The effects of photoperiod: Investigating the role of tshβb in early maturation in male Atlantic salmon (*Salmo salar*) at three different body sizes during photoperiod treatments during freshwater stages of development

Thesis for the degree

Master of Science in Aquaculture Biology

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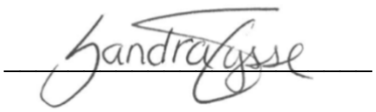
Preface

This thesis is written as the final work to complete my Master of Science degree in Aquaculture Biology at the University of Bergen (UiB). The work has been carried out at the Department of Biological science (BIO) and the Integrative Fish Biology Group (IFB) in the Norwegian Research Center AS (NORCE, Bergen). This experiment was funded by NORCE Research, with the contribution of projects SAFT I (Tidlig Modning hos Postsmolt fra RAS anlegg; 286597) and KABIS (Kapasistetsløft for Bærekraft og Innovativ Sjømatproduksjon; 280782), both projects founded by the Research Concil of Norway.



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S.T

Abstract

Early post-smolt maturation of male Atlantic salmon (*Salmo salar*) has become a growing concern in intensive land-based production systems causing enormous economic and welfare issues. New production protocols in intensive aquaculture facilities, such as manipulation of photoperiod regimes, temperature, and unlimited access to feed have been implemented in the industry to optimize growth. The aim of this research project was to use the well-established photoperiodic control of parr-smolt transformation as a model system to determine the roles of the tshβb gene and its involvement in the control of seasonal variation in salmon. Three different body sizes of Atlantic salmon (70, 110, and 180) were introduced to a 5-week winter signal period (WS) (LD12:12) to induce smoltification, and one group was introduced to continuous light (CL) (LD24) throughout the experiment. The four groups were maintained under a constant temperature of 12.5°C throughout the experiment. Body growth and condition factor (K) were used as an indicator of growth, and the ratio of gonadosomatic index (GSI) to follicle-stimulating hormone (FSH) was compared to determine maturation levels in the four experimental groups. The role of brain-pituitary-tshβb expression and its potential influence on maturation in Atlantic salmon was studied and investigated whether tshβb is involved in the BPG axis in the same way as PT-TSH in mammals and birds. The salmon in all experimental groups had a stable growth rate, and the condition factor showed that the 70 and 110 groups were smoltified, while the 180 and CL groups did not appear to complete smoltification and began to show signs of maturation. Photoperiod was the key regulator of pituitary tshβb expression levels that had a significant increase in the three body sizes (70, 110, and 180) after the introduction of WS. No tshβb peak was observed in the CL group, which may indicate that a continuous light regime may disrupt the potential physiology of developing Atlantic salmon in the freshwater stage under intensive aquaculture environments. The results induce that the introduction of light can cause a change in tshβb expression and that tshβb does not activate the BPG axis and therefore is not linked to maturation in Atlantic salmon as PT-TSH is in mammals and birds. No correlation between the peaks of tshβb was observed after the winter signal and the increase in GSI levels in this study. In mammals and birds, GSI levels and PT-TSH increases are closely correlated, whereas in salmon these parameters appear to be disconnected, and the tshβb peak most likely does not activate FSH for gonadal maturation. Based on our K values, our results suggest that a body size between 70g-110g may be a promising size to achieve healthy smoltification in Atlantic salmon.

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Glossary

Salmo salar	–	Atlantic salmon
Smoltification	–	Parr-smolt transformation

Abbreviations list

BPG	–	Brain-Pituitary-Gonad
cDNA	–	Complementary DNA
CL	–	Continuous light (CL24:0)
Cq	–	Quantification cycles
DIO2	–	Iodothyronine Deiodinase 2
DNA	–	Deoxyriboncleic acid
Ef1α	–	Elongation factor 1-alfa gene
FRW	–	Forward primer
FSH	–	Follicle-Stimulating Hormone
FTS	-	Flow-through systems
FW	–	Freshwater
GnIH	–	Gonadotropin-Inhibiting Hormone
GnRH	–	Gonadotropin-Releasing Hormone
GSI	–	Gonadosomatic Index (%)
K	–	Condition factor
Kiss	–	Kisspeptin
LD	–	Light, Darkness
LH	–	Luteinizing Hormone
LL	–	Long light
mRNA	–	Messenger RNA
NTC	–	No template control

PCR	–	Polymerase Chain Reaction
PN	–	Pars nervosa
PT	–	Pars tuberalis
qPCR	–	Quantitative Polymerase Chain Reaction
RAS	–	Recirculating Aquaculture System
REV	–	Reverse primer
RNA	–	Ribonucleic acid
SW	–	Seawater
T3	–	triiodothyronine
T4	–	thyroxine
TRH	–	Thyrotropin-releasing hormone
TSH	–	Thyroid-stimulating hormone
WS	–	Winter signal (LD12:12)

1. Introduction

1.1. History

Open cage farming of Atlantic salmon (*Salmo salar* Linnaeus, 1758) continues to expand along the coasts of the world, but growing concern in the Atlantic salmon industry related to early post-smolt maturation is a factor slowing the industry's expansion (Good & Davidson, 2016; Naylor et al., 2005). Atlantic salmon production in Norway was established in the early 1970s (Taranger et al., 2015), and has become the largest producer of farmed salmon in the world and the fourth largest export commodity in Norway after oil, gas, and metals (Hersoug, 2021; Liu et al., 2011). Already in 1989, the production of farmed salmon in Norway had increased to 118,000 tonnes, which in that year was close to 70% of the world's total production (Tilseth et al., 1991). For the aquaculture industry to continue to expand, it is crucial to address and resolve issues to meet the global demand for seafood products in a sustainable manner (Good & Davidson, 2016). The production of salmon in Norway increased between 10 and 20 percent annually in recent decades until 2012 when production stagnated (Sommerset et al., 2019). The stagnation of salmon production in Norway is due to several factors within environmental, production, and biological conditions and the potential production plateau in Norway is a severe concern for the industry.

The challenges associated with production have become limiting factors for the further development of the Norwegian aquaculture sector. To mitigate the current challenges with open cage farming, reducing the time fish spend in the open sea may be a key factor. This reduction can reduce the exposure period to disease outbreaks and salmon lice infestation and possibly upgrade the production cycle for Atlantic salmon. In addition, earlier findings have shown that larger smolt is more robust and able to handle the transfer to open cages in seawater (Handeland & Stefansson, 2001; Ytrestøyl et al., 2015). To solve these challenges and to increase production and profitability, the salmon industry is searching for alternative production systems (Liu et al., 2016; Terjesen et al., 2013; Weston, 2013). These challenges include intensive post-smolt production in recirculating aquaculture systems (RAS) and flow-through facilities (Dalsgaard et al., 2013).

During intensive post-smolt production in RAS and flow-through systems, the salmon is manipulated by various environmental parameters such as photoperiod regimes and high temperature to optimize production through increased growth and to be able to achieve the best

and relevant market sizes in the shortest possible time (Imsland et al., 2014; Martinez et al., 2023). Manipulating photoperiod and temperature has resulted in a growing concern in the salmon industry with the phenomenon of early post-smolt maturation and has become one of the most extensive biological challenges for the industry. "Post-smolt" is salmon that has undergone smoltification and transferred to seawater (Bjørndal & Tusvik, 2017; CtrlAQUA, 2023). In Norway, the definition of "post-smolt" is also often used to describe a large smolt of (Bjørndal & Tusvik, 2017) or salmon that are above a size range that is typical for a smolt (80-100 g) reared in land-based facilities up to 1000 g (CtrlAQUA, 2023).

1.2. Atlantic salmon life cycle

Atlantic salmon (*Salmo salar*) is known for their anadromous life history with life stages in both freshwater and seawater (Hansen & Quinn, 1998; McCormick et al., 2013). The salmon hatches in freshwater (FW) and then migrates to seawater (SW) as smolts to grow and feed before returning to the river and moving upstream to spawn in autumn (Thorstad et al., 2011) (Figur 1.2.). Mature salmon spawn in the river and sexual maturation in females are characterized by the development of ripe eggs (roe) in the ovaries and the formation of a redd (nest) in the gravel of a stream or river (Wallus & Simon, 2008). As the salmon approach the spawning grounds, the females will become increasingly plump, and their coloration will change to a deep red (Mobley et al., 2021). In males, sexual maturation is characterized by the development of ripe sperm in the testes and the formation of a hooked jaw, known as a kype (Aksnes et al., 1986; Witten & Hall, 2003). The males will also become increasingly plump, and their coloration will change to a deep red (Klemetsen et al., 2003). After spawing the female buries her eggs in the dark between gravel and stones until hatching in the spring (Jonsson & Jonsson, 2011; Mobley et al., 2021). The fertilized eggs hatch as alevins, which are small yolk sac fry that remain under the gravel sustained by a yolk sac for nutrition (Skoglund & Barlaup, 2006). When the yolk sac is consumed, they develop morphologically and physiologically and swim up to the surface to start their first feeding in the summer months as fry (Mobley et al., 2021). The fry develops into a juvenile salmon parr during the autumn, which is characterized by parr marks (vertical stripes along the sides of the fish) and spotted camouflage (McCormick et al., 2013). The parr lives in fresh water for at least one year (depending on environmental and growth conditions, and genetic factors) before migrating into the North Atlantic Ocean as smolt in early spring/summer (Mobley et al., 2021).

To become a smolt, the parr must undergo a process called smoltification, or parr-smolt transformation which is a series of morphological (e.g. reduced condition factor (K) and change in skin color (silvering)), physiological (e.g. such as shifts in osmoregulatory capacity and hormonal changes) and behaviors (e.g. negative rheotaxis, reduced territoriality, higher salinity, shoal behavior to avoid predators) processes that enable salmon to transition from FW to SW (Björnsson et al., 2012; Hoar, 1988; McCormick et al., 2013; McCormick & Saunders, 1987; Ytrestøyl et al., 2023). An indicator of smoltification is a reduction in condition factor (K), which is the ratio between weight and length (Björnsson et al., 1989; Mobley et al., 2021). Smoltification appears to be genetically determined (Bailey et al., 1980). Nevertheless, the timing and outcome are triggered and regulated by seasonal changes such as temperature (Johnston & Saunders, 1981) and the natural photoperiod, which includes short days in winter followed by longer day lengths in spring (McCormick & Saunders, 1987; Ytrestøyl et al., 2023). After smoltification, the Atlantic salmon spend several years growing until the salmon have sufficient energy stored to swim upstream to their native river and begin the spawning process (Mobley et al., 2021).

However, some males can develop mature gonads and participate in reproduction at the parr stage and avoid migration to the sea (Fleming, 1998; Mobley et al., 2021). The mature male parrs that mature earlier have required enough energy and are probably the most dominant parr in the river (Mobley et al., 2021). They eat the most, get enough resources to mature, and represent an alternative male reproductive strategy (Klemetsen et al., 2003). Females mature during or after smoltification but do not achieve reproductive capacity until after the fish have migrated to the sea (Fjelldal et al., 2018; Mobley et al., 2021). Nevertheless, the salmon growth capacity in the wild is determined by seasonal variations in temperature, photoperiod, and access to feed, which means that only a percentage of the males mature early (Thorpe, 1994). Under intensive aquaculture conditions such as RAS and flow-through systems, Atlantic salmon are light-manipulated and temperature-regulated, and feed access is unlimited (Martinez et al., 2023). These conditions represent a highly stimulating environment for growth and development, resulting in an increasing trend of early post-smolt maturation that has become a growing problem in the aquaculture industry (Good & Davidson, 2016; Martinez et al., 2023).

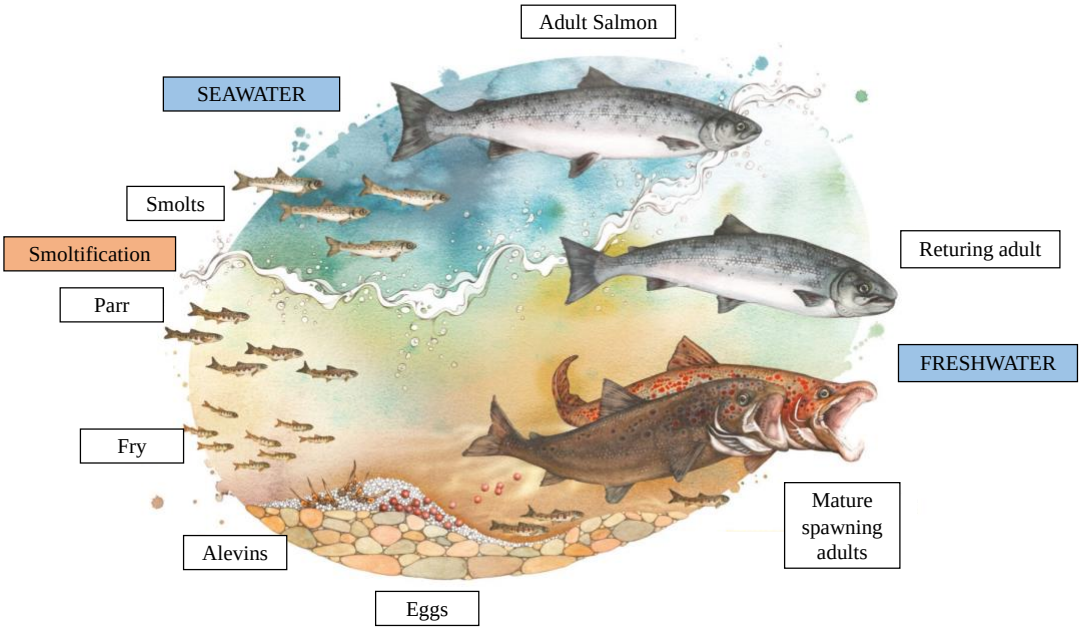

Figure 1.2. Atlantic salmon life cycle. The early stages of the fish's life take place in freshwater. Eggs (roe) in the freshwater hatch in the spring and develop into pairs. Pairs go through smoltification, and downstream migration to seawater takes place. The growth phase occurs in seawater where the fish develop into adults before returning to their original breeding area to spawn. The figure is slightly modified and borrowed from the State of North Atlantic Salmon Report (NASCO, 2019).

1.3. Recirculating Aquaculture Systems (RAS) and flow-through systems

Rearing of post-smolts up to a larger size is a strategy within RAS systems that has gained increasing interest in Norway in recent years (Fossmark et al., 2021). Land-based RAS facilities are a closed indoor aquaculture production system that has integrated control over the production parameters, allowing partial recycling of water through mechanical and biological treatment (Dalsgaard et al., 2013). RAS systems are increasingly using water recycling technologies that treat and recycle between 95-99.9% of the water (Bregnballe, 2015; CtrlAQUA, 2023).

Flow-through systems (FTS) are a more traditional and still dominantly used production method in Norway (Kolarevic et al., 2014). In contrast to RAS, flow-through has a high consumption of new water, which uses ambient water from a nearby water source (typically rivers), which is filtered coarsely before flowing through the hatchery systems and then back into the natural environment (Bergheim et al., 2009; Kolarevic et al., 2014).

Modern technology has enabled an expanded production of smolt on land which includes the early seawater phase. Production of post-smolt can potentially provide a more robust fish that is more adapted to seawater than smolt and can provide increased survival during the growth phase of production (Ytrestøyl et al., 2020). The production of post-smolt is growing and new production protocols that use continuous light (CL) are being implemented in the industry (Ytrestøyl et al., 2023). RAS facilities for post-smolt production provide high control over variables such as light regime (photoperiod, light intensity, and quality) and, among other things, water temperature (Summerfelt & Vinci, 2008). In flow-through systems, it has also become common to manipulate and optimize these parameters in combination with 24-hour intensive feeding regimes to maximize growth rate (Bergheim et al., 2009; Dalsgaard et al., 2013; Good & Davidson, 2016; Summerfelt & Vinci, 2008).

Manipulation of the photoperiod regime and water temperature has resulted in an increased incidence of early sexual maturation in farmed salmon at the post-smolt stage under intensive conditions (Imsland et al., 2014; Martinez et al., 2023). Early maturation is observed primarily in males, as the males require a lower physiological investment for testis maturation than for egg production (Good & Davidson, 2016; Myers et al., 1986; Saunders et al., 1982). The challenges associated with male maturation can cause significant biological and economic losses to the salmon industry, compromising the economic viability of RAS and flow-through facilities while also reducing fish welfare (Aksnes et al., 1986; Johnston & Saunders, 1981; McClure et al., 2007). The significant instigation of early sexual maturation is why we investigate and try and solve the problems associated with post-smolt maturation.

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

Sexual maturation is controlled by a complex interplay of several genetic, environmental, and internal factors (Good & Davidson, 2016) and in all cases, sexual maturation is initiated with activation of the brain-pituitary-gonadal (BPG) axis (Schulz et al., 2010; Taranger et al., 2010). Recent decades have discovered how the BPG axis is regulated in birds and mammals and to achieve sexual maturation several endocrine activities take place along the BPG axis (Taranger et al., 2010).

In long-day mammalian breeders, when exposed to increasing day length, the BPG axis is activated through it a complex interaction of neuropeptides which begins by stimulating the expression of the thyroid-stimulating hormone (TSH) in the pars tuberalis (PT) (Aizawa et al.,

2007; Yasuo et al., 2010). Pars-tuberalis TSH activates the hypothalamus and plays a key role in sexual development in mammals and other vertebrates (Nakao et al., 2008). TSH consists of two subunits: (1) an α-subunit and (2) a β-subunit (Yen, 2001). TSH shares the same α-subunit as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the β-subunit (tshβ) is unique to TSH and is hormone-specific (Yen, 2001).

Amphibians, birds, and mammals are found to have only a single TSH (a single tshβ gene), while recent studies show that in some fish the presence of duplicated tshβ paralogs has been discovered (Fleming et al., 2019; Maugars et al., 2014). In some teleost fish, two paralogs of tshβ exist (tshβa and tshβb) which is the result of a duplication of the genome that has occurred early in the development of the fish (3R event) (Irachi et al., 2021). In Atlantic salmon, a tshβ peak has been observed during smoltification, which suggests that tshβ may be an important regulator of life history transitions during smoltification or during sexual maturation (Fleming et al., 2019). Fleming et al. (2019) claim that the conserved regions of the gene tshβ are homologous to PT-TSH in mammals and birds that regulate maturation. In Atlantic salmon, tshβ expression has been shown to be stimulated by increased day length (Irachi et al., 2021). Despite knowing that tshβ expression is related to day length, we are unaware of how the photoperiodic signaling is transcribed by the BPG axis.

In mammals, the eyes are believed to be the only photoreceptor organ, and melatonin secretion triggers an endocrine signal that communicates photoperiod information to the pars tuberalis (PT) to regulate thyroid-stimulating hormone (TSH) in the pituitary (Nakane & Yoshimura, 2014). Melatonin is produced primarily from the pineal gland in the brain and is released in response to darkness (Dubocovich, 1988). Melatonin affects the biological and physiological rhythms associated with reproduction and the release of hormones (Arnao & Hernández-Ruiz, 2006). Birds perceive light via deep-brain photoreceptors, and the long day (LD) stimulus induces TSH from the pars tuberalis in the pituitary gland and causes local thyroid hormone activation within the mediobasal hypothalamus (MBH) (Ikegami et al., 2014; Yoshimura, 2013). In mammals, it has been established that there is a photoperiod-driven seasonal clock that regulates the synthesis and release of melatonin from the nearby pineal gland (Foulkes et al., 1997; Hastings & Herzog, 2004; Irachi et al., 2021; Klein, 1985). In mammals, melatonin is needed to activate TSH-producing cells of the pars tuberalis (PT) (PT-TSH) which cause a stimulatory pathway to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland, which further causing sexual maturation (Dardente et al., 2010;

Ikegami et al., 2014). Birds are directly stimulated by light stimuli that affect the deep brain receptors to have increased PT-TSH without the need for melatonin, and the pineal gland is thus not involved in the same way as in mammals (Ikegami & Yoshimura, 2012).

TSH-producing cells of the pars tuberalis (PT) show distinct characteristics that differ from the pars distalis (PD) (Ikegami et al., 2014). PD stimulates the thyroid gland to produce thyroid hormone (TH) and is regulated by negative feedback from thyroid hormones stimulated by TRH (Ertek, 2021). Pars tuberalis-derived TSH (PT-TSH) regulates seasonality by controlling the hypothalamus (Ikegami et al., 2014; Ikegami & Yoshimura, 2016). A high density of melatonin binding has been observed in PT, suggesting that PT-TSH may be a regulatory hub for photoperiodic signaling and seasonal reproduction in mammals (Chi et al., 2017; Hanon et al., 2008; Nakao et al., 2008) However, fish differ from mammals and birds in that the PT does not process an anatomically distinct (Nakane et al., 2013), and the hypothalamus has been considered by researchers to be the regulatory name for photoperiodism in fish (Chi et al., 2017).

PT-TSH is released from the pituitary PT and stimulates activation of the enzyme iodothyronine deiodinase 2 (DIO2) in the hypothalamus (Fleming et al., 2019; Ikegami et al., 2014; Nakao et al., 2008). DIO2 catalyzes the conversion of thyroxine (T4), which is the main thyroid hormone and is considered the "storage form" of thyroid hormone (Mansourian, 2011), to triiodothyronine (T3), which is the biologically active form of thyroid hormone (Yoshimura et al., 2003). T3 stimulates hypothalamic signaling and activates TH-regulated brain functions which provide signaling by increased gonadotropin-releasing hormone (GnRH) that further stimulates the onset of puberty and reproduction (Irachi et al., 2021).

In mammals, birds, and fish, the neurohypophysis kisspeptin/kisspeptin receptor (Kiss1/Kiss-r) system regulates the release of gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus (Ikegami & Yoshimura, 2016). The release of GnRH occurs through the mediation of internal and external signals to regulatory inputs for the activation of pituitary gonadotropin release (Weltzien et al., 2004). In birds, GnRH has been exhibited to be located in the same neurons found in vertebrates, suggesting a direct photoreceptive regulation of the onset of sexual maturation (Horne et al., 2023). The hypothalamus releases GnRH (Taranger et al., 2010; Weltzien et al., 2004) and stimulates the pituitary gland to release follicle-

stimulating hormone (FSH) and luteinizing hormone (LH) subsequently causing gonad development (Ciani et al., 2020; Whitlock et al., 2019).

1.5. Variation in the timing of Atlantic salmon maturation

Atlantic salmon undergo sexual maturation when it becomes adults, and to achieve maturation the fish must go through a series of endocrine activities along the brain-pituitary-gonadal (BPG) axis (Taranger et al., 2010), which stimulates by the photoperiod (Jonsson & Jonsson, 2011). In Atlantic salmon, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are released and act on the gonads to regulate the production of sex hormones such as estrogen and testosterone (Ciani et al., 2020; Schulz et al., 2010; Taranger et al., 2010). FSH stimulates the growth and development of ovarian follicles that produce estrogen when they mature in female salmon, while in male salmon FSH stimulates the development of sperm cells (Ciani et al., 2020; Taranger et al., 2010). FSH is correlated to the onset of sexual maturation and the timing of spawning, which is related to changes in gonadal somatic index (GSI) levels (Pino Martinez et al., 2021). The relationship between GSI and FSH in Atlantic salmon is central to sexual maturation and reproduction, and low levels of GSI and FSH reflect an immature salmon (Martinez, 2021). These parameters can therefore contribute to insight into the timing and regulation of spawning. LH occurs later and triggers ovulation and releases the egg in female salmon, while in male salmon it stimulates the production of testosterone in the testicles (Ciani et al., 2020; Taranger et al., 2010). The levels of these hormones vary depending on the stage of the reproductive cycle and the physiological state of the fish. When gonadotropins are produced, sex steroids together with growth factors send positive or negative feedback to the brain or pituitary gland, which stimulates or inhibits production (Taranger et al., 2010). The timing and levels of these hormones can vary depending on the salmon species, population, size, age, and environmental factors such as water temperature and food availability (Mobley et al., 2021; Nett et al., 2002; Taranger et al., 2010). Maturation is initiated by changes in environmental factors, such as decreasing water temperature and decreasing day length (Hansen et al., 1992), which signal the salmon to begin their migration back to their home stream or river to spawn (Mobley et al., 2021; Stefansson et al., 2020). Genetics, feeding ability, and body size may also play a role in the maturation of male parrs, as bulkier individuals may mature at a faster rate or larger size than smaller individuals (Mobley et al., 2021; Thorpe, 1994). Which brain regions are involved in sexual maturation in Atlantic salmon and how light information affects the neuroendocrine system is still not fully understood in teleosts (Horne et al., 2023).

1.5.1 Gonadosomatic index (%)

To quantify the degree to which the salmon is maturing the gonadosomatic index (GSI) is calculated. GSI is a relationship between the relative size of the gonads (ovaries or testes) and the total body weight of the fish (Devlaming et al., 1982). The index is often used as a measure of the reproductive activity of fish and other aquatic animals (Good & Davidson, 2016). The GSI level in the salmon increases rapidly from less than 0.1% up to between 5% and 10% when the salmon is smolt until it has become mature and ready to spawn (Taranger et al., 2010). The GSI levels used in this study are based on recent research in the laboratory at the Department of Biological science (BIO), and an indication of immature salmon is at a GSI $\leq 0.06\%$, early stage of maturation $0.06 < \text{GSI} \leq 0.1\%$, maturing $0.1 < \text{GSI} \leq 1\%$ and mature salmon at $1 < \text{GSI}$ (Martinez, 2021). GSI is calculated by dividing the weight of the gonads by the total weight of the fish and multiplying by 100 (Oppedal et al., 2003). A high GSI value indicates that the gonads are relatively large, and that the organism is probably in a reproductive state (Peterson & Harmon, 2005; Taranger et al., 2010). A low GSI value implies that the gonads are relatively small, and the organism is not in a reproductive condition (Volkoff & London, 2018).

1.5.2. The effect of temperature on sexual maturation

Atlantic salmon are ectothermic poikilothermic, meaning their body temperature adapts to their environment, and all biological processes in the fish are regulated by the ambient temperature (Angilletta Jr et al., 2002; Volkoff & Rønnestad, 2020). The effect of water temperature acts as a rate-controlling factor on the physiological responses to seasonal changes in photoperiod (Hoar, 1988; Imsland et al., 2014). Biological processes such as increased growth rate and appetite occur faster at higher water temperatures as metabolism boosts (Schmidt-Nielsen, 1997). Warmer temperatures can cause an increase in the production of hormones such as estrogen and testosterone, which stimulate the growth and development of reproductive organs leading to maturation (Mobley et al., 2021; Thyholdt, 2014). In addition, temperature also affects the timing of spawning (Taranger & Hansen, 1993; Taranger et al., 2003). Warmer water temperatures can cause the salmon to spawn earlier, while colder temperatures can cause the fish to spawn later (Taranger & Hansen, 1993; Taranger et al., 2003). This results in the timing of the salmon that run in a particular location can change from year to year depending on the water temperature (Graham et al., 1996; Rimmer & Paim, 1990).

1.5.3. The effect of photoperiod on sexual maturation

Photoperiod and temperature are the primary regulatory factors in a smoltification process (Byrne et al., 2004). Changes in the photoperiod (length of daylight) are highly reliable seasonal signals in the environment, and organisms use changes in the photoperiod as a calendar to adopt, among other things, their physiology, behavior, reproduction, migration, hibernation, and molting (Ikegami & Yoshimura, 2016). Salmon are known to have photoperiod-dependent developmental pathways, and changes in photoperiod be the key signal for the timing of reproduction, seaward migration, and the onset of spawning behavior (Beckman et al., 2007; Hoar, 1988; McCormick et al., 2002) aquaculture facilities, photoperiod regimes are manipulated, and the timing of maturation is therefore changed (Taranger et al., 2010; Taranger et al., 1998). Exposing the salmon to a long light regime (LL) between winter and early spring has been shown to inhibit maturation in the salmon (Oppedal et al., 1997; Taranger et al., 1998). Nevertheless, research shows that maturation is not only regulated by photoperiod in fish, as maturation can also occur under continuous light (CL) (Good & Davidson, 2016). This is different from other vertebrate species as photoperiod must be present for maturation to occur (Nicholls et al., 1988). The effect of constant temperatures and continuous light (CL) and their possible interaction on, among other things, early sexual maturation is still uncertain (Imsland et al., 2014).

How the changes in the photoperiod are recorded and signaled in Atlantic salmon is still poorly understood (Irachi et al., 2021), but the input pathway to the neuroendocrine output pathway has been suggested to be in the hypothalamus, which is the center of photoperiodic regulation, agreeing with the conserved function of this region throughout vertebrate evolution (Horne et al., 2023). Hypothalamus responds to changes in day length and releases GnRH (Mobley et al., 2021). In Atlantic salmon, two paralogs of DIO2 have been found: (1) DIO2a and (2) DIO2b which are present in the hypothalamus (Irachi et al., 2021). TSH and DIO2b increase in transcription upon exposure to increased day length (Lorgen et al., 2015), which means that it may play a central role in the seasonal variation of fish (Chi et al., 2017; Lorgen et al., 2015; Nakane et al., 2013).

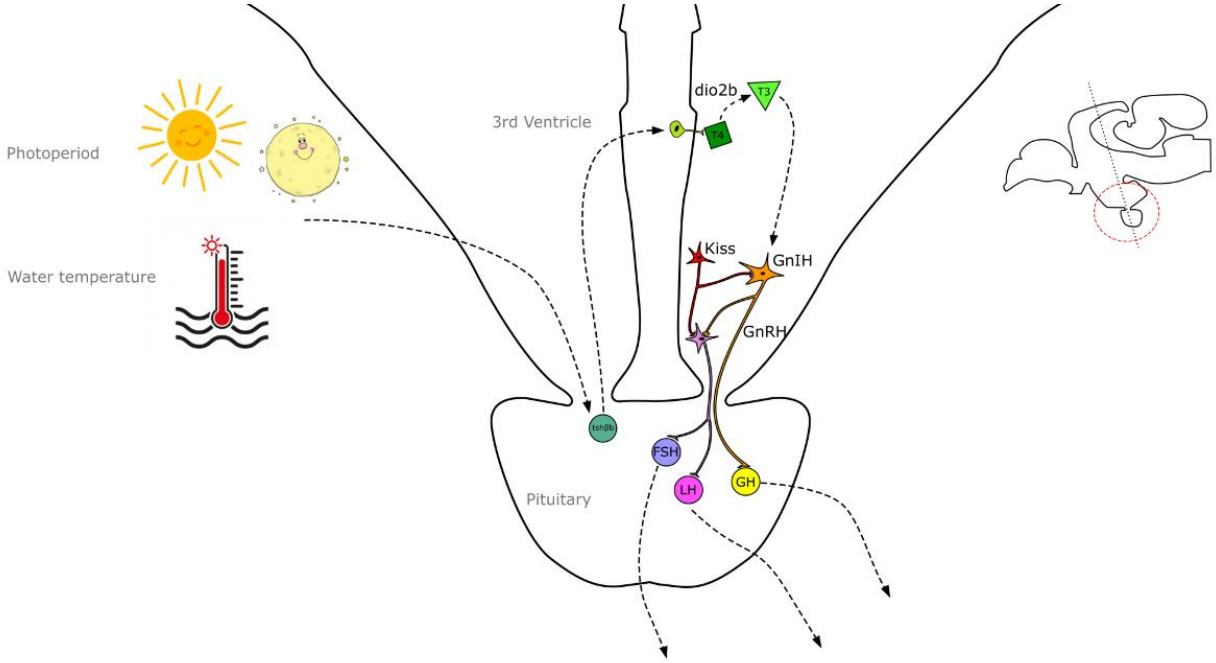

Figure 1.5. Illustration of the BPG signaling pathway in Atlantic salmon.
When exposed to increased day length and high water temperature, the gene tshβb is expressed by the TSH cells in the pituitary gland, which further activates DIO2b, which is present in the hypothalamus (Lorgen et al., 2015). During smoltification and downstream migration, an increase in DIO2b has been reported to catalyze the conversion of the thyroid hormone T4 to the biologically active form T3 (Fleming et al., 2019). T3 stimulates kisspeptin neurons which then stimulate gonadotropin-releasing hormone (GnRH) from the hypothalamus to produce the gonadotropic hormones luteinizing hormone (LH), follicle-stimulating hormone (FSH), and plasma growth hormone (GH) from the pituitary gland (Dickey & Swanson, 1998; Ertek, 2021; Tsutsui et al., 2010). Gonadotropin-inhibiting hormone (GnIH) acts as an inhibitory hormone and can interfere with the release of gonadotropin-releasing hormone (GnRH) (Tsutsui et al., 2010; Tsutsui et al., 2012).

The exact role and mechanism of tshβb in Atlantic salmon remain uncertain but tshβb involvement in smoltification and downstream migration behavior appears to be stimulated by brain DIO2b and T3 production, which is the same for PT-TSH involvement in seasonal regulation of life cycle characteristics in birds and mammals (Fleming et al., 2019). Transcription of tshβb is strongly stimulated in spring by increased day length, smolt development, and migration to the sea, and tshβb is present in distinct cells in the pituitary gland (Fleming et al., 2019). The Tshβb cells have recently been located dorsally in the pars nervosa (PN), suggesting that tshβb may be homologous to the β-subunit of TSH (PT-TSH) in mammals and birds that regulates sexual maturation (Fleming et al., 2019).

The effect of temperature and photoperiod has been in previous studies showing that early sexual maturation in Atlantic salmon reared at higher temperatures has a higher percentage of sexual maturation (Fjelldal et al., 2011; Good & Davidson, 2016; Imsland et al., 2014). In RAS and flow-through facilities, high temperature combined with exposure to continuous light regimes is used to maximize the growth rate of farmed fish, and this can affect the degree of sexual maturation in male Atlantic salmon after smolt (Davidson et al., 2021; Fang et al., 2021; Fjelldal et al., 2018; Handeland & Stefansson, 2001). When the temperature rises above the optimal range for growth and development in RAS and flow-through systems, it can accelerate the onset of puberty and lead to early maturation in Atlantic salmon (Good & Davidson, 2016). How water temperature affects the final phase of sexual maturation has been extensively studied (King et al., 2003; Taranger et al., 2010), but how temperature affects the beginning of maturation in Atlantic salmon is still limited (Imsland et al., 2014). The exact role of tshβb and its function related to photoperiod and sexual maturation are not fully understood. Therefore, this thesis wants to investigate tshβb in Atlantic salmon, especially its relationship with body size, smoltification, and sexual maturation.

1.6. Aim and research questions

This study aims to investigate and gain further knowledge about the problem of early sexual maturation and the effect of different photoperiod treatments in the commercial production of Atlantic salmon smolt reared in flow-through systems. Three different body sizes of Atlantic salmon were introduced to a 5-week winter signal (WS) photoperiod (LD12:12) and one group was introduced to continuous light (CL) (LD24:0). Each group was under a constant temperature of 12.5℃ throughout the experiment. In this study, the well-established photoperiodic control of parr-smolt transformation was used as a model system to determine whether the role of tshβb is involved in the control of seasonality in fish. Differences in growth, morphological and metabolic development between groups were assessed using various metrics; growth measurements (weight and length), morphometric calculations (conditional factor (CF)), and gonadotropin index (GSI%).

To test the research questions, transcription of tshβb was further measured in the pituitary to determine their potential role in photoperiodic signaling. This thesis wishes to investigate further the role of tshβb in Atlantic salmon, especially its relationship to body size and sexual maturation. The study was therefore based on the following research questions.

Research question 1: Do different body sizes of male Atlantic salmon that experience a 5-week WS affect growth rate and maturation occurrence?

Research question 2: Does the introduction of the WS at different body sizes affect the occurrence of smoltification and/or sexual maturation?

Research question 3: Does the activation of tshβb depend on body size and does this affect the occurrence of smoltification and/or sexual maturation?

2. Materials and methods

2.1. Fish stock

The experiment was carried out from the 19th of January 2021 to August 2021. The fish used in this experiment were juvenile Atlantic salmon parr (*Salmo salar* L.) from the commercial batch 737, obtained from the aquaculture company Bremnes Torvåg recirculating aquaculture facility (RAS) (Bremnes Seashore AS, Torvåg, Rogaland, Norway). Fertilized roe originated from the Salten strain and was acquired from SalmoSelect (Benchmark Genetics, Hafnarfjordur Iceland) and was a genomic selection for late maturation. Roe hatched on 03.07.2020 and maintained at 12.1°C from the first feeding. The fish were fed 4 mm pellets provided by Bremnes and were fed throughout daylight hours with automatic feeders. On 18.01.2021 when the pre-smolt was 40 g it was transferred from Bremnes Torvåg RAS to the flow-through system (FTS) in Bergen at Høyteknologisenteret (UiB, BIO).

2.2 Experimental design

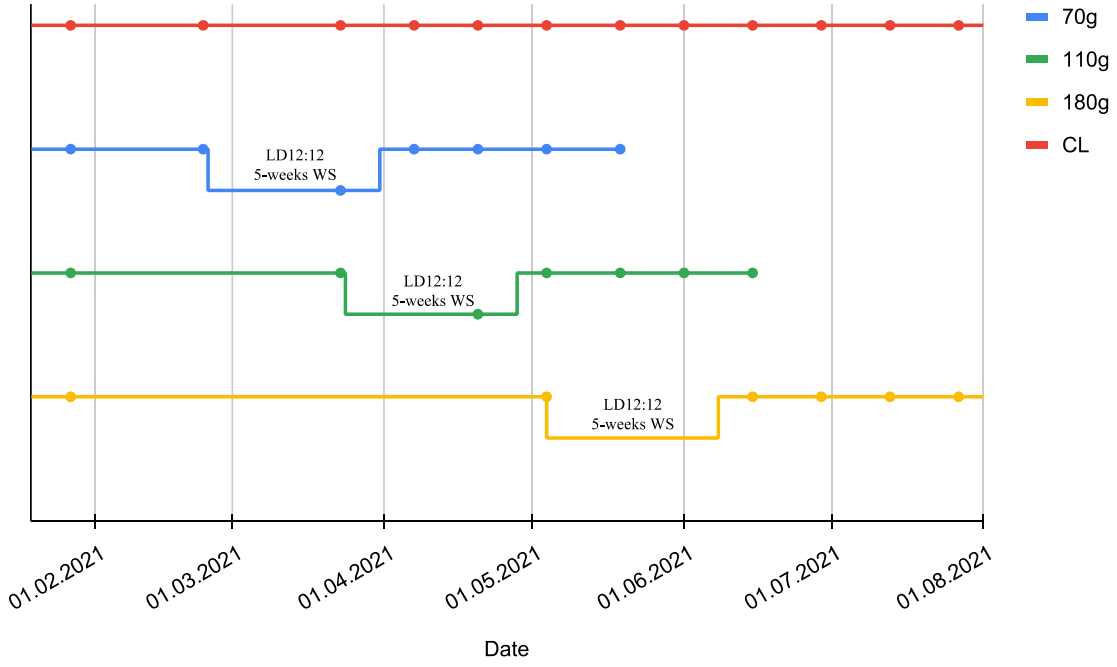

Figure 2.1. Illustration of photoperiod treatments for three different body size groups, continuous light (CL) and winter signal (WS). One group (CL) was treated with continuous light (LD24:0) from the start of the experiment to the end. Group 70, 100, and 180 was introduced to a 5-week winter signal (LD12:12) where 70-group were introduced to WS at 24/2 to 31/3, 110-group started WS 24/3 to 8/4, and the 180-group started WS 4/5 to 8/6. Before and after the five-week period with WS, the three groups were treated with continuous light (LD24:0). All dots marked on the figure indicate the sample date. Changed freshwater to seawater at the end of each group.

All the experimental fish were reared in freshwater tanks at Høyteknologisenteret in Bergen and the fish were divided equally into eight 1-meter tanks under a constant temperature of 12.5°C. The fish were let to acclimatize for 10 days before the trial began. The experiment consisted of three different groups and one group with continuous light (LD24:0) from start to end. The three groups were under continuous light (LD24:0) for 8 months, except for a 5-week winter signal photoperiod (LD12:12) introduced in all groups in February-May 2021 to allow for smoltification. The experiment took place in freshwater from 19.01.2021 and the first group of 70g was introduced to seawater on 01.06.2021 and the three other groups were transferred into seawater on 10.08.2021.

Table 2.1. Summary of experimental conditions of the three groups carried out during this experiment. The different groups receiving constant photoperiod (LD24:0) and winter signal (LD12:12) are shown in the table below.

Group	Location	Salmon (g) when WS (Initial weight)	Temperature	Condition LD (24:0) (CL)	Condition LD (12:12) (WS)
Group 1	Small scale Flow-through	35-40g	12.5°C	19/2 to 10-11/8	None
Group 2	Small scale Flow-through	60-70g	12.5°C	19/2 to 24/2	24/2 to 31/3
Group 3	Small-scale Flow-through	100-120	12.5°C	19/2 to 24/3	24/3 to 28/4
Group 4	Small-scale Flow-through	170-200	12.5°C	19/2 to 4/5	4/5 to 8/6

2.3. Dissecting the salmon brain and sampling protocol

8 samplings were carried out over a period from 23. February to 10-11. August 2021, except group nr. 4 which had 7 sampling since there was a lack of fish. In this experiment, only males were sampled, and the fish were sexed by opening the fish and checking for gonads. On 27 of January, before the first sampling, 12 male salmon from the (LD24:0) group were collected as a baseline sampling. Three males from each group were sampled before the introduction of the winter signal in group 2. For each sampling, at least 6 individuals were randomly collected from each tank (12 per experimental group). All fish were randomly caught with a hand net and quickly euthanized with an overdose of benzocaine by a bath (2ml/L; Benzaak vet. ® 20%, ACD Pharma AS, Norway). Blood was manually collected from the caudal vein with a sterile heparinized syringe. Blood samples were centrifuged for 4 minutes at 5000 rpm and plasma was collected in separate tubes and divided into two aliquots for 11-KT (11-ketotestosterone)

and Igf-1 (insulin-like growth factor 1). The plasma was then immediately frozen on dry ice and stored at -80°C. Before the dissection of the fish, body weight (g) and fork length (cm) were measured to the nearest 0.1 g and 0.1 cm, respectively, and used to calculate the condition factor (CF). When the fish was dissected, the gonads were examined to establish the gender of the fish and the degree of maturation. Only males were kept for sampling. Testes were removed and weighed to the nearest 0.001 g and used for the calculation of the gonadotropin index (GSI).

During the sampling, the brain of the fish was removed. Using a scalpel, the head was cut laterally from the side of the dorsal to the front of the pectoral fins. The fish head was then laid down and turned up where the skull then was opened. An incision was made in front of the nostrils and the scalpel was passed down the dorsal side of the eyes. The brain became visible by gently pushing the skull away at the same time as the incision was made. Using tweezers, the notochord was gently pulled in so that the brain could be tilted, and the pituitary gland became visible. The brain and pituitary gland were carefully removed, placed in tubes containing 1 ml of RNAlater® and incubated at 4°C for 24 hours. All samples that were carried out were stored at -80°C until further analysis.

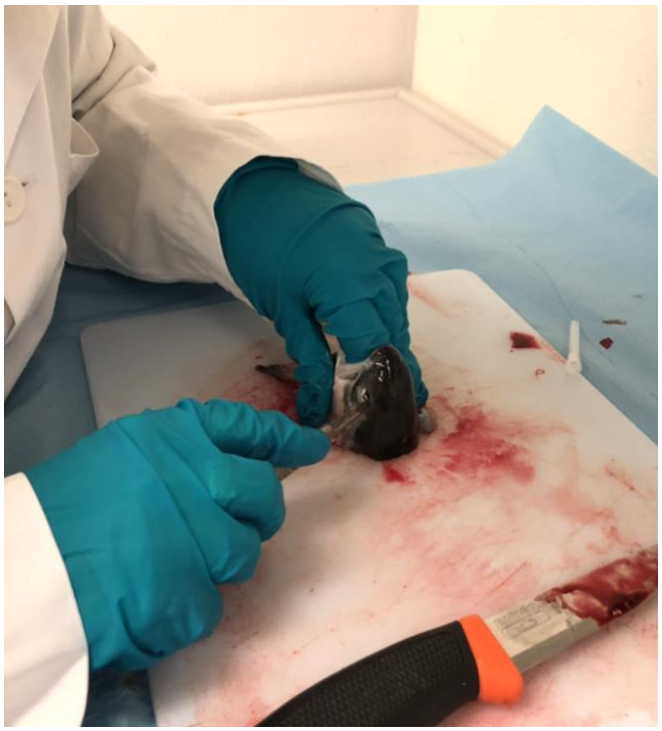

Figure 2.2. A photograph of the skull opened to reveal the brain and pituitary gland.

2.4. RNA extraction for pituitaries

Total RNA was manually isolated from 381 pituitary tissue samples using a standard RNA extraction with TRI Reagent® (TRIzol) protocol. Preparation before RNA extraction, microtubes with special caps (Sarstedt AG & CO. KG, Nümbrecht, Germany) labeled with sample numbers and filled with 8-10 zirconium oxide beads in each microtube (Betin Technologies, Montigny-le-Bretonneux, France). RNA free 1.5 ml tubes were also labeled for further use in the protocol. Samples were collected from the freezer and placed on ice in a styrofoam box. The tissue was transferred to a microtube filled with zirconia beads using a tweezer. 500 µl of TRI Reagent® (Sigma-Aldrich) was pipetted into each microtube and then homogenized twice with a Precellys® 24 lysis homogenizer (Bertin Technologies) using program 2 (5000-1x15-005). Left to rest for 5 minutes and then used a VWR Scientific Galaxy MiniStar Microcentrifuge to bring down the liquid before opening the lid. 100 µL of chloroform (Sigma-Aldrich) was added to each tube and vortexed for 8-10 seconds until the liquid was mixed to a light pink color. The tubes were then placed in an Eppendorf 5415 R Refrigerated Centrifuge at 4°C for 15 minutes at 13.200 RPM (maximum speed) and a g-force at 12.000 RCF.

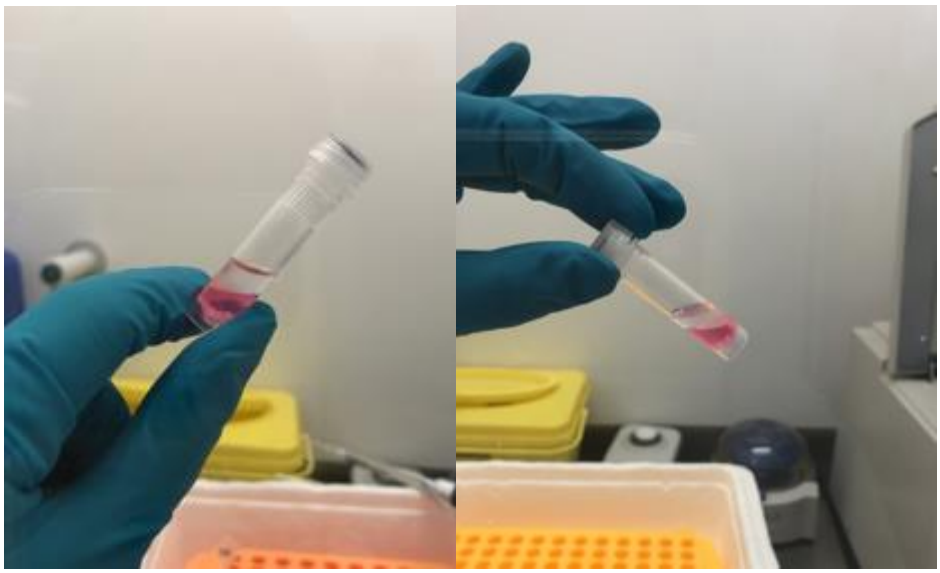

Figure 2.3. Photography of RNA precipitation. The aqueous phase, the first layer consists of RNA, then there is an interphase that consists of DNA. The pink layer is the organic phase and consists of proteins, lipids, and fats.

Tubes from the centrifuge were carefully taken out and placed on ice. The aqueous phase containing RNA was carefully pipetted out and into 1 ml RNase-free tubes. 300 µl of ice-cold 2-propanol (Sigma-Aldrich) was pipetted into each tube, inverted 5 times, and left at room

temperature for 20 minutesTubes were inverted 5 times and put back in the centrifuge for 10 minutes at 4℃ and 13.200 RPM. Tubes were taken out and quickly decanted in a can, leaving only the RNA pellet in the tube. 500-600 ml of 80% ethanol was added to the tubes to rinse the pellet. The tubes were inverted to ensure that the pellets were floating and well rinsed.

The RNA pellets were then decanted once more and the remaining EtOH was carefully removed with a pipette. Pellets were then dried for 20 minutes at room temperature. 10 µl of RNase-free H2O was transferred to each tube and left for 10-20 minutes or until the pellets were dissolved. The fluid was aspirated up and down 5 times before being transferred to a smaller sterile RNA free tube. The concentration of mRNA in each sample was quantified using a NanoDrop One spectrophotometer (Thermo Scientific). The samples were then stored at -80℃.

2.5. cDNA synthesis

First-strand complementary DNA (cDNA) synthesis was performed in a total volume of 20 µl by reverse-transcribing the total RNA using SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, USA) following the manufacturer protocol. RNA samples were mixed using Scientific Industries SI™ Vortex-Genie™ 2. First, RNA samples had to be normalized to equal RNA concentrations. A concentration of 750ng/ul was calculated for each sample by adding water to a final volume of 11ul. A mixture of 1 µl Oligo(dT)20 Primer (Invitrogen™, ThermoFisher Scientific Inc., USA) and 1 µl of Thermo Scientific™ dNTP Mix (Invitrogen™, ThermoFisher Scientific Inc., USA) was then added to each sample (total volume of 13 µl). The samples were incubated for 5 minutes at a heated mixture of 65°C using the C1000 Touch thermal cycler (Bio-Rad Laboratories, USA) and then put immediately on ice to limit the formation of secondary structures. Preparing a final mixture containing 4 µl first-strand buffer, 1 µl Dithiothreitol (DTT) and 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor, and 1 µl Superscript III Reverse Transcript (Invitrogen™, ThermoFisher Scientific Inc., USA) were added to each sample for a final volume of 20 ml. Mixing by pipetting gently up and down and then in Scientific Industries SI™ Vortex-Genie™ 2 before incubation of the cDNA synthesis at 50°C for 60 minutes. The temperature was increased to 70°C for 15 min to deactivate the reaction. cDNA was then stored at -20°C until further analysis.

Table 2.2. Tables of two examples of a mixture with 96 samples. The first column shows how much should be in each tube, while the second column shows the total amount of mix divided into 96 samples with a 10% addition.

Mix 1

Substance	**Amount per well**	**Total amount of mix 1**
dNTP	1 µl	1 µl * 96 + 10% = 106 µl
Oligo	1 µl	1 µl * 96 + 10% = 106 µl
Total	2 µl	212 µl

Mix 2

Substance	**Amount per well**	**Total amount of mix 1**
Buffer	4 µl	4 µl * 96 + 10% = 424 µl
DTT	1 µl	1 µl * 96 + 10% = 106 µl
RNaseOUT	1 µl	1 µl * 96 + 10% = 106 µl
SSIII	1 µl	1 µl * 96 + 10% = 106 µl
Total	7 µl	742 µl

2.6. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) was performed to quantify the expression of the gene tshβb and β-actin in the pituitary using the C1000 Touch Thermal Cycler, CFX96 Real-Time Detection System (Bio-Rad Laboratories, CA, US) and CFX Manager software (version 3.1) (Bio-Rad Laboratories) following the manufacture protocol. The RT-qPCR reactions were performed in duplicates in 96-well hard-shell PCR plates (Bio-Rad Laboratories, inc.) with a total reaction volume of 14 µL per well. 6.5 µl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.25 µl each of forward primer (FRW) and reverse primer (REV), 3.0 µl of UltraPure Distilled water, including 4 µl of 1/20 diluted cDNA. All plates contained two wells of a "no template control" (NTC) to detect contamination in the water and two wells of positive control which 1/20 diluted cDNA pooled from all samples.

Table 2.3. Calculation of substance amounts in SYBR-mixture for RT-qPCR.

Substance	**Amount per well**	**Total amount**
SYBR	6.5 µl	6.5 µl * number of wells + 10%
FRW	0.25 µl	0.25 µl * number of wells + 10%
REV	0.25 µl	0.25 µl * number of wells + 10%
H2O	3.0 µl	3.0 µl * number of wells + 10%
Total	10 µl + 2.5 µl Template for β-actin + 4 µl Template for tshβb	

The calculation of the amount of the substance is shown in table 2.3. 10% extra was included for possible pipetting errors. After pipetting the mixture in the wells, the plates were sealed with microseal® 'B' seals (Bio-Rad Laboratories, inc.) and spun down in the Eppendorf Centrifuge 5804 R for a couple of seconds to ensure that nothing was stuck under the microseal. The plates were then moved to the C1000 Touch Thermal Cycler. Each qPCR-run consisted of a 3-minute initiation denaturation at 95℃, followed by 36 cycles of denaturation at 95℃ for 15 seconds and annealing at 61℃ for 1 minute. For the melting curve denaturation, the temperature was set to 95℃ for 10 seconds following a melting curve re-hybridization, and the temperature was gradually increased from 65℃ to 95℃ (0.5℃ every 5s).

Table 2.4. Overview of primers used in RT-qPCR.

Gene	Primer	Primer sequence (5'→ 3')		Gene Accession number	Reference
β-actin	Ss_ β-actin	F	CCAAAGCCAACAGGGAGAAG	DQ924958	Oslvik et al., 2005
	Ss_ β-actin	R	AGGGACAACACTGCCTGGAT		
tshβb	tsh1bFW2	F	TTGCCGTCAACACCACCAT	MG948546	Fleming et at., 2019
	tsh1bRV2	R	GGGATGATAGACCAGGGAGTG		

Table 2.5. Thermal cycle program used in the present study.

Step	Process	℃	Timer pr. cycle	Cycles
1	Initiation denaturation	95	3 min	1
2	Denaturation	95	15 s	40
3	Annealing, extension, and fluorescence read	61	1 min	
4	Melting curve: Denaturation	95	10 s	1
5	Melting curve: Re-hybridization	65-95	5 s	60

Table 2.6. Example of plate nr 1. of my randomized plate setup for RT-qPCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	91	91	42	42	86	86	56	56	73	73
B	43	43	57	57	74	74	37	37	69	69	95	95
C	38	38	66	66	63	63	93	93	71	71	92	92
D	30	30	49	49	23	23	51	51	41	41	40	40
E	65	65	46	46	85	85	68	68	96	96	14	14
F	94	94	36	36	78	78	34	34	15	15	13	13
G	27	27	79	79	1	1	24	24	28	28	82	82
H	7	7	16	16	9	9	10	10	Cal	Cal	NTC	NTC

Before the mRNA expression analysis, serial dilutions were made of cDNA obtained from pooled samples with the ratios ⅕, 1/10, 1/20, 1/40, 1/80, 1/160, and 1/320 to test the efficiency of each primer and to determine which would give the best values for quantification cycles (Cq values). The cDNA pool was made for the template by pipetting 1 µl of each cDNA sample into an empty tube. The dilution of 1/20 (equating to ~1.25ng of RNA per reaction) gave the best Cq values and was therefore used to make a standard curve for each primer. Beta-actin was used as a reference gene.

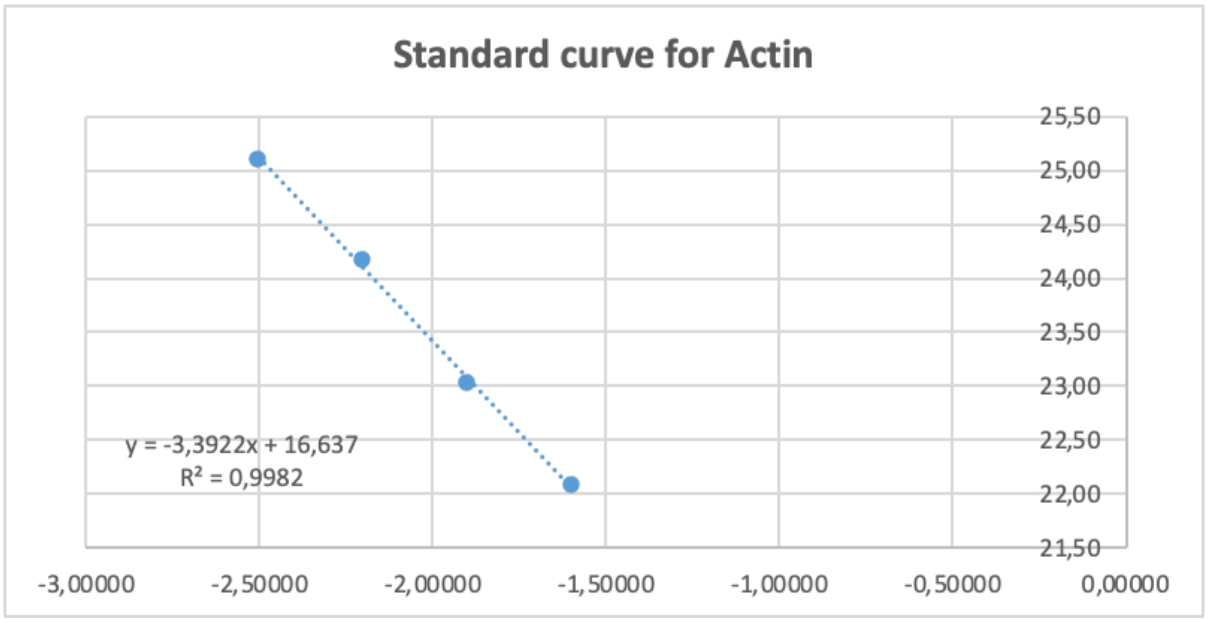

Figure 2.4. Standard curve for the primer β-actin.

2.7. Calculations

PCR efficiency (E)

The PCR efficiency (E) was determined from the slope of the regression line which was generated from the dilution curve. The formula for calculation of the primer efficiency that was used:

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

Condition factor (K)

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

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

Gonadosomatic index (GSI)

The gonadosomatic index (GSI) was used to measuring the ratio between gonad weight and body weight for each fish and was calculated by the following equation (Anderson and Gutreuter, 1983:

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

2.8. Data analysis

All data from the qPCR-runs were exported to a combined Microsoft Excel sheet. The dilutions of the pool samples were run in triplicates and generated for every tissue. Figure 2.4. showing a standard curve for primer β-actin. The slope of the regression line, generated from the dilution curves, was used to determine PCR efficiency. All figures and statistical analysis were carried out using GraphPad Prism 9 (California, USA). In all cases, we used Linear mixed effects models, with treatment, time and their interaction as fixed effects and tank as random effect. Tukey post hoc tests to find significant differences between groups at different times and within groups over time.

3. Results

3.1. Changes in mean body weight over time

All four experimental groups exhibited a gradual increase in body weight (mean ± SEM), and the growth rate was similar between the 70WS, 180WS, and CL groups throughout the experiment. 110WS group had a similar growth rate until the last two sampling groups where there was a larger variation between the weight. The samples taken on the 15th of June 2021 showed that the lowest growth in this group was 159.1 g, while the highest growth was 307.7 g. The last sample taken on the 29th of June 2021 also shows a large variation between the growth rates which explains a lower curve, where the lowest growth is 201 g, while the highest is 467.3 g. Average body weight for the 70WS, 110WS, 180WS, and CL groups all had an initial growth of ~41 grams (± 0.275). The 70WS group reached a final weight of 203 grams at the last sampling on the 17th of May 2021, 110-group reached 298.3 grams at the last sampling on the 29th of June 2021, 180WS group had a final body growth of 554.3 grams at the final sampling on the 27th of July 2021. The CL group ended up with 486.5 grams in average body weight for the last group on the 27th of July 2021. There were some minor significant differences in body weight at certain periods throughout the experiment, but no single treatment provided any major significant advantage in body weight.

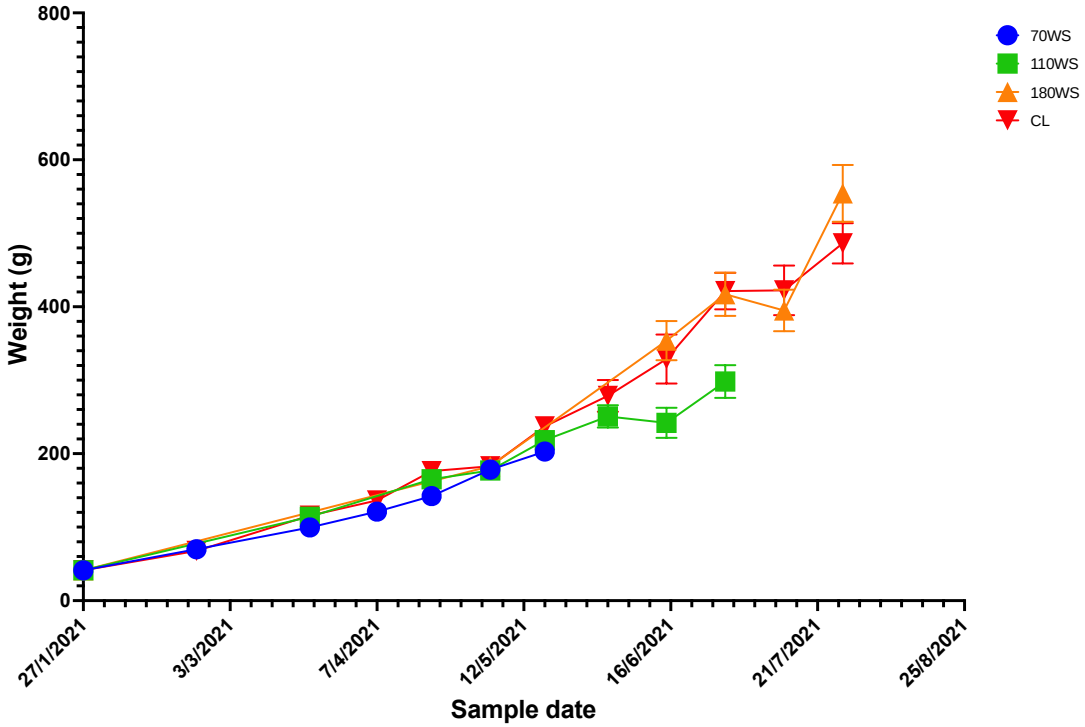

Figure 3.1.1. Average body weight (g): 70WS, 110WS, 180WS and CL. Average body weight measured in grams shown over a period with three different body sizes (70, 110 and 180) and a group with continuous light (CL). Average body weight represented by circles, squares, and triangles of different colors with upper and lower horizontal lines showing standard error of the mean (+/- SEM). Continuous light (CL) was included in each figure to show starting weight, but it was not included in the data analysis.

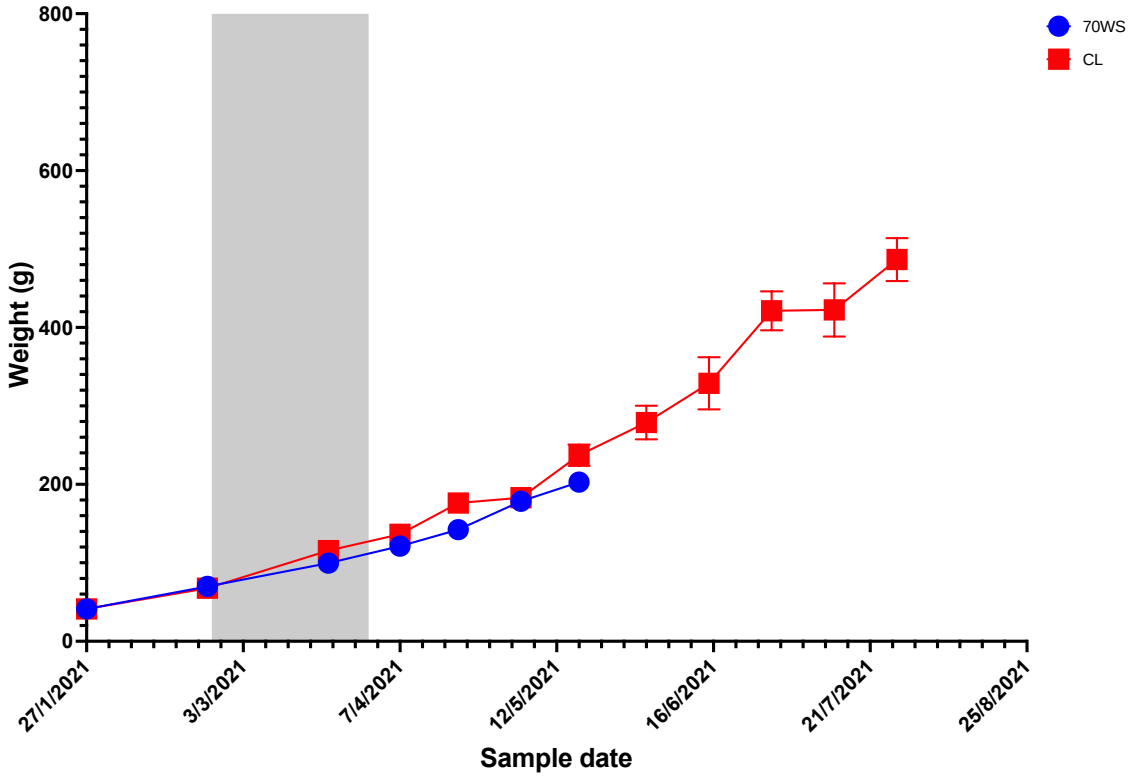

Figure 3.1.2. Average body weight (g): 70WS and CL. Average body weight (g) shown over a period. Blue line shows body size 70 and the red line shows continuous (CL). Average body weight is represented by circles and squares with upper and lower lines showing the standard error of the mean (+/- SEM). The grey area marked represents a 5-week winter signal (WS).

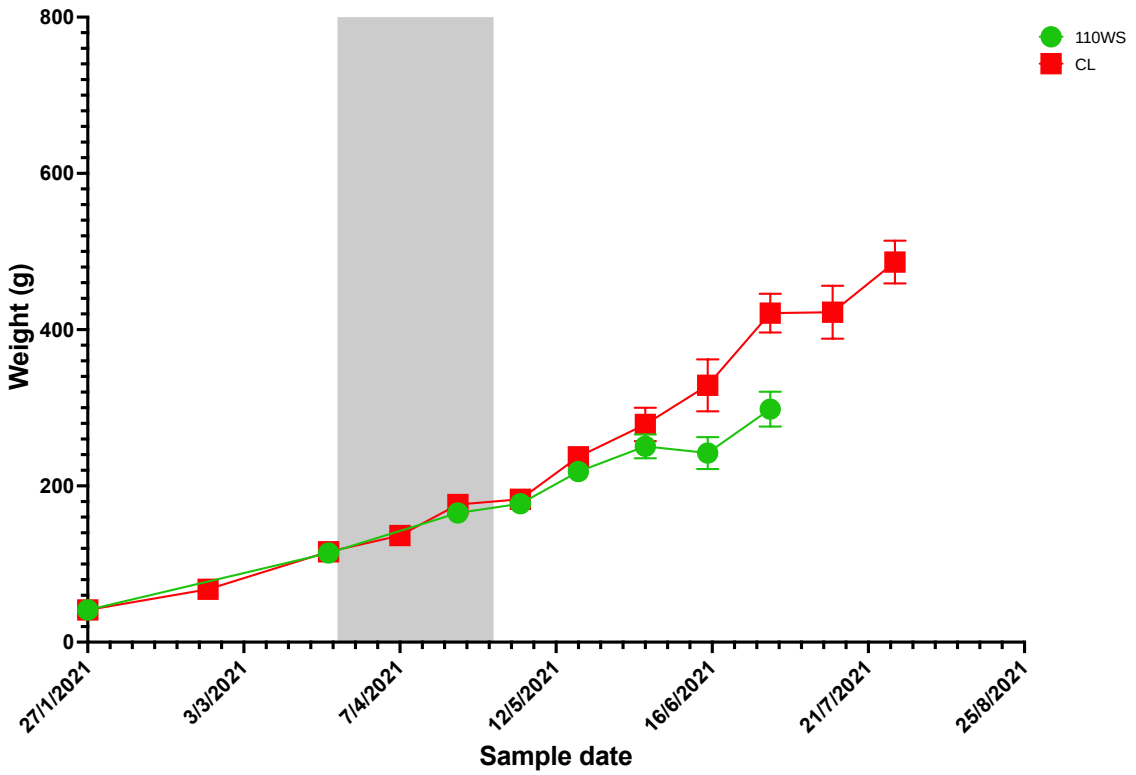

Figure 3.1.3. Average body weight (g): 110WS and CL. Green line show body size 110 and the red line shows continuous (CL). Average body weight is represented by circles and squares with upper and lower lines showing the standard error of the mean (+/- SEM). The grey area marked represents a 5-week winter signal (WS).

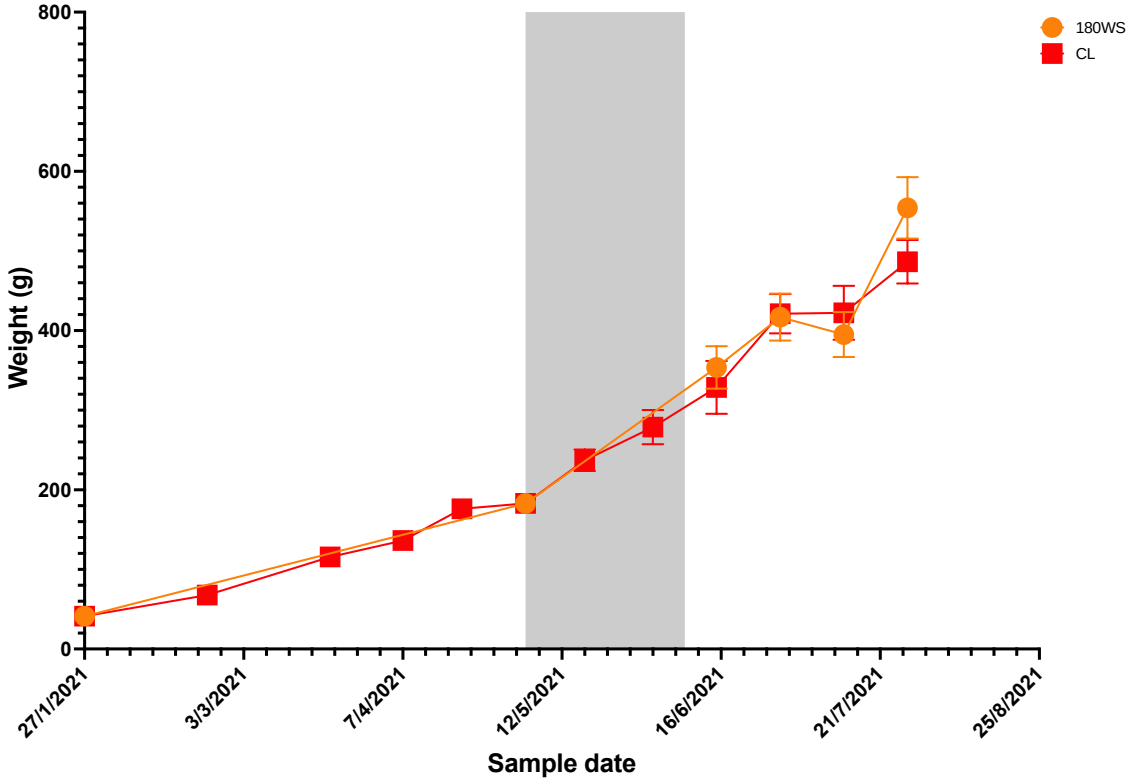

Figure 3.1.4. Average body weight (g): 180WS and CL. The orange line shows body size 180 and the red line shows continuous (CL). Average body weight is represented by circles and squares with upper and lower lines showing the standard error of the mean (+/- SEM). The grey area marked represents a 5-week winter signal (WS).

3.2. Changes in mean body length over time

The average length increase in the group of 70WS was 15 cm at the first sampling 27th of January 2021 to 25.6 cm at the last sampling 17th of May 2021. The group of 110WS showed an increase from 15 cm to 29.7 cm 29th of June 2021, while 180WS had an increase from 15 cm to 35.0 cm 27th of July 2021. The CL group started at 15 cm at the first sampling and 33.8 cm at the last sampling 27th of July 2021. No significant differences in length were observed between the four groups, nor any significant effect of the introduction of winter signal (WS) in any body size, and no growth advantage from CL.

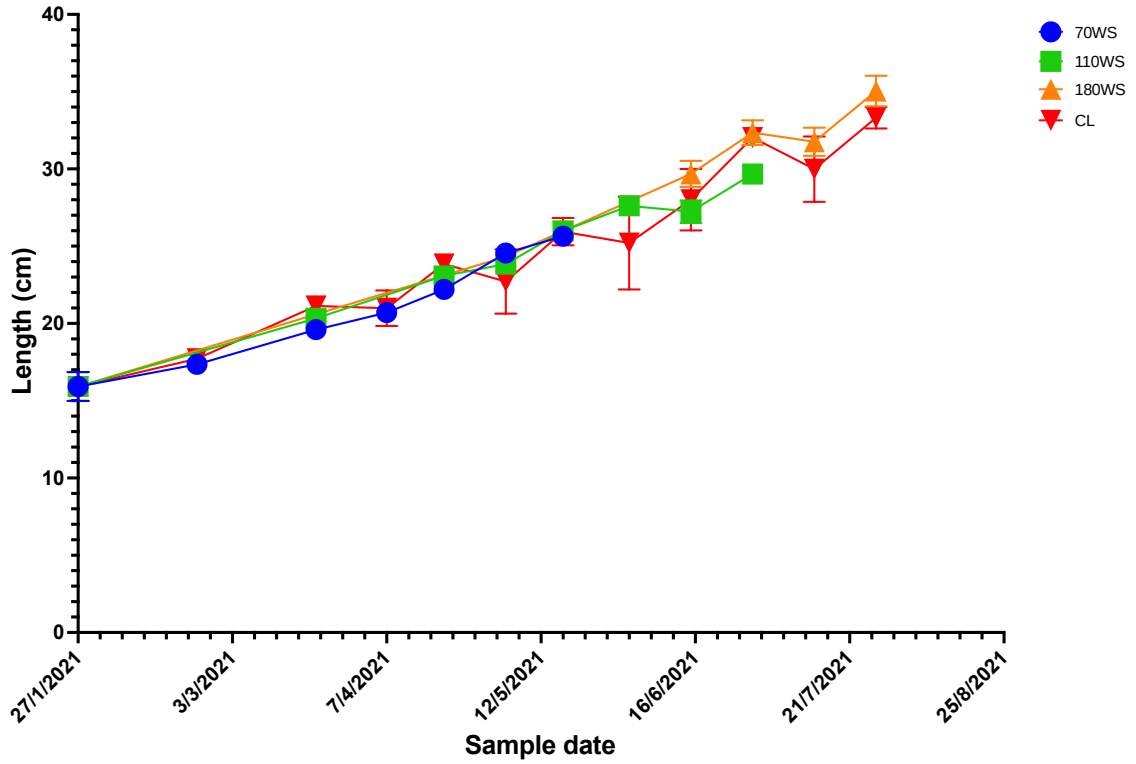

Figure 3.2.1. Average body length (cm): 70WS, 110WS, 180WS and CL. Average body length measured in centimeters shown over a period with three different body sizes (70, 110 and 180) and a group with continuous light (CL). Average body length represented by circles, squares, and triangles with upper and lower horizontal lines showing standard error of the mean (+/- SEM). Continuous light (CL) was included in each figure to show starting weight, but it was not included in the data analysis.

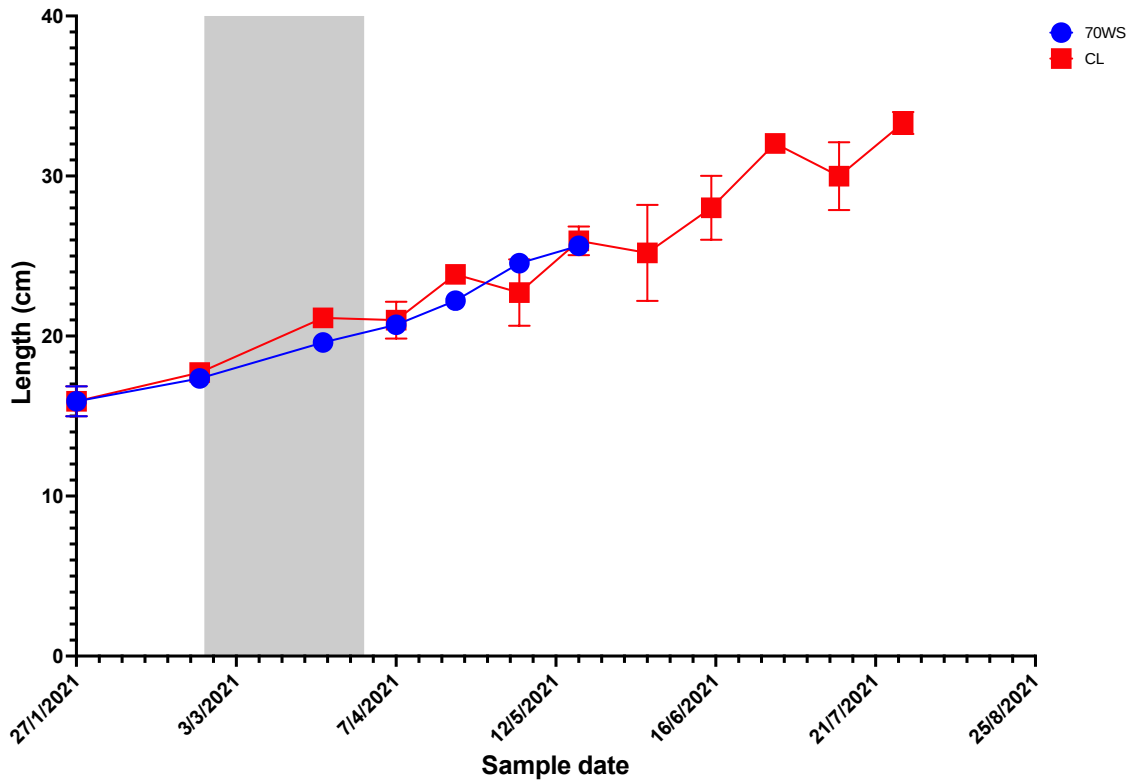

Figure 3.2.2. Average body length (cm): 70WS and CL. Blue line represents the body size of 70 and the red line represents continuous light (CL). Average body length represented by circles and squares with upper and lower horizontal lines showing standard error of the mean (+/- SEM). The grey area marked in the figure is the period for winter signal (WS).

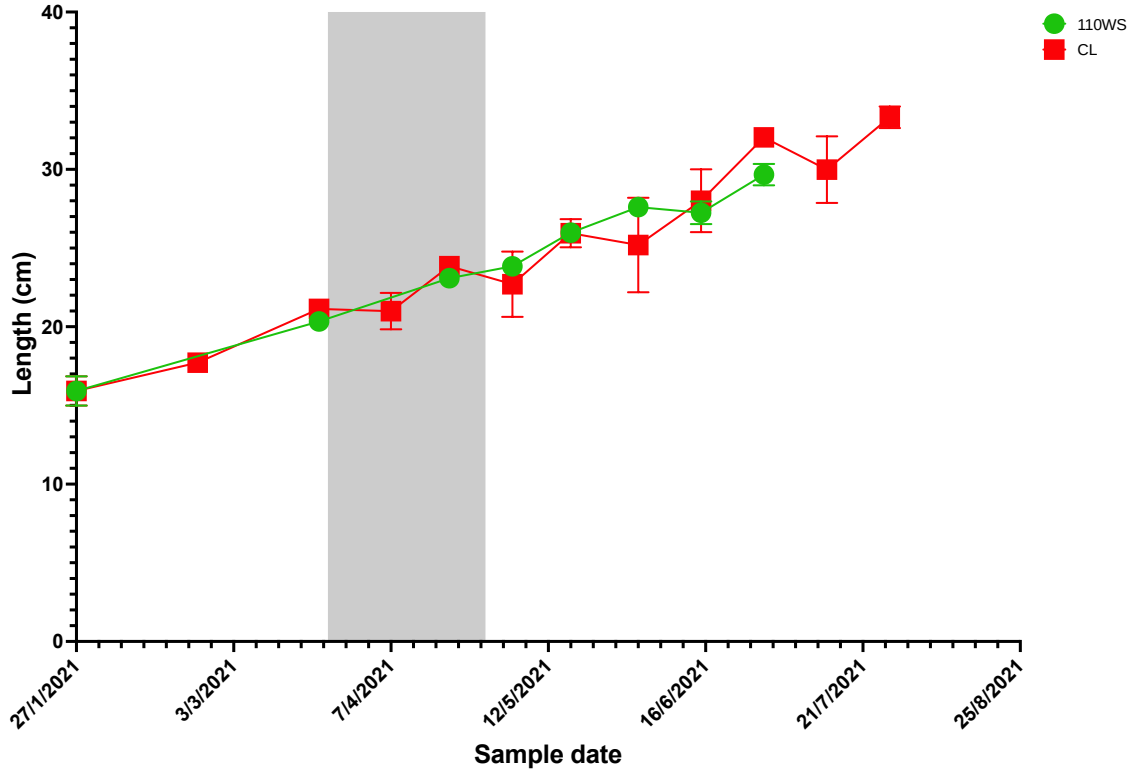

Figure 3.2.3. Average body length (cm): 110WS and CL. Green line represents the body size 110 and the red line represents continuous light (CL). Average body length represented by circles and squares with upper and lower horizontal lines showing standard error of the mean (+/- SEM). The grey area marked in the figure is the period for winter signal (WS).

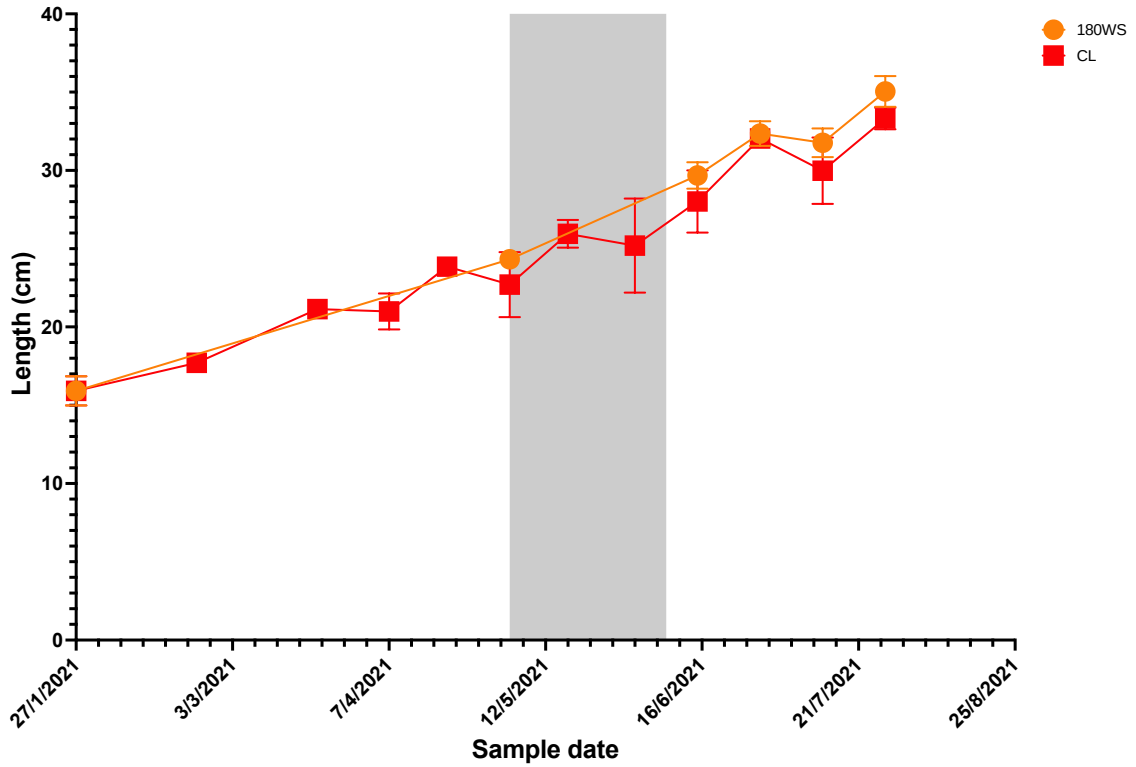

Figure 3.2.4. Average body length (cm): 180WS and CL. The orange line represents the body size of 180 and the red line represents continuous light (CL). Average body length represented by circles and squares with upper and lower horizontal lines showing standard error of the mean (+/- SEM). The grey area marked in the figure is the period for winter signal (WS).

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

The average mean condition (K) in all four groups fluctuated throughout the experiment shown in Figure 3.3.1. The 70WS group (blue line) started at ~1.22 (± 0.027), where it increased to ~1.34 (± 0.056) on the 23rd of February 2021, right before the winter signal was initiated. The 70WS remained at ~1.34 throughout the 5 weeks of WS, afterward significantly ($p<0.001$) decreasing to ~1.2 (± 0.017) by the end of the experiment. The 110WS group (green line) shows ~1.22 (± 0.027) at the first sampling, an increase to ~1.35 (± 0.036) on the 22nd of March 2021, right before the introduction of WS, and a gradual decrease to ~1.12 (± 0.026) on the last sampling. A significant drop ($p<0.0001$) in K was observed at the end of the experiment from sampling points 15th of June 2021 to the 29th of June 2021. The 180WS group (orange line) started at ~1.22 (± 0.027), where the highest point was in the middle of the experiment at ~1.34 (± 0.026) on the 15th of June 2021, right after the five weeks of WS and returning to continuous light. It then declined to ~1.27 (± 0.04) at the last sampling on the 27th of July 2021. Despite fluctuations throughout the experiment in the 180WS group, no significant changes in K in the 180WS were observed. CL (red line) started at ~1.22 (± 0.027), an elevated point after the second sampling to ~1.31 (± 0.017) on the 23rd of February 2021, and ended up at ~1.25 (± 0.041) at the final sampling on the 27th of July 2021. The CL group had small fluctuations throughout the experiment but only the decrease from sample 2-3 was considered significant ($p<0.05$); no other significant changes in K was observed.

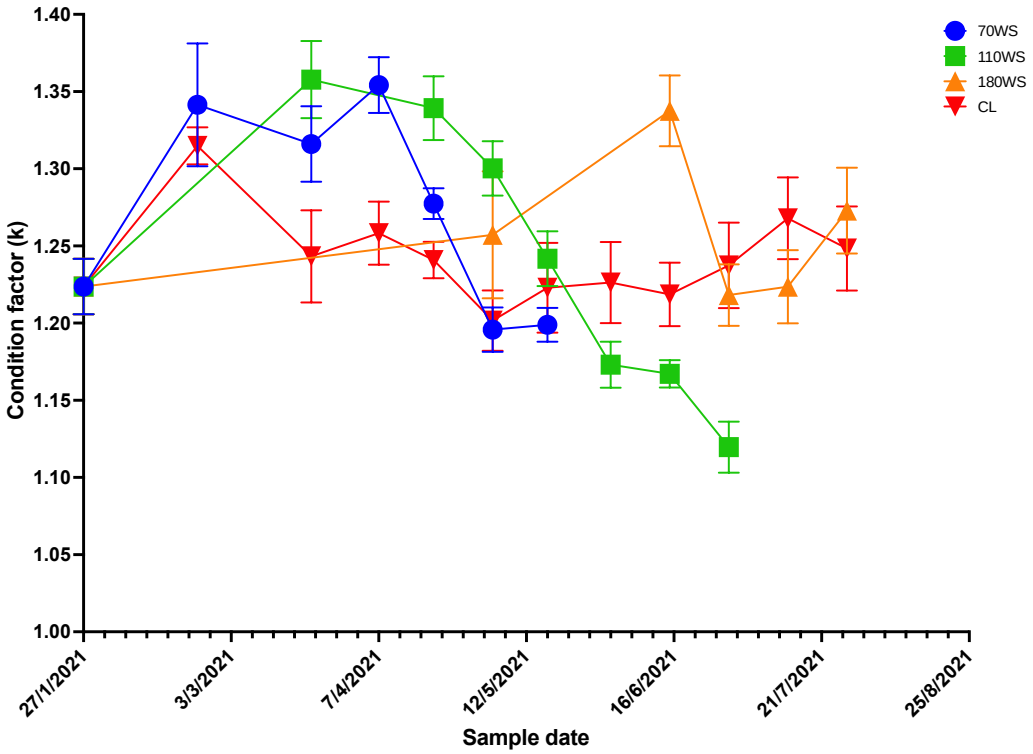

Figure 3.3.1. Average condition factor (K): 70WS, 110WS, 180WS, and CL. Average K showed over a period with three different body sizes (70, 110, and 180) and a group with continuous light (CL). The average condition factor (K) is represented by circles, squares, and triangles of different colors with upper and lower horizontal lines showing standard error of the mean (+/- SEM). Continuous light (CL) was included in each figure for comparison.

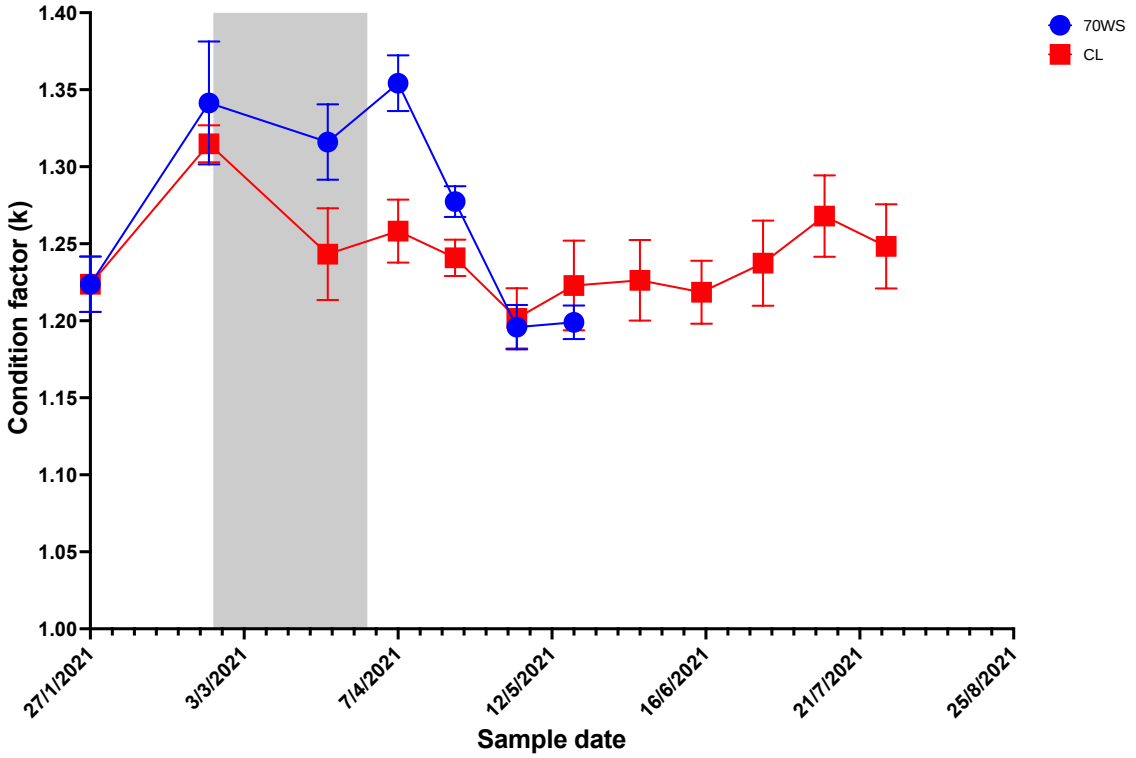

Figure 3.3.2. Average condition factor (K): 70WS and CL. The blue line shows the group of 70 and the red line shows the group of continuous light (CL). The average condition factor (K) is represented by circles and squares with upper and lower horizontal lines showing the standard error of the mean (+/- SEM). The grey area marked in the figure shows a 5-week winter signal (WS) period. Continuous light (CL) was included in each figure for comparison.

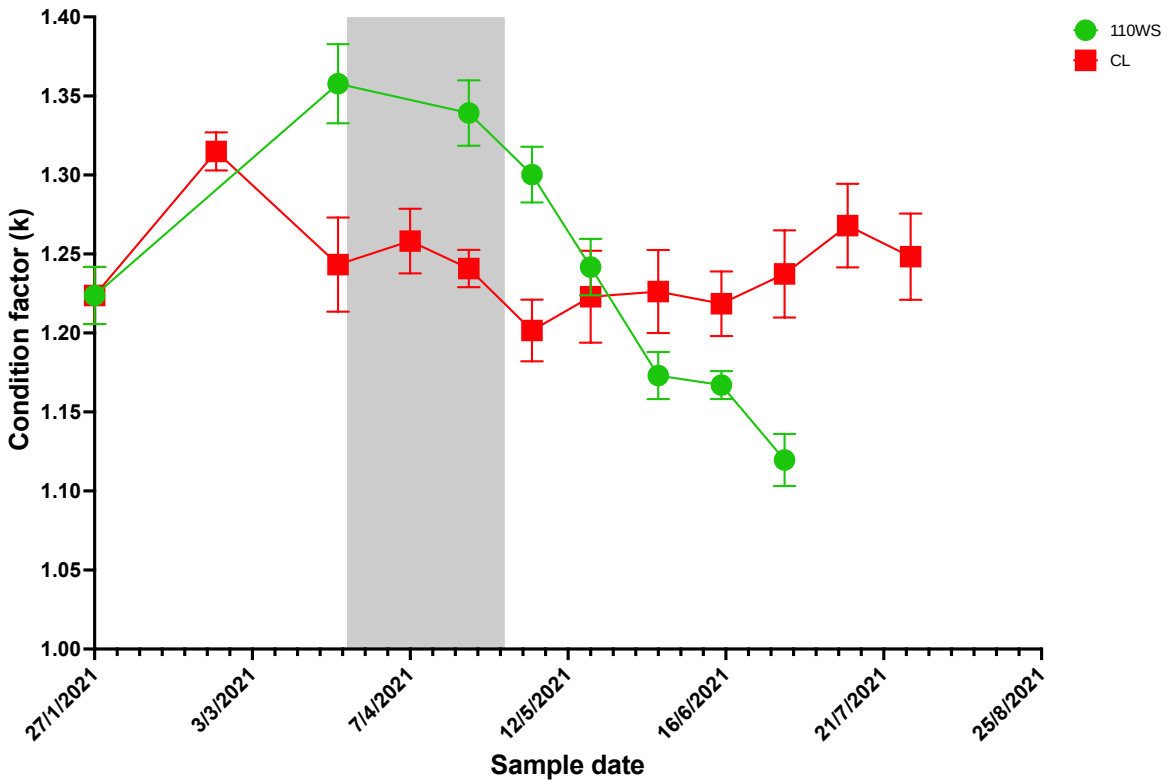

Figure 3.3.3. Average condition factor (K): 110WS and CL. Green line show the 110 group and the red line shows the group of continuous light (CL). The average condition factor (K) is represented by circles and squares with upper and lower horizontal lines showing the standard error of the mean (+/- SEM). Grey area marked in the figure shows a 5-week winter signal (WS) period. Continuous light (CL) was included in each figure for comparison.

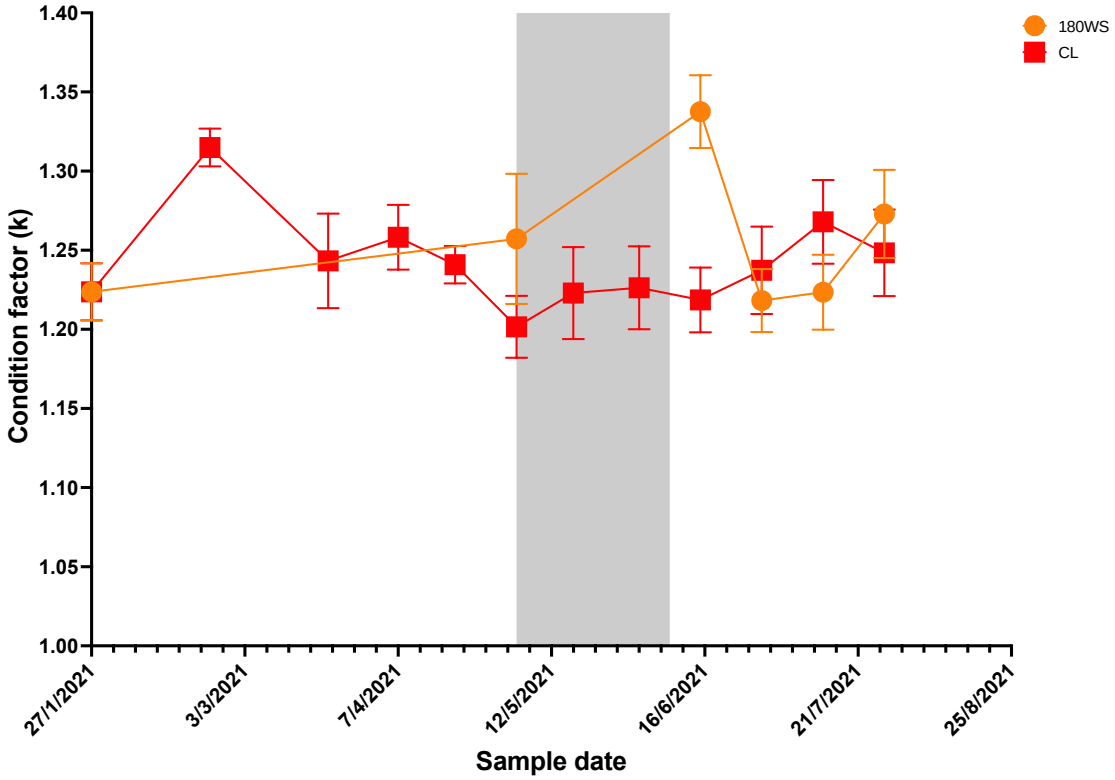

Figure 3.3.4. Average condition factor (K): 180WS and CL. The orange line shows the 180 group and red line shows the group of continuous light (CL). The average condition factor (K) is represented by circles and squares with upper and lower horizontal lines showing the standard error of the mean (+/- SEM). Grey area marked in the figure shows a 5-week winter signal (WS) period. Continuous light (CL) was included in each figure for comparison.

3.4. Changes in Mean Gonadosomatic Index (GSI) over time

The average gonadosomatic index (GSI) shows a low and consistent GSI of ~0.03 (± 0.013) throughout the experiment in the 70 and CL groups. The 110 group maintains a stable GSI of ~0.03 (± 0.013) until the last sampling on the 29th of June 2021, where the GSI of a single fish increased dramatically to ~0.68 (explaining the large error bars), bringing the average of the last group to ~0.086 (± 0.181). Other than the single individual, all remaining fish in the 110-group remained around ~0.03. The 180 group also maintains a low and steady GSI until the last samplings on the 13th of July 2021 and the 27th of July 2021, where the average GSI increases from ~0.04 (± 0.041) to ~0.06 (± 0.114). No significant changes in GSI were observed in any treatment however a strong trend of increasing maturation was observed in the 180WS group.

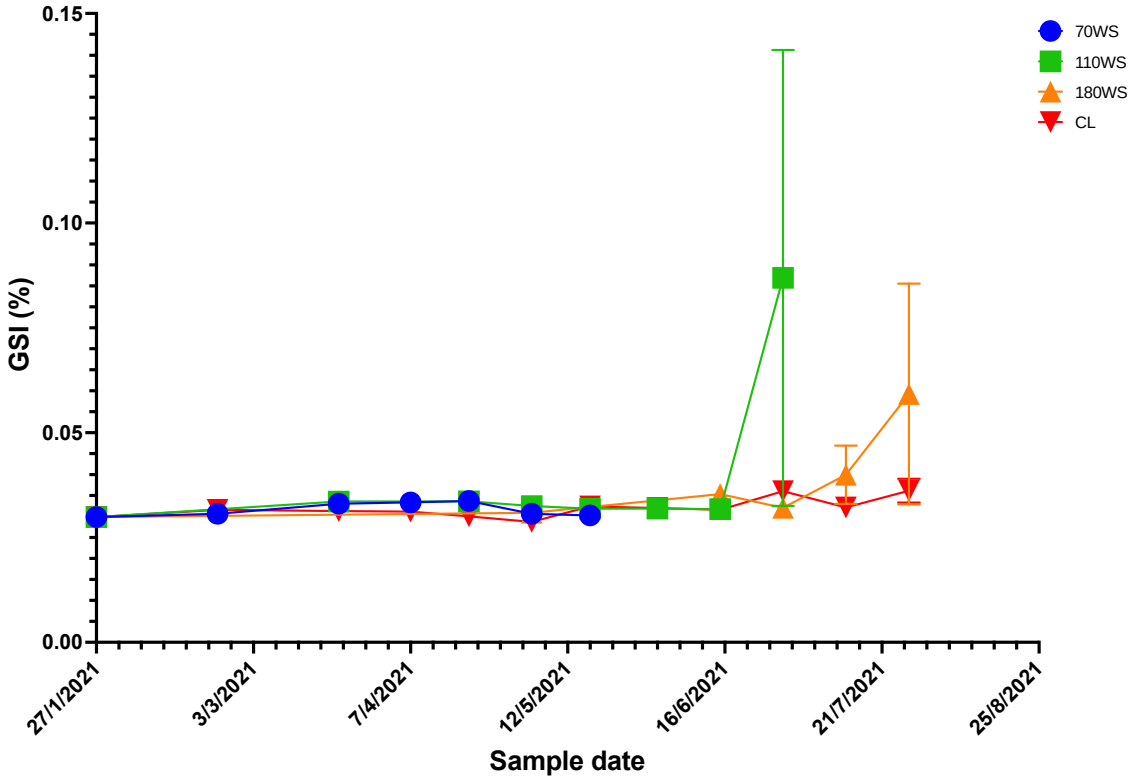

Figure 3.4.1. Average gonadosomatic index (GSI %): 70WS, 110WS, 180WS, and CL. Three different body sizes (70, 110, and 180) and a group with continuous light (CL). Average gonadosomatic index (GSI) of different colors with upper and lower horizontal lines showing

standard error of the mean (+/- SEM). Continuous light (CL) was included in each figure to show starting weight, but it was not included in the data analysis.

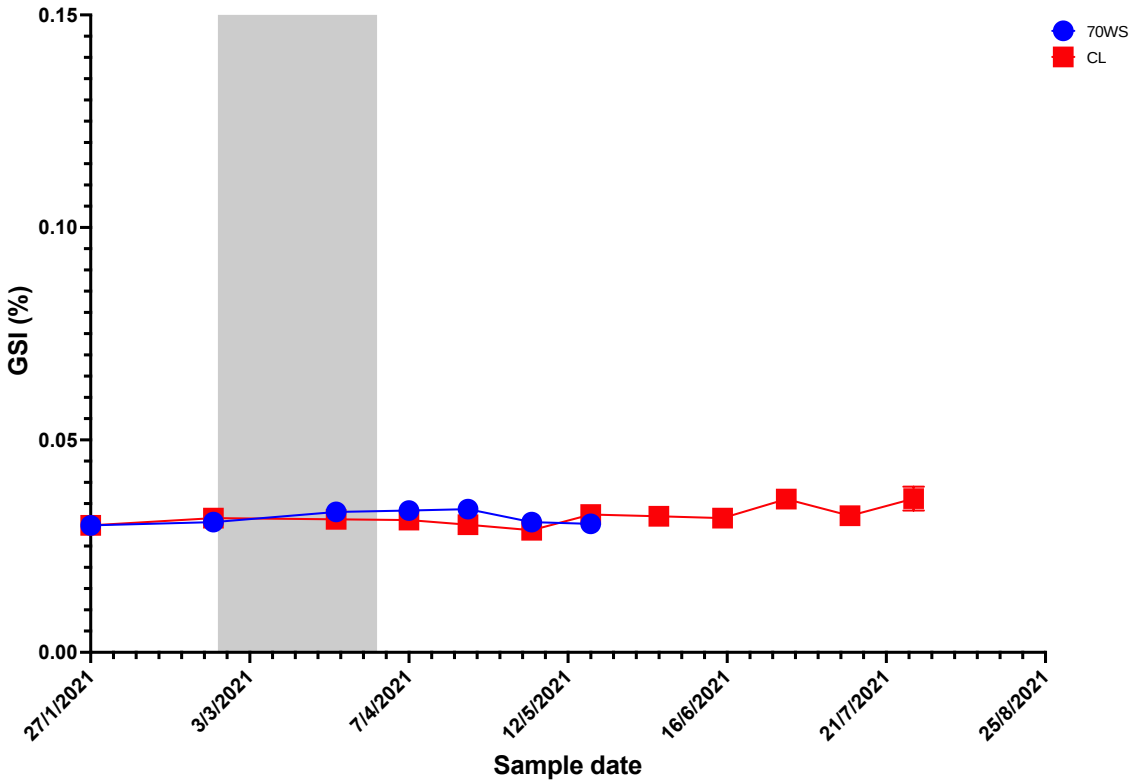

Figure 3.4.2. Average GSI (%): 70WS and CL. The blue line represents the 70-bodysize group and the red line is representing continuous light (CL). Average gonadosomatic index (GSI) is represented by circles and squares with upper and lower horizontal lines showing standard error of the mean (+/- SEM). The grey area marked in the figure is the period for winter signal (WS).

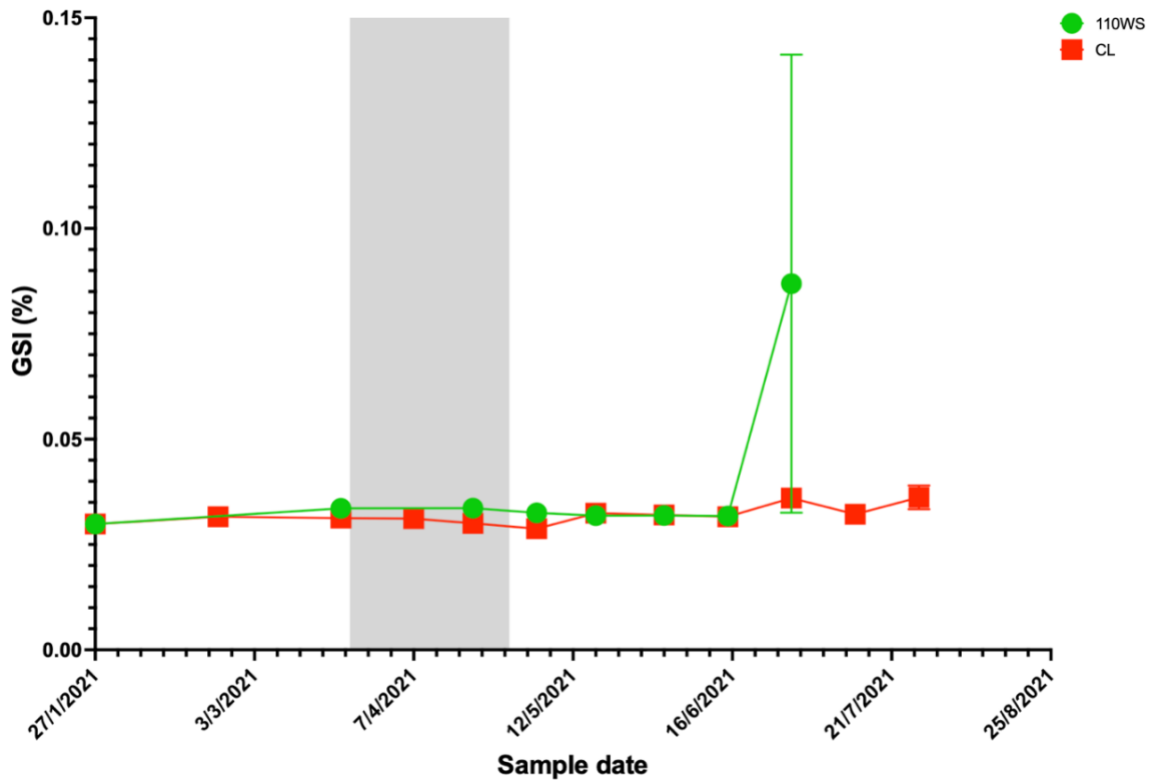

Figure 3.4.3. **Average GSI (%): 110 WS and CL.** Green line represents the 110-body size group, and the red line is representing continuous light (CL). The average gonadosomatic index (GSI) is represented by circles and squares with upper and lower horizontal lines showing the standard error of the mean (+/- SEM). The grey area marked in the figure is the period for winter signal (WS).

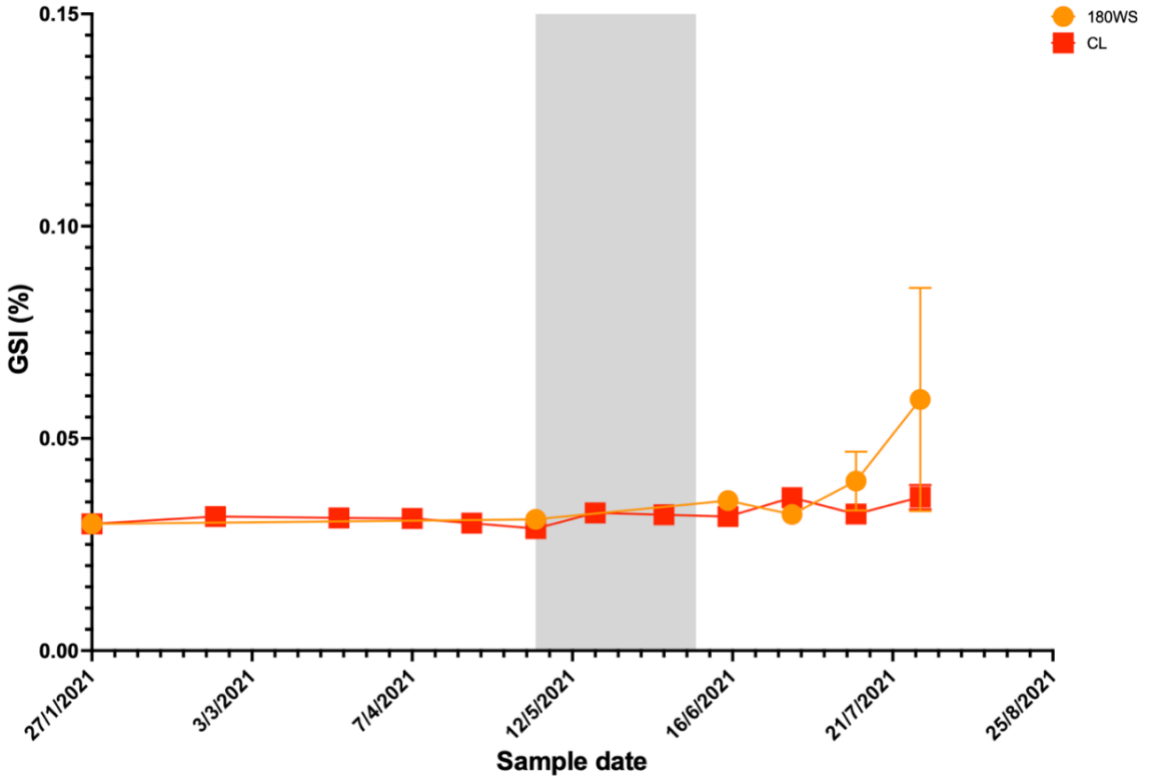

Figure 3.4.4. Average GSI (%): 180 WS and CL. The orange line represents the body size group of 180 and the red line is representing continuous light (CL). The average gonadosomatic index (GSI) is represented by circles and squares with upper and lower horizontal lines showing the standard error of the mean (+/- SEM). The grey area marked in the figure is the period for winter signal (WS).

3.5. Changes in the mean concentration of tshβb over time

The average concentration of tshβb had a rapid increase after returning to continuous light after the 5-week winter signal (WS) in the three different body size groups; while in the group with continuous light (CL), the concentration remained low throughout the entire experiment (figure 3.5.1.). The peaks in the different graphs represent the gene expression (tshβb) and could be seen in the different sizes of the fish. All three groups had a striking and significant ($p<0.0005$) increase in the mean concentration of tshβb following the return to CL after the 5 weeks of WS. 70WS group had a tshβb peak on the 7th of April 2021, 110WS had a peak 4th of May 2021, and 180WS on the 15th of June 2021; each occurring on the first sampling after the WS had ended. In all three treatments, tshβb levels quickly returned to basal levels, significantly declining ($p<0.0005$) following the peak of expression. The orange line shows the group of 180 g fish and shows a small difference from the blue and green lines as it is missing a date because there were no more fish left for sampling. The peak was highest in group 70 with an average of ~1.95 (± 0.273), and a lower peak was seen in the other two groups, where group 110 had an average of ~1.42 (± 0.254) and group 180 had an average of ~1.39 (± 0.257) where basal levels were at ~0.3.

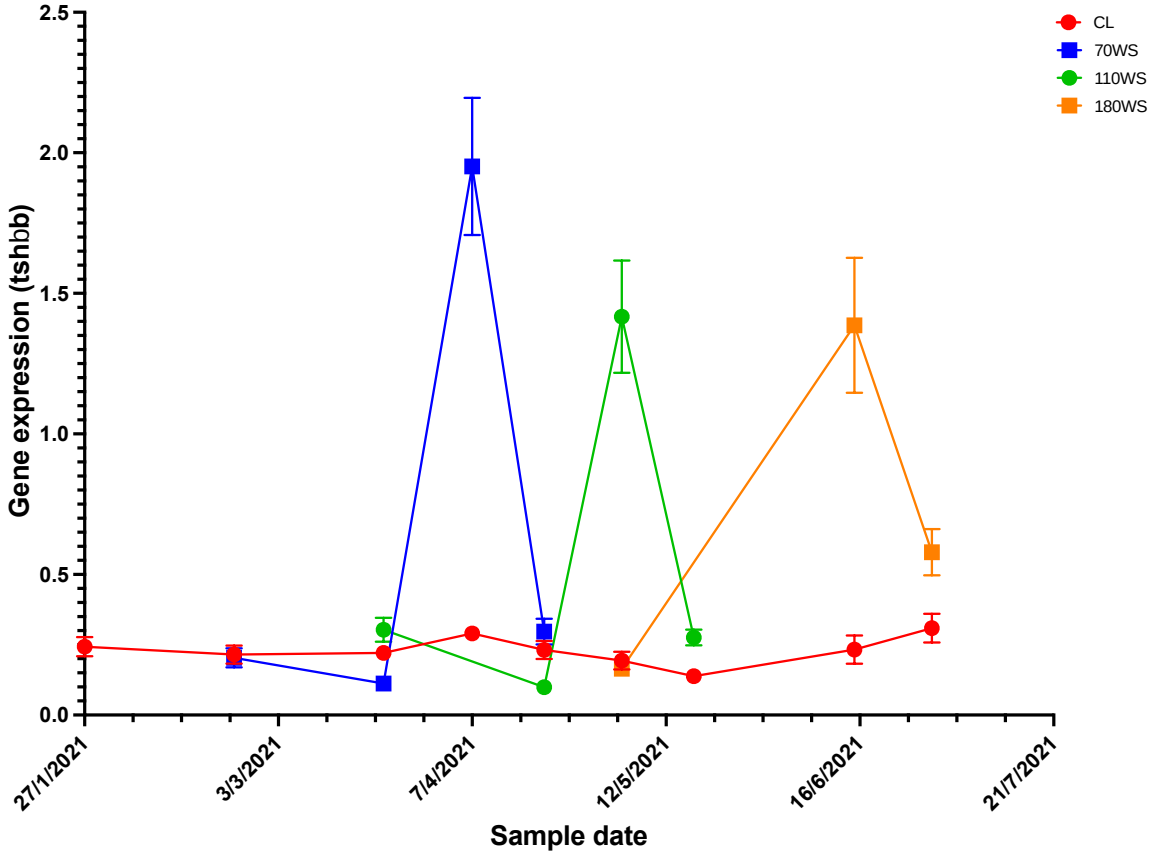

Figure 3.5.1 Tshβb-expression: 70WS, 110WS, 180WS and CL. The pituitary concentration of tshβb over a period, where the different colors represent different body sizes (70, 110, 180) of fish and the red line represents continuous light (CL). Circles, squares, and triangles show the average concentration at each sampling, and horizontal lines show standard mean error (+/- SEM). Continuous light (CL) was included in each figure, but it was not included in the data analysis.

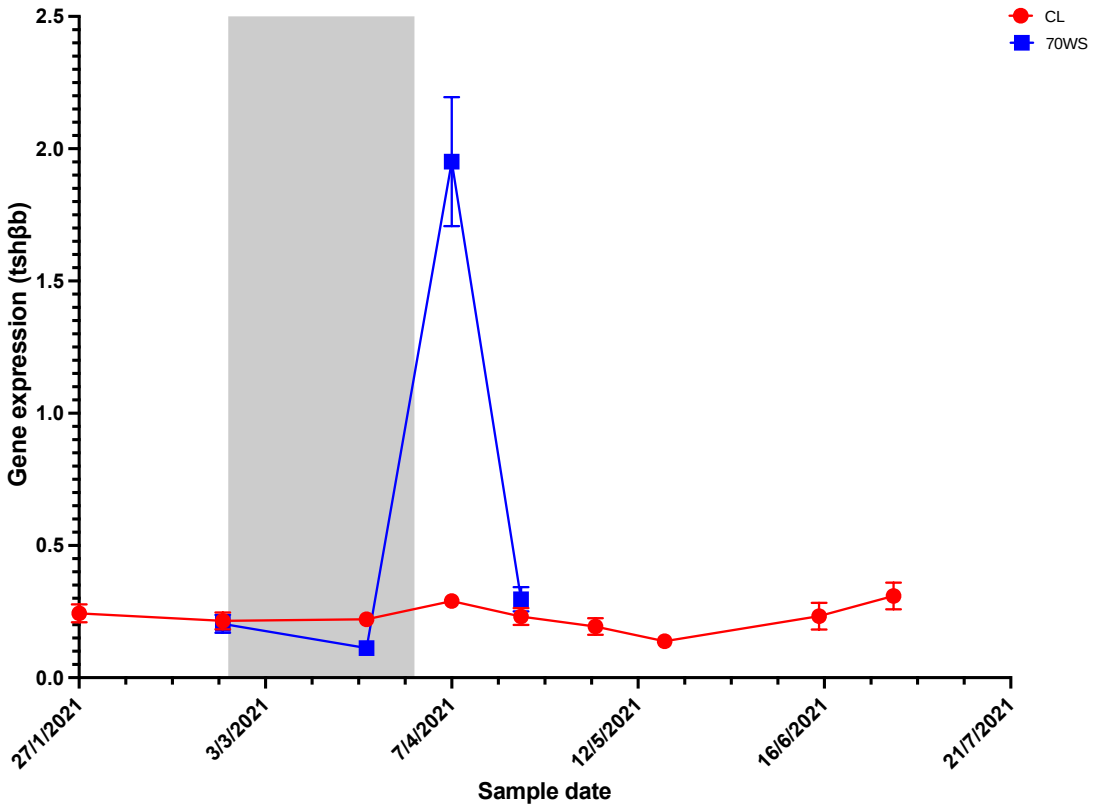

Figure 3.5.2. Tshβb-expression: 70WS and CL. The blue line represents body size 70 and the red line represents continuous light (CL). Circles and squares show the average concentration at each sampling and horizontal lines show standard mean error (+/- SEM). The gray area in the figure indicates a five-week winter signal period (WS).

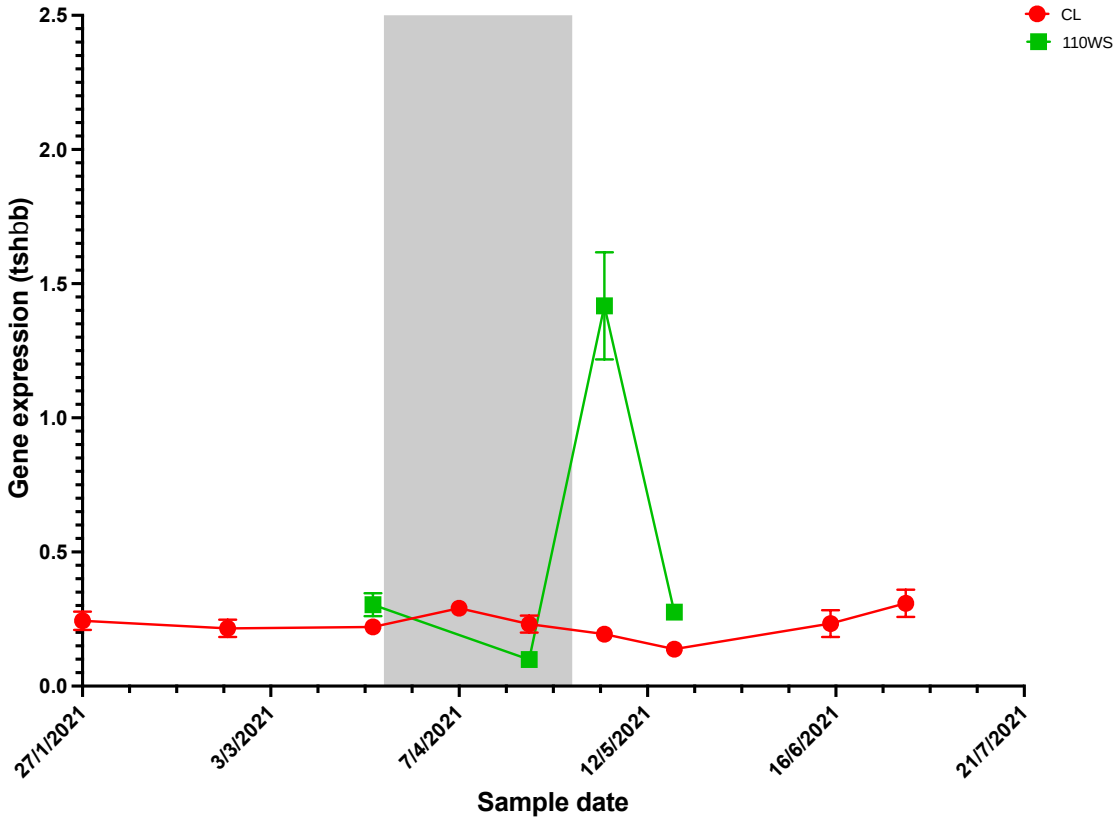

Figure 3.5.3. Tshβb-expression: 110WS and CL. Green line represents body size 110 and the red line represents continuous light (CL). Circles and squares show the average concentration at each sampling and horizontal lines show standard mean error (+/- SEM). The 5-week winter signal (WS) period is indicated by the grey area in the figure.

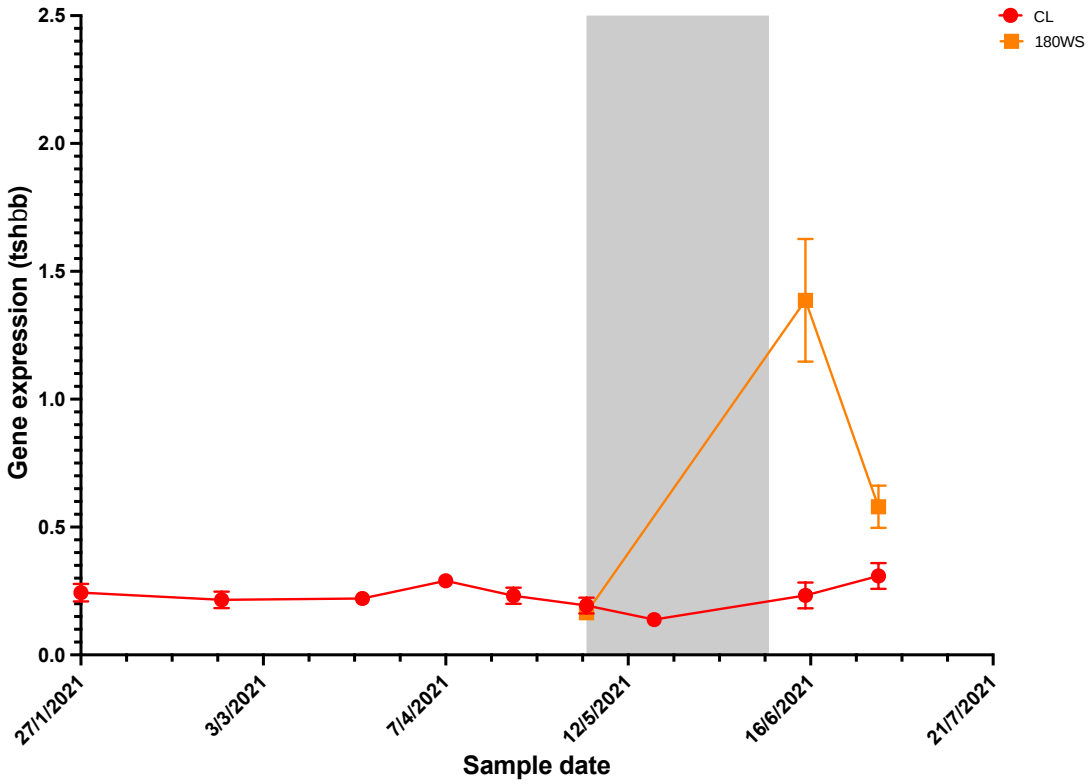

Figure 3.5.4. Tshβb-expression: 180WS and CL. The orange line represents body size 180 and the red line represents continuous light (CL). Circles and squares show the average concentration at each sampling and horizontal lines show standard mean error (+/- SEM). The 5-week winter signal (WS) period is indicated by the grey area in the figure.

4. Discussion

4.1. Discussion of results

In this project, we aim to gain a broader understanding of the role of the gene tshβb in Atlantic salmon, especially its role in three different body sizes and how photoperiod affects early sexual maturation and smoltification in freshwater. In the following sections, the main findings from the project and its implications are discussed.

4.1.1 Effects of different photoperiod regimes on changes in body weight and length over time

All four experimental groups had a steady growth rate throughout the experiment, with minor variations over time. In this experiment, introducing a 5-week winter signal (WS) had no significant effect in any of the three body sizes, nor was a growth advantage observed in the group with continuous light (CL). Contrary to our findings, Sigholt et al. (1998) showed in their research that rearing Atlantic salmon parrs in a light regime with short days (LD8.15:15.45) had a lower growth rate than those reared in a long light regime (LD24:0). The results also showed that fish bred in a light regime with short days continued to have a lower growth rate even after exposure to long light (LD24:0) for the remainder of the experiment compared to those reared in a long light regime throughout the study (Sigholt et al., 1998).

Imsland et al. (2014) reported similar findings to ours, where no differences in body weight were observed between the Atlantic salmon groups, although differences in the proportion of mature males were seen between the groups. Moreau & Fleming (2012) support our findings in that mechanisms that promote somatic growth in Atlantic salmon do not appear to play a causal role at the onset of maturation (Moreau & Fleming, 2012). Thorpe (2007) reports that the essential link between early maturation and the availability of appropriate resources such as internal signals such as temperature and photoperiod, enough energy, absolute size, or age must be close to achieving maturation (Thorpe, 2007). In our findings, it is possible to believe that both the photoperiod and absolute size of the fish help to disrupt smoltification and promote maturation. Recent research conducted by Pino Martinez et al. (2021) matches our results in that the photoperiod has not caused significant differences in the changes in body growth over time between the different groups. Pino Martinez et al. (2021) showed in their study that the introduction of a 5-week winter signal (LD12:12) did not result in a lower growth rate than in Atlantic salmon reared at LD24:0. Their research also indicated a slight

compensatory growth and slightly better smoltification in WS than it did in LL, although evidence of size-induced smoltification was also present in this group (Pino Martinez et al., 2021). Our results support this claim and further suggest that the introduction of WS at a body size between 80g-110g shows the best signs of smoltification, based on our K values in the results.

Two factors that could affect the growth rate in this study are temperature regimes and feeding regimes. Warmer temperatures can stimulate fish growth, while colder temperatures can stagnate growth (Mobley et al., 2021; Thyholdt, 2014). Pino Martinez et al. (2021) showed in their study that salmon reared at 12.5°C showed the best growth within the feeding regime. In this experiment, all fish were reared under a constant temperature of 12.5°C, but temperature regimes were not analyzed as a predictor in this experiment. The feeding regime may also affect growth rate as the groups introduced to the WS signal were fed during light hours during the winter signal period, while the CL group was continually fed. The feeding regime was also not analyzed as a predictor in our experiment, but as all the groups show a uniform growth rate, it does not appear that it has influenced changes in the body growth of the salmon throughout the experiment.

4.1.2 Effects of different photoperiod regimes on changes in condition factor (K) over time

The average condition factor (K) in all four groups fluctuated throughout the experiment shown in Figure 3.3.1. Photoperiod has been revealed in previous research to play a key role in controlling, among other things, the timing of reproduction, smoltification, and onset of spawning behavior in Atlantic salmon (Hoar, 1988; McCormick et al., 2002). Photoperiod affects changes in the condition factor and has linked manipulation of light regimes with condition factor and smoltification (Björnsson et al., 1989; Saunders et al., 1985; Strand et al., 2018). Exposing the salmon to a winter signal period (WS) has been shown in previous studies to be necessary to achieve successful smoltification in the salmon (Björnsson et al., 1989). In this study, three groups were introduced for a 5-week winter signal period, with the largest group (180WS) appearing not to complete smoltification and starting to show signs of maturation.

The current study used condition factor (K) to help indicate the developmental state of the fish. Our findings show that the 70WS and 110WS groups show typical K values for the

smoltification of salmon, which showed values that started low, increased before smoltification, and then decreased again throughout the smoltification period. These values are the typical condition factor observed for healthy smoltification fish (Samson et al., 1999) and, therefore, suggest that the fish in these groups had healthy smoltification. Sigholt et al. (1998) support our findings by reporting a significant reduction in K throughout the smoltification period in Atlantic salmon parrs that were exposed to long light after a short-day regime. Strand et al. 2018 also showed in their two experiments that K was significantly reduced after long light regimes after exposure to a winter signal period which also supports our findings in the 70WS and 110WS groups.

There was a big difference in the development of the condition factor in the 180WS and CL groups, where the K values indicated typical signs of maturation after smolt or indicated that the fish did not smoltify normally. Imsland et al. (2014) present in their study that salmon will develop smolt characteristics before it reaches 200 grams, and the best condition factor was reported in size ranges (112-162 grams) regardless of photoperiod. This may suggest that smoltification is size-driven (Imsland et al., 2014) and that the fish in our 180WS group was too large, which resulted in incomplete smoltification and the development of signs of maturation.

Handeland & Stefansson (2001) confirm our findings in the CL group by reporting disturbed development in smolts when the fish are exposed to continuous light. A decrease in growth was also reported in the group reared under LD24:0 after transfer to seawater (Handeland & Stefansson, 2001). An early commitment to maturation by activation of the BPG axis and highly stimulating rearing conditions may preclude smoltification as a life strategy in salmon (Martinez et al., 2023). When the fish are smoltified in our study seems to depend both on photoperiod regimes and body sizes.

In this study, we had a constant temperature of 12.5°C and as temperature regimes were not analyzed as a predictor in this experiment, we can bypass whether different temperatures affect the condition factor in a positive or negative direction.

4.1.3. Effects of different photoperiod regimes on changes in GSI levels over time

The mean gonadosomatic index (GSI) in the 70 and the CL groups remained low throughout the experiment, indicating that the fish remained immature. The group of 110 also showed a low GSI throughout the study except at the end, where the peak in the massive error bars comes from one mature fish. We decided to keep the data of the one fish that had become mature as a representation of the last group. The group of 180, on the other hand, had an increasing GSI trend toward the end of the experiment. By the last sampling, it was clear that many fish were becoming mature, with a GSI level approaching 1%. According to Martinez (2021), GSI levels of 0.0-0.06 indicates immaturity, 0.06-0.1 indicates early maturation, 0.1-1% imply maturing, and GSI < 1% is sexually mature salmon. The 70, 110, and CL groups maintain a GSI ≤ 0.06% throughout the attempt implying immature fish except for one fish in the 110-group that showed a 0.1 < GSI ≤ 1% meaning that the fish is maturing. The 180 group maintains a steady line before the last two groups show an early maturation stage of 0.06 < GSI ≤ 0.1% and mature salmon 0.1 < GSI ≤ 1%.

Some fish maturation was seen at the end of the group of 180 which may be correlated with the size of the fish as the GSI represents the ratio between the relative size of the gonads and the total body growth of the fish (Devlaming et al., 1982). We see trends that if you postpone the winter signal to larger body sizes, there is a chance to increase the probability of maturation (Imsland et al., 2014). Our results disagree with Hansen et al. (1992), who discovered that exposure to continuous light at the same time as a natural photoperiod resulted in fewer mature salmon compared to the groups of fish exposed only to a natural photoperiod.

GSI was investigated in this study to look at the maturity levels of the salmon. The GSI levels were used as an indicator factor for the activation of the BPG axis in this research, and maturation of the gonads comes from the activation of the axis. Changes in GSI levels correlate with the follicle-stimulating hormone (FSH), which is central to sexual maturation and reproduction (Pino Martinez et al., 2021). Low/high levels of GSI and FSH indicate an immature/mature salmon. Pituitary FSH levels were not analyzed in this study, but GSI levels make it possible to see if there is a relationship between TSH and GSI and if activation of the tshβb peak has a correlation with the increase of GSI (Pino Martinez et al., 2021).

4.1.4. Effects of different photoperiod regimes on changes in mean pituitary concentration of tshβb at three different body sizes

In this experiment, the mean concentration of tshβb increased in all body size groups (70, 110, and 180) after the end of the 5-week winter signal (WS) and a subsequent return to continuous light (CL). The group with continuous light (CL) showed no significant change in concentration throughout the experiment (Figure 3.5.1.), and no peak of tshβb was observed for this group in this study. The fact that we did not get any expression of tshβb has not been detected in any previous research, and it shows the potential physiological disturbance of continuous light. Results show a disruption of the physiology in the CL group and indicate that a continuous light regime can disrupt the physiology of developing Atlantic salmon in the freshwater stage under intensive aquaculture environments. The significant peaks observed in the 70WS, 110WS, and 180WS groups imply that exposing Atlantic salmon to a winter signaling period in intensive environments is of great matter. Based on our results, the interaction between photoperiod and time can have a meaningful effect on the changes in the mean concentration of tshβb and that photoperiod is a key regulator of the expression of tshβb in Atlantic salmon.

Irachi et al. 2021 support our findings by reporting in their study that an increasing photoperiod can directly cause changes in tshβb expression in Atlantic salmon, and exposure to increased day length (LD16:8) resulted in a fivefold increase in pituitary tshβb mRNA levels after only ten days. Since no peak of tshβb was seen in the CL group, it is possible to suggest that the use of a winter signal period in intensive farming environments is necessary to achieve the gene expression of tshβb (Irachi et al., 2021).

The temperature was not calculated as a predictor in this experiment, but since the temperature is an important part of development in salmon, future studies will be interesting to address the relationship between tshβb and temperature and how they are related.

To assess whether the expression of tshβb is related to smoltification and maturation in Atlantic salmon, comparisons between mean condition factor (K) levels, gonadosomatic index (GSI) levels, and tshβb expression were assembled. In our 70WS and 110WS groups, we see smoltified fish and a significant tshβb expression 7th of April 2021 in 70WS and the 4th of May 2021 in 110WS (figure 3.5.1.). A possible connection could be between healthy smolt and

the peaks of smoltification. However, no correlation factor has been seen between the peaks of tshβb after the winter signal and the increase in GSI in our findings.

Pituitary tshβb expression increased in early April in the 70WS group, in early May in the 110WS group, and in mid-June in the 180WS group. Maturation occurred in one fish in the 110WS group at the end of June, while the 180WS group began to show signs of maturation in mid-July (figure 3.4.1.). Pituitary tshβb expression was not present in our CL group but based on previous research by Martinez et al. (2023) and unpublished work here at UiB we expect to observe sexual maturation if the experiment had continued over a more extended period. Fleming et al. (2019) suggested in their study that tshβb and PT-TSH are homologous and due to the conserved role of PT-TSH, a conserved role of tshβb in regulating the BPG axis was assumed. In mammals and birds, PT-TSH activates the hypothalamus and plays a key role in sexual development (Yoshimura, 2013).

In this study, we wanted to investigate whether pituitary expression of tshβb plays a similar role in maturation in Atlantic salmon and whether the recent location of tshβb-expressing cells dorsally in the pars nervosa (PN) may be homologous to PT-TSH in mammals and birds that regulates maturation (Fleming et al., 2019).

Activation of PT-TSH in the hypothalamus in mammals and birds creates a stimulatory pathway where gonadotropins are released from the pituitary that induces gonadal development (Dardente et al., 2010). In mammals and birds, GSI levels and PT-TSH increases are closely correlated (Nakane et al., 2013). PT-TSH is the regulator of activation of GnRH and Kiss from the brain to release FSH in the pituitary gland to GSI levels for gonadal maturation (Ubuka et al., 2013; Yoshimura, 2013). An increase in GSI levels, on the other hand, appears to be disconnected from the expression of tshβb, and the tshβb peak presumably does not activate FSH as no increase in GSI levels was seen. However, our findings show that tshβb expression and the role of the brain-pituitary axis are not related to maturation in Atlantic salmon as they are in mammals and birds. It is now possible to see a clear difference between the developmental pattern we see in mammals and birds and Atlantic salmon, suggesting alternative or additional physiological signaling pathways regulating sexual maturation in salmon.

There are strong indications that the tshβb gene plays a role in smoltification, and we see an attention maturation relationship. The results of this study suggest that both things can happen independently but that tshβb seems to play a role in coordinating the events. It is possible to cause a peak of tshβb based on strict light, and in intensive farms such as RAS and flow-through facilities, the light can be turned on which provides the fish a long day, leading to an increase of tshβb. Tshβb may have a prominent role in life history transitions, but our results show that the peak of tshβb does not cause activation of the BPG axis as PT-TSH does in mammals and birds during sexual maturation. Mammals and birds depend on a significant expression of TSH to achieve maturation (Fleming et al., 2019). In this study, Atlantic salmon of three body sizes (70, 110, and 180) introduced to WS had a peak of tshβb, meaning that tshβb is not necessarily size driven. Even though the three body size groups had a significant expression of tshβb, not all fish matured. This may indicate that there is a disconnection between TSH and the reproductive pathway of salmon.

The results of this study suggest that the introduction of different light regimes results in an increased peak of tshβb, but it is not directly linked to maturation in the same way PT-TSH is in mammals and birds. In practical terms, this study shows that extended use of CL must be done with caution at increased temperatures, as this will promote early maturation in male salmon (Imsland et al., 2014).

5. Experimental design

In this experiment, the RNA extraction protocol was optimized for small/large pituitaries throughout the laboratory work. A standard curve was created for elf-1α prior to the mRNA expression analysis, but elf-1α was highly variable and therefore not a submission for a reference gene suggesting that elf-1α is not a suitable reference gene for the pituitary for this current experiment Beta-actin (β-actin) proved to work well and was therefore used as a reference gene in this study.

This research was conducted in the absence of commercial or financial circumstances that could be interpreted as a potential conflict of interest. If we had the opportunity to extend the experiment, we may have seen maturation in the CL group, similar to the maturation levels at 12.5°C seen in Martinez et al. (2023). We kept the salmon to 400-500 g, which may have

influenced our results by not showing maturation in the CL group, so interest for the next study could be to keep the experiment for a longer period.

6. Concluding marks

Early post-smolt maturation of male Atlantic salmon has been shown to be a growing problem in land-based aquaculture facilities such as RAS and flow-through and it has become a welfare and economic issue for the aquaculture industry. Farming of larger post-smolts proves to be more robust and able to handle the transfer to open seawater better. Nevertheless, rearing post-smolts is connected to biological challenges, and early ripening. Intensive production systems such as RAS and flow-through mainly consist of environmental parameters such as continuous light and warmer temperatures. These parameters together with the possibility of constant feeding, are the main reasons for the increased occurrence of early ripening in these closed systems. To reduce the cases of early maturation and to promote higher growth rates in Atlantic salmon, it is significant to identify a photoperiod and a temperature regime that optimizes these two factors. In this study, a photoperiod regime, a constant water temperature of 12.5°C and exposure to a winter signal period (WS) before continuous light (CL) in two different body sizes (70 and 110) presented to be the best cases to prevent early maturation while the groups promote an increased growth rate.

This study was conducted to gain a broader understanding of how the effects of the photoperiod regime influence the role of tshβb for smoltification and early maturation in male Atlantic parr salmon at three different stock sizes under photoperiod treatments in a flow-through system. The aim of this thesis was to gain a wider understanding of the role and mechanism of pituitary gene expression tshβb in Atlantic salmon and whether the gene is involved in the BPG axis in the same way as the β-subunit of TSH (PT-TSH) in mammals and birds.

In this research, three different body sizes of Atlantic salmon were introduced to a 5-week winter signal period before returning to continuous light, and one group was presented to continuous light throughout the experiment. In this experiment, the introduction of a 5-week WS had no significant effect on growth rate in any of the three body sizes, nor was a growth advantage observed in the group with CL. However, both photoperiod and body size may help to disrupt smoltification and promote maturation, as more mature males were seen in the largest body size (180).

Based on our findings, we can see that the introduction of a five-week WS may influence the 70 and 110 body sizes group to smoltify. Martinez et al. (2021) observed in their study that introducing WS is not necessary to achieve maturation, as high male maturation was seen in the group with CL. The results of this study show that smoltification may be size-driven and that the 180 and CL groups may have been too large, resulting in incomplete smoltification and the development of signs of maturation. Based on our K-values, our results suggest that a body size between 70g-110g may be a good size to achieve healthy smoltification in Atlantic salmon.

The activation of tshβb was observed in three body sizes (70, 110, and 180) introduced to WS, which may mean that the expression of tshβb is not necessarily based on the size of the fish. The continuous light (CL) group had no peak of tshβb, indicating a potential physiological disruption of exposure to a continuous light regime. Based on what we know about mammals and birds, a peak in PT-TSH leads to maturation. Contrary, the results in this study show that a tshβb-peak does not lead to maturation in Atlantic salmon. No correlation was observed between the peaks of tshβb after the winter signal and the increase in GSI levels in this study. In mammals and birds, GSI levels and PT-TSH increases are closely correlated, whereas in salmon these parameters appear to be disconnected, and the tshβb peak most likely does not activate FSH for gonadal maturation. In Atlantic salmon, it is possible to cause a change in the expression of tshβb based only on introducing light which is what the results show. Expression of tshβb does not activate the BPG axis and is therefore not linked to maturation in Atlantic salmon as it is in mammals and birds. Based on the results, it is now possible to see a clear difference between the role of TSH in mammals, birds, and fish.

7. Future perspectives

In this study, the three different body sizes of Atlantic salmon were exposed to a constant water temperature of 12.5°C, WS (LD 12:12), and CL (24:0) to analyze the parameters. Exposure to a water temperature of 12.5°C and the introduction of WS in a flow-through system led to fewer cases of early maturing without a reduction in growth rate. If this experiment were to be repeated, it would have been interesting to include more temperature regimes and further optimization of WS protocol. This could possibly help us to strengthen the role of temperature and WS on maturing obligations. As it has been known that Atlantic salmon reared at higher temperatures have a higher percentage of sexual maturation, rearing at a higher temperature would be interesting (Fjelldal et al., 2011; Good & Davidson, 2016; Imsland et al., 2014).

This study investigated the role of tshβb and whether it has the same role in fish as it does in mammals and birds. Although we see a peak in tsh in all body sizes, it does not appear to regulate sexual maturation in Atlantic salmon. For further study, it could be interesting to carry out the study in a RAS plant and see if recyclable water rather than changing fresh water would affect tshβb differently.

A peak in PT-TSH in mammals and birds is essential for maturation to occur (Yoshimura, 2013). The gene is extremely expressed during very important developmental times, so it is very important. It is highly conserved from an evolutionary point of view, which usually indicates that it is a very important molecule. To map the exact function of tshβb, it may be interesting in future studies to knock out the gene using the CRISPR method.

8. Bibliography

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Appendix I. Dataset and overview of values

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

fish_id	SAMPLING	tank_id	length (cm)	weight (g)	k	sex	gonad wht (g)	GSI (%)	date	exp_group
1	b	1	15.0	43.3	1.28296	M	0.01	0.023	2021-01-27	LL
2	b	1	14.7	39.5	1.24350	M	0.012	0.030	2021-01-27	LL
3	b	2	15.1	45.6	1.32445	M	0.01	0.022	2021-01-27	LL
4	b	2	15.5	44.9	1.20573	M	0.011	0.024	2021-01-27	LL
5	b	3	15.1	43.6	1.26636	M	0.015	0.034	2021-01-27	LL
6	b	3	15.1	41.0	1.19084	M	0.013	0.032	2021-01-27	LL
7	b	4	14.9	39.5	1.19258	M	0.014	0.035	2021-01-27	LL
8	b	4	15.5	43.0	1.15471	M	0.012	0.028	2021-01-27	LL
9	b	4	14.6	35.1	1.12624	M	0.013	0.037	2021-01-27	LL
10	b	4	14.7	36.8	1.15944	M	0.015	0.041	2021-01-27	LL
11	b	9	15.5	46.0	1.23527	M	0.01	0.022	2021-01-27	LL
12	b	9	14.2	37.3	1.30270	M	0.011	0.029	2021-01-27	LL
13	1	1	15.8	66.8	1.69358	M	0.026	0.039	2021-02-23	70WS
14	1	1	18.1	80.0	1.34913	M	0.032	0.040	2021-02-23	70WS
15	1	1	17.5	75.3	1.40408	M	0.025	0.033	2021-02-23	70WS
16	1	1	17.8	81.6	1.44687	M	0.016	0.020	2021-02-23	70WS
17	1	1	17.4	69.3	1.31548	M	0.019	0.027	2021-02-23	70WS
18	1	2	16.9	62.2	1.28864	M	0.023	0.037	2021-02-23	70WS
19	1	2	17.4	59.3	1.12566	M	0.023	0.039	2021-02-23	70WS
20	1	2	18.3	78.9	1.28743	M	0.021	0.027	2021-02-23	70WS
21	1	2	17.4	68.3	1.29650	M	0.02	0.029	2021-02-23	70WS
22	1	2	17.3	63.9	1.23413	M	0.017	0.027	2021-02-23	70WS
23	1	2	18.2	77.3	1.28223	M	0.02	0.026	2021-02-23	70WS
24	1	2	16.1	57.3	1.37302	M	0.014	0.024	2021-02-23	70WS
25	1	9	17.8	75.8	1.34403	M	0.024	0.032	2021-02-23	LL
26	1	9	17.2	68.5	1.34619	M	0.02	0.029	2021-02-23	LL
27	1	9	17.5	67.6	1.26134	M	0.019	0.028	2021-02-23	LL
28	1	9	17.8	71.3	1.26424	M	0.018	0.025	2021-02-23	LL
29	1	10	16.8	64.9	1.36873	M	0.023	0.035	2021-02-23	LL
30	1	10	16.8	65.7	1.38560	M	0.026	0.040	2021-02-23	LL
31	1	10	18.0	75.4	1.29287	M	0.021	0.028	2021-02-23	LL
32	1	9	17.9	74.7	1.30245	M	0.025	0.033	2021-02-23	LL
33	1	9	17.4	66.8	1.26803	M	0.02	0.030	2021-02-23	LL
34	1	9	16.5	60.2	1.34012	M	0.021	0.035	2021-02-23	LL
35	1	9	16.3	56.0	1.29308	M	0.022	0.039	2021-02-23	LL
36	1	10	17.1	65.6	1.31194	M	0.016	0.024	2021-02-23	LL
37	2	1	20.6	108.8	1.24414	M	0.036	0.033	2021-03-22	70WS
38	2	1	19.2	81.4	1.14978	M	0.041	0.050	2021-03-22	70WS
39	2	1	19.0	97.2	1.41770	M	0.025	0.026	2021-03-22	70WS
40	2	1	18.7	91.5	1.39986	M	0.036	0.039	2021-03-22	70WS
41	2	1	18.4	80.4	1.29063	M	0.026	0.032	2021-03-22	70WS
42	2	1	20.6	110.2	1.26107	M	0.033	0.030	2021-03-22	70WS
43	2	2	19.4	96.8	1.32509	M	0.04	0.041	2021-03-22	70WS
44	2	2	20.2	113.1	1.37217	M	0.03	0.027	2021-03-22	70WS
45	2	2	19.0	97.7	1.42441	M	0.029	0.030	2021-03-22	70WS
46	2	2	22.3	135.6	1.22259	M	0.04	0.030	2021-03-22	70WS
47	2	2	19.4	97.9	1.34043	M	0.026	0.027	2021-03-22	70WS
48	2	2	18.4	83.8	1.34457	M	0.027	0.032	2021-03-22	70WS
49	2	3	19.5	107.0	1.44237	M	0.044	0.041	2021-03-22	110WS
50	2	3	20.0	111.0	1.38725	M	0.033	0.030	2021-03-22	110WS
51	2	3	19.0	95.7	1.39525	M	0.033	0.034	2021-03-22	110WS
52	2	3	20.8	125.8	1.39761	M	0.036	0.029	2021-03-22	110WS
53	2	3	19.5	100.4	1.35403	M	0.031	0.031	2021-03-22	110WS
54	2	3	20.5	124.2	1.44165	M	0.045	0.036	2021-03-22	110WS
55	2	3	20.5	126.0	1.46254	M	0.046	0.037	2021-03-22	110WS
56	2	4	21.1	114.7	1.22100	M	0.031	0.027	2021-03-22	110WS
57	2	4	20.4	119.7	1.40995	M	0.038	0.032	2021-03-22	110WS
58	2	4	21.6	122.3	1.21357	M	0.044	0.036	2021-03-22	110WS
59	2	4	20.7	112.7	1.27005	M	0.044	0.039	2021-03-22	110WS
60	2	4	20.4	110.2	1.29793	M	0.035	0.032	2021-03-22	110WS
61	2	9	21.6	115.3	1.14411	M	0.033	0.029	2021-03-22	LL

62	2	9	21.5	115.1	1.15814	M	0.034	0.030	2021-03-22	LL
63	2	9	18.6	96.8	1.50431	M	0.04	0.041	2021-03-22	LL
64	2	9	21.6	130.6	1.29613	M	0.036	0.028	2021-03-22	LL
65	2	9	22.0	128.5	1.20718	M	0.04	0.031	2021-03-22	LL
66	2	9	21.4	115.6	1.17986	M	0.041	0.035	2021-03-22	LL
67	2	10	22.1	129.7	1.20189	M	0.038	0.029	2021-03-22	LL
68	2	10	20.1	94.2	1.16001	M	0.031	0.033	2021-03-22	LL
69	2	10	21.8	127.6	1.23163	M	0.043	0.034	2021-03-22	LL
70	2	10	21.8	124.9	1.20557	M	0.034	0.027	2021-03-22	LL
71	2	10	20.7	112.9	1.27287	M	0.035	0.031	2021-03-22	LL
72	2	10	19.3	97.6	1.35762	M	0.027	0.028	2021-03-22	LL
73	3	1	23.0	164.2	1.34955	M	0.044	0.027	2021-04-07	70WS
74	3	1	22.5	144.5	1.26859	M	0.041	0.028	2021-04-07	70WS
75	3	1	20.5	108.9	1.26406	M	0.034	0.031	2021-04-07	70WS
76	3	1	20.5	115.2	1.33718	M	0.038	0.033	2021-04-07	70WS
77	3	1	20.8	122.8	1.36461	M	0.034	0.028	2021-04-07	70WS
78	3	1	21.0	130.0	1.40374	M	0.052	0.040	2021-04-07	70WS
79	3	2	19.5	103.0	1.38910	M	0.04	0.039	2021-04-07	70WS
80	3	2	20.0	102.0	1.27500	M	0.039	0.038	2021-04-07	70WS
81	3	2	21.5	141.8	1.42679	M	0.049	0.035	2021-04-07	70WS
82	3	2	20.0	115.1	1.43875	M	0.037	0.032	2021-04-07	70WS
83	3	2	19.2	93.3	1.31819	M	0.032	0.034	2021-04-07	70WS
84	3	2	20.0	113.2	1.41500	M	0.04	0.035	2021-04-07	70WS
85	3	9	22.0	125.6	1.17956	M	0.033	0.026	2021-04-07	LL
86	3	9	22.5	146.0	1.28176	M	0.048	0.033	2021-04-07	LL
87	3	9	21.5	127.6	1.28391	M	0.04	0.031	2021-04-07	LL
88	3	9	22.5	149.0	1.30809	M	0.037	0.025	2021-04-07	LL
89	3	9	23.5	151.0	1.16352	M	0.045	0.030	2021-04-07	LL
90	3	9	22.6	136.0	1.17819	M	0.033	0.024	2021-04-07	LL
91	3	10	22.7	154.5	1.32084	M	0.06	0.039	2021-04-07	LL
92	3	10	22.3	138.4	1.24802	M	0.044	0.032	2021-04-07	LL
93	3	10	22.9	143.3	1.19327	M	0.046	0.032	2021-04-07	LL
94	3	10	20.7	117.7	1.32698	M	0.047	0.040	2021-04-07	LL
95	3	10	21.3	118.8	1.22936	M	0.028	0.024	2021-04-07	LL
96	3	10	21.0	128.3	1.38538	M	0.049	0.038	2021-04-07	LL
97	4	1	21.9	127.9	1.21769	M	0.048	0.038	2021-04-20	70WS
98	4	1	25.3	201.2	1.24241	M	0.058	0.029	2021-04-20	70WS
99	4	1	21.3	126.0	1.30417	M	0.041	0.033	2021-04-20	70WS
100	4	1	21.8	137.2	1.32401	M	0.054	0.039	2021-04-20	70WS
101	4	1	19.5	95.6	1.28890	M	0.032	0.033	2021-04-20	70WS
102	4	1	20.9	117.0	1.28180	M	0.042	0.036	2021-04-20	70WS
103	4	1	22.6	145.4	1.25988	M	0.047	0.032	2021-04-20	70WS
104	4	2	22.0	139.9	1.31358	M	0.043	0.031	2021-04-20	70WS
105	4	2	24.6	189.0	1.26923	M	0.055	0.029	2021-04-20	70WS
106	4	2	25.1	195.9	1.23877	M	0.074	0.038	2021-04-20	70WS
107	4	2	20.2	109.0	1.32279	M	0.039	0.036	2021-04-20	70WS
108	4	2	21.3	122.3	1.26537	M	0.038	0.031	2021-04-20	70WS
109	4	3	23.4	182.4	1.42356	M	0.071	0.039	2021-04-20	110WS
110	4	3	22.8	152.3	1.28498	M	0.042	0.028	2021-04-20	110WS
111	4	3	22.5	152.8	1.34145	M	0.051	0.033	2021-04-20	110WS
112	4	3	22.7	149.7	1.27981	M	0.043	0.029	2021-04-20	110WS
113	4	3	23.8	172.4	1.27844	M	0.07	0.041	2021-04-20	110WS
114	4	4	25.1	188.9	1.19431	M	0.069	0.037	2021-04-20	110WS
115	4	4	24.5	196.5	1.33638	M	0.074	0.038	2021-04-20	110WS
116	4	4	23.6	183.0	1.39217	M	0.05	0.027	2021-04-20	110WS
117	4	4	22.3	154.4	1.39212	M	0.051	0.033	2021-04-20	110WS
118	4	4	22.9	163.1	1.35807	M	0.051	0.031	2021-04-20	110WS
119	4	4	22.7	169.7	1.45053	M	0.073	0.043	2021-04-20	110WS
120	4	4	20.7	118.8	1.33927	M	0.03	0.025	2021-04-20	110WS
121	4	9	24.4	173.1	1.19145	M	0.05	0.029	2021-04-20	LL
122	4	9	26.7	249.5	1.31054	M	0.089	0.036	2021-04-20	LL
123	4	9	22.9	149.0	1.24074	M	0.039	0.026	2021-04-20	LL
124	4	9	25.2	199.9	1.24914	M	0.075	0.038	2021-04-20	LL

125	4	9	23.0	145.0	1.19175	M	0.039	0.027	2021-04-20	LL
126	4	10	25.0	192.6	1.23264	M	0.064	0.033	2021-04-20	LL
127	4	10	24.3	171.8	1.19702	M	0.051	0.030	2021-04-20	LL
128	4	10	22.0	138.6	1.30165	M	0.039	0.028	2021-04-20	LL
129	4	10	25.2	204.2	1.27582	M	0.06	0.029	2021-04-20	LL
130	4	10	24.0	167.0	1.20790	M	0.044	0.026	2021-04-20	LL
131	4	10	22.7	147.9	1.26476	M	0.042	0.028	2021-04-20	LL
132	4	10	24.3	176.0	1.22657	M	0.053	0.030	2021-04-20	LL
133	5	1	25.8	207.4	1.20767	M	0.054	0.026	2021-05-04	70WS
134	5	1	25.2	199.6	1.24727	M	0.042	0.021	2021-05-04	70WS
135	5	1	25.9	207.6	1.19489	M	0.057	0.027	2021-05-04	70WS
136	5	1	22.5	136.1	1.19484	M	0.045	0.033	2021-05-04	70WS
137	5	1	23.2	150.9	1.20844	M	0.041	0.027	2021-05-04	70WS
138	5	1	24.8	180.4	1.18272	M	0.057	0.032	2021-05-04	70WS
139	5	1	23.5	159.6	1.22979	M	0.046	0.029	2021-05-04	70WS
140	5	2	27.2	231.3	1.14939	M	0.063	0.027	2021-05-04	70WS
141	5	2	26.1	191.7	1.07820	M	0.056	0.029	2021-05-04	70WS
142	5	2	24.6	186.7	1.25412	M	0.087	0.047	2021-05-04	70WS
143	5	2	23.2	155.4	1.24480	M	0.052	0.033	2021-05-04	70WS
144	5	2	22.6	133.6	1.15739	M	0.048	0.036	2021-05-04	70WS
145	5	3	25.4	228.8	1.39622	M	0.065	0.028	2021-05-04	110WS
146	5	3	24.5	185.4	1.26070	M	0.066	0.036	2021-05-04	110WS
147	5	3	23.5	175.2	1.34999	M	0.055	0.031	2021-05-04	110WS
148	5	3	25.0	195.6	1.25184	M	0.053	0.027	2021-05-04	110WS
149	5	3	24.2	178.3	1.25807	M	0.065	0.036	2021-05-04	110WS
150	5	3	23.4	174.2	1.35988	M	0.058	0.033	2021-05-04	110WS
151	5	3	22.0	137.9	1.29508	M	0.064	0.046	2021-05-04	110WS
152	5	4	26.0	209.3	1.19083	M	0.065	0.031	2021-05-04	110WS
153	5	4	22.3	143.6	1.29491	M	0.042	0.029	2021-05-04	110WS
154	5	4	22.4	150.0	1.33423	M	0.043	0.029	2021-05-04	110WS
155	5	4	23.5	170.1	1.31069	M	0.051	0.030	2021-05-04	110WS
156	5	7	24.0	164.2	1.18779	M	0.049	0.030	2021-05-04	180WS
157	5	7	25.0	224.6	1.43744	M	0.082	0.037	2021-05-04	180WS
158	5	7	25.6	238.4	1.42097	M	0.067	0.028	2021-05-04	180WS
159	5	7	24.0	177.0	1.28031	M	0.049	0.028	2021-05-04	180WS
160	5	8	25.5	200.2	1.20738	M	0.056	0.028	2021-05-04	180WS
161	5	8	24.2	172.5	1.21701	M	0.048	0.028	2021-05-04	180WS
162	5	8	23.3	152.0	1.20164	M	0.04	0.026	2021-05-04	180WS
163	5	8	23.0	134.5	1.10537	M	0.058	0.043	2021-05-04	180WS
164	5	9	26.5	215.4	1.15747	M	0.052	0.024	2021-05-04	LL
165	5	9	22.5	143.9	1.26332	M	0.045	0.031	2021-05-04	LL
166	5	9	25.8	184.5	1.07433	M	0.05	0.027	2021-05-04	LL
167	5	9	25.5	200.9	1.21136	M	0.07	0.035	2021-05-04	LL
168	5	9	26.1	217.9	1.22534	M	0.068	0.031	2021-05-04	LL
169	5	10	25.0	189.1	1.21024	M	0.049	0.026	2021-05-04	LL
170	5	10	22.1	137.1	1.26998	M	0.032	0.023	2021-05-04	LL
171	5	10	23.5	154.6	1.19126	M	0.046	0.030	2021-05-04	LL
172	5	10	25.6	203.2	1.21105	M	0.063	0.031	2021-05-04	LL
173	6	1	25.1	185.7	1.17433	M	0.053	0.029	2021-05-17	70WS
174	6	1	25.1	180.7	1.14259	M	0.062	0.034	2021-05-17	70WS
175	6	1	26.6	226.8	1.20503	M	0.081	0.036	2021-05-17	70WS
176	6	1	26.0	211.6	1.20403	M	0.063	0.030	2021-05-17	70WS
177	6	1	24.9	179.2	1.16082	M	0.05	0.028	2021-05-17	70WS
178	6	1	24.8	185.3	1.21484	M	0.048	0.026	2021-05-17	70WS
179	6	2	24.0	172.4	1.24682	M	0.045	0.026	2021-05-17	70WS
180	6	2	26.9	240.3	1.23452	M	0.077	0.032	2021-05-17	70WS
181	6	2	27.0	223.1	1.13367	M	0.069	0.031	2021-05-17	70WS
182	6	2	27.0	242.1	1.23015	M	0.079	0.033	2021-05-17	70WS
183	6	2	25.3	195.1	1.20475	M	0.062	0.032	2021-05-17	70WS
184	6	2	25.0	193.1	1.23584	M	0.053	0.027	2021-05-17	70WS
185	6	3	23.8	185.9	1.37895	M	0.055	0.030	2021-05-17	110WS
186	6	3	26.4	220.9	1.20056	M	0.07	0.032	2021-05-17	110WS
187	6	3	27.0	254.6	1.29350	M	0.076	0.030	2021-05-17	110WS

188	6	3	24.4	181.1	1.24659	M	0.053	0.029	2021-05-17	110WS
189	6	3	25.7	207.8	1.22406	M	0.082	0.039	2021-05-17	110WS
190	6	3	26.2	221.0	1.22904	M	0.067	0.030	2021-05-17	110WS
191	6	3	26.4	214.0	1.16306	M	0.086	0.040	2021-05-17	110WS
192	6	4	26.0	229.9	1.30803	M	0.066	0.029	2021-05-17	110WS
193	6	4	25.9	205.2	1.18102	M	0.055	0.027	2021-05-17	110WS
194	6	4	28.4	272.1	1.18806	M	0.084	0.031	2021-05-17	110WS
195	6	4	25.4	200.0	1.22017	M	0.067	0.034	2021-05-17	110WS
196	6	4	26.2	227.9	1.26724	M	0.073	0.032	2021-05-17	110WS
197	6	9	26.0	219.1	1.24659	M	0.068	0.031	2021-05-17	LL
198	6	9	28.7	294.8	1.24696	M	0.109	0.037	2021-05-17	LL
199	6	9	26.5	211.8	1.13823	M	0.064	0.030	2021-05-17	LL
200	6	9	25.6	201.0	1.19823	M	0.051	0.025	2021-05-17	LL
201	6	10	27.0	246.0	1.24981	M	0.079	0.032	2021-05-17	LL
202	6	10	26.6	215.7	1.14600	M	0.077	0.036	2021-05-17	LL
203	6	10	26.3	215.3	1.18336	M	0.072	0.033	2021-05-17	LL
204	6	10	28.3	330.5	1.45818	M	0.133	0.040	2021-05-17	LL
205	6	10	25.7	202.6	1.19337	M	0.052	0.026	2021-05-17	LL
206	6	10	27.2	235.1	1.16833	M	0.079	0.034	2021-05-17	LL
207	7	3	26.5	222.4	1.19487	M	0.052	0.023	2021-06-01	110WS
208	7	3	28.5	282.7	1.22121	M	0.112	0.040	2021-06-01	110WS
209	7	3	26.0	211.9	1.20539	M	0.096	0.045	2021-06-01	110WS
210	7	3	24.9	165.3	1.07039	M	0.061	0.037	2021-06-01	110WS
211	7	3	24.1	156.7	1.11920	M	0.043	0.027	2021-06-01	110WS
212	7	4	28.2	272.7	1.21588	M	0.076	0.028	2021-06-01	110WS
213	7	4	28.6	270.6	1.15672	M	0.074	0.027	2021-06-01	110WS
214	7	4	30.4	330.0	1.17450	M	0.118	0.036	2021-06-01	110WS
215	7	4	28.7	278.9	1.17962	M	0.087	0.031	2021-06-01	110WS
216	7	4	29.5	304.3	1.18544	M	0.091	0.030	2021-06-01	110WS
217	7	4	27.0	245.6	1.24768	M	0.073	0.030	2021-06-01	110WS
218	7	4	28.9	267.1	1.10637	M	0.075	0.028	2021-06-01	110WS
219	7	9	30.2	347.0	1.25982	M	0.118	0.034	2021-06-01	LL
220	7	9	27.7	265.9	1.25097	M	0.102	0.038	2021-06-01	LL
221	7	9	29.6	332.9	1.28378	M	0.111	0.033	2021-06-01	LL
222	7	9	28.6	295.9	1.26500	M	0.109	0.037	2021-06-01	LL
223	7	10	28.0	280.1	1.27574	M	0.087	0.031	2021-06-01	LL
224	7	10	29.0	304.7	1.24929	M	0.099	0.032	2021-06-01	LL
225	7	10	24.1	150.1	1.07212	M	0.039	0.026	2021-06-01	LL
226	7	10	28.0	253.2	1.15329	M	0.061	0.024	2021-06-01	LL
227	8	3	31.3	352.4	1.14922	M	0.138	0.039	2021-06-15	110WS
228	8	3	25.6	201.7	1.20223	M	0.059	0.029	2021-06-15	110WS
229	8	3	26.2	198.3	1.10260	M	0.054	0.027	2021-06-15	110WS
230	8	3	24.0	159.1	1.15090	M	0.058	0.036	2021-06-15	110WS
231	8	3	24.7	175.8	1.16662	M	0.051	0.029	2021-06-15	110WS
232	8	3	27.4	243.1	1.18177	M	0.082	0.034	2021-06-15	110WS
233	8	3	27.0	231.1	1.17411	M	0.08	0.035	2021-06-15	110WS
234	8	4	31.2	370.7	1.22056	M	0.102	0.028	2021-06-15	110WS
235	8	4	29.2	285.5	1.14672	M	0.095	0.033	2021-06-15	110WS
236	8	4	29.7	308.2	1.17642	M	0.082	0.027	2021-06-15	110WS
237	8	4	25.4	188.0	1.14725	M	0.048	0.026	2021-06-15	110WS
238	8	4	25.2	189.9	1.18665	M	0.074	0.039	2021-06-15	110WS
239	8	7	26.1	251.1	1.41229	M	0.086	0.034	2021-06-15	180WS
240	8	7	30.5	408.6	1.44012	M	0.125	0.031	2021-06-15	180WS
241	8	7	30.4	351.0	1.24936	M	0.112	0.032	2021-06-15	180WS
242	8	7	30.3	363.4	1.30634	M	0.122	0.034	2021-06-15	180WS
243	8	8	31.5	420.3	1.34471	M	0.17	0.040	2021-06-15	180WS
244	8	8	29.6	337.2	1.30021	M	0.149	0.044	2021-06-15	180WS
245	8	8	26.2	244.9	1.36171	M	0.094	0.038	2021-06-15	180WS
246	8	8	32.8	453.8	1.28601	M	0.133	0.029	2021-06-15	180WS
247	8	9	27.2	259.2	1.28804	M	0.085	0.033	2021-06-15	LL
248	8	9	33.2	428.9	1.17204	M	0.128	0.030	2021-06-15	LL
249	8	9	28.1	272.2	1.22679	M	0.072	0.026	2021-06-15	LL
250	8	9	26.1	203.1	1.14232	M	0.049	0.024	2021-06-15	LL

251	8	10	33.0	426.2	1.18596	M	0.138	0.032	2021-06-15	LL
252	8	10	29.8	327.8	1.23868	M	0.104	0.032	2021-06-15	LL
253	8	10	31.1	383.9	1.27625	M	0.168	0.044	2021-06-15	LL
254	9	7	34.0	485.7	1.23575	M	0.144	0.030	2021-06-29	180WS
255	9	7	36.1	557.7	1.18544	M	0.18	0.032	2021-06-29	180WS
256	9	7	32.5	411.4	1.19843	M	0.134	0.033	2021-06-29	180WS
257	9	7	32.8	423.2	1.19929	M	0.132	0.031	2021-06-29	180WS
258	9	8	31.6	382.3	1.21155	M	0.129	0.034	2021-06-29	180WS
259	9	8	29.6	347.1	1.33838	M	0.128	0.037	2021-06-29	180WS
260	9	8	33.0	442.9	1.23243	M	0.12	0.027	2021-06-29	180WS
261	9	8	29.2	284.9	1.14431	M	0.095	0.033	2021-06-29	180WS
262	9	9	32.9	443.6	1.24567	M	0.174	0.039	2021-06-29	LL
263	9	9	33.7	517.1	1.35109	M	0.202	0.039	2021-06-29	LL
264	9	9	31.6	399.2	1.26511	M	0.144	0.036	2021-06-29	LL
265	9	9	30.0	349.5	1.29444	M	0.121	0.035	2021-06-29	LL
266	9	10	33.5	406.5	1.08125	M	0.159	0.039	2021-06-29	LL
267	9	10	34.5	507.7	1.23637	M	0.16	0.032	2021-06-29	LL
268	9	10	32.8	432.1	1.22451	M	0.147	0.034	2021-06-29	LL
269	9	10	29.7	314.5	1.20047	M	0.109	0.035	2021-06-29	LL
270	9	3	26.7	222.4	1.16843	M	0.095	0.043	2021-06-29	110WS
271	9	3	27.2	201.0	0.99882	M	0.043	0.021	2021-06-29	110WS
272	9	3	31.1	341.3	1.13463	M	0.116	0.034	2021-06-29	110WS
273	9	3	31.0	325.3	1.09194	M	0.081	0.025	2021-06-29	110WS
274	9	3	27.8	238.9	1.11194	M	0.132	0.055	2021-06-29	110WS
275	9	3	31.5	340.5	1.08940	M	0.123	0.036	2021-06-29	110WS
276	9	3	29.4	292.1	1.14945	M	1.999	0.684	2021-06-29	110WS
277	9	4	34.5	467.3	1.13799	M	0.131	0.028	2021-06-29	110WS
278	9	4	29.9	322.3	1.20572	M	0.083	0.026	2021-06-29	110WS
279	9	4	31.5	364.5	1.16618	M	0.098	0.027	2021-06-29	110WS
280	9	4	27.6	239.7	1.14009	M	0.08	0.033	2021-06-29	110WS
281	9	4	27.8	223.8	1.04166	M	0.067	0.030	2021-06-29	110WS
282	10	7	35.4	529.8	1.19427	M	0.163	0.031	2021-07-13	180WS
283	10	7	34.8	476.4	1.13040	M	0.135	0.028	2021-07-13	180WS
284	10	7	30.6	367.4	1.28226	M	0.309	0.084	2021-07-13	180WS
285	10	7	31.5	365.6	1.16970	M	0.104	0.028	2021-07-13	180WS
286	10	8	33.3	432.4	1.17099	M	0.112	0.026	2021-07-13	180WS
287	10	8	31.4	382.6	1.23582	M	0.13	0.034	2021-07-13	180WS
288	10	8	28.6	306.7	1.31104	M	0.161	0.052	2021-07-13	180WS
289	10	8	28.5	299.5	1.29379	M	0.107	0.036	2021-07-13	180WS
290	10	9	34.0	494.1	1.25712	M	0.136	0.028	2021-07-13	LL
291	10	9	35.1	620.2	1.43420	M	0.226	0.036	2021-07-13	LL
292	10	9	30.0	348.5	1.29074	M	0.119	0.034	2021-07-13	LL
293	10	9	32.0	408.2	1.24573	M	0.131	0.032	2021-07-13	LL
294	10	10	31.3	370.2	1.20727	M	0.111	0.030	2021-07-13	LL
295	10	10	32.9	439.7	1.23472	M	0.148	0.034	2021-07-13	LL
296	10	10	29.6	332.0	1.28016	M	0.11	0.033	2021-07-13	LL
297	10	10	31.3	366.0	1.19357	M	0.111	0.030	2021-07-13	LL
298	11	7	39.0	679.9	1.14618	M	0.243	0.036	2021-07-27	180WS
299	11	7	34.4	505.5	1.24178	M	0.158	0.031	2021-07-27	180WS
300	11	7	37.5	666.3	1.26350	M	0.33	0.050	2021-07-27	180WS
301	11	7	34.6	567.7	1.37054	M	1.975	0.348	2021-07-27	180WS
302	11	7	33.2	466.2	1.27397	M	0.135	0.029	2021-07-27	180WS
303	11	7	29.5	341.0	1.32828	M	0.122	0.036	2021-07-27	180WS
304	11	8	32.5	452.0	1.31670	M	0.18	0.040	2021-07-27	180WS
305	11	8	36.5	569.1	1.17033	M	0.191	0.034	2021-07-27	180WS
306	11	8	39.6	796.5	1.28263	M	0.258	0.032	2021-07-27	180WS
307	11	8	38.5	674.0	1.18108	M	0.195	0.029	2021-07-27	180WS
308	11	8	35.7	550.2	1.20925	M	0.129	0.023	2021-07-27	180WS
309	11	8	29.5	382.7	1.49071	M	0.088	0.023	2021-07-27	180WS
310	11	9	36.4	610.5	1.26585	M	0.154	0.025	2021-07-27	LL
311	11	9	34.3	475.2	1.17759	M	0.135	0.028	2021-07-27	LL
312	11	9	35.9	636.2	1.37502	M	0.347	0.055	2021-07-27	LL
313	11	9	31.7	413.7	1.29870	M	0.206	0.050	2021-07-27	LL

314	11	9	31.7	380.5	1.19447	M	0.117	0.031	2021-07-27	LL
315	11	10	31.5	384.2	1.22921	M	0.124	0.032	2021-07-27	LL
316	11	10	35.5	569.1	1.27205	M	0.171	0.030	2021-07-27	LL
317	11	10	36.5	569.8	1.17177	M	0.231	0.041	2021-07-27	LL
318	11	10	34.0	519.3	1.32124	M	0.227	0.044	2021-07-27	LL
319	11	10	32.4	349.9	1.02875	M	0.086	0.025	2021-07-27	LL
320	11	10	32.9	467.5	1.31279	M	0.199	0.043	2021-07-27	LL
321	11	10	32.6	461.7	1.33262	M	0.146	0.032	2021-07-27	LL

Table II. Overview of well, flour, target, fish if, sample date, condition, the concentration of tshβb, β-actin concentration, and int/ref.

Well	Fluor	Target	Fish id	Sample date	Condition	Concentration of tshbb	Actin concentration	int/ref
G05	SYBR	tshbb	1	2021-01-27	LL	0.103	0.244	0.424
G08	SYBR	tshbb	2	2021-01-27	LL	0.050	0.324	0.154
F10	SYBR	tshbb	3	2021-01-27	LL	0.059	0.318	0.185
D12	SYBR	tshbb	4	2021-01-27	LL	14121682.718	80311.168	175.837
F06	SYBR	tshbb	5	2021-01-27	LL	0.025	0.233	0.109
G02	SYBR	tshbb	6	2021-01-27	LL	0.031	0.231	0.134
H01	SYBR	tshbb	7	2021-01-27	LL	0.030	0.179	0.166
G06	SYBR	tshbb	8	2021-01-27	LL	0.046	0.184	0.249
H05	SYBR	tshbb	9	2021-01-27	LL	0.061	0.167	0.365
H07	SYBR	tshbb	10	2021-01-27	LL	0.039	0.179	0.215
G04	SYBR	tshbb	11	2021-01-27	LL	0.133	0.296	0.451
G12	SYBR	tshbb	12	2021-01-27	LL	0.081	0.278	0.290
F11	SYBR	tshbb	13	2021-02-23	70 WS	0.074	0.277	0.267
E11	SYBR	tshbb	14	2021-02-23	70 WS	0.068	0.318	0.213
F09	SYBR	tshbb	15	2021-02-23	70 WS	0.107	0.377	0.285
H03	SYBR	tshbb	16	2021-02-23	70 WS	0.027	0.330	0.080
D10	SYBR	tshbb	17	2021-02-23	70 WS	0.038	0.165	0.230
G10	SYBR	tshbb	18	2021-02-23	70 WS	0.024	0.371	0.065
C08	SYBR	tshbb	19	2021-02-23	70 WS	0.025	0.274	0.091
E02	SYBR	tshbb	20	2021-02-23	70 WS	0.066	0.291	0.225
E04	SYBR	tshbb	21	2021-02-23	70 WS	0.099	0.305	0.326
F02	SYBR	tshbb	22	2021-02-23	70 WS	0.088	0.492	0.179
D05	SYBR	tshbb	23	2021-02-23	70 WS	0.011	0.231	0.046
G07	SYBR	tshbb	24	2021-02-23	70 WS	0.116	0.282	0.409
D12	SYBR	tshbb	25	2021-02-23	LL	0.029	0.382	0.075
F04	SYBR	tshbb	26	2021-02-23	LL	0.039	0.456	0.085
G01	SYBR	tshbb	27	2021-02-23	LL	0.073	0.229	0.319
G09	SYBR	tshbb	28	2021-02-23	LL	0.117	0.359	0.325
H08	SYBR	tshbb	29	2021-02-23	LL	14121682.718	80311.168	175.837
D01	SYBR	tshbb	30	2021-02-23	LL	0.034	0.170	0.203
D08	SYBR	tshbb	31	2021-02-23	LL	0.018	0.006	3.246
E12	SYBR	tshbb	32	2021-02-23	LL	0.042	0.330	0.128
D08	SYBR	tshbb	33	2021-02-23	LL	0.075	0.347	0.215
F07	SYBR	tshbb	34	2021-02-23	LL	0.105	0.347	0.304
E02	SYBR	tshbb	35	2021-02-23	LL	14121682.718	80311.168	175.837
F03	SYBR	tshbb	36	2021-02-23	LL	0.079	0.281	0.280
B07	SYBR	tshbb	37	2021-03-22	70 WS	0.037	0.316	0.116
C01	SYBR	tshbb	38	2021-03-22	70 WS	0.016	0.171	0.093
C12	SYBR	tshbb	39	2021-03-22	70 WS	0.016	0.194	0.084
D11	SYBR	tshbb	40	2021-03-22	70 WS	0.026	0.139	0.184
D09	SYBR	tshbb	41	2021-03-22	70 WS	0.010	0.104	0.098
A05	SYBR	tshbb	42	2021-03-22	70 WS	0.027	0.156	0.173
B01	SYBR	tshbb	43	2021-03-22	70 WS	0.032	0.268	0.118
D10	SYBR	tshbb	44	2021-03-22	70 WS	0.004	0.032	0.127
E08	SYBR	tshbb	45	2021-03-22	70 WS	0.011	0.183	0.060
E03	SYBR	tshbb	46	2021-03-22	70 WS	0.019	0.165	0.113
H04	SYBR	tshbb	47	2021-03-22	70 WS	14121682.718	80311.168	175.837
C06	SYBR	tshbb	48	2021-03-22	70 WS	0.021	0.184	0.113
D03	SYBR	tshbb	49	2021-03-22	110 WS	0.051	0.144	0.351
A01	SYBR	tshbb	50	2021-03-22	110 WS	14121682.718	80257.710	175.954
D07	SYBR	tshbb	51	2021-03-22	110 WS	0.071	0.184	0.386
B08	SYBR	tshbb	52	2021-03-22	110 WS	0.032	0.330	0.097
B06	SYBR	tshbb	53	2021-03-22	110 WS	0.059	0.300	0.196
H06	SYBR	tshbb	54	2021-03-22	110 WS	14121682.718	80311.168	175.837
A08	SYBR	tshbb	55	2021-03-22	110 WS	0.092	0.248	0.373
A09	SYBR	tshbb	56	2021-03-22	110 WS	0.086	0.199	0.432
B03	SYBR	tshbb	57	2021-03-22	110 WS	0.029	0.162	0.178
B02	SYBR	tshbb	58	2021-03-22	110 WS	0.084	0.282	0.298

A12	SYBR	tshbb	59	2021-03-22	110 WS	0.163	0.310	0.527
C04	SYBR	tshbb	60	2021-03-22	110 WS	0.079	0.392	0.202
B12	SYBR	tshbb	61	2021-03-22	LL	0.034	0.270	0.124
B10	SYBR	tshbb	62	2021-03-22	LL	0.061	0.337	0.180
C05	SYBR	tshbb	63	2021-03-22	LL	0.054	0.355	0.153
A06	SYBR	tshbb	64	2021-03-22	LL	0.036	0.262	0.139
E01	SYBR	tshbb	65	2021-03-22	LL	0.061	0.280	0.217
C03	SYBR	tshbb	66	2021-03-22	LL	0.137	0.316	0.434
D06	SYBR	tshbb	67	2021-03-22	LL	0.071	0.310	0.228
E07	SYBR	tshbb	68	2021-03-22	LL	0.071	0.265	0.267
B09	SYBR	tshbb	69	2021-03-22	LL	0.052	0.244	0.214
A10	SYBR	tshbb	70	2021-03-22	LL	0.082	0.328	0.250
C09	SYBR	tshbb	71	2021-03-22	LL	0.073	0.301	0.243
B04	SYBR	tshbb	72	2021-03-22	LL	0.063	0.355	0.178
A11	SYBR	tshbb	73	2021-04-07	70 WS	1.339	0.393	3.410
B05	SYBR	tshbb	74	2021-04-07	70 WS	0.923	0.248	3.724
E10	SYBR	tshbb	75	2021-04-07	70 WS	0.622	0.236	2.639
D02	SYBR	tshbb	76	2021-04-07	70 WS	0.321	0.256	1.257
E06	SYBR	tshbb	77	2021-04-07	70 WS	0.348	0.276	1.258
F05	SYBR	tshbb	78	2021-04-07	70 WS	0.458	0.195	2.348
G03	SYBR	tshbb	79	2021-04-07	70 WS	0.352	0.229	1.539
F12	SYBR	tshbb	80	2021-04-07	70 WS	0.261	0.255	1.024
C10	SYBR	tshbb	81	2021-04-07	70 WS	0.598	0.326	1.831
G11	SYBR	tshbb	82	2021-04-07	70 WS	0.755	0.337	2.238
H02	SYBR	tshbb	83	2021-04-07	70 WS	14121682.718	80311.168	175.837
F08	SYBR	tshbb	84	2021-04-07	70 WS	0.384	0.301	1.277
E05	SYBR	tshbb	85	2021-04-07	LL	0.064	0.216	0.296
A07	SYBR	tshbb	86	2021-04-07	LL	0.048	0.181	0.265
C02	SYBR	tshbb	87	2021-04-07	LL	0.088	0.275	0.318
A04	SYBR	tshbb	88	2021-04-07	LL	0.046	0.201	0.230
D04	SYBR	tshbb	89	2021-04-07	LL	0.029	0.347	0.085
A02	SYBR	tshbb	90	2021-04-07	LL	0.103	0.341	0.302
A03	SYBR	tshbb	91	2021-04-07	LL	0.058	0.137	0.422
C11	SYBR	tshbb	92	2021-04-07	LL	0.068	0.219	0.309
C07	SYBR	tshbb	93	2021-04-07	LL	0.008	0.040	0.194
F01	SYBR	tshbb	94	2021-04-07	LL	0.082	0.266	0.309
B11	SYBR	tshbb	95	2021-04-07	LL	0.167	0.369	0.452
E09	SYBR	tshbb	96	2021-04-07	LL	0.065	0.228	0.286
F08	SYBR	tshbb	97	2021-04-20	70 WS	0.113	0.247	0.457
B06	SYBR	tshbb	98	2021-04-20	70 WS	0.098	0.199	0.490
G08	SYBR	tshbb	99	2021-04-20	70 WS	0.063	0.313	0.201
D02	SYBR	tshbb	100	2021-04-20	70 WS	0.043	0.222	0.192
H04	SYBR	tshbb	101	2021-04-20	70 WS	0.077	0.292	0.265
H02	SYBR	tshbb	102	2021-04-20	70 WS	0.048	0.304	0.159
E06	SYBR	tshbb	103	2021-04-20	70 WS	0.038	0.200	0.192
F12	SYBR	tshbb	104	2021-04-20	70 WS	0.088	0.280	0.312
D12	SYBR	tshbb	105	2021-04-20	70 WS	0.054	0.223	0.242
G02	SYBR	tshbb	106	2021-04-20	70 WS	0.034	0.280	0.123
E06	SYBR	tshbb	107	2021-04-20	70 WS	0.089	0.117	0.763
E04	SYBR	tshbb	108	2021-04-20	70 WS	0.064	0.195	0.329
D02	SYBR	tshbb	109	2021-04-20	110 WS	0.019	0.228	0.084
G08	SYBR	tshbb	110	2021-04-20	110 WS	0.045	0.291	0.153
E08	SYBR	tshbb	111	2021-04-20	110 WS	0.013	0.295	0.043
F10	SYBR	tshbb	112	2021-04-20	110 WS	0.026	0.513	0.051
G04	SYBR	tshbb	113	2021-04-20	110 WS	0.026	0.265	0.099
E10	SYBR	tshbb	114	2021-04-20	110 WS	0.088	0.295	0.297
G10	SYBR	tshbb	115	2021-04-20	110 WS	0.019	0.279	0.069
H04	SYBR	tshbb	116	2021-04-20	110 WS	0.007	0.346	0.021
G04	SYBR	tshbb	117	2021-04-20	110 WS	0.027	0.248	0.108
H06	SYBR	tshbb	118	2021-04-20	110 WS	0.007	0.145	0.051

H02	SYBR	tshbb	119	2021-04-20	110 WS	0.007	0.272	0.025
E08	SYBR	tshbb	120	2021-04-20	110 WS	0.019	0.077	0.249
G12	SYBR	tshbb	121	2021-04-20	LL	0.090	0.312	0.289
F02	SYBR	tshbb	122	2021-04-20	LL	0.022	0.262	0.086
G10	SYBR	tshbb	123	2021-04-20	LL	0.045	0.262	0.171
F12	SYBR	tshbb	124	2021-04-20	LL	0.013	0.121	0.112
F06	SYBR	tshbb	125	2021-04-20	LL	0.030	0.216	0.137
D04	SYBR	tshbb	126	2021-04-20	LL	0.090	0.293	0.307
E08	SYBR	tshbb	127	2021-04-20	LL	0.049	0.214	0.229
G02	SYBR	tshbb	128	2021-04-20	LL	0.053	0.241	0.219
E04	SYBR	tshbb	129	2021-04-20	LL	0.060	0.302	0.199
F06	SYBR	tshbb	130	2021-04-20	LL	0.042	0.224	0.187
G06	SYBR	tshbb	131	2021-04-20	LL	0.087	0.207	0.420
D12	SYBR	tshbb	132	2021-04-20	LL	0.119	0.275	0.431
D04	SYBR	tshbb	145	2021-05-04	110 WS	0.171	0.150	1.140
E04	SYBR	tshbb	146	2021-05-04	110 WS	0.536	0.183	2.932
H08	SYBR	tshbb	147	2021-05-04	110 WS	0.234	0.169	1.379
C02	SYBR	tshbb	148	2021-05-04	110 WS	0.369	0.298	1.237
F02	SYBR	tshbb	149	2021-05-04	110 WS	0.397	0.232	1.713
H06	SYBR	tshbb	150	2021-05-04	110 WS	0.072	0.184	0.393
F08	SYBR	tshbb	151	2021-05-04	110 WS	0.425	0.272	1.563
C10	SYBR	tshbb	152	2021-05-04	110 WS	0.416	0.259	1.606
E10	SYBR	tshbb	153	2021-05-04	110 WS	14121682.718	80311.168	175.837
G06	SYBR	tshbb	154	2021-05-04	110 WS	0.346	0.343	1.008
E12	SYBR	tshbb	155	2021-05-04	110 WS	0.204	0.177	1.149
C12	SYBR	tshbb	156	2021-05-04	180 WS	0.030	0.240	0.125
D08	SYBR	tshbb	157	2021-05-04	180 WS	0.030	0.154	0.194
A08	SYBR	tshbb	158	2021-05-04	180 WS	0.041	0.217	0.191
H08	SYBR	tshbb	159	2021-05-04	180 WS	0.052	0.210	0.249
D06	SYBR	tshbb	160	2021-05-04	180 WS	0.033	0.137	0.239
E02	SYBR	tshbb	161	2021-05-04	180 WS	0.044	0.271	0.163
F04	SYBR	tshbb	162	2021-05-04	180 WS	0.008	0.142	0.054
D10	SYBR	tshbb	163	2021-05-04	180 WS	0.030	0.284	0.105
B10	SYBR	tshbb	164	2021-05-04	LL	0.012	0.260	0.046
G12	SYBR	tshbb	165	2021-05-04	LL	0.042	0.168	0.250
A04	SYBR	tshbb	166	2021-05-04	LL	0.017	0.067	0.247
C04	SYBR	tshbb	167	2021-05-04	LL	0.036	0.191	0.189
E06	SYBR	tshbb	168	2021-05-04	LL	0.053	0.186	0.286
B06	SYBR	tshbb	169	2021-05-04	LL	0.032	0.155	0.209
F10	SYBR	tshbb	170	2021-05-04	LL	0.026	0.145	0.177
F04	SYBR	tshbb	171	2021-05-04	LL	0.007	0.153	0.048
B12	SYBR	tshbb	172	2021-05-04	LL	0.075	0.251	0.300
D10	SYBR	tshbb	185	2021-05-17	110 WS	0.065	0.206	0.313
C08	SYBR	tshbb	186	2021-05-17	110 WS	0.074	0.301	0.245
B08	SYBR	tshbb	187	2021-05-17	110 WS	0.058	0.232	0.249
E02	SYBR	tshbb	188	2021-05-17	110 WS	0.029	0.172	0.167
B02	SYBR	tshbb	189	2021-05-17	110 WS	0.062	0.205	0.300
B02	SYBR	tshbb	190	2021-05-17	110 WS	0.049	0.248	0.198
B04	SYBR	tshbb	191	2021-05-17	110 WS	0.052	0.198	0.264
B08	SYBR	tshbb	192	2021-05-17	110 WS	0.050	0.095	0.529
C04	SYBR	tshbb	193	2021-05-17	110 WS	0.051	0.247	0.208
A10	SYBR	tshbb	194	2021-05-17	110 WS	0.070	0.192	0.366
C06	SYBR	tshbb	195	2021-05-17	110 WS	0.056	0.232	0.242
B10	SYBR	tshbb	196	2021-05-17	110 WS	0.047	0.178	0.265
E10	SYBR	tshbb	197	2021-05-17	LL	0.014	0.338	0.042
A12	SYBR	tshbb	198	2021-05-17	LL	0.059	0.256	0.229
C12	SYBR	tshbb	199	2021-05-17	LL	0.051	0.334	0.154
C08	SYBR	tshbb	200	2021-05-17	LL	0.013	0.182	0.070
C06	SYBR	tshbb	201	2021-05-17	LL	0.025	0.152	0.167
A06	SYBR	tshbb	202	2021-05-17	LL	0.067	0.336	0.200

D08	SYBR	tshbb	203	2021-05-17	LL	0.020	0.272	0.072
B04	SYBR	tshbb	204	2021-05-17	LL	0.024	0.221	0.110
C02	SYBR	tshbb	205	2021-05-17	LL	0.034	0.304	0.113
A06	SYBR	tshbb	206	2021-05-17	LL	0.050	0.223	0.223
C10	SYBR	tshbb	239	2021-06-15	180 WS	0.337	0.322	1.047
A04	SYBR	tshbb	241	2021-06-15	180 WS	0.815	0.307	2.653
A12	SYBR	tshbb	242	2021-06-15	180 WS	0.479	0.585	0.818
A02	SYBR	tshbb	243	2021-06-15	180 WS	0.236	0.157	1.505
A08	SYBR	tshbb	244	2021-06-15	180 WS	0.117	0.170	0.691
E12	SYBR	tshbb	245	2021-06-15	180 WS	0.394	0.388	1.014
D06	SYBR	tshbb	246	2021-06-15	180 WS	0.512	0.259	1.975
A02	SYBR	tshbb	246	2021-06-15	180 WS	0.243	0.205	1.187
B12	SYBR	tshbb	247	2021-06-15	LL	0.016	0.256	0.064
A10	SYBR	tshbb	248	2021-06-15	LL	0.065	0.227	0.287
C10	SYBR	tshbb	249	2021-06-15	LL	0.003	0.035	0.097
C08	SYBR	tshbb	250	2021-06-15	LL	0.022	0.057	0.389
A10	SYBR	tshbb	251	2021-06-15	LL	0.011	0.041	0.270
C04	SYBR	tshbb	252	2021-06-15	LL	0.036	0.082	0.441
D06	SYBR	tshbb	253	2021-06-15	LL	0.017	0.081	0.207
D04	SYBR	tshbb	254	2021-06-29	180 WS	0.041	0.062	0.670
A04	SYBR	tshbb	255	2021-06-29	180 WS	0.056	0.073	0.766
A06	SYBR	tshbb	256	2021-06-29	180 WS	0.028	0.050	0.560
A12	SYBR	tshbb	257	2021-06-29	180 WS	0.068	0.067	1.008
B04	SYBR	tshbb	258	2021-06-29	180 WS	0.025	0.041	0.608
C12	SYBR	tshbb	259	2021-06-29	180 WS	0.039	0.061	0.639
C02	SYBR	tshbb	260	2021-06-29	180 WS	0.021	0.092	0.233
B06	SYBR	tshbb	261	2021-06-29	180 WS	0.028	0.061	0.451
B12	SYBR	tshbb	262	2021-06-29	LL	0.015	0.038	0.407
C06	SYBR	tshbb	263	2021-06-29	LL	0.011	0.088	0.128
B08	SYBR	tshbb	264	2021-06-29	LL	0.018	0.037	0.479
D02	SYBR	tshbb	265	2021-06-29	LL	0.010	0.066	0.157
B02	SYBR	tshbb	266	2021-06-29	LL	0.069	0.166	0.417
A02	SYBR	tshbb	267	2021-06-29	LL	0.013	0.053	0.241
A08	SYBR	tshbb	268	2021-06-29	LL	0.044	0.090	0.487
B10	SYBR	tshbb	269	2021-06-29	LL	0.013	0.053	0.246
H11	SYBR	tshbb	NTC			14121682.718	80311.168	175.837
H12	SYBR	tshbb	NTC			14121682.718	80311.168	175.837
H12	SYBR	tshbb	NTC			14121682.718	80311.168	175.837
H12	SYBR	tshbb	NTC			14121682.718	80311.168	175.837
H12	SYBR	tshbb	NTC			14121682.718	80311.168	175.837
H09	SYBR	tshbb	Pos Ctrl			0.147	0.213	0.688
H10	SYBR	tshbb	Pos Ctrl			0.101	0.239	0.424
H10	SYBR	tshbb	Pos Ctrl			0.080	0.323	0.247
H10	SYBR	tshbb	Pos Ctrl			0.119	0.263	0.454
H10	SYBR	tshbb	Pos Ctrl			0.182	0.207	0.876

Appendix II. Overview of a standard curve

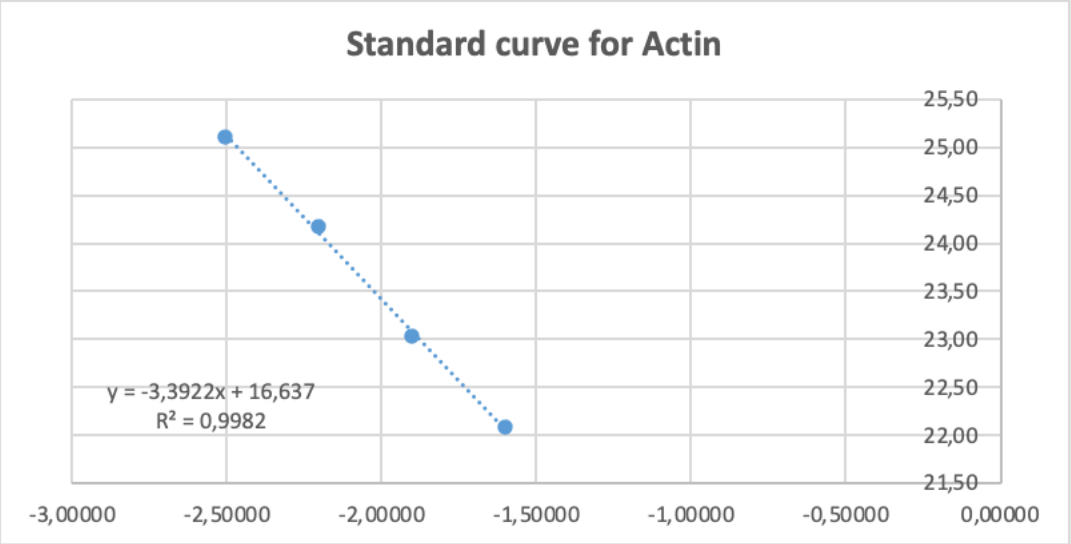

Figure I. Standard curve for β-actin.

	Fluor	Content	Sample	Concentration qPCR 2uL	Cq	Cq Mean	Cq Std. Dev	Log copies	Log	Concentration	
B10	SYBR	Std	D5		22,09	22,09	0,00	1,344	-1,60206	0,025	22,09
B11	SYBR	Std	D5		22,09				-1,90309	0,0125	23,03
B12	SYBR	Std	D5		22,09				-2,20412	0,00625	24,18
C10	SYBR	Std	D6		23,03	23,03	0,00	1,362	-2,50515	0,003125	25,11
C11	SYBR	Std	D6		23,03						
C12	SYBR	Std	D6		23,03						
D10	SYBR	Std	D7		24,18	24,18	0,00	1,383			
D11	SYBR	Std	D7		24,18						
D12	SYBR	Std	D7		24,18						
E10	SYBR	Std	D8		25,11	25,11	0,00	1,400			
E11	SYBR	Std	D8		25,11						
E12	SYBR	Std	D8		25,11						
				m	-3,3922						
				b	16,637						
				E	97,16 %						
				R2	0,9982						

Figure II. Overview of data used to make the standard curve for β-actin.