Norwegian University of Life Sciences

Master's Thesis 2021 60 ECTS

Faculty of Veterinary Medicine
Department of Preclinical Sciences and Pathology

Stunting- A Physiological and Molecular Characterization of Atlantic salmon *(Salmo salar)* with reduced growth in the sea cages

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Aquaculture

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M.Sc. Aquaculture Thesis

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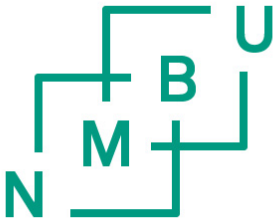

Norwegian University of Life Sciences

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Norwegian University of Life Sciences (NMBU) 2021

Acknowledgements

I have no words or phrases to express my gratitude to my thesis supervisor Kjetil Hodne for his constant supervision, valuable suggestion, scholastic guidance for conducting the research and writing this manuscript since day one to final days. I am greatly indebted to you since without your help, the completion of this work would not have been possible.

I am extremely thankful of my co-supervisor, professor Ian Mayer, for his insightful comments during the writing period of manuscript and making me part of FHF project.

I would like to extend my heartfelt gratitude to Rasoul Nourizadeh-Lillabadi for the help on my practical work in laboratory. Without his help, I could have difficult time on doing qPCR. Also, I am grateful to Ida Beitnes Johansen for making me acquaint with hormonal assay.

I would like extends my heartfelt gratitude to Marit Svendby for her help in RNA extraction. I would like to thank all members of Physiology Department (specially Romain Fontaine) for providing such a warm and encouraging environment during my thesis period.

Sincere thanks to the FHF project for all the funding without which I could not have been able to complete this thesis.

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Abstract

Atlantic salmon *(Salmon salar*) has become the dominating fish species in Norwegian aquaculture industry. Having an anadromous life cycle, salmon spend the first part of their lifecycle in freshwater, then migrates to the sea before returning to freshwater to spawn. Before migration to seawater, the freshwater parr undergo series of morphological, physiological, and behavioural changes known as smoltification, which pre-adapts the fish (now called smolts) for life in the sea. Typically, Gh, cortisol, and thyroxine together with *tshbb* peak during smoltification before returning to baseline levels around sea transfer. In nature, the hormone profile observed during salmon smoltification determines the preferred or optimal window for sea transfer.

Stunting where smolts stop growing after sea transfer has been a major economic and welfare issue for the industry. The aim of this thesis was to conduct molecular and physiological characterization of stunts to provide insight about mechanism behind stunting. In total 30 fish were sampled eight weeks after transfer to sea cages. The fish were categorized into stunts (20) and normal growing fish (10) based on weight and condition factor. Plasma Gh levels were measured using ELISA. Gene expression of all pituitary hormone, and gill Na^+K^+-ATPase were investigated using qPCR. Plasma levels of Gh was significant elevated in stunts. The qPCR results from pituitaries showed significant upregulation of *gh, pomca2*, *tshbb* in stunts. In contrast, *smlta, prl* and *tshba* were down regulated in stunts. The expression levels of gill *nka3* and *nkab1* were significantly downregulated in stunts. Based on the gene expression pattern and elevated levels of plasma Gh, the stunts might have been transferred prematurely to sea. Furthermore, as the plasma osmolality in stunts were the same as in normal growing fish it strongly suggests that stunts can maintain their ability to hypoosmoregulate for a period after sea transfer.

1. Introduction

Norway has a long history in aquaculture production dating back to the 1850s'. Norway started the commercial aquaculture of Atlantic salmon *(Salmo salar)* already in the early 1960s and is today the world's leading producer of farmed Atlantic salmon with 1.45 million tons of salmon produced in 2019 for NOK 72.5 billion (StatisticsNorway, 2020). The introduction of sea cage production of salmon in the early 1970s was a major break-through, leading to a rapid increase in the production of farmed salmon (Venvik, 2005). Today, Norway's aquaculture industry is dominated by Atlantic salmon, which accounts for 93.9% of total Norway's aquaculture production, while Rainbow trout *(Oncorhynchus mykiss)* accounts for 5.7% of production. In addition to being Norway's third most valuable export, the salmon industry employs 8.370 people directly, while many more are employed indirectly in a range of business, from feed production to fish health. Norwegian production of farmed salmon in 2019 increased by 6.4% compared to 2018, and by 58.18% over the last decade. Norwegian production accounted for over 50% of the global production of farmed salmon, exported to 111 countries globally. About 75% of salmon is exported to European countries, with Poland, Denmark, France, Spain, and the Netherlands being the main importers (StatisticsNorway, 2020).

1.1. Life Cycle of Atlantic Salmon

The Atlantic salmon is a teleost fish belonging to the subfamily Salmoninae under family Salmonidae (Mills, 1989). It is native to the temperate and subarctic regions of the North Atlantic Ocean (Aas et al., 2010). Atlantic salmon is an anadromous fish, that begin its life in freshwater and migrates to the ocean to feed and grow before returning to freshwater to spawn (Figure 1) (Hoar, 1988). Young Atlantic salmon (parr) spend first two to three years of their life in their home river and migrate towards North Atlantic ocean (Aas et al., 2010). Before migrating downstream to the sea, freshwater juvenile (parr) undergoes a pronounced metamorphosis, termed parr-smolt transformation (smoltification), which pre-adapts the juvenile salmon for life in the marine environment. Somltification is a complex process involving a series of morphological, physiological, and behavioral changes (Hoar, 1988). Physiologically, smolts develop progressive changes in the major osmoregulatory organs, the gill, gut, and kidney, enabling them to keep internal osmotic concentration constant in ocean through the absorption of more water and secretion of excess salts from body. Hence, through physiological changes, smolts develop hypoosmoregulation and associated ion regulation abilities that in turn make them adaptable for seawater (McCormick, 2012). Silvering and

disappearance of vertical parr marks (Hoar, 1988; McCormick et al., 1998), darkening of fin margins and reduction in condition factor (weight to length ratio) making parr slimmer and streamlined are distinct morphological changes occurring during smoltification. In term of behavioral changes, smolts develop affinity towards salinity, becomes less territorial and start to swim downstream along with water current forming a school (McCormick et al., 1998; McCormick, 2012). Smolts migratory journey towards ocean usually begins in the spring. In food supply rich marine environment (Good & Davidson, 2016), where they spend 1-5 years and grow into an adult. Then, they return to their native river to spawn. Salmon spawn during late autumn-early winter and hatch the following spring (Aas et al., 2010). A special case regarding maturation age of male can be observed in Atlantic salmon. Some males achieve maturation soon after spending one year in sea while some male mature even at fresh water parr stage (Whalen & Parrish, 1999). It can be observed in both natural and farm condition (Berrill et al., 2003) and could be the reproductive strategy evolved to maintain biodiversity and genetic contribution of cohort over a several years (Saunders & Schom, 1985). Although precocious maturation occurs predominantly in male Atlantic salmon, it exists also in females (Berrill et al., 2003; McClure et al., 2007).

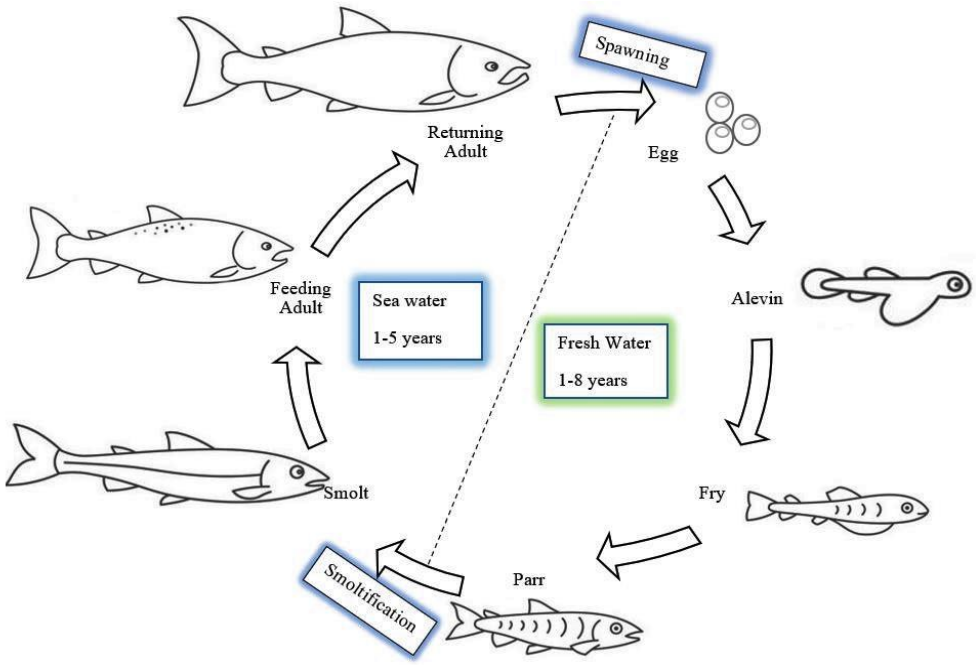


Figure 1. Summary of life cycle of Atlantic salmon in natural habitat. Constructed based on the information provided by Aas et al. (2010).

1.2. Salmon aquaculture-production cycle

Commercial salmon farming involves a 2-stage production cycle. The first stage is the freshwater production of smolts, and the second stage is the grown-on stage following sea water transfer of smolts to the sea cages. In comparison to natural growth condition, the time taken to complete a lifecycle is compressed in commercial aquaculture. Thus, fish size of 4-5 kg can be produced in less than 2 years from hatching. Salmon rearing beings with hatcheries receiving fertilized eggs from a commercial breeding company. When water temperature is kept 8 ^{o}C, fertilized eggs turn into eyed egg stage after 230 day degrees (after fertilization) and hatch into yolk sac larvae after 510 day degrees. The newly hatched larvae are called alevins, and following yolk sac absorption the alevins, now termed fry, start to swim freely and actively feed. During the whole process the temperature, pH and oxygen level must be regulated to provide optimal growth condition. As soon as fry start to accept starter dry feed, they are reared in smaller indoor tanks until they reach of size of 5 g. Once they reach this size, the juveniles, now termed parr are transferred to larger tanks and kept supplied with through flowing freshwater until smoltification. Smoltification usually occurs after 2 years for wild fish (Asche & Bjorndal, 2011; Trygve, 1993). In contrast, due to light and temperature manipulation it has been possible to produce larger smolts during short duration under aquacultural conditions (Lysfjord et al., 2004). At the time of sea cage transfer, smolt attains the weight of 100-250 g which mainly takes place during autumn and spring (Bergheim et al., 2009). Following sea water transfer, the post-smolts grow rapidly, and attain marketable size (4-5 kg) after 12 to 18 months (Sandvold & Tveterås, 2014).

1.3. Stunting

It has been long observed that following sea cage transfer, some of the smolts fail to grow normally (Figure 2), display poor hypoosmoregulatory ability and eventually die. These affected fish are termed as “stunts”. Knowledge gaps still exist in our understanding of the underlying causes of stunt development, which occurs in post-smolts following sea water transfer. The condition of stunting is associated with reduced growth, loss of appetite, repressed immune system and osmoregulatory failure of fish to acclimatize in saltwater causing eventual death (Folmar et al., 1982; Martin et al., 1999; Van Rijn et al., 2021). Paradoxically, earlier physiological studies found that Atlantic salmon stunts display elevated plasma level of growth hormone (Gh), the main hormone responsible for growth and development, even though these stunts showed poor growth. It was hypothesized that elevated Gh levels was due to defects in

the hormone receptor mechanism of intermediating system instead of a direct effect of Gh per se (Björnsson et al., 1988). Although plasma ion profile was found to be normal (Clarke & Nagahama, 1977), stunting is usually linked to premature sea transfer of smolts, during which the fish still has underdeveloped hypoosmoregulatory capacity (Martin et al., 1999). Stunting has been a common phenomenon in salmon aquaculture and found to be affect up to one fourth of the stock, in some cases up to 40%, causing heavy economic loss as well as being a major welfare concern of salmon industry (Clarke & Nagahama, 1977; Vindas et al., 2016). In this scenario, molecular and physiological characterization of stunts is necessary to provide some clues about the cause of stunting phenomenon.

Figure 2. Stunts and normal fish. Difference in body length and weight can be visually observed between normal and stunts. Stunts fail to grow which makes them shorter in fork length and having low body weight as compared to normal fish of same age raised under same rearing environment. After the two months of sea cage transfer, average weight of stunts was 172.5 ± 13.63 g while normal fish has reached average weight of 12,132 ± 83.36 g. Average fork length of stunts was 29.30 ± 0.60 cm while normal fish has 45.10 ± 1.06 cm fork length.

1.4. Smolt production in recirculation aquaculture system (RAS)

While recirculation aquaculture systems (RAS) have been extensively used in aquaculture globally for number of decades, their use for salmon smolt production has only just started (Summerfelt et al., 2016). Compared to traditional flow-through smolt production, RAS offers great control over the rearing environment and significantly reduces environmental impacts by reducing the amount of freshwater consumption. As such, an increase in RAS production would lead to more sustainable salmon production. The number of RAS farms in Norway is now increasing rapidly. Until 2011, less than 10% Norwegian smolt farmers had adopted RAS

technology (Dalsgaard et al., 2013) which doubled in less than a decade period reaching around 20% at the end of 2018 (Meriac, 2019). More than 60 RAS companies (AKVA group, Kruger Kaldnes) are involved in production and installation of RAS technologies in Norway (Badiola et al., 2018; Hagspiel et al., 2018). The Norwegian government recently lifted the ban on producing smolts larger than 250 g, so Norway's major salmon producers are now planning to use RAS facilities to produce larger smolts (up to 1 kg) for stocking in sea cage (Hagspiel et al., 2018). In recent years, there are undergoing several ambitious projects to promote land based production of salmon using RAS expecting to produce 10% of Norway's salmon to produce on land (Meriac, 2019).

Using RAS makes it possible to provide optimal growth conditions for fish by regulating water quality parameters such as dissolved gas level, pH, and suspended solids (Dalsgaard et al., 2013). The light and temperature regime are maintained precisely to manipulate timing of smoltification and thus to attain year-round production of smolts. Photoperiod manipulation is done by changing duration of light and darkness period within fish tank. During the photoperiod manipulation phase, the parr are kept at continuous photoperiod (LD 24:0-24 hours light,0 hours dark) until they reach the mean size of around 40 g, then the artificial winter signal is provided by manipulating photoperiod of (LD 12:12-12 hours light, 12 hours dark) for six weeks. Subsequently, they are grown under the continuous photoperiod regime (LD: 24: 0) (Holan et al., 2020) and the parr smoltify within the period of 3 to 4 weeks as a result of received spring signal as in natural environment (Figure 3) (Good & Davidson, 2016). The temperature is usually kept stable between 12-14 °C in the RAS facility (Dalsgaard et al., 2013) and 3000-4500 day degree is sufficient for smoltification (Bergheim et al., 2009). In early 1970s until 1980s production of 2-year-old smolt (S2) had dominated salmon industry which shifted towards more profitable alternative, production of 1-year old (S1) which took approximately 15 months from hatching to delivery. With the introduction of artificial light and temperature manipulation (Lysfjord et al., 2004) under-yearling (S0) can be delivered 9-10 months after hatching. Time for sea cage transfer for the 1-year-old smolt is mostly from April to May, while the under-yearlings are transferred to sea from September to October. The RAS facility provides the opportunities to maintain higher temperature (Dalsgaard et al., 2013) throughout winter and made possible the production of larger smolts at the time of sea cage transfer; 100-200 g under-yearling and 150-250 g 1-year old smolt (Bergheim et al., 2009).

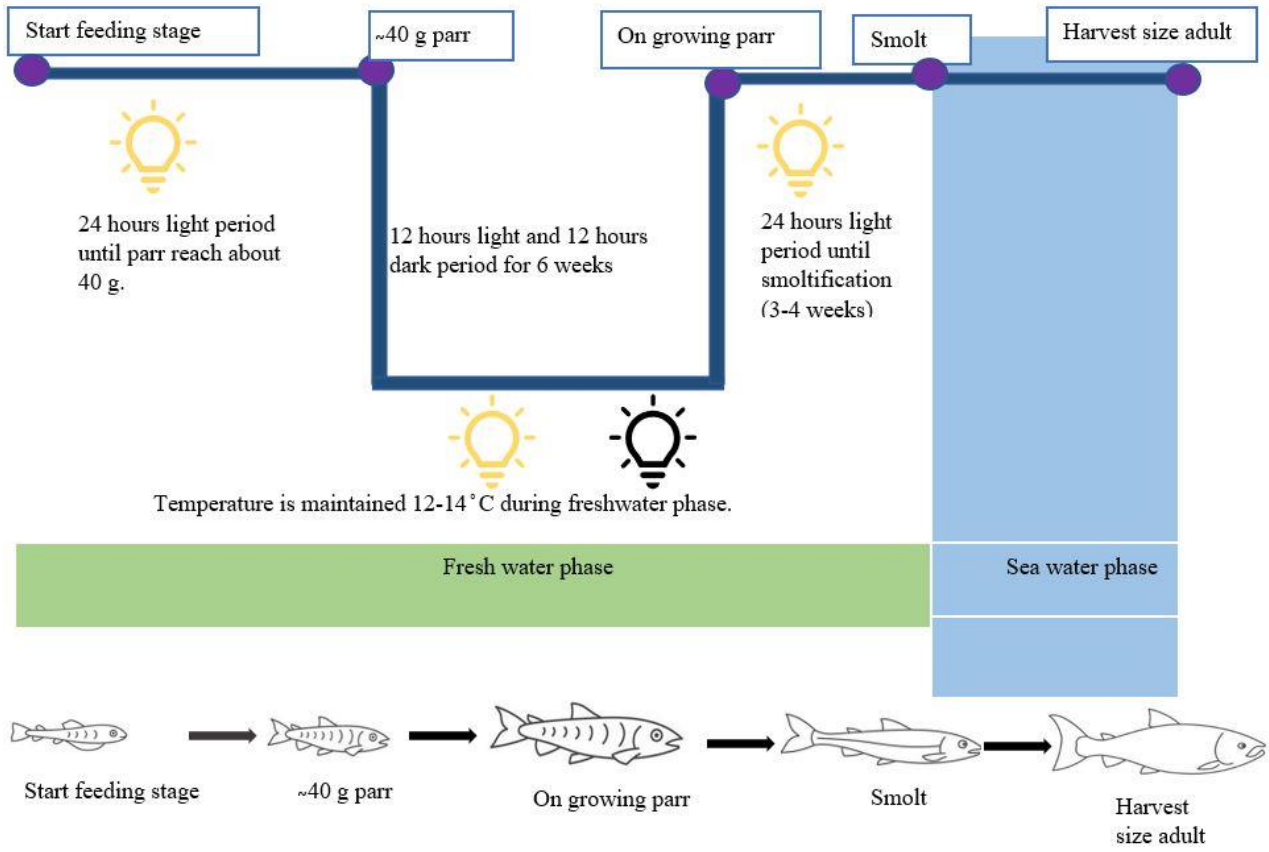


Figure 3. Typical Production technique used by commercial farms for the smolt production in a RAS facility. Constructed based on the information provided by Good & Davidson (2016).

1.5. Hormonal changes during smoltification process

Both developmental and external factors are driving forces for the smoltification process. Laboratory studies show that the juveniles have a bimodal size distribution (lower and upper growth modes) at the end of growth period. The parr belonging to upper size mode class, having attained critical length at the end of winter or early spring typically undergo smoltification next spring and lower mode fish wait one more year for smoltification. Elson (1957) proposed the concept of critical length for initiation of smoltification and concluded minimum length of 10 cm for Atlantic salmon (Björnsson & Bradley, 2007; Elson, 1957; Hoar, 1988; Skilbrei, 1991). Photoperiod and temperature are known to be the major environmental cues for the timing of smoltification (Björnsson et al., 2011). Temperature works in concert with photoperiod and it affects the rate of development (McCormick et al., 2002). It has been observed that warmer

temperature tends to increase development rate and advancing the timing of smoltification process (McCormick & Shrimpton, 1997). The circadian system responsible for transmission of environmental signal to brain, is less linearly organized in teleost as compared to mammals (Falcón et al., 2007). The transfer of the environmental photoperiod signal into the light-brain-pituitary axis involves neural pathways between brain and pituitary, with the hormone melatonin acting as the hormonal signal of photoperiodic information (Ebbesson et al., 2003). Melatonin is produced primarily in the pineal organ, which in fish, unlike in mammals, is directly light sensitive (Maitra et al., 2015; Simonneaux & Ribelayga, 2003). Increased daylength leads to the activation of the major neuroendocrine axis' known to be involved in the control of parr-smolt transformation. The activation ultimately results in a fine-tuned release of including but not limited to thyroid hormones, (Ths), including (growth hormone, (Gh), and cortisol) (Ebbesson et al., 2003; McCormick et al., 2013) (Figure 4)

The key hormones regulating smoltification includes, thyroid hormones (Ths), thyroxine (T_3) and triiodothyronine (T_4), prolactin (Prl), growth hormone (Gh), the insulin-like growth factor-I (Igf-i), and cortisol (McCormick, S. et al., 2009; Prunet et al., 1989). The thyroid hormones (T_3/T_4) are believed to be involved in metabolism, morphological changes, and imprinting (McCormick, 2012). The increase in plasma levels of Gh, Igf-i, and cortisol leads to increased hypo-osmoregulatory capacity through the cellular and biochemical changes in the osmoregulatory organs, the gill, gut, and kidney, resulting increased salinity tolerance, besides influence in growth and metabolism. Prior to the sea water entry, there comes a phase during smoltification, when the hormones levels reach at their peak (McCormick, S. et al., 2009; Prunet et al., 1989) (Figure 4) and plasma levels of Gh and T_4 start to decline following sea water entry (McCormick et al., 2019).

As stunting has been often linked with osmoregulatory and physiological abnormality resulting premature transfer, the study of these hormones is necessary to provide insight as to why stunting occurs. In addition, MacCormick (2009), suggested that ideal balance between favorable environmental conditions or, "ecological" smolt window and limited period of readiness or, "physiological" window for the survival of smolts in seawater (Figure 4).

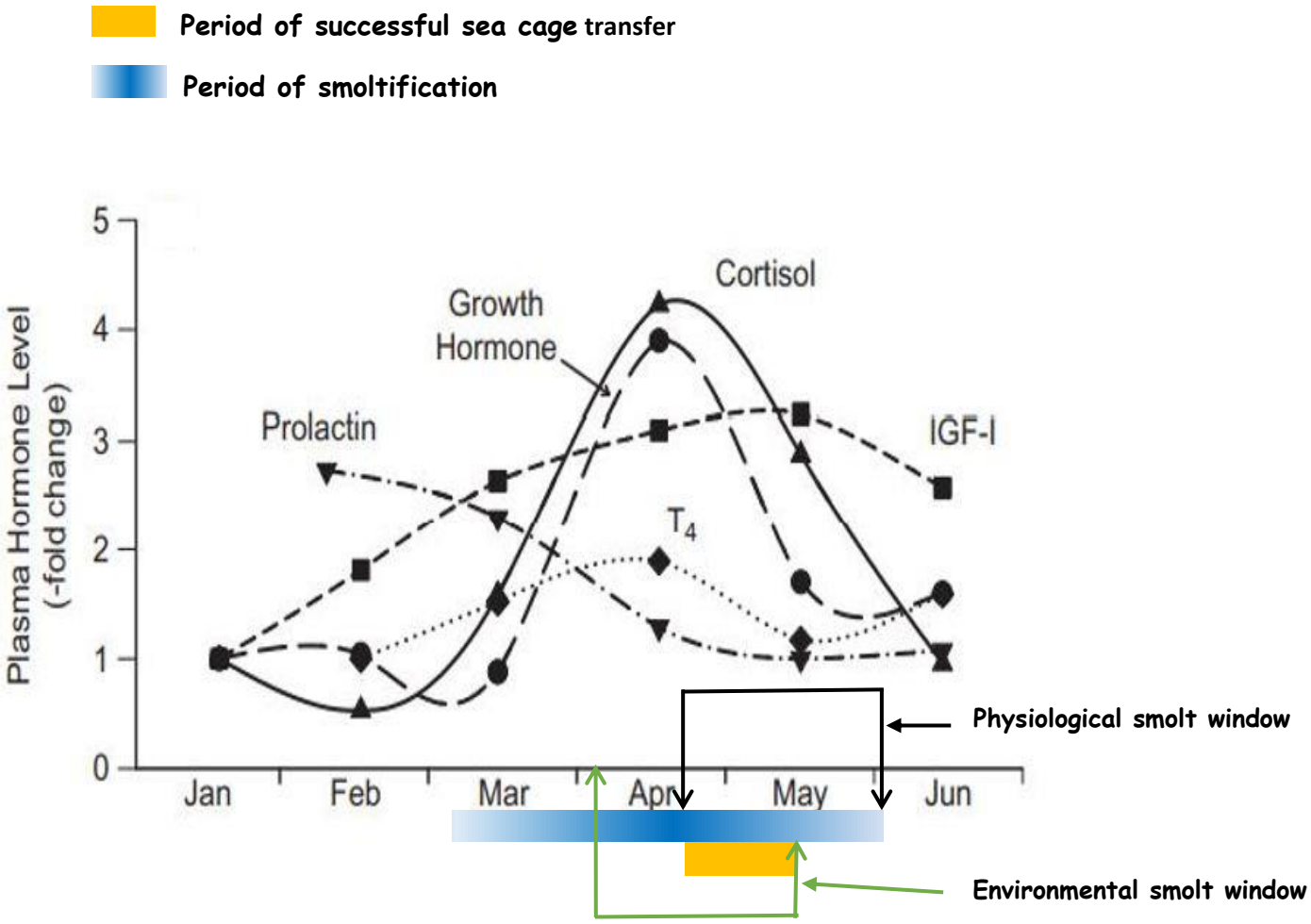


Figure 4. Plasma hormone level changes during the smoltification process. At the beginning of spring, an elevation in plasma T_4 levels can be observed with a simultaneous increase in Igf-i and decrease in prolactin levels. Plasma levels of cortisol and Gh increases shortly after the rise in T_4 levels. With exception of prolactin, peak hormone levels can be observed during April and May with concurring peak phase in smoltification (McCormick, S. et al., 2009; Prunet et al., 1989). The overlapping of physiological and environmental smolt windows results the successful transfer to the sea (Fleming, 2018).

1.6. Precocious maturation and hormonal changes during maturation

It has recently been reported that the incidence of early sexual (precocious) maturation in male Atlantic salmon has increased with the intensification of rearing conditions in RAS systems (Good & Davidson, 2016). The early stages of sexual maturation are characterized by an increase in appetite and accumulation of energy reserves. In contrast, during the final stages of maturation, the stored energy is utilized for physical activities, body maintenance and gonadal maturation process due to which reduction of weight, fat and protein content occurs (Aksnes

et al., 1986). While this diversion of energy resources from somatic growth to gonadal growth is common to all fish, it is particularly pronounced in Atlantic salmon. For example, the mature gonads represent 20-25% and 3-9% of total body weight in females and males respectively (Fleming, 1996). Early sexual maturation is associated with both a reduction in feed intake (appetite) and the mobilization of energy reserves for testicular development, resulting in a poor feed conversion ratio and reduced growth (McClure et al., 2007). Besides, they cannot be easily identified for culling out, hence, end up being transferred to sea water (Good & Davidson, 2016). In sea water, they can suffer high mortality and reduced welfare most probably due to their poor osmoregulatory ability (Taranger et al., 2010). Hence, apart from premature sea cage transfer of smolt, precocious maturation aspect will be explored considering as a probable reason behind stunting.

Like during smoltification, hormonal changes occur during maturation phase of Atlantic salmon. In common with all vertebrates, salmonids have two types of gonadotropins, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh). In both sexes, Fsh is mostly involved in the regulation of the early stages of gonadal development while Lh is primarily involved in regulating the final gonad maturation (ovulation and spermiation), final gamate maturation and release. At the start of gametogenesis (oogenesis and spermatogenesis), Fsh levels elevates gradually over several months, before declining at the time of final maturation. In contrast, plasma Lh levels increase sharply at the time of final maturation (ovulation and spermiation) and gamete release during spawning (Baker et al., 2000; Oppen-Berntsen et al., 1994).

1.7. The pituitary

The pituitary gland acts as a functional bridge connecting nervous system and endocrine system. It receives the signals from the hypothalamus and secrets the hormones which in turn regulates vital physiological processes such as growth, reproduction, metabolism and maintain homeostasis (Pogoda & Hammerschmidt, 2009). Based on ontogeny and anatomy, the pituitary can be divided into two main compartments: the anterior pituitary or adenohypophysis (AH), having oral ectodermal origin, and the posterior pituitary or neurohypophysis (NH), having neuroectodermal origin. The adenohypophysis originates as an ectodermal upgrowth from the roof of the embryonic oral cavity or stomodeum, whereas the neurohypophysis, is ventral extension of the diencephalon floor (Daniel, 1966). In most of the mammals, the adenohypophysis is located anterior to the neurohypophysis, while in mice, rat and teleost it is often ventrally located to the adenohypophysis (Fontaine et al., 2020a).

In teleost, preoptic-hypothalamic neurons directly project into the adenohypophysis, follow the blood vasculature and release their neurohormones into blood vessels or directly at target cells in an economic way, while in mammals, the hypothalamo-hypophysial portal system of the median eminence stalk receives neurohormones and convey them to pituitary (Fontaine et al., 2020a; Levavi-Sivan et al., 2010; Pogoda & Hammerschmidt, 2007). Different from adult tetrapods, where different types of hormone-producing cells are organized in mosaic pattern, in teleosts, group of cells responsible for production of each specific hormone are localized in distinct domain, reflecting embryonic compartmental organization (Fig. 5) (Pogoda & Hammerschmidt, 2007; Weltzien et al., 2004).

According to the nomenclature of Green (1951), the teleostean adenohypophysis can be divided into the anteriorly located *pars distalis* (PD) and the posteriorly located *pars intermedia* (PI). The PD can be further sub-divided into the *rostral pars distalis* (RPD) and the *proximal pars distalis* (RPD) (Weltzien et al., 2004). The neurohypophysis alternatively known as *pars nervosa* (PN) comprises mainly nerve endings of neuroendocrine cells projected from preoptic-hypothalamic region of brain (Fontaine et al., 2020b; Pogoda & Hammerschmidt, 2009), as well as collagen fiber, and glial pituicytes cells having tropic and supportive function (Ferrandino & Grimaldi, 2008). Arginine vasotocin and isotocin are produced by neurohypophysis which are homologous to mammalian vasopressin and oxytocin respectively (Murphy et al., 1998).

The teleost adenohypophysis comprises secretory cells. The RPD possess of lactotrophes producing prolactin (Prl), somatotrophes producing growth hormone (Gh), gonadotrophes producing luteinizing hormone (Lh) or follicle stimulating hormones (Fsh), thyroptropes producing thyroid stimulating horomone (Tsh) and corticotropes producing adrenocorticotropin (Acth). The melanotropes produces melanocyte-stimulating hormone (α-Msh) and somatolactotropes produces somatolactin (Sl) (Kasper et al., 2006; Weltzien et al., 2003). Growth hormone, somatolactin, adrenocorticotropic hormone, thyroid stimulating hormone and prolactin are produced by the pituitary. The adrenocorticotropic hormone acts on inter-renal tissue and stimulates the production and release of cortisol. Similarly, thyroid stimulating hormone (Tsh) activates thyroid to produce thyroxine (T_4) which can be converted into the more biological active form T_3 by the iodothyronine deiodinase enzyme present in all target tissues. Fsh and Lh are the two main hormones controlling sexual maturation. These hormones are also secreted from pituitary gland, are transported to gonads for the production of sex steroids and gametes, thus regulating reproduction (Cowan et al., 2017). The

hypophysiotropic system, the central regulator of pituitary functions, receives information about change in photoperiod, which could stimulate or alter the rhythms of hormones production and trigger the initiation of smoltification process (Ebbesson et al., 2003). As a result, there can be observed clear change in production pattern of Gh, T_4, T_3, cortisol and prolactin during the smoltification process (McCormick, 2012). These changes are not only limited at physiological level but also can be observed in molecular level. There are evidence of change in mRNA expression level of pituitary genes governing hormone production during parr-smolt transformation (Ágústsson et al., 2001; Ágústsson et al., 2003; O'Keeffe et al., 2008). For example, gradual increase in pituitary *tshbb* mRNA expression during smoltification period and peaking at the time initiation of downstream migration by smolts (Fleming et al., 2019). Hence, hormone production functionality connects pituitary directly with smoltification and reproduction process.

Single-cell gene expression analysis shows the expression of several genes: *pomc, prl, smlta, gh, tshb, fshb, lhb,* a common *tsh*, *fsh*, and *lh a*-subunit genes (Siddique et al., 2020). Occurance of higher number of genes and their paralogs is the result of whole-genome duplication event occurred between 50 and 100 million years ago in common ancestor of salmonids. In addition, they have high paralog retention rate (Warren et al., 2014). For instance, the pituitary glycoprotein hormones: Tsh, Lh and Fsh in Atlantic salmon, are composed of a common *alpha(a)*-subunit and a hormone specific *beta (b)* subunit. These glycoprotein hormone *b*-subunits were generated by successive duplications starting from an ancestral glycoprotein hormone *b*-subunit gene *(ancgph)* through two rounds of genomic duplications (first round and second round). The first round (1R) duplication resulted in the formation of two paralogous genes, one evolutionary precursor of gonadotropin *b* subunit genes *(pregthb)* and the other, precursor of the *tshb* subunit gene *(pretshb)*. The subsequent second round (2R) duplication resulted the production of *lhb* and *fshb* from *pregthb* (Maugars et al., 2014) and two paralogs of *tshb (tshba and tshbb)* from *pretshb* (Fleming et al., 2019). There are several pituitary genes found in salmonids. There have been discovered three paralogs of proopiomelanocortin genes (*pomc-a1, -a2, b*) and one splice variant *(-a2s*) (Murashita et al., 2011) , two growth hormone genes (*gh-1* and *gh-2*) (Von Schalburg et al., 2008) , two prolactin genes (*prl1 and prl2*) (Yasuda et al., 1986) , two somatolactin gene (*smlt-a and smlt-b*) (Benedet et al., 2008) and two thyroid stimulating hormone beta gene (*tshba and tshbb*) in salmonids (Fleming et al., 2019). In gene expression studies, it is common to target the specific *beta (b)* subunit when measuring the levels of *tsh, lh* and *fsh*. In my study, I will, therefore, use the term *tshba, tshbb,*

fshb and *lhb* when describing the expression of pituitary glycoproteins. Apart from this, I follow nomenclature recommendations from Zebrafish Nomenclature Conventions for fish genes and hormones. Fish genes are written with all lowercase letters and italicized *(gh)*; and fish proteins are non-italicized and first letter uppercase (Gh).

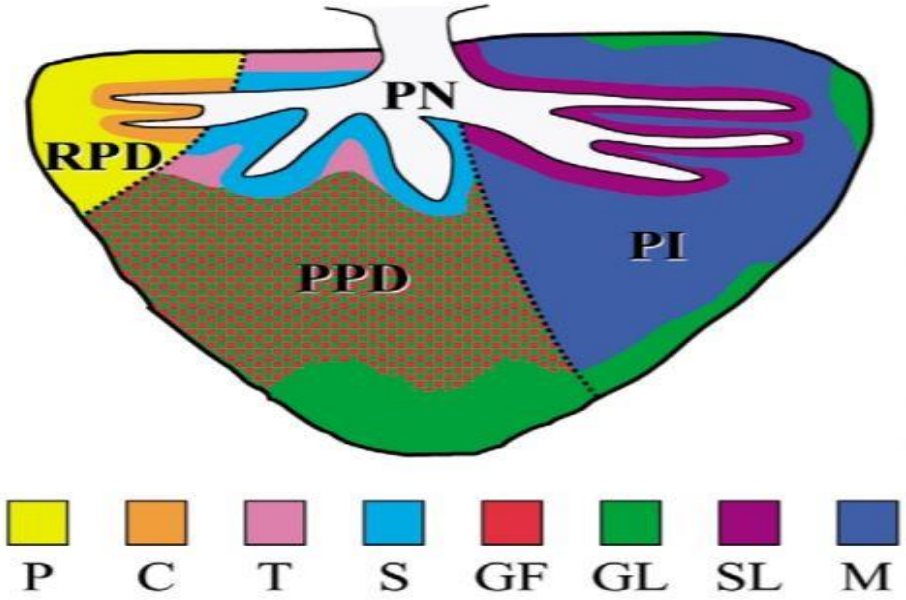


Figure 5. Schematic representation of pituitary hormone producing cells localization in teleost (male Atlantic halibut). PN, pars intermedia, RPD, rostral pars distalis, PPD, proximal pars distalis, PI, pars intermedia, P, lactotropes, C, corticotropes, T, thryotropes, S, somatotropes, GF, Fsh producing gonadotropes, GL, luteinizing hormone producing cells, SL, somatolactotropes and M, mealnotropes (Weltzien et al., 2003).

1.8. Gills

For all animals, it is vital to maintain a stable plasma osmolality and salt composition. Even small changes in the extra- or intra- cellular salt balance can disrupt normal cellular functions. Fish lives in direct contact with water, and the salinity of water determines whether the fish needs to active remove or to retain excess salt from the body. Thus, migration from fresh water to sea water and vice versa require extraordinary osmoregulatory abilities and is unique to anadromous fish species. The main organs controlling salt balance in fish are the gills, kidney, and intestine (McCormick, 2012).

Gills are multifunctional but gaseous exchange and ion regulation are the two main functions. In addition, the gills play a significant role in acid-base regulation and excretion of nitrogenous waste (Evans et al., 2005). The functional unit of the gills are the highly folded filaments on dorsal and ventral surfaces giving rows of plate-like structure known as lamellae. The lamellae has large surface area and highly vascularized with blood vessels covered with thin epithelium designed for gaseous exchange. The position of lamellae offers the counter-current flow of water over the gills with respect to blood flow inside the gill. With the help of specific ion transporters residing in highly specialized cells known as the chloride cells (ionocytes) in the filament epithelium ions can be actively transported across the gill epithelium and thereby facilitate water movement (Hwang et al., 2011; Wilson & Laurent, 2002). Among several ion specific transporters responsible for moving the ions across the gills, Sodium pump, Na^+/K^+-ATPase (Nka) is facilitating both reabsorption and secretion of ions in hypotonic and hypertonic water environments respectively as well as helping in acid-base regulation and ammonia excretion (Foskett & Scheffey, 1982; Hwang et al., 2011). In seawater, sodium and chloride secretion is aided by two additional ion transport proteins $Na^+/K^+/2Cl^-$ cotransporter (Nkcc) and cystic fibrosis transmembrane conductance regulator (Cftr). Nka creates an electrochemical gradient inside the chloride cell by pumping out three sodium ions while pumping in two potassium ions. The low sodium gradient allows the transport of chloride ions into Nkcc which is subsequently pumped out of chloride cells on favorable electrical gradient through an apical membrane Cftr anion channel (Marshall, 2002). Due to the very reason, N^+/K^+-ATPase activity is used to measure the osmoregulatory preparedness in salmonids smolt (Zydlewski & Zydlewski, 2012) and increased in N^+/K^+-ATPase activity has been taken as the indication of readiness of smolts for sea water transfer (McCormick, 1993). In teleost, Nka is composed of two essential subunits. The main catalytic *alpha (a)* subunit, and *beta (b)* subunit, for folding and placement of the complete unit at the cell membrane (McCormick, S. D. et al., 2009). Several isoforms of *a*-subunits have been found to expressed in the salmonids (Richards et al., 2003). In Atlantic salmon, four isoforms of *nkaa* genes *(-1a, -1b, -1c, -3)* and one *nkab1* gene has been found to express in gill tissues (Nilsen et al., 2007). *nkaa-1a* is found to be highly expressed in freshwater whereas *nkaa-1b* in saltwater (McCormick, S. D. et al., 2009; Nilsen et al., 2007). The expression of *nka* isomers are found to be regulated by Gh and cortisol eventually regulating N^+/K^+-ATPase activity (McCormick, 2012).

1.9. Aims of the study

While the occurrence of stunting has long been observed in the salmon industry, recent years has witnessed an increasing number of stunts reported in some production facilities. As such, stunting is increasingly being recognized as one of the major production limitations in the salmon industry, resulting in significant economic losses as well as impacting fish welfare, however, our understanding of the causes of stunting remains largely unknown. Few studies have investigated the underlying molecular and physiological mechanisms controlling stunting in salmon. The results of this study hope to provide new insights into the mechanism behind stunting and help to promote economic and welfare status of salmon fish industry.

The aims of this study were to:

- Investigate potential changes in gene expression of all pituitary hormones and compare plasma level of growth hormone.

- Investigate gene expression analysis of gill sodium potassium ATPase.

2. Methodology

In my master thesis I used mainly two methods. One for measuring gene expression levels and one plasma protein levels. These two methods were real time quantitative polymerase chain reaction (qPCR) and the enzyme linked immunosorbent assay (ELISA).

2.1. qPCR

qPCR was first introduced in 1992 by Huguchi and his co-workers. The first use of qPCR was in the detection of human Y-chromosome specific sequences. In this work Huguchi and co-workers monitored the amplification using ethidium bromide (EtBr) as a fluorescence dye. The EtBr intercalates between the stacked bases of the DNA double-helix and increase in fluorescence occurs with each proceeding PCR cycle. The increase in fluorescence was measured with the help of a fiber optic device (Higuchi et al., 1992). Since then, it has been broadly used in the quantification of genetic material including genotyping (Cheng et al., 2004; Gibson, 2006; Wilson et al., 2005), quantification and detection of pathogen (Ward et al., 2004), single nucleotide polymorphism analysis, chromosome aberrations analysis, protein detection (Kubista et al., 2006), and oncogene quantification in cancer cells (Kindich et al., 2005; Konigshoff et al., 2003). However, this technique has been most extensively used to study gene expression (Fraga et al., 2008; Liss, 2002; Livak & Schmittgen, 2001).

qPCR offers several advantages over traditional or end point detection methods used for quantification of genetic material. In traditional methods, PCR products were used to be collected at the end of PCR assay, and later detected by EtBr gel staining, radioactivity labelling, fluorescence labelling, high performance liquid chromatography, southern blotting, densitometric analysis or other post-amplification methods (Pfaffl, 2004). These post-PCR handling of samples are time consuming, increases the experimental variation, carryover contamination and leads to potential error (Foy & Parkes, 2001). In contrast, qPCR offers advantages of highly accurate and reproducible data, less time consuming, high sensitivity, and can be used for both absolute and relative quantification over other traditional methods (Fraga et al., 2008). Because of high sensitivity, it is highly applicable in the detection and quantification of gene expression level in context of low abundance of RNA concentrations in samples and for elucidation of small changes in mRNA expression level (Pfaffl, 2004). In present situation where oligonucleotides synthesis has been available at affordable prices, enzymes are more reliable and cheaper, and thermal cyclers are being more affordable and user

friendly (Bustin & Huggett, 2017), qPCR has become relatively less costly even compared to ELISA test (Perestam et al., 2017).

qPCR involves the reverse transcription of RNA to cDNA followed by PCR, and detection and quantification of amplified genetic materials in the same assay (Higuchi et al., 1992; Higuchi et al., 1993; Pfaffl, 2004). Reverse transcription results in the synthesis of a complementary (c) DNA molecule from RNA template in the presence of a reverse transcriptase enzyme. In addition to the RNA template, the reverse transcription reaction consists of oligonucleotide primers (the most used are oligo(dT), random hexamers and sequence-specific primers), nucleotides, RNase inhibitor and reaction buffer. With a constant temperature between 50 and 60 °C, the reverse transcription is finalized after about 1 hour. During incubation, the primers anneal to the RNA and cDNA synthesis process begins from the end of the primer, using the RNA sequence as a template. The reverse transcriptase facilitates the synthesis process and is a determinant of the incubation temperature. Nucleotides (mixture of dATP, dTTP, dCTP, and dGTP) provides bases for cDNA synthesis, RNase inhibitor protect RNA template from RNA-degrading ribonucleases present in the laboratory surrounding and reaction buffer provides optimal working condition for enzyme. The samples are heated after completion of reverse transcription reaction to inactivate reverse transcriptase which prevents further reaction (Best & Roberts, 2014).

Technically, each real time PCR cycle consists of three primary steps that is repeated for 36-42 times: melting, annealing and extension (Figure 6 A). In melting step, the double-stranded DNA molecules samples are subjected to a high temperature (94-96 °C) allowing the double stranded DNA to dissociate into single strands. During annealing, the primer pairs defining the amplification area anneals to the complementary sequence of the single dissociated DNA stands. The annealing temperature is primer dependent but usually between 58 and 61 °C. The amplification is performed by a heat stable polymerase enzyme at 72 °C and starts from the complementary primer pairs (Best & Roberts, 2014; Mehra & Hu, 2005).

During the whole process of qPCR amplification, there are four kinetic phases early ground phase, exponential growth phase, linear growth phase and plateau phase (Tichopad et al., 2003). The amount of product formed during course of each amplification cycle can be monitored with the help of fluorescence intensity emitted by fluorescent reporter dyes introduced into the reaction (Kubista et al., 2006). The intensity of fluorescence continuously increases with increase in PCR product after each PCR cycle and reaching a peak at the plateau

phase (Higuchi et al., 1993; Pfaffl, 2004). When amplification curve is plotted using fluorescence emitted verses number of PCR cycles, we can obtain a sigmoidal amplification curve. The curve can be divided into three phases. Phase I, Phase II and Phase III (Figure 6 B). Phase I is early ground phase, phase III consists exponential phase and linear growth phase, and plateau phase fall under Phase III. During the early phase I, amplification cannot be detected because of insufficient level of fluorescence generated from low amount of PCR product in reaction. During phase III, there is no formation of product in exponential rate and hence product formed is no longer proportional to the initial concentration of RNA (Karlen et al., 2007). More than one factors are believed to attribute decrease in reaction rate at phase III. Most common attributes are depletion of reagents (mostly primers), annealing of PCR products with each other, denaturation of DNA polymerase enzyme (Jansson & Hedman, 2019; Karlen et al., 2007), blocking of DNA polymerase activity due accumulation of high amount of PCR product (Karlen et al., 2007). During exponential phase, the amount of amplified target is directly proportional to the input amount of target. Therefore, qPCR measurement of PCR product is taken within exponential phase (Pfaffl, 2004).

The most preferred fluorescent reporter for real-time PCR is SYBR Green I (Kindich et al., 2005), an intercalating dye which binds to all double stranded DNA. Dye gets intercalated between the adjacent base pairs of DNA strands and enhance fluorescent signal upon binding (Figure 7) (Kubista et al., 2006; Smith & Osborn, 2009). It is comparatively cost effective and easy to carry out than other fluorescent dyes (Giglio et al., 2003; Tajadini et al., 2014). Being is highly sensitive, it can detect small amount of DNA (Singer et al., 1999). SYBR has minimum intrinsic fluorescence but upon binding with DNA, it exhibits 800 to 1000-fold fluorescence enhancement. It has high affinity towards ds DNA than that of traditional EtBr dye. Apart from having comparatively low DNA affinity, EtBr is highly toxic to organism. It can be easily absorbed through skin and cause irritation in eyes, mouth and upper respiratory tract and extra expenses needed for managing EtBr waste is needed (Saeidnia & Abdollahi, 2013). The probable problem associated with SYBR is it binds with any unspecific DNA strand. The unspecific binding can occur if there is presence of non-target DNA in reaction, which can be easily detected by melting curve analysis without further analysis and such defective reaction process can be discarded (Ririe et al., 1997).

The calculation of amount of DNA/cDNA present initially is done based on crossing point (C_p) determination between the PCR fluorescence and a chosen benchmark (Figure 6 B). It can be set manually by experimenter or automatically by software present in real-time PCR

instrument. The commonly used form of benchmark is threshold fluorescence. The number of cycles need to reach the threshold fluorescence is denoted as C_t. At crossing point the amount of PCR product in each sample and the fluorescence emitted from them is the same. To simplify, the lower the RT-PCR template amount, the more amplification cycles are needed to reach the threshold fluorescence and lower expression of target gene (Čikoš et al., 2007; Peirson et al., 2003; Ramakers et al., 2003). Both C_t and C_p are now known as "cycle of quantification" C_q (Bustin et al., 2009), are proportional to the log base 2 (log_2) of the number of target molecules present (Forootan et al., 2017).

Housekeeping or reference is used to normalize the expression levels of mRNA and expression level of target gene is presented relative to expression level of housekeeping gene. Commonly used reference genes are *β-actin*, glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*, *β-tubulin*, 18S ribosomal RNA *(18S rRNA)*. The reference gene acts as internal control and normalizes the errors that might occur during experimental process such as pipetting error, difference in the efficiency of enzymes used during reverse transcription and amplification steps. No or only minimal changes in expression levels between the individual samples and experimental conditions makes any reference gene ideal for qPCR assay (Rebouças et al., 2013).

As assay performance depends on experimental conditions, variation can occur depending on extraction methods used to purify the templates, reagents used for the PCR reaction and thermal cycler used, validation and optimization of assay condition before performing own assays is crucial to obtain precise and reliable results (Bustin & Huggett, 2017). To confirm there has been annealing of only primer to specific gene of target, melt curve analysis should be performed at the end of the PCR cycles. The sharp single peak proves amplification of only specific product during PCR reaction. All reagents used during PCR should be free of foreign DNA. A duplicate non-template control (NTC) should be included in every run for each primer pair to examine the purity of used reagents and melting curve analysis should be performed. The curve without any melting peak validates the purity of reagents. Next important step in validation of PCR reaction is to evaluate PCR efficiency. Under ideal experimental condition, with each cycle there will be 2-fold increase in PCR product. In such case the efficiency of primer becomes 2 and reaction is considered 100 % efficient. In contrast, certain experimental errors such as poor pipetting, presence of inhibitors in reagents, poorly designed primers and suboptimal annealing temperature can lead to deviation of efficiency of reaction from 2. To determine efficiency of each primer pair used in reaction, a standard curve is used. A cDNA

sample with known concentration is taken and PCR is performed over a series of dilution starting from most concentrated cDNA solution to dilute one. C_q value for each dilution is calculated and plotted against the log of the respective concentration (Taylor et al., 2010). Based on plotted data, regression line is obtained. The 10^{slope} of regression line gives the efficiency of reaction (Kontanis & Reed, 2006). Selection of ideal reference gene is crucial for successful qPCR. There is no ideal housekeeping gene and evaluation studies should be conducted to identify ideal housekeeping gene for any qPCR study. Depending on tissue it has also been suggested to use three to five different control genes and use their geometric mean (Rebouças et al., 2013). The measurement of qPCR only gives idea measurement of transcription level (McCormick et al., 2019) therefore, for the detection and quantification of proteins and hormones ELISA test should be performed (Fukada et al., 1997; Schuurs & Van Weemen, 1977).

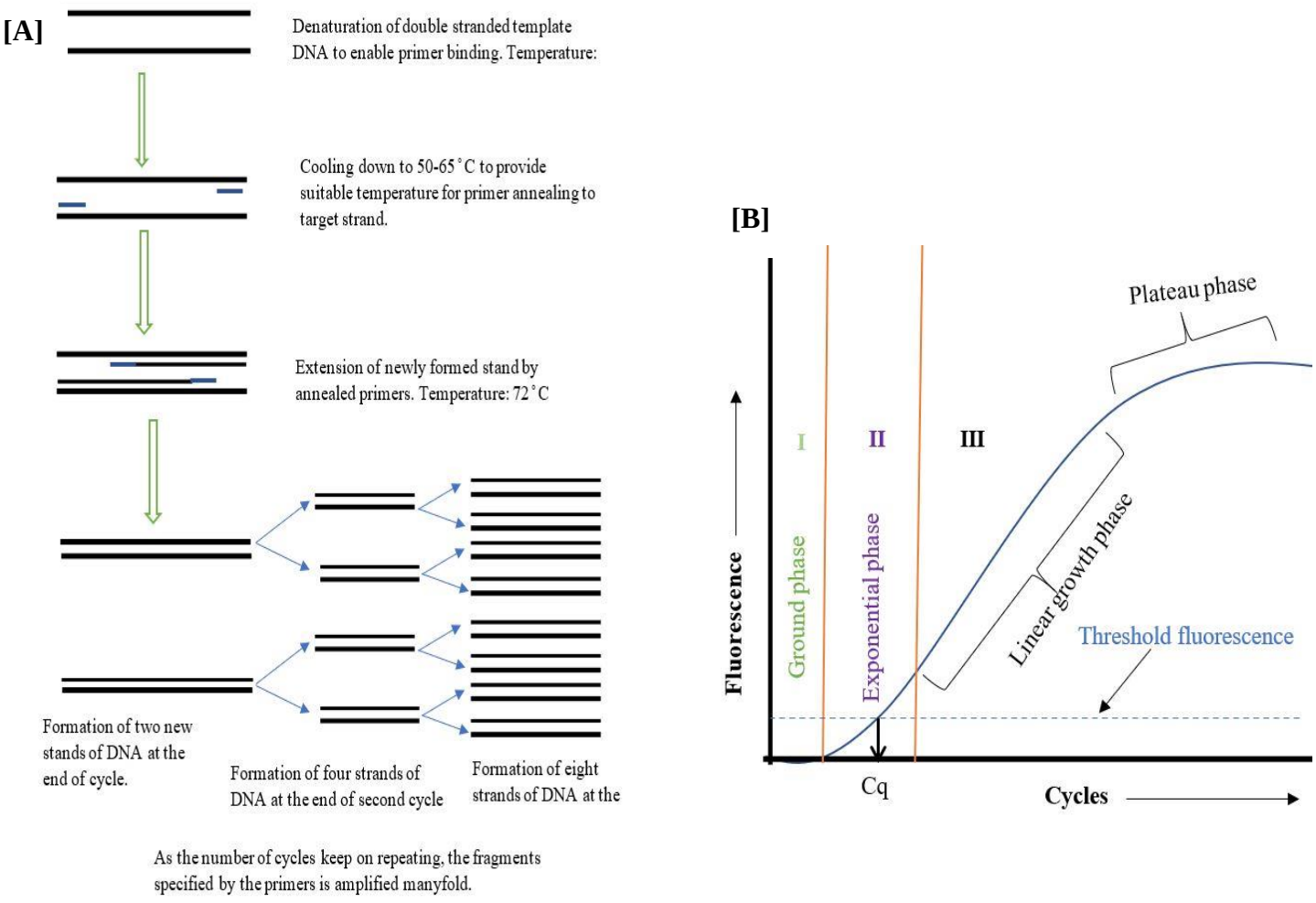


Figure 6. A) Schematic illustration of exponential amplification of a DNA templet using the polymerase chain reaction, B) Amplification curve showing different phases. In phase

I there is no detection of fluorescence. In phase II, there occurs exponential increase in PCR product. Threshold fluorescence is set in this phase to obtain C_q value. In phase III, occurs the amplification of product in a linear manner and at the end of reaction plateau stage is reached.

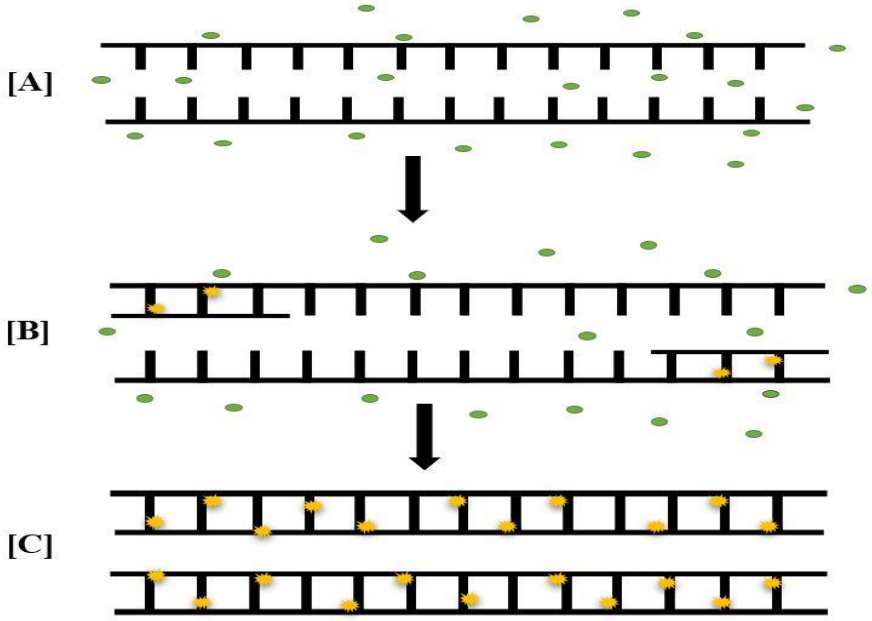


Figure 7. SYBR Green Assay. [A] Denaturation: separates the DNA strands providing space for primer binding. [B] Annealing: the primer binds to their specific ends (3' or 5') and starts extension of strand. The SYBR green molecule also starts to bind with extending DNA segment. [C] Extension and detection: At the end of cycle, extension completes duplicating the number of initial DNA present in sample. The SYBR present in the sample gets attached to the minor grooves of newly formed DNA segments and emit fluorescence signal.

2.2. ELISA

ELISA was first developed by Engvall and Perlmann in 1971 for the quantitative determination of immunoglobulin G (IgG), an antigen from rabbit serum (Engvall & Perlmann, 1971). There occurs a special chemical interaction between antibodies and antigen to protect body from pathogen and toxins. ELISA is based on the principle of radioimmunoassay of specific antigen-antibody reaction using enzymes as reporter label (Lequin, 2005; Sakamoto et al., 2018). It has been a widely used bioanalytical tool in the detection of antigens such as serum protein, hormones, tumor antigens and diagnosis of infectious diseases. It uses the antigen and antibody reaction mechanism (Schuurs & Van Weemen, 1977).

In this study, the indirect competitive ELISA was used for the detection and quantification of Gh plasma levels. In competitive ELISA, there occurs a competition reaction between targets (antigen or antibody) in the sample and enzyme-labeled targets (antigen or antibody) against corresponding immobilized antibody or antigen coated in the ELISA plate. The higher the amount of antigen in the sample, the lower the amount of enzyme-labeled antigen that binds to the antibody (Sakamoto et al., 2018). Upon addition of the fluorogenic substrate, these enzymes degrade the substrate and turns them into a stable and soluble colored compound. The fluorescence emitted by colored compound is easily quantified using a spectrophotometer (O'Beirne & Cooper, 1979). Hence, sample with higher amount of target antigen will give lower level of fluorescence signal and vice versa (Figure 8) (Sakamoto et al., 2018). The commonly used enzyme labels are alkaline phosphatase, horse-radish peroxidase, glucose oxidase and β-D- galactosidase (O'Beirne & Cooper, 1979). Optical densities can be measured directly from the well plate using a colorimeter (Caulfield & Shaffer, 1984). To increase the sensitivity of ELISA biotin/avidin system was incorporated into competitive ELISA (Kendall et al., 1983). In this method, target antigen labeled with biotin was supplied to compete with target antigen in sample (Gould et al., 1985). Avidin acts as a bridge connecting biotin and enzyme as it can directly conjugated with enzyme and as well as strongly bind with biotin molecules (Kendall et al., 1983). Avidin-biotin interaction is more specific and stable interaction as compared to antigen-antibody interaction used in normal ELISA technique. In addition, it does not affect the other bio molecules present in reaction (Jain & Cheng, 2017).

The estimation of the concentration of target antigen in the sample is done with the help of an equation derived from a calibration curve/standard curve. Therefore, along with the sample, a series of dilutions of known standards are included on the plate (Raghava et al., 1992). The standard curve is obtained by plotting concentration of standard against measured corresponding optical density. In most of the cases the logarithmic concentration scale is used and the calibration curve appears sigmoidal, can be easily fitted in logistic model (DeSilva et al., 2003; Findlay & Dillard, 2007). The unknown concentration of target antigen is estimated based on location of the resulting optical density on the standard curve (Herman et al., 2008). The ELISA has advantages of high sensitivity, speed, specificity, economy, and efficiency in antigen and reagent use (Crowther, 2001).

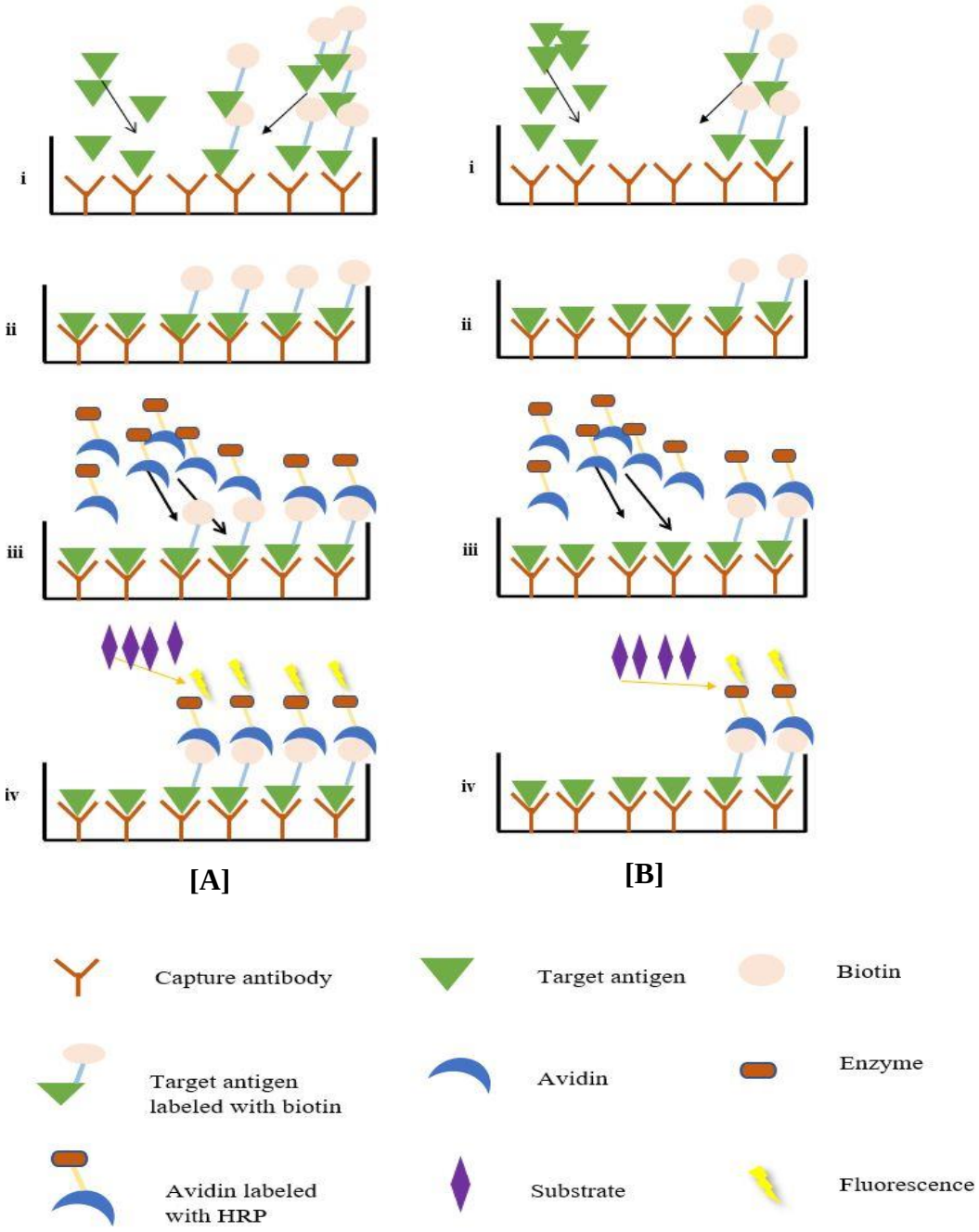


Figure 8. Schematic illustration of competitive ELISA in samples with various amount of target antigen in sample. [A] Sample with low amount of target antigen, and [B] Sample with comparatively high amount of target antigen. Target antigen from sample and target antigen labeled with biotin provided with ELISA kit are added to assay plate coated with antigen in equal quantity. Competition occurs between them to attach with antigen. Avidin labeled with enzyme is added to the plate after the washing of unbound antigen. Avidin bounds with biotin. Upon addition of substate the fluorescence signal is emitted due to the action of enzyme on substrate. Higher amount of fluorescence will be generated in the sample with low amount of target antigen.

2.3. Sample collection

Atlantic salmon were sampled in October 2019, 8 weeks after sea transfer from a commercial fish farm located at the West-coast of Norway. Before sea transfer the salmon were maintained at a recirculating aquaculture system (RAS) facility. From first feeding stage, the salmon were kept at a constant water temperature between 14 and 14.5 °C and with continuous light (24L:0D). When the fry reach at about 30 g, 8 weeks long "winter signal" (12L:12D) was provided to initiated smoltification. Following, the continuous light (24L:0D) for 4 weeks to complete smoltification. Altogether 50 fish were sampled randomly. Out of which 25 individuals were discarded based on their health defects and only healthy individuals were selected. The remaining 25 fish were classified into two groups as judged by condition factor (CF) as stunts (0.66 ± 0.23) and normal (1.32 ± 0.03). The individuals (n= 15) having CF less than 1 were categorized under stunts. Similarly, the individuals (n=10) having CF greater than 1 were placed into normal group.

2.4. Tissue collection

Fishes were anesthetized using benzocaine (ChemCenter, Canada) immediately after they were harvested from the sea cage. Blood samples were taken before decapitation. Heparinized vacutainer was used to draw blood from the caudal vessels and centrifuged for 5-10 minutes at 1000-2000 g to isolate plasma. The plasma was stored on dry ice during transportation (about 5-8 hours) before transfer to -80 °C until hormonal assay. Single pituitaries were collected after the brain was removed from the skull and transferred to 2 ml polypropylene tubes (Fisher scientific, UK) containing 300 µl TRIzol Reagent (Life Technologies, Maryland, USA) and 5-6 cheremic beads. Immediately after securing the pituitaries, the vials were submerged in liquid nitrogen and stored on dry ice for about 12 hours. After returning to the laboratory the pituitaries were store at -80 °C until gene expression analysis. Gill filaments were clipped and transferred to 2 ml polypropylene tubes containing 300 µl TRIzol Reagent (Life Technologies).

2.5. mRNA extraction

For safety purpose (avoid respiration of toxic chemicals) and to avoid contamination of DNA and RNA of samples, all the chemical and sample handling procedures were conducted inside a fuming hood.

2.5.1. mRNA extraction from pituitary

The pituitary samples were taken out from the -80 °C and thawed for 5 minutes. Then, 200 µl TRIzol was added to the sample and homogenized using FastPrep®-24 homogenizer (MP Biomedicals, California, USA) for 20 s at speed of 4 m/s. Homogenization causes tissue and cellular lysis of the pituitary and release the cellular components including RNA. After homogenization, 200 µl chloroform (Sigma-Aldrich, Missouri, USA) was added and the mixture was again vortexed at speed of 4 m/s for 20 s using FastPrep®-24 for complete homogenization. The mixture was then centrifuged at 17, 900 g for 15 minutes and 4 °C using Kubota centrifuge Model 3500 (KUBOTA, Tokyo, Japan). Centrifugation separates the TRIzol-chlorform homogenate into three phases. An upper transparent aqueous layer containing all the RNA, a middle white solid layer containing denatured proteins and some genomic DNA, and the lower layer containing organic components. Thus, the upper aqueous phase rich in RNA carefully pipetted off, without touching the middle or lower layer, and transferred to RNAse-free 1.5 ml centrifuge tubes (brand and country) containing 2µl Glycogen Blue Coprecipitant (Thermofisher Scientific, Maryland, USA). The tubes were vortexed for 15s to mix dye and RNA properly using Bio Vortex V1 (Biosan, Riga, Lativa).

Isopropanol (Sigma-Aldrich, Missouri, USA) was added to the aqueous phase in proportion of 1:1 (aqueous phase: isopropanol). Subsequently, the mixture was centrifuged for 30 minutes, at 4 °C, 17, 900g. Glycogen blue is an inert blue dye covalently linked to glycogen used in alcohol precipitation. The dye precipitates together with RNA and helps in visualization of the RNA pellet for further analysis. RNA present in the samples and glycogen blue get coprecipitated as a visible blue pellet at the bottom of the tube due to centrifugation. The supernatant was removed leaving only the blue RNA pellet at the bottom of the tube.

The RNA pellet washed with 500 µl of 75 % ethanol (Antibac AS, Asker, Norway) and centrifuged for 30 minutes, at 4 °C and 17,900 g. All ethanol was removed without touching the pellet. The tubes were left opened for 15 minutes at room temperature so that traces of ethanol present inside the tube evaporates ensuring complete dryness of the RNA pellet. 15µl of deionized water (Gibco^{TM}, UK) was added to dissolve the RNA, with gentle and repeated in and out pipetting of water within the tube. To finalize the process, the dissolved RNA was heated at 55 °C for 5 minutes allowing complete solubilization of the RNA using dry bath (Labnet International, New Jersey, USA). The heated tubes were immediately placed on ice bath to avoid of RNA degradation.

2.5.2. mRNA extraction from gills tissue

mRNA from gills tissue was extracted by using automatic system with the help of Biomek 4000 robot (Beckman Coulter, California, USA). Gills sample (~32 to 60 g) were placed inside a screw cap 2 ml micro tubes (Sarstedt As, Oslo, Norway) with two stainless steel beads inside. 1-Thioglycerol homogenization solution (Promega Cooperation, Wisconsin, USA) was added at the rate of 125ml/mg of sample. The homogenization 1-thiol solution was prepared 600 µl 1-Thioglycerol (Promega Cooperation) to 30 ml of homogenization solution. The samples were homogenized twice using Fastprep (MP Biomedicals) at the speed of 6.5 m/s for 20 s. In between each homogenization, samples were placed on ice for 60 s to avoid heating and subsequent degradation of RNA. The homogenized solution was centrifuged for 5 minutes at 4 °C, 2,000 g and 230 µl of homogenized gill tissue solution was transferred into a clean Therma deep well working plate of Biomek 4000. Other reagents: cell lysis solution, binding buffer III, DNase I solution, proteinase K, nuclease free water, binding buffer, wash buffer and 80 % ethanol (Antibac) were added to their respective troughs based on the calculation made by Biomek Software v4.0 (Beckman Coulter, California, USA) according to the number of samples loaded for extraction. DNase I solution and ethanol were prepared freshly in laboratory. 80 ml of absolute alcohol was added to 20 ml nuclease free water to make 80% ethanol solution. Addition of 1,060 µl of DNase I to 20,140 ul of yellow core buffer gave DNase I solution. In other hand, other reagents were used as it was supplied by manufacturer. Except ethanol, all other chemical used in mRNA extraction were manufactured by Promega Cooperation, Wisconsin, USA. It took 3 hours for Biomek 4000 (Beckman Coulter) to extract RNA.

2.6. Validation and quantification of mRNA

The purity and concentration of extracted mRNA was evaluated by Nanodrop fluorescence spectrometry using BioTek® spectrophotometer (Bio Tek Instruments, Vermont, USA) and software Gen5 v3.10. The RNA amount present in solution was quantified as ng RNA per µl solution. The purity of RNA was measured by using 260/280 ratio (ratio of absorbance/optical density). Nitrogen bases absorb UV light at 260 nm spectrum, whereas proteins and organic solvents are detected at 280 and 230 nm respectively. Optical density (OD) 260/280 ratio value 1.8 - 2.1 and OD (260/230) of 1.8 or higher is considered as good quality RNA. The RNA qualifying quality test were selected and stored at -80 °C for the further cDNA synthesis.

2.7. cDNA synthesis:

2.7.1. Preparation of dNTP and random primer mixture

The dNTP reaction mixture was prepared by using commercially available 100 mM dNTP stocks (dCTP, dGTP, dATP and dTTP) (Life Technologies, Maryland, USA). 10µl of each 100 mM dNTP stocks were mixed with 60µl water to give 10 mM dNTP reaction mixture. Secondly, dNTP and random primers of concentration 50 ng/µl (Life Technologies, Maryland, USA) were mixed at the ratio of 1:1. The mixture was vortexed for 20 s.

2.7.2. Annealing of random primers

Sterilization of the chamber, pipettes and tube holder with spirit was done before the performance of experiment. The stored RNA samples were kept at room temperature for 5 minutes and then vortexed for 20 s before transferring it into new PCR tubes (VWR International, Pennsylvania, USA).

The concentration of the RNA present in each sample was not uniform in every sample. Therefore, the concentration of the RNA on each sample was maintained 3000 ng (in pituitary samples) and 1000 ng (in gills sample) per 11µl solution to maintain uniformity in the initial amount of RNA quantity. During the quantification procedure, the amount of RNA was calculated as ng per unit µl solution. Hence, required amount of RNA extract solution was calculated using simple unitary method (For instance, according to spectrophotometry quantification, if the RNA extract sample had 3000 ng of RNA amount per 1 µl extract solution, then 1 µl of RNA extract solution was taken and 10 µl of deionized water was added). Hence, the total quantity of RNA used for cDNA synthesis becomes 3000 ng and total volume of sample 11µl. First, the predetermined amount of RNA extract was added to 0.2 ml PCR tubes (VWR International) and then required amount of water was added. Then, tubes were sealed completely using lid after the addition 2µl of mixture of dNTP and random primers (Life Technologies) and vortexed for 10 s with the help of mini centrifuge (VWR International, Korea). Random primers are oligo deoxyribonucleotides which binds randomly throughout the entire of RNA during the reverse transcription process ensuring reverse transcriptase of all RNA sequences. Then, RNA extract solution was subjected to 65 °C for 5 minutes for annealing on PCR Express Thermal Cycler (Hybaid Limited, UK). After annealing, tubes were placed on ice bath to check further denaturation. A reaction mixture was prepared using Dithiothretiol (DTT) (Life Technologies, California, USA), SuperScript III Reverse Transcriptase (SS III RT) (Life Technologies, California, USA), Ribonuclease inhibitor (Life

Technologies, California, USA) and reaction buffer (Life Technologies, California, USA) at the proportion of (1:1:1:4) (DTT: SS III RT: RNase inhibitor: buffer). 7 ul of reaction mixture was added to the previously heated RNA extract and subjected to 75 °C for 90 minutes for reverse transcription process.

2.8. qPCR

The prepared cDNA was diluted before qPCR. For that purpose, 80µl of water was added in 20µl cDNA solution to make 5X dilution of cDNA. The dilution facilitates efficient action of enzymes used during qPCR. The concentration of each specific primers used was 50 ng/µl.

Real Time qPCR was performed using LightCycler® 96 Instrument (Roche Life Science, Germany) with LightCycler® 480 SYBR® Green I Master mix (Roche Diagnostics GmbH, Germany) in final volume of 10µl. The PCR reactions consisted of 3µl of cDNA sample, 1µl of forward primer, 1µl of reverse primer and 5µl of LightCycler® 480 SYBR® Green I Master mix in each well. In parallel, non-template control for each gene was performed in the same plate. Non-template control consisted of the same reaction mixture but substituting cDNA with nuclease free water. Each sample and non-template control were performed in duplicate. The qPCR was conducted on 96 well plate (BIOplastics BV, The Netherlands). Transparent Opti-Seal Optical Sealing Sheet (BIOplastics BV) covered the plate and qPCR was performed.

The thermo parameters adjusted during qPCR for each gene were as follows:

One Cycle of pre-incubation at 95 °C for 300s to activate the polymerase followed by 40 cycles of amplification; each cycle composes 3 steps: cDNA denaturation: 95 °C for 10 s, primer annealing: 60 °C for 10 s, extension period was different for specific primer based on their length (~1s/25bp) at 72 °C. Melting curve analysis was done immediately at the end of PCR cycle with one melting cycle having 3 steps: 95 °C for 5s, 60 °C for 60s, 97 °C for 1 s followed by one cooling cycle at 40 °C.

Beta actin (*β-actin*) was used as the reference gene. There was no drastic deviation in its expression level along individual sample making it stable reference gene for the calculation of relative mRNA expression of target genes (Appendix Figure 3 A & B). Dissociation or melting curve analysis was done immediately after PCR through LightCycler® 96 Software v1.1. Single melt peak was observed in all reactions which confirmed the formation of only specific product during PCR (Appendix Figure 1 & 2). The C_q value for each sample was obtained using LightCycler® 96 Software v1.1. As there were two replications for each sample, hence

mean C_q value was calculated. The efficiency of the PCR reaction was determined by using following formula from standard curve slope: Efficiency (E) = $10^{[-1/slope]}$. In the study of pituitary genes, precalculated efficiency of respective primers were taken from previous experiment conducted on our laboratory. The software has considered E value 2 (theoretical) while calculating C_q value. For that reason, the mean C_q was multiplied with correction factor (Log_2 E) to adjust C_q value corresponding with calculated efficiency of primer. In case of gill genes, theoretical value 2 was taken as efficiency of primers (Appendix Table 1). Hence, expression of pituitary and gills genes are relative to *β-actin* expression in corresponding sample. The relative amount of expression of target gene was calculated based on following equation.

$$ratio(r) = Et^{-CqT} \times Er^{CqR}$$

where, *Et*= efficiency of target gene

C_qT= corrected C_q of target gene

Er= efficiency of reference gene

C_qR=corrected C_q of reference gene

Table 1. List of primers used in qPCR

Gene	Reference ID	Sequence (5'-3')	Size(bp)
lhb	AF146151	**F**:GACGTCCGCTATGAAACGAT	74
		R:GTCACAGCTCAGAGCCACAG	
fshb	AF146152	**F**: TACCTGGAAGGCTGTCCATC	118
		R:TATGCGATCACAGTCGGTGT	
β-actin	Prepared in own lab for Atlantic salmon	**F**:AGCCAACAGGGAGAAGATGA	97
		R:AGAGGCGTACAGGGACAACA	
smlt	NM_001123604.1/ DQ412570.1	**F**:CTTGGATCGCTATGACAACGC	120
		R:TCATCGTCCAGCATCTTCCTG	
tshba	NM_001123528.1	**F**:ATGGGCTTCTGCTACTCAAGG	104
		R: ACTGTTCGGTACTCCACCTG	
prl	NM_001123668.1	**F**: CTAGTTGTGTCCTGTCATGCC	85
		R:TTGGTGGAAAGTGAGAGTCCAG	
gh	NM_001123676.1	**F**:AACGCAGACAGCTGAACAAG	115

		R: ATATGGAGCAGCTTCAGGACTG	
pomcb	NM_001128604.1	**F**: GTGATTCCATTCTGCGCTGTG	106
		R:GACGCACACACCATATTCAACC	
tshbb	MG948546	**F**: TTGCCGTCAACACCACCAT	125
		R: GGGATGATAGACCAGGGAGTG	
pomca2	ENSSSAT00000124177.1	**F**: GAGCGCAGTCAGAAACCACT	110
	(E201)	**R**: TCCTTTCCCTCAATGCCCTC	
pomca1	ENSSSAT00000010436.1	**F**: GGTTAAAGGTCAGTGCTGGGA	141
	(E202)	**R**: TTGGGTTGGAGATGCACCTC	
smltb	NM_001141618	**F**: CCATCCAACATGCTGAGCTC	108
		R: TCCCTGTTTGAGTCAACGGA	
prl x-	XM_014192393.1	**F**: TGTACTTGGGTCTGTGTGCG	101
variant	(LOC100136580)	**R**: GGCATCATCACTCGTCCCAT	
nkaa1a	CK878443	**F**: CCAGGATCACTCAATGTCACTCT	93
		R:GCTATCAAAGGCAAATGAGTTTAATATC	
nkaa1b	CK879688	**F**: GCTACATCTCAACCAACAACATTACAC	91
		R: TGCAGCTGAGTGCACCAT	
nkaα1c	CK 885259	**F**: AGGGAGACGTACTACTAGAAAGCAT	85
		R: CAGAACTTAAAATTCCGAGCAGCAA	
nkaa3	CK170270	**F**: GGAGACCAGCAGAGGAACAG	58
		R: CCCTACCAGCCCTCTGAGT	
nkab1	CK886866	**F**: CGTCAAGCTGAACAGGATCGT	68
		R: CCTCAGGGATGCTTTCATTGGA	

F, forward; **R**, reverse.

2.9. Gh ELISA

2.9.1. Preparation of reagents

Plasma Gh levels from Atlantic salmon were assayed using a commercially available ELISA Kit (MyBioSource, California, USA). At first, all reagents were heated to room temperature for 2 hours to assure that the products were completely dissolved and free of crystals.

The standard solution was prepared according to the protocol. First, a 40 ng/ml Gh stock was made by dissolving the Gh in1 ml of sample buffer provided with the ELISA kit. From this stock, I made a series of 2x dilutions between 20 and 0.625 ng/ml. The dilutions were made by transferring 500 µl from each dilution to a subsequent 2 ml tube containing 500 ul of sample buffer provided along with the kit. Plasma samples were diluted into 1:2 times, 1:4 times and

1:6 times with sample buffer to make sure the optical density values for these dilution falls within the range of the standard curve. Sample buffer alone was used as a negative control for checking non-specific binding and false positives.

Other reagents provided with the ELISA kit, namely wash buffer, working solution of detection reagent A and B were prepared as follows. Wash buffer was prepared by mixing 30 ml of concentrated wash buffer with 720 ml of Ambion® nuclease-free water (Life Technologies, Texas, USA). Biotin-labeled target antigen and Avidin conjugated to Horseradish Peroxidase (HRP) were diluted by using assay diluents A and B, respectively, to obtain working concentrations of 1:100.

2.9.2. Assay procedure

The ELISA assay comes with a 96 well plate precoated with capture antibody. All samples were run in duplicates. The whole experiment procedure was protected from direct light after wards to avoid its probable negative effect on substrate solution. Immediately after the plate was taken from -20 °C to room temperature 50 µl of the different samples including the standards and negative control were added to each of the wells followed by immediate addition of 50 µl working solution of biotin labeled target antigen. The plate was gently tapped on the side to ensure thorough mixing of all solutions in the wells. The plate was covered with Plate sealer (MyBioSource, California, USA) and incubated for 1 hour at 37 °C. After incubation, each well was emptied completely by inverting. Subsequently, 350 µl of wash buffer was added. The plate was allowed to rest for 2 minutes before decanting. The washing procedure was repeated for three times. Between each wash the plate was inverted and blotted against clean paper towel to completely removal any trace of liquid. After washing, 100 µl of the Avidin conjugated to horseradish peroxidase (HRP) was added to each well and re-sealed with a new plate sealer. Thereafter, the plate was incubated for 45 minutes at 37 °C. A second wash step was conducted after the incubation using same procedure as the first washing, but this time repeated 5 times. Then, 90 µl of Tertramethyl benzidine (TMB) substrate solution was added to each well and covered with a new plate sealer before incubating for 20 minutes at 37 °C. Finally, 50 µl of Sulphuric acid (H_2SO_4) was added to each well as a stop solution to terminate further enzyme substrate reaction. The plate was gently tapped to ensure thorough mixing of all solutions. After achieving a uniform colour in each well, the optical density of each well was measured at of 450 nm using a multi microplate from BioTek spectrophotometer (Bio Tek Instruments).

2.10. Plasma osmolality measurement:

Plasma osmolality was determined using a freeze point osmometer (KNAUER Semi-micro Osmometer K07400, Berlin, Germany) and reported as mOsmol Kg^{-1}. Plasma from Atlantic salmon was diluted 1: 2 with nuclease free water (Life Technologies) before measuring the osmolality. The instrument is calibrated to handle exactly 150 µl solution.

2.11. Data analysis

All statistical analyses were performed using the software GraphPad Prism V9.0.0 (GraphPad Software, LLC, San Diego, California, USA). Outliers were identified and removed using ROUT method (Q=5%). Mann-Whitney test was used to examine the significance of observed differences between stunts and normal groups. For all tests, significance was set at the $p<0.05$ level. Data are presented as mean ± standard error of mean (SEM).

3. Results

In present study, fish were categorized into normal and stunts group based on the condition factor calculated using their respective length and body weight. qPCR was conducted to analyse expression level of genes and quantified relative to *β-actin* gene. As reproduction and smoltification are two distinct events in Atlantic salmon lifecycle, pituitary genes governing reproduction and smoltification were presented separately. ELISA test was conducted to measure plasma Gh levels. Sodium potassium ATPase genes expression in gills were assessed using qPCR. In addition, plasma osmolality was also measured to assess ionic status in body fluid of fish. Obtained results were compared in between two groups.

3.1. Weight and condition factor

Table 2. Mean ± SEM values for total weight (g), fork length (cm) and condition factor (k). W is weight expressed in gram and L is fork length of fish expressed in centimeters.

Group	n	Length (cm)	Weight (g)	K (W/L^3) × 100
Stunts	20	29.30 ± 0.60***	172.5 ± 13.63***	0.66 ± 0.23***
Normal	10	45.10 ± 1.06	12131 ± 83.36	1.32 ± 0.03

The average fork length of stunts was found to be 29.30 ± 0.60cm with average weight of 172.5 ± 13.63 g. Normal fish had 45 ± 1.06 cm average fork length with average weight of 12,131 ± 83.36 g. The condition factor of stunts was lower than that of normal fish (Table 2). Mann Whitney test showed, stunts were significantly smaller and thinner as compared to the normal fish ($P < 0.05$). *** indicates significance levels with P<0.001.

3.2. Gh levels

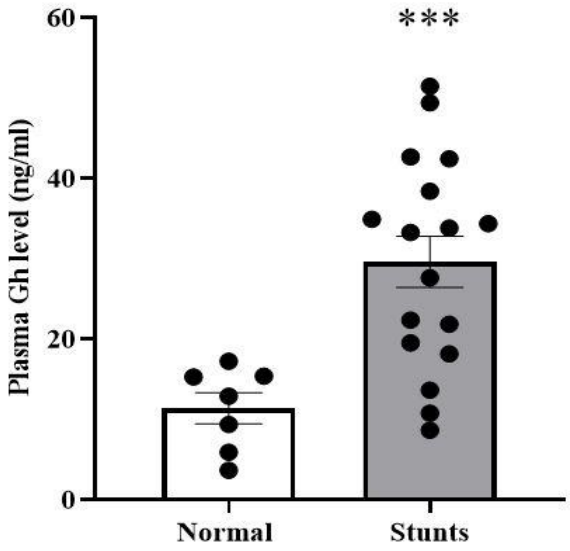


Figure 9. Plasma Gh levels (ng/ml) of stunts and normal fish.

The ELISA showed plasma Gh levels in stunts were significantly higher than in normal growing smolts ($p<0.05$) (Figure 9). *** indicates significance levels with $P<0.001$.

3.3. Osmolality

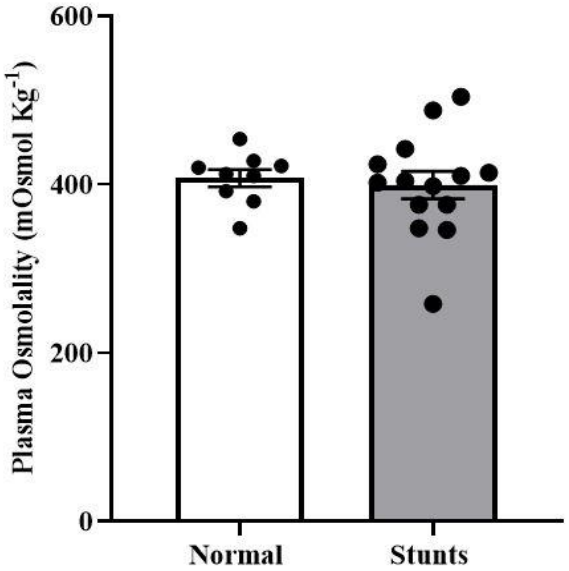


Figure 10. Osmolality of plasma (mOsmol Kg^{-1}) of stunts and normal fish

Osmolality gives the insight about ionic status inside body fluid. Hence, to compare hypoosmoregulatory capacity of fish to maintain ionic balance, plasma osmolality was measured. The result showed that osmolality of the blood plasma of stunts was found to be

lower whereas osmolality of the normal fish, but the difference was not statistically significant ($p>0.05$) (Figure 10).

3.4. Gene expression analysis

3.4.1. Expression of pituitary genes

3.4.1.1. Expression of gonadotropins

To assess reproductive status in stunts compared to normal growing fish, a visual inspection of the gonads was performed. In addition, I analysed the expression levels of the two hormones regulating gonadal maturation, *fshb* and *lhb.* The two groups (normal vs stunts) had no gonadal development (gonadosomatic index < 0.1). The expression level of *fshb* and *lhb* mRNA in pituitary of normal and stunts were not significant ($p>0.05$) (Figure 11 A). However, separation of the sexes show that normal growing males had significantl higher *fshb* expression compared to male stunts (Figure 11 B). Despite separation between males and females there were neither any significant differences in *fshb* expression in females (Figure 11 C) nor *lhb e*xpression in both males and females (Figure 12 B & C). In normal fish, significant upregulation of *smlta* was noted as compared to stunts ($p<0.05$) (Figure 13 A). In other hand, there was not any significant differences in the expression levels of *smltb* between both normal and stunts groups ($p>0.05$) (Figure 13 B).

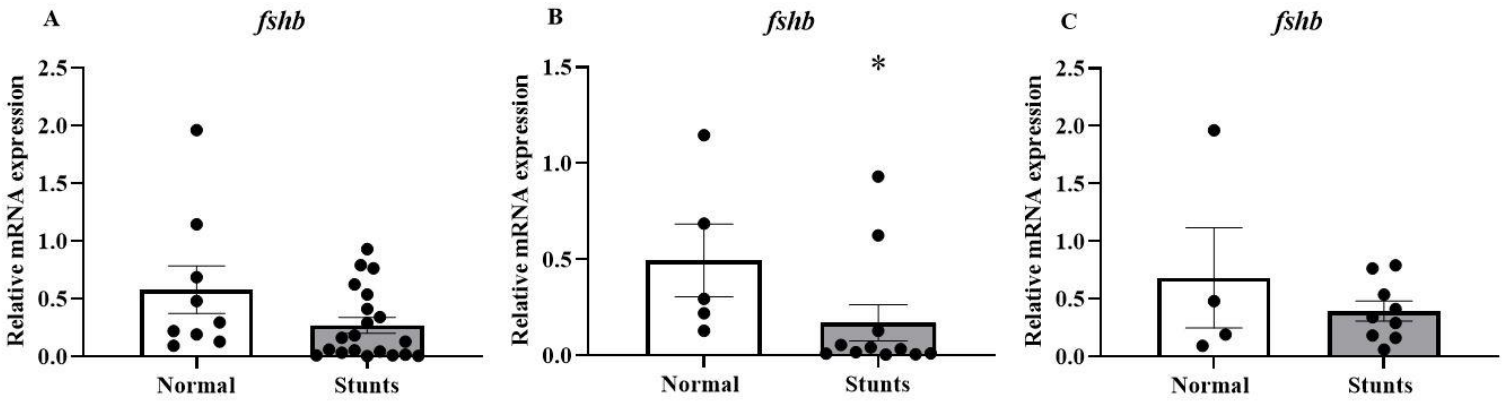


Figure 11. The level of *fshb* mRNA expression in the pituitary of Atlantic salmon between various groups [A] normal and stunts when both sexes are mixed; [B] normal male and stunts male; [C] normal female and stunts female. * indicates significance levels with, $P<0.05$.

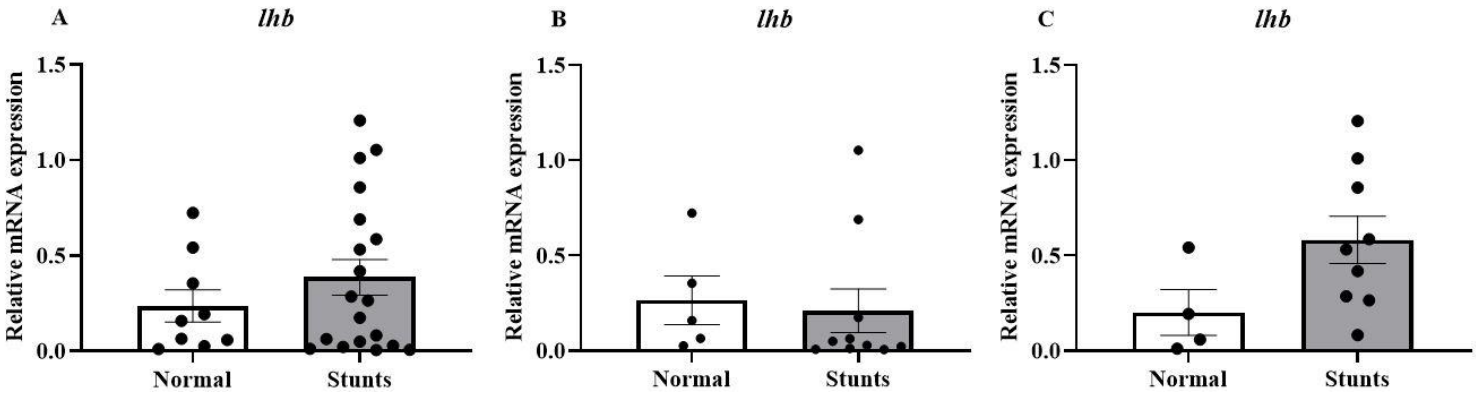


Figure 12. The level of *lhb* mRNA expression in the pituitary of Atlantic salmon between various groups: [A] normal and stunts; [B] normal male and stunts male; [C] normal female and stunts female**.**

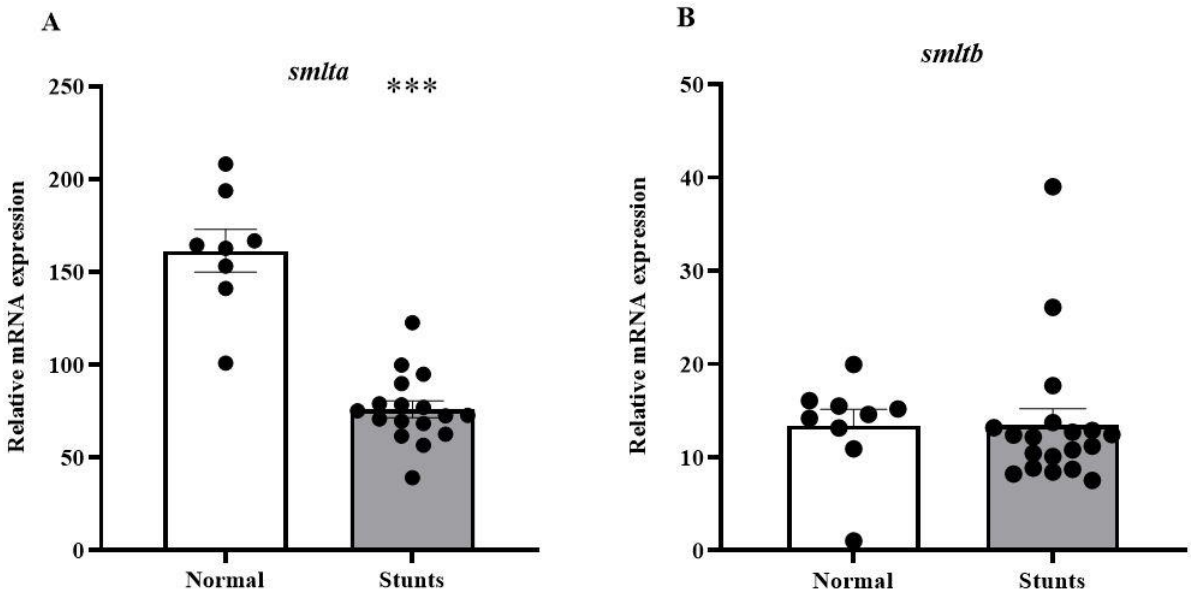
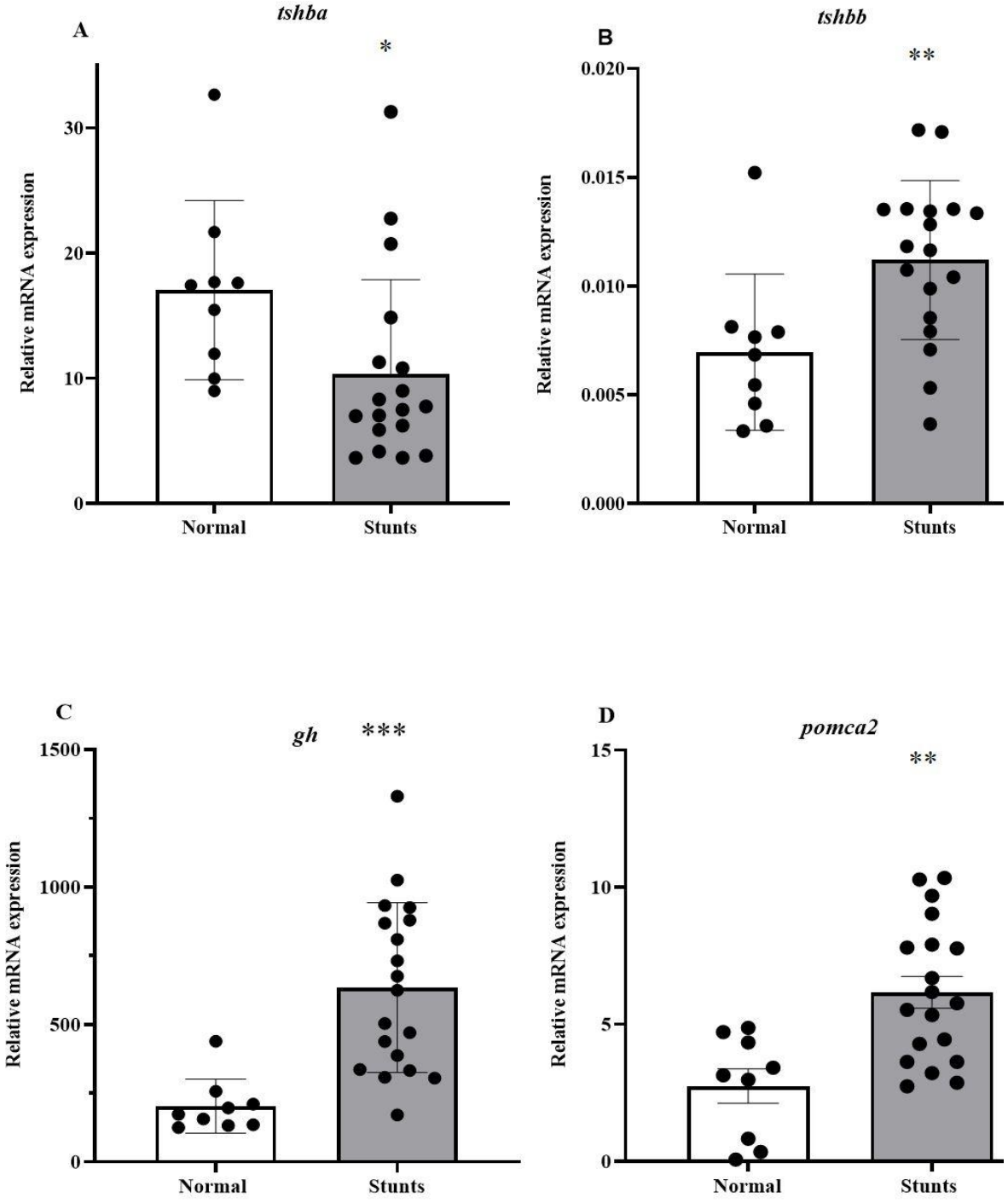


Figure 13. The level of mRNA expression in the pituitary of normal and stunted Atlantic salmon; [A] *smlta* and [B] *smltb*. *** indicates significance levels with P<0.001.

3.4.1.2. Expression of genes regulating smoltification

To assess the status of smoltification in stunts, I analyzed genes governing smoltification: *tshba, tshbb, gh, pomca2, pomca1, pomcb, prl,* and *prl x-variant*. Stunts showed significant up regulation of *tshbb* in comparison to normal fish whereas significant ($p<0.05$) (Figure 14 B) opposite result was obtained in expression level of *tshba* (Figure 14 A). In terms of *gh*, significantly high *gh* mRNA expression was observed in stunts as compared to normal fish (Figure 14 C). Similarly, *pomca2* mRNA levels were two-fold greater ($p<0.05$) compared to normal fish (Figure 14 D). However, there was not any significant differences in the expression

level of *pomca1* (Figure 14 E) and *pomcb* between both stunts and normal groups (p>0.05) (Figure 14 F). Lower expression of *prl* mRNA (p<0.05) was observed compared to normal fish (Figure 14 G) whereas *prl x-variant* was non significantly upregulated in stunts (p>0.05) (Figure 14 H).



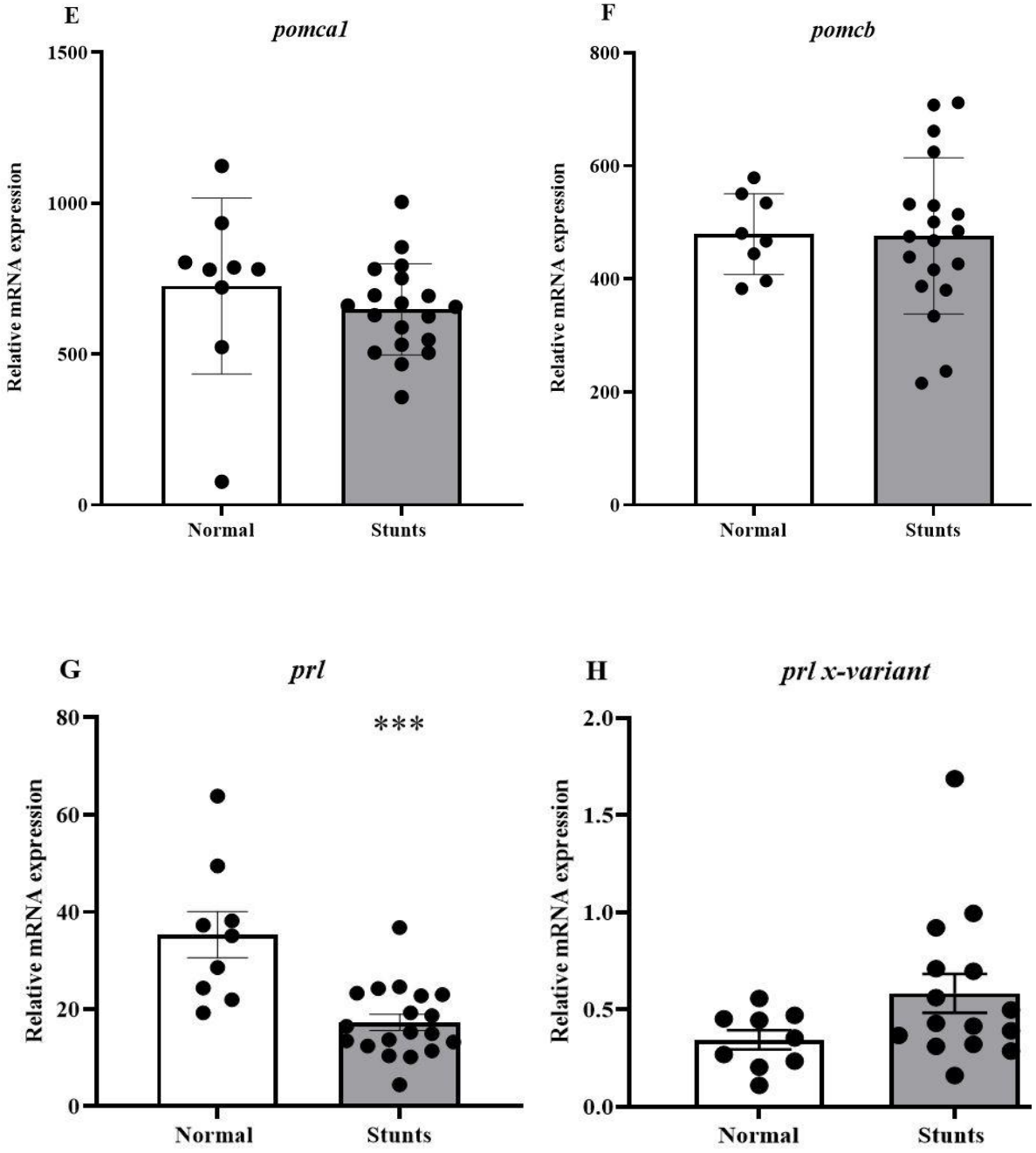
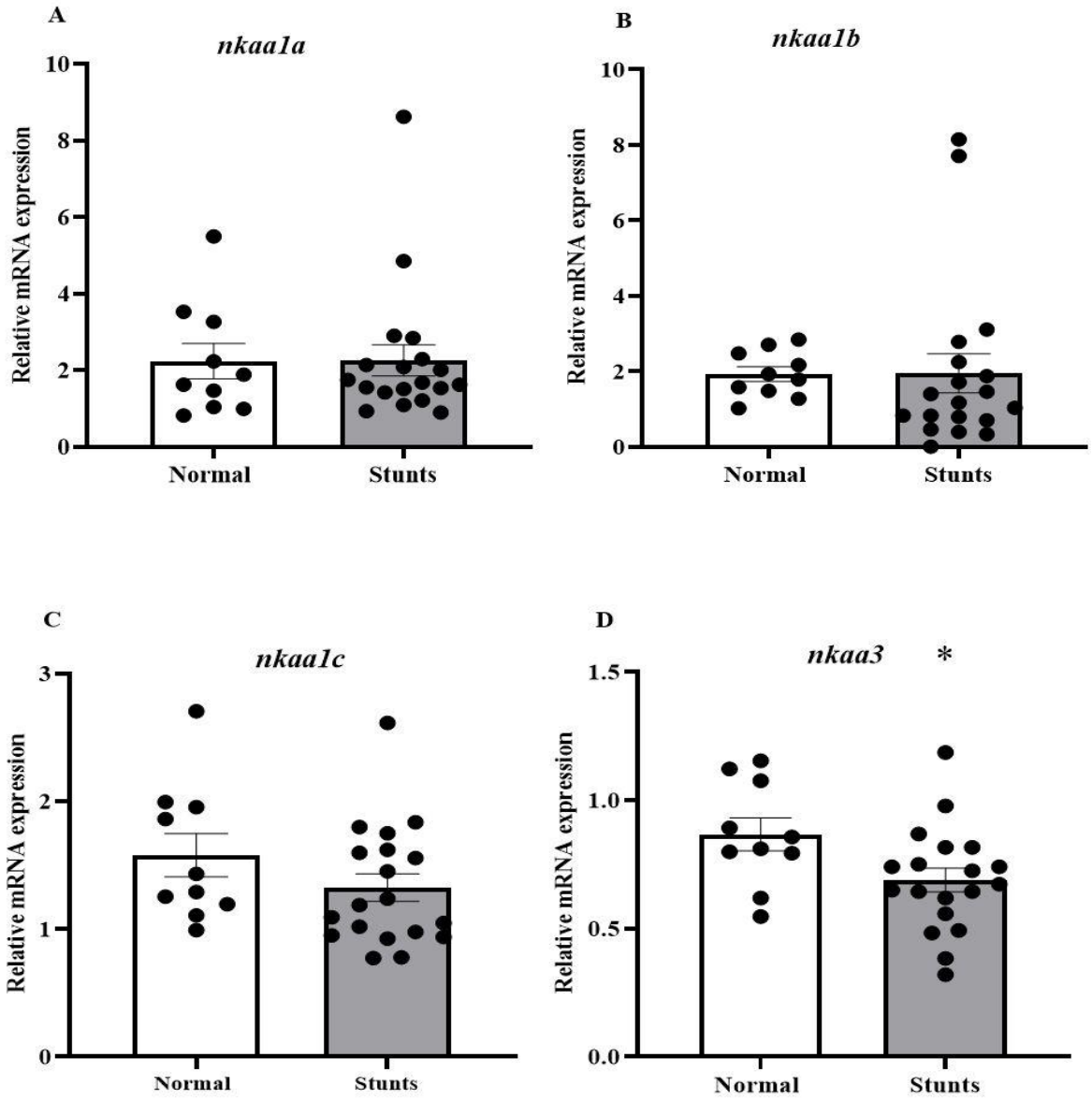


Figure 14. The level of mRNA expression in pituitary of normal and stunted Atlantic salmon. [A] *tshba*, [B] *tshbb*, [C] *gh*, [D] *pomca2*, [E] *pomca1*, [F] *pomcb*, [G] *prl*, [H] *prl x-variant*. * indicates significance levels with P<0.05, ** indicates significance levels with P<0.01, *** indicates significance levels with P<0.001.

3.4.2. Expression of sodium potassium ATPase genes

To assess the osmoregulatory ability stunts as compared to normal fish, expression level of five genes governing Nka activity, *nkaa1a*, *nkaa1b*, *nka1c*, *nka3*, and *nkab1* were analysed. In normal fish, *nkab1* and *nkaa3* mRNA levels were significantly higher than in stunts ($P<0.05$) (Figure 15 D and E). The mRNA levels of other isomers of *nkaa (1a, 1b* and 1c) were not significantly different between stunts and normal groups ($P>0.05$) (Figure 15 A, B and C).



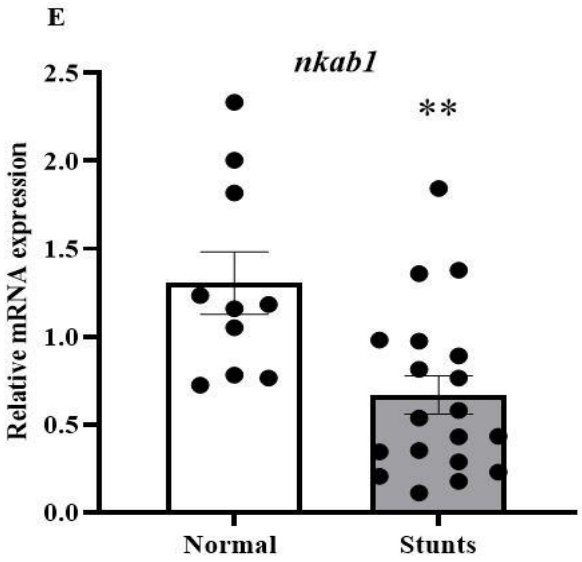


Figure 15. The level of mRNA expression in gills of normal and stunted Atlantic salmon. [A] *nkaa1a*, [B] *nkaa1b*, [C] *nkaa1c*, [D] *nkaa3*, and [E] *nkab1*. * indicates significance levels with P<0.05, ** indicates significance levels with P<0.01.

4. Discussion

The present study reveals differences between the stunts and normal fish at both molecular and physiological level. The plasma level of hormones depends on both secretion rate and clearance rates. The levels of pituitary hormone mRNA expression can give an indication of the potential hormonal secretion rate in Atlantic salmon (Ágústsson et al., 2001). The mRNA expression level of *gh* in the pituitary and plasma hormone levels measured in this study are positively correlated (Appendix Figure 4) and corresponds with the assumption of Ágústsson et al. (2001).

4.1. Expression of pituitary genes

4.1.1. Expression of gonadotropins

A comparison between pituitary expression levels of *fshb* mRNA in pituitary between male and female salmon showed that normal males had significantly higher expression levels of *fshb* compared to normal female (Figure 11 B). In a situation where there was not any observation of gonadal development in normal male and limitation of samples available for analysis (normal male (n=5) and normal female (n=11), we cannot conclude the occurrence of gonadal development in normal male. There was no significant variation in expression levels of *fshb* between male and female groups of stunted fish (Figure 11 C). Likewise, the non-significant difference in expression level of *lhb* between male and female within normal and stunts fish group (Figure 12 B & C).

Although there is lack of clarity regarding the specific function of Sl in fish, it is found to be involved in several physiological functions including maturation, calcium regulation, stress response, acid-base regulation, fat metabolism, and body colour regulation (Kaneko, 1996). Sl has been linked to reproduction in salmonids. For example, Rand-Weaver et al. (1995) reported that plasma Sl were significantly higher in mature compared to immature rainbow trout (Rand-Weaver et al., 1995). Similarly, it was reported that plasma Sl levels increased in both male and female coho salmon, *Oncorhynchus kisutch*, during sexual maturation, with reaching peak level at spawning (Rand-Weaver et al., 1992; Rand-Weaver & Swanson, 1993) and elevated plasma Sl levels were reported in chum salmon, *Oncorhynchus keta*, during spawning migration (Kakizawa et al., 1995). A study on mature and immature Atlantic salmon parr, showed higher plasma Sl levels in mature compared to immature males, while gonadectomy or androgen implants in castrated males resulted in a reduction or increase in plasma Sl levels respectively (Mayer et al., 1998). From this last study, it was proposed that the testes involve in the synthesis and release of pituitary Sl (Mayer et al., 1998). Hence, significantly lower

pituitary *smlta* mRNA expression levels in stunts (Figure 13 A) along with no visual sign of gonadal development suggests absence of precocious sexual maturation in stunts.

4.1.2. Expression of genes regulating smoltification

Plasma levels of number of key hormones, including growth hormone, T_4, T_3, cortisol and prolactin, show pronounced changes during the period of parr-smolt transformation, strongly supporting their active role in regulating smoltification occurs. Early studies confirmed that growth hormone not only played a key role in regulating growth but also in development of hyposmorgulatory ability in salmon smolts (Björnsson, 1997). Paradoxically, even though stunts show poor growth, in the present study we found that stunts had significantly higher levels of pituitary *gh* mRNA expression (Figure 14 C) as well as higher plasma Gh levels compared to normal (Figure 9). It is well established that pituitary hormones can be stored in cells prior to release. As such, while mRNA level measurement gives a measure of transcription level and it does not verify the release of the hormone into blood stream. However, high activity of mRNA levels can indicate the probability of eventual release (McCormick et al., 2019), and our results show the release of Gh in accordance with the increased *gh* mRNA transcription indicating absence of any defects in Gh releasing mechanism.

The involvement of *tshba* in smoltification is still unclear as pituitary *tshba* mRNA expression in Atlantic salmon smolts was found unaffected by photoperiod (Irachi et al., 2020) and unchanged throughout the smoltification period (Fleming et al., 2019). In this study, pituitary *tshba* mRNA level was significantly higher in normal compared to stunts (Figure 14 A). In this context suggestion can be made that *tshba* might have potential role in normal growth and development in seawater phase.

The observation of high plasma Gh level in stunts in this study (Figure 9) is consistent with an earlier study on Atlantic salmon stunts following premature sewater transfer (Björnsson et al., 1988), and in both hatchery-derived and wild coho salmon stunts (Bolton et al., 1987; Varnavsky et al., 1992; Young et al., 1989). In contrast, the low plasma Gh levels measured in fast growing Atlantic salmon smolts following seawater transfer most likely reflects high Gh clearance rates in these rapidly growing fish (Arnesen et al., 2003). In addition, down regulation of Gh-receptors in liver, gill, and kidney of stunts had been observed as compared to smolts (Fryer & Bern, 1979; Gray et al., 1990). In the previous study on salmonids has shown high level of Gh in smolts which are still not ready for seawater life. In wild coho salmon, the plasma Gh levels in stunts measured around four weeks after seawater transfer and it was found

to be as high as “migrating smolts” (Varnavsky et al., 1992). Other studies conducted in Atlantic salmon under natural environment showed similar results. The peak level of Gh has been reported in the smolts at the time of seawater entry (McCormick et al., 2013), and high Gh level was observed in the smolts migrating towards bay as compared to post smolts (Stefansson et al., 2012).

Higher expression of the *tshbb* pituitary mRNA was measured in stunts as compared to normal fish (Figure 14 B). Previous work on Atlantic salmon have reported an increase in *tshbb* expression during parr-smolt transformation, peaking at the start of active downstream migration and start declining with commencement of winter (Fleming et al., 2019). The initiation of active downstream migration corresponds to the period of peak smoltification, also indicates that the smolts are optimally adapted for life in the sea (Hoar, 1988).

The cortisol has inhibitory effect on the production of the Prl in both long and short run. There are clear evidences of role of cortisol in reduction of Prl production (Borski et al., 2001; Yoshikawa, 1992). In vitro study conducted in tilapia has shown the inhibitory effect of cortisol on the release of Prl involving both genomic and non-genomic pathway. The immediate inhibition, within 10-20 minutes of cortisol treatment, on Prl release is done with the interactions with plasma membrane whereas, after 4-6 hours of cortisol exposure *prl* mRNA expression is reduced and leads to subsequent decrease in Prl synthesis (Borski et al., 2001; Yoshikawa, 1992). The high level of pituitary *pomca2* mRNA (Figure 14 D) and low expression of *prl* mRNA (Figure 14 G) in stunts in present study could indicate the higher production of cortisol in stunts. Plasma profile of cortisol also follows the same pattern as Gh and *tshbb* during smoltification. It peaks during parr-smolt transformation, and then return to basal levels after the smolts have entered seawater (McCormick, 2012). Hence, the elevated of *gh*, *tshbb* and *pomca2* pituitary mRNA level indicates the stunts has not reached the hormonal peak (or smoltification peak) phase at the time of sea cage transfer. Besides, there is probability of poor seawater growth in case of late seawater transfer after passing physiological ‘smolt window’. In our study, there is least probability of late transfer as smolts used in this study were held for 4 weeks under continuous light period for smoltification. Highest number of Atlantic salmon post smolt with reduced growth was reported when continuous light phase provided for smoltification was two weeks. The incidence of reduced growth kept on decreasing with increase in duration provided for smoltification (5.2% with 4 weeks, 1.4% with 6 weeks and 0% with 8 weeks) (Van Rijn et al., 2021).

4.2. Effect of cortisol on growth and development

Cortisol reduces the growth and development of vertebrates affecting several physiological processes. Growth inhibition in response to cortisol treatment has been several previous studies rainbow trout (Madison et al., 2015), channel catfish (*Ictalurus punctatus*) (Aydin, 2015), largemouth bass (*Micropterus salmoides*) (O'Connor et al., 2011), brook trout (*Salvelinus fontinalis*) and Atlantic salmon (Vargas-Chacoff et al., 2021). It affects the Gh/Igf-i system by down regulating Gh receptors mRNA levels (Small et al., 2006) and/or activating intracellular pathways that affect Gh receptor signal transduction (Philip & Vijayan, 2015). Increased plasma Gh and reduced plasma Igf-i, in cortisol treatment induced growth reduction in brook trout and Atlantic salmon (Vargas-Chacoff et al., 2021). In other hand, cortisol increases the resting metabolic rate in fish including Atlantic salmon (Hvas & Oppedal, 2019), steelhead trout (*Oncorhynchus mykiss*) (Motyka et al., 2017), Atlantic cod (*Gadus morhua*) (Thorarensen et al., 2010) and Nile tilapia (*Oreochromis niloticus*) (Li et al., 2018) leading to lower conversion efficiency and decrease growth (Vargas-Chacoff et al., 2021). Cortisol is related with deviation of energy from growth related anabolic activities towards more immediate metabolic demanding process such as homeostasis (Liebert & Schreck, 2006). The plasma level of cortisol gives the measurement of magnitude of stress response (Peter, 2011).

It was clearly observed that both stunts and non-stunts had an osmolality above the optimal osmolality range of 290-340 mOsmol Kg^{-1} (Figure 10) (McCormick & Saunders, 1987). In present study, where *pomca2* was upregulated in stunts indicates stunts fish are under osmotic stress, although plasma osmolality of both groups, normal and stunts were not significantly different (Figure 10) suggesting the stressed stunts used their metabolic energy towards maintaining body osmolarity optimum at the cost of growth inhibition.

4.3. Expression of sodium potassium ATPase genes

The *nkaa1a* and *nkaa1b* are considered as fresh water and sea water isomers respectively, based on their increased expression levels in their respective environments (Madsen et al., 2009; Tipsmark & Madsen, 2009). Based on this assumption, distinct function of has been suggested for these two different isomers: *nkaa1a* is responsible for ion uptake in freshwater while *nkaa1b* is responsible for ion excretion in saltwater (Bystriansky et al., 2006). The similarity in plasma osmolality levels and assumed seawater isomers *(-1b)* of gill *nkaa* between stunts and normal fish (Figure 15 B), suggests a normal osmoregulatory process in stunts. We observed a significant down regulation of *nkaa3* in stunts (Figure 15 D) which in contradictory

to previous studies in different teleost species. For example, in rainbow trout, there was no change in *nkaa3* mRNA expression levels after transfer to 80% saltwater, even after 15 days (Richards et al., 2003). Similarly, in tilapia *(Oreochromis mossambicus)* there was no change in of *nkaa3* mRNA expression between freshwater and sea water (Lee et al., 1998). The abundance of *nkaa3* isomers is low as compared to other *nkaa* isomers in the gills of Atlantic salmon. Despite its low abundance, transfer to seawater resulted in a significant decrease in *nkaa3* compared to the levels found in fresh water (Madsen et al., 2009).

In contrast to the catalytic *a*-subunit, the *b*-subunit of *nka* has a supporting role. It is essential for stabilization of the *a*-subunit, membrane targeting, structural and functional maturation of the pump (Madsen et al., 2009). The level of *b*-unit is significantly lower (nearly 1/50) than the total α-subunits. The level *nkab1* expression was found to be higher in saltwater than in fresh water suggesting that the production rate of *nkab1* subunit might be a limiting step in the generation of new pumps controlling the overall pump abundance (Madsen et al., 2009). The lower expression of *nkaa3* and *nkab1* (Figure 15 D, E) indicates an effect of *nkab1* on the overall abundance by affecting *nkaa3*.

5. Conclusion

In conclusion, in order to gain a better understanding of the underlying mechanisms controlling stunting in Atlantic salmon, the molecular and physiological characterization of stunts were performed. Following conclusions can be derived based on the results of present study:

- Stunting is not the result of precocious maturation.
- Stunts are capable of maintaining hypoosmoregulatory capacity in sea cages.
- The main reason behind stunting is premature sea cage transfer of smolts which causes osmotic stress in fish considered as sub-optimal smolts. As a result, a deviation of metabolic energy and modification in physiological processess occurs in order to maintain osmotic balance, but at a cost of normal growth and development.

References

Aas, Ø., Klemetsen, A., Einum, S. & Skurdal, J. (2010). *Atlantic salmon ecology*: John Wiley & Sons.

Ágústsson, T., Sundell, K., Sakamoto, T., Johansson, V., Ando, M. & Bjornsson, B. T. (2001). Growth hormone endocrinology of Atlantic salmon (Salmo salar): pituitary gene expression, hormone storage, secretion and plasma levels during parr-smolt transformation. *Journal of endocrinology*, 170 (1): 227-234.

Ágústsson, T., Sundell, K., Sakamoto, T., Ando, M. & Björnsson, B. T. (2003). Pituitary gene expression of somatolactin, prolactin, and growth hormone during Atlantic salmon parr–smolt transformation. *Aquaculture*, 222 (1-4): 229-238.

Aksnes, A., Gjerde, B. & Roald, S. O. (1986). Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, Salmo salar. *Aquaculture*, 53 (1): 7-20.

Arnesen, A. M., Toften, H., Agustsson, T., Stefansson, S. O., Handeland, S. O. & Björnsson, B. T. (2003). Osmoregulation, feed intake, growth and growth hormone levels in 0+ Atlantic salmon (Salmo salar L.) transferred to seawater at different stages of smolt development. *Aquaculture*, 222 (1-4): 167-187.

Asche, F. & Bjorndal, T. (2011). *The economics of salmon aquaculture*, vol. 10: John Wiley & Sons.

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, 72: 4-15.

Badiola, M., Basurko, O., Piedrahita, R., Hundley, P. & Mendiola, D. (2018). Energy use in recirculating aquaculture systems (RAS): a review. *Aquacultural engineering*, 81: 57-70.

Baker, D. M., Davies, B., Dickhoff, W. W. & Swanson, P. (2000). Insulin-like growth factor I increases follicle-stimulating hormone (FSH) content and gonadotropin-releasing hormone-stimulated FSH release from coho salmon pituitary cells in vitro. *Biology of Reproduction*, 63 (3): 865-871.

Benedet, S., Björnsson, B. T., Taranger, G. L. & Andersson, E. (2008). Cloning of somatolactin alpha, beta forms and the somatolactin receptor in Atlantic salmon: seasonal expression profile in pituitary and ovary of maturing female broodstock. *Reproductive biology and endocrinology*, 6 (1): 1-17.

Bergheim, A., Drengstig, A., Ulgenes, Y. & Fivelstad, S. (2009). Production of Atlantic salmon smolts in Europe—current characteristics and future trends. *Aquacultural Engineering*, 41 (2): 46-52.

Berrill, I. K., Porter, M. J., Smart, A., Mitchell, D. & Bromage, N. R. (2003). Photoperiodic effects on precocious maturation, growth and smoltification in Atlantic salmon, Salmo salar. *Aquaculture*, 222 (1-4): 239-252.

Best, D. & Roberts, K. (2014). In Vitro Amplification Techniques. *Pathobiology of human disease: a dynamic encyclopedia of disease mechanisms*: Elsevier: 4064-4073

Björnsson, B. T., Ogasawara, T., Hirano, T., Bolton, J. P. & Bern, H. A. (1988). Elevated growth hormone levels in stunted Atlantic salmon, Salmo salar. *Aquaculture*, 73 (1-4): 275-281.

Björnsson, B. T. (1997). The biology of salmon growth hormone: from daylight to dominance. *Fish Physiology and Biochemistry*, 17 (1): 9-24.

Björnsson, B. T. & Bradley, T. M. (2007). Epilogue: Past successes, present misconceptions and future milestones in salmon smoltification research. *Aquaculture*, 273 (2-3): 384-391.

Björnsson, B. T., Stefansson, S. O. & McCormick, S. D. (2011). Environmental endocrinology of salmon smoltification. *General and comparative endocrinology*, 170 (2): 290-298.

Bolton, J. P., Young, G., Nishioka, R. S., Hirano, T. & Bern, H. A. (1987). Plasma growth hormone levels in normal and stunted yearling coho salmon, Oncorhynchus kisutch. *Journal of experimental zoology*, 242 (3): 379-382.

Borski, R. J., Hyde, G. N., Fruchtman, S. & Tsai, W. S. (2001). Cortisol suppresses prolactin release through a non-genomic mechanism involving interactions with the plasma membrane. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 129 (2-3): 533-541.

Bustin, S. & Huggett, J. (2017). qPCR primer design revisited. *Biomolecular detection and quantification*, 14: 19-28.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W. & Shipley, G. L. (2009). *The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments*: Oxford University Press.

Bystriansky, J., Richards, J., Schulte, P. & Ballantyne, J. (2006). Reciprocal expression of gill Na+/K+-ATPaseα-subunit isoforms α1a and α1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. *Journal of Experimental Biology*, 209 (10): 1848-1858.

Caulfield, M. J. & Shaffer, D. (1984). A computer program for the evaluation of ELISA data obtained using an automated microtiter plate absorbance reader. *Journal of immunological methods*, 74 (2): 205-215.

Cheng, J., Zhang, Y. & Li, Q. (2004). Real-time PCR genotyping using displacing probes. *Nucleic acids research*, 32 (7): e61-e61.

Čikoš, Š., Bukovská, A. & Koppel, J. (2007). Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *BMC molecular biology*, 8 (1): 1-14.

Clarke, W. C. & Nagahama, Y. (1977). Effect of premature transfer to sea water on growth and morphology of the pituitary, thyroid, pancreas, and interrenal in juvenile coho salmon (Oncorhynchus kisutch). *Canadian Journal of Zoology*, 55 (10): 1620-1630.

Cowan, M., Azpeleta, C. & López-Olmeda, J. F. (2017). Rhythms in the endocrine system of fish: a review. *Journal of Comparative Physiology B*, 187 (8): 1057-1089.

Crowther, J. R. (2001). *The ELISA guidebook*, vol. 149: Springer Science & Business Media.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K. & Pedersen, P. B. (2013). Farming different species in RAS in Nordic countries: Current status and future perspectives. *Aquacultural engineering*, 53: 2-13.

Daniel, P. M. (1966). The anatomy of the hypothalamus and pituitary gland. *Neuroendocrinology*, 1: 15-80.

DeSilva, B., Smith, W., Weiner, R., Kelley, M., Smolec, J., Lee, B., Khan, M., Tacey, R., Hill, H. & Celniker, A. (2003). Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharmaceutical research*, 20 (11): 1885-1900.

Ebbesson, L. O., Ekström, P., Ebbesson, S. O., Stefansson, S. O. & Holmqvist, B. (2003). Neural circuits and their structural and chemical reorganization in the light–brain–pituitary axis during parr–smolt transformation in salmon. *Aquaculture*, 222 (1-4): 59-70.

Elson, P. (1957). The importance of size in the change from parr to smolt in Atlantic salmon. *Canadian Fish Culturist*, 21: 1-6.

Engvall, E. & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry*, 8 (9): 871-874.

Evans, D. H., Piermarini, P. M. & Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological reviews*, 85 (1): 97-177.

Falcón, J., Besseau, L., Sauzet, S. & Boeuf, G. (2007). Melatonin effects on the hypothalamo–pituitary axis in fish. *Trends in Endocrinology & Metabolism*, 18 (2): 81-88.

Ferrandino, I. & Grimaldi, M. C. (2008). Ultrastructural study of the pituicytes in the pituitary gland of the teleost Diplodus sargus. *Brain research bulletin*, 75 (1): 133-137.

Findlay, J. W. & Dillard, R. F. (2007). Appropriate calibration curve fitting in ligand binding assays. *The AAPS journal*, 9 (2): E260-E267.

Fleming, I. (1996). Reproductive strategies ecology and evolution of Atlantic salmon. *Rev. Fish Biol. Fish.*, 6 (4): 379-416.

Fleming, M. (2018). *Environmental and neuroendocrine control of smoltification in long-river (Loire-Allier) Atlantic salmon*: Museum national d'histoire naturelle-MNHN PARIS.

Fleming, M. S., Maugars, G., Lafont, A.-G., Rancon, J., Fontaine, R., Nourizadeh-Lillabadi, R., Weltzien, F.-A., Yebra-Pimentel, E. S., Dirks, R. & McCormick, S. D. (2019). Functional divergence of thyrotropin beta-subunit paralogs gives new insights into salmon smoltification metamorphosis. *Scientific reports*, 9 (1): 1-15.

Folmar, L. C., Dickhoff, W. W., Mahnken, C. V. & Waknitz, F. W. (1982). Stunting and parr-reversion during smoltification of coho salmon (Oncorhynchus kisutch). *Aquaculture*, 28 (1-2): 91-104.

Fontaine, R., Ciani, E., Haug, T. M., Hodne, K., Ager-Wick, E., Baker, D. M. & Weltzien, F.-A. (2020a). Gonadotrope plasticity at cellular, population and structural levels: A comparison between fishes and mammals. *General and comparative endocrinology*, 287: 113344.

Fontaine, R., Royan, M. R., von Krogh, K., Weltzien, F.-A. & Baker, D. M. (2020b). Direct and indirect effects of sex steroids on gonadotrope cell plasticity in the teleost fish pituitary. *Frontiers in Endocrinology*, 11.

Forootan, A., Sjöback, R., Björkman, J., Sjögreen, B., Linz, L. & Kubista, M. (2017). Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular detection and quantification*, 12: 1-6.

Foskett, J. K. & Scheffey, C. (1982). The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science*, 215 (4529): 164-166.

Foy, C. A. & Parkes, H. C. (2001). Emerging homogeneous DNA-based technologies in the clinical laboratory. *Clinical Chemistry*, 47 (6): 990-1000.

Fraga, D., Meulia, T. & Fenster, S. (2008). Real-time PCR. *Current protocols essential laboratory techniques* (1): 10.3. 1-10.3. 34.

Fryer, J. & Bern, H. (1979). Growth hormone binding to tissues of normal and stunted juvenile coho salmon, Oncorhynchus kisutch. *Journal of fish biology*, 15 (5): 527-533.

Fukada, H., Hiramatsu, N., Gen, K. & Hara, A. (1997). Development of an ELISA for chum salmon (Oncorhynchus keta) growth hormone. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 117 (3): 387-392.

Gibson, N. J. (2006). The use of real-time PCR methods in DNA sequence variation analysis. *Clinica Chimica Acta*, 363 (1-2): 32-47.

Giglio, S., Monis, P. T. & Saint, C. P. (2003). Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. *Nucleic Acids Research*, 31 (22): e136-e136. doi: 10.1093/nar/gng135 %J Nucleic Acids Research.

Good, C. & Davidson, J. (2016). A review of factors influencing maturation of Atlantic salmon, Salmo salar, with focus on water recirculation aquaculture system environments. *Journal of the World Aquaculture Society*, 47 (5): 605-632.

Gould, E., Buckley, A. & Cammack, N. (1985). Use of the biotin-streptavidin interaction to improve flavivirus detection by immunofluorescence and ELISA tests. *Journal of virological methods*, 11 (1): 41-48.

Gray, E. S., Young, G. & Bern, H. A. (1990). Radioreceptor assay for growth hormone in coho salmon (Oncorhynchus kisutch) and its application to the study of stunting. *Journal of Experimental Zoology*, 256 (3): 290-296.

Hagspiel, V., Hannevik, J., Lavrutich, M., Naustdal, M. & Struksnæs, H. (2018). Real options under technological uncertainty: A case study of investment in a post-smolt facility in Norway. *Marine Policy*, 88: 158-166.

Herman, R. A., Scherer, P. N. & Shan, G. (2008). Evaluation of logistic and polynomial models for fitting sandwich-ELISA calibration curves. *Journal of immunological methods*, 339 (2): 245-258.

Higuchi, R., Dollinger, G., Walsh, P. S. & Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Bio/technology*, 10 (4): 413-417.

Higuchi, R., Fockler, C., Dollinger, G. & Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Bio/technology*, 11 (9): 1026-1030.

Hoar, W. (1988). 4 The physiology of smolting Salmonids. In vol. 11 *Fish physiology*, pp. 275-343: Elsevier.

Holan, A. B., Good, C. & Powell, M. D. (2020). Health management in recirculating aquaculture systems (RAS). In *Aquaculture Health Management*, pp. 281-318: Elsevier.

Hvas, M. & Oppedal, F. (2019). Physiological responses of farmed Atlantic salmon and two cohabitant species of cleaner fish to progressive hypoxia. *Aquaculture*, 512: 734353.

Hwang, P.-P., Lee, T.-H. & Lin, L.-Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 301 (1): R28-R47.

Irachi, S., Hall, D. J., Fleming, M. S., Maugars, G., Björnsson, B. T., Dufour, S., Uchida, K. & McCormick, S. D. (2020). Photoperiodic regulation of pituitary thyroid-stimulating hormone and brain deiodinase in Atlantic salmon. *Molecular and Cellular Endocrinology*: 111056.

Jain, A. & Cheng, K. (2017). The principles and applications of avidin-based nanoparticles in drug delivery and diagnosis. *Journal of controlled release*, 245: 27-40.

Jansson, L. & Hedman, J. (2019). Challenging the proposed causes of the PCR plateau phase. *Biomolecular detection and quantification*, 17: 100082.

Kakizawa, S., Kaneko, T., Ogasawara, T. & Hirano, T. (1995). Changes in plasma somatolactin levels during spawning migration of chum salmon (Oncorhynchus keta). *Fish physiology and biochemistry*, 14 (2): 93-101.

Kaneko, T. (1996). Cell biology of somatolactin. *International review of cytology*, 169: 1-24.

Karlen, Y., McNair, A., Perseguers, S., Mazza, C. & Mermod, N. (2007). Statistical significance of quantitative PCR. *BMC bioinformatics*, 8 (1): 1-16.

Kasper, R. S., Shved, N., Takahashi, A., Reinecke, M. & Eppler, E. (2006). A systematic immunohistochemical survey of the distribution patterns of GH, prolactin, somatolactin, β–TSH, β–FSH, β–LH, ACTH, and α–MSH in the adenohypophysis of Oreochromis niloticus, the Nile tilapia. *Cell and tissue research*, 325 (2): 303-313.

Kendall, C., Ionescu-Matiu, I. & Dreesman, G. R. (1983). Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). *Journal of immunological methods*, 56 (3): 329-339.

Kindich, R., Florl, A. R., Jung, V., Engers, R., Muller, M., Schulz, W. A. & Wullich, B. (2005). Application of a modified real-time PCR technique for relative gene copy number quantification to the determination of the relationship between NKX3. 1 loss and MYC gain in prostate cancer. *Clinical chemistry*, 51 (3): 649-652.

Konigshoff, M., Wilhelm, J., Bohle, R. M., Pingoud, A. & Hahn, M. (2003). HER-2/neu gene copy number quantified by real-time PCR: comparison of gene amplification, heterozygosity, and immunohistochemical status in breast cancer tissue. *Clinical chemistry*, 49 (2): 219-229.

Kontanis, E. J. & Reed, F. A. (2006). Evaluation of real-time PCR amplification efficiencies to detect PCR inhibitors. *Journal of forensic sciences*, 51 (4): 795-804.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B. & Strömbom, L. (2006). The real-time polymerase chain reaction. *Molecular aspects of medicine*, 27 (2-3): 95-125.

Lee, T.-H., Tsai, J.-C., Fang, M.-J., Yu, M.-J. & Hwang, P.-P. (1998). Isoform expression of Na+-K+-ATPase α-subunit in gills of the teleost Oreochromis mossambicus. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 275 (3): R926-R932.

Lequin, R. M. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical chemistry*, 51 (12): 2415-2418.

Levavi-Sivan, B., Bogerd, J., Mañanós, E. L., Gómez, A. & Lareyre, J. J. (2010). Perspectives on fish gonadotropins and their receptors. *General and comparative endocrinology*, 165 (3): 412-437.

Li, M., Wang, X., Qi, C., Li, E., Du, Z., Qin, J. G. & Chen, L. (2018). Metabolic response of Nile tilapia (Oreochromis niloticus) to acute and chronic hypoxia stress. *Aquaculture*, 495: 187-195.

Liebert, A. M. & Schreck, C. B. (2006). Effects of acute stress on osmoregulation, feed intake, IGF-1, and cortisol in yearling steelhead trout (Oncorhynchus mykiss) during seawater adaptation. *General and Comparative Endocrinology*, 148 (2): 195-202.

Liss, B. (2002). Improved quantitative real-time RT–PCR for expression profiling of individual cells. *Nucleic acids research*, 30 (17): e89-e89.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, 25 (4): 402-408.

Lysfjord, G., Jobling, M. & Solberg, C. (2004). Atlantic salmon, Salmo salar L., smolt production strategy affects body composition and early seawater growth. *Aquaculture*, 237 (1-4): 191-205.

Madison, B. N., Tavakoli, S., Kramer, S. & Bernier, N. J. (2015). Chronic cortisol and the regulation of food intake and the endocrine growth axis in rainbow trout. *J Endocrinol*, 226 (2): 103-19.

Madsen, S. S., Kiilerich, P. & Tipsmark, C. K. (2009). Multiplicity of expression of Na+, K+–ATPaseα-subunit isoforms in the gill of Atlantic salmon (Salmo salar): cellular localisation and absolute quantification in response to salinity change. *Journal of Experimental Biology*, 212 (1): 78-88.

Maitra, S. K., Mukherjee, S. & Hasan, K. N. (2015). Melatonin: endogenous sources and role in the regulation of fish reproduction. In *Indoleamines: sources, role in biological processes and health effects*, pp. 43-77: Nova Science Publishers Inc New York (NY).

Marshall, W. S. (2002). Na+, Cl−, Ca2+ and Zn2+ transport by fish gills: retrospective review and prospective synthesis. *Journal of experimental zoology*, 293 (3): 264-283.

Martin, S. A. M., Wallner, W., Youngson, A. & Smith, T. (1999). Differential expression of Atlantic salmon thyrotropin β subunit mRNA and its cDNA sequence. *Journal of fish biology*, 54 (4): 757-766.

Maugars, G., Dufour, S., Cohen-Tannoudji, J. & Quérat, B. (2014). Multiple thyrotropin β-subunit and thyrotropin receptor-related genes arose during vertebrate evolution. *PloS one*, 9 (11): e111361.

Mayer, I., Rand-Weaver, M. & Borg, B. (1998). Effects of gonadectomy and steroids on plasma and pituitary levels of somatolactin in Atlantic salmon, Salmo salar. *General and comparative endocrinology*, 109 (2): 223-231.

McClure, C. A., Hammell, K. L., Moore, M., Dohoo, I. R. & Burnley, H. (2007). Risk factors for early sexual maturation in Atlantic salmon in seawater farms in New Brunswick and Nova Scotia, Canada. *Aquaculture*, 272 (1-4): 370-379.

McCormick, S. & Shrimpton, J. (1997). JD ZYDLEWSKI Temperature effects on osmoregulatory physiology of juvenile anadromous fish. *Global warming: implications for freshwater and marine fish* (61): 279.

McCormick, S., Lerner, D. T., Monette, M. Y., Nieves-Puigdoller, K., Kelly, J. T. & Björnsson, B. T. (2009). *Taking it with you when you go: how perturbations to the freshwater environment, including temperature, dams, and contaminants, affect marine survival of salmon*. American Fisheries Society Symposium: Citeseer.

McCormick, S. D. & Saunders, R. L. (1987). *Preparatory physiological adaptations for marine life of salmonids: osmoregulation, growth, and metabolism*. Am. Fish. Soc. Symp.

McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurement of Na+, K+-ATPase activity. *Canadian Journal of Fisheries and Aquatic Sciences*, 50 (3): 656-658.

McCormick, S. D., Hansen, L. P., Quinn, T. P. & Saunders, R. L. (1998). Movement, migration, and smolting of Atlantic salmon (Salmo salar). *Canadian Journal of Fisheries and Aquatic Sciences*, 55 (S1): 77-92.

McCormick, S. D., Shrimpton, J. M., Moriyama, S. & Björnsson, B. T. (2002). Effects of an advanced temperature cycle on smolt development and endocrinology indicate that temperature is not a zeitgeber for smolting in Atlantic salmon. *Journal of Experimental Biology*, 205 (22): 3553-3560.

McCormick, S. D., Regish, A. & Christensen, A. (2009). Distinct freshwater and seawater isoforms of Na+/K+-ATPase in gill chloride cells of Atlantic salmon. *Journal of Experimental Biology*, 212 (24): 3994-4001.

McCormick, S. D. (2012). Smolt physiology and endocrinology. In vol. 32 *Fish physiology*, pp. 199-251: Elsevier.

McCormick, S. D., Sheehan, T. F., Björnsson, B. T., Lipsky, C., Kocik, J. F., Regish, A. M. & O'Dea, M. F. (2013). Physiological and endocrine changes in Atlantic salmon smolts during hatchery rearing, downstream migration, and ocean entry. *Canadian Journal of Fisheries and Aquatic Sciences*, 70 (1): 105-118.

McCormick, S. D., Regish, A. M., Ardren, W. R., Björnsson, B. T. & Bernier, N. J. (2019). The evolutionary consequences for seawater performance and its hormonal control when anadromous Atlantic salmon become landlocked. *Scientific reports*, 9 (1): 1-10.

Mehra, S. & Hu, W. S. (2005). A kinetic model of quantitative real-time polymerase chain reaction. *Biotechnology and bioengineering*, 91 (7): 848-860.

Meriac, A. (2019). Smolt production and the potential for solid waste collection in Norway. *Nofima rapportserie*.

Mills, D. (1989). *Ecology and management of Atlantic salmon*: Springer Science & Business Media.

Motyka, R., Norin, T., Petersen, L. H., Huggett, D. B. & Gamperl, A. K. (2017). Long-term hypoxia exposure alters the cardiorespiratory physiology of steelhead trout (Oncorhynchus mykiss), but does not affect their upper thermal tolerance. *Journal of thermal biology*, 68: 149-161.

Murashita, K., Jordal, A.-E. O., Nilsen, T. O., Stefansson, S. O., Kurokawa, T., Björnsson, B. T., Moen, A.-G. G. & Rønnestad, I. (2011). Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 158 (1): 79-86.

Murphy, D., Si-Hoe, S. L., Brenner, S. & Venkatesh, B. (1998). Something fishy in the rat brain: molecular genetics of the hypothalamo-neurohypophysial system. *Bioessays*, 20 (9): 741-749.

Nilsen, T. O., Ebbesson, L. O., Madsen, S. S., McCormick, S. D., Andersson, E., Björnsson, B. T., Prunet, P. & Stefansson, S. O. (2007). Differential expression of gill Na+, K+-ATPaseα-and β-subunits, Na+, K+, 2Cl-cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon Salmo salar. *Journal of Experimental Biology*, 210 (16): 2885-2896.

O'Beirne, A. J. & Cooper, H. R. (1979). Heterogeneous enzyme immunoassay. *Journal of Histochemistry & Cytochemistry*, 27 (8): 1148-1162.

O'Connor, C. M., Gilmour, K. M., Arlinghaus, R., Matsumura, S., Suski, C. D., Philipp, D. P. & Cooke, S. J. (2011). The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (Micropterus salmoides). *Canadian Journal of Fisheries and Aquatic Sciences*, 68 (4): 693-705.

O'Keeffe, A., Hubert, S., Voisin, M., Houeix, B., Cotter, D. & Cairns, M. (2008). Somatolactin mRNA expression during the parr–smolt transformation in hatchery-reared Atlantic salmon Salmo salar smolts. *Journal of Fish Biology*, 73 (2): 436-443.

Oppen-Berntsen, D., Olsen, S., Rong, C., Taranger, G., Swanson, P. & Walther, B. (1994). Plasma levels of eggshell zr-proteins, estradiol-17β, and gonadotropins during an annual reproductive cycle of Atlantic salmon (Salmo salar). *Journal of Experimental Zoology*, 268 (1): 59-70.

Peirson, S. N., Butler, J. N. & Foster, R. G. (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic acids research*, 31 (14): e73-e73.

Perestam, A. T., Fujisaki, K. K., Nava, O. & Hellberg, R. S. (2017). Comparison of real-time PCR and ELISA-based methods for the detection of beef and pork in processed meat products. *Food Control*, 71: 346-352.

Peter, M. S. (2011). The role of thyroid hormones in stress response of fish. *General and Comparative Endocrinology*, 172 (2): 198-210.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. *AZ of quantitative PCR*, 1: 89-113.

Philip, A. M. & Vijayan, M. M. (2015). Stress-immune-growth interactions: cortisol modulates suppressors of cytokine signaling and JAK/STAT pathway in rainbow trout liver. *PLoS One*, 10 (6): e0129299.

Pogoda, H.-M. & Hammerschmidt, M. (2007). *Molecular genetics of pituitary development in zebrafish*. Seminars in cell & developmental biology: Elsevier.

Pogoda, H.-M. & Hammerschmidt, M. (2009). How to make a teleost adenohypophysis: molecular pathways of pituitary development in zebrafish. *Molecular and cellular endocrinology*, 312 (1-2): 2-13.

Prunet, P., Boeuf, G., Bolton, J. P. & Young, G. (1989). Smoltification and seawater adaptation in Atlantic salmon (Salmo salar): plasma prolactin, growth hormone, and thyroid hormones. *General and comparative endocrinology*, 74 (3): 355-364.

Raghava, G., Joshi, A. & Agrewala, J. (1992). Calculation of antibody and antigen concentrations from ELISA data using a graphical method. *Journal of immunological methods*, 153 (1-2): 263-264.

Ramakers, C., Ruijter, J. M., Deprez, R. H. L. & Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience letters*, 339 (1): 62-66.

Rand-Weaver, M., Swanson, P., Kawauchi, H. & Dickhoff, W. W. (1992). Somatolactin, a novel pituitary protein: purification and plasma levels during reproductive maturation of coho salmon. *Journal of Endocrinology*, 133 (3): 393-403.

Rand-Weaver, M. & Swanson, P. (1993). Plasma somatolactin levels in coho salmon (Oncorhynchus kisutch) during smoltification and sexual maturation. *Fish Physiology and Biochemistry*, 11 (1): 175-182.

Rand-Weaver, M., Pottinger, T. & Sumpter, J. (1995). Pronounced seasonal rhythms in plasma somatolactin levels in rainbow trout. *Journal of endocrinology*, 146 (1): 113-119.

Rebouças, E. d. L., Costa, J. J. d. N., Passos, M. J., Passos, J. R. d. S., Hurk, R. v. d. & Silva, J. R. V. (2013). Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. *Brazilian Archives of Biology and Technology*, 56 (1): 143-154.

Richards, J. G., Semple, J. W., Bystriansky, J. S. & Schulte, P. M. (2003). Na+/K+-ATPase α-isoform switching in gills of rainbow trout (Oncorhynchus mykiss) during salinity transfer. *Journal of Experimental Biology*, 206 (24): 4475-4486.

Ririe, K. M., Rasmussen, R. P. & Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical biochemistry*, 245 (2): 154-160.

Saeidnia, S. & Abdollahi, M. (2013). *Are other fluorescent tags used instead of ethidium bromide safer?*: Springer.

Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H. & Morimoto, S. (2018). Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *Journal of natural medicines*, 72 (1): 32-42.

Sandvold, H. N. & Tveterås, R. (2014). Innovation and productivity growth in Norwegian production of juvenile salmonids. *Aquaculture Economics & Management*, 18 (2): 149-168.

Saunders, R. L. & Schom, C. B. (1985). Importance of the variation in life history parameters of Atlantic salmon (Salmo salar). *Canadian Journal of Fisheries and Aquatic Sciences*, 42 (3): 615-618.

Schuurs, A. & Van Weemen, B. (1977). Enzyme-immunoassay. *Clinica Chimica Acta*, 81 (1): 1-40.

Siddique, K., Ager-Wick, E., Fontaine, R., Weltzien, F.-A. & Henkel, C. V. (2020). Characterization of hormone-producing cell types in the teleost pituitary gland using single-cell RNA-seq. *bioRxiv*.

Simonneaux, V. & Ribelayga, C. (2003). Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacological reviews*, 55 (2): 325-395.

Singer, V. L., Lawlor, T. E. & Yue, S. (1999). Comparison of SYBR® Green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in the Salmonella/mammalian microsome reverse mutation assay (Ames test). *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 439 (1): 37-47.

Skilbrei, O. T. (1991). Importance of threshold length and photoperiod for the development of bimodal length–frequency distribution in Atlantic salmon (Salmo salar). *Canadian Journal of Fisheries and Aquatic Sciences*, 48 (11): 2163-2172.

Small, B. C., Murdock, C. A., Waldbieser, G. C. & Peterson, B. C. (2006). Reduction in channel catfish hepatic growth hormone receptor expression in response to food deprivation and exogenous cortisol. *Domestic animal endocrinology*, 31 (4): 340-356.

Smith, C. J. & Osborn, A. M. J. F. m. e. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. 67 (1): 6-20.

StatisticsNorway. (2020). *Aquaculture (terminated in Statistics Norway)*. Available at: https://www.ssb.no/en/fiskeoppdrett/.

Stefansson, S. O., Haugland, M., Björnsson, B. T., McCormick, S. D., Holm, M., Ebbesson, L. O., Holst, J. C. & Nilsen, T. O. (2012). Growth, osmoregulation and endocrine changes in wild Atlantic salmon smolts and post-smolts during marine migration. *Aquaculture*, 362: 127-136.

Summerfelt, S. T., Mathisen, F., Holan, A. B. & Terjesen, B. F. (2016). Survey of large circular and octagonal tanks operated at Norwegian commercial smolt and post-smolt sites. *Aquacultural engineering*, 74: 105-110.

Tajadini, M., Panjehpour, M. & Javanmard, S. H. (2014). Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. *Advanced biomedical research*, 3.

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.-A., Dufour, S., Karlsen, Ø. & Norberg, B. (2010). Control of puberty in farmed fish. *General and comparative endocrinology*, 165 (3): 483-515.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M. & Nguyen, M. (2010). A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. *Methods*, 50 (4): S1-S5.

Thorarensen, H., Gústavsson, A., Mallya, Y., Gunnarsson, S., Árnason, J., Arnarson, I., Jónsson, A. F., Smáradóttir, H., Zoega, G. T. & Imsland, A. K. (2010). The effect of oxygen saturation on the growth and feed conversion of Atlantic halibut (Hippoglossus hippoglossus L.). *Aquaculture*, 309 (1-4): 96-102.

Tichopad, A., Dilger, M., Schwarz, G. & Pfaffl, M. W. (2003). Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic acids research*, 31 (20): e122-e122.

Tipsmark, C. K. & Madsen, S. S. (2009). Distinct hormonal regulation of Na+, K+-atpase genes in the gill of Atlantic salmon (Salmo salar L.). *Journal of Endocrinology*, 203 (2): 301-310.

Trygve, G. (1993). Fiskeoppdrett. *Vekstnæring for distrikts-Norge*.

Van Rijn, C. A., Jones, P. L., Evans, B. S. & Afonso, L. O. (2021). Physiological and growth responses of juvenile Atlantic salmon (Salmo salar) transferred to seawater during different stages of smolt development. *Aquaculture*, 538: 736527.

Vargas-Chacoff, L., Regish, A. M., Weinstock, A., Björnsson, B. T. & McCormick, S. D. (2021). Effects of long-term cortisol treatment on growth and osmoregulation of

Atlantic salmon and brook trout. *General and Comparative Endocrinology*, 308: 113769.

Varnavsky, V. S., Sakamoto, T. & Hirano, T. (1992). Stunting of wild coho salmon (Oncorhynchus kisutch) in seawater: patterns of plasma thyroid hormones, cortisol, and growth hormone. *Canadian journal of fisheries and aquatic sciences*, 49 (3): 458-461.

Venvik, T. (2005). National Aquaculture Sector Overview, Norway. *FAO Fisheries and Aquaculture Department. Disponible online en: http://www. fao. org/fishery/countrysector/naso_norway/en [último acceso: 4/2/2017]*.

Vindas, M. A., Johansen, I. B., Folkedal, O., Höglund, E., Gorissen, M., Flik, G., Kristiansen, T. S. & Øverli, Ø. (2016). Brain serotonergic activation in growth-stunted farmed salmon: adaption versus pathology. *Royal Society open science*, 3 (5): 160030.

Von Schalburg, K. R., Yazawa, R., De Boer, J., Lubieniecki, K. P., Goh, B., Straub, C. A., Beetz-Sargent, M. R., Robb, A., Davidson, W. S. & Devlin, R. H. (2008). Isolation, characterization and comparison of Atlantic and Chinook salmon growth hormone 1 and 2. *BMC genomics*, 9 (1): 1-12.

Ward, C., Dempsey, M., Ring, C., Kempson, R., Zhang, L., Gor, D., Snowden, B. & Tisdale, M. (2004). Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *Journal of clinical virology*, 29 (3): 179-188.

Warren, I. A., Ciborowski, K. L., Casadei, E., Hazlerigg, D. G., Martin, S., Jordan, W. C. & Sumner, S. (2014). Extensive local gene duplication and functional divergence among paralogs in Atlantic salmon. *Genome biology and evolution*, 6 (7): 1790-1805.

Weltzien, F.-A., Norberg, B., Helvik, J. V., Andersen, Ø., Swanson, P. & Andersson, E. (2003). Identification and localization of eight distinct hormone-producing cell types in the pituitary of male Atlantic halibut (Hippoglossus hippoglossus L.). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 134 (2): 315-327.

Weltzien, F.-A., Andersson, E., Andersen, Ø., Shalchian-Tabrizi, K. & Norberg, B. (2004). The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 137 (3): 447-477.

Whalen, K. G. & Parrish, D. L. (1999). Effect of maturation on parr growth and smolt recruitment of Atlantic salmon. *Canadian Journal of Fisheries and Aquatic Sciences*, 56 (1): 79-86.

Wilson, J. M. & Laurent, P. (2002). Fish gill morphology: inside out. *Journal of experimental Zoology*, 293 (3): 192-213.

Wilson, P. E., Kazadi, W., Kamwendo, D. D., Mwapasa, V., Purfield, A. & Meshnick, S. R. (2005). Prevalence of pfcrt mutations in Congolese and Malawian Plasmodium falciparum isolates as determined by a new Taqman assay. *Acta tropica*, 93 (1): 97-106.

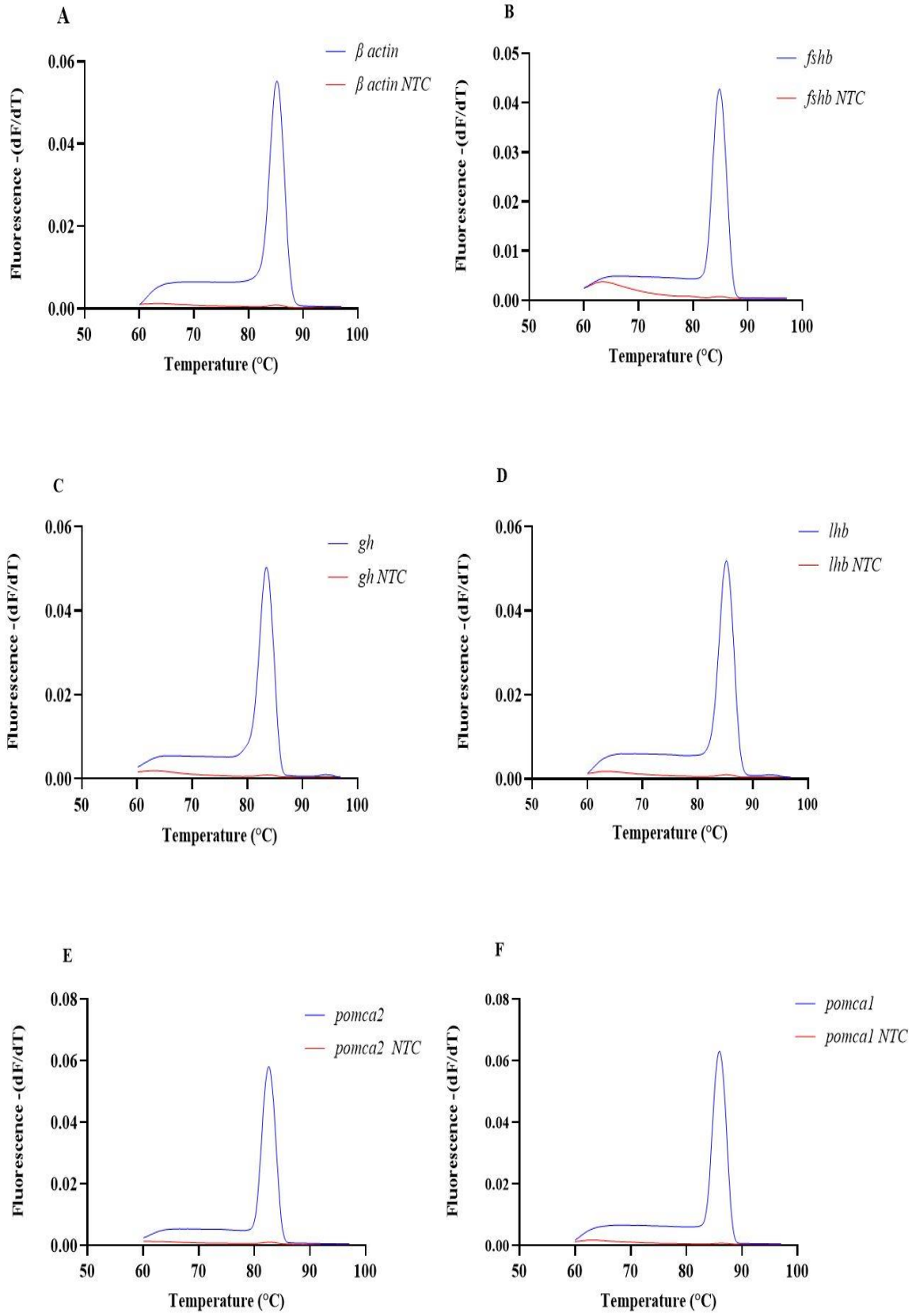
Yasuda, A., Itoh, H. & Kawauchi, H. (1986). Primary structure of chum salmon prolactins: Occurrence of highly conserved regions. *Archives of biochemistry and biophysics*, 244 (2): 528-541.

Yoshikawa, J. S. M. (1992). *Regulation of Messenger RNA Transcription and Translation of the Two Prolactins of the Tilapia, Oreochromis Mossambicus, by Salinity, Osmotic Pressure, and Cortisol*: University of Hawaii at Manoa.

Young, G., Prunet, P., Ogasawara, T., Hirano, T. & Bern, H. A. (1989). Growth retardation (stunting) in coho salmon: plasma hormone levels in stunts in seawater and after transfer to fresh water. *Aquaculture*, 82 (1-4): 269-278.

Zydlewski, G. B. & Zydlewski, J. (2012). Gill Na+, K+-ATPase of Atlantic salmon smolts in freshwater is not a predictor of long-term growth in seawater. *Aquaculture*, 362: 121-126.

Appendix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Temperature (°C)
B
fshb
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Fluorescence -(dF/dT)
Temperature (°C)
C
gh
gh NTC
Fluorescence -(dF/dT)
Temperature (°C)
D
lhb
lhb NTC
Fluorescence -(dF/dT)
Temperature (°C)
E
pomca2
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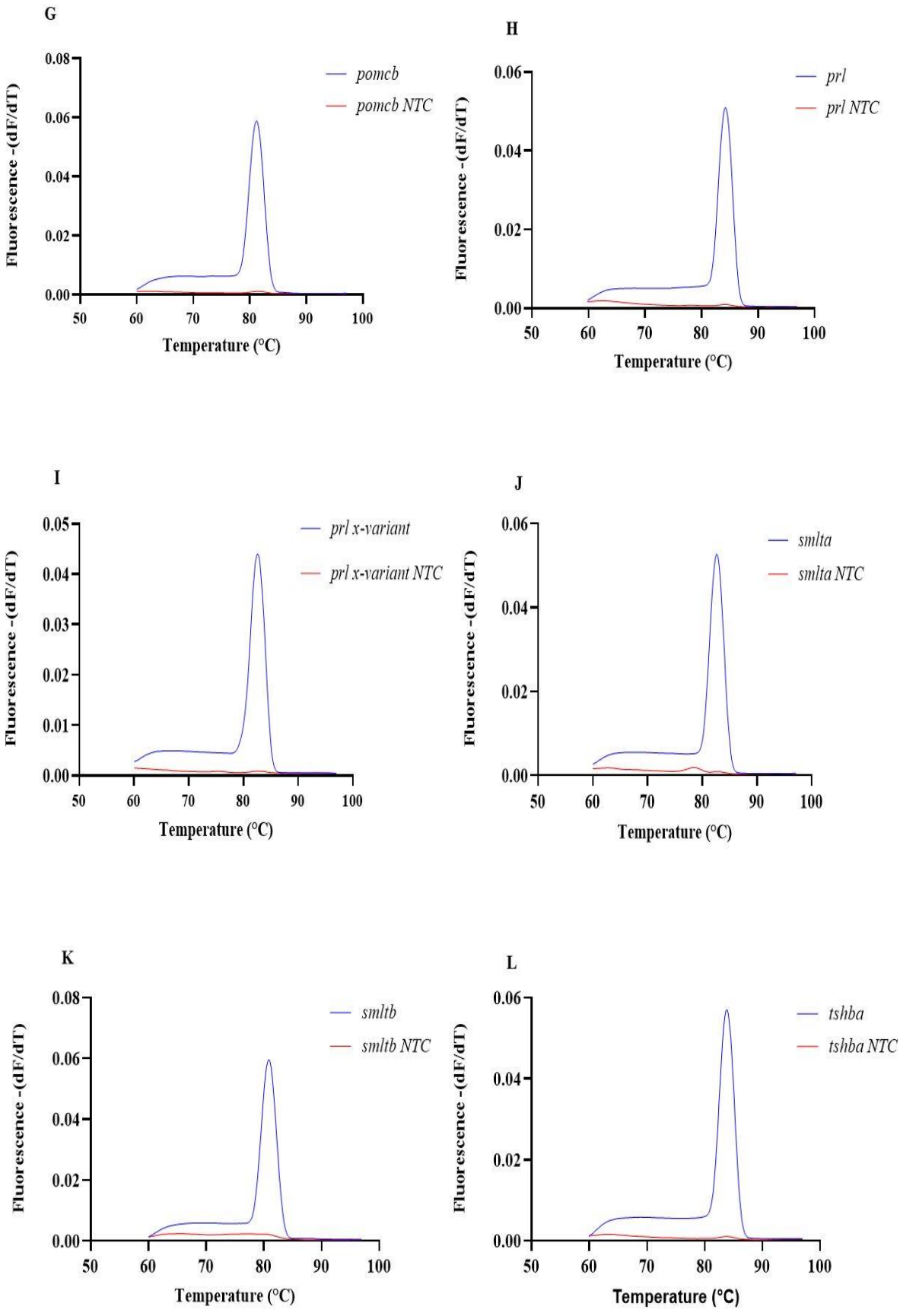
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prl x-variant NTC
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Fluorescence -(dF/dT)
Temperature (°C)
K
smltb
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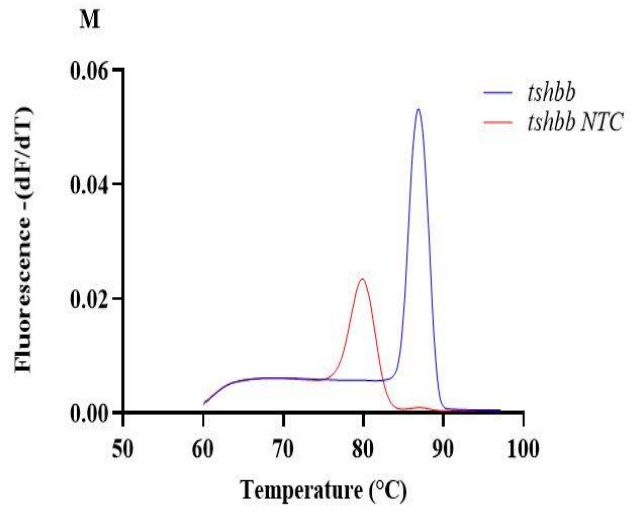
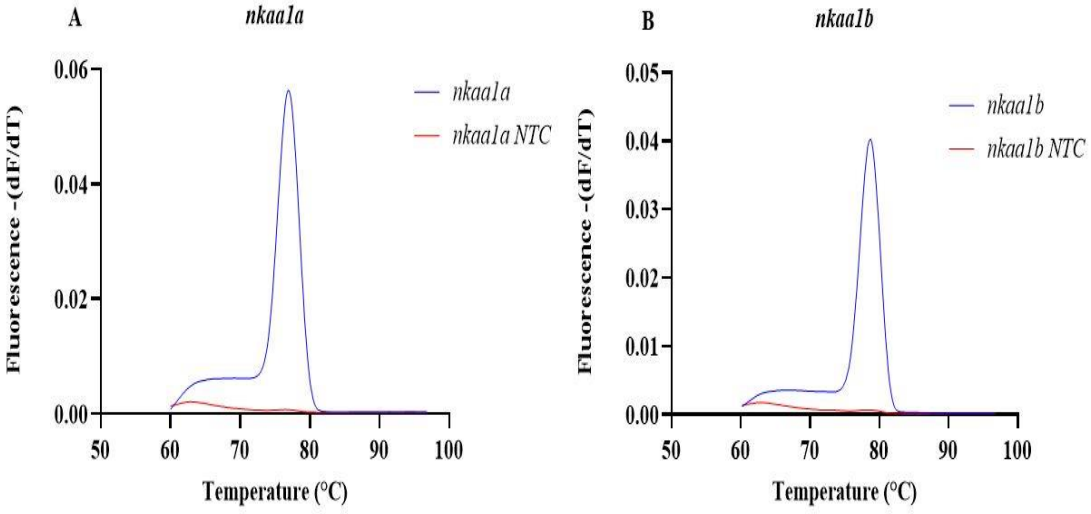


Figure 1. Melting curve analysis of each gene of pituitary along with their respective negative control. A) *β-actin*, B) *fshb*, C) *gh*, D) *lhb*, E) *pomca2*, F) *pomca1*, G) *pomcb*, H) *prl*, I) *prl x-variant*, J) *smlta*, K) *smltb*, L) *tshba*, and M) *tshbb*.



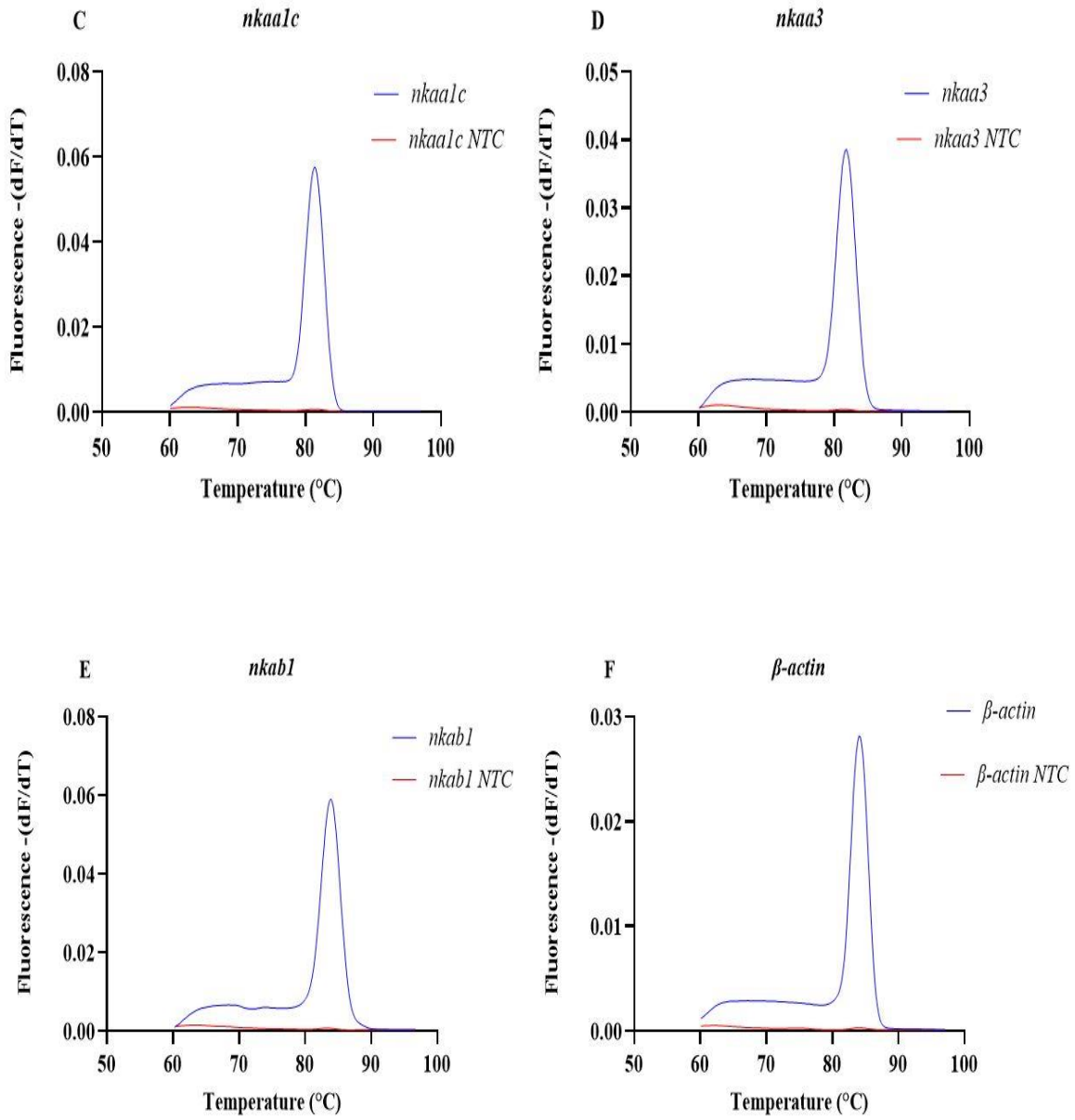


Figure 2. Melting curve analysis of each sodium potassium ATPase genes of gills along with their respective negative control. A) *nkaa1a*, B) *nkaa1b*, C) *nkaa1c*, D) *nkaa3*, E) *nkab1*, and F) *β-actin*.

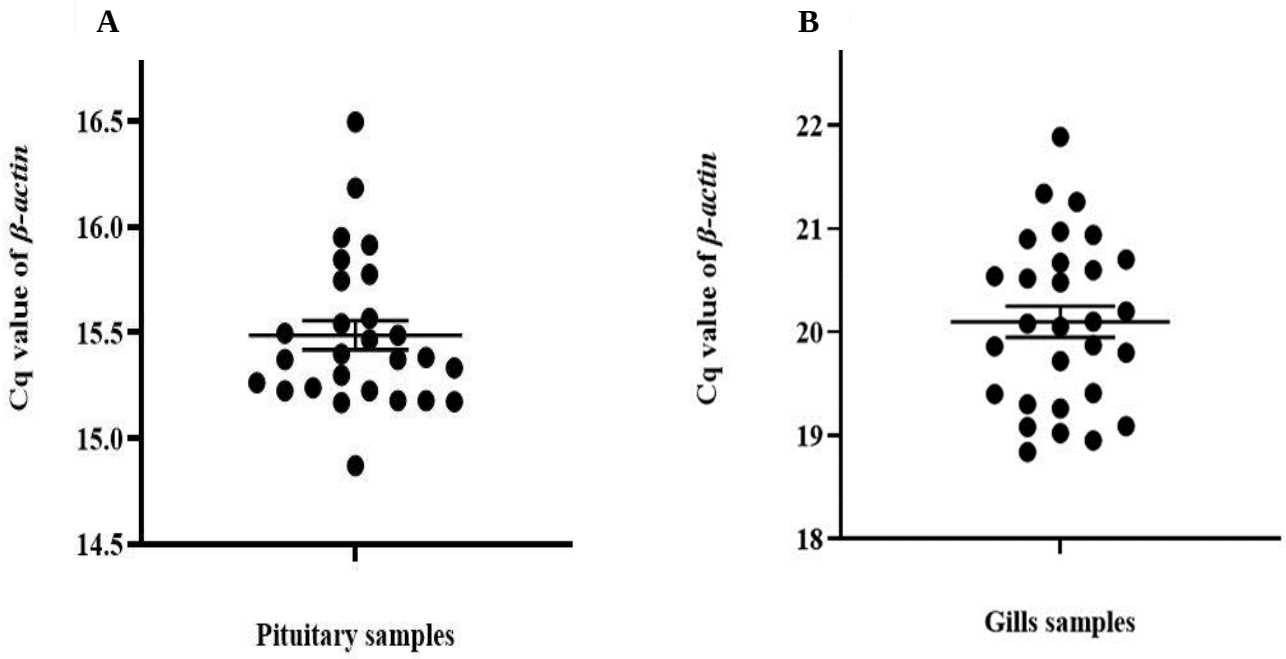


Figure 3. Distribution of C_q value of *β-actin* along [A] pituitary samples and, [B] gills samples.

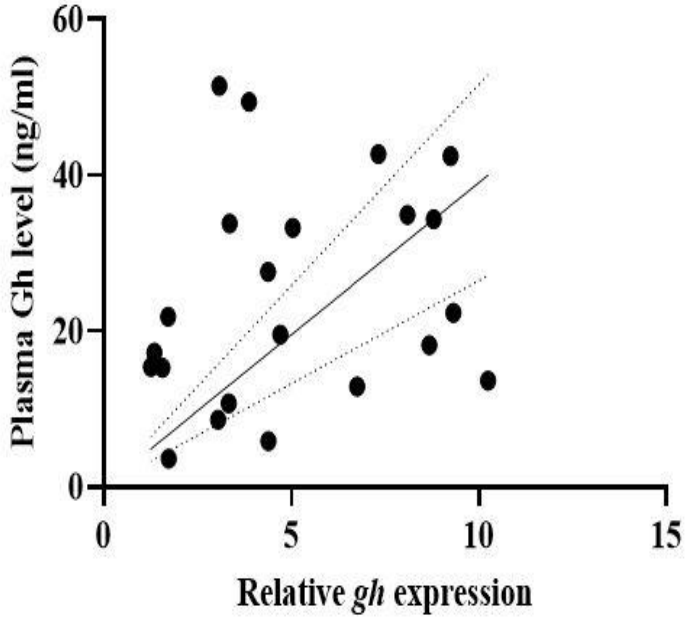


Figure 4. Correlation between relative expression of gh in pituitary and plasma Gh level. The line with increasing slope shows the positive correlation between level of *gh* expression in pituitary and plasma Gh level. It indicates with the increase in *gh* expression in pituitary, there occurs increase in plasma Gh levels.

Table 1. List of efficiency of primers used in qPCR.

Gene target of primer	Reference ID	Efficiency of primer
lhb	AF146151	1.99
fshb	AF146152	1.99
β-actin	Prepared in own lab for Atlantic Salmon	1.99
smlta	NM_001123604.1/ DQ412570.1	1.98
tshba	NM_001123528.1	2.04
prl	NM_001123668.1	2.02
gh	NM_001123676.1	2.01
pomcb	NM_001128604.1	2.03
tshbb	MG948546	2
pomca2	ENSSSAT00000124177.1 (E201)	2.05
pomca1	ENSSSAT00000010436.1 (E202)	1.99
smltb	NM_001141618	2.01
prl x-variant	XM_014192393.1 (LOC100136580)	2.02
nkaa1a	CK878443	2
nkaa1b	CK879688	2
nkaa1c	CK 885259	2
nkaa3	CK170270	2
nkab1	CK886866	2

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