 2005).

In mammals and some teleosts, thyrotropin-releasing hormone (TRH) is produced in the hypothalamus and stimulate the production of thyroid stimulating hormone (TSH) by thyrotrophic cells in the anterior pituitary (Fleming et al., 2020; Kryvi and Poppe, 2016; Staurnes and Stefansson, 1998). TSH then regulates the production in and release of T_3 and T_4 from the thyroid gland into the blood plasma, where increasing concentrations coincide with processes of high energetic costs (Staurnes and Stefansson, 1998). TSH consists of two subunits which are an α subunit and a β subunit, where FSH and LH share the same alpha subunit as TSH and the beta subunit (TSHβ) is specific to the hormone (Fleming et al., 2019; Pierce and Parsons, 1981).

Two paralogues of *tshβ* exist in some teleosts after 3R (*tshβa* and *tshβb),* and two paralogues of *tshβa* have been identified in Atlantic salmon following Ss4R, where evidence suggests that *tshβaα* has been conserved and *tshβaβ* is a pseudogene and has been silenced (Fleming et al., 2019). Only one *tshβb* paralogue has been conserved in Atlantic salmon after Ss4R, indicating early loss of the other paralogue sometime after this WGD event (Fleming et al., 2019).

Unlike mammals and birds, teleosts do not have a distinct pars tuberalis (Nakane et al., 2013), and a recent study found that TRH did not affect either *tshβa* or *tshβb* in Atlantic salmon, whereas multiple other hormones (e.g. cortisol, T_3, and T_4) were found to have either different or similar effects on these paralogues (Fleming et al., 2020). Both *tshβa* and *tshβb* are primarily expressed in the pituitary, wherein expressing cells have been found in specified regions in the Atlantic salmon (Fleming et al., 2019). The *tshβb* cells are expressed in the dorsal pars nervosa (PN) of Atlantic salmon, and the location of these cells indicate that *tshβb* potentially have similar roles as the PT-TSH in mammals (Fleming et al., 2019).

An increase of pituitary *tshβb* expression has been found to coincide with smoltification in Atlantic salmon, where its specific role was suggested to be related to the change of rheotaxi during smoltification and the start of downstream migration, whereas no increases in pituitary expression of *tshβa* were found during the experiment (Fleming et al., 2019). This, along with *tshβb* potentially being homologous to PT-TSH in mammals, raises the possibility that *tshβb* may similarly play a role in gonad development in Atlantic salmon and was therefore included in the present study.

Two paralogues *dio2* family (*dio2a* and *dio2b*) have been identified in Atlantic salmon and are thought to have originated from Ss4R (Lorgen et al., 2015). High mRNA levels of *dio2b* have previously been observed in the thalamus, hypothalamus, and the optic tectum in the Atlantic salmon brain (Lorgen et al., 2015). Evidence supporting that a system between TSH and DIO2 plays a significant role in signaling photoperiod in Atlantic salmon was found recently, where mRNA levels of *tshβb* increased in the pituitary followed by an increase *dio2b* in the hypothalamus, medulla oblongata and optic tectum in smolts exposed to a longer light regime (Irachi et al., 2021). Therefore, there might be a similar signaling system in Atlantic salmon to that of mammals where melatonin and the TSH-DIO2 pathway may affect maturation and reproduction.

1.6. Aim of the present study

Horne et al. recently located *gnrh2* expressing cells in the midbrain tegmentum and *kiss2*, *gniha* and *gnihb* expressing cells in the diencephalon of Atlantic salmon (Horne et al., under revision). Diencephalon expression profiles of *gnrh2*, *gniha* and *gnihb* and their potential roles in maturation in Atlantic salmon was therefore analyzed in the present thesis. The diencephalon expression of *gpr54* was analyzed instead of *kiss2* due to difficulties with synthesizing primers for *kiss2*.

The aim of the present study was to gain a better understanding of how both temperature and photoperiod affects Atlantic salmon and whether different photoperiod and temperature regimes can cause or inhibit early maturation. Body weight and condition factor were measured and calculated to examine whether and how the different regimes affected growth and smoltification in the salmon, and GSI was calculated to see if the fish were maturing early. Gene expression of *gnrh2*, *gpr54*, *gniha*, *gnihb*, and *dio2b* in the diencephalon and pituitary gene expression of *tshβb* was used to see whether these genes played stimulating or inhibiting roles in maturation in the experiment.

Research question 1: How does exposing Atlantic salmon parrs to different photoperiod and temperature regimes affect their development in freshwater in terms of changes in body weight (growth) and condition factor (K)?

Research question 2: Can exposing Atlantic salmon parrs to different photoperiod and temperature regimes inhibit or stimulate early maturation in freshwater in terms of increasing their gonadosomatic index (GSI)?

Research question 3: How does exposing Atlantic salmon parrs to different photoperiod and temperature regimes in freshwater affect neuroendocrine changes, and how do these changes relate to early sexual maturation?

2. Materials and methods

2.1. Experimental design

The experiment took place from October 28th, 2019 – May 7th, 2020. The salmon were placed in eight separate tanks and were acclimated for two weeks, where there was a control group for each of the four experimental conditions (Fig. 2.1). Bremnes Trovåg Recirculating Aquaculture System (RAS) (Bremnes Seashore AS, Trovåg, Rogaland, Norway) supplied the parrs, which were of the Erfjord strain. Parrs were kept in daylength (LD) 24:0 and 12°C in the RAS facility prior to delivery. Mean weight of the salmon before the experiment began was 48 grams. There were 1000 fish in total and 125 individuals in each tank, where the sex ratio was approximately 50/50. Volume of the tanks was 0,5m^3 and BioMar 4,5mm commercial pellets (BioMar, Brande, Denmark) were used as feed. The fish were fed in excess during light hours, meaning feeding was done continuously for the long light (LL) groups. Initial temperature in all tanks were 12.5°C, and was increased to 15°C in the relevant four tanks November 6th, 2019. Winter signal (WS) of LD 12:12 was introduced February 1st, 2020 and lasted for 5 weeks until March 9th, 2020. Afterwards, all tanks returned to LD 24:0.

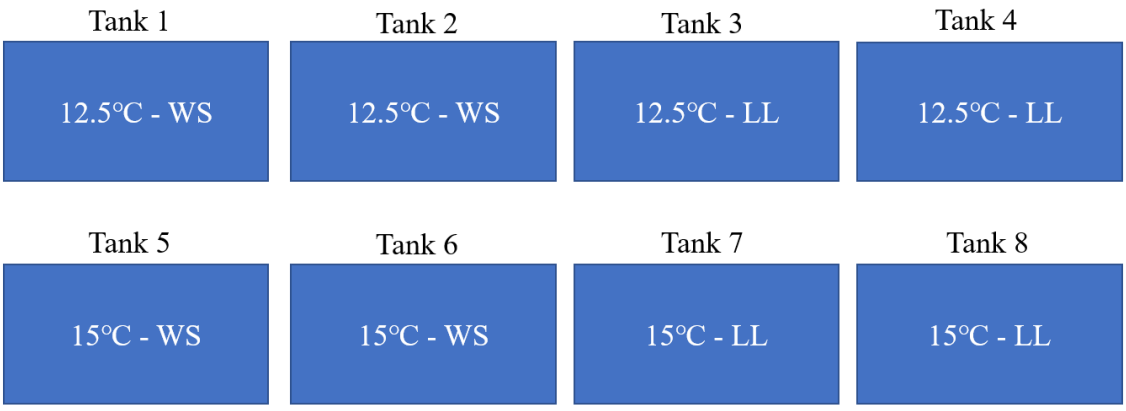

Figure 2.1. Overview of tank setup.

2.2. Sampling

The fish were sampled in eight samplings, which took place November 11th, 2019, January 28th, 2020, February 11th, 2020, February 25th, 2020, March 15th, 2020, March 29th, 2020, April 16th, 2020, and May 7th, 2020. During the first sampling, 12 fish from the 12,5°C-LL group were sampled as a baseline. Three salmon were sampled from each tank in the samplings done prior

to the introduction of winter signal. In the last samplings following the end of the winter signal period, six fish were sampled from each tank. All salmon were anesthetized with an overdose of MS-222 (10g/L; buffered with sodium bicarbonate 28g/L in a 2:1 ratio), and then weighed and measured prior to the dissections for later calculations of GSI and condition factor. Blood was drawn from the caudal vein using heparinized syringes and centrifuged for four minutes at 5000 rpm at 4°C. The blood was then spun down into plasma and stored on dry ice. Sex determination was done by opening the fish and examining the gonads, where male gonads were dissected out, weighed, stored in tubes containing RNAlater™ (Sigma-Aldrich, Burlington, Massachusetts, United States) and frozen at -80°C.

During the samplings, the heads were cut off laterally from the dorsal side and down the anterior side of the pectoral fins by using a scalpel. By putting a finger through the mouth of the fish and placing the head down facing up, the cranium was opened with an incision anterior to the nostrils down the dorsal side of the eyes (Fig. 2.2). The cranium was then opened by slowly pushing it away from the eyes while doing the incision, making the whole brain visible. A pair of tweezers was used to carefully tug at the notochord and tilting the brain over to make the pituitary gland visible, which was carefully removed. Brains were subsequently separated from the cranium. Pituitary glands and brains were placed in tubes containing 1mL of RNAlater™ for tissue preservation. All tubes were incubated at 4°C for 24 hours, then frozen at -80°C until further analysis.

Figure 2.2. Photograph showing how the cranium was opened for sampling of brains and pituitary glands.

2.3. Post dissection

Samples were taken out of the freezer and placed on ice in a polystyrene box to thaw and keep cool. Each brain was taken out of the tube and gently placed on parafilm under a light microscope. Brain dissections were carried out using a pair of tweezers, a pair of forceps and a scalpel. Firstly, the saccus vasculosus was removed by gently pushing it downwards towards the anterior side of the brain by using a pair of closed tweezers, making sure not to squeeze it or the rest of the brain (Fig. 2.3). The brains were then turned over to give a better overview of the telencephalon, which was then dissected (Fig. 2.4). Removal of the membrane was done after the removal of the saccus vasculosus and telencephalon as to not risk damaging them and was done by selecting a good start point (often a chunk of membrane under the cerebellum) and pulling it carefully off the brain (Fig. 2.5). Tectum opticum was then separated by gently opening the middle and gently pushing each side outwards and dissected, which was done

slowly to avoid causing tearing damage to the diencephalon (Fig. 2.6 and Fig. 2.7). Turning the brain over to its side and gently pushing the cerebellum and valvula cerebelli dorsally gave room for the dissection of the cerebellum (Fig. 2.8). Lastly, the medulla oblongata was separated from the diencephalon (Fig. 2.9). An overview of the diencephalon region is shown in figure 2.10. All tubes were labeled and RNA free prior to use, and the samples were placed in a freezer at -80°C.

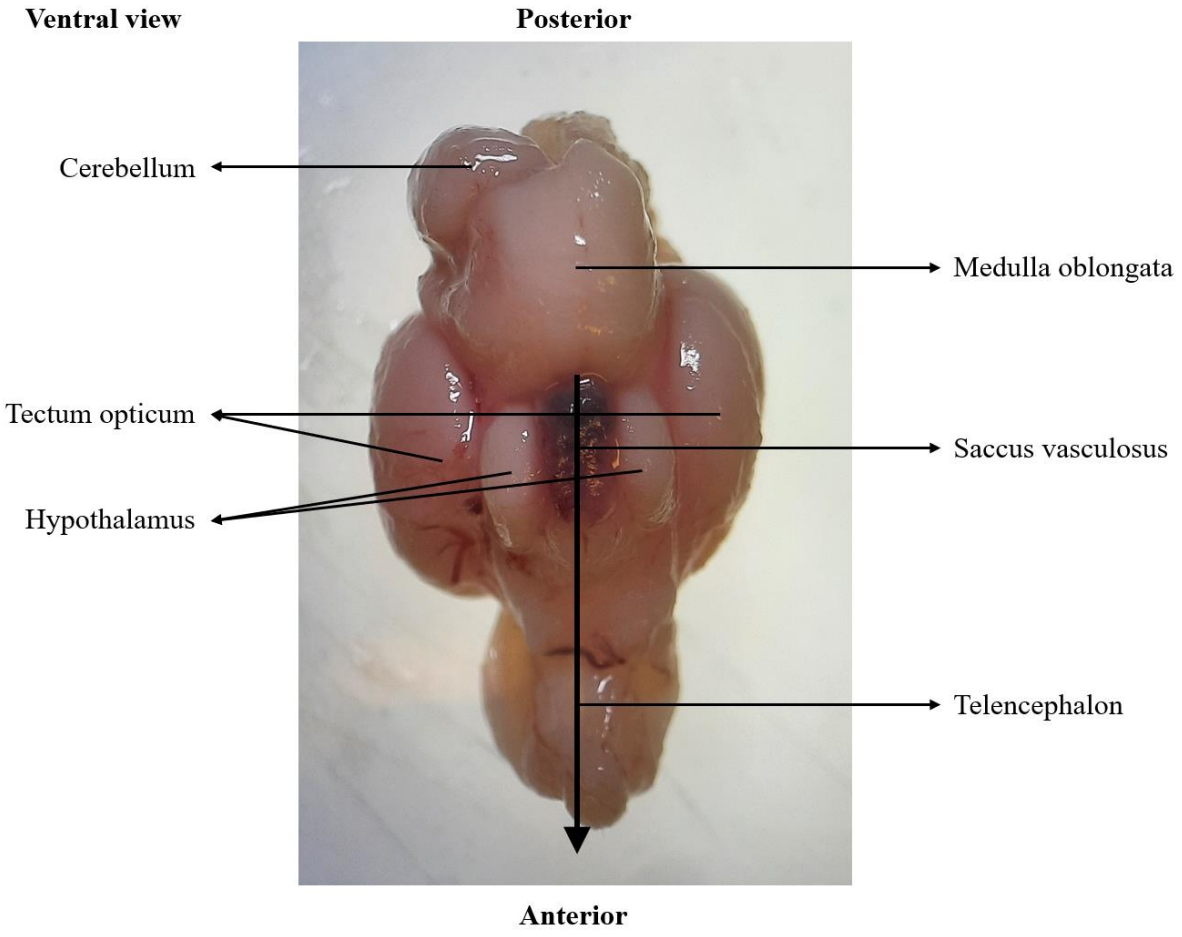

Figure 2.3. Ventral view of a brain sampled from a male Atlantic salmon, where the bigger arrow illustrates the start point and direction of the removal of saccus vasculosus.

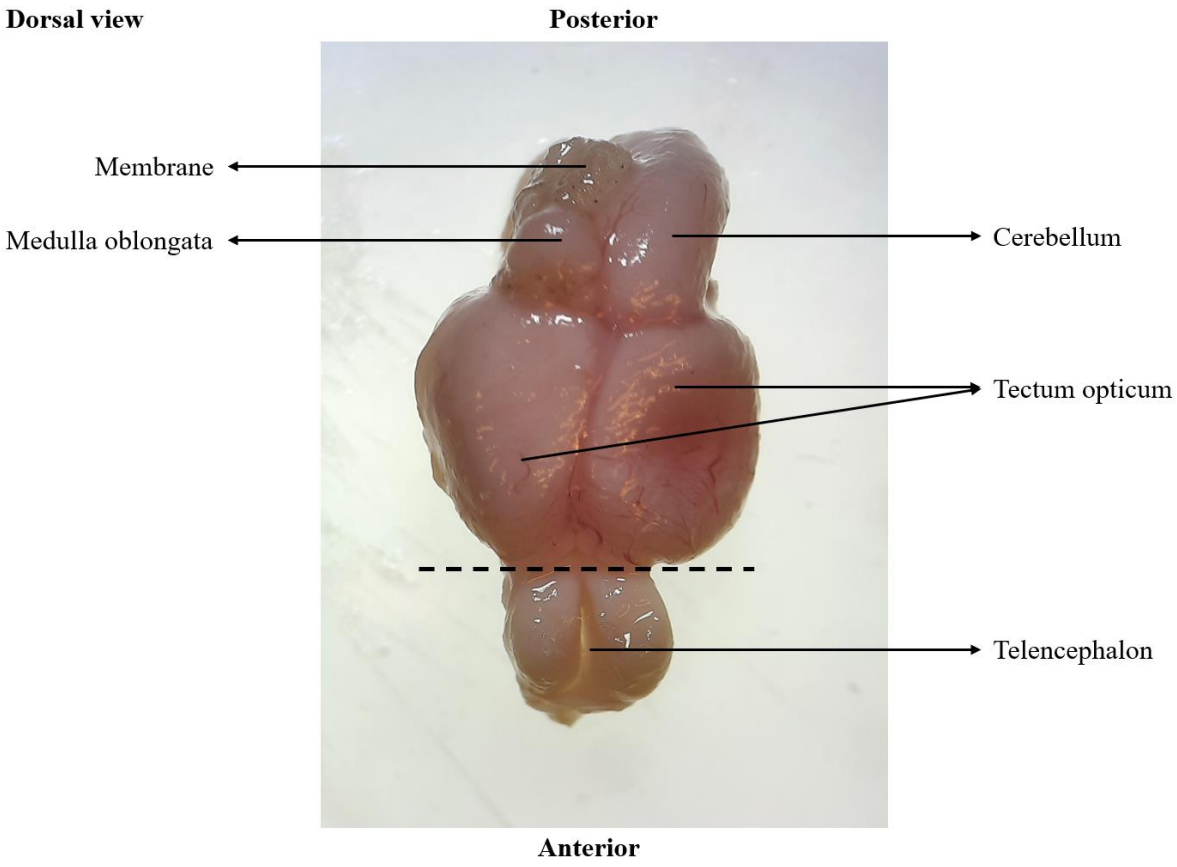

Figure 2.4. Dorsal view of the brain after removal of saccus vasculosus, where the dotted line shows where the cut was made.

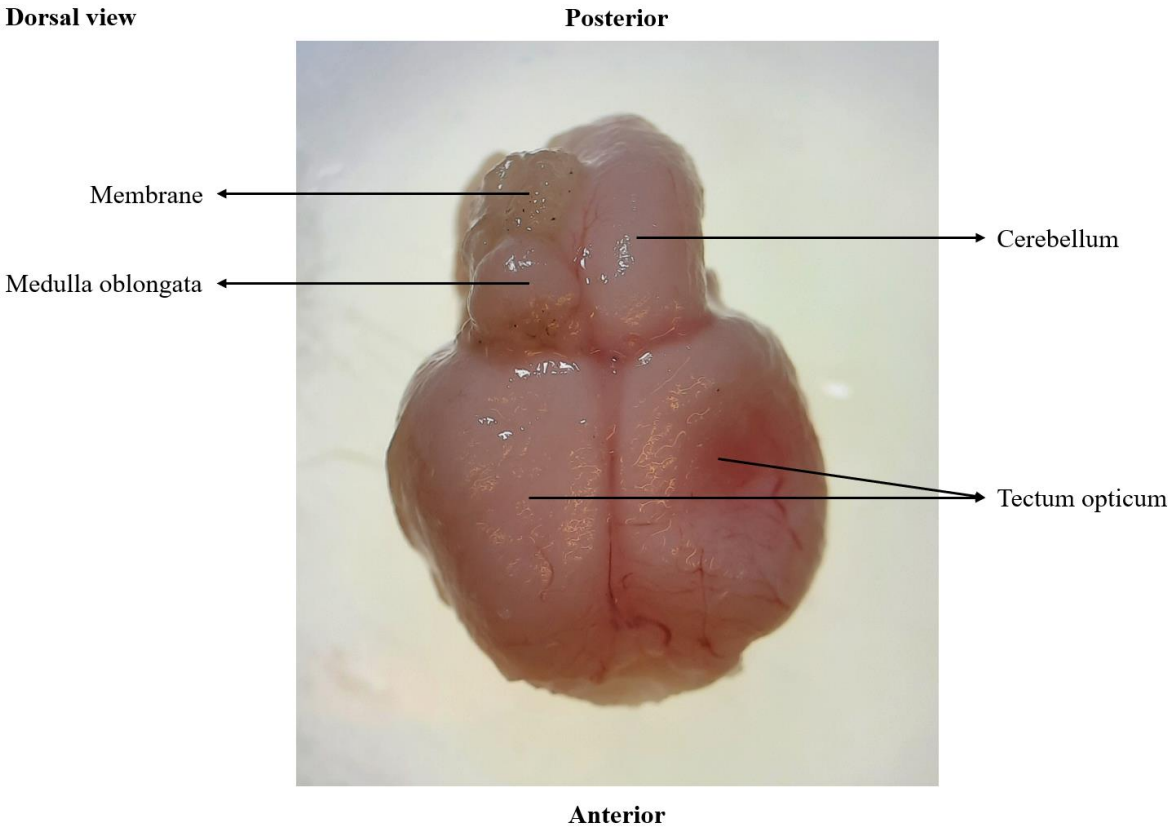

Figure 2.5. Dorsal view of the brain after dissection of the telencephalon, where the arrow to membrane shows a possible start point for membrane removal.

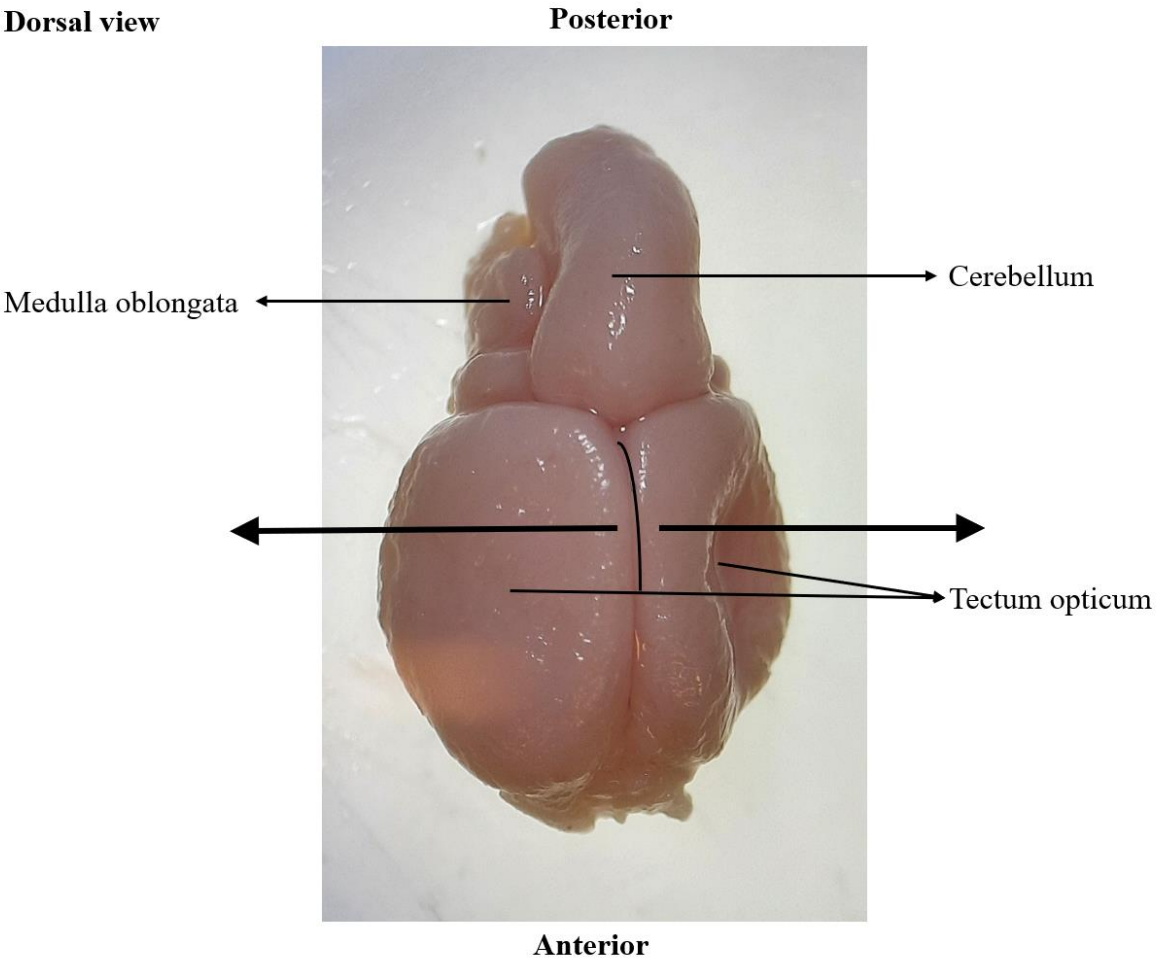

Figure 2.6. Dorsal view after removal of the membrane, where the line in the middle illustrates wherefrom the tectum opticum was separated and the thicker arrows show the direction they were pulled.

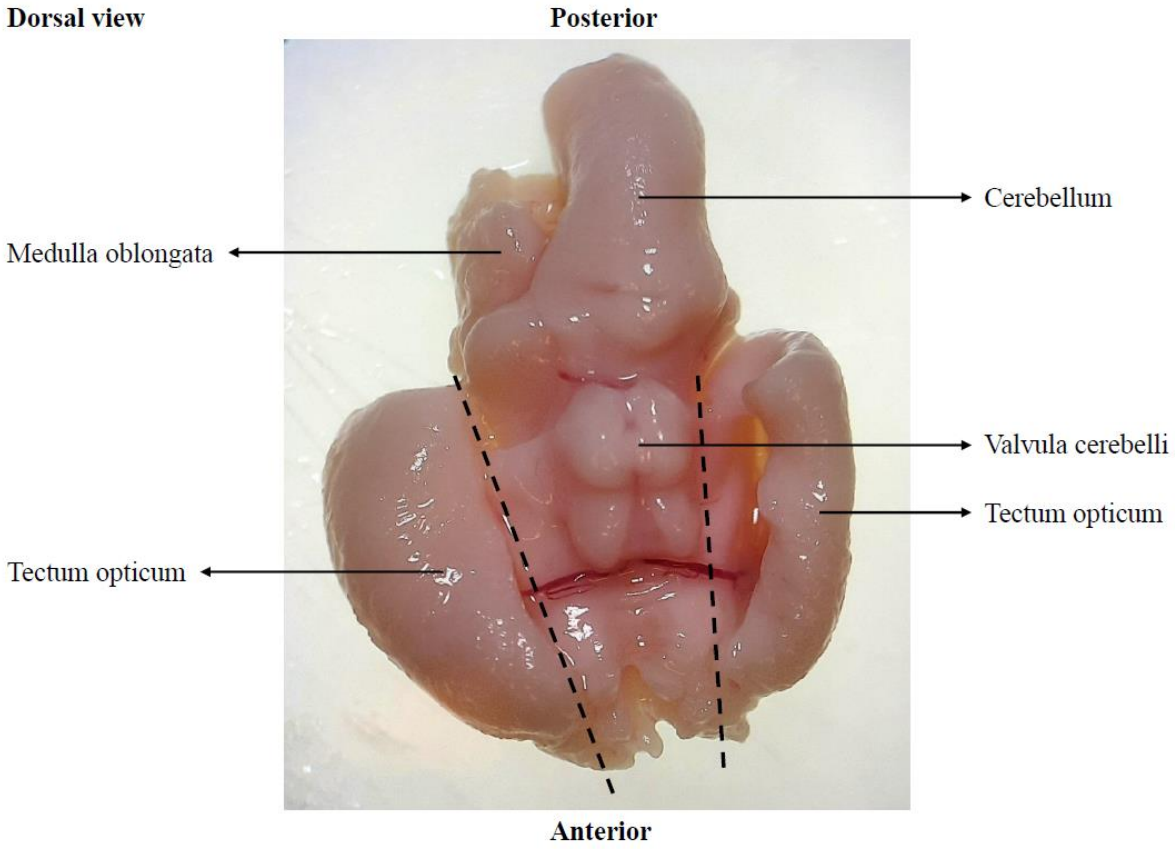

Figure 2.7. Dorsal view of the brain after the tectum opticum had been separated, where the dotted lines illustrate where the dissections were made.

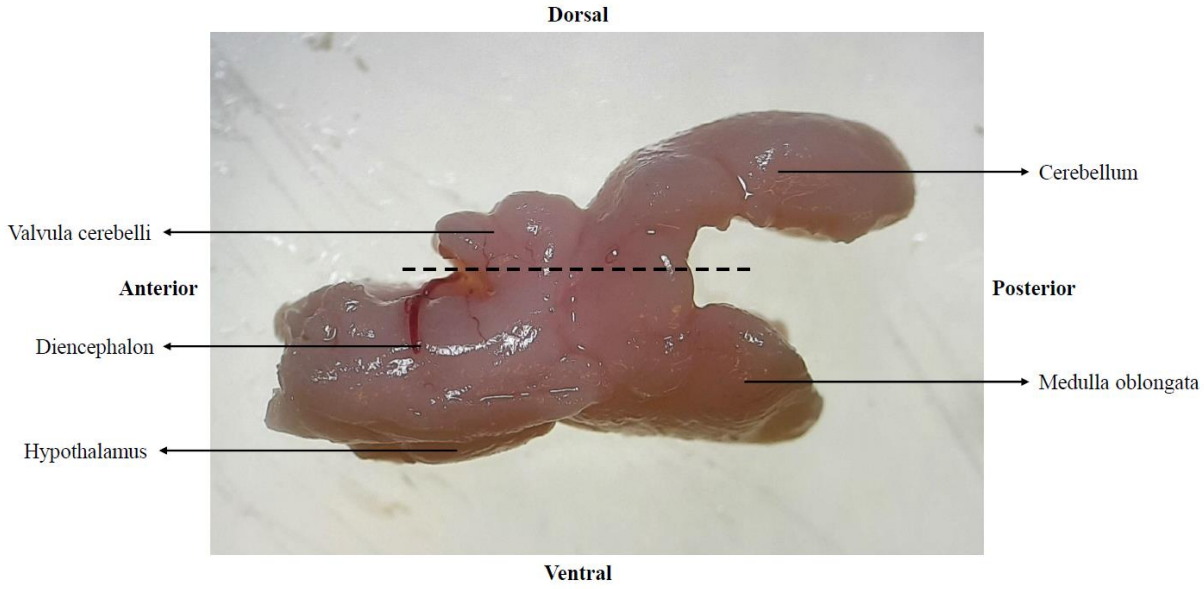

Figure 2.8. Brain after dissection of the tectum opticum and turning it over, where the dotted line demonstrates where the cut was made.

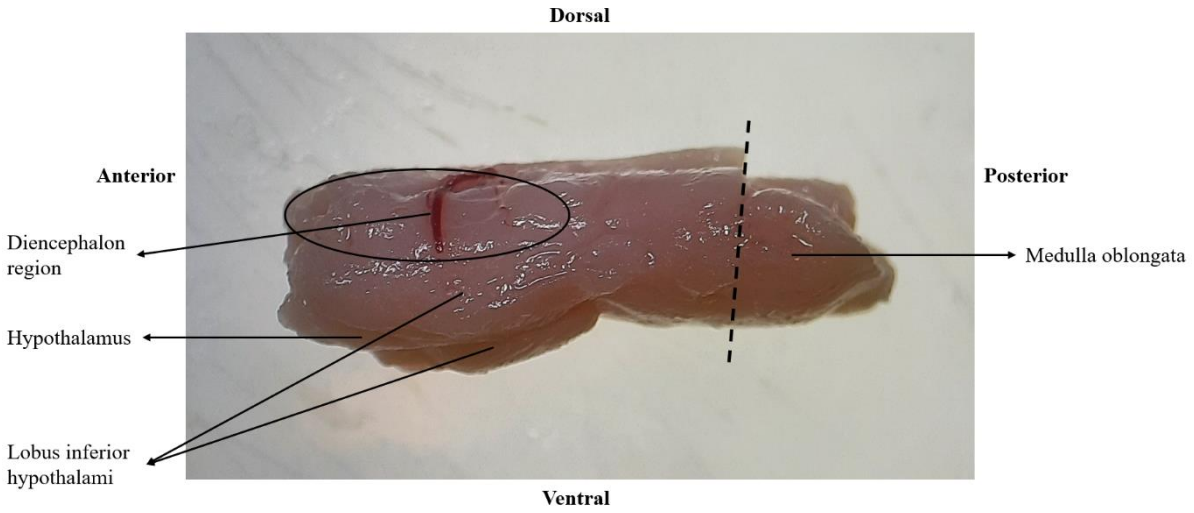

Figure 2.9. Brain after removal of the cerebellum, where the dotted line shows where the cut was made to separate the medulla oblongata from the diencephalon.

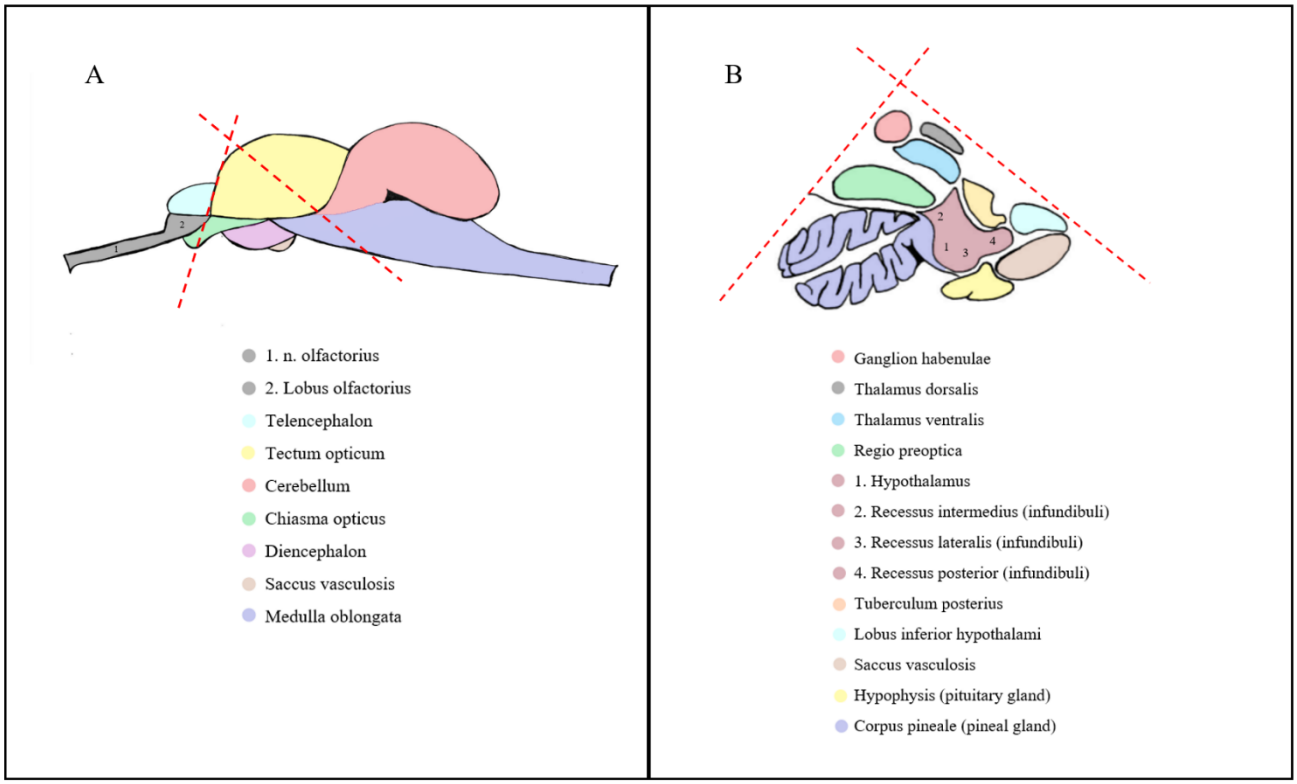

Figure 2.10. Schematic figures of the salmon brain where A. shows the whole brain and dotted lines indicate the approximate location of the diencephalon dissection and B. illustrate the parts of the diencephalon region. Inspired by Kryvi and Poppe (2016) and Nieuwenhuys et al. (1998).

2.4. mRNA extraction

Before starting the mRNA-extraction protocol, 2mL micro tubes with screw tops (Sarstedt AG & CO. KG, Nümbrecht, Germany) were labeled with the sample numbers and filled with 0.6g of Precellys® ceramic bulk beads each (Bertin Technologies, Montigny-le-Bretonneux, France). RNA-free 1,5ml tubes were also labeled for later use in the protocol. Diencephalon samples were taken out of the freezer and placed in aluminum foil on ice in a Styrofoam box. Some of the samples required extra dissection to ensure that the medulla oblongata was removed from the diencephalon before they were placed in labeled tubes. Each diencephalon was then cut into two pieces and placed in their own 2,5mL tubes (Corning Inc.). 1,5mL of TRI Reagent® (Sigma-Aldrich) was pipetted into each tube, which were then left on ice for 5 minutes. Samples were homogenized twice using a Precellys® homogenizer (Bertin Technologies) using program 2 (5000-1x15-005), left to rest for 5 minutes until the foam went down, and were then homogenized two or more times until all tissue had been homogenized. 300µl of chloroform (Sigma-Aldrich) was pipetted into each tube, and then vortexed for 15-30

seconds until the contents had a light pink color. Tubes were then placed equidistant in an Eppendorf centrifuge 5415 R (Eppendorf, Eppendorf, Hamburg, Germany) at 4℃ for 15 minutes at 15,000g. Afterwards, the tubes were carefully taken out and placed on ice. The upper aqueous layer was carefully pipetted out and into the 1,5mL tubes, making sure not to pipette anything from the other layers. 750µl of 2-propanol (Sigma-Aldrich) was pipetted into each tube, which were turned over at least 5 times to mix the contents in intervals during a 20-minute period at room temperature. From sampling 6, the volume of mRNA pipetted out increased to be over 700µl, so the amount of isopropanol was decreased to 600µl. Tubes were then centrifuged again at 4℃ for 10 minutes and were decanted swiftly afterwards, leaving the pellet behind. 1mL of 80% ethanol was pipetted into each tube to rinse the pellet. All tubes were decanted and washed with ethanol once more to ensure that the pellets were clean. Samples were either placed in a freezer at -20℃ overnight or decanted and dried for the remaining parts of the protocol. Prior to the next steps, the pellets were left to dry for a couple of minutes, ensuring that the ethanol had evaporated completely.

After drying the pellets, 50µl of nuclease free water was pipetted into each tube dissolving the pellet. Samples were then DNase treated using TURBO DNase (Thermo Fisher Scientific, Waltham, Massachusetts, United States) following the manufacturer's protocol, to remove any genomic DNA from the samples. 0,2mL PCR-tubes (Corning Inc.) were labeled and prepared. 5µl of 10X DNase Buffer (0,1µl x 50µl mRNA) and 1µl of DNase was pipetted into each prepared tube. Then, 50µl of mRNA was pipetted into the tubes. Contents were mixed by carefully pipetting up and down whilst adding the mRNA. All tubes were incubated at 37℃ for 25 minutes in a C1000 Touch^{TM} Thermal Cycler (Bio-Rad Laboratories, inc., Hercules, California, USA) with a lid temperature of 65℃. 5µl of resuspended DNase Inactivation Reagent (0,1µl x 50µl mRNA) was pipetted and mixed into each tube and left to incubate 5 minutes at room temperature, where the contents were mixed periodically during the incubation time when needed. Afterwards, the tubes were spun down and the upper layer was then carefully pipetted out and into the clean prepared tubes, which were put on ice. mRNA concentration in each sample was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Samples were then stored at -80℃.

cDNA was synthesized by my supervisor using Superscript III (Invitrogen, Massachusetts, USA) following the manufacturer's protocol. For removal of genomic DNA, cDNA was treated with Turbo DNA-free (Applied Biosystems, Massachusetts USA).

2.5. Quantitative polymerase chain reaction (qPCR)

A series of dilutions for all genes were made to determine which one would provide the best values for quantification cycles (Cq values < 30) and to test primers. For the template, a pool of cDNA from each temperature group was made by pipetting 1µl from each cDNA sample into empty 1,5mL tubes, and were thoroughly vortexed to ensure proper mixing of the contents. RNA-free water was used for the dilutions and for the no template control (NTC). To check that no other circumstances caused gene expression, elongation factor 1-alpha (*ef1a*) was used as a reference gene and serial dilutions were also ran for this gene for later normalization of the interest genes. These dilution series were later used to make standard curves, which were used to calculate the values of gene expression (further discussed in section 2.6). For all genes, a dilution of 1/20 gave the best Cq-values, so positive controls were prepared by mixing RNA-free water and the cDNA pools into 1/20 dilutions. 1/10 dilutions were also prepared for the primer pairs for each gene, mixing RNA-free water and forward primer (FRW) and reverse primer (REV) in their own labeled tubes.

Table 2.1. Calculation of substance amounts in SYBR-mixture for qPCR.

Substance	Amount per well	Total amount in SYBR-mixture
SYBR	6.5µl	6.5µl * number of wells + 10%
FRW	0.25µl	0.25µl * number of wells +10%
REV	0.25µl	0.25µl * number of wells +10%
H2O	3.0µl	3.0µl * number of wells +10%
Total	10µl	

The volumes of SYBR, forward primer, reverse primer and RNA-free water needed in the mixture was then calculated by the number of wells * 10% to account for possible pipetting errors (Table 2.1). Before pipetting, the sample numbers were randomized to avoid bias (Table 2.3). 10µl of SYBR-mixture and 5,0µl of each cDNA template and RNA-free water was pipetted in duplicates into their corresponding wells in a 96-well hard-shell PCR plate (Bio-Rad Laboratories, inc.). All plates were sealed with microseal® 'B' seals (Bio-Rad Laboratories, inc.) and quantitative polymerase chain reaction (qPCR) ran in a CFX96^{TM} Real Time System Thermal Cycler (Bio-Rad Laboratories, inc.). Plates were prepared and wrapped in aluminum foil to keep them protected from light and placed in a fridge at 4℃ until analyzis.

Each qPCR run started with an initial denaturation at 95°C for 3 minutes, whereafter 37 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute was done. For *tshβb*, the annealing temperature was set as 62°C. Following the cycles, denaturation ran for 10 seconds at 95°C, elongation at 65°C for 5 seconds and ended with an increasing temperature to 95°C with 0,5°C increments per second.

Table 2.2. Overview of primers used for each interest gene.

Gene name	Primer name	Primer sequence 5'--3'	Product size (bp)
elf-1α	Ef1FW	CAAGGATATCCGTCGTGGCA	
	Ef1RV	CCTCTTGGTCGTTTCGCTGT	317
GnRH2	ssGnRH2-1 FRW	ACCTGAGACCACAGCGAAGG	
	ssGnRH2-1 REV	AGGGTAAAGAAGGGATGCGACA	218
GnIHa	ssGnIHa-FRW	CCATGACCAACGACAACGACGG	
	ssGnIHa-REV	TTGACAGGTGGCGGGTAGAGT	172
GnIHb	ssGnIHb-FRW	TGACCGACGACAACAACAGA	
	ssGnIHb-REV	TTGGCGTGAAGGTGTAAAGG	190
dio2b	dio2bFW	GGATGTGAGGCAGTATCTGGAACAG	
	dio2bRV	GCCTGTCATTTGTGGTCAGA	184
GPR54	sGPR54-1-FRW	ACCCTTTAAAGTCCCTACGCC	
	sGPR54-1-REV	GGTGGATAGAATGAAGGAACCGAT	89
tshβb	tsh1bFW2	TTGCCGTCAACACCACCAT	
	tsh1bRV2	GGGATGATAGACCAGGGAGTG	124

Gene name	Primer name	Annealing temp (°C)	Reference
elf-1α	Ef1FW	60	Lorgen, 2015
	Ef1RV		Lorgen, 2015
GnRH2	ssGnRH2-1 FRW	60	Maugars and Fleming, no pub
	ssGnRH2-1 REV		Maugars and Fleming, no pub
GnIHa	ssGnIHa-FRW	60	Maugars G, no pub
	ssGnIHa-REV		Maugars G, no pub
GnIHb	ssGnIHb-FRW	60	Maugars G, no pub

	ssGnIHb-REV		Maugars G, no pub
dio2b	dio2bFW	60	Lorgen, 2015
	dio2bRV		Lorgen, 2015
GPR54	sGPR54-1-FRW	60	Fleming, unpublished
	sGPR54-1-REV		Fleming, unpublised
tshβb	tsh1bFW2	62	Fleming, 2019
	tsh1bRV2		Fleming, 2019

The primers used for each gene are listed in Table 2.2.

Table 2.3. Example of a randomized well setup for qPCR. Shown here is the setup for a plate of the 12,5℃ samples.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	127	127	81	81	121	121	76	76	126	126	123	123
B	119	119	124	124	131	131	128	128	122	122	158	158
C	111	111	160	160	162	162	163	163	155	155	115	115
D	221	221	159	159	118	118	71	71	28	28	63	63
E	34	34	317	317	179	179	258	258	114	114	320	320
F	212	212	27	27	312	312	113	113	269	269	223	223
G	61	61	310	310	32	32	253	253	298	298	166	166
H	31	31	254	254	271	271	35	35	NTC	NTC	Cal	Cal

2.6. Data analysis

Data from all qPCR-runs were exported as Microsoft Excel sheets. Standard curves were made in Excel using values from the serial dilutions of each gene, where gene concentrations were set as the amount of cDNA in each dilution. Scatterplots were then made the Cq-values according to the serial dilution of choice (in order from highest to lowest) (Appendix II). Using the scatterplot, we extrapolated the slope of the scatterplot using the equation ($y = mx + b$). The slope of the line indicates the efficiency of the primers (Fig. 2.11).

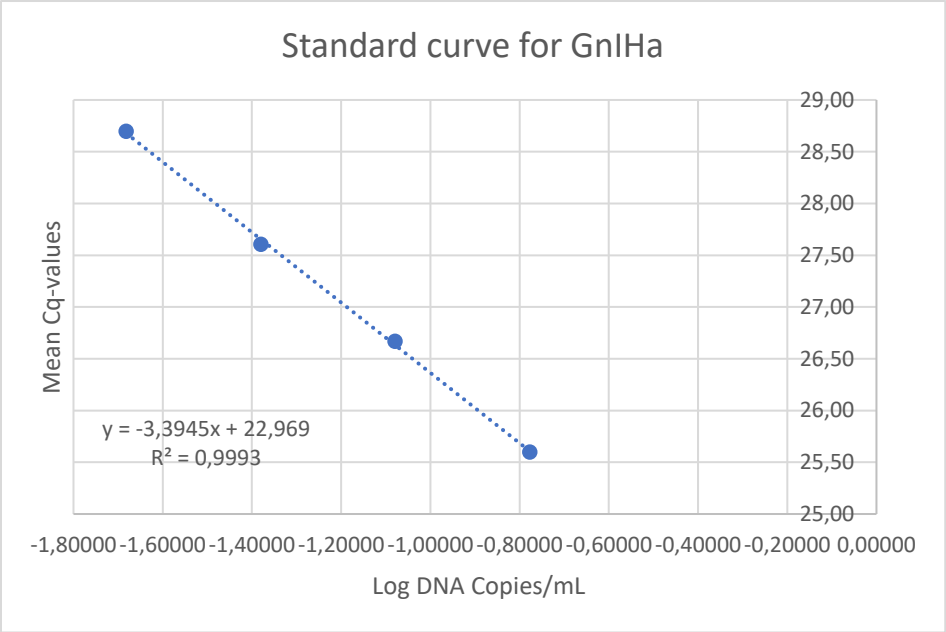

Figure 2.11. Example of a standard curve, here showing the standard curve for GnIHa.

PCR efficiency (E) was calculated in two steps. The first step was to divide -1 by the slope (m) and put it in the power of 10:

$$PCR\ efficiency = 10^{(-1/m)}$$

To convert the PCR efficiency into a percentage, the following equation was used:

$$\%\ PCR\ Efficiency = (PCR\ efficiency - 1) * 100$$

Table 2.4. Overview of standard curve values and calculated PCR efficiency (%) for all genes.

Gene	Slope (m)	Intercept (b)	% PCR efficiency (E)
ef1a	-3,5215	18,369	92,8
gnrh2	-3,3849	23,34	97,44
gniha	-3,3945	22,969	97,06
gnihb	-3,3424	23,875	99,17
dio2b	-3,2985	23,417	100,99
tshβb	-3,5182	25,483	93,2
gpr54	-3,7793	26,789	84

The PCR efficiency was then calculated for all genes (Table 2.4). Concentrations of all genes for each sample were then calculated in Excel using the values from the standard curves. As the standard curves were made using logarithmic values for the concentrations from the serial dilutions, the equation was set in the power of 10 to calculate the concentrations (x):

$$y = mx + b \quad \rightarrow \quad x = \frac{y - b}{m} \quad \rightarrow \quad x = 10^{(y-b)/m}$$

All concentrations were then normalized by the reference gene by dividing the concentrations of the interest genes (*gnrh2*, *gniha*, *gnihb*, *dio2b*, *tshβb* and *gpr54*) by the corresponding concentrations of the reference gene (*ef1a*) for each sample (Appendix I).

The condition factor (K) of each fish was calculated using the Fulton formula (Froese, 2006):

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

The gonadosomatic index (GSI) for each fish was calculated by the following equation (Fjelldal et al., 2011):

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

RStudio was used for statistical analysis of the data. Various histograms were made to check the distribution of values, where different transformations of data were tested to identify which one gave the most normal distribution and would be used for data analysis. To check for normal distribution of data, Shapiro-Wilk's test and Lilliefors' (Kolmogorov-Smirnov) test was done, where the distribution was assumed to be normal when the significance level was higher than 0.05 (p-value > 0.05). Outliers and missing values were removed for data analysis. Predictors in this experiment were date, sampling, photoperiod, and temperature, and the responses were mean body weight (g), mean condition factor (K), mean gonadosomatic index (GSI), and the mean concentrations of *gnrh2*, *gniha*, *gnihb*, *gpr54*, *dio2b* and *tshβb*. Because the fish were divided into tanks for the experiment, the data was clustered. As such, linear mixed effects models were used for all genes for data analysis, with tanks as a random effect. The models used for normality tests, summary, random variance in tanks, nested ANOVA tests (analysis of

variance) and Levene's test included the interaction between the predictors date (time), temperature and photoperiod. Random variance in tanks (%) was calculated by using the intercept and residual values from the summary of the model, where percentages < 0.05% were considered to be low and acceptable:

$$Random\ variance\ (\%)\ \frac{Intercept^2 * 100}{Intercept^2 + Residual^2}$$

Nested ANOVA tests were performed to check whether the predictors and the interaction between them had significantly affected changes in each of the responses. Levene's test was used to check the homogeneity of variance, where F-values under 20 were considered acceptable. Tukey's Honest Significant Difference (HSD) post-hoc tests were used to compare the experimental groups with each other and see whether there were significant differences between them, and to identify significant changes within each experimental group throughout the experiment. For the post-hoc tests, sampling was used as a predictor in the models instead of date. The significance level used in ANOVA and the post-hoc tests was 0.05.

3. Results

Table 3.1. Overview of data transformations and random tank variances (%) for all responses.

Response	Data transformation	Random tank variance
Body weight	Logarithmic (log10)	3.5 %
Condition factor (K)	None	0.5 %
Gonadosomatic index (GSI)	Inverse (1/x)	3.3e-07%
[*gnrh2*]	Square root (sqrt)	7.8 %
[*gniha*]	Logarithmic (log10)	1.7 %
[*gnihb*]	Logarithmic (log10)	2.6 %
[*dio2b*]	Logarithmic (log10)	2.3 %
[GPR54]	Logarithmic (log10)	2.0 %
[*tshβb*]	Logarithmic (log10)	1.4e-07%.

Various forms of data transformations were chosen for the responses, except for condition factor (K) where none was used (Table 3.1). Random tank variance varied between the responses, where all but the random tank variance for the concentration of *gnrh2* as a response were under 5% (Table 3.1).

3.1. Changes in mean body weight over time

Mean body weight increased in all experimental groups throughout the experiment, and the growth rate was similar between groups (Fig. 3.1). The salmon started with a body weight of ~52 grams and grew steadily through the experiment. In the 12.5℃-LL group, mean body weight had increased to ~635 grams at the end of the experiment. The 12.5℃-WS group had a slower growth than the others in mid-April, but had the highest end mean body weight at ~717 grams (Fig. 3.1). Mean end body weight in the 15℃-LL group was ~669 grams, and in the 15℃-WS group it was ~650 grams. Individual variance between fishes was generally low but started to increase from the end of March (shown by +/- SEM bars) (Fig. 3.1). The different photoperiod and temperature regimes did not significantly affect changes in mean body weight in this experiment, and neither photoperiod nor temperature were found to have had a significant effect on changes in mean body weight (p-value > 0.05).

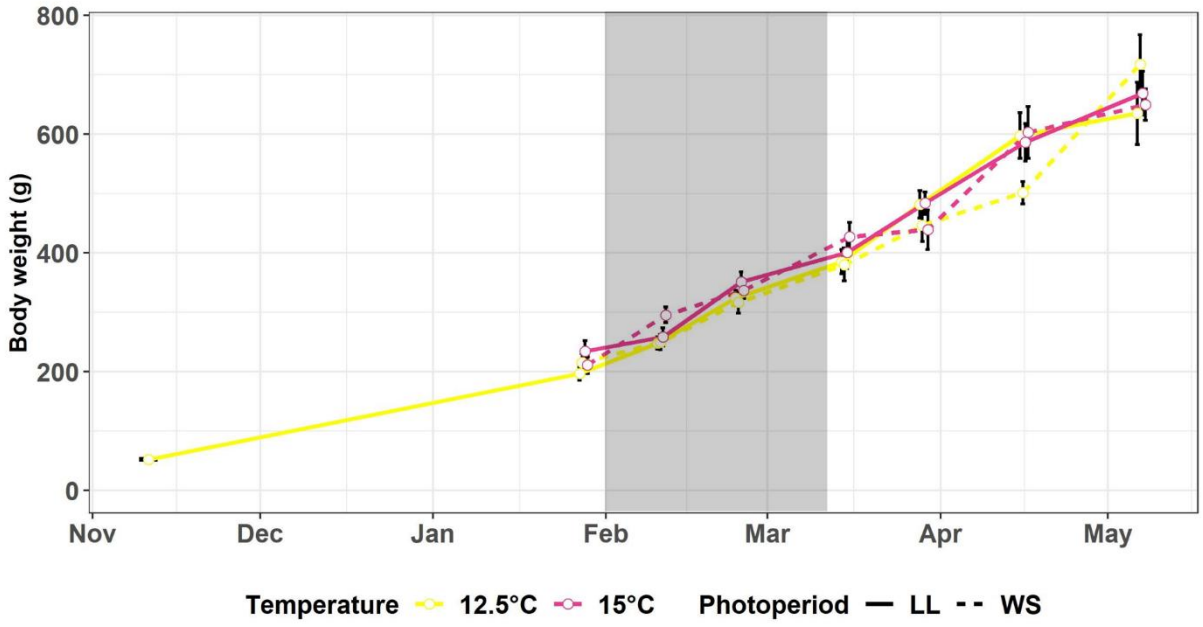

Figure 3.1. Body weight shown over time, where mean body weight at each sampling is indicated by white circles with whiskers showing standard mean error (+/- SEM). Winter signal period is indicated by grey shadowing, and samplings where significant differences were found for one or more groups are indicated by numbering over the lines. The first sampling was included to show start weight but was not included in data analysis.

3.2. Changes in mean condition factor (K) over time

Mean condition factor (K) generally increased in all experimental groups, where all had fluctuations throughout the experiment (Fig. 3.2). The fish started with a mean K of ~1,2 and all groups ended up with a higher mean K at the end of the experiment (Fig. 3.2). Mean K at the end of the experiment was ~1,3 in the 12.5°C-LL group and ~1,4 in the 12.5°C-WS group, 15°C-LL group and 15°C-WS group. The general increasing trend in all groups indicates that the salmon did not smoltify normally during this experiment in either of the experimental conditions. The 12.5°C-LL group, 12.5°C-WS group and 15°C-LL group followed similar trends halfway through the experiment, where there was an initial decrease in mean K near mid-February, followed by increases until mid-March (Fig. 3.2.). Mean K then fluctuated a bit until mid-April in the 15°C-LL group before increasing at the end of the experiment (Fig. 3.2). In the 12.5°C-LL group, mean K continued to increase until mid-April, followed by a decrease at the end of the experiment (Fig. 3.2). In the 12.5°C-WS group, mean K decreased until mid-April following the end of the winter signal and increased again by the end of the experiment

(Fig. 3.2). Mean K in the 15℃-WS group increased from the end of January until the end of the winter signal period in March, whereafter it decreased (Fig. 3.2), This was followed by a significant increase by mid-April (p-value < 0.05) and a decrease at the end of the experiment (Fig. 3.2). Individual variance between fishes mostly decreased throughout the experiment and was highest at the samplings from the end of January until the end of February (indicated by +/- SEM bars) (Fig. 3.2). A significant difference between the photoperiod regimes was found within the groups given 15℃ in the end of March and in the groups given 12.5℃ in mid-April (p-value < 0.05). There was also a significant difference found between the temperature regimes in the groups given WS in mid-April (p-value < 0.05).

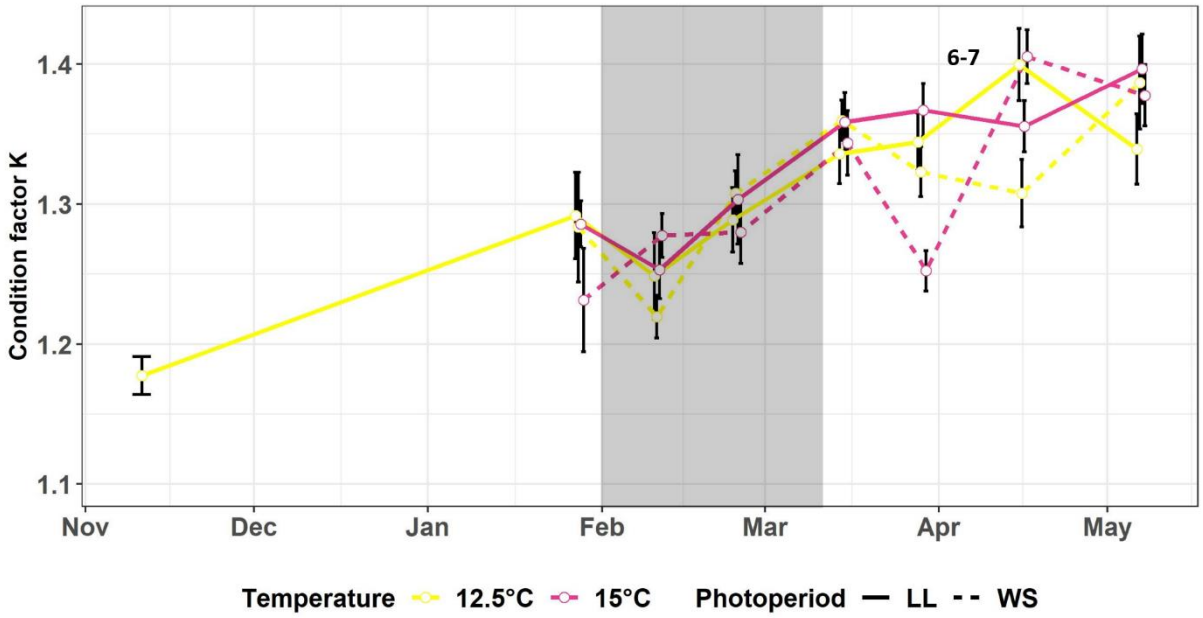

Figure 3.2. Condition factor (K) shown over time, where the circles indicate the mean condition factor at each sampling with whiskers showing standard mean error (+/- SEM). The first sampling is included to show start condition factor but was not included in data analysis. Numeration over the lines show where there was a significant difference between samplings in the 15℃-WS group. Grey shadowing indicates the winter signal period.

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

Mean GSI began to increase in mid-February in the 15℃-LL group to almost 0.3 which indicates that the salmon were maturing early in the experiment (Fig. 3.3). Mean GSI kept increasing until the end of the experiment in the 15℃-LL group (Fig. 3.3). The mean GSI in mid-March was still lower than 1.0 and indicated that the salmon were still maturing at this point, and because the mean GSI levels from the end of March until the end of the experiment were higher than 1.0 the salmon were classified to be mature at these three last samplings (Fig. 3.3).

Mean GSI remained low throughout the winter signal period 12.5℃-LL group, 12.5℃-WS group and 15℃-WS group where mean concentrations in these groups indicated that the salmon were immature from the beginning of the experiment until the end of March (Fig. 3.3). Mean GSI began rising from the end of March until the end of the experiment in the 12.5℃-LL group, 12.5℃-WS group and 15℃-WS group (Fig. 3.3). The mean increases in GSI between the end of March and mid-April and between mid-April and the end of the experiment were significant in the 15℃-WS group (p-value < 0.05). In the 15℃-WS group, mean GSI was higher than 0.1 in mid-April where the salmon were classified as maturing, and mean GSI increased to be higher than 1.0 at the end of the experiment, meaning the salmon were mature at this stage (Fig. 3.3). In the 12.5℃-WS group, the salmon were classified as maturing in mid-April as the mean was a bit higher than 0.1 and were classified as being mature at the end of the experiment as the mean was higher than 1.0. In the 12.5℃-WS group, mean GSI had increased to be over 0.06 in mid-April where the salmon were defined as being in the early stage of maturation (Fig. 3.3). By the end of March, mean GSI in the 12.5℃-WS group had increased to around 0.3 and the salmon were maturing (Fig. 3.3). Salmon in the 12.5℃-WS group did not become mature throughout the experiment (Fig. 3.3).

Large individual variances were observed in the 15℃-LL group from the end of February until the end of March and in the 12.5℃-LL group in mid-April and at the end of the experiment (indicated by large +/- SEM bars). Within both photoperiod groups, significant differences were found between 12.5℃ and 15℃ in mid-April and at the end of the experiment (p-value < 0.05). No significant differences were found when comparing the photoperiod regimes (p-value > 0.05). Temperature, time, and the interaction between temperature and time were found to have significantly affected changes in mean GSI in the overall experiment (ANOVA, p-value < 0.05).

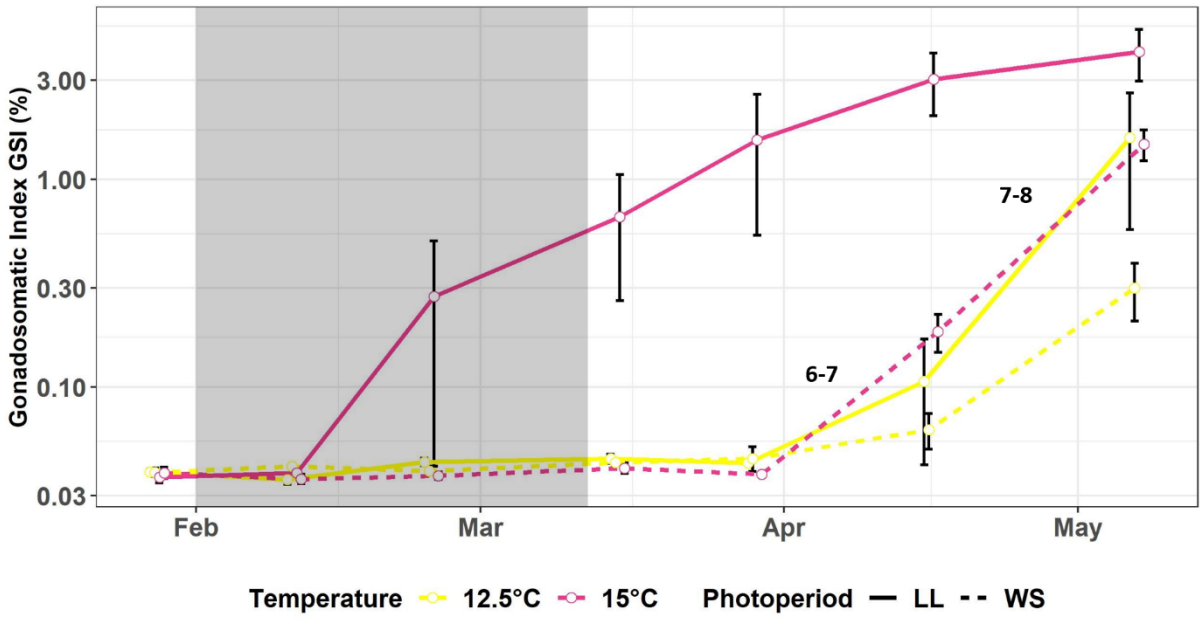

Figure 3.3. GSI (%) shown over time, where circles indicate mean GSI at each sampling and whiskers show standard mean error (+/- SEM). Numbering above lines show where significant differences between samplings were found for the 15°C-WS group. The winter signal period is marked by the grey area.

3.4. Changes in mean concentrations of *gnrh2* over time

Mean concentration of *gnrh2* was overall higher in the 15°C groups throughout the experiment, where the 15°C-LL and 15°C-WS groups had an initial increase near mid-February (Fig. 3.4). The initial increase in the 15°C groups was followed by a rapid decrease in the end of February, which was significant for the 15°C-LL group (p-value < 0.05) (Fig. 3.4). Mean concentration of *gnrh2* then increased again in the 15°C groups by the end of March after the end of the winter signal period, before decreasing in mid-April until the end of the experiment (Fig. 3.4). In the 15°C-WS group, mean concentration of *gnrh2* increased again at the end of the experiment, whereas it significantly decreased in the 15°C-LL group (p-value < 0.05) (Fig. 3.4).

In the 12°C groups, the mean concentration of *gnrh2* remained low with some fluctuations during the experiment (Fig. 3.4). The fluctuations in the 12°C groups were much less drastic than in the 15°C groups, where mostly minor changes were observed in the 12°C groups throughout the experiment (Fig. 3.4). In the 12°C groups, the concentration of *gnrh2* decreased until the end of February, followed by an increase after the end of the winter signal period in mid-March and another decrease in the end of March. Mean concentration of *gnrh2* then started

to rise again in mid-April, where the increase was significant in the 12.5°C-WS group (p-value < 0.05) and ended with a decrease at the end of the experiment in both groups (Fig. 3.4).

Individual variation between fishes was highest for the 15°C groups throughout and for the 12.5°C-LL group in the end of March (indicated by +/- SEM bars) (Fig. 3.4). All groups except for the 12.5°C-LL showed significant differences in the concentration of *gnrh2* between samplings over time (p-value < 0.05). For the groups given LL, significant differences between the temperature regimes were found at all samplings between mid-February and mid-April (p-value < 0.05). In the groups given WS, significant differences between 12.5°C and 15°C were found at the samplings in mid-February, the end of February and the end of March (p-value < 0.05). Comparing photoperiod regimes did not reveal any significant differences (p-value > 0.05). In the overall experiment, temperature, date and the interaction between temperature and date were found to have significantly affected changes in the mean concentration of *gnrh2* (ANOVA, p-value < 0.05).

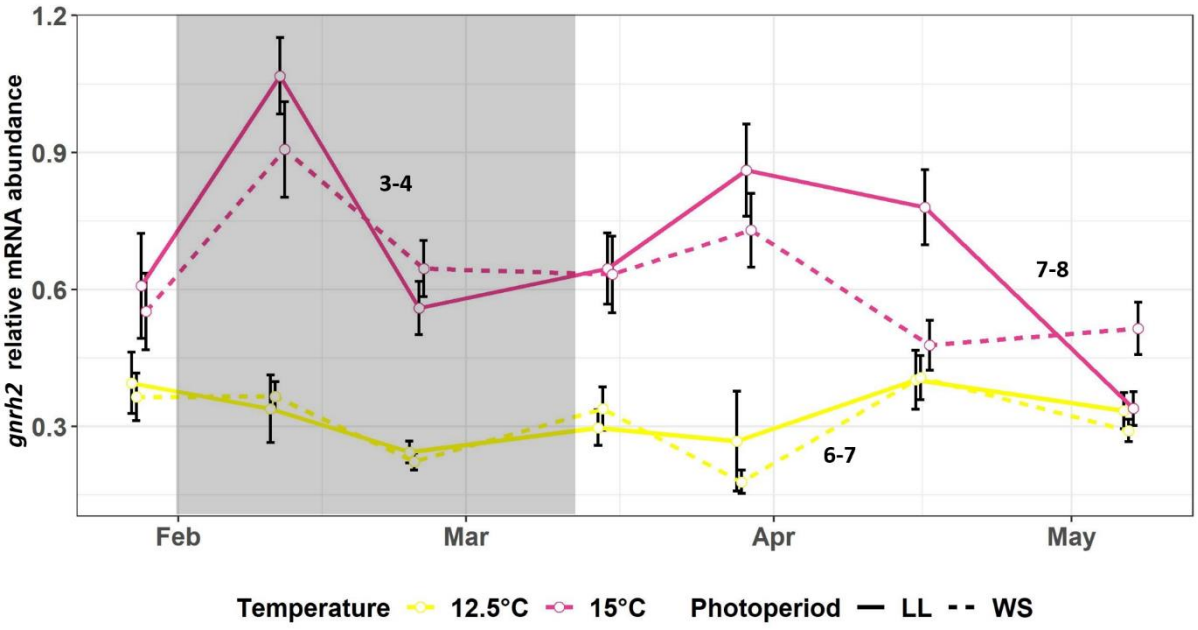

Figure 3.4. Diencephalon concentration of *gnrh2* shown over time, where circles indicate the mean concentration at each sampling with whiskers showing standard mean error (+/- SEM). Significant differences between samplings are indicated by numbering above the trend lines, and the winter signal period is illustrated by shadowing.

3.5. Changes in mean concentrations of *gpr54* over time

Mean concentration of *gpr54* was overall higher in the 15℃ groups, whereas it remained low in the 12.5℃ groups throughout the experiment (Fig. 3.5). In the 15℃-WS group, there was an initial significant increase in the concentration of *gpr54* near mid-February (p-value < 0.05), followed by a significant decrease in the end of February (p-value < 0.05) (Fig. 3.5). Mean concentration of *gpr54* then decreased until mid-March before increasing in the end of March after the end of the winter signal period (Fig. 3.5). The mean concentration of *gpr54* then decreased in mid-April before increasing again in the end of the experiment (Fig. 3.5). In the 15℃-LL group, mean concentration of *gpr54* initially increased in mid-February, followed by a decrease by the end of February (Fig. 3.5). Mean concentration of *gpr54* then increased again by the end of March before decreasing until the end of the experiment (Fig. 3.5). In both 12.5℃ groups, there were only small fluctuations in the mean concentration of *gpr54* from the end of January until the end of March (Fig. 3.5). There was a small increase in the mean concentration of *gpr54* in mid-April in both 12.5℃ groups, whereafter it remained quite stable until the end of the experiment (Fig. 3.5).

There were large individual variances between fishes in the 15℃ groups at multiple samplings, where they were largest for the 15℃-WS group in mid-February and in the end of February, and in the 15℃-LL group in the end of January, mid-February and in the end of March (indicated by large +/- SEM bars). In the groups given WS, there was a significant difference between the temperature regimes in mid-February and in the end of March (p-value > 0.05). In the groups given LL, there was a significant difference between the temperature regimes in mid-March, in the end of March and in mid-April (p-value > 0.05). No significant differences were found when comparing the photoperiod groups (p-value > 0.05). In the overall experiment, temperature and time were shown to have significantly affected changes in the mean concentration of *gpr54* (ANOVA, p-value < 0.05).

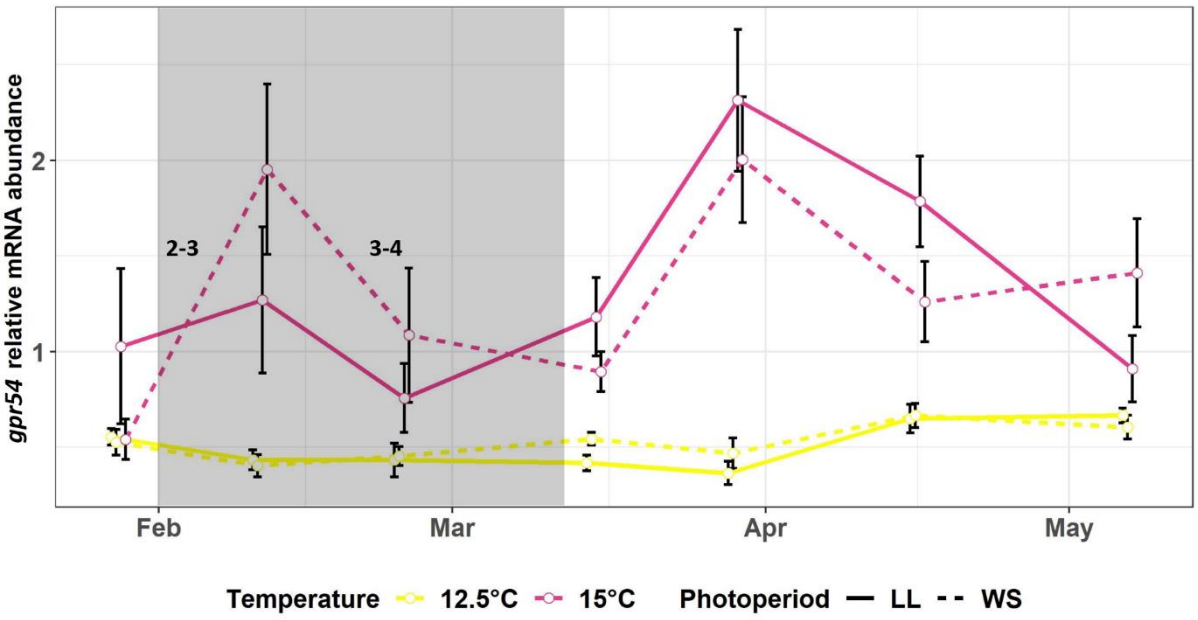

Figure 3.5. Diencephalon concentration of *gpr54* over time. Circles indicate mean concentration at each sampling, where whiskers show standard mean error (+/- SEM). Numbers over trend lines show where significant differences were found between samplings. Winter signal period is indicated by grey shadowing.

3.6. Changes in mean concentrations of *gniha* over time

Mean concentration of *gniha* was generally higher in the 15°C groups compared to the 12.5°C groups throughout the experiment, with fluctuations over time in all groups (Fig. 3.6). The 15°C groups followed similar trends until mid-April, with an initial increase in the mean concentration of *gniha* followed by a decrease in the end of February (Fig. 3.6). Mean concentration of *gniha* then increased again until the end of March in the 15°C groups, where the increase was smaller in the 15°C-WS group after the end of the winter signal period in the end of March (Fig. 3.6). This was followed by a decrease in the mean concentration of *gniha* in both 15°C groups in mid-April, whereafter it increased in the 15°C-WS group and significantly decreased in the 15°C-LL group (p-value < 0.05) by the end of the experiment (Fig. 3.6). In the 12.5°C-WS group, the mean concentration of *gniha* was quite stable with minor fluctuations until the end of February (Fig. 3.6). Mean concentration of *gniha* decreased some near mid-February in the 12.5°C-LL group, followed by an increase in the end of February (Fig. 3.6). The 12.5°C groups followed similar trends from the end of February, with a decrease in mean concentration of *gniha* in mid-March (Fig. 3.6). There was an increase in the mean

concentration of *gniha* in both 12.5℃ groups by the end of March following the end of the winter signal period, which was a bit bigger in the 12.5℃-WS group, whereafter it decreased until the end of the experiment (Fig. 3.6).

There were large individual variances between fishes at multiple samplings, where it was highest for the 15℃ groups near mid-February and in the end of March and for the 12.5℃ groups in the end of March (indicated by large +/- SEM bars) (Fig. 3.6). In the groups given LL, there was a significant difference between the temperature regimes in the end of March (p-value < 0.05). No significant differences were found between the photoperiod groups (p-value > 0.05). In the overall experiment, temperature and the interaction between temperature and time were found to have significantly affected changes in the mean concentration of *gniha* (ANOVA, p-value < 0.05).

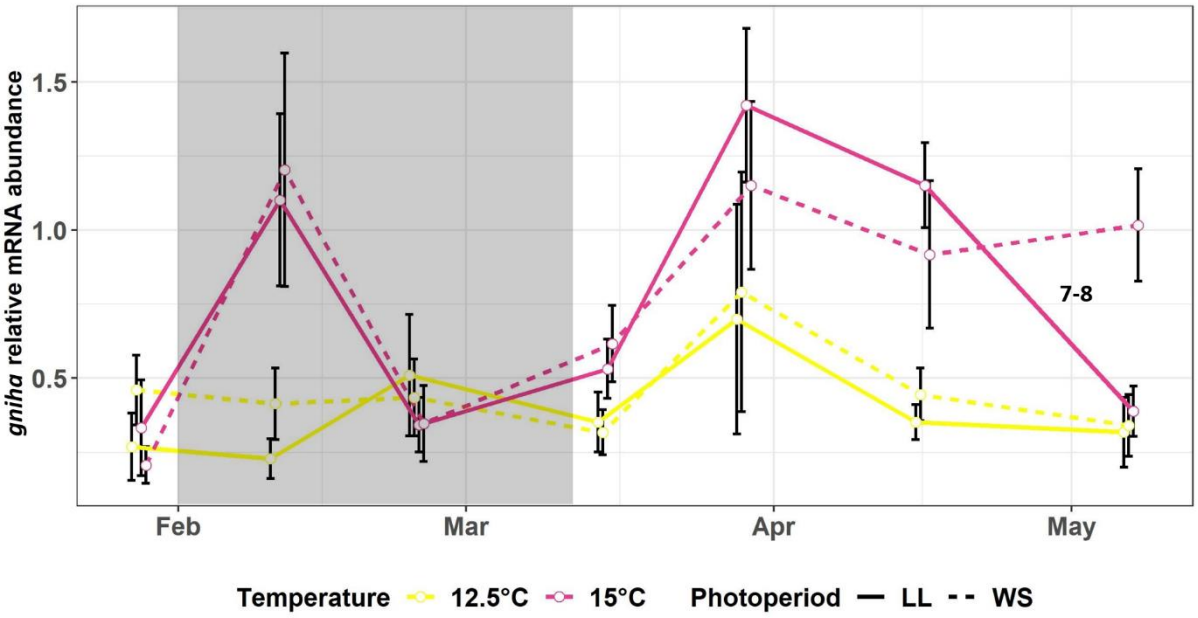

Figure 3.6. Diencephalon concentration of *gniha* over time. Circles illustrate mean concentration at each sampling, where whiskers show standard mean error (+/- SEM). Numeration above the trend line show where there was a significant difference in the 15℃-LL group. Shadowing indicates the winter signal period.

3.7. Changes in mean concentrations of *gnihb* over time

The mean concentration of *gnihb* in the 15℃ groups showed similar expression patterns until mid-April, whereafter the mean concentration of *gnihb* in the 15℃-LL group decreased and the mean concentration of *gnihb* in the 15℃-WS group increased by the end of the experiment (Fig. 3.7). Mean concentration of *gnihb* in both 15℃ groups initially increased near mid-February and subsequent decreased in the end of February, where both were significant in the 15℃-WS group (p-value < 0.05) (Fig. 3.7). Mean concentration of *gnihb* then increased until the end of March in the 15℃ groups following the end of the winter signal period, before decreasing in mid-April (Fig. 3.7). In the 15℃-WS group, the mean concentration of *gnihb* increased again by the end of the experiment, whereas it significantly decreased by the end of the experiment in the 15℃-LL group (p-value < 0.05) (Fig. 3.7).

In the 12.5℃-WS group, mean concentration of *gnihb* also initially increased near mid-February before decreasing in the end of February (Fig. 3.7). Another increase in mean concentration of *gnihb* was observed in the 12.5℃-WS group mid-March, which was followed by a significant decrease in the end of March after the end of the winter signal period (p-value < 0.05) (Fig. 3.7). Mean concentration of *gnihb* in the 12.5℃-WS group then significantly increased in mid-April (p-value < 0.05) and decreased again by the end of the experiment (Fig. 3.7). In the 12.5℃-LL group, the mean concentration of *gnihb* slowly increased until mid-March and decreased by the end of March (Fig. 3.7). Mean concentration of *gnihb* then significantly increased in mid-April (p-value < 0.05) and decreased again in the end of the experiment (Fig. 3.7).

Large individual variances between fishes were observed at multiple samplings, where it was biggest in the 12.5℃-WS group in the end of January and in the 12.5℃-WS group, 15℃-WS group and 15℃-LL group in mid-February (indicated by large +/- SEM bars) (Fig. 3.7). There was a significant difference between the temperature regimes in the end of March both within the groups given LL and within the groups given WS (p-value < 0.05). No significant differences were found between the photoperiod regimes (p-value > 0.05). In the overall experiment, time was found to have significantly affected changes in the mean concentration of *gnihb* (ANOVA, p-value < 0.05).

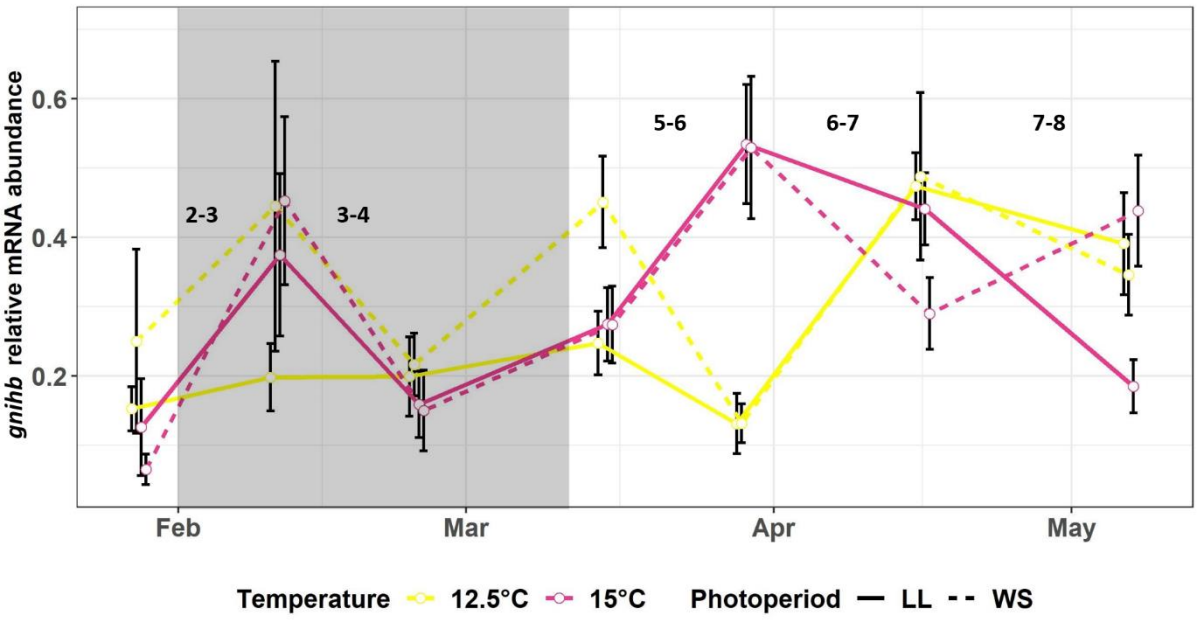

Figure 3.7. Diencephalon concentration of *gnihb* over time, where circles show mean concentration at each sampling and whiskers show standard mean error (+/- SEM). Numeration between samplings show where significant differences were found for multiple groups. Winter signal period is indicated by the grey area.

3.8. Changes in mean concentrations of *dio2b* over time

Mean concentration of *dio2b* was generally higher in the 12.5°C groups than in the 15°C groups, where it decreased by the end of the experiment in all groups (Fig. 3.8). In the 12.5°C-WS group, mean concentration of *dio2b* increased near mid-February, followed by a significant decrease in the end of February (p-value < 0.05) (Fig. 3.8). Mean concentration of *dio2b* then significantly increased in mid-March (p-value < 0.05), followed by a further increase in the end of March after the end of the winter signal period (Fig. 3.8). The mean concentration of *dio2b* then decreased some by mid-April before significantly decreasing in the end of the experiment (p-value < 0.05) (Fig. 3.8). In the 12.5°C-LL group, mean concentration of *dio2b* initially increased near mid-February before decreasing until mid-March. There was a minor increase in mean concentration of *dio2b* in the end of March, whereafter it decreased until the end of the experiment (Fig. 3.8). In the 15°C-WS group, the mean concentration of *dio2b* decreased until the end of February, followed by a small increase mid-March after the end of the winter signal period. The mean concentration of *dio2b* then rapidly increased by the end of March (p-value < 0.05) followed by a significant decrease mid-April (p-value < 0.05), whereafter it remained

stable until the end of the experiment (Fig. 3.8). In the 15℃-LL group, mean concentration of *dio2b* initially decreased near mid-February, followed by a minor increase in the end of February and another decrease in mid-March (Fig. 3.8). Mean concentration of *dio2b* then increased a bit until mid-April before decreasing again at the end of the experiment (Fig. 3.8).

A large individual variance between fishes was observed at multiple samplings, where it was especially large in both 12.5℃ groups in mid-February and in the 12.5℃-WS group in the end of March and in mid-April (indicated by large +/- SEM bars) (Fig. 3.8). There was a significant difference between the temperature regimes within the groups given LL in mid-March and within the groups given WS in mid-April (p-value < 0.05). In the fish given 15℃, there was a significant difference between WS and LL the end of March (p-value < 0.05). In the overall experiment, temperature and time were found to have significantly affected changes in the mean concentration of *dio2b* (ANOVA, p-value < 0.05).

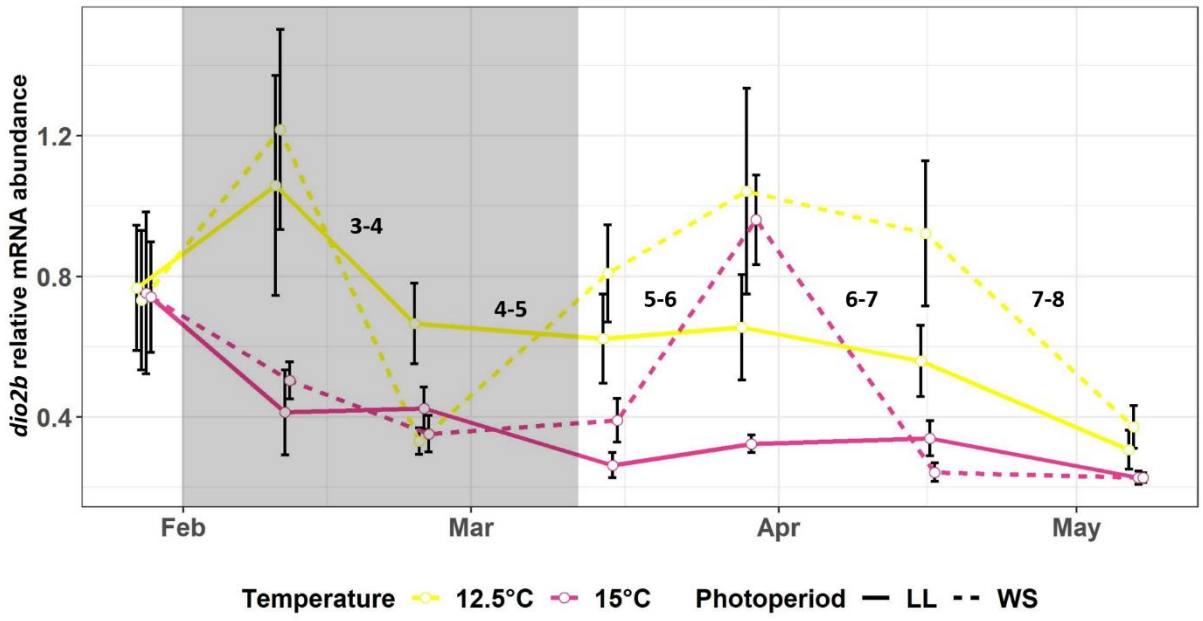

Figure 3.8. Diencephalon concentration of *dio2b* over time, where circles marks mean concentration at each sampling and whiskers show standard mean error (+/- SEM). Significant differences between samplings are indicated by numbering in the graph. Winter signal period is indicated by shadowing.

3.9. Changes in mean concentrations of *tshβb* over time

Mean concentration of *tshβb* rapidly increased in mid-March in both WS groups before decreasing until mid-April, whereas it remained low throughout the experiment in the LL groups (Fig. 3.9). The WS groups followed similar trends, with only small fluctuations until a significant increase in the mean concentration of *tshβb* in mid-March after the end of the winter signal period and exposure to LL (p-value < 0.05) (Fig. 3.9). This was followed by a significant decrease in the end of March in both WS groups (p-value < 0.05) and a decrease in mid-April, which was significant in the 15℃-WS group (p-value < 0.05) (Fig. 3.9). No dramatic changes in the mean concentration of *tshβb* were observed in any of the LL groups at any time during the experiment (Fig. 3.9). A large individual variance was observed in the 15℃-WS group in mid-March (indicated by large +/- SEM bars) (Fig. 3.9). In the groups given 12.5℃, there were significant differences between the photoperiod regimes in the end of February, in mid-March and in the end of March (p-value < 0.05). Within the groups given 15℃, there were significant differences between the photoperiod regimes in mid-February, the end of February, in mid-March and in the end of March (p-value < 0.05). No significant differences were found when comparing the temperature groups (p-value > 0.05). In the overall experiment, photoperiod and the interaction between photoperiod and time were found to have significantly affected changes in the mean concentration of *tshβb* (ANOVA, p-value < 0.05).

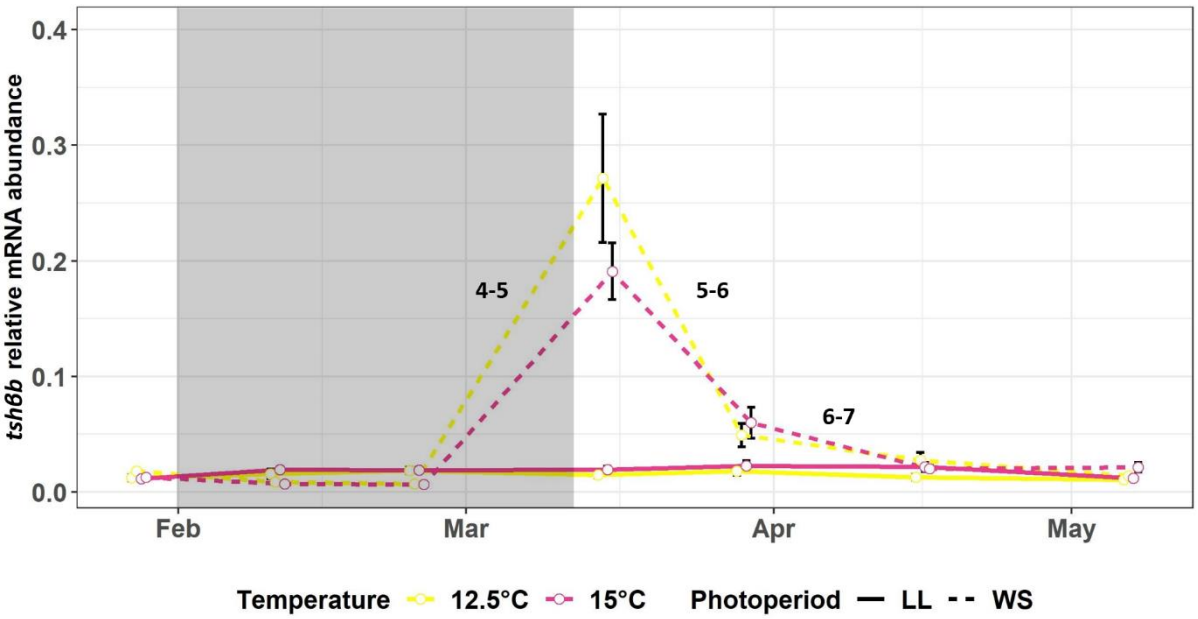

Figure 3.9. Pituitary concentration of *tshβb* over time, where circles show mean concentration at each sampling and whiskers mark standard mean error (+/- SEM). Numeration above the

lines show significant changes between samplings. Winter signal period is indicated by the grey area.

4. Discussion

4.1. Developmental effects, genes and brain regions selected for this study

To track growth in the Atlantic salmon in this study, changes in body weight and condition factor over time was chosen. Changes in body weight was used to track the growth rate to see whether the different experimental conditions would cause varying growth rates in the different groups. Condition factor was used as a smoltification parameter to see whether the salmon were smoltifying instead of maturing early under the different photoperiod and temperature regimes. Both body weight and condition factor are developmental parameters that have been used in previous studies (Björnsson et al., 1989; Saunders et al., 1985; Strand et al., 2018).

GSI was used as a parameter in this study to see whether and when the salmon were maturing. Using GSI as an indicator of maturation in Atlantic salmon has been previously used in multiple studies, but the definition of when the salmon is maturing in terms of GSI varies between studies (Ciani et al., 2021; Fjelldal et al., 2011; Good et al., 2015; Melo et al., 2014) Melo et al. considered the Atlantic salmon males to be immature at a GSI < 0.04% and as maturing at a GSI > 0.05% (Melo et al., 2014). Ciani et al. also considered the males to be maturing at a GSI > 0.05 where testicular development had begun, and that they were defined as being immature at a GSI ≤ 0.05 (Ciani et al., 2021). Good et al. defined the male Atlantic salmon as grilse (mature) at a GSI ≥ 1.0% along with morphological characteristics, and that they otherwise were immature (Good et al., 2015). Fjelldal et al. considered the Atlantic salmon males to be maturing at a GSI of 1.5 ± 0.2% (Fjelldal et al., 2011).

In the present study, recent research in our lab set the basis for the definition of maturation stages and GSI levels, where the salmon were defined as immature at a GSI ≤ 0.06%, in early stage of maturation at a GSI > 0.06% and GSI ≤ 0.1%, as maturing at a GSI > 0.1% and GSI ≤ 1% and as mature at a GSI > 1% (Martinez et al., 2021).

The recent localization of *gnrh2*, *kiss2*, *gniha* and *gnihb* in Atlantic salmon (Horne et al., under revision), prompted us to investigate the expression in male Atlantic salmon brains at different stages of maturation during the use of intense aquaculture environmental conditions.

As *dio2b* expression has been found in the thalamus and hypothalamus, along with a suggested TSH-DIO2 system similar to that found in mammals and birds, the diencephalon *dio2b* expression profiles in the salmon were analyzed to see whether it is involved in maturation in Atlantic salmon. The recent localization of *tshβb* cells dorsally in the PN and its possible similar

roles to that of PT-TSH in mammals, prompted us to analyze pituitary *tshβb* expression to find out if it may be involved in the onset of maturation in Atlantic salmon.

4.2. Discussion of results

Random tank variance was generally low for the different responses, where it was below 5% for almost all responses (Table 3.1). The variance between tanks were considered to not have affected the changes in the responses in these cases. The only exception was the random tank variance for the mean concentration of *gnrh2*, where it was 7.8% (Table 3.1). Although it was a bit higher than for the other responses, the variance between tanks were considered to only have had a minor effect on the changes in the mean concentration of *gnrh2*.

4.2.1. Effects of different photoperiod and temperature regimes on changes in body weight

All groups had a steady growth rate throughout the experiment, with some minor variations over time (Fig 3.1). In this study, exposing the Atlantic salmon to different photoperiod and temperature regimes was not shown to have caused different growth rates in the salmon, which is contradicting to multiple previous studies which have found effects of photoperiod (Saunders et al., 1985; Sigholt et al., 1998; Strand et al., 2018) and temperature on growth rate (Austreng et al., 1987; Björnsson et al., 1989; Sigholt et al., 1998; Handeland et al., 2000; Handeland et al., 2004; Handeland et al., 2008).

Saunders et al. found that Atlantic salmon parrs that were only reared in natural light had a lower growth rate than the ones that were subjected to long light (LD 24:0) for two months (Saunders et al., 1985). Sigholt et al. had similar findings, where they found that Atlantic salmon parrs reared in a regime of short days (LD 8.15:15.45) had a lower growth rate than the ones reared in a long light regime (LD 24:0) (Sigholt et al., 1998). They also found that the fish reared in a short-day regime kept having a lower growth rate even after exposure to long light for the rest of the experiment compared to the ones that were only reared in long light (Sigholt et al., 1998). Similarly, the findings from two experiments conducted by Strand et al. were that photoperiod had a significant effect on growth in Atlantic salmon parrs, where the groups reared in long light (LD 20:4 and LD 24:0) had significantly higher growth rates than the groups reared in photoperiods with a shorter daylength after exposure to a winter signal period (Strand et al., 2018). In the present study however, photoperiod was not found to have significantly caused differences in the changes in body weight over time between the groups, and no significant

differences between the LL-groups and WS-groups were found under either of the temperature regimes (Fig. 3.1).

Temperature is an important external cue that influences feeding and growth in Atlantic salmon (Hansen et al., 1998). In a study conducted by Björnsson et al., they found that growth rates were significantly higher in Atlantic salmon juveniles reared in 11℃ compared to the ones reared at 6℃ (Björnsson et al., 1989). Temperature has also been found to significantly affect the growth rate in freshwater of two strains of fast-growing Atlantic salmon (Mowi and AquaGen), where growth rate increased with higher temperatures in both and were significantly higher in the salmon reared at 12℃ than in the ones reared at 8,9℃ (Handeland et al., 2004). No such difference between temperature regimes were observed in the current study, where the fish grew steadily in both the 12.5℃ groups and the 15℃ groups (Fig. 3.1).

An important aspect to consider is the thermal range in which an increase in growth rate is promoted. Austreng et al. found that growth rate increased with temperature (4-16℃) in young Atlantic salmon in freshwater, as well as in sea cages (2-14℃) based on data from their previous experiments (Austreng et al., 1987). They suggested that the optimum temperature for growth in freshwater was in the range of 16℃ or higher (Austreng et al., 1987).

In the study by Sigholt et al. mentioned above, they also tested three temperature regimes after all photoperiod groups were exposed to a long light regime (Sigholt et al., 1998). They observed that fish reared in the lowest and highest temperature ranges (4.5℃-10.8℃ and 10.5℃-17.3℃ respectively) had lower growth rates than the ones reared in a medium temperature range (7.6℃-13.7℃) in both light regimes (Sigholt et al., 1998). This suggests that there is an optimum thermal range for growth close to the medium range that they tested, where growth rates are reduced in lower or higher temperature ranges.

Handeland et al. found in 2000 that mean weight increased with increases in temperature in Atlantic salmon smolts reared in 4.6℃, 9.1℃ and 14.4℃, but not in the ones reared at 18.9℃. They suggested that there is an upper thermal limit between 14.4℃ and 18.9℃ for growth and development at this stage (Handeland et al., 2000). Handeland et al. found in 2008 that optimum temperature varied both between processes (e.g. growth and feed intake) and between different body weights in Atlantic salmon post-smolts, where the growth rate was highest at 14℃ (Handeland et al., 2008). They also found that the growth rate was lower in fish kept at 18℃ and that it was not significantly different from the one observed in fish kept in 10℃ (Handeland et al., 2008), indicating that the optimum temperature for growth was in the range around 14℃.

Based on these studies, one would have expected that there would be a higher growth rate in the 15℃ groups in the current study compared to the ones in the 12.5℃ groups. However, no significant differences between the temperature groups were found (Fig. 3.1). One possible explanation is that the difference between the temperatures wasn't big enough to have caused significant differences in growth rate between the two temperature regimes. It's also possible that both 12.5℃ and 15℃ are within the optimum temperature range for growth and that both temperatures similarly stimulated the increases in growth rates in the Atlantic salmon observed in this study.

A factor that could have affected growth rate in this study is feeding regime, as the LL-groups were fed continuously, and the WS-groups were only fed during light hours in the winter signal period. Feeding regime was not analyzed as a predictor in this experiment, but it generally does not seem to have affected changes in body weight as all experimental groups grew steadily through the experiment (Fig. 3.1).

4.2.2. Effects of different photoperiod and temperature regimes on changes in condition factor (K)

Because the mean condition factor generally increased in all four experimental groups (Fig 3.2), it is probable that the fish did not smoltify normally in any of the groups in this study. Previous research indicate that photoperiod is a key factor affecting changes in condition factor and has connected the manipulation of light regime with condition factor and smoltification (Björnsson et al., 1989; Saunders et al., 1985; Strand et al., 2018), where it has been suggested that exposing Atlantic salmon to a winter signal period is necessary for the fish to successfully smoltify (Björnsson et al., 1989). The finding that there was no clear decrease in K in the LL groups in the present study is consistent with previous research, whereas the findings from the WS-groups in the present study are not (Björnsson et al., 1989; Björnsson et al., 2000; Saunders et al., 1985; Sigholt et al., 1998; Stefansson et al., 2007; Strand et al., 2018).

In the study by Sigholt et al., they found no significant effect of temperature on changes in condition factor in Atlantic salmon (Sigholt et al., 1998). Their findings about temperature are mostly congruent with the present study, where temperature was not found to have influenced the overall experiment. However, temperature seems to have had some effect on the changes in the mean condition factor, as a significant difference was found between the temperature regimes in in mid-April in the groups exposed to WS.

McCormick et al. exposed Atlantic salmon juveniles to two different photoperiod regimes and two different temperature regimes and found that both photoperiod and temperature had significant effects on condition factor, where there was a coupled effect of the two factors on the changes in condition factor and that temperature alone did not cause these changes (McCormick et al., 2002). The findings of the present study also suggest that the temperature effect may be coupled with a photoperiod as no clear trends in the temperature groups were observed and no significant differences were found between the temperature regimes in the groups given LL (Fig. 3.2). Exposure to a higher temperature may have affected the significant increase in mean K observed in the 15°C-WS group in mid-April, and exposure to a lower temperature may have affected the longer period of a decreasing mean K in the 12.5°C-WS group in the end of March after the end of the winter signal period (Fig. 3.2). Exposure to different temperature regimes can therefore not be concluded to have caused significant differences in the present experiment, but the findings indicate a possible coupled effect with photoperiod.

Saunders et al. found that Atlantic salmon juveniles reared in natural light at two different temperature regimes had a decrease in condition factor from winter to spring, whereas no clear reduction trends in condition factor were observed in the groups reared in long light (LD 24:0) at two different temperatures and suggested that few or none became smolts (Saunders et al., 1985). Similarly, Björnsson et al., 1989 exposed Atlantic salmon juveniles to four different combinations of different temperatures and photoperiod regimes and one group to 11°C and continuous light (LD 24:0) and found that condition factor was lower at the end of the experiment in all groups except for the group exposed to 11°C and continuous light where it increased during the experiment and remained high (Björnsson et al., 1989). Stefansson et al. also reported that that the condition factor was higher in Atlantic salmon juveniles exposed to continuous light compared to the ones exposed to a natural photoperiod (Stefansson et al., 2007). Their findings from the groups of the Atlantic salmon exposed to a long light regime are consistent with the present study where no significant or clear decreases in mean K were observed in the LL-groups (Fig. 3.2).

Sigholt et al. found that condition factor significantly decreased in Atlantic salmon parrs that were exposed to a photoperiod of long light after a photoperiod of short days (Sigholt et al., 1998). Strand et al. found in two experiments that condition factor significantly decreased in Atlantic salmon exposed to long light regimes following a winter signal period (Strand et al.,

2018). In the present study however, no significant decreases were observed in the WS-groups following the end of the winter signal period and exposure to LL (Fig. 3.2).

In 2000, Björnsson et al. exposed Atlantic salmon juveniles to different photoperiod regimes, where condition factor generally increased in the group only exposed to continuous light throughout the experiment, whereas the ones exposed to a winter signal period for six weeks (LD 12:12) displayed a sharp decline in condition factor after exposure to continuous light (Björnsson et al., 2000). Their findings are both different and similar to those of the present one. Like with their study, no significant decreases or clear decreasing trends in condition factor were observed in the LL-groups at either temperature (Fig. 3.4). A decrease in mean condition factor was also observed in the WS-groups in the present study but they were not very clear and sharp (Fig. 3.4).

In the present study, a decrease in mean K was observed in both WS-groups in the end of March after the end of the winter signal period and exposure to LL, where there was a significant difference between the photoperiod regimes within the groups given 15℃ (Fig. 3.2). Additionally, there was significant difference between photoperiod regimes in the 12.5℃-groups in mid-April (Fig. 3.2). However, mean K then significantly increased in the 15℃-WS group in mid-April before decreasing again at the end of the experiment (Fig 3.2). In the 12.5℃-WS group, mean K also started increasing again at the end of the experiment (Fig. 3.2). These findings suggest that the mean K in the WS-groups may have decreased as a response to exposure to LL and indicate that some individuals may have started smoltifying, but that the right conditions weren't met for a normal smoltification process in this experiment.

4.2.3. Effects of different photoperiod and temperature regimes on changes in GSI levels

Mean GSI quickly increased early in the experiment in the 15℃-LL group, where some of the salmon in this group were maturing from the end of February and mature from the end of March (Fig. 3.3). Salmon in the 15℃-WS group and 12.5℃-LL group were maturing in mid-April and mature in the end of the experiment (Fig. 3.3). In the 12.5℃-WS group, the salmon were in the early stages of maturation in mid-April and maturing at the end of the experiment (Fig. 3.3). The individual variation was large in the 15℃-LL group in the end of February until the end of March, and in the 12.5℃-LL group in mid-April and at the end of the experiment, indicating that the maturation levels varied between individuals at these points of the experiment in these groups (Fig. 3.4).

In the present study, photoperiod was not found to have had a significant effect on the overall experiment, and no significant differences between the photoperiod regime groups were found at either of the temperatures. These findings are dissimilar to those of Hansen et al., who found that exposing Atlantic salmon to continuous light along with a natural photoperiod led to a lower number of maturing Atlantic salmon the second year compared to the ones only exposed to a natural photoperiod (Hansen et al., 1992). The early onset of maturation in the 15°C-LL group and lack of similar observations in the 15°C-WS group do however suggest that photoperiod influences the onset of maturation, but that it is coupled with temperature (Fig. 3.3).

Temperature and the interaction between temperature and time was found to have a significant effect on changes in mean concentration of *gnrh2* in the overall experiment, and significant differences were found between 12.5°C and 15°C in mid-April and at the end of the experiment within both photoperiod groups. These findings suggests that temperature has a strong regulating effect on the onset of maturation in Atlantic salmon post-smolts, as has been suggested previously (Fjelldal et al., 2011; Fjelldal et al., 2018; Imsland et al., 2014).

In a study conducted by Imsland et al., they exposed Atlantic salmon parrs in freshwater and post-smolts in seawater to four different photoperiod and temperature regimes, which were combinations of natural photoperiod, continuous light, and water temperatures of 8.3°C and 12.7°C (Imsland et al., 2014). They found that exposing Atlantic salmon to continuous light and a higher water temperature led to a much higher number of mature individuals than in the other three experimental groups, and that exposure to a natural photoperiod and a higher temperature led to the second highest number of mature individuals (Imsland et al., 2014). Although maturation was only assessed in post-smolts in seawater, they suggested that their findings support the notion that photoperiod and temperature together influence the onset of early maturation because the number of mature individuals was highest in the group exposed to long light and a higher temperature (Imsland et al., 2014). Their findings support the ones of the present study as both early maturation and the highest end mean GSI was observed in the 15°C-LL group (Fig 3.3), whereas maturation this early was not observed in salmon in the 15°C-WS group who were exposed to the same temperature or in the 12.5°C-LL group where they were exposed to the same photoperiod (Fig. 3.3).

Fjelldal et al. conducted an experiment where they exposed Atlantic salmon pre-smolts to a winter signal period (LD 12:12) before exposing them to different combinations of temperature (5°C, 10°C and 16°C) and photoperiod (LD 24:0 and LD 12:12) regimes and found that 47%

+/- 7% of the Atlantic salmon smolts exposed to the highest water temperature and continuous light had started maturing, whereas none had started maturing in the other experimental groups (Fjelldal et al., 2011). In 2018, Fjelldal et al. exposed Atlantic salmon pre-smolts to a photoperiod regime of a simulated natural photoperiod and temperature (5℃) followed by continuous light and a higher temperature (16℃) after rearing them in tanks with a natural photoperiod and temperature and found that the altered photoperiod and temperature regime stimulated the onset of maturation in the salmon (Fjelldal et al., 2018). Their findings thereby indicate a coupled effect of temperature and photoperiod on the onset of maturation in Atlantic salmon (Fjelldal et al., 2011; Fjelldal et al., 2018).

In the present study, the salmon in the 15℃-WS group also started maturing in mid-April following exposure to LL (Fig. 3.3). However, the salmon in the 12.5℃-LL group also started maturing at this stage of the experiment, and the salmon in the 12.5℃-WS group were in the early stages of maturation (Fig. 3.3). Fjelldal et al. on the other hand reported no maturing individuals in the other temperature groups exposed to continuous light after a winter signal period (Fjelldal et al., 2011). The difference between their findings and the findings of the present study is probably due to the temperatures used in their experiment being lower than in the current study.

Based on the findings of these previous studies and the present study, it is indicated that Atlantic salmon should be exposed to temperatures lower than 12.5℃ and a winter signal period to inhibit the early onset of maturation. Additionally, the finding that Atlantic salmon started maturing early in the 15℃-LL group in the present study further suggests a coupled effect of photoperiod and temperature on the onset of maturation (Fig. 3.3). Exposure to a photoperiod regime existing only of LL and a warmer temperature regime is suggested to trigger the early onset of maturation in Atlantic salmon in the current study and should be avoided in intense aquaculture settings to avoid early maturation.

4.2.4. Effects of different photoperiod and temperature regimes on changes in mean diencephalon concentration of *gnrh2*

In the present study, the highest mean concentrations of *gnrh2* in the diencephalon of the Atlantic salmon were observed in the 15℃ groups, where the overall means were higher in the 15℃-LL group (Fig. 3.4). Mean concentrations in both the 12.5℃ groups remained at lower levels with some fluctuations throughout the experiment, displaying similar expression patterns

(Fig. 3.4). Temperature was found to have significantly affected changes in the mean concentration of *gnrh2* in the overall experiment and exposing Atlantic salmon to different temperature regimes caused different *gnrh2* expression profiles between the 12.5℃ groups and the 15℃ groups, where significant differences were found between the temperature groups within both WS and LL in mid-April and at the end of the experiment (Fig. 3.4).

The findings of the present study are different to what has been reported in other teleosts (Shahjahan et al., 2013; Shahjahan et al., 2017). Shahjahan et al. exposed zebrafish (*Danio rerio*) to three different temperature regimes (15℃, 27℃ and 35℃) and found no significant differences in the brain mRNA levels of *gnrh2* between the three temperature groups (Shahjahan et al., 2013). Likewise, exposing male grass puffer (*Takifugu niphobles*) to three different temperature regimes (14℃, 21℃ and 28℃) did not cause significant differences in brain mRNA levels of *gnrh2* between the temperature groups (Shahjahan et al., 2017).

In the present study however, diencephalon mRNA levels were higher in the Atlantic salmon exposed to 15℃ compared to the ones exposed to 12.5℃ (Fig. 3.4). These findings suggests that temperature is an external factor that plays a key role on changes in diencephalon expression of *gnrh2* in Atlantic salmon posts-smolts reared in freshwater, where exposure to a higher temperature seems to induce *gnrh2* expression and exposure to a lower temperature may inhibit *gnrh2* expression. The differences between the present findings and those in studies on other teleosts might be due to the other species having a broader thermal range in which *gnrh2* expression is not significantly affected by exposure to different temperatures, and that diencephalon gene expression of *gnrh2* in Atlantic salmon has a thermal range which is more susceptible to lower temperature changes compared to that of zebrafish and grass puffer.

Photoperiod did not seem to be a key regulator of diencephalon gene expression of *gnrh2* expression in Atlantic salmon (Fig. 3.4), and no significant difference was found between the photoperiod groups within each temperature group. Additionally, photoperiod was not found to have had a significant effect on the overall experiment. There were some observed differences between the photoperiod regimes within the 15℃-groups, where the diencephalon mean concentrations of *gnrh2* were generally higher in the 15℃-LL group compared to the 15℃-WS group (Fig. 3.4). These findings suggests that there might be an effect of photoperiod on diencephalon expression of *gnrh2* in Atlantic salmon, but that it is associated to temperature and does not cause significant changes alone.

These findings are both similar and different to what has been reported in other teleost species (Amano et al., 2004; Hildahl et al., 2013; Miranda et al., 2009). Miranda et al. exposed pejerrey (*Odontesthes bonariensis*) to two different photoperiod regimes (LD 8:16 and LD 16:8) and temperature regimes (12℃ and 20℃) and found that brain expression levels of *gnrh2* were significantly higher in the fish exposed to the longer photoperiod regime than in the ones exposed to the shorter photoperiod regime in both temperature groups (Miranda et al., 2009). Their findings are thereby different than the present study, where the diencephalon *gnrh2* expression was not found to be significantly affected by exposure to different photoperiod regimes in Atlantic salmon and signifies a different photoperiod response than the one reported in pejerrey.

Amano et al. exposed male barfin flounder (*Verasper moseri*) to two different photoperiod regimes (LD 8:16 and LD 16:8) in two experiments where the fish were exposed to different temperature ranges in the two experiments (Amano et al. 2004). They found that there were no significant differences in brain concentrations of *gnrh2* between the two photoperiod regime groups in either of the experiments (Amano et al., 2004). Similarly, Hildahl et al. found that exposing Atlantic cod (*Gadus morhua*) to four different photoperiod regimes did not cause significant changes in brain *gnrh2* expression (Hildahl et al., 2013). Their findings are similar to the present study, which indicates that photoperiod does not significantly affect diencephalon *gnrh2* expression in Atlantic salmon. The observed differences between the 15℃-LL group and 15℃-WS group suggest that the different photoperiod regimes may play a contributing role in changes in *gnrh2* expression in Atlantic salmon exposed to a higher temperature as the expression levels were generally higher in the 15℃-LL group, but that temperature is the key regulator of diencephalon *gnrh2* expression.

Miranda et al. found elevated brain expression of *gnrh2* in pejerrey that had higher GSI levels and suggested that *gnrh2* expression might be involved in reproduction in pejerrey (Miranda et al., 2009). Additionally, GnRH2 has been found to significantly increase the pituitary mRNA levels of gonadotrophs in mature goldfish (Khakoo et al., 1994).

Previous findings of GnRH2 research related to maturation in salmonids has led to various results (Amano et al., 1998; Okuzawa et al., 1990). Okuzawa et al. found that the brain concentrations of GnRH2 in rainbow trout (*Oncorhynchus mykiss*, previously *Salmo gairdneri*) were not significantly different in various brain regions between immature and mature individuals and suggested that it is not involved in the BPG axis in this species (Okuzawa et al., 1990). Amano et al. found that brain levels of GnRH2 increased during maturation in the masu

salmon (*Oncorhynchus masou*) but could not detect pituitary levels of GnRH2, suggesting a potential but unknown role of GnRH2 in the BPG axis in salmonids (Amano et al., 1998).

Diencephalon expression profiles of *gnrh2* were compared to the stage of maturation (mean GSI levels) in the different experimental groups to see whether *gnrh2* is expressed during the maturation stages (Fig 3.3 and Fig. 3.4). At the start of the experiment, the salmon in all groups were immature (Fig 3.3), where higher expression levels of *gnrh2* were observed in the 15°C groups at the beginning of the experiment compared to the 12.5°C groups (Fig. 3.4). All parrs were exposed to LL and the different temperature regimes for almost three months prior to the experiment, so the increases near mid-February in both 15°C groups might therefore have been caused by the exposure to a higher water temperature. Following the initial increase in diencephalon *gnrh2* expression, salmon in the 15°C-LL group started maturing in the end of February, which indicates that *gnrh2* expression may have affected the early stages of the BPG axis in the salmon (Fig 3.3 and Fig. 3.4). However, the salmon in the 15°C-WS group did not start maturing following the initial increase in *gnrh2* expression. In the 15°C-WS group, there was another increase in *gnrh2* expression in the end of March prior to the salmon maturing in mid-April (Fig 3.3 and Fig. 3.4). These findings suggest that exposure to a winter signal period inhibits the potential effect of *gnrh2* expression on the BPG-axis and subsequent maturation in Atlantic salmon, whereas exposure to LL may stimulate the potential effect when exposed to a warmer temperature regime.

In the 12.5°C groups, similar diencephalon *gnrh2* expression trends of were observed, where the expression levels were lower than in the 15°C groups throughout the experiment (Fig. 3.4). Salmon in the 12.5°C-LL group started maturing in mid-April, but no significant increase in *gnrh2* expression was observed prior to this (Fig 3.3 and Fig. 3.4). In the 12.5°C-WS group, the fish were in early stages of maturation in mid-April and maturing at the end of the experiment (Fig. 3.3). There was a significant increase in *gnrh2* expression in the 12.5°C-WS group at this stage of the experiment, but none were observed in the stages prior to the onset of maturation (Fig 3.3 and Fig. 3.4). These findings indicate that *gnrh2* expression did not affect the onset of maturation in the 12.5°C groups. The overall findings are therefore somewhat conflicting, and the potential role of diencephalon *gnrh2* expression on the BPG axis and onset of maturation in Atlantic salmon remains to be elucidated.

4.2.5. Effects of different photoperiod and temperature regimes on changes in mean diencephalon concentration of *gpr54*

In the present study, mean concentrations of *gpr54* in the diencephalon were overall higher in the 15°C-groups where the expression pattern changed over time in both the 15°C-WS group and the 15°C-LL group (Fig. 3.5). Diencephalon *gpr54* expression remained at lower levels in the 12.5°C-groups. Temperature was found to have a significant effect on the overall experiment, and there were significant differences between the temperature regimes within the groups exposed to LL in mid-March, the end of March and in mid-April.

Different temperature regimes have previously been reported to influence the expression of kisspeptin (*kiss2*) and kisspeptin receptor (*kiss2r*) mRNA levels in male grass puffer (Shahjahan et al., 2017). Shahjahan et al. found that *kiss2* and *kiss2r* expression significantly decreased when the fish were exposed to temperatures lower and higher than the normal temperature and suggested that the lower and higher temperatures inhibited expression of these genes in male grass puffer (Shahjahan et al., 2017).

Various temperature regimes have also been found to affect the expression of *kiss1* and its receptor *kissr1* and the expression of *kiss2* and the correlating receptor *kissr2* in the zebrafish (Shahjahan et al., 2013). Shahjahan et al. found that the expression of *kiss1* and *kissr1* were significantly higher in zebrafish exposed to a lower temperature regime compared to those exposed to a normal and high temperature regime, and that the expression of *kiss2* and *kissr2* were significantly lower in zebrafish exposed to a low and high temperature regime than in the ones exposed to a normal temperature regime (Shahjahan et al., 2013).

Similarly, exposing Atlantic salmon parrs to two different temperature regimes also caused different expression profiles of *gpr54* in the present study, where *gpr54* expression was higher in the 15°C-groups compared to the 12.5°C-groups (Fig. 3.5). These findings suggests that *gpr54* expression is inhibited when Atlantic salmon are exposed to a lower temperature and induced when exposed to a higher temperature and indicate that temperature is a key regulator of *gpr54* expression in Atlantic salmon.

Photoperiod was not found to have a significant effect on the different changes in mean concentration of *gpr54* between experimental groups. However, the higher increase in the mean concentration of *gpr54* observed near mid-February in the 15°C-WS group does suggest that photoperiod may have had some effect on the diencelaphon *gpr54* expression in this study.

These findings are different from what has previously been found in Atlantic salmon (Chi et al., 2017).

In a study conducted by Chi et al. they found that photoperiod may influence the expression of *gpr54* in the hypothalamus and saccus vasculosus (Chi et al., 2017). They found that the highest *gpr54* expression levels in the hypothalamus were observed in salmon exposed to the longest light regimes, which were LL and LL followed by WS (Chi et al., 2017). Like their findings, high expression levels of *gpr54* were also observed in the 15°C-LL group and the 15°C-WS group early in the experiment in the present study (Fig. 3.5). However, they did not find high expression levels of *gpr54* in the group exposed to WS prior to LL (Chi et al., 2017), whereas the mean concentration of *gpr54* increased in the 15°C-WS group in the end of March following exposure to LL (Fig. 3.5). Additionally, they found significant differences when comparing these groups to the other photoperiod regimes used in their study (Chi et al., 2017) whereas no significant differences were found when comparing the photoperiod regimes used in the present study. This may be due to the photoperiod regimes used being too different from each other as they slowly altered the photoperiod in the LL to WS group and LL to WS group by five minutes every day of the experiment (Chi et al., 2017), whereas the 15°C-WS group was exposed to LL right after the winter signal period in the present study. Therefore, photoperiod may have a stronger effect on *gpr54* expression when the change in photoperiod is slow.

Kisspeptin and the kisspeptin receptor are thought to regulate GnRH secretion in mammals, thereby starting maturation (Somoza et al., 2020; Taranger et al., 2010). Nocillado et al. found that brain expression of both *gpr54* and *gnrh2* increased in the early stages of maturation in female grey mullet (*Mugil cephalus*) and suggested that the expression levels are related to each other (Nocillado et al., 2007). Findings from a previous study has also indicated a role of kisspeptin and the kisspeptin receptor in the BPG axis in Atlantic salmon in regulating GnRH secretion (Chi et al., 2017). Therefore, we wanted to investigate whether diencephalon *gpr54* expression could be related to *gnrh2* expression, and the expression profile trends of *gpr54* and *gnrh2* were compared to see whether the expression of *gpr54* was related to *gnrh2* expression in the present study (Fig 3.4 and Fig. 3.5).

In both the 12.5°C-groups and the 15°C-groups, the expression profiles of both *gnrh2* and *gpr54* were similar throughout the experiment (Fig 3.4 and Fig. 3.5). The main differences were observed in the 15°C-WS group, where the mean concentration of *gpr54* was higher in the 15°C-WS group near mid-February, but mean expression of *gnrh2* was lower in the 15°C-WS group at this stage of the experiment (Fig 3.4 and Fig. 3.5). Additionally, the increase in mean

concentration of *gpr54* seen at this point was significant in the 15°C-WS group (Fig. 3.5). In the end of February, the decrease in mean concentration of *gpr54* was only significant in the 15°C-WS group, whereas the decrease in mean concentration of *gnrh2* was only significant in the 15°C-LL group. Additionally, the decrease in mean concentration of both genes in the in the 15°C-LL group at end of the experiment was only significant for *gnrh2* (Fig 3.4 and Fig. 3.5).

The findings of the present study are that the diencephalon expression profiles of *gpr54* mainly seem to be similar to those of diencephalon expression of *gnrh2*, which suggests that diencephalon *gpr54* expression may stimulate *gnrh2* expression and maturation in Atlantic salmon.

4.2.6. Effects of different photoperiod and temperature regimes on changes in mean diencephalon concentration of *gniha*

Research on the GnIH system in teleosts species is still in its early stages and little is known about how photoperiod and temperature affect this system in teleosts (Di Yorio et al., 2019). In mammals, photoperiod has previously been found to affect the expression of GnIH (Dardente et al., 2008; Ubuka et al., 2012). Ubuka et al. found that the expression levels of GnIH in Siberian hamsters (*Phodopus sungorus*) were higher in hamsters exposed to a photoperiod regime of long days (LD 16:8) than in the ones compared to short days (LD 8:16) (Ubuka et al., 2012). Similarly, Dardente et al. found that the expression levels of GnIH in the hypothalamus in Soay sheep (*Ovis aires*) were high in the sheep that were exposed to a photoperiod of long days (LD 16:8) but were barely detectable in the ones exposed to short days (LD 8:16) (Dardente et al., 2008).

In the present study, photoperiod was not found to have had a significant effect on the mean concentration of *gniha* in the overall experiment and no significant differences in the mean concentration *gniha* were found between the photoperiod groups in either of the temperature regimes tested in this study. The findings of the present study indicate that photoperiod does not have the effect on *gniha* expression in Atlantic salmon as has been reported in mammals.

There were some observed differences in the mean concentrations of *gniha* between the 12.5°C-LL group and the 12.5°C-WS group, as well as between the 15°C-LL group and 15°C-WS group at multiple stages of the experiment where the biggest difference was observed at the end of the experiment (Fig. 3.6). This suggests that photoperiod might have a contributing effect on the

diencephalon *gniha* expression in Atlantic salmon, but that it is connected to and may be dependent on the temperature effect on *gniha* expression. The individual variation was large at in the 15℃-groups near mid-February, and in all experimental groups in the end of March, suggesting that the *gniha* expression levels varied between individuals at these points of the experiment in these groups (Fig. 3.6).

How temperature affect *gnih* expression in teleosts is not yet understood, but a recent study found that temperature affected *gnih* expression in European sea bass (*Dicentrarchus labrax*) differently during the developmental stages after hatching (Paullada-Salmerón et al., 2017). Paullada-Salmerón et al. exposed two groups of newly hatched sea bass to a low and high temperature regime and found that there was a significant effect of temperature on the *gnih* expression during the development in this species, where the higher temperature regime led to lower *gnih* expression in the early developmental stages (Paullada-Salmerón et al., 2017). Their findings suggests that temperature may regulate *gnih* expression in teleosts.

In the present study, temperature and the interaction between temperature and time were found to have significantly affected changes in mean concentration of *gniha* in the overall experiment, and a significant difference between the temperature regimes within the groups given LL in the end of March. These findings suggests that temperature is a key regulator of diencephalon *gniha* expression in Atlantic salmon. The mean concentrations of *gniha* were generally higher in the 15℃-groups compared to the 12.5℃-groups.

Previous research has found both inhibitory and stimulatory effects of GnIH on different stages of the BPG axis in other teleost species (Aliaga-Guerrero et al., 2017; Amano et al., 2006; Qi et al., 2013a; Shahjahan et al., 2011). To assess the potential role of diencephalon *gniha* expression in maturation, the changes in mean concentration of *gniha* were compared to the GSI levels observed in the four experimental groups (Fig. 3.3 and Fig. 3.6). The mean concentration of *gniha* initially increased in both 15℃-groups in mid-February, before decreasing in the end of February (Fig. 3.6). The increase in *gniha* expression preceded the increase in mean GSI observed in the maturing salmon in the 15℃-LL group in the end of February, suggesting that *gniha* expression may have stimulated the onset of maturation in this group (Fig. 3.3 and Fig 3.6). Interestingly, the mean concentration of *gniha* decreased significantly in the 15℃-LL group both in the end of February after the salmon had started maturing and again at the end of the experiment after the salmon had become mature (Fig. 3.3 and Fig 3.6). This suggests that the mean *gniha* concentrations decrease when the salmon have become mature and indicate that a possible stimulatory effect of diencephalon *gniha* expression

on the BPG axis happens in the early stages of maturation where the *gniha* expression levels decrease at the later stages.

Salmon in the 15°C-WS group were however immature until the end of March following the initial increase in *gniha* expression (Fig. 3.3 and Fig 3.6). This suggests that the possible stimulating effect of *gniha* expression on maturation may be inhibited by exposure to a winter signal period in male Atlantic salmon post-smolts. The mean concentration of *gniha* increased in all experimental groups in the end of March following the end of the winter signal period, where the highest mean concentrations of *gniha* were observed in the 15°C-groups (Fig. 3.6). Following these increases, the salmon in the 15°C-WS group and the 12.5°C-LL group were maturing in mid-April and mature at the end of the experiment (Fig. 3.3). The fish in the 12.5°C-WS group were in the early stages of maturation in mid-April and maturing at the end of the experiment (Fig. 3.3). The increases in mean *gniha* concentrations were observed after exposure to LL in the end of March prior to maturing in both WS-groups (Fig. 3.6).

The overall findings of comparing *gniha* expression profiles with stages of maturation in this experiment suggest that *gniha* expression may have a stimulating effect on the BPG axis and onset of maturation in male Atlantic salmon post-smolts and not an inhibiting effect as has been observed in goldfish (Qi et al., 2013a) and the Senegalese sole (*Solea senegalensis*) (Aliaga-Guerrero et al., 2017). A stimulatory effect generally seems to be induced by exposure to a higher temperature and LL, whereas exposure to a winter signal period and a colder water temperature seem to inhibit the possible role of diencephalon *gniha* expression levels on the onset of maturation.

Shahjahan et al. found that the brain expression of *gnih* significantly increased in grass puffer (*Takifugu niphobles*) that were between the pre-spawning and spawning phase of reproduction and found that exposure to gfGnIH1 caused significantly higher pituitary expression levels of maturation-related genes (*fshβ* and *lhβ*) (Shahjahan et al., 2011). Similarly, the diencephalon expression of *gniha* increased in the maturing salmon in the 15°C-LL group and decreased when they were mature at the end of the experiment in this study.

Amano et al. found that all three existing forms of GnIH in Sockeye salmon had a stimulating effect on the release of FSH and LH and may play a similar role as to that of GnRH (Amano et al., 2006). Their findings are congruent with the ones from the present study, where the diencephalon expression of *gniha* seemed to have a stimulating role on the onset of maturation

and not an inhibitory one. The findings of Amano et al. and the present study suggests that GnIH may also have stimulating effects on maturation in other salmonids.

To assess the potential inhibiting effect of GnIH on GnRH, the expression levels of *gniha* were compared to expression levels of *gnrh2*. Interestingly, in the 15°C-groups the expression profile of *gniha* (Fig. 3.6) is very similar to that of *gnrh2* (Fig. 3.4). This suggests that diencephalon expression of these genes have similar roles in maturation in Atlantic salmon. The main differences between the two besides the values of the mean concentrations is that the mean concentration of *gniha* in the 15°C-WS group is higher than in the 15°C-LL group in mid-February (Fig. 3.6), where the opposite is true when looking at the mean expression of *gnrh2* (Fig. 3.4). The increases in the mean concentrations of *gniha* from the end of February until the end of March are also much higher in the 15°C-groups (Fig. 3.6) compared to the observed increases of *gnrh2* at these stages in the experiment (Fig. 3.4).

In the 12.5°C-groups, the expression profiles of *gniha* are different than the ones of *gnrh2*. The main differences in the 12.5°C-groups are that the mean concentration of *gniha* follow opposite trends from mid-February until mid-April in the mean concentration of *gnrh2*, whereas they have similar expression profiles from the beginning of the experiment until mid-February and from mid-April until the end of the experiment. The findings from the 15°C-groups suggest that *gniha* expression does not inhibit *gnrh2* expression in Atlantic salmon parrs exposed to higher temperatures in the present study, and that the role of GnIH is comparable to that of GnRH2 in Atlantic salmon. The fact that the expression trends observed for *gniha* are opposite to the ones observed for *gnrh2* in the 12.5°C-groups from mid-February until mid-April may suggest that *gniha* expression may have inhibited *gnrh2* expression at these stages of the experiment. Thus, *gniha* expression may inhibit *gnrh2* expression in Atlantic salmon when they are exposed to lower temperatures.

4.2.7. Effects of different photoperiod and temperature regimes on changes in mean diencephalon concentration of *gnihb*

Photoperiod was not found to have significantly affected changes in mean concentration of *gnihb* in the overall experiment, and no significant differences were found between the photoperiod regimes. These findings suggest that diencephalon expression of *gnihb* is not regulated by photoperiod, similar to what was found for the expression of *gniha* in this study. As with the diencephalon expression of *gniha*, a clear effect of photoperiod on the expression

of *gnihb* was not observed as in the ones found in research on the Siberian hamster and Soay sheep where the expression levels of GnIH were higher in animals that were exposed to photoperiods of long days (Dardente et al., 2008; Ubuka et al., 2012).

Photoperiod did however seem to affect the expression of *gnihb* in the 12.5℃-groups as the increases observed in the 12.5℃-WS group in mid-February and mid-March prior to and right after the end of the winter signal period were much higher than in the 12.5℃-LL group. These findings indicate that a possible effect of photoperiod on the diencephalon expression of *gnihb* in Atlantic salmon is higher when they are exposed to lower temperatures, and that a higher temperature acts as a stronger factor in *gnihb* expression trumping that of photoperiod. As with the diencephalon expression of *gniha*, a clear effect of photoperiod on the expression of *gnihb* was not observed as the ones found in research Siberian hamsters and Soay sheep (Dardente et al., 2008; Ubuka et al., 2012).

In contrast to what was found for diencephalon expression of *gniha*, temperature was not found to have significantly affected changes in the mean concentration of *gnihb* in the overall experiment. However, the significant difference between the temperature regimes both within salmon exposed to LL and salmon exposed to WS in the end of March does suggest that temperature influenced the diencephalon expression of *gnihb.*

Interestingly, the increase in the mean concentration of *gnihb* was much higher in the 12.5℃-WS group than the 12.5℃-LL group near mid-February and in mid-March (Fig. 3.7). This suggests a combined effect of photoperiod and temperature, where exposure to a winter signal period and a lower temperature may stimulate *gnihb* expression in Atlantic salmon. This is further suggested by the fact that the 12.5℃-groups had similar *gnihb* expression patterns when both groups were exposed to LL from the end of March until the end of the experiment after the end of the winter signal period (Fig. 3.7).

The expression profiles of *gnihb* were similar in the 15℃-groups from the beginning of the experiment until mid-April, whereafter the mean concentration of *gnihb* increased in the 15℃-WS group and decreased in the 15℃-LL group (Fig. 3.7). Interestingly, the expression profile of *gniha* is very similar to that of *gnihb* in the 15℃-groups, suggesting that the expression of these genes is similar in Atlantic salmon exposed to a warmer water temperature (Fig. 3.6 and Fig. 3.7).

Expression profiles of *gnihb* in all four experimental groups were compared to the corresponding mean GSI values to assess the potential stimulating or inhibitory role of

diencephalon expression of *gnihb* in Atlantic salmon maturation (Fig. 3.3 and Fig. 3.7). Initial increases in the mean concentrations of *gnihb* were observed in the 12.5℃-WS group, 15℃-LL group and 15℃-WS group where the mean GSI levels indicated that the salmon in these groups were immature (Fig. 3.3 and Fig. 3.7). In the 15℃-LL group, the salmon were maturing in the end of February, suggesting that the initial increase in the expression of *gnihb* may have stimulated the onset of maturation in this group like what was (Fig. 3.3 and Fig. 3.7). However, the salmon in the 12.5℃-WS group and 12.5℃-LL group were not maturing following the initial increase in *gnihb* expression (Fig. 3.3 and Fig. 3.7). This indicates that exposure to a winter signal period suppresses the possible stimulating role of *gnihb* expression on the onset of maturation in Atlantic salmon like what was observed for the *gniha* expression.

The mean concentration of *gnihb* increased in the end of March in the 15℃-WS group prior to salmon maturing in mid-April (Fig. 3.3 and Fig. 3.7). In the 12.5℃-groups, *gnihb* expression increased at the same time as the salmon were maturing in the 12.5℃-LL group and in the early stages of maturation in the 12.5℃-WS group (Fig. 3.3 and Fig. 3.7). These findings further suggests that diencephalon *gnihb* expression may have a stimulating effect on the onset of maturation in Atlantic salmon and does not inhibit it.

When comparing the *gnihb* expression profiles with stages of maturation in this experiment, the findings suggest that *gnihb* expression may have a stimulating effect on the BPG axis, where the potential stimulating effect seem to be stimulated in Atlantic salmon that are exposed to a higher water temperature and continuous light but inhibited by exposure to a lower water temperature or a combination of a winter signal period and a higher water temperature. Like with the diencephalon expression of *gniha*, these findings are different to the inhibitory effects of GnIH on maturation that has been reported in the Senegalese sole (Aliaga-Guerrero et al., 2017) and goldfish (Qi et al., 2013a), but similar to the stimulating effect on maturation as reported in the Sockeye salmon (Amano et al., 2006) and the grass puffer (Shahjahan et al., 2011).

As the expression profiles of *gniha* and *gnihb* were so similar to each other in the 15℃-groups (Fig 3.6 and Fig. 3.7), comparing the expression profiles of *gnihb* to the expression profiles of *gnrh2* revealed similar findings (Fig. 3.4 and Fig. 3.7). Interestingly, the expression profiles of all these genes are very similar in the 15℃-groups, suggesting that they play similar roles to each other in maturation in Atlantic salmon and that their diencephalon expression levels are similarly affected by photoperiod and temperature (Fig. 3.4, Fig. 3.6, and Fig. 3.7).

The expression profiles of *gnihb* are very different than the ones of *gnrh2* in the 12.5℃-groups. In the 12.5℃-LL group, there was an increasing trend in *gnihb* expression from the start of the experiment until mid-March whereas there was a decreasing trend in the expression of *gnrh2* until the end of February in the 12.5℃-LL group, but there are no obvious stimulatory or inhibitory influences of *gnihb* expression on *gnrh2* expression at these stages (Fig. 3.4 and Fig. 3.7). Similarly, the increases in *gnihb* expression in the 12.5℃-WS group near mid-February and in mid-March does not seem to have caused comparative increases or decreases in the *gnrh2* expression in this group (Fig. 3.4 and Fig. 3.7). Similar expression profiles are observed in both 12.5℃-groups from the end of March until the end of the experiment after the end of the winter signal period, suggesting a possible stimulating influence of *gnihb* expression on *gnrh2* expression at these stages (Fig. 3.4 and Fig. 3.7).

These findings suggest a complex system where the diencephalon expression of *gnihb* may have a stimulating influence on both *gnrh2* and maturation in Atlantic salmon, and that its influence is affected by both photoperiod and temperature.

4.2.8. Effects of different photoperiod and temperature regimes on changes in mean diencephalon concentration of *dio2b*

In this study, the mean diencephalon concentration of *dio2b* increased after the end of the winter signal period and exposure to LL until the end of March in both the 12.5℃-WS group and the 15℃-WS group (Fig. 3.8). Similar increases were not observed at these stages of the experiment in the LL-groups (Fig. 3.8). The largest difference between within the temperature groups was observed between the 15℃-groups following the increase in mean *dio2b* expression in the end of March after the end of the winter signal period, where the difference between WS and LL was significant (Fig. 3.8). These findings suggest that a change in photoperiod from a winter signal period to continuous light stimulates expression of *dio2b* in male Atlantic salmon. This is congruent with findings from previous studies conducted on the photoperiodic effect on *dio2b* expression Atlantic salmon (Irachi et al., 2021; Lorgen et al., 2015; Strand et al., 2018).

Lorgen et al. exposed Atlantic salmon juveniles to a photoperiod regime of long light (LD 16:8) after a photoperiod regime of short days (LD 8:16) and found that brain *dio2b* expression was low during exposure to short days and increased following exposure to long light, where they suggested that *dio2b* expression is induced by a change in photoperiod regime (Lorgen et al., 2015). Similarly, Strand et al. found that Atlantic salmon juveniles exposed to LL (LD 24:0)

after a photoperiod with short days (LD 6:18) had significantly higher brain *dio2b* expression levels than the other photoperiod regimes tested in their experiment (Strand et al., 2018). In a study conducted by Irachi et al., they found that mRNA levels of *dio2b* in the hypothalamus were 75% higher in Atlantic salmon that had been exposed to a photoperiod of long days (LD 16:8) for 20 days after a photoperiod of short days (LD 10:14) than in the ones reared only in a photoperiod of short days (Irachi et al., 2021).

The findings of these studies are consistent with the ones from the present study, where the mean diencephalon concentration of *dio2b* significantly increased in both WS-groups following exposure to LL (Fig. 3.8). Although photoperiod was not found to have significantly affected changes in mean concentrations of *dio2b* in the overall experiment, the significant differences between WS and LL within the fish exposed to 15°C in the end of March following the end of the winter signal period suggest that *dio2b* expression is induced by the changing photoperiod regime as suggested in previous studies (Lorgen et al., 2015).

Interestingly, the findings of the present experiment also suggests that temperature is a regulator of *dio2b* expression in Atlantic salmon. Temperature was found to have had a significant effect on the overall experiment, and there was a significant difference between the temperature groups within the groups given WS in mid-April and the groups given LL in mid-March. When the winter signal period was introduced in the beginning of February, differences between the 15°C-groups and the 12.5°C-groups were observed, where the mean diencephalon concentrations of *dio2b* were higher in the 12.5°C-groups than in the 15°C-groups (Fig. 3.8). There was a large individual variation in both 12.5°C-groups near mid-February and in the 12.5°C-WS group in the end of March and mid-April, which suggests that the *dio2b* expression varied between the individuals at these stages of the experiment (Fig. 3.8). Even so, these findings suggests that exposure to a colder water temperature stimulates *dio2b* expression in Atlantic salmon as the mean concentrations of *dio2b* were generally higher in the 12.5°C-groups within both photoperiod groups throughout the experiment (Fig. 3.8).

In the previous studies on *dio2b* expression in Atlantic salmon, Lorgen et al. kept the salmon in a temperature of 7°C, Strand et al. kept the salmon in 7°C during the winter signal period and 10°C during LL and Irachi et al. kept the salmon in a temperature between 10.7°C and 12.1°C (Irachi et al., 2021; Lorgen et al., 2015; Strand et al., 2018). This suggests that a temperature between 7°C and 12.5°C may stimulate *dio2b* expression in Atlantic salmon, whereas a higher temperature may inhibit it.

In the beginning of the experiment, the four experimental groups had similar mean concentrations of *dio2b*, indicating that exposure to the different temperature regimes for three months prior to the experiment did not affect changes in mean concentration of *dio2b* between the groups (Fig. 3.8). The observed changes in diencephalon expression of *dio2b* after the different photoperiod regimes were implemented in both the 15°C-groups and the 12.5°C-groups thereby suggests that there is a coupled effect of temperature and photoperiod on diencephalon (Fig. 3.8).

Irachi et al. also found that the increase of mRNA levels of *dio2b* in response to increased day length coincided with increases in levels of indicators related to smoltification and suggested that both smoltification and reproduction can be linked to photoperiodic signaling and a TSH/DIO pathway (further discussed in 4.2.9) (Irachi et al., 2021). DIO2 has also been related to maturation in birds, where DIO2-regulated conversion of T_4 to T_3 stimulates GnRH secretion (Yoshimura, 2013). Additionally, *Dio2* increases have been observed before secretion of LH in the Japanese quail (Yasuo et al., 2005). To see whether *dio2b* expression was related to maturation in a similar manner to that of birds, the expression profiles in the four experimental groups (Fig. 3.8) were compared to the mean GSI-levels (Fig. 3.3) and expression profiles of *gnrh2* in the groups (Fig. 3.4).

The overall lowest expression of *dio2b* was observed in the 15°C-LL group where there was a decreasing trend throughout the experiment (Fig. 3.8). However, early maturation was observed in the salmon in the 15°C-LL group (Fig. 3.3). In the 15°C-WS group, there were two increases in *dio2b* expression near mid-February and the end of March (Fig. 3.8). Although the salmon in the 15°C-WS group started maturing in mid-April following the increase in *dio2b* expression in the end of March, the fact that they did not start maturing after the increased *dio2b* expression near mid-February and that the salmon in the 12.5°C-LL group also started maturing at this point where no increase in *dio2b* expression was observed in the end of March suggests that *dio2b* expression was not related to the salmon maturing in these groups (Fig. 3.3 and Fig. 3.8). Lastly, the highest overall mean concentrations of *dio2b* were observed in the 12.5°C-WS group which had the lowest overall mean GSI levels (Fig. 3.3 and Fig. 3.8).

When comparing the diencephalon expression profiles of *dio2b* and *gnrh2*, it does not seem like *dio2b* expression and *gnrh2* expression are related (Fig. 3.4 and Fig. 3.8). In the 15°C-LL group, the overall mean concentrations of *dio2b* were lowest whereas the overall mean concentrations of *gnrh2* were highest (Fig. 3.4 and Fig. 3.8). In the 15°C-WS group, the mean concentration of *dio2b* initially increased, whereas the mean concentration of *gnrh2* decreased

(Fig. 3.4 and Fig. 3.8). The following expression profiles of *dio2b* and *gnrh2* were also different from each other through the rest of the experiment in the 15℃-WS group. The expression profile of *gnrh2* were similar in both 12.5℃-groups throughout the experiment, whereas there was an initial increase in *dio2b* expression in these groups and significant increases and decreases in mean *dio2b* during the experiment in the in the 12.5℃-WS group (Fig. 3.4 and Fig. 3.8). These findings suggests that diencephalon *dio2b* expression may affect *gnrh2* expression and maturation in Atlantic salmon, but its role remains to be elucidated.

4.2.9. Effects of different photoperiod and temperature regimes on changes in mean pituitary concentration of *tshβb*

In this experiment, mean concentration of *tshβb* significantly increased in both WS-groups in mid-March right after the end of the winter signal period and exposure to LL (Fig. 3.9). Mean concentration of *tshβb* then significantly decreased afterwards in both WS-groups in the end of March (Fig. 3.9). No significant changes in *tshβb* expression were found in the LL groups (Fig. 3.9). Photoperiod and the interaction between photoperiod and time was found to have a significant effect on the changes in mean concentration of *tshβb* and significant differences were found at multiple stages of the experiment between the photoperiod regimes within the groups given 12.5℃ and 15℃. These findings suggest that changes in photoperiod is a key regulator of diencephalon *tshβb* in Atlantic salmon where exposure to only LL inhibits *tshβb* expression and exposure to LL following a winter signal period stimulates *tshβb* expression. The fact that no significant increases or peaks were observed in *tshβb* expression in the groups exposed to LL indicate that the continuous light regime disrupts the physiology of Atlantic salmon during development in the freshwater under these intense aquaculture settings. The significant peaks observed in the WS groups indicate that exposing Atlantic salmon to a winter signal period in these settings is of high importance.

Similarly, Irachi et al. found that the mRNA levels of *tshβb* in the pituitary were much higher in Atlantic salmon after being exposed to a photoperiod regime with long days (LD 16:8) for 10 days compared to the ones only reared under short days (LD 10:14), and that the mRNA levels of *tshβb* decreased some but remained high in the salmon exposed to long days after 20 days at the end of the experiment (Irachi et al., 2021). They suggested that an increasing photoperiod directly causes changes in *tshβb* expression in Atlantic salmon (Irachi et al., 2021), which is supported by the findings in the present study.

Temperature was not found to have significantly affected changes in mean concentration of *tshβb* and no significant differences were found when comparing the temperature groups. Also, pituitary mean concentration of *tshβb* increased in both the 12.5℃-WS group and the 15℃-WS group (Fig. 3.9). Therefore, temperature does not seem to be a key regulator of *tshβb* expression in Atlantic salmon. The increase in *tshβb* expression was however observed to be higher in the in mid-March, which suggests that exposure to a colder temperature regime may help stimulate the pituitary *tshβb* expression in Atlantic salmon (Fig. 3.9).

In mammals, melatonin-stimulated release of TSH from the PT creates a stimulatory pathway where gonadotropins are released from the pituitary inducing gonad development (Dardente et al., 2010). The recent location of *tshβb* expressing cells dorsally in the PN suggests that the *tshβb* paralogue may have similar roles as the PT-TSH in mammals (Fleming et al., 2019). Therefore, we wanted to investigate if pituitary expression of *tshβb* similarly plays a role in maturation in Atlantic salmon.

Comparisons between the mean GSI levels and *tshβb* expression were made to assess if *tshβb* expression is related to maturation in Atlantic salmon (Fig. 3.9). Pituitary *tshβb* expression remained low in the LL-groups, but the salmon started maturing in the end of February in the 15℃-LL group and in mid-April in the 12.5℃-LL group (Fig. 3.3 and Fig. 3.9). Pituitary *tshβb* expression increased in mid-March in the WS-groups and the salmon started maturing in the 15℃-WS group and were in early stages of maturation in the 12.5℃-WS group in mid-April (Fig. 3.3 and Fig. 3.9). No increases in pituitary *tshβb* expression were observed prior to the salmon in the LL-groups maturing (Fig. 3.3 and Fig. 3.9). These findings thereby indicate that *tshβb* expression is not related to maturation in Atlantic salmon.

Irachi et al. also found that the hypothalamus *dio2b* mRNA levels increased following an increase in pituitary *tshβb* mRNA levels in the Atlantic salmon exposed to a photoperiod of long days and suggested that there is a pituitary TSH – brain DIO2 pathway connected to photoperiodic signaling (Irachi et al., 2021). To investigate the possible TSH-DIO2 pathway in the present study, the expression profiles of *dio2b* in the diencephalon and *tshβb* in the pituitary were compared (Fig. 3.8 and Fig. 3.9).

Despite an initial increase in the 12.5℃-LL group, the diencephalon *dio2b* expression profiles in the LL-groups displayed decreasing trends through the experiment where the pituitary *tshβb* expression remained low in these groups (Fig. 3.8. and Fig. 3.9). A similar initial increase in mean concentration of diencephalon *dio2b* expression was observed in the 12.5℃-WS group

where none was observed for pituitary mean concentration of *tshβb* (Fig. 3.8. and Fig. 3.9). Interestingly, the significant increase in pituitary *tshβb* expression in the 12.5℃-WS group happened simultaneously as the significant increase in diencephalon *dio2b* expression in mid-April following the end of the winter signal period (Fig. 3.8 and Fig. 3.9). The *dio2b* expression level kept increasing in the after pituitary *tshβb* expression had significantly decreased in the 12.5℃-WS group (Fig. 3.8. and Fig. 3.9). Additionally, there was a significant increase in diencephalon *dio2b* expression in the end of March following the significant increase in pituitary *tshβb* expression in the 15℃-WS group (Fig. 3.8 and Fig. 3.9).

These results findings support that there is a TSH-DIO2 signaling system in Atlantic salmon that is induced by a change in photoperiod regime from a winter signal period to continuous light. The role of a TSH-DIO2 system in relation to maturation is however not as clear as in mammals and birds.

4.3. Experimental design and future perspectives

In the third sampling near mid-February, the Atlantic salmon reared in the warmer temperature regimes had fatty brains. This affected the mRNA extraction and caused issues when quantifying the mRNA concentrations, which led to fewer viable mRNA samples in the salmon from these groups. Therefore, the mRNA extraction protocol was altered where the diencephalon was cut in half and each half was placed in their own tubes. Additionally, increased volumes of TRI Reagent®, 2-propanol and chloroform were used to account for the higher fat contents in the brains. These changes resolved the issues, and the altered protocol was used for the remaining samples from the other sampling dates. Why the warm water temperature causes the brains to be fattier in Atlantic salmon is an interesting topic for future research.

In the present study, the salmon were exposed to combinations of 12.5℃, 15℃, WS (LD 12:12) and LL (24:0) to analyze the parameters. Exposure to 12.5℃ and WS generally led to fewer instances of early maturation without negatively affecting growth. Further investigation of other combinations of a lower temperature (e.g. 10℃) and other winter signal photoperiods (e.g. LD 8:16) could provide better insight into how temperature and photoperiod can affect the onset of maturation in Atlantic salmon and find a regime which leads to even fewer instances of early maturation without loss of growth.

This study analyzed the diencephalon expression of *gnrh2*, *gpr54*, *gniha*, *gnihb* and revealed similar expression profiles in the 15℃-groups, suggesting that there is an interaction between these genes in Atlantic salmon. Future research investigating where the neurons of these genes innervate could provide more insight into the possible communication between these genes.

The results of this study suggest that GnIH might play a stimulating or inhibitory role on the BPG axis and have similar roles as GnRH in Atlantic salmon, and further investigation into the effect of GnIH on the production and release of FSH and LH would be very interesting.

Future research on the potential role of KISS2, a KISS2/GPR54 system and GnRH3 in early maturation in Atlantic salmon should also be conducted.

5. Conclusions

Early maturation in Atlantic salmon has proved to be a serious issue in land-based aquaculture system like RAS, especially in males. The main concern regarding early maturation is decreased animal welfare due to a decreased health and increased instances of infections and higher mortality rates. Additionally, early maturation causes big economic losses for the Aquaculture industry as it decreases fillet quality and alters production schedules. The altered regimes used in intense aquaculture settings mainly consist of a warmer temperature and continuous light but are also the main causes for the increased instances of early maturation found in these systems. Therefore, identifying a photoperiod and temperature regime that both promotes higher growth rates and decreases instances of early maturation is important to better animal welfare and economic profits in the aquaculture industry. In this study, a photoperiod regime of colder water 12.5℃ and exposure to a winter signal period prior to continuous light presented as the best option to prevent instances of early maturation whilst still promoting an increased growth rate.

This study was conducted to gain a broader understanding of how manipulating the temperature and photoperiod regime affect Atlantic salmon growth and development from the parr to post-smolt stage and whether these manipulations can help increase growth and inhibit early sexual maturation in males. The aim of the present thesis was also to gain a broader understanding of the complicated neuroendocrine control of the BPG axis maturation and whether diencephalon gene expression of *gnrh2*, *gpr54*, *gniha*, *gnihb*, and *dio2b*, and pituitary gene expression of *tshβb* are involved in the BPG axis and if they play stimulating or inhibiting roles in Atlantic salmon maturation.

Research question 1: Exposing Atlantic salmon to different photoperiod and temperature regimes did not significantly cause differences in body weight over time (growth) in this experiment. No clear decreasing trends in condition factor were observed in either experimental group, meaning the salmon did not smoltify normally. Exposure to a winter signal period led to the highest observed decreases in K and indicating the highest occurrence of smoltification in these photoperiod groups.

Research question 2: Exposing the Atlantic salmon to different photoperiod and temperature regimes led to different maturation (GSI levels) between the experimental groups. A photoperiod regime of continuous light and a warmer temperature regime stimulated early maturation of Atlantic salmon males in freshwater and exposing the salmon to a colder water temperature and a winter signal period inhibited early maturation.

Research question 3: Exposing Atlantic salmon to a warmer temperature regime in freshwater stimulated diencephalon gene expression of *gnrh2*, *gpr54*, *gniha* and *gnihb* in both photoperiod groups. Diencephalon gene expression of *gnrh2*, *gpr54*, *gniha* and *gnihb* may be involved in the neuroendocrine regulation of maturation, where the effects can be stimulated by exposing the salmon to continuous light and inhibited by exposure to a winter signal period. Exposing the salmon to a colder water temperature stimulated diencephalon gene expression of *dio2b*. Exposing the salmon to continuous light after a winter signal period stimulated pituitary gene expression of *tshβb* and diencephalon gene expression of *dio2b*, supporting a possible photoperiodic TSH/DIO2 signaling pathway in Atlantic salmon. The roles of diencephalon gene expression of *dio2b* and pituitary gene expression of *tshβb* in maturation in Atlantic salmon is still uncertain.

This study is only part of the beginning of understanding the complicated neuroendocrine control of the BPG axis and maturation in Atlantic salmon and the neuronal pathway and specific roles of the selected genes in this study remains to be elucidated. The results suggest a different pathway than what has been observed in mammals and birds, as well as in other teleost species. Further research on the interplay of these genes, their neuronal pathway, possible co-expression and involvement of other genes and paralogues in the BPG axis will further help us to understand how this system works in Atlantic salmon.

6. References

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Appendix I. Dataset and overview of values

Table I. Overview of fish id, sampling, tank id, temperature, photoperiod, length (cm), weight (g), K, sex, gonad weight (g), GSI (%), date and experimental group.

fish_id	SAMPLING	tank_id	Temp (°C)	Photoperiod	length (cm)	weight (g)	k	sex	gonad wht (g)	GSI (%)	date	exp_group
1	1	0	12,5	LL	16,5	54,4	1,21101	M	0,016	0,029	2019-11-11	12.5-LL
2	1	0	12,5	LL	17,0	58,6	1,19275	M	0,022	0,038	2019-11-11	12.5-LL
3	1	0	12,5	LL	16,0	46,1	1,12646	M	0,012	0,026	2019-11-11	12.5-LL
4	1	0	12,5	LL	17,0	58,3	1,18665	M	0,024	0,041	2019-11-11	12.5-LL
5	1	0	12,5	LL	16,0	47,7	1,16455	M	0,016	0,034	2019-11-11	12.5-LL
6	1	0	12,5	LL	16,0	48,6	1,18652	M	0,014	0,029	2019-11-11	12.5-LL
7	1	0	12,5	LL	15,6	43,7	1,15109	M	0,012	0,027	2019-11-11	12.5-LL
8	1	0	12,5	LL	16,4	54,4	1,23330	M	0,021	0,039	2019-11-11	12.5-LL
9	1	0	12,5	LL	16,7	52,7	1,13152	M	0,018	0,034	2019-11-11	12.5-LL
10	1	0	12,5	LL	17,2	57,6	1,13198	M	0,019	0,033	2019-11-11	12.5-LL
11	1	0	12,5	LL	15,5	47,6	1,27824	M	0,02	0,042	2019-11-11	12.5-LL
12	1	0	12,5	LL	17,0	55,8	1,13576	M	0,016	0,029	2019-11-11	12.5-LL
13	2	1	15	LL	27,5	275,5	1,32472	M	0,102	0,037	2020-01-28	15-LL
14	2	1	15	LL	26,5	246,0	1,32190	M	0,113	0,046	2020-01-28	15-LL
15	2	1	15	LL	23,0	160,0	1,31503	M	0,057	0,036	2020-01-28	15-LL
16	2	2	15	WS	25,0	189,1	1,21043	M	0,065	0,034	2020-01-28	15-WS
17	2	2	15	WS	26,8	236,1	1,22657	M	0,074	0,031	2020-01-28	15-WS
18	2	2	15	WS	26,5	214,3	1,15134	M	0,087	0,041	2020-01-28	15-WS
19	2	3	15	WS	26,6	260,0	1,38143	M	0,097	0,037	2020-01-28	15-WS
20	2	3	15	WS	25,2	205,3	1,28288	M	0,097	0,047	2020-01-28	15-WS
21	2	3	15	WS	24,3	163,0	1,13598	M	0,069	0,042	2020-01-28	15-WS
22	2	4	15	LL	25,5	206,4	1,24465	M	0,081	0,039	2020-01-28	15-LL
23	2	4	15	LL	27,2	248,8	1,23636	M	0,083	0,033	2020-01-28	15-LL
24	2	4	15	LL	27,7	270,4	1,27242	M	0,08	0,030	2020-01-28	15-LL
25	2	7	12,5	LL	26,1	217,3	1,22219	M	0,086	0,040	2020-01-28	12.5-LL
26	2	7	12,5	LL	18,3	85,1	1,38892	M	7,189	8,446	2020-01-28	12.5-LL
27	2	7	12,5	LL	24,8	188,2	1,23386	M	0,071	0,038	2020-01-28	12.5-LL
28	2	8	12,5	WS	27,4	286,6	1,39324	M	0,11	0,038	2020-01-28	12.5-WS
29	2	8	12,5	WS	24,4	186,4	1,28315	M	0,087	0,047	2020-01-28	12.5-WS
30	2	8	12,5	WS	26,8	214,4	1,11357	M	0,08	0,037	2020-01-28	12.5-WS
31	2	9	12,5	WS	24,1	189,7	1,35524	M	0,066	0,035	2020-01-28	12.5-WS
32	2	9	12,5	WS	24,7	192,0	1,27412	M	0,067	0,035	2020-01-28	12.5-WS
33	2	9	12,5	WS	25,9	222,7	1,28180	M	0,092	0,041	2020-01-28	12.5-WS
34	2	10	12,5	LL	24,1	180,0	1,28594	M	0,076	0,042	2020-01-28	12.5-LL
35	2	10	12,5	LL	25,8	228,1	1,32821	M	0,081	0,036	2020-01-28	12.5-LL
36	2	10	12,5	LL	20,0	170,4	2,13000	M	19,376	11,371	2020-01-28	12.5-LL
37	3	1	15	LL	25,0	192,0	1,22880	M	0,09	0,047	2020-02-11	15-LL
38	3	1	15	LL	30,5	394,0	1,38866	M	0,162	0,041	2020-02-11	15-LL
39	3	1	15	LL	28,0	267,0	1,21629	M	0,094	0,035	2020-02-11	15-LL
40	3	1	15	LL	26,5	217,0	1,16606	M	0,077	0,035	2020-02-11	15-LL
41	3	1	15	LL	28,5	272,0	1,17499	M	0,092	0,034	2020-02-11	15-LL
42	3	1	15	LL	27,5	271,0	1,30308	M	0,101	0,037	2020-02-11	15-LL
43	3	2	15	WS	30,0	354,0	1,31111	M	0,126	0,036	2020-02-11	15-WS
44	3	2	15	WS	29,5	351,0	1,36723	M	0,103	0,029	2020-02-11	15-WS
45	3	2	15	WS	29,0	305,0	1,25056	M	0,082	0,027	2020-02-11	15-WS
46	3	2	15	WS	28,5	291,0	1,25707	M	0,119	0,041	2020-02-11	15-WS
47	3	2	15	WS	28,0	274,0	1,24818	M	0,087	0,032	2020-02-11	15-WS
48	3	2	15	WS	27,5	260,0	1,25019	M	0,103	0,040	2020-02-11	15-WS
49	3	3	15	WS	30,0	343,0	1,27037	M	0,113	0,033	2020-02-11	15-WS
50	3	3	15	WS	27,5	275,0	1,32231	M	0,103	0,037	2020-02-11	15-WS
51	3	3	15	WS	27,0	250,0	1,27013	M	0,088	0,035	2020-02-11	15-WS
52	3	3	15	WS	26,0	203,0	1,15498	M	0,061	0,030	2020-02-11	15-WS
53	3	3	15	WS	29,0	324,0	1,32847	M	0,142	0,044	2020-02-11	15-WS
54	3	3	15	WS	29,0	317,0	1,29977	M	0,154	0,049	2020-02-11	15-WS
55	3	4	15	LL	26,5	241,0	1,29503	M	0,144	0,060	2020-02-11	15-LL
56	3	4	15	LL	27,0	236,0	1,19900	M	0,078	0,033	2020-02-11	15-LL
57	3	4	15	LL	28,0	278,0	1,26640	M	0,102	0,037	2020-02-11	15-LL
58	3	4	15	LL	27,5	252,0	1,21172	M	0,095	0,038	2020-02-11	15-LL
59	3	4	15	LL	24,5	201,0	1,36678	M	0,058	0,029	2020-02-11	15-LL
60	3	4	15	LL	28,6	286,0	1,22255	M	0,103	0,036	2020-02-11	15-LL

61	3	7	12,5	LL	28,5	289,0	1,24843	M	0,081	0,028	2020-02-11	12.5-LL
62	3	7	12,5	LL	27,5	227,0	1,09151	M	0,058	0,026	2020-02-11	12.5-LL
63	3	7	12,5	LL	27,0	243,0	1,23457	M	0,099	0,041	2020-02-11	12.5-LL
64	3	7	12,5	LL	27,5	254,0	1,22134	M	0,109	0,043	2020-02-11	12.5-LL
65	3	7	12,5	LL	26,7	219,0	1,15056	M	0,085	0,039	2020-02-11	12.5-LL
66	3	8	12,5	WS	28,0	265,0	1,20718	M	0,108	0,041	2020-02-11	12.5-WS
67	3	8	12,5	WS	27,2	263,0	1,30692	M	0,096	0,037	2020-02-11	12.5-WS
68	3	8	12,5	WS	29,5	327,0	1,27374	M	0,118	0,036	2020-02-11	12.5-WS
69	3	8	12,5	WS	29,0	280,0	1,14806	M	0,112	0,040	2020-02-11	12.5-WS
70	3	8	12,5	WS	28,0	262,0	1,19351	M	0,104	0,040	2020-02-11	12.5-WS
71	3	8	12,5	WS	26,5	222,0	1,19293	M	0,096	0,043	2020-02-11	12.5-WS
72	3	9	12,5	WS	24,0	179,0	1,29485	M	0,069	0,039	2020-02-11	12.5-WS
73	3	9	12,5	WS	27,0	223,0	1,13296	M	0,094	0,042	2020-02-11	12.5-WS
74	3	9	12,5	WS	28,5	285,0	1,23115	M	0,128	0,045	2020-02-11	12.5-WS
75	3	9	12,5	WS	28,5	282,0	1,21819	M	0,134	0,048	2020-02-11	12.5-WS
76	3	9	12,5	WS	26,5	228,0	1,22517	M	0,101	0,044	2020-02-11	12.5-WS
77	3	9	12,5	WS	24,5	178,0	1,21038	M	0,076	0,043	2020-02-11	12.5-WS
78	3	10	12,5	LL	27,8	283,0	1,31720	M	0,106	0,037	2020-02-11	12.5-LL
79	3	10	12,5	LL	28,0	260,0	1,18440	M	0,085	0,033	2020-02-11	12.5-LL
80	3	10	12,5	LL	27,0	260,0	1,32094	M	0,122	0,047	2020-02-11	12.5-LL
81	3	10	12,5	LL	26,5	231,0	1,24129	M	0,076	0,033	2020-02-11	12.5-LL
82	3	10	12,5	LL	24,0	171,8	1,24277	M	0,051	0,030	2020-02-11	12.5-LL
83	3	10	12,5	LL	27,0	292,0	1,48351	M	0,111	0,038	2020-02-11	12.5-LL
84	4	1	15	LL	29,5	334,3	1,30218	M	0,173	0,052	2020-02-25	15-LL
85	4	1	15	LL	29,4	324,0	1,27498	M	0,156	0,048	2020-02-25	15-LL
86	4	1	15	LL	32,4	395,8	1,16370	M	0,162	0,041	2020-02-25	15-LL
87	4	1	15	LL	31,5	448,8	1,43589	M	0,17	0,038	2020-02-25	15-LL
88	4	1	15	LL	28,2	283,7	1,26506	M	0,121	0,043	2020-02-25	15-LL
89	4	1	15	LL	29,0	320,2	1,31289	M	8,291	2,589	2020-02-25	15-LL
90	4	2	15	WS	30,3	366,7	1,31821	M	0,109	0,030	2020-02-25	15-WS
91	4	2	15	WS	29,5	328,5	1,27959	M	0,137	0,042	2020-02-25	15-WS
92	4	2	15	WS	30,4	338,1	1,20344	M	0,136	0,040	2020-02-25	15-WS
93	4	2	15	WS	29,8	342,2	1,29310	M	0,131	0,038	2020-02-25	15-WS
94	4	2	15	WS	30,6	381,6	1,33182	M	0,147	0,039	2020-02-25	15-WS
95	4	2	15	WS	31,0	412,0	1,38297	M	0,182	0,044	2020-02-25	15-WS
96	4	3	15	WS	28,5	314,1	1,35685	M	0,095	0,030	2020-02-25	15-WS
97	4	3	15	WS	27,6	258,1	1,22761	M	0,098	0,038	2020-02-25	15-WS
98	4	3	15	WS	29,4	321,5	1,26514	M	0,098	0,030	2020-02-25	15-WS
99	4	3	15	WS	32,4	398,2	1,17076	M	0,149	0,037	2020-02-25	15-WS
100	4	3	15	WS	29,5	297,2	1,15766	M	0,108	0,036	2020-02-25	15-WS
101	4	3	15	WS	27,4	282,1	1,37136	M	0,127	0,045	2020-02-25	15-WS
102	4	4	15	LL	30,4	346,5	1,23334	M	0,092	0,027	2020-02-25	15-LL
103	4	4	15	LL	30,6	443,9	1,54925	M	0,195	0,044	2020-02-25	15-LL
104	4	4	15	LL	29,5	335,2	1,30568	M	0,111	0,033	2020-02-25	15-LL
105	4	4	15	LL	29,3	311,9	1,23997	M	0,153	0,049	2020-02-25	15-LL
106	4	4	15	LL	29,5	321,9	1,25388	M	0,13	0,040	2020-02-25	15-LL
107	4	7	12,5	LL	28,1	316,2	1,42509	M	0,184	0,058	2020-02-25	12.5-LL
108	4	7	12,5	LL	30,0	323,9	1,19963	M	0,121	0,037	2020-02-25	12.5-LL
109	4	7	12,5	LL	27,6	282,8	1,34509	M	0,127	0,045	2020-02-25	12.5-LL
110	4	7	12,5	LL	28,1	302,0	1,36109	M	0,151	0,050	2020-02-25	12.5-LL
111	4	7	12,5	LL	31,8	406,9	1,26534	M	0,2	0,049	2020-02-25	12.5-LL
112	4	7	12,5	LL	27,7	257,8	1,21295	M	0,1	0,039	2020-02-25	12.5-LL
113	4	8	12,5	WS	28,3	293,6	1,29538	M	0,089	0,030	2020-02-25	12.5-WS
114	4	8	12,5	WS	30,6	410,3	1,43198	M	0,155	0,038	2020-02-25	12.5-WS
115	4	8	12,5	WS	29,2	321,6	1,29172	M	0,127	0,039	2020-02-25	12.5-WS
116	4	8	12,5	WS	29,0	309,5	1,26901	M	0,118	0,038	2020-02-25	12.5-WS
117	4	8	12,5	WS	25,6	213,7	1,27375	M	0,091	0,043	2020-02-25	12.5-WS
118	4	8	12,5	WS	26,8	235,8	1,22501	M	0,107	0,045	2020-02-25	12.5-WS
119	4	9	12,5	WS	30,2	362,2	1,31501	M	0,153	0,042	2020-02-25	12.5-WS
120	4	9	12,5	WS	28,4	296,0	1,29222	M	0,103	0,035	2020-02-25	12.5-WS

121	4	9	12,5	WS	31,2	389,7	1,28312	M	0,144	0,037	2020-02-25	12.5-WS
122	4	9	12,5	WS	27,0	270,4	1,37377	M	0,122	0,045	2020-02-25	12.5-WS
123	4	9	12,5	WS	31,4	395,9	1,27878	M	0,15	0,038	2020-02-25	12.5-WS
124	4	9	12,5	WS	28,1	302,1	1,36154	M	0,129	0,043	2020-02-25	12.5-WS
125	4	10	12,5	LL	27,2	280,9	1,39587	M	0,122	0,043	2020-02-25	12.5-LL
126	4	10	12,5	LL	29,3	304,8	1,21175	M	0,1	0,033	2020-02-25	12.5-LL
127	4	10	12,5	LL	30,8	334,9	1,14621	M	0,144	0,043	2020-02-25	12.5-LL
128	4	10	12,5	LL	30,5	367,3	1,29456	M	0,136	0,037	2020-02-25	12.5-LL
129	4	10	12,5	LL	30,5	361,2	1,27306	M	0,161	0,045	2020-02-25	12.5-LL
130	4	10	12,5	LL	31,3	390,0	1,27184	M	0,16	0,041	2020-02-25	12.5-LL
131	4	10	12,5	LL	27,7	287,1	1,35081	M	0,136	0,047	2020-02-25	12.5-LL
132	5	1	15	LL	32,0	438,0	1,33667	M	0,154	0,035	2020-03-15	15-LL
133	5	1	15	LL	29,0	335,0	1,37357	M	3,436	1,026	2020-03-15	15-LL
134	5	1	15	LL	31,5	412,0	1,31815	M	0,167	0,041	2020-03-15	15-LL
135	5	1	15	LL	28,5	291,0	1,25707	M	0,099	0,034	2020-03-15	15-LL
136	5	1	15	LL	31,5	421,0	1,34695	M	0,122	0,029	2020-03-15	15-LL
137	5	2	15	WS	29,0	332,0	1,36127	M	0,176	0,053	2020-03-15	15-WS
138	5	2	15	WS	30,2	376,0	1,36511	M	0,165	0,044	2020-03-15	15-WS
139	5	2	15	WS	34,0	597,0	1,51893	M	0,179	0,030	2020-03-15	15-WS
140	5	2	15	WS	31,0	400,0	1,34269	M	0,117	0,029	2020-03-15	15-WS
141	5	2	15	WS	33,0	522,0	1,45254	M	0,228	0,044	2020-03-15	15-WS
142	5	2	15	WS	30,0	345,0	1,27778	M	0,129	0,037	2020-03-15	15-WS
143	5	3	15	WS	29,5	340,0	1,32438	M	26,97	7,932	2020-03-15	15-WS
144	5	3	15	WS	31,5	411,0	1,31495	M	0,132	0,032	2020-03-15	15-WS
145	5	3	15	WS	32,0	454,0	1,38550	M	0,181	0,040	2020-03-15	15-WS
146	5	3	15	WS	31,5	414,0	1,32455	M	0,185	0,045	2020-03-15	15-WS
147	5	3	15	WS	35,0	551,0	1,28513	M	0,239	0,043	2020-03-15	15-WS
148	5	3	15	WS	29,0	327,0	1,34077	M	0,121	0,037	2020-03-15	15-WS
149	5	3	15	WS	34,5	482,0	1,17379	M	0,266	0,055	2020-03-15	15-WS
150	5	4	15	LL	29,0	312,0	1,27927	M	0,128	0,041	2020-03-15	15-LL
151	5	4	15	LL	34,0	575,0	1,46296	M	0,456	0,079	2020-03-15	15-LL
152	5	4	15	LL	30,0	374,0	1,38519	M	0,213	0,057	2020-03-15	15-LL
153	5	4	15	LL	32,0	475,0	1,44958	M	18,99	3,998	2020-03-15	15-LL
154	5	4	15	LL	30,0	372,0	1,37778	M	4,65	1,250	2020-03-15	15-LL
155	5	7	12,5	LL	28,0	258,0	1,17529	M	0,084	0,033	2020-03-15	12.5-LL
156	5	7	12,5	LL	30,5	372,0	1,31112	M	0,179	0,048	2020-03-15	12.5-LL
157	5	7	12,5	LL	31,5	451,0	1,44293	M	0,181	0,040	2020-03-15	12.5-LL
158	5	7	12,5	LL	32,0	434,0	1,32446	M	0,202	0,047	2020-03-15	12.5-LL
159	5	7	12,5	LL	32,5	507,0	1,47692	M	0,192	0,038	2020-03-15	12.5-LL
160	5	7	12,5	LL	28,5	292,0	1,26139	M	0,102	0,035	2020-03-15	12.5-LL
161	5	8	12,5	WS	33,5	507,0	1,34857	M	0,226	0,045	2020-03-15	12.5-WS
162	5	8	12,5	WS	30,5	370,0	1,30407	M	0,14	0,038	2020-03-15	12.5-WS
163	5	8	12,5	WS	29,5	357,0	1,39060	M	0,176	0,049	2020-03-15	12.5-WS
164	5	8	12,5	WS	34,5	560,0	1,36374	M	0,242	0,043	2020-03-15	12.5-WS
165	5	8	12,5	WS	29,5	353,0	1,37502	M	0,147	0,042	2020-03-15	12.5-WS
166	5	8	12,5	WS	32,0	430,0	1,31226	M	0,193	0,045	2020-03-15	12.5-WS
167	5	9	12,5	WS	30,0	356,0	1,31852	M	0,194	0,054	2020-03-15	12.5-WS
168	5	9	12,5	WS	26,5	252,0	1,35414	M	0,09	0,036	2020-03-15	12.5-WS
169	5	9	12,5	WS	28,5	297,0	1,28299	M	0,131	0,044	2020-03-15	12.5-WS
170	5	9	12,5	WS	31,5	438,0	1,40134	M	0,165	0,038	2020-03-15	12.5-WS
171	5	9	12,5	WS	30,5	411,0	1,44858	M	0,177	0,043	2020-03-15	12.5-WS
172	5	9	12,5	WS	25,5	235,0	1,41725	M	0,1	0,043	2020-03-15	12.5-WS
173	5	10	12,5	LL	29,0	325,0	1,33257	M	0,129	0,040	2020-03-15	12.5-LL
174	5	10	12,5	LL	30,0	370,0	1,37037	M	0,178	0,048	2020-03-15	12.5-LL
175	5	10	12,5	LL	33,0	490,0	1,36350	M	0,312	0,064	2020-03-15	12.5-LL
176	5	10	12,5	LL	30,0	352,0	1,30370	M	0,155	0,044	2020-03-15	12.5-LL
177	5	10	12,5	LL	30,0	346,0	1,28148	M	0,182	0,053	2020-03-15	12.5-LL
178	5	10	12,5	LL	30,5	390,0	1,37456	M	0,175	0,045	2020-03-15	12.5-LL
179	5	10	12,5	LL	31,5	421,0	1,34695	M	0,221	0,052	2020-03-15	12.5-LL
180	6	1	15	LL	32,0	427,0	1,30310	M	0,154	0,036	2020-03-29	15-LL

181	6	1	15	LL	32,5	480,0	1,39827	M	0,188	0,039	2020-03-29	15-LL
182	6	1	15	LL	32,5	488,0	1,42157	M	0,199	0,041	2020-03-29	15-LL
183	6	1	15	LL	32,0	438,0	1,33667	M	0,17	0,039	2020-03-29	15-LL
184	6	1	15	LL	31,0	429,0	1,44003	M	1,735	0,404	2020-03-29	15-LL
185	6	1	15	LL	33,0	472,0	1,31341	M	34,543	7,318	2020-03-29	15-LL
186	6	2	15	WS	31,5	390,0	1,24777	M	0,131	0,034	2020-03-29	15-WS
187	6	2	15	WS	34,0	488,0	1,24160	M	0,202	0,041	2020-03-29	15-WS
188	6	2	15	WS	34,5	535,0	1,30286	M	0,195	0,036	2020-03-29	15-WS
189	6	2	15	WS	31,0	370,0	1,24199	M	0,126	0,034	2020-03-29	15-WS
190	6	3	15	WS	32,5	408,0	1,18853	M	0,159	0,039	2020-03-29	15-WS
191	6	3	15	WS	34,5	489,0	1,19083	M	0,196	0,040	2020-03-29	15-WS
192	6	3	15	WS	31,0	356,0	1,19499	M	0,12	0,034	2020-03-29	15-WS
193	6	3	15	WS	36,0	593,0	1,27100	M	0,252	0,042	2020-03-29	15-WS
194	6	3	15	WS	26,5	235,0	1,26279	M	0,084	0,036	2020-03-29	15-WS
195	6	3	15	WS	30,5	371,0	1,30760	M	0,138	0,037	2020-03-29	15-WS
196	6	3	15	WS	35,5	593,0	1,32547	M	0,265	0,045	2020-03-29	15-WS
197	6	4	15	LL	35,0	559,0	1,30379	M	0,313	0,056	2020-03-29	15-LL
198	6	4	15	LL	31,5	450,0	1,43973	M	0,183	0,041	2020-03-29	15-LL
199	6	4	15	LL	35,5	642,0	1,43499	M	0,34	0,053	2020-03-29	15-LL
200	6	4	15	LL	34,5	535,0	1,30286	M	0,197	0,037	2020-03-29	15-LL
201	6	4	15	LL	32,5	439,0	1,27883	M	0,155	0,035	2020-03-29	15-LL
202	6	4	15	LL	31,5	448,0	1,43333	M	47,048	10,502	2020-03-29	15-LL
203	6	7	12,5	LL	30,0	350,0	1,29630	M	0,151	0,043	2020-03-29	12.5-LL
204	6	7	12,5	LL	34,0	510,0	1,29758	M	0,226	0,044	2020-03-29	12.5-LL
205	6	7	12,5	LL	33,0	466,0	1,29671	M	0,233	0,050	2020-03-29	12.5-LL
206	6	7	12,5	LL	31,5	427,0	1,36614	M	0,192	0,045	2020-03-29	12.5-LL
207	6	7	12,5	LL	32,5	453,0	1,31962	M	0,225	0,050	2020-03-29	12.5-LL
208	6	7	12,5	LL	32,5	485,0	1,41284	M	0,178	0,037	2020-03-29	12.5-LL
209	6	7	12,5	LL	33,0	468,0	1,30228	M	0,202	0,043	2020-03-29	12.5-LL
210	6	8	12,5	WS	32,5	475,0	1,38371	M	0,165	0,035	2020-03-29	12.5-WS
211	6	8	12,5	WS	33,0	509,0	1,41637	M	0,508	0,100	2020-03-29	12.5-WS
212	6	8	12,5	WS	34,0	529,0	1,34592	M	0,218	0,041	2020-03-29	12.5-WS
213	6	8	12,5	WS	36,5	615,0	1,26473	M	0,252	0,041	2020-03-29	12.5-WS
214	6	9	12,5	WS	30,5	367,0	1,29350	M	0,146	0,040	2020-03-29	12.5-WS
215	6	9	12,5	WS	32,5	441,0	1,28466	M	0,168	0,038	2020-03-29	12.5-WS
216	6	9	12,5	WS	30,0	348,0	1,28889	M	0,145	0,042	2020-03-29	12.5-WS
217	6	9	12,5	WS	30,5	352,0	1,24063	M	0,142	0,040	2020-03-29	12.5-WS
218	6	9	12,5	WS	31,5	423,0	1,35335	M	0,155	0,037	2020-03-29	12.5-WS
219	6	9	12,5	WS	31,0	405,0	1,35947	M	0,157	0,039	2020-03-29	12.5-WS
220	6	10	12,5	LL	35,0	571,0	1,33178	M	0,246	0,043	2020-03-29	12.5-LL
221	6	10	12,5	LL	33,5	534,0	1,42039	M	0,213	0,040	2020-03-29	12.5-LL
222	6	10	12,5	LL	35,5	642,0	1,43499	M	0,27	0,042	2020-03-29	12.5-LL
223	6	10	12,5	LL	32,5	495,0	1,44197	M	0,229	0,046	2020-03-29	12.5-LL
224	6	10	12,5	LL	31,5	379,0	1,21257	M	0,133	0,035	2020-03-29	12.5-LL
225	7	1	15	LL	35,0	586,0	1,36676	M	35,84	6,116	2020-04-16	15-LL
226	7	1	15	LL	35,2	599,0	1,37341	M	0,262	0,044	2020-04-16	15-LL
227	7	1	15	LL	37,0	681,0	1,34444	M	0,325	0,048	2020-04-16	15-LL
228	7	1	15	LL	32,0	449,0	1,37024	M	36,448	8,118	2020-04-16	15-LL
229	7	1	15	LL	33,5	513,0	1,36453	M	48,246	9,405	2020-04-16	15-LL
230	7	1	15	LL	34,5	555,0	1,35156	M	28,331	5,105	2020-04-16	15-LL
231	7	1	15	LL	34,5	528,0	1,28581	M	0,446	0,084	2020-04-16	15-LL
232	7	2	15	WS	39,0	845,0	1,42450	M	4,284	0,507	2020-04-16	15-WS
233	7	2	15	WS	33,0	512,0	1,42472	M	1,385	0,271	2020-04-16	15-WS
234	7	2	15	WS	40,0	886,0	1,38438	M	0,295	0,033	2020-04-16	15-WS
235	7	2	15	WS	32,0	434,0	1,32446	M	0,517	0,119	2020-04-16	15-WS
236	7	2	15	WS	34,0	599,0	1,52402	M	1,09	0,182	2020-04-16	15-WS
237	7	2	15	WS	34,0	523,0	1,33065	M	1,13	0,216	2020-04-16	15-WS
238	7	3	15	WS	36,0	650,0	1,39318	M	0,886	0,136	2020-04-16	15-WS
239	7	3	15	WS	37,0	738,0	1,45697	M	1,326	0,180	2020-04-16	15-WS
240	7	3	15	WS	31,5	420,0	1,34375	M	0,235	0,056	2020-04-16	15-WS

241	7	3	15	WS	34,0	594,0	1,51130	M	1,841	0,310	2020-04-16	15-WS
242	7	3	15	WS	34,0	550,0	1,39935	M	0,96	0,175	2020-04-16	15-WS
243	7	3	15	WS	33,0	484,0	1,34680	M	0,176	0,036	2020-04-16	15-WS
244	7	4	15	LL	34,5	588,0	1,43192	M	0,289	0,049	2020-04-16	15-LL
245	7	4	15	LL	35,5	587,0	1,31206	M	17,068	2,908	2020-04-16	15-LL
246	7	4	15	LL	36,5	655,0	1,34698	M	0,296	0,045	2020-04-16	15-LL
247	7	4	15	LL	38,5	847,0	1,48423	M	0,319	0,038	2020-04-16	15-LL
248	7	4	15	LL	33,0	444,0	1,23550	M	19,955	4,494	2020-04-16	15-LL
249	7	7	12,5	LL	36,5	674,0	1,38606	M	0,349	0,052	2020-04-16	12.5-LL
250	7	7	12,5	LL	33,5	522,0	1,38847	M	4,234	0,811	2020-04-16	12.5-LL
251	7	7	12,5	LL	32,5	472,0	1,37497	M	0,214	0,045	2020-04-16	12.5-LL
252	7	7	12,5	LL	34,5	517,0	1,25902	M	0,176	0,034	2020-04-16	12.5-LL
253	7	7	12,5	LL	37,5	723,0	1,37102	M	0,262	0,036	2020-04-16	12.5-LL
254	7	7	12,5	LL	32,0	454,0	1,38550	M	0,192	0,042	2020-04-16	12.5-LL
255	7	7	12,5	LL	39,0	912,0	1,53745	M	0,449	0,049	2020-04-16	12.5-LL
256	7	7	12,5	LL	33,0	492,0	1,36906	M	0,202	0,041	2020-04-16	12.5-LL
257	7	8	12,5	WS	32,0	489,2	1,49292	M	0,683	0,140	2020-04-16	12.5-WS
258	7	8	12,5	WS	35,4	533,7	1,20306	M	0,44	0,082	2020-04-16	12.5-WS
259	7	8	12,5	WS	36,1	665,8	1,41521	M	1,02	0,153	2020-04-16	12.5-WS
260	7	8	12,5	WS	35,2	535,8	1,22850	M	0,21	0,039	2020-04-16	12.5-WS
261	7	8	12,5	WS	33,4	455,8	1,22331	M	0,183	0,040	2020-04-16	12.5-WS
262	7	8	12,5	WS	32,1	420,0	1,26980	M	0,145	0,035	2020-04-16	12.5-WS
263	7	9	12,5	WS	33,5	500,0	1,32995	M	0,17	0,034	2020-04-16	12.5-WS
264	7	9	12,5	WS	33,0	469,0	1,30506	M	0,201	0,043	2020-04-16	12.5-WS
265	7	9	12,5	WS	33,5	501,0	1,33261	M	0,324	0,065	2020-04-16	12.5-WS
266	7	9	12,5	WS	33,5	485,0	1,29005	M	0,172	0,035	2020-04-16	12.5-WS
267	7	9	12,5	WS	32,5	432,0	1,25844	M	0,138	0,032	2020-04-16	12.5-WS
268	7	9	12,5	WS	34,0	528,0	1,34337	M	0,268	0,051	2020-04-16	12.5-WS
269	7	10	12,5	LL	36,0	661,0	1,41675	M	0,282	0,043	2020-04-16	12.5-LL
270	7	10	12,5	LL	35,0	568,0	1,32478	M	0,249	0,044	2020-04-16	12.5-LL
271	7	10	12,5	LL	34,5	656,0	1,59752	M	0,234	0,036	2020-04-16	12.5-LL
272	7	10	12,5	LL	33,5	521,0	1,38581	M	0,217	0,042	2020-04-16	12.5-LL
273	8	1	15	LL	36,0	594,0	1,27315	M	0,244	0,041	2020-05-07	15-LL
274	8	1	15	LL	37,0	701,0	1,38393	M	70,55	10,064	2020-05-07	15-LL
275	8	1	15	LL	36,0	712,0	1,52606	M	50,58	7,104	2020-05-07	15-LL
276	8	1	15	LL	31,5	414,0	1,32455	M	31,07	7,505	2020-05-07	15-LL
277	8	1	15	LL	36,5	652,0	1,34082	M	48,44	7,429	2020-05-07	15-LL
278	8	2	15	WS	35,5	623,2	1,39297	M	0,913	0,147	2020-05-07	15-WS
279	8	2	15	WS	38,5	750,0	1,31425	M	6,783	0,904	2020-05-07	15-WS
280	8	2	15	WS	36,0	644,0	1,38032	M	8,21	1,275	2020-05-07	15-WS
281	8	2	15	WS	32,5	478,0	1,39244	M	5,602	1,172	2020-05-07	15-WS
282	8	2	15	WS	35,5	654,0	1,46182	M	18,866	2,885	2020-05-07	15-WS
283	8	3	15	WS	35,5	643,0	1,43723	M	12,259	1,907	2020-05-07	15-WS
284	8	3	15	WS	35,0	658,0	1,53469	M	12,453	1,893	2020-05-07	15-WS
285	8	3	15	WS	36,5	629,0	1,29352	M	20,08	3,192	2020-05-07	15-WS
286	8	3	15	WS	37,5	723,0	1,37102	M	7,156	0,990	2020-05-07	15-WS
287	8	3	15	WS	40,5	839,0	1,26298	M	11,2	1,335	2020-05-07	15-WS
288	8	3	15	WS	35,0	586,0	1,36676	M	5,841	0,997	2020-05-07	15-WS
289	8	3	15	WS	35,0	568,0	1,32478	M	5,74	1,011	2020-05-07	15-WS
290	8	4	15	LL	38,5	872,0	1,52804	M	0,308	0,035	2020-05-07	15-LL
291	8	4	15	LL	34,0	571,0	1,45278	M	43,9	7,688	2020-05-07	15-LL
292	8	4	15	LL	38,0	817,0	1,48892	M	56,47	6,912	2020-05-07	15-LL
293	8	4	15	LL	38,5	795,0	1,39311	M	19,352	2,434	2020-05-07	15-LL
294	8	4	15	LL	37,5	724,0	1,37292	M	0,645	0,089	2020-05-07	15-LL
295	8	4	15	LL	34,5	568,0	1,38322	M	0,593	0,104	2020-05-07	15-LL
296	8	4	15	LL	36,0	603,0	1,29244	M	0,263	0,044	2020-05-07	15-LL
297	8	7	12,5	LL	37,5	676,0	1,28190	M	0,32	0,047	2020-05-07	12.5-LL
298	8	7	12,5	LL	37,0	590,0	1,16479	M	0,27	0,046	2020-05-07	12.5-LL
299	8	7	12,5	LL	31,0	408,0	1,36954	M	2,033	0,498	2020-05-07	12.5-LL
300	8	7	12,5	LL	36,5	697,0	1,43336	M	0,335	0,048	2020-05-07	12.5-LL

301	8	7	12,5	LL	34,5	548,0	1,33451	M	0,208	0,038	2020-05-07	12.5-LL
302	8	7	12,5	LL	33,0	509,0	1,41637	M	43,161	8,480	2020-05-07	12.5-LL
303	8	8	12,5	WS	37,0	737,0	1,45500	M	5,276	0,716	2020-05-07	12.5-WS
304	8	8	12,5	WS	35,5	555,0	1,24053	M	0,215	0,039	2020-05-07	12.5-WS
305	8	8	12,5	WS	37,0	671,0	1,32470	M	0,316	0,047	2020-05-07	12.5-WS
306	8	8	12,5	WS	39,5	847,0	1,37433	M	0,385	0,045	2020-05-07	12.5-WS
307	8	9	12,5	WS	33,5	449,0	1,19430	M	0,14	0,031	2020-05-07	12.5-WS
308	8	9	12,5	WS	31,5	396,0	1,26251	M	0,175	0,044	2020-05-07	12.5-WS
309	8	9	12,5	WS	37,0	755,0	1,49053	M	3,176	0,421	2020-05-07	12.5-WS
310	8	9	12,5	WS	40,0	918,0	1,43438	M	0,493	0,054	2020-05-07	12.5-WS
311	8	9	12,5	WS	40,0	898,0	1,40313	M	2,953	0,329	2020-05-07	12.5-WS
312	8	9	12,5	WS	38,0	849,0	1,54724	M	5,085	0,599	2020-05-07	12.5-WS
313	8	9	12,5	WS	39,0	816,0	1,37561	M	2,5	0,306	2020-05-07	12.5-WS
314	8	9	12,5	WS	36,0	718,0	1,53892	M	7,024	0,978	2020-05-07	12.5-WS
315	8	10	12,5	LL	33,0	428,0	1,19097	M	0,138	0,032	2020-05-07	12.5-LL
316	8	10	12,5	LL	32,5	489,0	1,42449	M	47,57	9,728	2020-05-07	12.5-LL
317	8	10	12,5	LL	40,0	891,0	1,39219	M	0,429	0,048	2020-05-07	12.5-LL
318	8	10	12,5	LL	39,0	818,0	1,37898	M	0,326	0,040	2020-05-07	12.5-LL
319	8	10	12,5	LL	41,5	968,0	1,35435	M	0,432	0,045	2020-05-07	12.5-LL
320	8	10	12,5	LL	35,5	595,0	1,32994	M	0,238	0,040	2020-05-07	12.5-LL

Table II. Overview of fish id, sampling, tank id, experimental group, [GPR54/ef1a], [dio2b/ef1a], [GnIHa/ef1a], [GnRH2/ef1a], [GnIHb/ef1a] and [tshβb/ef1a].

fish_id	SAMPLING	tank_id	exp_group	[GPR54/ef1a]	[dio2b/ef1a]	[GnIHa/ef1a]	[GnRH2/ef1a]	[GnIHb/ef1a]	[tshβb/ef1a]
1	1	0	12.5-LL	0,3891	0,4205	0,4457	0,3162	0,2045	0,0151
2	1	0	12.5-LL	0,5321	0,8562	0,7547	0,4004	0,6385	0,0205
3	1	0	12.5-LL	0,5853	3,5465	0,5062	0,2204	0,9904	0,0122
4	1	0	12.5-LL	0,5846	1,4307	0,2369	0,4432	0,2995	0,0230
5	1	0	12.5-LL	0,5472	1,5136	0,2702	0,3152	0,1121	0,0226
6	1	0	12.5-LL	0,5677	1,7375	0,2330	0,1941	0,2344	0,0344
7	1	0	12.5-LL	0,4617	1,1392	0,1341	0,3862	0,0805	0,0247
8	1	0	12.5-LL	0,2944	1,9692	0,4240	0,2774	0,2502	0,0491
9	1	0	12.5-LL	0,6636	1,5904	0,3266	0,3542	0,4444	0,0090
10	1	0	12.5-LL	0,4950	0,7749	0,1996	0,3010	0,1446	0,0019
11	1	0	12.5-LL	0,5953	2,0045	0,3883	0,2588	0,4349	0,0163
12	1	0	12.5-LL	0,4562	2,2870	0,0973	0,3631	0,1350	0,0087
13	2	1	15-LL	0,3309	0,2773	0,1078	0,5107	0,0628	0,0107
14	2	1	15-LL					2,9242	0,0116
15	2	1	15-LL					0,0486	0,0099
16	2	2	15-WS	0,9660	0,8742	0,4542	0,8612	0,1156	0,0119
17	2	2	15-WS	0,2757	0,5630	0,0514	0,5124	0,0196	0,0190
18	2	2	15-WS	0,3276	1,0042	0,0837	0,6102	0,0406	0,0080
19	2	3	15-WS	0,5916	0,2768	0,3145	0,2579	0,1487	0,0161
20	2	3	15-WS	0,4071	0,4297	0,1741	0,4250	0,0303	0,0148
21	2	3	15-WS	0,6816	1,2983	0,1607	0,6429	0,0335	0,0077
22	2	4	15-LL	0,8618	1,3424	0,1645	0,4099	0,0352	0,0094
23	2	4	15-LL	2,1953	0,8626	0,8109	0,9384	0,3349	0,0197
24	2	4	15-LL	0,7204	0,5276	0,2472	0,5725	0,0709	0,0080
25	2	7	12.5-LL	0,6373	0,6997	0,2517	0,2778	0,1010	0,0120
26	2	7	12.5-LL	0,4426	1,2885	0,2309	0,5872	0,1592	0,0156
27	2	7	12.5-LL	0,6109	0,5728	0,5700	0,3412	0,2397	0,0195
28	2	8	12.5-WS	0,4413	0,5416	0,5259	0,2871	0,1219	0,0145
29	2	8	12.5-WS	0,6983	1,7117	0,7462	0,5233	0,9095	0,0151
30	2	8	12.5-WS	0,6138	0,4401	0,6204	0,1651	0,1662	0,0210
31	2	9	12.5-WS	0,3183	0,6021	0,0827	0,4179	0,0894	0,0191
32	2	9	12.5-WS	0,3855	0,4534	0,1170	0,3427	0,0637	0,0247
33	2	9	12.5-WS	0,6983	0,6458	0,6682	0,4491	0,1498	0,0128
34	2	10	12.5-LL	0,5244	0,5065	0,0200	0,3733	0,1097	0,0024
35	2	10	12.5-LL						0,0163
36	2	10	12.5-LL						0,0068
37	3	1	15-LL	2,0271	0,5911	1,4624	1,0791	0,5600	0,0145
38	3	1	15-LL						0,0077
39	3	1	15-LL						0,0239
40	3	1	15-LL						0,0186
41	3	1	15-LL						0,0378
42	3	1	15-LL						0,0286
43	3	2	15-WS	2,9653	0,4438	2,5161	0,9900	0,7817	0,0055
44	3	2	15-WS						0,0018
45	3	2	15-WS						0,0045
46	3	2	15-WS						0,0184
47	3	2	15-WS	1,3415	0,5598	0,9315	1,1884	0,3859	0,0009
48	3	2	15-WS						0,0108
49	3	3	15-WS	1,1397	0,5108	1,1430	0,9946	0,5033	0,0037
50	3	3	15-WS						0,0101
51	3	3	15-WS						0,0020
52	3	3	15-WS	1,2042	0,3470	1,3598	0,5792	0,5512	0,0040
53	3	3	15-WS						0,0092
54	3	3	15-WS	3,1114	0,6597	0,0689	0,7800	0,0413	0,0149
55	3	4	15-LL	0,8005	0,6479	1,5635	1,1957	0,5491	0,0167
56	3	4	15-LL	0,9803	0,1699	1,0987	1,1677	0,3281	0,0128
57	3	4	15-LL		0,2422	0,2838	0,8285	0,0613	0,0215
58	3	4	15-LL						0,0119
59	3	4	15-LL						0,0122
60	3	4	15-LL						0,0247

61	3	7	12.5-LL	0,5071	0,4739	0,0820	0,4018	0,1303	0,0072
62	3	7	12.5-LL	0,3609	0,6195	0,4351	0,3256	0,0459	0,0035
63	3	7	12.5-LL	0,4643	0,5217	0,0163	0,4463	0,5068	0,0131
64	3	7	12.5-LL	0,7947	1,5950	0,3449	0,2505	0,2434	0,0071
65	3	7	12.5-LL	0,3815	3,5351	0,0642	0,3775	0,1108	0,0180
66	3	8	12.5-WS	0,6018	0,9777	1,2522	0,4699	0,9189	0,0066
67	3	8	12.5-WS	0,3805	3,0438	0,1245	0,4629	0,2291	0,0125
68	3	8	12.5-WS	0,4205	1,2566	0,1015	0,3203	0,0465	0,0033
69	3	8	12.5-WS	0,4462	1,7698	0,1443	0,3823	0,0896	0,0100
70	3	8	12.5-WS	0,3290	1,3423	0,1603	0,3061	0,0909	0,0072
71	3	8	12.5-WS	0,4623	0,5061	0,4529	0,3641	0,2769	0,0129
72	3	9	12.5-WS	0,7219	2,1147	0,4926	0,4743	0,4283	0,0091
73	3	9	12.5-WS	0,2242	0,5818		0,2563	0,0265	0,0179
74	3	9	12.5-WS						0,0055
75	3	9	12.5-WS						0,0031
76	3	9	12.5-WS	0,3829	0,5615	0,4384	0,1737	0,1723	0,0041
77	3	9	12.5-WS	0,0632	0,0263	0,5579	0,4437	2,1707	0,0101
78	3	10	12.5-LL	0,4675	0,6593	0,6503	0,1843	0,2300	0,0396
79	3	10	12.5-LL	0,0000	0,1520	0,0810	0,9268	6,1254	0,0070
80	3	10	12.5-LL	0,2633	1,1251	0,1763	0,1938	0,1789	0,0084
81	3	10	12.5-LL	0,3332	0,4159	0,0438	0,1318	0,0394	0,0430
82	3	10	12.5-LL	0,3339	1,4836	0,3928	0,1483	0,2962	0,0161
83	3	10	12.5-LL						0,0091
84	4	1	15-LL	0,0912	0,3078	0,2266	0,4906	0,0806	0,0143
85	4	1	15-LL	0,0658	0,3265	0,0380	0,3016	0,0179	0,0126
86	4	1	15-LL	0,3686	0,6019	0,0297	0,4271	0,0122	0,0145
87	4	1	15-LL	0,7707	0,1922	0,0609	0,2977	0,0327	0,0224
88	4	1	15-LL	1,2549	0,2906	0,7032	0,8464	0,4634	0,0255
89	4	1	15-LL	0,6047	0,2606	0,6246	0,6942	0,1372	0,0142
90	4	2	15-WS	3,5865	0,7488	0,1071	0,8338	0,0482	0,0044
91	4	2	15-WS	0,1933	0,4072	1,1877	0,9810	0,3964	
92	4	2	15-WS	1,2435	0,2870	0,1544	0,8900	0,0671	0,0067
93	4	2	15-WS	0,8728	0,3610	1,3073	0,7522	0,6967	0,0073
94	4	2	15-WS	0,8635	0,3141	0,3458	0,7654	0,1066	0,0082
95	4	2	15-WS	0,0818	0,1787	0,2726	0,4225	0,1435	0,0043
96	4	3	15-WS	0,6788	0,2165	0,0438	0,4643	0,0193	0,0105
97	4	3	15-WS	0,0398	0,2193	0,1072	0,7751	0,0677	0,0019
98	4	3	15-WS	0,1679	0,2579	0,0350	0,4236	0,0169	0,0083
99	4	3	15-WS	1,9408	0,5882	0,0547	0,6125	0,0225	0,0034
100	4	3	15-WS	0,1577	0,1591	0,4956	0,4561	0,1719	0,0106
101	4	3	15-WS	3,1935	0,4821	0,0583	0,3685	0,0406	0,0066
102	4	4	15-LL	1,0622	0,2941	0,5391	0,5704	0,2388	0,0170
103	4	4	15-LL	0,3410	0,5888	0,2671	0,6134	0,2339	0,0253
104	4	4	15-LL	1,1164	0,3524	0,0748	0,4028	0,0240	0,0364
105	4	4	15-LL	2,0851	0,8169	0,3019	0,6796	0,1010	0,0078
106	4	4	15-LL	0,5720	0,6380	0,9040	0,8249	0,4070	0,0168
107	4	7	12.5-LL	0,3893	0,7086	0,1135	0,3063	0,1586	0,0401
108	4	7	12.5-LL	0,4165	1,6689	0,2099	0,3449	0,3018	0,0236
109	4	7	12.5-LL	0,7491	0,2896	2,2472	0,2064	0,4124	0,0110
110	4	7	12.5-LL	0,3141	1,3801	0,1268	0,2868	0,2721	0,0142
111	4	7	12.5-LL	0,1881	0,2734	0,2310	0,1606	0,0523	0,0277
112	4	7	12.5-LL	1,3884	0,5023	0,1628	0,4266	0,7626	0,0053
113	4	8	12.5-WS	0,1712	0,1128	0,0036	0,1113	0,0777	0,0069
114	4	8	12.5-WS	0,4547	0,2988	0,4255	0,2736	0,2344	0,0021
115	4	8	12.5-WS	0,5038	0,2456	0,2065	0,2242	0,2904	0,0033
116	4	8	12.5-WS	0,8348	0,3304	0,0094	0,3495	0,6236	0,0085
117	4	8	12.5-WS	0,4039	0,3104	1,0371	0,2291	0,1540	0,0058
118	4	8	12.5-WS	0,5493	0,2749	1,4574	0,2398	0,2355	0,0090
119	4	9	12.5-WS	0,1681	0,6176	0,2088	0,2193	0,0282	0,0078
120	4	9	12.5-WS	0,4908	0,3370	0,6655	0,3030	0,0610	0,0025

121	4	9	12.5-WS	0,4317	0,4207	0,2373	0,1655	0,1998	0,0078
122	4	9	12.5-WS	0,4751	0,2631	0,7035	0,1831	0,3336	0,0050
123	4	9	12.5-WS	0,4869	0,4870	0,1693	0,2020	0,2108	0,0168
124	4	9	12.5-WS	0,4665	0,2725	0,0941	0,1745	0,1487	0,0069
125	4	10	12.5-LL	0,2958	0,4873	0,1845	0,2263	0,0684	0,0104
126	4	10	12.5-LL	0,2948	0,7810	2,0093	0,2224	0,0420	0,0280
127	4	10	12.5-LL	0,3268	0,5100	0,0537	0,1967	0,0219	0,0151
128	4	10	12.5-LL	0,3500	0,4347	0,6159	0,1223	0,1914	0,0119
129	4	10	12.5-LL	0,3164	0,5837	0,0837	0,1674	0,0952	0,0178
130	4	10	12.5-LL	0,2939	0,6912	0,0540	0,3071	0,0516	0,0253
131	4	10	12.5-LL	0,3048	0,3443	0,5394	0,1893	0,1579	0,0111
132	5	1	15-LL	0,4818	0,1556	0,0985	0,2493	0,0560	0,0013
133	5	1	15-LL	1,7413	0,1756	0,3925	0,5382	0,1609	0,0272
134	5	1	15-LL	1,5562	0,2250	0,8852	0,8221	0,5082	0,0168
135	5	1	15-LL	0,4023	0,1136	0,9224	0,5486	0,4550	0,0242
136	5	1	15-LL	0,4008	0,2330	0,5277	0,7658	0,1704	0,0097
137	5	2	15-WS	1,4254	0,1542	0,1891	0,8816	0,0847	0,0314
138	5	2	15-WS	0,6405	0,1433	0,3607	0,6374	0,1811	0,1385
139	5	2	15-WS	1,1376	0,2833	1,3679	1,0967	0,6473	0,0885
140	5	2	15-WS	1,1263	0,3427	1,0201	0,7966	0,2989	0,2628
141	5	2	15-WS	1,2006	0,2025	0,6962	0,7930	0,3001	0,3002
142	5	2	15-WS	1,4064	0,9500	1,0967	1,0673	0,3719	0,1919
143	5	3	15-WS	0,8320	0,1992	1,1934	0,7623	0,6257	0,3195
144	5	3	15-WS	0,6358	0,4030	0,9967	0,5840	0,4413	0,2015
145	5	3	15-WS	1,1637	0,5395	0,2865	0,3048	0,1536	0,1360
146	5	3	15-WS	0,3142	0,4945	0,4371	0,5467	0,2124	0,2789
147	5	3	15-WS	0,8388	0,4082	0,0296	0,1837	0,0213	0,1324
148	5	3	15-WS	0,5415	0,6133	0,2425	0,3243	0,1292	0,1491
149	5	3	15-WS	0,3859	0,3447	0,1018	0,2507	0,0938	0,2519
150	5	4	15-LL	1,2715	0,3491	0,0388	0,5004	0,0379	0,0310
151	5	4	15-LL	2,0170	0,3602	0,3366	0,6624	0,2348	0,0244
152	5	4	15-LL	0,6439	0,2092	0,8699	1,1260	0,4435	0,0173
153	5	4	15-LL	1,3582	0,3289	0,6738	0,4291	0,3286	0,0106
154	5	4	15-LL	1,9368	0,4747	0,5669	0,8137	0,3513	0,0300
155	5	7	12.5-LL	0,4890	0,3753	0,1910	0,1438	0,3956	0,0245
156	5	7	12.5-LL	0,4252	0,3295	0,9701	0,2024	0,1121	0,0176
157	5	7	12.5-LL	0,1369	0,2706	0,1754	0,1203	0,0770	0,0101
158	5	7	12.5-LL	0,3749	0,3518	0,4119	0,1381	0,2771	0,0046
159	5	7	12.5-LL	0,2296	0,4697	0,1788	0,1243	0,1865	0,0273
160	5	7	12.5-LL	0,5425	0,3004	1,2996	0,2769	0,3296	0,0212
161	5	8	12.5-WS	0,3952	0,2400	0,1419	0,1262	0,1540	0,1953
162	5	8	12.5-WS	0,4618	0,4220	0,3119	0,2435	0,2556	0,5833
163	5	8	12.5-WS	0,4198	0,4262	0,1920	0,2553	0,3161	0,0075
164	5	8	12.5-WS	0,5518	0,3788	0,3988	0,1533	0,4926	0,1937
165	5	8	12.5-WS	0,4545	1,2587	0,3986	0,2388	0,3289	0,3013
166	5	8	12.5-WS	0,6306	0,4397	0,0680	0,2466	0,3738	0,3943
167	5	9	12.5-WS	0,7317	1,4723	0,9970	0,4029	0,8747	0,3111
168	5	9	12.5-WS	0,5520	0,8284	0,1187	0,4300	0,1532	0,0535
169	5	9	12.5-WS	0,4209	1,4650	0,4077	0,7237	0,4586	0,4395
170	5	9	12.5-WS	0,6819	0,3513	0,0288	0,4299	0,6534	0,1287
171	5	9	12.5-WS	0,5440	1,2394	0,4993	0,3643	0,6945	0,5554
172	5	9	12.5-WS	0,6759	1,1784	0,2423	0,4508	0,6543	0,0930
173	5	10	12.5-LL	0,3280	0,4422	0,2316	0,4268	0,1731	0,0053
174	5	10	12.5-LL	0,4704	0,6847	0,1580	0,3842	0,1460	0,0077
175	5	10	12.5-LL	0,5713	1,8234	0,2905	0,5388	0,6539	0,0223
176	5	10	12.5-LL	0,3743	1,2771	0,2549	0,4572	0,1989	0,0193
177	5	10	12.5-LL	0,2984	0,6931	0,1656	0,3655	0,0610	0,0051
178	5	10	12.5-LL	0,5544	0,7748	0,1772	0,3738	0,1935	0,0059
179	5	10	12.5-LL	0,6375	0,3016	0,0734	0,3100	0,4127	0,0215
180	6	1	15-LL	1,8984	0,3494	1,2905	0,8350	0,4758	0,0307

181	6	1	15-LL	1,7226	0,1407	0,2362	0,3708	0,1301	0,0435
182	6	1	15-LL	1,0225	0,3169	1,2955	0,4464	0,6563	0,0124
183	6	1	15-LL	0,4588	0,2830	1,3783	0,6334	0,8047	0,0467
184	6	1	15-LL	2,6411	0,2520	0,0965	0,3433	0,0329	0,0105
185	6	1	15-LL	12,1883	0,4148	1,1133	1,1183	0,4554	0,0050
186	6	2	15-WS	2,1387	1,4443	2,3478	1,0429	0,8356	0,0405
187	6	2	15-WS	0,6117	0,5807	2,8066	0,6530	1,2054	0,0862
188	6	2	15-WS	4,3289	0,5655	0,1707	0,3922	0,0930	0,1810
189	6	2	15-WS	1,5789	1,3563	2,3975	1,1331	0,8137	0,0430
190	6	3	15-WS	1,4026	0,7564	0,7382	0,4362	0,5434	0,0747
191	6	3	15-WS	0,6446	0,4984	0,1863	0,4236	0,1521	0,0434
192	6	3	15-WS	2,4180	1,7168	0,4715	1,0039	0,2254	0,0309
193	6	3	15-WS	2,2363	0,8963	0,7764	0,8459	0,4864	0,0340
194	6	3	15-WS	1,3985	0,7992	1,3676	0,7479	0,7205	0,0233
195	6	3	15-WS	2,0283	1,3224	0,7440	0,8348	0,4686	0,0624
196	6	3	15-WS	3,2432	0,6341	0,6584	0,5106	0,2818	0,0387
197	6	4	15-LL	2,1078	0,3064	1,7818	1,3378	0,5802	0,0399
198	6	4	15-LL	2,1695	0,3095	1,5707	1,3589	0,6562	0,0134
199	6	4	15-LL	2,0586	0,3093	2,8762	0,8940	0,6285	0,0369
200	6	4	15-LL	5,1175	0,3130	0,5562	0,9027	0,1699	0,0149
201	6	4	15-LL	3,0101	0,4600	1,9256	1,1048	0,8073	0,0093
202	6	4	15-LL	3,2334	0,4258	2,9392	0,9881	1,0184	0,0085
203	6	7	12.5-LL	0,3696	0,6164	0,0938	0,3904	0,1204	0,0344
204	6	7	12.5-LL	0,2814	0,6482	0,0772	0,1416	0,1140	0,0068
205	6	7	12.5-LL	0,6034	2,0627	0,4054	0,2511	0,3262	0,0121
206	6	7	12.5-LL	0,1219	0,4965	0,0407	0,0479	0,1257	0,0275
207	6	7	12.5-LL	0,0868	0,3308	0,0541	0,1310	0,0447	0,0244
208	6	7	12.5-LL	0,4370	0,5414	0,0703	0,1563	0,0980	0,0111
209	6	7	12.5-LL	0,3632	1,0369	0,0408	0,1058	0,0274	0,0043
210	6	8	12.5-WS	0,5237	0,5762	0,1308	0,2012	0,1778	0,0287
211	6	8	12.5-WS	0,6216	0,7355	0,0704	0,2248	0,0783	0,0287
212	6	8	12.5-WS	0,1433	0,3483	3,6278	0,0822	0,0410	0,0283
213	6	8	12.5-WS	0,4995	0,9882	0,0519	0,1965	0,2486	0,0735
214	6	9	12.5-WS	0,3409	1,9342	0,0550	0,2692	0,1812	0,1288
215	6	9	12.5-WS	0,4408	3,3496	0,1444	0,3308	0,2870	0,0477
216	6	9	12.5-WS	0,1524	0,8006	0,2565	0,1171	0,0288	0,0502
217	6	9	12.5-WS	0,8168	0,7421	0,5334	0,1260	0,1388	0,0474
218	6	9	12.5-WS	0,8583	0,5154	0,3420	0,0795	0,0665	0,0250
219	6	9	12.5-WS	0,2897	0,4336	2,6981	0,1583	0,0665	0,0332
220	6	10	12.5-LL	0,5110	0,1829	0,4384	0,0318	0,0189	0,0271
221	6	10	12.5-LL	0,0501	0,3293	3,3225	0,1706	0,0425	0,0100
222	6	10	12.5-LL	0,3533	0,4064	3,7762	0,3187	0,0504	0,0419
223	6	10	12.5-LL	0,4385	0,9774	0,0642	1,4223	0,5353	0,0074
224	6	10	12.5-LL	0,7590	0,2298	0,0130	0,0462	0,0695	0,0095
225	7	1	15-LL	1,2496	0,2907	1,9052	0,4952	0,7266	0,0063
226	7	1	15-LL	0,9347	0,2506	0,9122	0,3802	0,3669	0,0230
227	7	1	15-LL	2,8873	0,2382	0,2909	0,3505	0,2360	0,0166
228	7	1	15-LL	0,8557	0,1451	1,4774	0,8740	0,6118	0,0193
229	7	1	15-LL	2,5757	0,2156	1,3242	0,8501	0,5003	0,0162
230	7	1	15-LL	2,3035	0,5497	1,0387	0,8863	0,3473	0,0270
231	7	1	15-LL	1,1764	0,1257	1,6918	1,3430	0,6287	0,0121
232	7	2	15-WS	1,4918	0,1788	1,0888	0,4392	0,4201	0,0071
233	7	2	15-WS	0,4907	0,2675	1,0220	0,3960	0,3975	0,0272
234	7	2	15-WS	0,8241	0,2968	0,4399	0,4854	0,1708	0,0169
235	7	2	15-WS	1,8175	0,1754	0,1663	0,2020	0,1197	0,0360
236	7	2	15-WS	2,6737	0,1543	1,5321	0,7607	0,5191	0,0198
237	7	2	15-WS	2,1783	0,2095	2,1943	0,7539	0,5772	0,0248
238	7	3	15-WS	0,7399	0,3157	2,8162	0,7680	0,4738	0,0163
239	7	3	15-WS	0,6830	0,1247	0,2636	0,4260	0,1457	0,0078
240	7	3	15-WS	0,2663	0,2565	0,4021	0,3521	0,1501	0,0271

241	7	3	15-WS	1,7064	0,3625	0,0998	0,3075	0,0290	0,0135
242	7	3	15-WS	1,1991	0,1566	0,5069	0,3366	0,2317	0,0122
243	7	3	15-WS	1,0593	0,4084	0,4790	0,5060	0,2464	0,0343
244	7	4	15-LL	1,4441	0,3265	1,4026	0,6772	0,6214	0,0175
245	7	4	15-LL	3,0551	0,5898	0,8593	0,8554	0,3584	0,0312
246	7	4	15-LL	2,0163	0,4921	1,6162	0,9817	0,4607	0,0188
247	7	4	15-LL	2,1949	0,2456	0,7958	0,6565	0,2960	0,0116
248	7	4	15-LL	0,7209	0,5983	0,5042	1,0043	0,1381	0,0601
249	7	7	12.5-LL	0,1837	1,0849	0,5071	0,6900	0,1462	0,0024
250	7	7	12.5-LL	0,4971	0,3493	0,6323	0,3421	0,6969	0,0091
251	7	7	12.5-LL	0,7926	0,9733	0,3524	0,1457	0,6887	0,0290
252	7	7	12.5-LL	0,2242	0,9917	0,7151	0,6136	0,6592	0,0248
253	7	7	12.5-LL	0,9699	0,2094	0,0627	0,3687	0,3108	0,0063
254	7	7	12.5-LL	0,7660	0,3536	0,0951	0,2753	0,3799	0,0172
255	7	7	12.5-LL	0,6637	0,1601	0,1982	0,2046	0,4877	0,0051
256	7	7	12.5-LL	1,0450	0,2150	0,1988	0,1567	0,4605	0,0079
257	7	8	12.5-WS	0,4421	0,8465	0,7155	0,6844	0,4053	0,0135
258	7	8	12.5-WS	0,8497	0,4159	0,3605	0,4344	0,5447	0,0124
259	7	8	12.5-WS	0,9886	0,2560	0,1444	0,2154	0,2152	0,0076
260	7	8	12.5-WS	0,4900	1,5908	0,9060	0,2795	1,7592	0,0765
261	7	8	12.5-WS	0,7297	0,8805	0,1216	0,6033	0,4022	0,0171
262	7	8	12.5-WS	0,7465	0,5347	0,0859	0,3516	0,4711	0,0385
263	7	9	12.5-WS	0,9831	0,7473	0,2153	0,2308	0,3911	0,0700
264	7	9	12.5-WS	0,3672	1,0727	0,6840	0,5979	0,4677	0,0235
265	7	9	12.5-WS	0,7770	0,2465	0,3095	0,3786	0,1918	0,0239
266	7	9	12.5-WS	0,3892	0,2649	0,2165	0,3032	0,1681	0,0088
267	7	9	12.5-WS	0,5400	2,6491	0,8007	0,5630	0,3401	0,0152
268	7	9	12.5-WS	0,6884	1,5572	0,7843	0,2378	0,5010	0,0217
269	7	10	12.5-LL	0,7426	0,5094	0,2290	0,4843	0,4907	0,0121
270	7	10	12.5-LL	0,5938	0,3242	0,4524	0,3562	0,3810	0,0049
271	7	10	12.5-LL	0,5493	0,5583	0,4146	0,8680	0,3745	0,0142
272	7	10	12.5-LL	0,7677	0,9810	0,3577	0,3212	0,6092	0,0183
273	8	1	15-LL	0,2599	0,2060	0,3558	0,3644	0,1636	0,0108
274	8	1	15-LL	2,1845	0,1477	0,0585	0,2616	0,0188	0,0146
275	8	1	15-LL		0,2109	0,7463	0,3561	0,3635	0,0097
276	8	1	15-LL						0,0136
277	8	1	15-LL	0,4604		0,5577	0,2879	0,2859	0,0075
278	8	2	15-WS	1,3556	0,1806	1,1243	0,4926	0,6496	0,0169
279	8	2	15-WS	1,7089	0,2041	1,1908	0,6314	0,2896	0,0164
280	8	2	15-WS	1,8405	0,2811	0,5331	0,5371	0,2862	0,0135
281	8	2	15-WS	0,2940	0,2524	0,3966	0,4711	0,1765	0,0118
282	8	2	15-WS	1,6997	0,1792	1,4707	0,2881	0,6550	0,0082
283	8	3	15-WS	1,4505	0,2215	0,5398	0,4167	0,3195	0,0267
284	8	3	15-WS	0,8145	0,1964	0,6545	0,6696	0,2575	0,0196
285	8	3	15-WS	1,9731	0,1597	1,3775	0,3556	0,6307	0,0390
286	8	3	15-WS	3,6389	0,2259	0,7039	0,8113	0,2774	0,0535
287	8	3	15-WS	0,1395	0,2836	2,1918	0,7467	0,7809	0,0110
288	8	3	15-WS	1,8113	0,3109	0,0293	0,1238	0,0145	0,0073
289	8	3	15-WS	0,2056	0,2275	1,9940	0,6236	0,9248	0,0342
290	8	4	15-LL	0,9434	0,1643	0,0332	0,1271	0,0176	0,0114
291	8	4	15-LL	0,7980	0,2997	0,5350	0,4221	0,2449	0,0099
292	8	4	15-LL	0,8802	0,2448	0,0414	0,1679	0,0322	0,0116
293	8	4	15-LL	0,5600	0,3201	0,2927	0,4305	0,1686	0,0265
294	8	4	15-LL	1,3867	0,1826	0,8054	0,5357	0,3420	0,0156
295	8	4	15-LL	1,0127	0,1930	0,2397	0,4517	0,1213	0,0071
296	8	4	15-LL	0,6125	0,2980	0,6152	0,3235	0,2779	0,0038
297	8	7	12.5-LL	0,6864	0,1263	0,3157	0,0613	0,2591	0,0173
298	8	7	12.5-LL	0,6196	0,2170	0,0718	0,2541	0,3191	0,0056
299	8	7	12.5-LL	0,7259	0,2085	0,6235	0,4001	0,3383	0,0190
300	8	7	12.5-LL	0,7518	0,2238	0,0565	0,3372	0,5644	0,0098

301	8	7	12.5-LL	0,5895	0,1677	0,3254	0,1563	0,1120	0,0254
302	8	7	12.5-LL	0,7355	0,8745	1,3688	0,3567	1,0464	0,0034
303	8	8	12.5-WS	0,4945	0,2971	0,0336	0,1361	0,2304	0,0159
304	8	8	12.5-WS	0,9267	0,2042	0,3829	0,2890	0,6861	0,0224
305	8	8	12.5-WS	0,6947	0,1972	0,2179	0,2452	0,2887	0,0090
306	8	8	12.5-WS	0,7615	0,2767	1,4301	0,2641	0,3232	0,0111
307	8	9	12.5-WS	0,8233	0,4113	0,2486	0,2273	0,5033	0,0144
308	8	9	12.5-WS	0,3865	0,3222	0,2220	0,3946	0,2519	0,0222
309	8	9	12.5-WS	0,6154	0,3239	0,2788	0,2702	0,2516	0,0158
310	8	9	12.5-WS	0,3314	0,2007	0,1976	0,2229	0,1487	0,0016
311	8	9	12.5-WS	0,2622	0,9700	0,0826	0,4361	0,0539	0,0223
312	8	9	12.5-WS	0,8639	0,4408	0,2377	0,3311	0,7332	0,0145
313	8	9	12.5-WS	0,4962	0,3036	0,3408	0,3748	0,3509	0,0074
314	8	9	12.5-WS	0,6061	0,5062	0,4137	0,2999	0,3292	0,0115
315	8	10	12.5-LL	0,7635	0,2418	0,0088	0,3627	0,5135	0,0093
316	8	10	12.5-LL	0,8132	0,3577	0,0277	0,5664	0,5072	0,0050
317	8	10	12.5-LL	0,4159	0,3562	0,0126	0,2657	0,1159	
318	8	10	12.5-LL	0,8388	0,3156	0,1710	0,3989	0,3417	0,0087
319	8	10	12.5-LL	0,4472	0,2768	0,7124	0,3535	0,1702	0,0080
320	8	10	12.5-LL	0,6056	0,3112	0,1140	0,4966	0,4027	0,0056

Appendix II. Overview of standard curves.

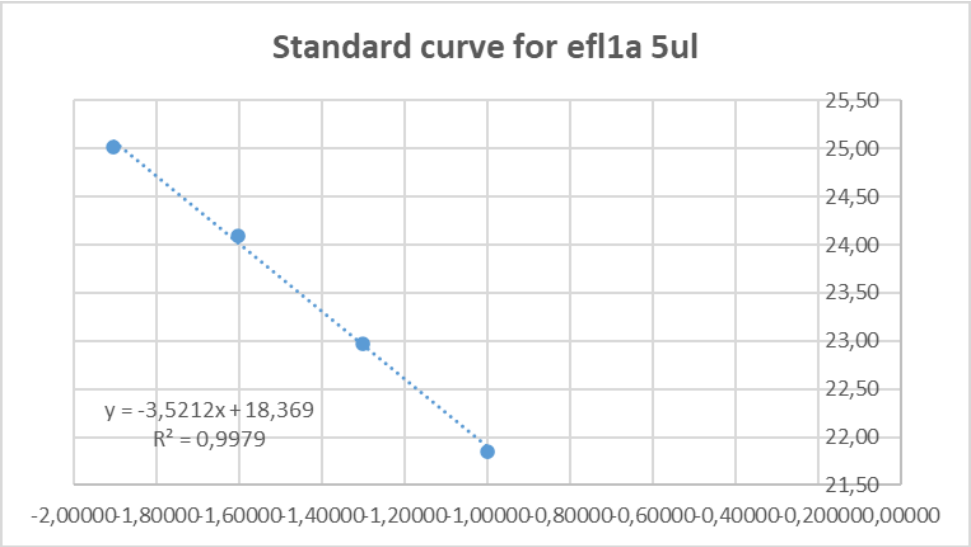

Figure I. Standard curve for elf-1α.

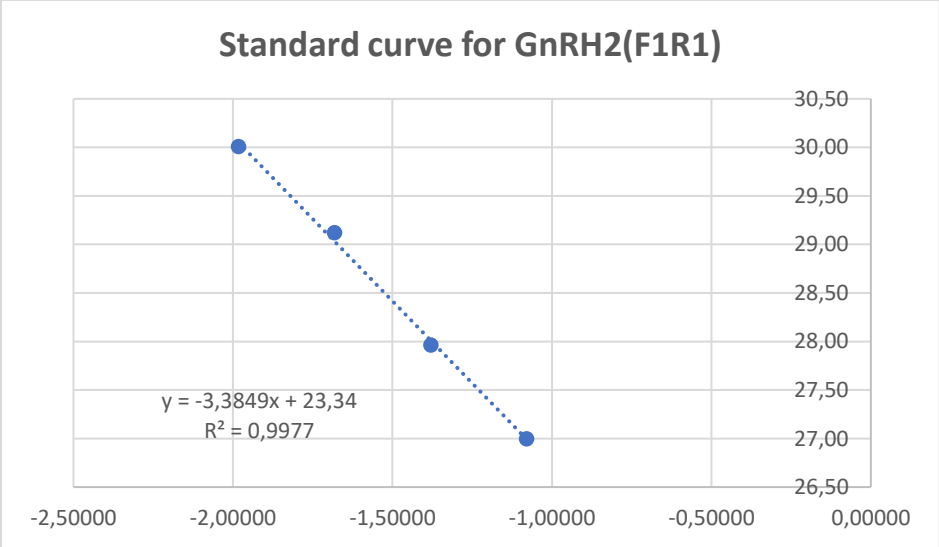

Figure II. Standard curve for GnRH2.

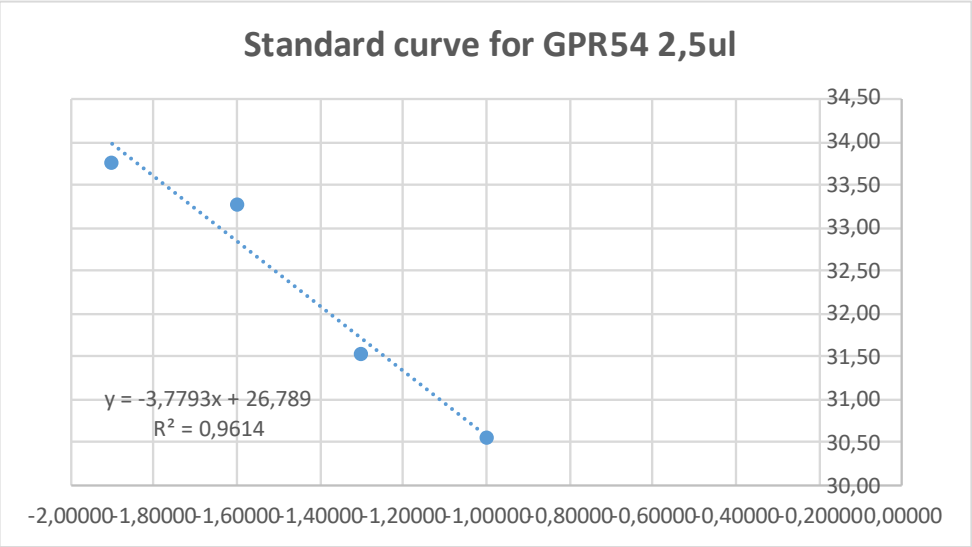

Figure III. Standard curve for GPR54.

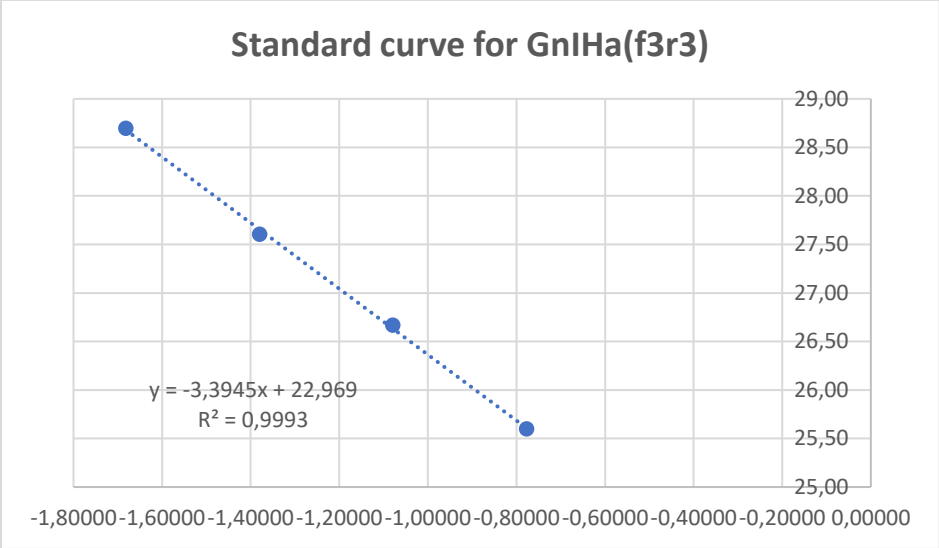

Figure IV. Standard curve for GnIHa.

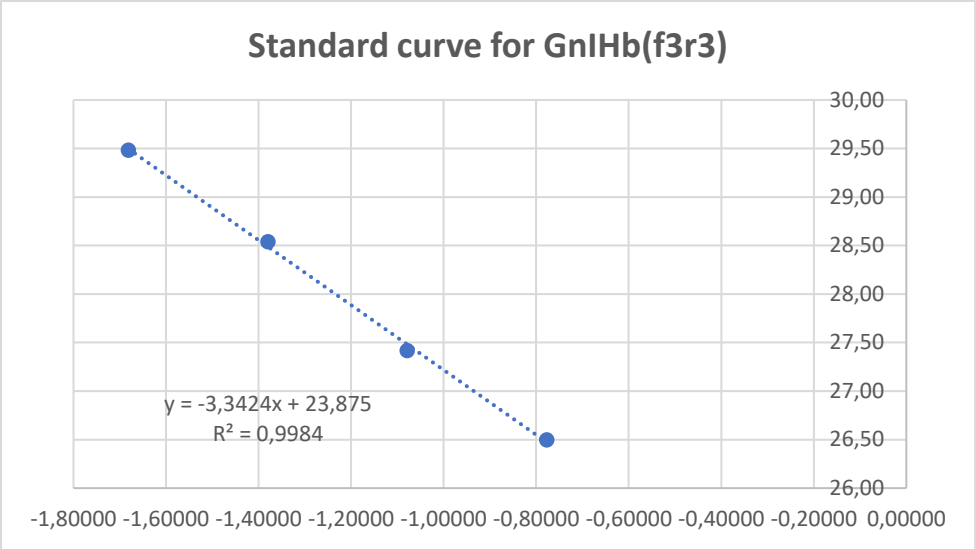

Figure V. Standard curve for GnIHb.

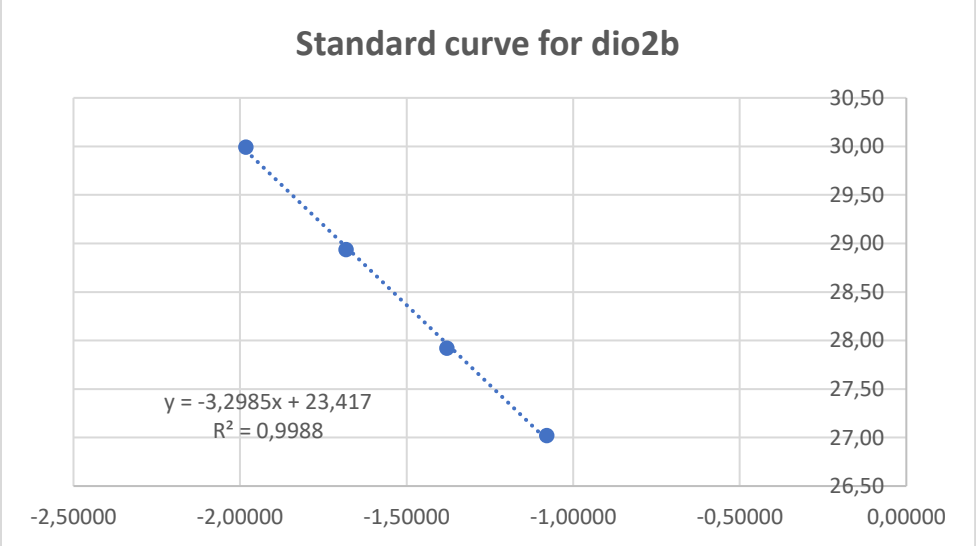

Figure VI. Standard curve for dio2b.

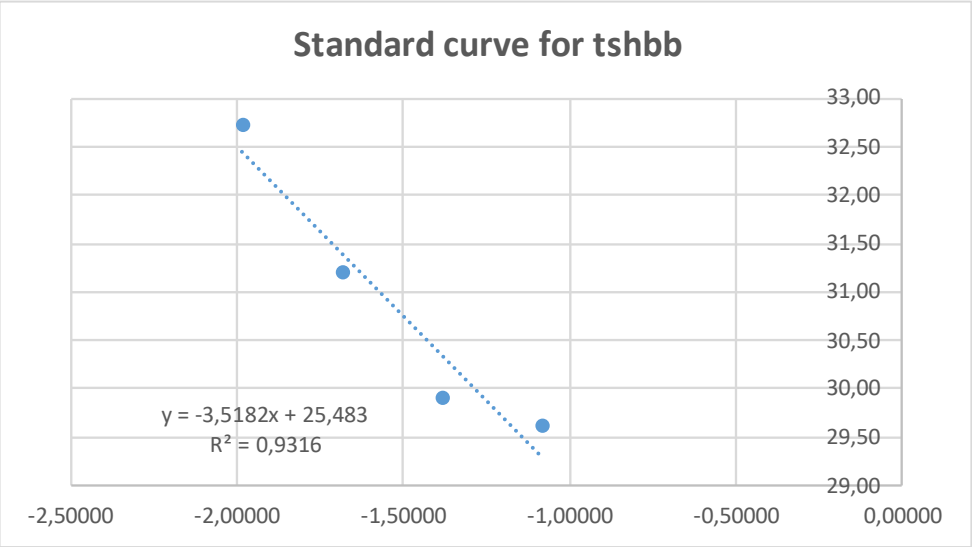

Figure VI. Standard curve for *tshβb*.