Environmental and neuroendocrine control of smoltification in long-river (Loire - Allier) Atlantic salmon

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Abbreviations

1R	First round of whole genome duplication
2R	Second round of whole genome duplication
3R	Third round of whole genome duplication
4R	Fourth round of whole genome duplication
ap4b1	Adaptor Related Protein Complex 4 Subunit Beta 1
ACTH	Adrenocorticotropic hormone
Bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
CFTR	Cystic fibrosis transmembrane regulator
Cq	Quantitation cycle
CNSS	Conservatoire national du saumon sauvage
CRH	Corticotropin-releasing hormone
cRNA	Complementary ribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
Dio2	Deiodinase type-2 enzyme
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
FISH	Fluorescent *in situ* hybridization
FSH	Follicle stimulating hormone
FW	Freshwater
GH	Growth hormone
GpHβ	Glycoprotein hormone β-subunit
HPT	Hypothalamus-pituitary-thyroid axis
HW	Hybridization wash
IGF-1	Insulin like growth factor-1
K	Condition factor
kcna10	Potassium voltage-gated channel subfamily A member 10
LBP	Light-Brain-Pituitary pathway
LD	Long-day photoperiod
LH	Luteinising hormone
MR	Mitochondrion-rich cells
mRNA	Messenger ribonucleic acid
N5	Natural photoperiod with warm water (+5°C)
NKA	Na^+-K^+-ATPase (Sodium-potassium adenosine triphosphatase)
NKA α1a	Na^+-K^+-ATPase α-subunit 1a paralog
NKA α1b	Na^+-K^+-ATPase α-subunit 1b paralog
NKCC	Na^+-K^+-$2Cl^-$ cotransporter
NN	Natural photopeiod with natural water temperature
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PD	Pars distalis
PFA	Paraformaldehyde

PN	Pars nervosa
PPD	Proximal pars distalis
PRL	Prolactin
PT	Pars tuberalis
PT-TSH	Pars tuberalis thyrotropin
PTU	Propylthiouracil
PVN	Periventricular nucleus
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPD	Rostral pars distalis
rplp2	Ribosomal Protein Lateral Stalk Subunit P2
SD	Short-day photoperiod
s.e.m	Standard mean error
slc16a1	Solute Carrier Family 16 Member
SSWGD	Salmonid specific whole genome duplication
SW	Seawater
T3	Triiodothyronine
T4	Thyroxine
TH	Thyroid hormones
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin (Thyroid stimulating hormone)
Tshβa	Thyroid stimulating hormone β-subunit a paralog
Tshβb	Thyroid stimulating hormone β-subunit b paralog
TSH-R	TSH receptor
tspan	Tetraspanin
TSWGD	Teleost specific whole genome duplication
WGD	Whole genome duplication
W5	Winter photoperiod with warm water (+5°C)
WN	Winter photoperiod with natural water temperature

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Introduction

This PhD has been carried out in the frame of the European ITN project IMPRESS "Improved Production Strategies for Endangered Freshwater Species". This European wide Horizon 2020 project seeks to understand and provide solutions to the decline of wild populations of Europe's most economically and socially important diadromous fish species: European eel (*Anguilla anguilla*), European sturgeon (*Acipenser sturio*) and Atlantic salmon (*Salmo salar*). Together, this project aims to provide novel knowledge on life histories, broodstock management and hatchery management practices in order to help conserve these unique species throughout European waterways.

1. Model of research: Loire-Allier Atlantic salmon

There are few species in the world that have become as important to human civilization as the Atlantic salmon. Etched into the walls of the Valley of Gorge d'Enfer (Southwest France, 44° 56' 39" north, 0° 59' 54" east) roughly 22,000 years ago, the depiction of a male spawning Atlantic salmon narrates the importance of this species since the dawn of human history (Figure 1).

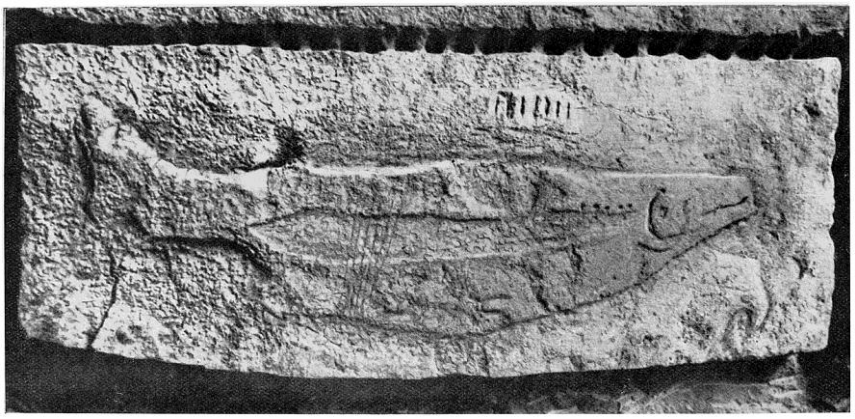

Figure 1: Depiction of male spawning salmon etched into the walls of the Valley of Gorge d'Enfer in Southwest France, 44° 56' 39" north, 0° 59' 54" east

Today, Atlantic salmon are an enormous part of our economy, recreation and society. In 2016, the EU had a total market volume of over 1.5 million tonnes of Atlantic salmon and France was ranked the 5th largest consumer of Atlantic salmon in the European Union (Global Trade Tracker, 2017). 99.9% of all imported salmon into EU countries comes from

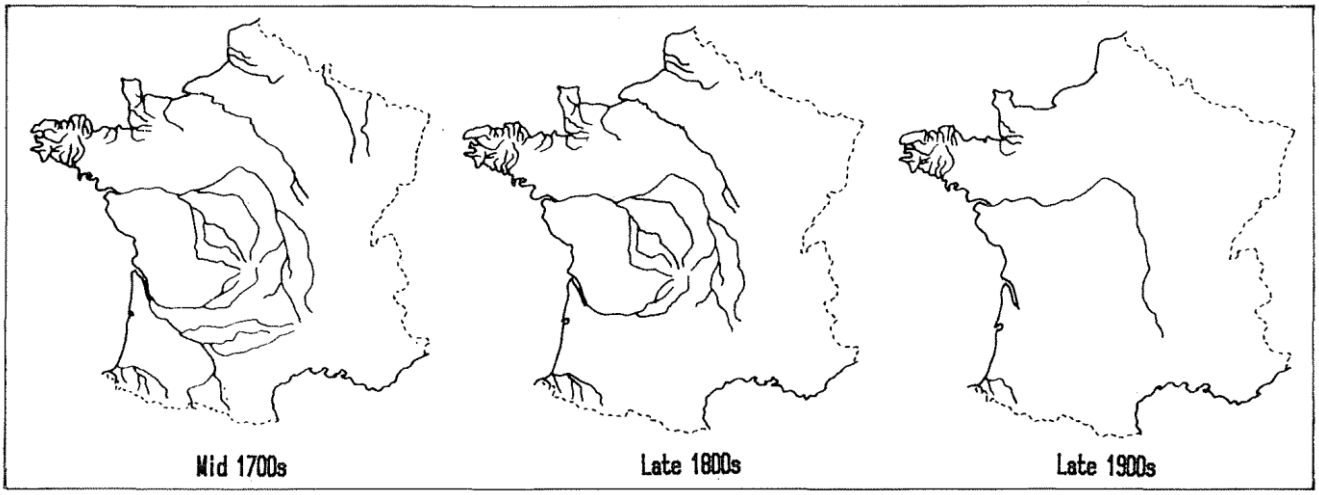

Figure 2: Rivers colonized in France by Atlantic salmon in the mid-eighteenth century, end of the nineteenth century and end of the twentieth century (Thibault, 1994)

Norwegian aquaculture production of Atlantic salmon. Fish produced in aquaculture are very different to that of their wild counterparts and in fact, as we witness a global increase of Atlantic salmon production, we are simultaneously experiencing a massive decline in wild populations. Rivers throughout Europe and North America are being subjected to human globalization as well as anthropogenic and climate related threats, pushing many Atlantic salmon populations towards extinction (Parrish *et al.*, 1998).In France, the historical runs of Atlantic salmon were enormous, reaching estimated levels of over 800,000 returning adults in the mid-1800s. However, due to the disconnection of river continuity by hydroelectric dams and the degradation of juvenile habitat, French populations of Atlantic salmon have greatly diminished (Figure 2) (Thibault, 1994). In addition, river modification for navigation and flood control left many of the natural river ways no longer suitable for Atlantic salmon (Prouzet, 1990).

The Loire River is the largest river in France, spanning over 1,000km long, and is home to a diverse range of flora and fauna. The river winds through 1/5 of the metropolitan regions of France and once had a carry capacity of over 100,000 Atlantic salmon. Catch records indicate a severe decline of Atlantic salmon from 1928-1975, dropping from 15,000 fish per month to less than 1,000 fish per season (Cohendet, 1993). In 1984, as Atlantic salmon were on the brink of extinction, the French government banned all Atlantic salmon fishing in the Loire River and its tributaries. Despite this, wild returning populations of the Loire went extinct by

the mid-1990s and its main tributary, the Allier, became the last remaining tributary of the Loire with wild returning Atlantic salmon.

1.1 The Loire-Allier River

The top of the Allier rests in Lozère, 1485 meters above sea level in the Northern regions of the Languedoc-Roussillon. The upper regions of the river, known as the "Haut-Allier", remains pristine, weaving in between rocks and forests draped by large steep-sided volcanic plateaus. The river itself winds 421 km north before it joins the Loire River at the Bec d'Allier in Nevers and continues another 550 km to the estuary in Nantes. This puts the upmost spawning grounds roughly 950 km from the ocean, making the Loire-Allier the longest run for Atlantic salmon in mainland Europe.

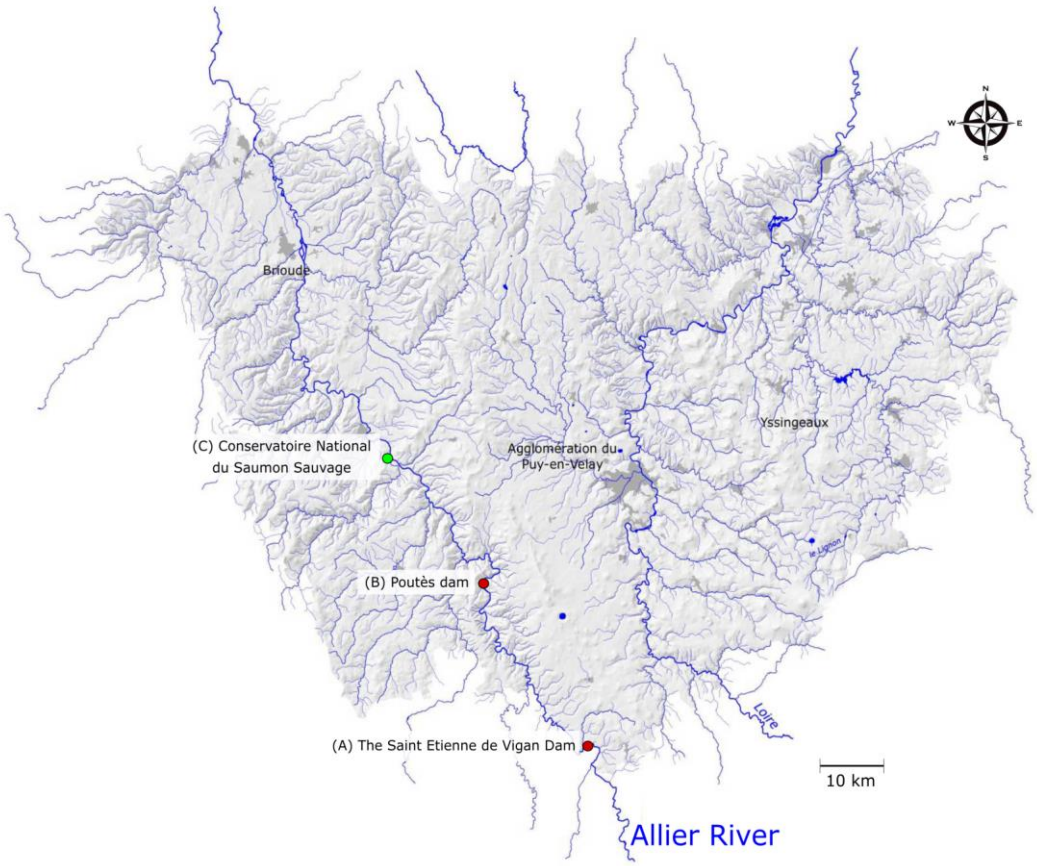

Figure 3: Haut-Allier region of France. (A) Historic location of The Saint Etienne de Vigan dam (B) Current location of the Poutès dam (C) Location of the Conservatoire national du saumon sauvage (map modified from www.ode43.fr)

Unfortunately the Allier has had hydroelectric dams, weirs and other obstructions which have restricted access to spawning grounds since the late 1800s. One dam in particular, The Saint Etienne de Vigan (Figure 3 A), was located near the source of the Allier and completely blocked Atlantic salmon access to 70 acres of the best spawning grounds the region had to offer. Another severe obstruction, the Poutès hydroelectric dam (Figure 3 B), was built in 1941 cutting off all salmon migration to the Haut-Allier until a salmon elevator was installed in 1986. This region, prior to the dam, was estimated to produced nearly 10 tonnes of salmon per year, which contributed heavily to the local economy and exemplifies the population health prior to anthropogenic interference. Despite recent efforts to facilitate upstream migration, only few salmon make it above Poutès dam each year (24 fish in 2017; CNSS data). After years of conservational efforts, The Poutès dam is undergoing a reconstruction project in hopes to better facilitate the down and upstream passage of Atlantic salmon.

1.2 Restoration projects

In the late 1980s, the SOS Loire Vivante ("Living Loire") was created to oppose the creation of the Seree de la Fare dam project, which would have been detrimental to the pristine environment of the upper Loire basin. Backed by the WWF and international media coverage, the SOS Loire Vivante fought diligently against the project and finally won the battle with the cancellation of all 4 dams, confirmed by the French government in January of 1994. The SOS Loire Vivante brought forward, through large demonstrations, strong scientific research, alternatives to damming and novel approaches to river management which sparked the creation of "Plan Loire Grandeur Nature" in 1994. The plan coordinated a series of projects aimed at preserving the Loire River ecosystem. The plan had a particular focus on saving the remaining French Atlantic salmon population by the removal of poorly designed or inefficient dams, suspension of all Atlantic salmon fishing and the construction of the Conservatoire national du saumon sauvage (CNSS). The SOS Loire Vivante network won the Nobel Prize for environment protectors in 1992 for their relentless efforts of preserving the Loire River.

1.3 Conservatoire National du Saumon Sauvage

The Conservatoire national du Saumon sauvage (CNSS) began operation on July 10, 2001 and has dedicated the last 17 years to maintain the Loire-Allier Atlantic salmon population (http://www.saumon-sauvage.org/). One of Europe's largest freshwater hatcheries, CNSS has the capacity to produce over 2 million eggs, 600,000 alevins and over 200,000 smolts, all of which come from the intensive crossing of wild brood stock caught at the Vichy counting station. Different to typical hatchery protocols, CNSS uses natural water composition and temperature, natural photoperiod and custom feed in order to mimic that of the natural environment in a pursuit to produce juvenile salmon best fit for survival in the wild. Despite its efforts, the Loire-Allier population remains at risk with less than 1,000 adults being detected each year at the Vichy dam since 2006, and even less able to complete upstream migration and reach the spawning grounds of the Haut-Allier (counted at the Poutès dam fish elevator) (Figure 4).

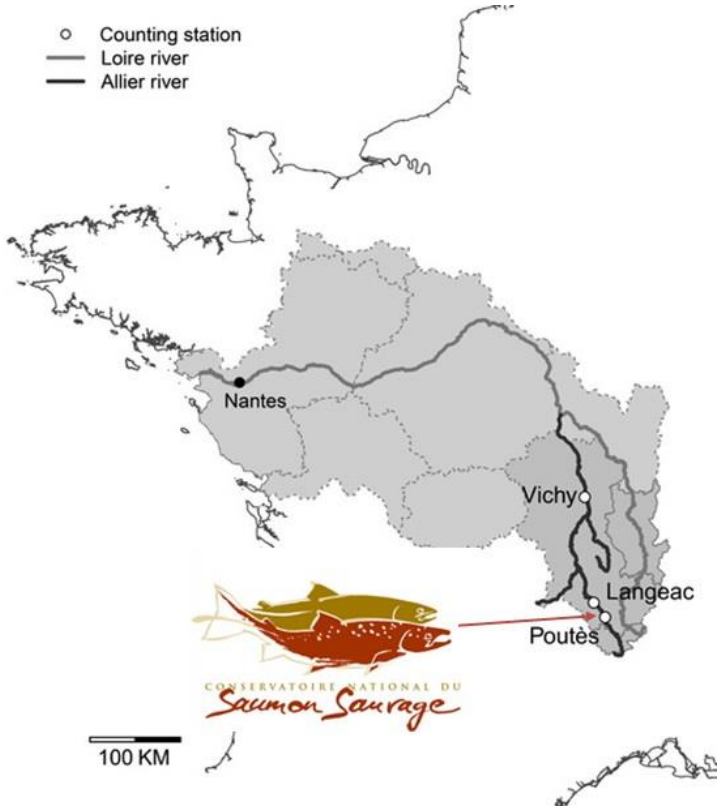

Figure 4: The Loire-Allier River. At over 1000km long the Loire-Allier is one of the longest rivers in France. Salmon migrate over 900 km to the pristine spawning grounds above Poutes dam. Adult salmon are caught at the Vichy counting station for production of smolts at CNSS.

1.4 Atlantic salmon life cycle

Atlantic salmon is an anadromous fish species able to live in both freshwater and saltwater environments (Figure 5) (For review on life history: Klemetsen *et al.*, 2003). Spawning and most of the early developmental stages of the Atlantic salmon life cycle takes place in freshwater (FW). Eggs, or ova, are released into small nests built by adult spawning females. The ova are fertilized by one or more males and will develop throughout the winter, hatching in the spring. Just-hatched fish are called alevins and have a nutrient sack attached to them, which they will absorb throughout the coming months staying hidden between pebbles and small substrate. Once the yolk sack is absorbed and the fish have become strong enough to swim to the surface, they will do so to fill their air bladders and gain better control in the moving streams. Now referred to as fry, the young fish will develop throughout the summer and into the fall feeding on microscopic invertebrates and seeking refuge in slower moving water. Over the autumn, fry will develop into parr which have distinct colouration and behaviours associated with them. Dark green and brown body colour and dark vertical bands along the side of the body provide camouflage from predators as the young parr begin to scavenge for aquatic insects. They are much more territorial during this stage than their other early life stages and will aggressively compete for food. Once a certain growth stage is reached, juvenile salmon will undergo a dramatic life history transition referred to as smoltification. This is when the salmon develop from their parr stage into the smolt stage and encompasses many behavioural, morphological and physiological changes which pre-adapt the salmon for life in seawater (SW). The environmental and neuroendocrine control of this metamorphic event will be covered extensively in the following sections. During this period, downstream migration is initiated and the juvenile fish, now referred to as smolts, will begin their journey to the sea. During this stage, smolts give up their territorial behaviour in favour of schooling and through a series of environmental and physiological cues, will begin to swim downstream to the ocean. Smolts will remain in the estuary for a short period for acclimatization to changes in temperature and salinity before making the journey to the North Sea feeding grounds.

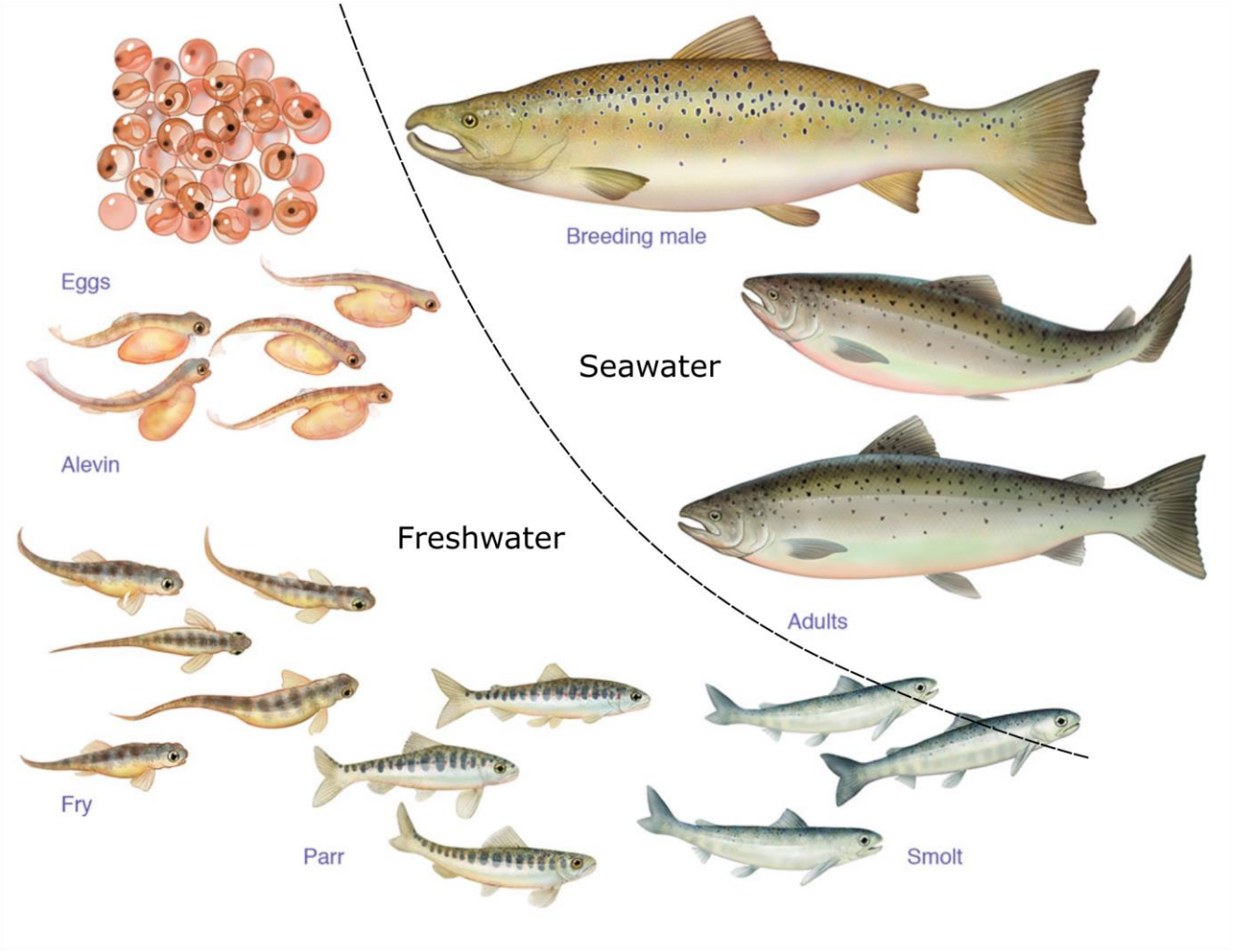

Figure 5: Atlantic salmon life cycle. Early stages of life take place in freshwater. Eggs or ova are layed in nests called reds which they incubate through the winter and hatch in the spring. Once juvenile salmon develop into parr, smotlfication and downstream migration occurs in which salmon transition to life in seawater. Feeding and growth phase takes place in the Oceans where they develop into Adults. During upstream migration adult salmon return to their native rearing grounds and spawn (image modified from: Life Cycle of Atlantic Salmon, Barbara Harmon, 2011)

Salmon will feed and mature in the North Sea ranging from 1-5 years depending on the specific population. Salmon that mature after one sea winter are referred to as griles and will return to their native stream or river for spawning after just one year of feeding. These fish tend to come from shorter rivers or rivers that have an overall relatively short migration length. Salmon populations whose migration to the southernmost limit, like those in France, will spend upwards of 3-4 winters feeding in the open ocean and tend to be much larger in size when beginning their spawning migration (Martin and Verspoor, 2011).

1.5 The Loire-Allier Atlantic salmon

The Loire-Allier Atlantic salmon have many unique characteristics which separate them from most short river Atlantic salmon stocks. The long 900 km+ migration results in a unique upstream migration consisting of multiple phases, separated by stopping periods according to the salmon cohort and date of arrival to the estuary (Martin and Verspoor, 2011). Spawning migration begins from mid-September to mid-November and involves large salmon which have spent at least 3 winters in the oceans feeding. These salmon will migrate upriver until they are halted by low winter temperatures, recommencing once again when water temperatures rise around mid-March. The salmon will continue migrating upriver until mid-June when temperatures reach too high for migration forcing salmon to take refuge until the final leg in the autumn where salmon will reach the upper catchment and spawning areas of the Loire-Allier (Martin and Verspoor, 2011)..

The Loire-Allier Atlantic salmon are notoriously large fish, reaching approximately 5-7kg and over a meter in length at spawning. Females will typically lay between 1200-2000 eggs per kg of weight. Spawning in the Loire-Allier takes place in late autumn and the majority of adults will die after spawning, due to the enormous energetic demands of the long migration. However, if the salmon is of good health, multiple migration and spawning events can take place. Adult salmon used as broodstock at CNSS will be nursed back to good health after spawning and multiple spawning events are common within the hatchery. Eggs develop throughout the winter and will hatch the following spring (Martin and Verspoor, 2011).

The majority of Loire-Allier parr will undergo smoltification after 1 year of growth in the river and begin their downstream migration around March-April. In comparison, salmon from Scotland or Norway will smoltify around May-June and as late as August for some of the most northern latitude rivers (Davidsen *et al.*, 2005; Antonsson and Gudjonsson, 2002) The earlier smoltification and downstream migration of the Loire-Allier population is most likely combination of genetic component of long river migrating fish as well as the response to southern environmental conditions. The period in which salmon reach the estuary must be coordinated with their body's physiology and thus the synchronization of downstream migration with physiological development is imperative. Estuary arrival data for the Loire-Allier salmon is scarce as conservation programs prevent scientific research downstream of

the Vichy dam. However the SALSEA-Merge project estimates the Loire-Allier population should arrive at the Nantes estuary around the end of April in order to complete successful seaward migration within tolerable environmental conditions (SALSEA-MERGE, 2012).

1.6 Physiological and environmental "windows" of opportunity

In order to describe the period in which smolts can enter the sea successfully during downstream migration, physiological and environmental windows or "smolt windows" are often discussed. These temporal timing windows are used to describe the period in which both the environment and the physiology of the fish are in the correct state to make a successful transition to the sea (Figure 6) (Boeuf and Prunet, 1985; Prunet *et al.*, 1989; McCormick *et al.*, 1998). The windows of opportunity are highly variable between rivers and populations and thus may be considered a local adaptation to the area's prevailing environmental conditions (for review: Boeuf, 1993; McCormick, 2012).

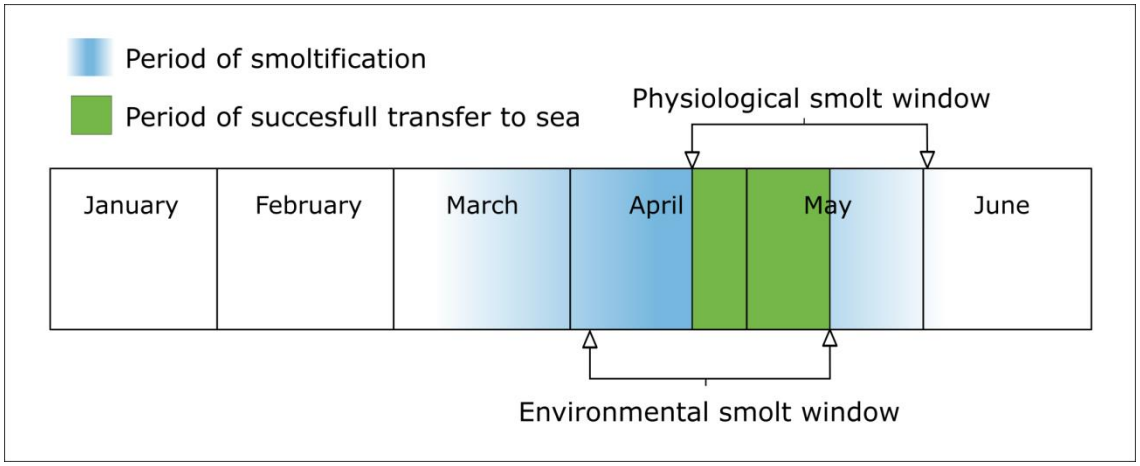

Figure 6: Example of physiological and environmental smolt windows. Successful transfer to the sea relies on the overlapping of physiological and environmental smolt windows

Salmon which arrive to the estuaries late run the risk of losing their seawater adeptness (McCormick *et al.*, 1998). A process referred to as desmolting or desmoltification is the loss of seawater adaptation and smolt characteristics when salmon are kept in unfavourable (freshwater) conditions past their physiological smolt window. Therefore, in long rivers such as the Loire-Allier with multiple migratory barriers which can cause delays during downstream migration, the risk of missing the opportunity for successful seawater transfer is

much higher than in short rivers (Martin *et al.*, 2012). Therefore, the time in which juvenile salmon smoltify and initiate downstream migration in long rivers has a direct effect on the long term survivability. Understanding the environmental and endocrine mechanisms which regulate this metamorphosis will elucidate a better understanding of the smoltification process and lead to better conservational efforts for high risk long river Atlantic salmon populations such as the Loire-Allier.

1.7 Genome duplication events

Nearly 50 years ago, Ohno (1970) presented evidence that whole genome duplication events (WGD) supply genetic raw material for the emergence of new functions through natural selection forces and allow for large-scale adaptation to new environments. Generally speaking, there are three fates for a gene after it has been duplicated: one copy will become silenced due to degenerative mutations (nonfunctionalization), one copy will acquire a completely new and beneficial function which then will be passed on through natural selection (neofunctionalization) or copies of the duplicated gene will adopted sub functions of the original role with the combined capacity equal to that of the original functions (subfunctionalization) (Ohno, 1970; Lynch and Conery, 2000). The majority of genes after duplication events will acquire deleterious mutations which render them non-functional and if still present in the genome can be characterized as pseudogene.

Teleosts belong to the Actinopterygian infraclass which is amazingly diverse with over 26,000 discovered species. Three rounds of whole genome duplication events have taken place in the teleost lineage; 1R and 2R before the divergence of lamprey from jawed vertebrates and a third teleost-specific (3R; TSWGD) WGD at the base of teleosts roughly 320 million years ago (Meyer and Van De Peer, 2005; Smith *et al.*, 2013). The diversification of the teleost infraclass has been hypothesized to be a consequence of the 3R supported by the fact that classes which diverged from Aactinopterygians before the 3R event are extremely species-poor in comparison (Meyer and Van De Peer, 2005).

Recent advances in sequencing technology have concluded that salmonids have undergone a 4^{th} WGD event. The salmonid-specific whole genome duplication (4R; SSWGD) is an additional genome duplication event that took place roughly 80 million years ago in the common ancestor of the salmonid species. Interestingly, Lein and collaborators, using the

gene expression patterns of Atlantic salmon compared to a pre-4R outgroup, concluded that the instances of neofunctionalization were much higher than that of subfunctionalization (Lien *et al.*, 2016). Thus there seems to be the potential for a number of genes duplicated *via* the 4R which have acquired novel functions not yet discovered.

2. Smoltification

The parr-smolt transformation of salmonid species has captivated scientists for decades. Fuelled by the economic importance of this family of teleosts, the research into the metamorphic event of smoltification has been robust (for reviews: (Folmar and Dickhoff, 1980; Wedemeyer, Saunders and Clarke, 1980; Hoar, 1988; Boeuf, 1993; Björnssone *et al*., 2011; Rousseau *et al.*, 2012; McCormick, 2012). Despite this, the discussion of to what extent smoltification is regulated by exogenous or endogenous factors remains unclear. Smoltification is a complex series of the morphological, behavioural, and physiological changes managed by the endocrine system and suggested to be synchronized with environmental factors such as photoperiod, temperature, lunar phase, salinity, turbidity and river flow (Hoar, 1988).

2.1 Morphology

2.1.1 Early growth

Within a freshlyl hatched cohort of juvenile Atlantic salmon, referred to as 0+, differential growth rates between individuals will result in a bimodal size-frequency distribution that is related to smolt development (Kristinsson *et al*., 1985). The upper mode represents the potential pre-smolts which will continue to eat and grow throughout the autumn and winter in preparation for smoltification and migration in the spring. The lower mode will enter a state of anorexia during the winter ceasing growth and development until the following spring (Thorpe *et al.*, 1982; Metcalfe and Thorpe, 1992). Juvenile salmon that develop into smolts after 1 full winter are called +1 smolts, 2 winters are called 2+ smolts and so forth (Thorpe, 1989). The majority of the Loire-Allier population smoltify and migrate at +1 or 2+, however 3+ is seen on occasion.

2.1.2 Colouration

There are distinct morphological differences between parr and smolt of Atlantic salmon, which can lend to the visual observation of the smoltification process. Parr have dark pigmented bands along the side of the body referred to as “parr marks”, which slowly fade during the smolting process, as deposition of crystalline purines (guanine and hypoxanthine) in the skin and scales causes extensive silvering of the body (Johnston and Eales, 1967). Synchronous to silvering, caudal, dorsal and pectoral fins become significantly darker throughout the smolting process due to the diffusion of melanin granules in melanophore cells (Figure 7) (Mizuno *et al.*, 2004).

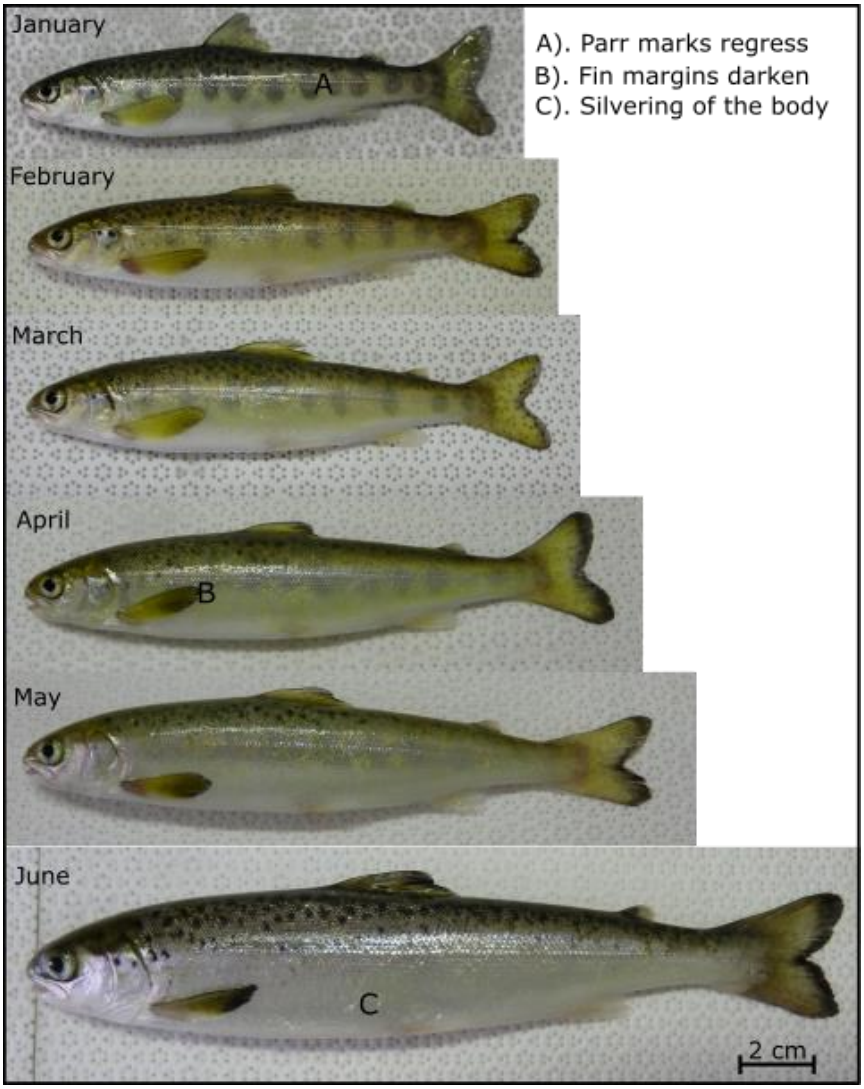

Figure 7: Morphological changes of the Loire-Allier Atlantic salmon during the smoltification metamorphosis. (A) Parr marks regress, (B) Fin margins darken and (C) extensive silvering of the body occurs during this metamorphic event which helps preadapt the salmon for life at sea.

2.1.4 Condition factor

The body shape of a developing smolt is different to that of a parr. Smolts become elongated as their linear growth rate increases relative to total growth, leading to a reduction in condition factor [k=(L/w^3)*100]. Condition factor is often used for fish to help indicate the overall health of a fish; indicating such things as feeding condition, parasitic infections and physiological factors (Le Cren, 1951; for review: Froese, 2006). Elongation during the parr-smolt transformation is expected and thus condition factor can be used to characterize the smolting process. Parr tend to increase condition factor during the spring as feeding increases, meanwhile maintaining low lipid demands. During smoltification, increased linear growth rate, metabolic rate and reduced lipid intake result in a decreasing condition factor (Mccormick *et al.*, 1987).

2.1.5 Other organs and tissues

As smoltification is a metamorphosis, many organs and tissues are affected during the process. Although not the focus of this thesis, these changes are worth mentioning: the eye, brain, gills, intestine and kidney all undergo some sort of adjustment to coincide with the change of habitats during smoltification (for review: Rousseau *et al.*, 2012)

2.2 Metabolism

Overall standard metabolic rates are 30-50% greater in smolts than in parr when body weight is accounted for (Maxime *et al.*, 1989; Baraduc and Fontaine; 1955). The increased metabolism is characteristic of smoltification and due in part to the increased growth, feeding, sodium-potassium adenosine triphosphatase enzyme (Na^+-K^+-ATPase : NKA) activity and concentration of mitochondrial rich (MR) cells (Blake *et al.*, 1984; Hoar, 1988; Maxime *et al.*, 1989). Increased haemoglobin concentrations and complexity has been reported during smoltification due to the increased oxygen demands during migration (Zaugg and McLain, 1976; Sullivan *et al.*, 1985). Overall, lipid concentrations decrease during smoltification, mediated by lipase enzymes, which in part may be regulated by endocrine factors such as thyroxine (T4), cortisol, growth hormone (GH) and prolactin (PRL) (Sheridan, 1988, 1989; Bell *et al.*, 1997).

2.3 Behaviour

Parr have a distinct territorial behaviour, which they exhibit during their residence in rivers and streams. They remain near the bottom of the water column hidden between the substrate, and use fast bursts of swimming to feed on passing food particles. As the winter ends and the parr begin the smolt transformation, they reduce their territorial behaviour in favour of forming shoals. Increased social behaviour coincides with a reduction in fast swimming activity and an increase in migratory readiness behaviour (Hoar, 1988; Boeuf, 1993). As spring approaches, smoltification will commence and migratory behaviour will change from predominantly facing the stream (positive rheotaxis) to predominantly facing downstream (negative rheotaxis), and begin their journey downward to the sea (Boeuf, 1993; McCormick *et al.*, 1998)

The factors contributing to the change in rheotaxis may be the most critical in determining the overall timing of smolt migration, yet these factors remain poorly understood (for review: McCormick, 2012). Laboratory studies have demonstrated environmental factors contributing to a change in rheotaxis such as advanced photoperiod (Muir *et al.*, 1994), accumulated thermal units (Zydlewski *et al*., 2005; Sykes and Shrimpton, 2009), water temperature (Jonsson and Ruud-Hansen, 1985), water discharge (Thorpe *et al.*, 1981; Thorpe, 1989) and endocrine factors such as thyroid hormones (TH) (Ojima and Iwata, 2007) and growth hormone (Prunet *et al.*, 1989); however no single entity takes full responsibility of migration onset (McCormick *et al.*, 1998). Due to the variability in river geographic location, topography and length of the initiation of migration, a genetic component that is unique to river populations is likely involved. Furthermore, the onset of migration may also depend on things such as social cues or increased exothermic predation (Hansen and Jonsson, 1985). Nevertheless, the change in rheotaxis during the smoltification period is a marked characteristic of the smolting process (Boeuf, 1993; McCormick *et al.*, 1998). Arriving to the estuary at the correct period depends on the correct time of departure from rearing grounds and therefore indicative to smolt survival.

2.4 Osmoregulation and increased salinity preference

One of the most important changes that occur during smoltification is the change in osmoregulatory function as salmon go from a freshwater to salt water environment (for

reviews: (Sakamoto *et al*., 1993; McCormick, 2012). In freshwater, fish normally do not drink and will excrete excess water through diluted urine while absorbing salt through the gills. In salt water, fish drink seawater to compensate for passive water loss and actively secrete excess salt *via* gills and kidney. For salmon migrating down river, it is important that they maintain the ability to absorb ions while they are still in freshwater meanwhile preparing for the increasing salinity as they approach the estuary.

Smolts develop increased salinity tolerance over several weeks prior to SW entry. Many tissues including the gut, intestine, kidney and bladder manage the internal salt and water balance using the gills for major source of ion uptake in freshwater and salt secretion in seawater (For review: Evans *et al.*, 2005). Mitochondrion-rich cells, also known as chloride cells, are specialized ionocytes responsible for the active transport of ions across the gills. Three main transport proteins are localized in gill ionocytes cells: NKA, Na^+-K^+-$2Cl^-$ cotransporter (NKCC) and the cystic fibrosis trans membrane regulator (CFTR) (Evans et al., 2005). The NKA enzyme is a solute pump that actively pumps sodium out of the cell while pumping potassium into the activity of which increase during the smoltification process (Rainbow trout (*Oncorhynchus mykiss*): Richards, 2003; Arctic char (*Salvelinus alpinus*): Bystriansky, LeBlanc and Ballantyne, 2006; Atlantic salmon: (Nilsen *et al.*, 2007; McCormick *et al*., 2009)). In Atlantic salmon, two isoforms of NKA α are present; NKA α1a and NKA α1b, which have shown to be expressed in variable abundance depending on the aquatic environment the fish are in (McCormick *et al.*, 2009). In freshwater, NKA α1a expression is highly abundant in distinct chloride cells while NKA α1b becomes the dominant isoform after seawater acclimation. Albeit low numbers, NKA α1b mRNA is detectable in chloride cells while in freshwater suggesting the mechanism for seawater tolerance is available before fish experience an increase in salinity (Nilsen *et al.*, 2007).

Two NKCC isoforms (NKCC1 and NKCC2) are present in teleosts; one for secretory function (NKCC1) and the other for absorptive function (NKCC2) (for review: McCormick, 2012). In Atlantic salmon, mRNA expression of the NKCC1 isoform increases during smolt development and especially in gill ionocyte cells (Nilsen *et al.*, 2007). Juvenile Atlantic salmon kept under continuous light disrupts smolt development and inhibits NKCC transcription and development of seawater tolerance (Stefansson *et al.*, 2007). In brown

trout (*Salmo trutta*), NKCC and NKA abundance increases gradually with smolt development and dramatically after seawater exposure (Tipsmark *et al.*, 2002)

There have been two confirmed CFTR isoform in Atlantic salmon which has differential expression during smolt development. CFTR I mRNAs increase during smolt development and after seawater transfer, whereas CFTR II expression shows no increase and only a slight increase, respectively (Singer *et al.*, 2003; Nilsen *et al.*, 2007).

2.5 Environmental regulation of smoltification

Environmental conditions act through the endocrine system to relay vital information for the timing of physiological and behavioural changes during smoltification (Hoar, 1976, 1988; Björnsson, Stefansson and McCormick, 2011).

2.5.1 Photoperiod

Similar circannual rhythms of smoltification characteristics such as increased growth rate, silvering and reduced condition factor can be achieved under vastly different photoperiods; however, correct light and dark conditions are needed for normal and healthy smolt development able to complete successful migrations and transfer to the sea (Eriksson and Lundqvist, 1982). Parr exposed to continuous light experienced less retinal innervation to the preoptic nucleus than controls (Stefansson *et al.*, 2007). These fibres contribute to the light-brain-pituitary (LBP) pathway and demonstrate a direct or indirect regulation of pituitary hormones such as GH, thyroid stimulating hormone (TSH) or adrenocorticotropic hormone (ACTH) (Holmqvist *et al*., 1994; Ebbesson *et al.*, 2003).

In salmonids, as like other teleosts, melatonin profiles accurately reflect the prevailing light/dark cycles and are known to be involved in the synchronization of a number of rhythmic physiological processes (for review: Korf, 1994). Melatonin synthesis increases during hours of darkness and is produced from photoreceptor cells in the pineal gland (Falcon et al., 1992). Pinealectomized Atlantic salmon still undergo smoltification, but with a delay of nearly 3 weeks as compared to controls, while pinealectomized salmon given intramuscular injection of melatonin have advanced smoltification (Porter *et al.*, 1998). Additionally, melatonin injections caused increased growth rate of smolting salmon and thus

suggest that melatonin is an important component of somatic growth and smoltification, while not necessary for the metamorphosis to occur (Porter *et al.*, 1998; Mayer, 2000).

For Atlantic salmon, the vast knowledge concerning the effect of photoperiod on physiological development during smoltification comes from salmon aquaculture research and the pursuit to consistently produce healthy 0+ smolts. Using artificial photoperiod, hastened and abrupt simulations of summer and winter, 0+ smolts able to advance elevated plasma GH (Björnsson *et al.*, 2000), cortisol (Sundell *et al.*, 2003) and increased NKA activity (Berge *et al.*, 1995) to levels similar to a natural +1 smolt. Juvenile salmon raised under continuous light have increased growth rate and NKA activity while in freshwater as compared to controls (Mccormick *et al.*, 1987; Stefansson *et al.*, 1991). These studies emphasis the entrainment of smoltification with photoperiod and concluded that photoperiod is one of the, if not the most, important environmental factor for the initiation of smoltification.

Photoperiod however is not the only primary factor when determining the timing of smoltification and does not contribute to smoltifying characteristics when body growth or water temperature are too low (Thorpe *et al*., 1982; McCormick *et al*., 2000). Thus it has been suggested that photoperiod determines the temporal range in which the fish become sensitive to factors such as water temperature, flow, turbidity and lunar cycle in order to synchronize the endocrine system with environmental factors (for review: McCormick, 2012)

2.5.2 Temperature

Increased water temperatures can hasten the rate of smolt development (Duston *et al*., 1991; Sigholt *et al*., 1998). Thus, European stocks of wild Atlantic salmon from southern populations typically smoltify at 1-2+ whereas cooler northern populations will smoltify at 3-5+ (Martin *et al.*, 2012; Erkinaro, 1997). It has been suggested that a determining factor for smoltification is related to the degree days the fish are exposed to (Zydlewski *et al*., 2005); whereas others suggest a critical temperature threshold must be met in order for smolting and migration to take place (Hansen and Jonsson, 1985). Reported temperature thresholds vary between populations and species, likely being genetically specific to populations of certain river geographic location (Handeland *et al.*, 2004; Handeland *et al*., 2008). For example, river Orkla in Northern Norway has shown migration to commence when water

temperatures range between 1.7 and 4.4°C (Hvidsten et al., 1995), while migration onset was observed at temperatures between 8.5-13°C for the Varzuga River in Russia (Davidsen *et al.*, 2005).

For the Loire-Allier, downstream movements has been suggested to commence when water temperatures ranged from 7.5-13°C with maximum swimming speed at 10.5°C; however, downstream migration quickly diminishes when water temperatures exceed 17°C and completely halt above 20°C (Martin *et al.*, 2012). Generally speaking, salmonids are negatively impacted by temperatures outside their thermal range, which can inhibit smolt development, halt migration and hasten the loss of some smoltification characteristics (de-smoltifying) (Duston, Saunders and Knox, 1991; Handeland *et al.*, 2004). Physiological and morphological parameters such as NKA activity and silvering can be advanced with increased rearing temperatures; however, loss of seawater adeptness and smolt characterises are also advanced at temperatures above thermal range of the species (Duston *et al.*, 1991).

Increased temperature can hasten the NKA activity during smoltification, but the date of which peak activity occurs is the same between experimental groups of Atlantic salmon raised at two different temperature regimes (McCormick *et al.*, 2002). In coho salmon (*Oncorhynchus kisutch*), Larsen et al. (2001) found no difference in the timing of NKA activity increase at salmon reared throughout winter at 2.5 and 10˚C. Interestingly, advanced silvering and NKA activity induced *via* increased water temperature are nullified without photoperiod stimulus (Sigholt *et al.*, 1998; McCormick *et al.*, 2002). Thus a common conclusion is that temperature is an important modulator of smoltification and downstream migratory behaviour, but not sufficient as directive factor alone (Clarke *et al*., 1978; Wedemeyer *et al*., 1980; Boeuf, 1993; McCormick *et al.*, 1998; Sigholt *et al.*, 1998; Martin *et al.*, 2012).

2.5.3 Water flow and turbidity

Early investigations into the downstream movements of smolts related migratory behaviour to increased water flow and concluded that smolt movement is the passive displacement primarily controlled by flow rate (Thorpe *et al.*, 1981; Jonsson and Ruud-Hansen, 1985; Thorpe, 1989; Thorpe *et al*., 1994). However, further studies argue that smolt migration is a mix of passive and active swimming, using water flow as a facilitating mechanism to

downstream movements (Hansen and Jonsson, 1985; Aarestrup *et al*., 2002; Davidsen *et al.*, 2005; Martin *et al.*, 2012). The migratory strategies used by different populations of migrating smolts may depend on the length and geographic region of the river and could be a heritable trait. Nevertheless, water flow seems to play an important role in the movement of smolts once smoltification has commenced but does not regulate downstream movement alone (McCormick *et al.*, 1998).

2.5.4 Lunar cycle

Compelling evidence for the influence of lunar cycle and migratory behaviour was first reported in coho salmon, by Grau et al (1981) who observed a tight correlation to new moon events with T4 surges during smoltification. Further studies observed similar correlations between new moon events and T4 surges in Atlantic salmon (Boeuf and Prunet, 1985), Chinook salmon (Nishioka *et al.*, 1985) and masu salmon (Yamauchi *et al.*, 1984) and suggest that T4 surges during smoltification may be regulated by exogenous factors (for review: (Grau *et al.*, 1982)

3. Endocrinology of smoltification

3.1. Hypothalamus-pituitary-thyroid (HPT) or Thyrotropic axis

Thyroid hormones (TH) are responsible for several critical biological phenomena and are important throughout growth and development of most organisms. In adult vertebrates, TH control basal metabolic rates and energy metabolism whereas in developing vertebrates, nearly all processes concerning neurogenesis and central nervous system maturation are depending on the availability of thyroid hormones (Tata, 2006). The thyrotropic axis is the main neuroendocrine axis involved in the regulation of salmonid smoltification, as in the control of amphibian and flatfish metamorphosis.

3.1.1. Major actors of HPT axis

3.1.1.1. Mammals

In mammals, hypothalamic thyrotropin-releasing hormone (TRH) located in the periventricular nucleus (PVN) of the brain have been shown to be responsible for the regulation of TSH from the pars distalis (PD) of the anterior pituitary, which subsequently

stimulates the synthesis and release of T4 from the thyroid gland (for reviews: Fekete and Lechan, 2007; Zoeller, Tan and Tyl, 2007). T4 gets then converted to bioactive triiodothyronine (T3) by the deiodinase type-2 enzyme (dio2) in various tissues. Interestingly, TRH has also been shown to have multiple stimulatory effects on other pituitary hormones such as PRL and GH in vertebrates (Galas *et al.*, 2009). TRH neurons in the PVN are regulated by complex web of neuronal axons and humoral signalling, which allow the HPT axis to respond to environmental stimuli (Lechan and Fekete, 2006; Ortiga-Carvalho *et al.*, 2016).

3.1.1.2. Other vertebrates

The involvement of TRH as the hypothalamic factor controlling the thyrotropic axis has been subject to debate among vertebrates other than mammals. Studies on non-mammalian vertebrates showed that TRH does not always act as a TSH releasing factor and led to the discovery that corticotropin-releasing hormone (CRH) was acting as a potent TSH stimulator in amphibians, reptiles, birds and teleosts (Denver, 1988; Denver and Licht, 1989; De Groef *et al.*, 2006). CRH is generally known as the central regulator of the corticotropic axis (or stress axis), which produces corticosteroids. With its dual action, CRH may coordinate both and thyrotropic axes during vertebrate life stage transitions (Watanabe *et al*, 2016).
In amphibians, Denver and Licht (1989) showed that American bullfrog (*Rana pipiens*) tadpole pituitaries increase TSH secretion after ovine CRH treatment, but did not respond to TRH. In addition, Denver (1988) showed pituitaries from adult *Rana pipiens*, *Hyla regilla*, and *Xenopus laevis* increased TSH secretion in a dose dependent manner with exposure to TRH. These studies suggest that TRH has a TSH-releasing action only in metamorphosed amphibians but not larval amphibians (for review: De Groef et al. 2006).

In reptiles, *in vivo* and *in vitro* studies on adult turtles (*Chrysemys picta*) and lizards (*Anolis carolinesis*) observed an increase of TSH after TRH stimulation (Preece and Licht, 1987; Licht and Denver, 1988), but also after CRH treatment in turtle (*Pseudemys scripta*: Denver and Licht, 1989).

Similar results have been reported in birds in which CRH acts as a potent TSH stimulator. In chicken embryos, ovine CRH caused both an increase in T4 as well as corticosterone levels (Meeuwis et al., 1989). Furthermore, Geris and collaborators confirmed the direct action of

CRH as a TSH-releasing factor on domestic fowl (*Gallus domesticus*) pituitaries both *in vitro* and *in vivo* (Geris *et al.*, 1996, 1999).

In basal teleosts, lungfish and hagfish, TRH does not stimulate the release of TSH (African lungfish *Protopterus ethiopicus*: Gorbman and Mohamed, 1971); Pacific hagfish *Eptatretus stouti*: Dickhoff, Crim and Gorbman, 1978). However some more recent studies have shown an increase of *tsh* mRNA expression in Japanese eel (*Anguilla japonica*; Han *et al.*, 2004) and bighead carp (*Aristichthys nobilis*; Chatterjee, Hsieh and Yu, 2001) after TRH treatment. In coho salmon, Larsen *et al* (1998) discovered a potent stimulation of TSH release by CRH in pituitary cell cultures, whereas TRH did not induce any changes. In supporting evidence for the interaction between CRH and TSH in salmonids, Matz and Hofeldt (1999) showed that CRH-immunoreactive fibres terminated in pituitary regions containing TSH immunopositive cells in Chinook salmon. In flatfish (Pleuronectiformes), few studies have investigated the hypothalamic regulation of TSH. Exposure to the goitrogen, thiourea, or to T4 did not induce any changes in TRH mRNA levels, indicating that TRH do not participate in the regulation of HPT in the sole (Iziga *et al.*, 2010). In addition, TRH expression was shown to decrease progressively from 2-3 days after hatching till metamorphosis in Senegalese sole, suggesting that TRH was not involved in the induction of flatfish metamorphosis (Iziga et al., 2010). More recently, Campinho and collaborators hypothesize that flatfish metamorphosis may be an hypothalamic independent process as neither *trh* nor *crh* expression changes during metamorphosis, and hypothalamus does not respond to an anti-thyroid drug blockade whereas pituitary and thyroid do (Senegalese sole (*Solea senegalensis*): Campinho et al. 2015).

3.1.2. HPT and the control of major developmental processes

3.1.2.1. HPT and circannual rhythms

Birds and mammals have developed a novel role for HPT in the regulation of seasonal changes in physiological functions such as reproduction (for reviews: Ikegami and Yoshimura, 2017). Recent studies in the quail (Nakao *et al.*, 2008), sheep (Hanon *et al.*, 2008) and mouse (Ono *et al.*, 2008) revealed that TSH could also be produced by the pars tuberalis (PT) of the anterior pituitary under long day stimulus. This PT-TSH production is independent

of the HPT axis, but is instead regulated by melatonin from the pineal gland in mammals (Malpaux *et al.*, 2001) and direct photoperiodic information through deep brain photoreceptors in birds (Nakane and Yoshimura, 2010). PT-TSH locally activates thyroid hormones in the hypothalamus, which in turn induces the activation of the gonadotropic axis involved in the control of reproduction (for reviews: Yoshimura, 2013; Ikegami and Yoshimura, 2017). For now, in fish, it is the saccus vasculosus, an organ located on the ventral side of the diencephalon posterior to the pituitary, which has been described as the seasonal sensor (Nakane *et al.*, 2013).

In birds, thyroid hormones are related to many circannual processes including reproduction, molting and migratory readiness (for reviews: Dawson *et al.*, 2001; Kuenzel, 2003). Exogenous treatment of T4 can mimic the reproductive effects of long photoperiod in a variety of bird species measured by increased levels of LH and FSH secretion and testicular growth (tree sparrow: Wilson and Reinert, 1996; Japanese quail: Follett and Nicholls, 1988; European starling: Goldsmith and Nicholls, 1992). Removal of the thyroid gland in redheaded bunting (*Emberiza bruniceps*) was found to inhibit the fat deposition and nocturnal restlessness, which are related to pre-migratory disposition; both were restored with exogenous treatment of T4 or T3 (Pant and Chandola-Saklani, 1993).

In seasonal mammals, thyroid hormones are also involved in the control of seasonal processes such as reproduction. In sheep, a short day (SD) breeder, thyroidectomy blocks the suppression of gonadotropin induced by long photoperiod, and hypothalamic T4 microimplants reverse this blocking effect (Anderson *et al.*, 2003). In Siberian hamster, a long day breeder (LD), daily T3 injections to short-day animals induce testicular growth, mimicking thus long day length (Freeman *et al.*, 2007). Similarly, T3 intrahypothalamic microimplants prevent the onset of testicular regression normally observed in short photoperiod (Barrett *et al.*, 2007).

3.1.2.2. HPT and metamorphosis

Metamorphosis is a term used to define a remarkable body change during development that is normally accompanied by a change in habitat or behaviour. Metamorphic events ranging from mild to extreme have been described in a variety of groups including cnidarian, insects, crustacean, molluscs, tunicates and some vertebrates (amphibians and some fish; for review:

Laudet, 2011; Rousseau *et al.*, 2012). Although the molecular mechanism involved in metamorphosis throughout various chordate lineages are largely unknown, THs and the HPT axis seem to predominantly regulated metamorphosis in amphibians and teleost fishes (Paris and Laudet, 2008). In fish, two types of metamorphoses have been described; First or larval metamorphosis is a true metamorphosis (as amphibian metamorphosis) which occurs for example in pleuronectiforms (flatfish). Secondary metamorphosis is observed in some diadromic migratory teleosts and compared to first/larval metamorphosis involves less drastic morphological changes in post-embryonic juveniles; the smoltification metamorphosis has been debated as a secondary metamorphosis (Björnsson *et al*. 2012; Rousseau *et al.*, 2012).

3.1.2.2.1. HPT and amphibian metamorphosis

Amphibian metamorphosis is the best example of dramatic/extensive morphological, biochemical and cellular changes occurring during development and its regulation has been extensively studied and documented. This post-embryonic metamorphosis is obligatorily initiated and sustained by TH (Figure 8). The involvement of thyroid gland in the induction of metamorphosis has been suggested as early as 1912. At that time, Gudernatsch observed that common frog (*Rana temporaria*) tadpoles were turning into frogs when given powdered sheep thyroid gland (Gudernatsch, 1912). In addition, when surgical or chemical ablation of the thyroid gland was performed by Allen (1917), the larvae, instead of metamorphosing, became giant tadpoles (Allen, 1917). Further studies of early exposure to TH confirmed these first observations (for review: Tata, 2006). External environmental factors and stressors, from higher brain centers, influence hypothalamic CRH as the main stimulator of TSH and ultimately TH (Denver et al., 2002). TH then target *via* their receptors, a wide variety of tissues causing a series of anatomical and functional changes including the simultaneous emergence of limbs and loss of larval tails, gills and digestive system. CRH and TSH regulation of TH during metamorphosis has been clearly demonstrated in a variety of studies (for reviews: (Denver *et al*., 2002; Manzon and Denver, 2004; Tata, 2006; Grimaldi *et al.*, 2013).

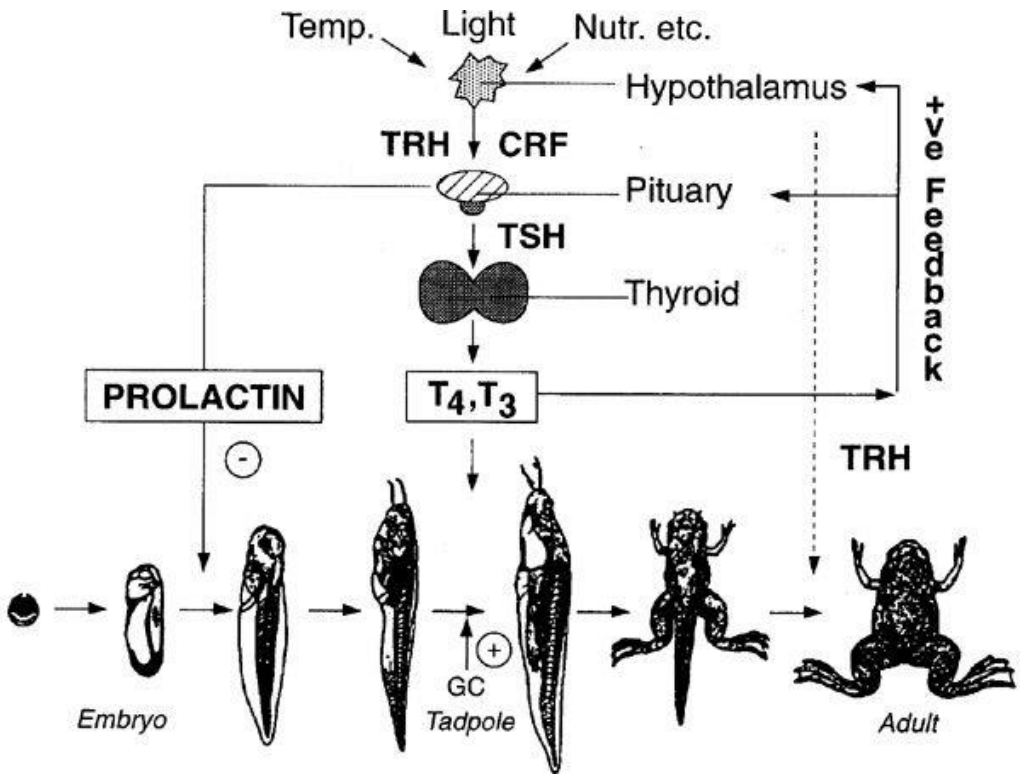

Figure 8: Proposed hormonal regulation of amphibian metamorphosis (Tata, 1993)

3.1.2.2.2. HPT and teleost metamorphoses

3.1.2.2.2.1. HPT and flatfish larval metamorphosis

Flatfish (Pleuronectiformes) have an extreme asymmetry with both eyes being on the same side of the head after metamorphosis. Similar to amphibians, larval metamorphosis in flatfish is primarily regulated by the HPT axis (for reviews: (Inui and Miwa, 1985; Schreiber and Specker, 1998; Power *et al.*, 2008). During this metamorphic event, many changes happen to the body and include the dramatic migration of one eye to the contralateral side of the head resulting in both eyes located on the same side of the head. Radioimmunoassay studies have shown a peak of T4 and histology studies have shown activation of TSH cells during the climax of the metamorphosis (Miwa *et al.*, 1988). Injection of bovine TSH in Japanese flounder can increase T4 levels and accelerate metamorphosis concluding that TSH is the main stimulatory factor of T4 and thus the major regulatory of metamorphosis (for review: Inui and Miwa, 2012).

3.1.2.2.2.2. HPT and salmonid smoltification (secondary metamorphosis)

Thyroid hormones

As early as 1939, thyroid hormones have been hypothesized to be involved in the control of smoltification. Hoar discovered *via* histological studies that the thyroid follicles of Atlantic salmon became highly active during the period of smoltification (Hoar, 1939). Since then, surges of T4 during the period of smolitfication has been shown in a variety of salmonid species (coho salmon: Dickhoff *et al*., 1978; Larsen *et al*., 2011; masu salmon, (*Oncorhynchus masou*): Nishikawa *et al*., 1979; Atlantic salmon: Boeuf and Prunet, 1985; Prunet *et al.*, 1989)

These peaks of T4 led scientists to suggest that T4 surge during smoltification is the critical component to the timing of the smoltification metamorphosis. Further studies concluded through treatments with thyroid hormones or anti-thyroid drugs such as propylthiouracil (PTU) (immersion, oral administration, intraperitoneal implants etc) that thyroid hormones could stimulate silvering and fin margination, metabolism and downstream migration (for review: Rousseau *et al.*, 2012). However they could not induce other changes of smoltification such as increased osmoregulatory ability (Refstie, 1982; Brown *et al.*, 1987). Thyroid hormones seem crucial for smoltification; however the mechanisms that regulate TH availability are not fully understood.

CRH

Administration of human CRH increased plasma T4 levels in coho salmon and stimulated downstream movements in coho, Chinook (*Oncorhynchus tshawytscha*) and chum (*Oncorhynchus keta*) salmon when administered during the smoltification period (Clements and Schreck, 2004; Ojima and Iwata, 2009, 2010). At the time of smoltification, neurogenesis of CRH neurons is activated in Atlantic salmon (Ebbesson *et al.*, 2011).

TSH

Among teleosts, pituitary content of TSH is characteristically low in relation to thyroid hormone activity (for review: MacKenzie, Jones and Miller, 2009). Within salmonids, the data obtained for TSH has been contradictory. Fridberg *et al*. (1981) reported in the Atlantic salmon that pituitary TSH-cells were more numerous and had increased activity in presmolts and smolts than in parr; yet Nishioka *et al.* (1982) showed no ultra-structural changes of TSH cells in coho salmon. No change or a slight decrease in pituitary *tsh* mRNA levels were

measured in smolts as compared to parr in Atlantic salmon (Martin *et al.*, 1999; Robertson and McCormick, 2012) and coho salmon (Larsen *et al*. 2011). No variations in pituitary and plasma TSH protein levels were reported in coho salmon throughout smoltification (Larsen *et al*., 2011). More work is needed to understand the role of TSH in teleosts.

TSH is comprised of two subunits, a common alpha subunit shared with the gonadotropins, luteinizing hormone and follicle-stimulating hormone, and a beta subunit (TSHβ) conferring hormone specificity (Pierce and Parsons 1981). Recent work by Maugars and collaborators has outlined the detailed discovery and characterization of thyrotropin beta subunit paralogs in the teleost lineage (Maugars *et al.*, 2014). Through detailed phylogeny and synteny analysis, the authors presented TSH paralogs in various teleost species and used the European eel to display differential expression of the two paralogs throughout peripheral tissues (Maugars *et al.*, 2014). In addition, the authors used the deduced amino acid sequence to confirm the conservation of 12 cysteine residues essential for proper folding and functionality of thyrotropin suggesting a functional role of these paralogs in multiple species. Functional paralogs of TSH have already been observed in stickleback. Kitano *et al.* (2010) discovered paralogs of the tshß subunit (named tshß1 and tshß2 by the authors) in stickleback and reported higher pituitary transcripts of tshß2 in populations migrating to the sea compared to stream-resident populations, meanwhile expressing similar transcript levels of tshß1.

3.2 Other factors involved in the control of smoltification

3.2.1 GH and IGF-1

Growth hormone and insulin like growth factor -1 (IGF-1) are important regulators of growth and metabolism in vertebrates including teleost fish (for reviews: Moriyama *et al*., 2000; Wood *et al*., 2005). Both have been shown to be crucial components during the smolting process of salmonid species (Sakamoto *et al*., 1993; Larsen *et al.*, 1995; Agustsson *et al.*, 2001). Pituitary GH is the main stimulator of IGF-1, which is primarily secreted from the liver.

In Atlantic salmon parr, plasma GH and IGF-1 remain low and increase with smolt development peaking slightly before the peak of NKA activity (Prunet *et al.*, 1989; McCormick *et al.*, 2007). The mRNA expression of the two genes for GH (GH1 and GH2)

present in Atlantic salmon increases during the parr-smolt transformation and peaks concomitantly to that of TSH (for review: Rousseau *et al.*, 2012). In coho salmon, plasma GH concentrations increase at the end of the smoltification period, and exposure to seawater causes increase in GH circulating levels (Sweeting *et al*., 1985).

Exogenous treatment of GH in Atlantic salmon pre-smolts increases NKA activity and salinity tolerance, while causing no affect in smolts (Prunet *et al.*, 1994). In coho salmon, GH also induces gill corticosteroid receptor abundance, leading to increased responsiveness of gills to cortisol and partially accounting for the increase in NKA activity (Shrimpton *et al.*, 1995). Interestingly, the natural increase of GH during smoltification is absent in landlocked populations of salmon, which is associated with their poor salinity tolerance (Nilsen *et al.*, 2008). The strong induction of NKA activity by GH and IGF-1 during smoltification has been suggested to play a role in behaviour change during smolting as they cause an increased salinity preference in smolts (for review: Iwata 1995). Injections of GH in Atlantic salmon parr cause increased Na^+–K^+–$2Cl^-$ cotransporter abundance in gill chloride cells (Pelis and McCormick, 2001). Low levels of GH receptors in the liver and subsequent low levels of plasma IGF-1 have been related to extreme reduced growth ("stunting" phenomenon) after seawater exposure suggesting the importance of increased GH and IGF-1 in proper SW tolerance (Duan *et al*., 1995).

In addition to its role in the control of salinity tolerance, GH possesses also its "classical" strong growth promoting action in salmonids (Björnsson, 1997), which can be associated with increased food intake (Johnsson and Björnsson, 1994). GH usually stimulates length growth over weight growth resulting in leaner elongated fish (Johnsson and Björnsson, 1994). Increased growth could be due to the stimulation of lipid mobilisation and protein synthesis (Björnsson, 1997). In Atlantic salmon, the increased metabolic rate in smolts when compared to parr could come from the increased NKA activity and mitochondrial cell proliferation as a consequence of increased GH (Maxime *et al.*, 1989; Handeland, Imsland and Stefansson, 2008).

3.2.2 Prolactin

In 1928, Stricker and Grueter discovered that pituitary prolactin (PRL) could stimulate milk in rabbits (Stricker and Grueter, 1928). Over the following decades, PRL was accredited with over 300 biological activates among various vertebrae species (for reviews: Bole-Feysot *et al.*, 1998; Manzon, 2002). In fish, PRL was first demonstrated by Pickford and Phillips to be part of the ion regulation mechanism in freshwater Killifish (*Fundulus geteroclitus*) in 1959 (Pickford and Phillips, 1956). Further studies reported high levels of PRL in winter and spring, which decreased with the progression of smoltification in Atlantic and coho salmon (Prunet *et al.*, 1989; Young *et al.*, 1989). In Atlantic salmon, Morin et al., (1994) observed no changes in PRL over the period of smoltification. However if smolts are kept in freshwater for extended periods of time, PRL levels will begin to increase (Boeuf and Prunet, 1985; Morin and Haug, 1994). PRL in parr may be a smolt inhibitory mechanism supported by the fact that PRL inhibits the development of seawater chloride cells meanwhile promoting ion uptake cells in sea-water adapted tilapia (*Oreochromis niloticus*) (Herndon, McCormick and Bern, 1991; Pisam *et al.*, 1993). Atlantic salmon PRL promotes acclimation to freshwater while GH promotes acclimation to seawater mediated by the dual osmoregulatory function of cortisol (Pelis and McCormick, 2001).

3.2.3 ACTH and Cortisol

Commonly called the stress axis, the corticotropic axis involves hypothalamic CRH, which stimulates the pituitary to release ACTH, which subsequently acts on the interrenal to stimulate the production and release of cortisol. The activation of this axis during smoltification was first reported in 1957 by Fontaine and Olivereau who observed hypertrophy of the interrenal in smolting Atlantic salmon (Fontaine and Olivereau, 1957)

Cortisol is suggested to be involved in the control of smoltification since 1954 when Fontaine and Hatey first observed increased plasma cortisol levels in Atlantic salmon smolts (Fontaine and Hatey, 1954). Further studies confirmed the dramatic increase of circulating cortisol during smoltification of multiple Atlantic salmon populations, which experience an increase in salinity, while is absent in non-anadromous populations (McCormick *et al.*, 2007; Nilsen *et al.*, 2008). In coho salmon, plasma cortisol increases at the same time as plasma GH during smoltification (Young *et al.*, 1989). Treatment with cortisol stimulates salinity tolerance in

Atlantic salmon smolts and has been shown to increase the major ion transport proteins (Na^+-K^+-$2Cl^-$-cotransporter) in the gill and gut (Pelis and McCormick, 2001).

There seems to be a tight relationship between GH, IGF-1 and cortisol and the increased salinity tolerance during smoltification. Cortisol treatment stimulates the production of both NKA isoforms, while in the presence of GH it only increases the NKA α1b isoform responsible for ion excretion in seawater (for review: McCormick, 2012). In addition, Pelis and McCormick observed an increase of Na^+-K^+-$2Cl^-$cotransporter abundance after injections of GH and cortisol separately, and greater increase when GH and cortisol were given in combination (Pelis and McCormick, 2001). Therefore, the peak of GH early in smoltification may lead to an increased salinity preference driven by the switch in NKA isoforms by cortisol regulation. In addition, cortisol then in turn increases the transcription of GH and IGF-1 receptors in the gill, which may further increases salinity preference of smolts (Tipsmark *et al.*, 2008).

4. Study Objectives

The primary objective of this thesis is to investigate the environmental and neuroendocrine regulation of smoltification and downstream migratory behaviour of the endangered Atlantic salmon population from the Loire-Allier River in France. Such knowledge will help better understand the complex life cycle of the Atlantic salmon and bring new insights to the smoltification metamorphosis including the crucial period of downstream migration initiation. The results of this study hope to better understand the dynamics of long river migration in order to help preserve the last remaining long river population of Atlantic salmon in western Europe as well as provide new knowledge to the restocking programs of other long rivers throughout Europe.

1. To characterize and investigate the presence and functional divergence of thyrotropin beta subunits and their relationship to smoltification and change of rheotaxis behaviour (Chapter 1)
2. To investigate the modulation of pituitary expression of *tsh* beta subunits and change in rheotaxis behaviour by increased water temperature and short day photoperiod conditions (Chapter 2)

Together, these objectives will seek to give new insights to the neuroendocrine regulation of smoltification and in particular the functional divergence of the thyrotropic pathway in Atlantic salmon. In addition, modulation of environmental conditions and how they affect *tsh* and downstream migratory behaviour may foreshadow the consequences of global climate change and bring attention to precautionary measures that must be taken in order to preserve the unique Loire-Allier Atlantic salmon population.

Methodology

Experimental design

The experimental design included 4 different conditions which juvenile salmon were raised in throughout the period of smoltification. The 4 conditions were as follows; Natural photoperiod with natural water temperature (NN), Natural photoperiod with warm water (goal of +5°C; N5), winter photoperiod with natural water temperature (WN) and winter photoperiod with warm water (W5) (Figure 9).

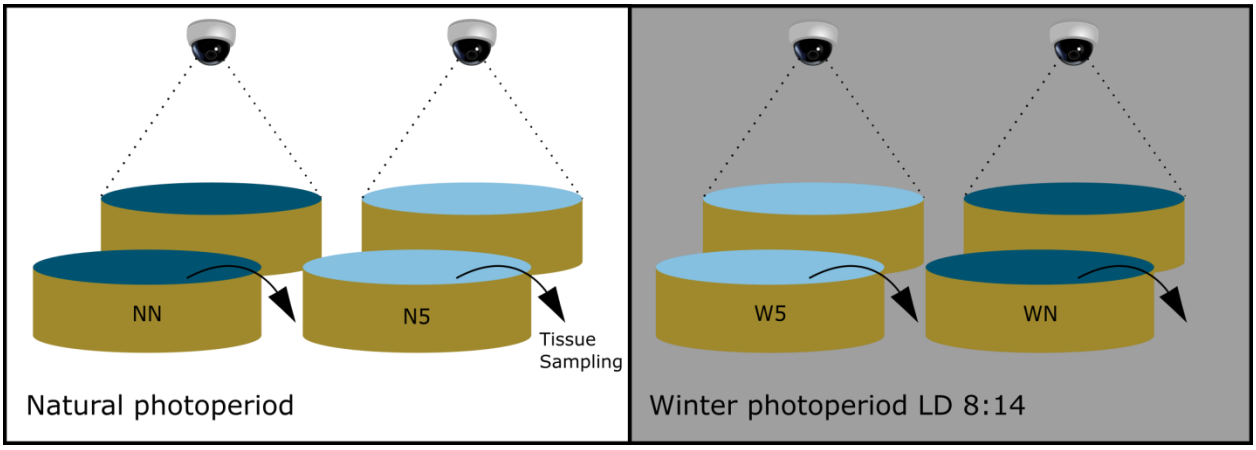

Figure 9: Experimental design. Four conditions were investigated: Natural photoperiod with natural water temperature (NN), Natural photoperiod with warm water (goal of +5°C; N5), winter photoperiod with natural water temperature (WN) and winter photoperiod with warm water (W5). Two tanks for each condition, one dedicated to video monitoring and the other to sampling. Fish were raised from parr to smolt stage from December to June of 2016.

Natural photoperiod was obtained using sensors outside which synchronized the turning on/off of the tank lights with that of sunrise and sunset. This produced a “natural” light regime for the fish that mimiced the natural photoperiod. Winter photoperiod was obtained by maintaining the Light/Dark (LD) hours to that of winter solstice of the current year which fell on December 21, 2015. From this point on, lights above the tank remained at the specific light/dark regime of December 21, 2015 which was equivalent to a LD regime of 8:14 .

Natural river water is extracted from the Desges river, small tributary of the Allier river (Figure 10), and pumped directly to the tanks passing only through a small UV filter to removed potential bacteria. To obtain “warm water”, natural water from the Desges River passes through a heat exchange system with that of the local well water of Chanteuges. River water passing through his system draws heat from the local well water bring the temperature up to a goal of +5°C to that of the natural water temperature. Due to the nature of the design, larger differences in water temperature were more easily achievable during periods of cooler temperatures when the difference between the river water and well water are at its greatest. As the river temperature rises throughout the spring the efficiency of the heat exchange system decreases and the difference between the well and river water is reduced. Water temperatures were measure on an hourly basis using temperature probes in each experimental tank providing a detailed profile of water temperature throughout the experiment.

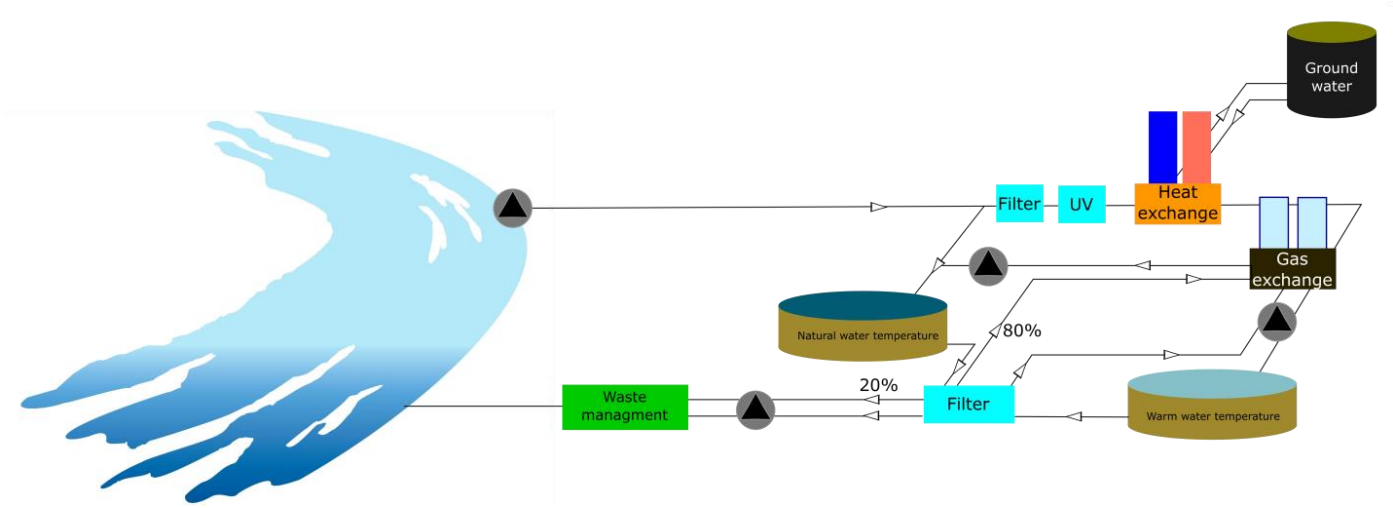

Figure 10: Simplified schematic of the recirculating and heat exchange system at the Conservatoire national du Saumon Sauvage. Natural water is drawn from the Desges river, a tributary of the Allier. For increased water temperature, natural water is passed through a heat exchange system. The system runs on a 80/20% recirculating system.

Smolt production

All Atlantic salmon smolts were raised at the Conservatoire National du Saumon Savage in Chanteuges France (south central France: N 45°04’48, E03°31’55). The conservatory uses

wild returning adult salmon caught at the Vichy dam to produce F1 progeny used for restocking of the Allier and tributaries of the Allier and Loire in part of the Plan Loire Grandeur Nature restoration project. For the current experiment, 390 under-yearling fish at approximately ~8 months old and exceeding 145mm ("upper mode": (Thorpe *et al*., 1982)) were transferred to experimental tanks on December 1st, 2015 and were let to acclimatize to the tanks for 3 weeks. The number of fish used was decided by accounting for sampling frequency and growth in order to maintain an acceptable density of fish not to disturb normal behaviour. In addition, the low rearing density (3-4kg/m^3) compared to fish farms (25kg/m^3) is a strategy used by CNSS to increase fish health and post-release survival rates.
Each tank was equipped with tangentially oriented water inlet which produced an anti-clockwise flow. Water flow was maintained at 1.5L/s until the start of April where the water flow increased to 2L/s in order to maintain a dissolved oxygen concentration of 7mg/L. Fish were fed a custom blend (Turbot label Rouge, Le Gouessant, France) by automatic feeders 5 times a day. Feeding times were distributed evenly throughout daylight hours.

Tissue collection

Fish were anesthetized with an overdose of ms222 (0.4 ml/l; Sigma-Aldrich, St Louis, MI, USA) and total body length, fork length and total body weight were recorded. Fish were then killed by decapitation. For qPCR analyses of the tissue distribution of *tshβa* and *tshβb* transcripts, 10 fish (5 males and 5 females) were sampled in March 2015; the following organs were individually collected and stored in RNALater (Ambion Inc, Austin, USA) at -20°C until RNA extraction: retina, brain (dissected into olfactory bulbs, telencephalon, epiphysis, optic lobes, hypothalamus, saccus vasculosus, cerebellum, medulla oblongata), pituitary, as well as samples of gill filaments, kidney, liver, spleen, muscle, skin, abdominal fat, testis or ovary. For qPCR analyses of pituitary *tshβa* and *tshβb* expression profiles throughout smoltification, 20 fish (mixed sex) were sampled once a month from January to June in 2013 and 2014, and 8 fish were sampled at more frequent intervals from December 2015 to June 2016; individual pituitaries were collected in RNALater and stored at -20°C until RNA extraction. For *in situ* hybridization of *tshβa*- and *tshβb*-expressing pituitary cells, 8 fish were sampled in April and 8 in June 2017; individual pituitaries were collected, fixed in

paraformaldehyde (PFA) overnight at 4°C, dehydrated in increasing series of ethanol (EtOH) concentration and stored in 98% methanol at -20°C before further processing.

Identification of *tshβ* paralogs in the Atlantic salmon

Atlantic salmon *tshβ* loci were identified in the recent Atlantic salmon genome assembly (ICSASG_v2, GCA_000233375.4) (Lien *et al.*, 2016) after interrogation of the Atlantic salmon annotated gene database in GenBank. The presence of additional *tshβ* genes were investigated by blasting salmon *tshβa* and *tshβb* against the Atlantic salmon genome. Gene sequences were examined with CLC Main Workbench 8 (Qiagen Bioinformatics, Hilden, Germany) for prediction of exons, introns, coding sequence (CDS) and signal peptide.

Cloning and sequencing of partial cDNA of Atlantic salmon *tshβa and tshβb* paralogs

Cloning primers for Atlantic salmon *tshβa* and *tshβb* were designed on predicted mRNA sequences of corresponding genes (LOC100136355 and LOC106572976) using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/, Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, US)(Untergasser *et al.*, 2012) (Table 1). PCR was performed using cDNA of smolt pituitaries collected in April using Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) for *tshβa* and Platinum Taq DNA polymerase (Invitrogen) for *tshβb* according to the manufacturer's instructions. Purified PCR fragments were subcloned into PCRII vectors (Thermo-Fisher, Wahtham, MA, USA) before sequencing by GATC Biotech (Brussels, Belgium). Plasmids were used for preparing cRNA probes (section fluorescence *in situ* hybridization).

Gene Name	Primer name	Purpose	Primer Sequence	Annealing Temperature	Primer Reference
tshβa	tshβa-CL-F	PCR	GTCTCCTTTGCCTGCTCTTC	55°C	This paper
	tshβa-CL-R	PCR	GTCTCCTTTGCCTGCTCTTC	55°C	This paper
tshβb	tshβb-CL-F	PCR	CTGGTCGCTGTGTGGATAGG	55°C	This paper
	tshβb-CL-R	PCR	CGCTGTGTGGATAGGCAGTT	55°C	This paper
tshβa	tshβa-F	qPCR	CTCCTTTGCCTGCTCTTCAG	60°C	This paper
	tshβa-R	qPCR	GGCCAGCTCCTTCATGTTAC	60°C	This paper

tshßb	tshßb-F	qPCR	TTGCCGTCAACACCACCAT	62°C	This paper
	tshßb-R	qPCR	GGGATGATAGACCAGGGAGTG	62°C	This paper
ß-actin	actin-F	qPCR	CCAAAGCCAACAGGGAGAAG	60°C	[56]
	actin-R	qPCR	AGGGACAACACTGCCTGGAT	60°C	[56]

Table 1. Primer sequences. The table provides the sequences of Forward and Reverse primers used for PCR of Atlantic salmon *tshβa* and *tshβb* (cloning and FISH) and for qPCR of Atlantic salmon *tshβa*, *tshβb* and *β-actin*.

Phylogeny analysis.

Phylogeny analysis of 39 vertebrate TSHβ amino-acid sequences was performed using a part of dataset from (Maugars *et al.*, 2014), enriched with additional teleost TSHβ sequences, including TSHβ paralogs of salmonids identified in this study. New *tshβ* genes were either retrieved from GenBank or were identified by blasting (TBLASTN algorithm) genome assembly databases when genes were not annotated in GenBank. The amino-acid sequences of TSHβ were deduced and signal peptides were predicted using CLC Main Workbench 8.

The sequence alignment was performed on CLC Main workbench 8 and manually adjusted. Phylogenetic tree was constructed using Maximum Likelihood algorithm with PhyML:3.0(Guindon *et al.*, 2010) combined to the SMS model selection(Lefort, Longueville and Gascuel, 2017) and SPR as tree improvement on ATGC browser (http://www.atgc-montpellier.fr/phyml/). Tree topology was assessed by bootstrapping on 1000 replicates.

Synteny analysis.

Synteny analysis was performed on *tshβ* genomic region in actinopterygians, using a holostean, the spotted gar as a reference (LepOcu1 (GCA_000242695.1)). Comparisons were made with *tshβ* parlogons in the pike (Eluc_V3 (GCA_000721915.3)), and in two salmonid representatives, Atlantic salmon (ICSASG_v2 (GCA_000233375.4)) and rainbow trout (Omyk_1.0 (GCA_002163495.1)). Neighbouring genes of *tshβ* loci were identified and compared manually using chromosome annotation. Blast analyses on the genomes were

performed to search for un-annotated genes and additional paralogs. Genes fractionated, showing a frameshift mutation or missing exon were considered pseudogenes.

RNA extraction and cDNA synthesis.

Total RNA was extracted by homogenizing tissues in TRIzol (Thermo-Fisher) according to the manufacturer's protocol, using TissueLyser II (Qiagen). After a chloroform separation step, RNA was precipitated in ice cold isopropanol with 1 µl of glycoblue (Ambion). Total RNA was treated with DNase I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. RNA concentration was measured using Nanopore 2000c/2000 (Thermo-Fisher).
Reverse transcription was performed using 75 ng random hexamer primers (Invitrogen) and SuperScriptIII First Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocol. For pituitaries, 250 ng of total RNA were used and 750 ng for brain and peripheral tissues.

Quantitative RT-PCR.

Specific quantitative real-time PCR (qPCR) primers for *tshβa* and *tshβb* were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/)(Untergasser *et al.*, 2012) with forward and reverse primers on two different exons to prevent amplification of genomic DNA (Supplementary Table S3); primers were purchased from Eurofins scientific (Luxembourg). Specificity of the primers was controlled by sequencing PCR product. *β-actin* was used as reference gene using previously published primers(Olsvik *et al.*, 2005).

Quantitative PCR assays were performed using LightCycler 1.2 (Roche Diagnostics) and LightCycler FastStart Master plus SYBR Green I kit (Roche Diagnostics). Each reaction contained: 4 µl of diluted cDNA template, 2 µl of SYBR green master mix and 1µl of specific primers (500 nM final concentration). The following thermal cycling steps were used for each qPCR run: initial denaturation 94°C for 10 min followed by 41 cycles of 10 s of denaturation at 95°C, 5 s of annealing temperature (60°C or 62°C; Supplementary Table S3) and 6 s of elongation at 72°C. The program ended by slowly increasing temperatures (0.1°C/s) from 68-95°C for amplification specificity controlled by melting curve analysis. Relative quantification

was performed using standard curves created for each gene with serial dilutions of pooled pituitary cDNA. One dilution of the cDNA pool was added in each run as a calibrator. All samples were analysed in duplicates and each qPCR run contained a negative control using water in substitute for template cDNA. Calculations of sample concentrations were made using the Roche LightCycler 1.2 manufacturer's software.

Fluorescence *in situ* hybridization (FISH).

Antisense and sense cRNA probes for FISH were synthesized by *in vitro* transcription from *tshβa* or *tshβa* plasmids using T7 and SP6 RNA polymerase (Promega, Madison, Wisconsin USA) and labelled with digoxigenin-11 UTP (Roche Diagnostics) at 37°C for 2 h. Probes were purified using Nucleospin RNA clean-UP kit (Machere-Nagal, Hoerdt, France) and controlled by gel electrophoresis.

Whole fixed pituitaries were rehydrated (96, 70, 50, 25% EtOH), included in 3% RNase free agarose gel and sliced into 70 µm parasagittal sections using VT1000S Leica vibratome (Leica, Wetzlar, Germany). Sections were permeabilized using proteinase K (Sigma-Aldrich, 1 µg/ml in PBS with 0.1% Tween 20, PBST) for 45 min at 37°C, then proteinase K was inactivated using glycine (Sigma-Aldrich, 2 mg/ml in PBST) for 30 min at room temperature (RT), followed by a post fixation step in 4% PFA for 15 min and washing in PBST.

Prior to FISH, sections were incubated with hybridization buffer (HB: 50% formamide, SSC 5X, 0.1% Tween 20, 15 ng/ml Heparin, Sigma-Aldrich; 80 µg/ml Torula yeast tRNA, Sigma-Aldrich; pH 6.5) at 55°C for 4 h. FISH was performed in fresh HB containing 300 ng/ml *tshβa* probe or 600 ng/ml *tshβb* probe at 55°C. After 18 h, sections were washed with a series of 4 different hybridization washes (HW): HW1 (50% formamide, SSC 5X, 0.1% Tween 20) 2 x 30 min, HW2 (50% HW1, 50% SSC 2X) 2 x 30 min, HW3 (SSC 2X, 1% Tween 20) 2 x 30 min, HW4 (SSC 0.2X, 0.1% Tween 20) 2 x 2 min. Sections were soaked in TNE buffer (10 mM Tris HCL pH 7.6, 500 mM NaCl, 1 mM EDTA) at 37°C for 30 min then treated with RNaseA (Sigma-Aldrich, 20 µg/ml in TNE buffer) for 30 min at 37°C. Sections were washed in TNE buffer, 2 x 10 min at 37°C followed by SSC 0.2X 0.1% Tween 20, 2 x 30 min at 55°C. Sections were washed in PBST for 10 min with agitation at RT and incubated in PBST with 2% H2O2 for 30 min in order to inactivate endogenous peroxidases, followed by washes in PBST, 3 x 10 min with agitation. Blocking was performed using 1% Blocking Reagent (Roche Diagnostics) in Maleic

acid buffer (MAB) for 2 h with agitation. Sections were incubated in blocking/MAB buffer with anti-digoxigenin peroxidase-conjugated antibody (1/250, Roche Diagnostics) overnight at 4°C, and then washed in PBST for 2 h with agitation. Tyramide revelation was carried out using green FITC conjugated tyramide (Sigma-Aldrich, 1/200 in PBST with 0.01% H2O2) for 30 min in darkness at RT. After washing in PBST for 5 x 20 min, sections were let overnight in PBST at 4°C in darkness. Cell nuclei were stained using DAPI staining (Sigma-Aldrich, 1/1000 in PBST) for 20 min at RT. Sections were mounted in Vectashield H-1000 Mounting Medium (Vector, Eurobio/Abcys, CA, USA). Confocal images were obtained using a confocal microscope (Zeiss LSM710, Oberkochen, Germany). Channels were acquired sequentially to avoid signal crossover between the different filters. Images were processed using the ZEN software (version 2009, Zeiss). Z-plan and Z-projection images were obtained using Image J software (Fiji software; http://rsbweb.nih.gov/ij/).

Chapter 1

ABSTRACT

Smoltification is a metamorphic event in salmon life history, which initiates downstream migration and pre-adapts juvenile salmon for seawater entry. While a number of reports concern thyroid hormones and smoltification, few and inconclusive studies have addressed the potential role of thyrotropin (TSH). TSH is composed of a α-subunit common to gonadotropins, and a β-subunit conferring hormone specificity. We report the presence and functional divergence of duplicated TSH β-subunit paralogs (*tshβa* and *tshβb*) in Atlantic salmon. Phylogeny and synteny analyses allowed us to infer that they originated from teleost-specific whole genome duplication. Expression profiles of both paralogs in the pituitary were measured by qPCR throughout smoltification in Atlantic salmon from the endangered Loire-Allier population raised at the Conservatoire National du Saumon Sauvage. This revealed a striking peak of *tshβb* expression in April, concomitant with downstream migration initiation, while *tshβa* expression remained relatively constant. *In situ* hybridization showed two distinct pituitary cell populations, *tshβa c*ells in the anterior adenohypophysis, and *tshβb* cells near to the pituitary stalk, a location comparable to the pars tuberalis TSH cells involved in seasonal physiology and behaviour in birds and mammals. Functional divergence of *tshβ* paralogs in Atlantic salmon supports a specific role of *tshβb* in smoltification.

Introduction

The Atlantic salmon (*Salmo salar*), like other salmonids, has a complex life cycle with the reproduction occurring in the upper part of the rivers, while the growth phase taking place in the ocean. A crucial life history transition, called smoltification (or parr-smolt transformation) initiates downstream migration and pre-adapts the juvenile salmon to

seawater entry[1-3]. With multiple morphological, physiological, and behavioural changes, smoltification can be viewed as a metamorphosis[4,5]. In amphibians, extensive research has demonstrated that larval metamorphosis is triggered mainly by thyroid hormones (TH, thyroxine T4 and triiodothyronine T3), the production of which is stimulated by a pituitary hormone, thyrotropin (or thyroid-stimulating hormone, TSH). TSH is comprised of two subunits, a common alpha subunit shared with the gonadotropins, luteinizing hormone and follicle-stimulating hormone, and a beta subunit (TSHβ) conferring hormone specificity[6].

A histological study by Hoar first reported an activation of thyroid follicles in Atlantic salmon during smoltification[7]. An increase in T4 plasma levels was then shown during smoltification in various salmonids (coho salmon, *Oncorhynchus kisutch*[8,9]; masu salmon, *Oncorhynchus masou*[10]; Atlantic salmon[11]), even though changes in T4 plasma levels were relatively limited as compared to other hormones such as cortisol, insulin-like growth factor-1 and growth hormone[3,5]. Experimental hormonal treatments suggested that TH may be responsible for smoltification-related change in rheotaxis and swimming behaviour[12] and olfactory imprinting[13], but would be insufficient to induce preadaptation to osmoregulation in seawater[14].

While many studies addressed TH, little is known on TSH and smoltification, and contradictory data have been obtained. In the Atlantic salmon, pituitary TSH-cells were more numerous and had increased activity in presmolts and smolts than in parr[15], but no ultra-structural changes of TSH cells were observed in coho salmon during smoltification[16]. No change or a slight decrease in pituitary *tshβ* mRNA levels were measured in smolts as compared to parr in Atlantic salmon[17,18] and coho salmon[9]. No variations in pituitary and plasma TSH protein levels were reported in coho salmon throughout smoltification[9].

While extant amphibians, birds and mammals possess only a single TSH (a single *tshβ* gene) recent studies revealed the presence of duplicated *tshβ* paralogs in some other vertebrates[19]. Duplicated *tshβ* genes originated from whole genome duplication events that occurred in early vertebrates ("1R/2R", for "1st and 2nd rounds of whole genome duplication") and in early teleosts ("3R", for "3rd round of whole genome duplication")[19]. Thus, chondrichthyans, such as the elephant shark, *Callorhinchus milii*, and basal sarcopterygians, such as the coelacanth, *Latimeria chalumnae*, have two *tshβ* paralogs issued from 2R (*tshβ* and *tshβ2*), while tetrapods have lost the *tshβ2* paralog and conserved only a single *tshβ* gene[19]. *Tshβ2* has also been lost in the actinopterygian lineage, but various teleost species possess two *tshβ* paralogs as a result of 3R-duplication of *tshβ*[19,20], named in the present study *tshβa* and *tshβb*, according to the most common nomenclature "a and b" for teleost 3R-paralogs. In the present study, we searched for *tshβ* paralogs in the Atlantic salmon, considering also the additional genome duplication that occurred in the salmonid lineage ("4R") [21].

We revealed the presence and expression of two *tshβ* paralogs in Atlantic salmon and brought new knowledge on the evolutionary history of *tshβ* genes in salmonids. We investigated the potential involvement of the *tshβ* paralogs in smoltification, using the Atlantic salmon from the Loire-Allier basin as a model. This population is the last extant salmon population able to migrate long rivers in Western Europe and is currently endangered. Samplings were performed at the "Conservatoire National du Saumon Sauvage" (CNSS), Chanteuges, France, whom breeds wild brood stock and produces juvenile salmon which are released at different developmental stages, as part of a conservation programme. Quality and timing of smoltification are key issues for this population, as smolts need to achieve a 900 km-downstream migration before reaching the Loire estuary in a

narrow window of suitable physiological and environmental conditions. This requires new research advances on environmental and neuroendocrine regulatory mechanisms of smoltification and initiation of downstream migration. With the demonstration of a striking expression peak of one of the *tshβ* paralog, this study provides the first evidence of the involvement of TSH in smoltification.

Results

Two thyrotropin beta subunit (*tshβ*) paralogs in the Atlantic salmon.

Using the recent Atlantic salmon genome assembly (GCA_000233375.4)[22], we retrieved two genes with two exons each, encoding complete TSH β-subunit sequences: one gene (named in the present study *tshβa*) located on the chromosome ssa22 and corresponding to the *tshβ* sequence previously isolated[17], and the second gene (named in the present study *tshβb*) located on ssa15 and coding for another *tshβ*; this *tshβb* sequence encompassed two exons, as all vertebrate *tshβ*, and included a previously identified exon 1[19]. In order to assess that the two *tshβ* paralogs are transcribed, cDNA sequences were successfully cloned using pituitary RNA from Atlantic salmon sampled during the parr-smolt transformation. A partial *tshβa* mRNA sequence (401 bp) was cloned and its sequence shared 100% identity with already characterized *tshβ* (AF060566)[17]. A *tshβb* mRNA sequence (517 bp) including the full length CDS was cloned (MG948546, this study); it presented 100% identity with the corresponding predicted *tshβb* sequence in the genome. A third putative *tshβ* locus was identified on ssa12, corresponding to a partial exon 2 with a frameshift mutation resulting in an early stop codon. Therefore this locus was identified as a *tshβ*-pseudogene.

Comparison of TSHβ deduced amino-acid sequences (Supplementary Fig. S1) showed that Atlantic salmon paralogs TSHβa and TSHβb shared 31.5% identity and 47.5% similarity and

that both have conserved the twelve cysteine residues that have been shown to be required for proper folding and functional activity of TSH in mammals[23]. Both paralogs also shared the typical N-glycosylation site conserved among vertebrate glycoprotein hormone beta subunits; in addition, Atlantic salmon TSHβb presented a second N-glycosylation site located between the two first cysteine residues, as with the other teleost TSHβb[19].

Phylogeny analysis of TSHβ.

Molecular phylogeny analysis was performed on 38 gnathostome TSH β-subunit sequences and using lamprey, *Petromyzon marinus,* glycoprotein hormone β-subunit (GpHβ) as outgroup (Figure 1; Supplementary Fig. S1). As previously shown[19], most sequences grouped into a "classical TSHβ" clade, whereas a few chondrichthyan and basal sarcopterygian sequences formed a small TSHβ2 sister clade. Among the TSHβ clade, the analysis also supported the two sister clades for teleost sequences resulting from teleost 3R, named here TSHβa and TSHβb following the teleost 3R paralogs nomenclature (previously named TSHβ and TSHβ3[19]). The two Atlantic salmon TSHβ branched into the two teleost TSHβa and TSHβb clades, respectively (Figure 1) allowing us to classify and name the Atlantic salmon TSHβ paralogs, TSHβa and TSHβb. The pike, *Esox lucius*, representative species of a sister group of Salmoniforms, the Esociforms which have not undergone the salmonid 4R, also possessed the two teleost *tshβ* 3R paralogs, encoding for TSHβa and TSHβb (Figure 1). In contrast, we identified up to three *tshβ* genes in the genomes of rainbow trout, *Oncorhynchus mykiss* (GCA_002163495.1) and coho salmon (GCF_002021735.1): a single *tshβb* and two *tshβa* genes (*tshβaα* and *tshβaβ* according to the current nomenclature "*α* and *β*" for salmonid 4R paralogs[21]). They encode for three putative TSHβ (TSHβaα, TSHβaβ and TSHβb, Figure 1, Supplementary Fig. S1).

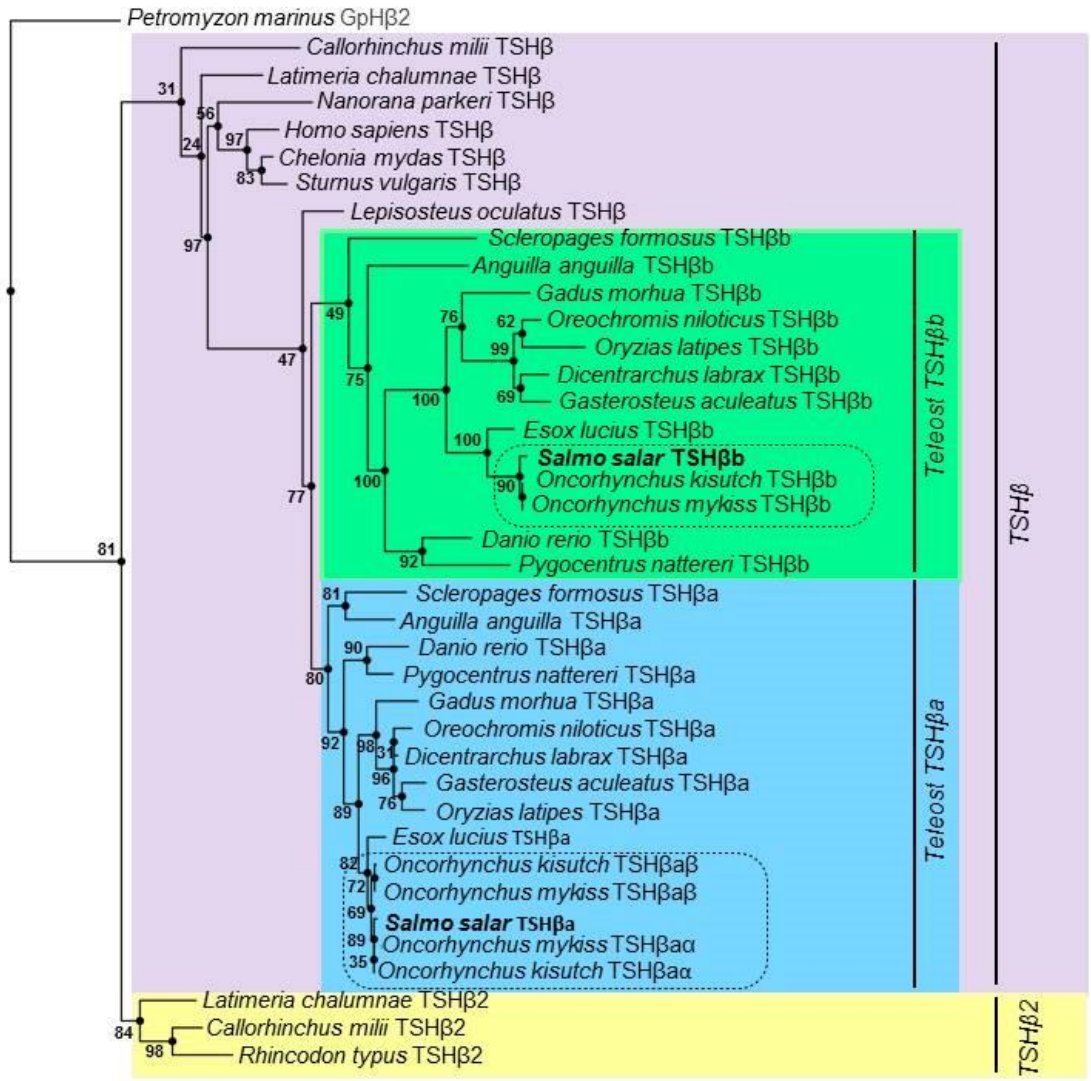

Figure 1. Consensus phylogenetic tree of TSHβ amino-acid sequences. Analysis was performed on 38 gnathostome TSHβ amino-acid sequences using the Maximum likelihood method, with 1000 bootstrap replicates. The tree was rooted using lamprey (*Petromyzon marinus*) GpHβ as outgroup. Bootstrap values are indicated at each node. The gnathostome TSHβ and TSHβ2 sister clades are highlighted in purple and yellow, and the teleost TSHβa and TSHβb sister clades, in blue and green, respectively. For sequence alignment, see Supplementary Fig. S1 and for sequence references, see Supplementary Table S1.

Synteny analysis of *tshβ* genomic region.

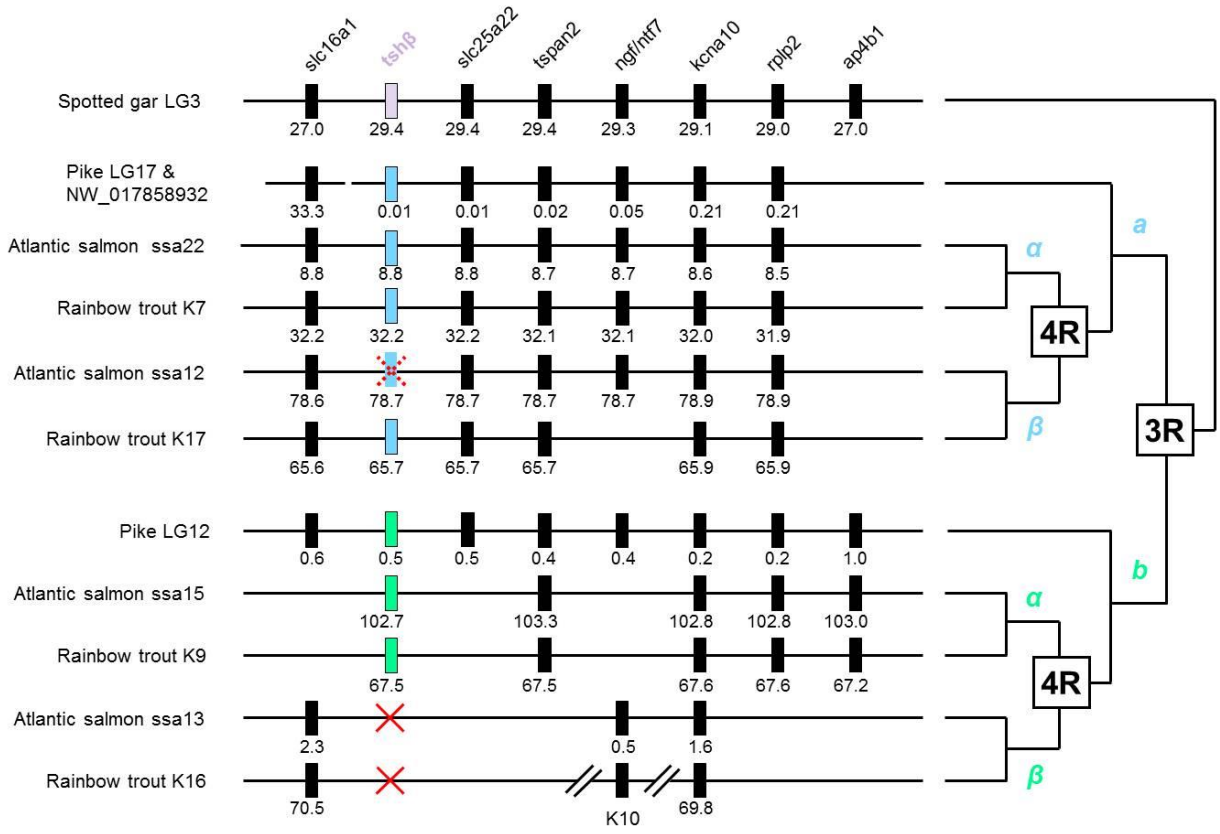

Figure 2. Synteny analysis of *tshβ* genomic region in actinopterygians. *Tshβ* genomic region of a non-teleost actinopterygian, a holostean, the spotted gar (*Lepisosteus oculatus*) was used as a reference. *Tshβ* genomic region was duplicated by teleost-specific whole genome duplication (3R) as seen in the pike (*Esox lucius*), resulting in two paralogons (a and b), and further duplicated by salmonid-specific whole genome duplication (4R) as seen in the Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), resulting in four paralogons (aα, aβ, bα, bβ). For each species, chromosome or scaffold number is indicated. Gene positions are given (in Mega base) below the genes. Full names and references of *tshβ* and neighbouring genes are given in Supplementary Table S2. Red cross indicates loss of *tshβ* paralog; dotted red cross indicates *tshβ* pseudogene.

To further assess the origin and nomenclature of salmonid *tshβ* paralogs, we performed a synteny analysis (Figure 2) of the *tshβ* genomic region of the pike and of two salmonids, the Atlantic salmon and the rainbow trout. We used as a reference the *tshβ* genomic region of

the spotted gar, *Lepisosteus oculatus*, holostean basal actinopterygian, which has not undergone the teleost 3R. Synteny analysis confirmed that the *tshβ* genomic region has been duplicated into two paralogons in the pike, in agreement with the teleost 3R, and further duplicated into four paralogons in the Atlantic salmon and rainbow trout, in agreement with the salmonid 4R. As an example, *kcna10,* one of *tshβ* neighbouring genes, was present as a single gene in the spotted gar, as two paralogs in the pike, and as four paralogs in the Atlantic salmon and rainbow trout, reflecting full conservation of 3R- and 4R-duplicated paralogs (Figure 2). Other *tshβ* neighbouring genes, such as *rplp2*, *tspan*, or *slc16a1*, showed full conservation of 3R duplicated paralogs, but incomplete conservation of 4R duplicated paralogs leading to only three paralogs in salmonids (Figure 2). Further paralog gene losses were observed for other neighbouring genes, and in particular for *ap4b1*, with a single gene present in the pike and salmonids, as in the spotted gar, indicating losses of 3R- and 4R-duplicated paralogs (Figure 2). For *tshβ,* synteny analysis confirmed that teleost 3R gave rise to *tshβa* and *tshβb* paralogs, as shown in the pike (Figure 2). Concerning *tshβa*, it assessed that salmonid 4R further gave rise to *tshβaα* and *tshβaβ* paralogs, both conserved in the rainbow trout, while only *tshβaα* was conserved in the Atlantic salmon. For simplicity, in this study we named the *tshβaα* paralog conserved in the Atlantic salmon*, tshβa*. Concerning the 4R duplicated *tshβb* paralogs, synteny supported that only one paralog was conserved in the rainbow trout and Atlantic salmon, named here *tshβb.*

Differential tissue distribution of *tshβa* and *tshβb* transcripts in the Atlantic salmon.

We developed specific qPCRs for each Atlantic salmon *tshβa* and *tshβb* paralogs and compared the tissue distribution of their expression in smolts (Figure 3). Both *tshβa* and *tshβb* paralogs were mainly expressed in the pituitary. While salmon *tshβa* transcript was

exclusively found in the pituitary, *tshβb* was expressed also at low levels in various brain regions, and at lower but detectable levels in some peripheral tissues such as gills, kidney, liver, muscle, fat and gonads; *tshβb* transcripts were not detectable in heart, spleen and skin (Figure 3).

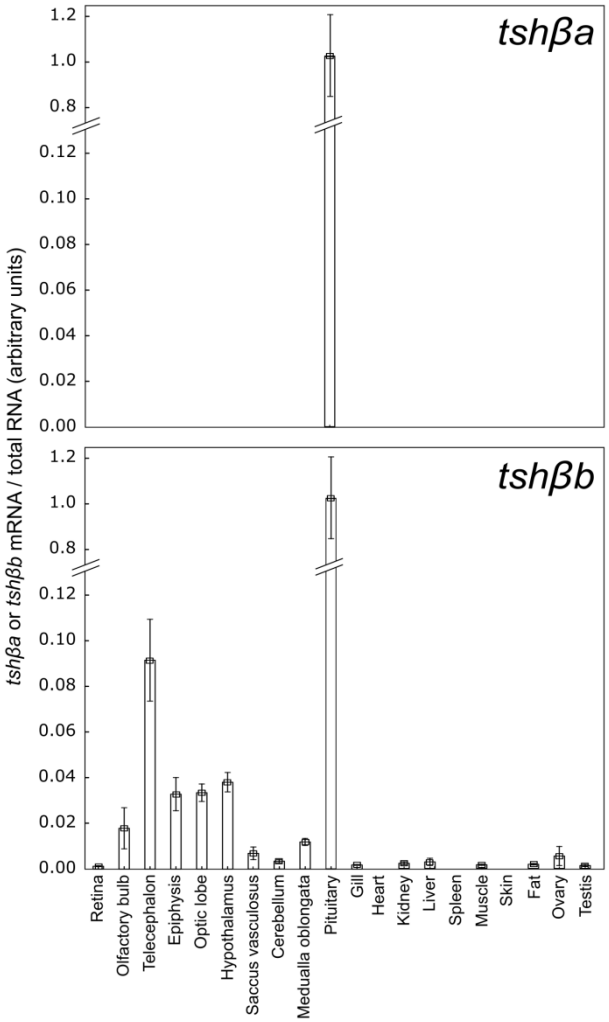

Figure 3. Tissue distribution of *tshβa* and *tshβb* transcripts in the Atlantic salmon. Messenger RNA levels of *tshβa* and *tshβb* paralogs were measured by qPCR in various tissues from smolts sampled in March 2015. Owing to the different nature of the tissues, transcripts levels were normalized to the amount of total RNA, and expressed as arbitrary units. Results are means ± s.e.m (n= 5 females for ovary; n= 5 males for testis; n= 10 mixed sex for the other tissues as there was no differences between sex).

Peak expression of pituitary *tshβb* paralog during smoltification.

In 2013 and 2014, under-yearling juveniles were sampled every month from January to June, which covers the smoltification period that occurs in early spring (end of March-April) for the Loire-Allier population (Figure 4a, b). Smoltification was marked by a drastic change in swimming behaviour, as visually recorded during day time, from positive rheotaxis (facing the current) in parr to negative rheotaxis (facing downstream) in smolt, which triggers the onset of downstream migration (Figure 4a, b). Pituitary *tshβa* and *tshβb* mRNA levels were measured by qPCR. While the expression profile of *tshβa* remained relatively constant throughout the sampling period, a dramatic peak in the expression of the other paralog, *tshβb,* was measured in March-April, during the smoltification period (Figure 4a, b).

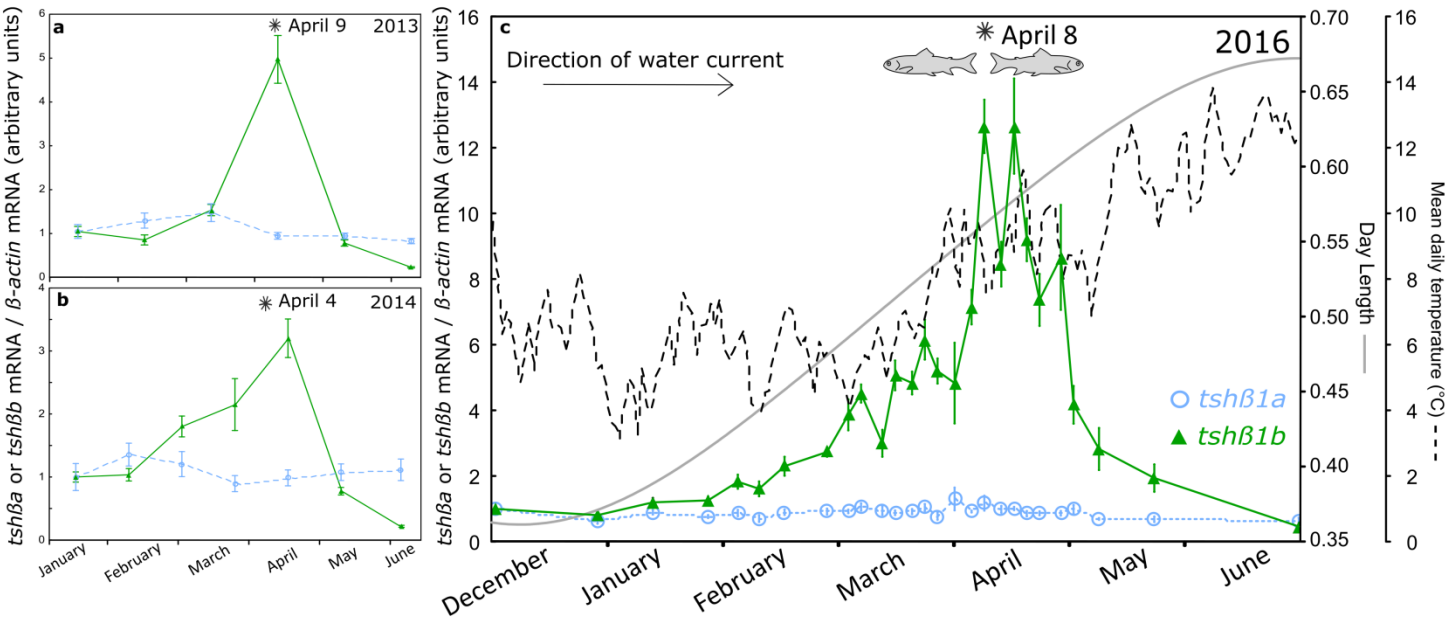

Figure 4. Expression profiles of pituitary *tshβa* and *tshβb* transcripts in the Atlantic salmon throughout the smoltification period. Three independent experiments (a, b, c) were performed. Under-yearling Atlantic salmon produced at CNSS were transferred in December, for each experimental year, to the experimental tanks under natural river water, temperature and photoperiod, and with circular water flow. For experiments a and b, monthly fish samplings were made from January to June in 2013 and in 2014. The third experiment (c) was performed with more frequent fish samplings, from December 2015 to June 2016. Messenger RNA levels of *tshβa* and *tshβb* paralogs were measured by qPCR, normalized to beta-actin as reference gene, and expressed as arbitrary units. Results are

means ± s.e.m (experiment a, n= 20 individual pituitaries; experiment b, n= 10 pools of 2 pituitaries; experiment c, n= 8 individual pituitaries). Photoperiod and mean daily water temperature are indicated for experiment c. Fish swimming behaviour and rheotaxis was observed during daytime. For experiment c, water current is indicated by an arrow and fish orientation symbolised. * indicates date of inversion of fish rheotaxis from positive to negative, in experiments a, b and c.

In order to confirm the differential regulation of the pituitary expression of *tshβa* and *b* paralogs, we performed a third experiment from December 2015 to June 2016, with high frequency sampling during the smoltification period (Figure 4c). The results were in full agreement with the previous experiments. Pituitary expression of *tshβa* remained stable throughout the experiment, while a large expression peak of *tshβb* was recorded during the smoltification period. Pituitary *tshβb* transcript levels started to rise during February, reached a dramatic peak in early April and then dropped at the end of April until June, to reach lower levels than in December (Figure 4c). It is noteworthy that qPCR results (Figure 4) were expressed as arbitrary units for each paralog; however comparison of quantification cycle values (Cq) between paralogs suggested lower pituitary levels of *tshβb* than *tshβa* in December and June (8 Cq mean difference), and even in April at the time of *tshβb* peak (4 Cq mean difference). We observed again that the change in rheotaxis from positive to negative, a characteristic of smoltification, was concomitant with the expression peak of *tshβb* paralog (Figure 4c).

Distinct populations of *tshβa*- and *tshβb*-expressing cells in the Atlantic salmon pituitary.

In order to get more insight into the pituitary expression of the two paralogs, we compared the localization of *tshβa* and *tshβb* transcripts by fluorescent *in situ* hybridization (FISH), using pituitaries from smolts sampled in April and June. *Tshβa*-expressing cells could be well

detected by FISH in April as well as in June, while *tshβb*-expressing cells could be observed only in April. The lack of detection of *tshβb*-expressing cells by FISH in June is in agreement with the very low expression of this paralog outside of the smoltification period, as shown by qPCR. *Tshβa* cells were located in the anterior adenohypophysis, in the rostral pars distalis (RPD) close to the follicles formed by the prolactin cells, at the border with the proximal pars distalis (PPD) (Figure 5). In contrast, *tshβb* cells were observed in the dorsal region of the PPD, boarding the dorsal region of the pars nervosa (PN) near the pituitary stalk (Figure 5). No labelling was observed when using control sense probes (Supplementary Fig. S2). FISH results demonstrated that salmon *tshβa* and *tshβb* paralogs are expressed by two distinct cell populations within the pituitary.

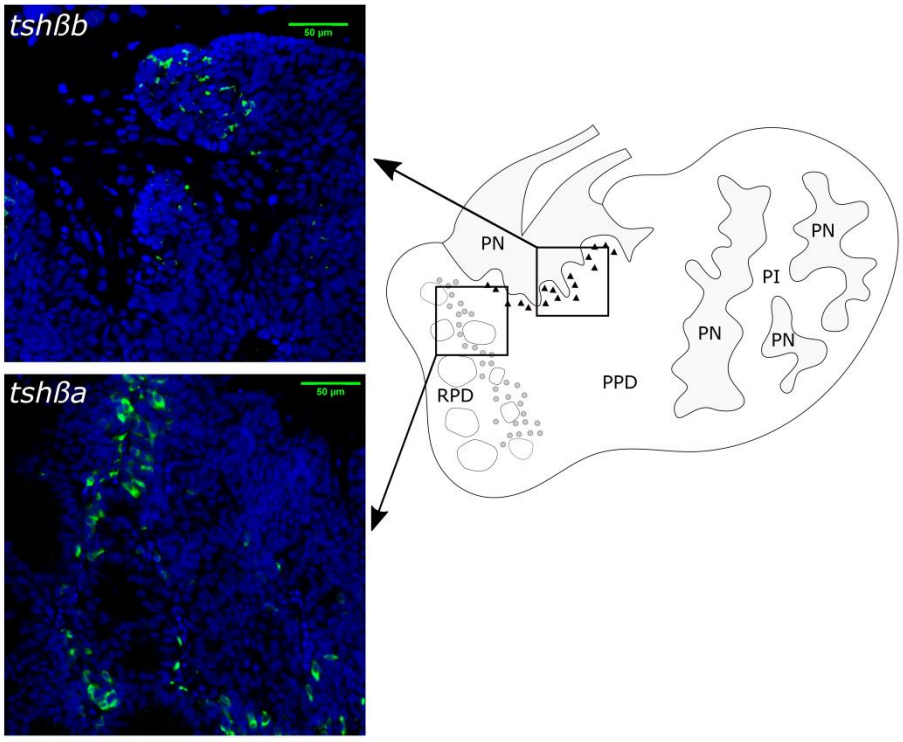

Figure 5. Localization by FISH of *tshβa* and *tshβb* transcripts in the Atlantic salmon pituitary. Fluorescent *in situ* hybridization (FISH) of *tshβa* and *tshβb* was performed on 70 µm parasagittal sections of pituitaries of smolts sampled in April, 2017. FISH photos: *tshβa* and *tshβb* cells are labelled in green (FITC); cell nuclei are labelled in blue (DAPI); upper: *tshβb* labelling: Confocal Z-projection from 3µm Z-stack; lower: *tshβa* labelling: Confocal Z-plan image. Diagram: representation of the localization of *tshβa*- and *tshβb*-expressing cell

populations; *tshβa*-expressing cells are located in the rostral pars distalis (RPD) close to the prolactin follicles (white circles), at the border with the proximal pars distalis (PPD); *tshβb*-expressing cells are less numerous and located in the dorsal PPD close to the pars nervosa (PN) of the pituitary stalk. No FITC labelling was observed in the pars intermedia (PI). Controls were performed using FITC sense probes and showed no labelling (see Supplementary Fig. S2).

Discussion

The present study revealed that two paralogous genes, named *tshβa* and *tshβb* based on phylogeny and synteny analyses, are expressed in the Atlantic salmon. The deduced protein sequences TSHβa corresponded to the subunit previously characterized in Atlantic salmon[17], while TSHβb corresponded to a novel not yet investigated subunit. A third homologous gene sequence was identified as a pseudogene. Atlantic salmon TSHβa and b sequences shared the 12 cysteine residues and the N-glycosylation site conserved among vertebrate TSHβ. Yet, they were largely different with only 47.5% amino-acid similarity reflecting their divergence since the teleost 3R genome duplication event. Differently to TSHβa, Atlantic salmon TSHβb possessed a second glycosylation site, as previously observed for other teleost TSHβb[19], which may confer differential biological properties. Recent work in mice has shown that tissue specific glycosylation of TSH produced in pars distalis *versus* in pars tuberalis, induces differential bioactivity in the blood[24,25].

Phylogeny and synteny analyses brought new advances on the evolutionary scenario of *tshβ* genes, as illustrated in Figure 6. As previously indicated[19] the duplicated paralogs *tshβ* and *tshβ2* originated from vertebrate 2R, but only *tshβ* was conserved in the actinopterygian lineage as observed in an extant holostean, the spotted gar. Teleost 3R duplicated *tshβ* into *tshβa* and *tshβb*, both conserved in various extant teleosts. Salmonid 4R further duplicated *tshβa* into *tshβaα* and *tshβaβ* paralogs, both conserved in *Oncorhynchus* species; however,

tshβaβ is undergoing a loss in Atlantic salmon, where it was detected as a pseudogene. Salmonid 4R would have also duplicated *tshβb* into two paralogs, but a single *tshβb* gene has been conserved in the *Oncorhynchus* and *Salmo* species investigated in this study; this suggests that one of the *tshβ* 4R paralogs would have been lost in the salmonid lineage, shortly after the 4R, before the divergence between *Oncorhynchus* and *Salmo* species (Figure 6).

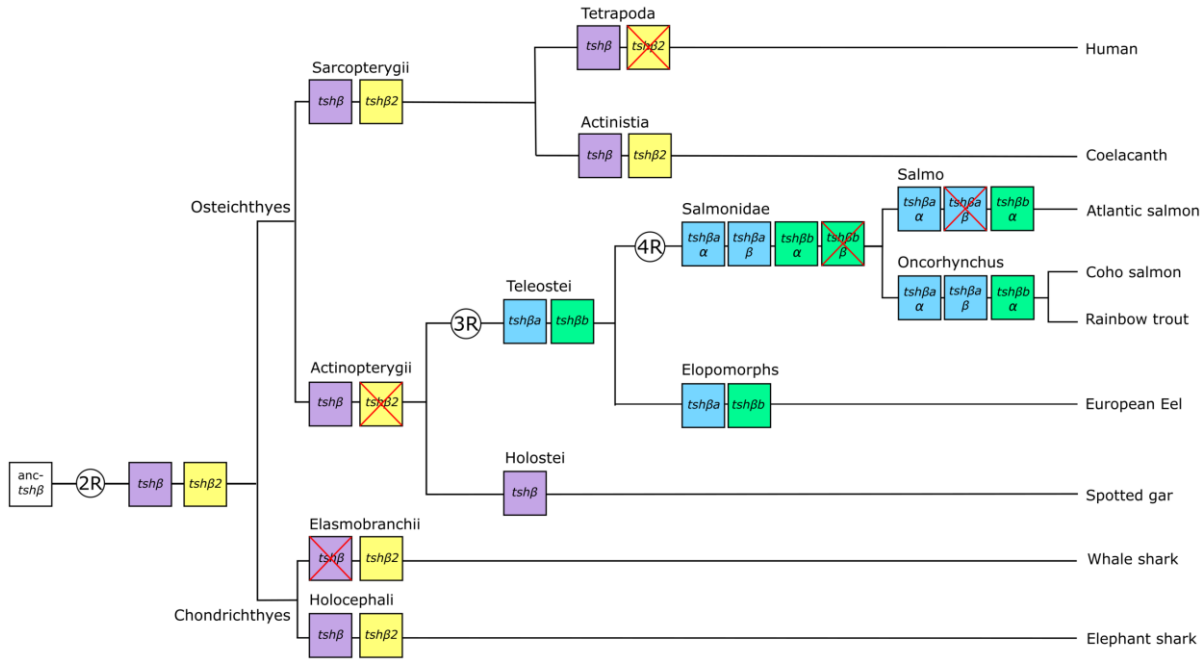

Figure 6. Proposed evolutionary scenario of vertebrate *tshβ* with a special focus on salmonids. The evolutionary scenario is based on phylogeny and synteny analyses ([19] and the present study). Examples of extant representative species are listed on the right. *Tshβ* and *tshβ2* arose from the 2nd round of whole genome duplication in early vertebrates (2R). *Tshβ2* paralog was lost early in the actinopterygian lineage. *Tshβa* and *tshβb* arose from the teleost-specific 3rd round of whole genome duplication (3R) and were further duplicated by the salmonid-specific 4th round of whole genome duplication (4R). One of the 4R parlogs, *tshβbβ*, was lost early in the salmonid lineage, before the divergence between the Onchorynchus and Salmo lineages. Another 4R paralog, *tshβaβ*, still present in the rainbow trout (*Onchorynchus mykiss*) and coho salmon (*Onchorynchus kisutch*), is undergoing a loss in Atlantic salmon (*Salmo salar*) where it is detected as a pseudogene.

Development of specific qPCR for Atlantic salmon *tshβa* and *tshβb* allowed us to show that both genes are mainly expressed in the pituitary, and indicated that *tshβa* transcript levels were much higher than *tshβb* transcript levels. The expression of both *tshβa* and *tshβb* paralogs in the pituitary has been previously reported in two other teleost species, the stickleback, *Gasterosteus aculeatus* (named *tshβ1* and *tshβ2* by the authors[20]) and the European eel, *Anguilla anguilla* (named *tshβ* and *tshβ3* by the authors[19]).

As measured by qPCR in the Atlantic salmon, *tshβa* was exclusively expressed in the pituitary, while *tshβb* was also expressed at lower levels in different brain regions, and in various peripheral tissues. A similar result was previously obtained in the European eel, with a pituitary-only expression of *tshβa* and a more ubiquitous tissue distribution of *tshβb (tshβ* and *tshβ*3 respectively[19]). In amphibians, reptiles and birds, which possess a single *tshβ* gene, most studies have reported the expression of *tshβ* mRNA only in the pituitary (chicken[26]; bullfrog[27]; quail[28]; turtle[29]; duck[30,31]) while investigating a wide range of other tissues (brain, gonads, liver, thyroid, muscle, lung, heart, intestine, kidney and spleen). However in mammals, which also possess a single *tshβ* mainly expressed in the pituitary, *tshβ* transcript or TSH protein were reported at low levels in the brain[32] and in some peripheral tissues, such as myometrium and skin[33].

We further investigated by FISH the respective localization of *tshβa* and *tshβb* in the Atlantic salmon pituitary and revealed that the paralogs were expressed by distinct pituitary cell populations. Numerous *tshβa*-expressing cells were located at the border between the RPD and antero-ventral PPD, while fewer *tshβb*-expressing cells were observed in the dorsal PPD close to the pituitary stalk. Early histological works in salmonids using radiothyroidectomy already reported the localization of TSH cells mainly in the RPD at the junction with PPD (Atlantic salmon[34]; chinook salmon, *Oncorhynchus tshawytscha*[35]). Immunocytochemical

studies, using an antibody against human TSHβ, revealed TSH cells in the ventral PPD adjacent to the RPD (chum salmon, *Oncorhynchus keta* and rainbow trout[36,37]). A similar localization was observed using an antibody raised against purified coho salmon TSH (rainbow trout[38]; chinook salmon[39]). In light of our present study, these previous investigations likely observed the localization of the abundant TSHβa.

Two distinct populations of TSH cells have also been described in the pituitary of birds and mammals, but both expressing the same single gene present in tetrapods (*tshβ*). The "classical" TSH cell population is located in the pars distalis (PD), while a less numerous TSH cell population is located in the pars tuberalis (PT) which surrounds the pituitary stalk (quail[40]; Soy sheep[41]; mice[42]; European hamster[43]). We may relate the localization of salmon *tshβa*- and *tshβb*-expressing cells to that of amniote PD- and PT-*tshβ* cells, respectively, and infer that the specific expression of *tshβa* and *tshβb* in these distinct pituitary cell populations would represent a typical case of subfunctionalization of duplicated paralogs.

The potential involvement of *tshβa* and *tshβb* paralogs in smoltification was investigated by measuring their pituitary expression profiles in juvenile Atlantic salmon from Loire-Allier basin. Three independent sampling experiments were performed in 2013, 2014 and 2016 at CNSS. Remarkably, a striking peak in the expression of *tshβb*, with no change in *tshβa,* was recorded in April, at the period of smoltification, in each yearly experiment. This is the first demonstration of a surge in pituitary *tshβ* in relation to smoltification metamorphosis in salmonids. In contrast, previous studies reported slightly lower[9,17] or no change[18] in pituitary *tshβ* transcript levels during smoltification; these investigations were in fact targeting the *tshβa* paralog, the expression of which remains relatively stable as shown in the present study. The demonstration of a differential regulation of the pituitary expression of salmon

tshβa and *tshβb* paralogs, with a specific peak of *tshβb*, revealed a marked functional divergence of the two paralogs, conferring a specific role in smoltification to *tshβb* paralog.

In birds and mammals, TSH produced by PT cells plays a key role in the seasonal regulation of major steps of life cycles, including reproduction, migration, hibernation[41,44,45]. Photoperiodic regulation of PT-TSH production is mediated *via* melatonin in mammals, or *via* deep brain photoreceptors in birds, but PT-TSH exerts a similar action on neighbouring hypothalamus in both birds and mammals; PT-TSH stimulates the expression of type 2 deiodinase (DIO2), which catalyses the conversion of T4 into the more biologically active T3, thus leading to the activation of TH-regulated brain functions (Figure 7). In a recent study[46], a salmonid specific 4R-issued DIO2 paralog (*dio2b*) has been identified in the Atlantic salmon, the expression of which increases in circumventricular brain area of cell proliferation, during experimental photoperiod-induced smoltification. The authors proposed a specific role of DIO2b in promotion of TH-dependent brain development during smoltification[46].

In the line of PT-TSH role and action mechanism in amniotes, we propose that TSHb produced by dorsal PPD cells in Atlantic salmon may stimulate brain DIO2b expression and promote TH-activated brain functions related to smoltification (Figure 7). As a support to this hypothesis, in our three yearly experiments, the expression peak of *tshβb* paralog occurred simultaneously with smoltification-related changes in rheotaxis, which triggers the onset of downstream migration.

In the Atlantic salmon, *tshβb* was expressed not only in the pituitary but also at lower levels in various brain regions, so that additional regulatory pathways may also occur locally in some brain regions. Such a local signalling between light sensors, TSH and DIO2, has been proposed in the *saccus vasculosus* for the photoperiodic regulation of reproduction in masu

salmon[47,48]. In our study, *tshβb* transcripts were detectable in the *saccus vasculosus* of the Atlantic salmon smolt but were at least 100 times less expressed than in the pituitary; *tshβ1a* transcripts were not detectable outside the pituitary.

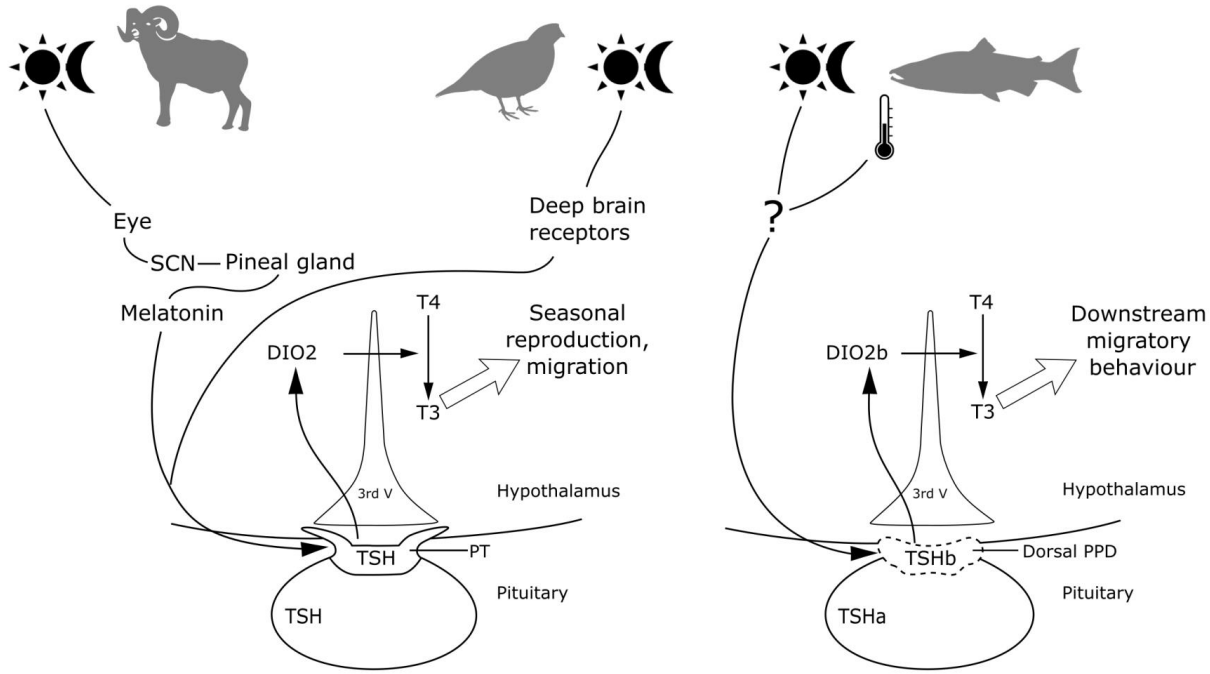

Figure 7. Proposed subfunctionalization of TSHa and TSHb, with potential role of TSHb in Atlantic salmon smoltification. In birds and mammals, the same gene (*tshβ*) is expressed by the "classical" TSH cells of the adenohypophysis and by the fewer TSH cells from the pars tuberalis (PT). PT-TSH is involved in the photoperiodic regulation of seasonal life traits[40,41,44,45] by increasing brain deiodinase 2 (DIO2) and stimulating the conversion of thyroxin (T4) into triiodothyronine (T3). Salmon *tshβa* and *tshβb* paralogs are expressed by distinct cell populations as shown by FISH. We propose that *tshβb* cells located in the dorsal region of the pars proximal distalis (PPD) may be related to the PT-TSH cells in birds and mammals, and infer a subfunctionalization of salmon *tshβa* and *tshβb* paralogs. We revealed a peak expression of *tshβ* at smoltification, concomitant to the inversion of rheotaxis initiating downstream migration. An increase in brain DIO2b paralog was reported at smoltification in Atlantic salmon[46], and we hypothesize that the peak expression of *tshβb* may be responsible for this activation. Salmon TSHb may play a key-role in the environmental and internal regulation of smoltification and downstream migration, via similar signalling pathway as bird and mammal PT-TSH.

In mammals, PT-TSH has been suggested to act not only on the basal hypothalamus but also to exert some paracrine effects in the pituitary[49], so that we may also hypothesize pituitary actions of *tshβb* paralog during salmon smoltification. Using a basal teleost, the European eel, as a model, we previously identified 3R duplicated TSH receptor (TSHR) paralogs and revealed the specific expression of *tshrb* paralog in various brain regions as well as in the pituitary, supporting brain and pituitary actions of TSH in teleosts[19]. Further studies should aim at investigating TSH receptor paralog number and tissue distribution in the Atlantic salmon.

In the present study, we propose a specific role of Atlantic salmon *tshβb* paralog at smoltification, related to the change in rheotaxis and triggering of downstream migration. The remarkable expression peak of pituitary *tshβb* demonstrated in our study may be of high relevance for deciphering the internal and environmental regulation of salmonid smoltification and initiation of downstream migration; especially concerning the endangered long-river Loire-Allier salmon population, understanding these mechanisms may provide new basis for its conservation. A specific role in migration of *tshβb* paralog (named *tshβ2* by the authors[20]*)* was also suggested in the stickleback[20], based on higher pituitary transcript levels of *tshβb* in populations migrating to the sea, as compared to stream-resident populations, with no difference in *tshβa* paralog (named *tshβ1* by the authors[20]*)* between the two ecotypes. The authors suggested that genetic differences in cis-regulatory regions of *tshβb* gene may convey this adaptive divergence in migratory behaviour during stickleback radiation[20,50]. Similarly, there is large diversity of migration strategies among salmonid species and populations, from long-river anadromous to landlocked. Our present findings open new research avenues for comparing *tshβb* expression during smoltification, as well *tshβb* regulatory genomic sequences, between salmonid species and ecotypes.

In conclusion, two *tshβ* paralogs are expressed in the Atlantic salmon by distinct pituitary cell populations, and exhibit a striking functional divergence, with a large expression peak of *tshβb*, but not of *tshβa*, during smoltification. This is the first demonstration of an involvement of TSH in salmonid metamorphic event of smoltification. A specific role of *tshβb* paralog is suggested in the onset of smoltification-related downstream migratory behaviour, possibly mediated by the stimulation of brain DIO2 and T3 production, as shown for PT-TSH involved in the seasonal regulation of life cycle traits in birds and mammals. The remarkable functional divergence of *tshβa* and *tshβb* in salmon may have represented selective forces for the conservation of these duplicated paralogs.

Methods

Fish. The study was carried on juvenile Atlantic salmon (*Salmo salar*) of the Loire-Allier population raised indoor under natural water, temperature, and photoperiod conditions, at the Conservatoire National du Saumon Sauvage (CNSS), Chanteuges, France (Agreement N° B43 056 005; according to the ARRETE N° DDCSPP/CS/2016/40), which breeds wild returning adult Atlantic salmon genitors caught at the Vichy dam, 620km from the Loire estuary. The research project was performed in accordance with guidelines and regulations according to the protocol approved by Cuvier Ethic Committee France.

For each experimental year, 390 under-yearling fish were transferred in December into two circular tanks (3 m diameter; depth range 0.5 m) supplied with UV filtered natural running water from the Desges River (tributary of the Allier). Water temperature was measured using probes (Johnson control, Colombes, France; TS 9101: accuracy ± 0.2°C). An anti-clockwise flow was achieved by a tangentially oriented water inlet at the periphery of the

tank and a central drain as previously described[51]. Photoperiod regime mimicked the natural photoperiod by using an outside light sensor that controlled the light above each tank. Each tank had a LedBulb (D 14-75W E27 827 A67; Philips, Amsterdam, Netherlands) 3 m above the water surface. Fish were fed automatically with a custom fish diet (Turbot label Rouge, Le Gouessant, Lamballe France) in excess five times a day at equal intervals during daylight hours. Fish swimming behaviour was visually observed during daytime: positive rheotaxis for fish facing the water current *versus* negative rheotaxis for fish swimming with the current.

Tissue collection. Fish were anesthetized with an overdose of ms222 (0.4 ml/l; Sigma-Aldrich, St Louis, MI, USA) and killed by decapitation. For qPCR analyses of the tissue distribution of *tshβa* and *tshβb* transcripts, 10 fish (5 males and 5 females) were sampled in March 2015; the following organs were individually collected and stored in RNALater (Ambion Inc, Austin, USA) at -20°C until RNA extraction: retina, brain (dissected into olfactory bulbs, telencephalon, epiphysis, optic lobes, hypothalamus, saccus vasculosus, cerebellum, medulla oblongata), pituitary, as well as samples of gill filaments, kidney, liver, spleen, muscle, skin, abdominal fat, testis or ovary. For qPCR analyses of pituitary *tshβa* and *tshβb* expression profiles throughout smoltification, 20 fish (mixed sex) were sampled once a month from January to June in 2013 and 2014, and 8 fish were sampled at more frequent intervals from December 2015 to June 2016; individual pituitaries were collected in RNALater and stored at -20°C until RNA extraction. For *in situ* hybridization of *tshβa*- and *tshβb*-expressing pituitary cells, 8 fish were sampled in April and 8 in June 2017; individual pituitaries were collected, fixed in paraformaldehyde (PFA) overnight at 4°C, dehydrated in increasing series of ethanol (EtOH) concentration and stored in 98% methanol at -20°C before further processing.

Identification of *tshβ* paralogs in the Atlantic salmon. Gene and transcript names are in lower case and italics (e.g. *tshβ*) and protein names are in upper case (e.g. TSHβ).

Atlantic salmon *tshβ* loci were identified in the recent Atlantic salmon genome assembly (ICSASG_v2, GCA_000233375.4)[22] after interrogation of the Atlantic salmon annotated gene database in GenBank. The presence of additional *tshβ* genes were investigated by blasting salmon *tshβa* and *tshβb* against the Atlantic salmon genome. Gene sequences were examined with CLC Main Workbench 8 (Qiagen Bioinformatics, Hilden, Germany) for prediction of exons, introns, coding sequence (CDS) and signal peptide.

Cloning and sequencing of partial cDNA of Atlantic salmon *tshβa and tshβb* paralogs. Cloning primers for Atlantic salmon *tshβa* and *tshβb* were designed on predicted mRNA sequences of corresponding genes (LOC100136355 and LOC106572976) using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/, Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, US)[52] (Supplementary Table S3). PCR was performed using cDNA of smolt pituitaries collected in April using Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) for *tshβa* and Platinum Taq DNA polymerase (Invitrogen) for *tshβb* according to the manufacturer's instructions. Purified PCR fragments were subcloned into PCRII vectors (Thermo-Fisher, Wahtham, MA, USA) before sequencing by GATC Biotech (Brussels, Belgium). Plasmids were used for preparing cRNA probes (section fluorescence *in situ* hybridization).

Phylogeny analysis. Phylogeny analysis of 39 vertebrate TSHβ amino-acid sequences was performed using a part of dataset from Maugars et al., 2014[19], enriched with additional teleost TSHβ sequences, including TSHβ paralogs of salmonids identified in this study. New *tshβ* genes were either retrieved from GenBank or were identified by blasting (TBLASTN algorithm) genome assembly databases when genes were not annotated in GenBank. The amino-acid sequences of TSHβ were deduced and signal peptides were predicted using CLC Main Workbench 8.

The sequence alignment was performed on CLC Main workbench 8 and manually adjusted. Phylogenetic tree was constructed using Maximum Likelihood algorithm with PhyML:3.0[53] combined to the SMS model selection[54] and SPR as tree improvement on ATGC browser (http://www.atgc-montpellier.fr/phyml/). Tree topology was assessed by bootstrapping on 1000 replicates.

Synteny analysis. Synteny analysis was performed on *tshβ* genomic region in actinopterygians, using a holostean, the spotted gar as a reference (LepOcu1 (GCA_000242695.1)). Comparisons were made with *tshβ* parlogons in the pike (Eluc_V3 (GCA_000721915.3)), and in two salmonid representatives, Atlantic salmon (ICSASG_v2 (GCA_000233375.4)) and rainbow trout (Omyk_1.0 (GCA_002163495.1)). Neighbouring genes of *tshβ* loci were identified and compared manually using chromosome annotation. Blast analyses on the genomes were performed to search for un-annotated genes and additional paralogs. Genes fractionated, showing a frameshift mutation or missing exon were considered pseudogenes.

RNA extraction and cDNA synthesis. Total RNA was extracted by homogenizing tissues in TRIzol (Thermo-Fisher) according to the manufacturer's protocol, using TissueLyser II (Qiagen). After a chloroform separation step, RNA was precipitated in ice cold isopropanol with 1 µl of glycoblue (Ambion). Total RNA was treated with DNase I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. RNA concentration was measured using Nanopore 2000c/2000 (Thermo-Fisher).

Reverse transcription was performed using 75 ng random hexamer primers (Invitrogen) and SuperScriptIII First Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocol. For pituitaries, 250 ng of total RNA were used and 750 ng for brain and peripheral tissues.

Quantitative RT-PCR. Specific quantitative real-time PCR (qPCR) primers for *tshβa* and *tshβb* were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/)[52] with forward and reverse primers on two different exons to prevent amplification of genomic DNA (Supplementary Table S3); primers were purchased from Eurofins scientific (Luxembourg). Specificity of the primers was controlled by sequencing PCR product. *β-actin* was used as reference gene using previously published primers[55].

Quantitative PCR assays were performed using LightCycler 1.2 (Roche Diagnostics) and LightCycler FastStart Master plus SYBR Green I kit (Roche Diagnostics). Each reaction contained: 4 µl of diluted cDNA template, 2 µl of SYBR green master mix and 1µl of specific primers (500 nM final concentration). The following thermal cycling steps were used for each qPCR run: initial denaturation 94°C for 10 min followed by 41 cycles of 10 s of denaturation at 95°C, 5 s of annealing temperature (60°C or 62°C; Supplementary Table S3) and 6 s of elongation at 72°C. The program ended by slowly increasing temperatures (0.1°C/s) from 68-

95°C for amplification specificity controlled by melting curve analysis. Relative quantification was performed using standard curves created for each gene with serial dilutions of pooled pituitary cDNA. One dilution of the cDNA pool was added in each run as a calibrator. All samples were analysed in duplicates and each qPCR run contained a negative control using water in substitute for template cDNA. Calculations of sample concentrations were made using the Roche LightCycler 1.2 manufacturer's software.

Fluorescence *in situ* hybridization (FISH). Antisense and sense cRNA probes for FISH were synthesized by *in vitro* transcription from *tshβa* or *tshβa* plasmids using T7 and SP6 RNA polymerase (Promega, Madison, Wisconsin USA) and labelled with digoxigenin-11 UTP (Roche Diagnostics) at 37°C for 2 h. Probes were purified using Nucleospin RNA clean-UP kit (Machere-Nagal, Hoerdt, France) and controlled by gel electrophoresis.

Whole fixed pituitaries were rehydrated (96, 70, 50, 25% EtOH), included in 3% RNase free agarose gel and sliced into 70 µm parasagittal sections using VT1000S Leica vibratome (Leica, Wetzlar, Germany). Sections were permeabilized using proteinase K (Sigma-Aldrich, 1 µg/ml in PBS with 0.1% Tween 20, PBST) for 45 min at 37°C, then proteinase K was inactivated using glycine (Sigma-Aldrich, 2 mg/ml in PBST) for 30 min at room temperature (RT), followed by a post fixation step in 4% PFA for 15 min and washing in PBST.

Prior to FISH, sections were incubated with hybridization buffer (HB: 50% formamide, SSC 5X, 0.1% Tween 20, 15 ng/ml Heparin, Sigma-Aldrich; 80 µg/ml Torula yeast tRNA, Sigma-Aldrich; pH 6.5) at 55°C for 4 h. FISH was performed in fresh HB containing 300 ng/ml *tshβa* probe or 600 ng/ml *tshβb* probe at 55°C. After 18 h, sections were washed with a series of 4 different hybridization washes (HW): HW1 (50% formamide, SSC 5X, 0.1% Tween 20) 2 x 30

min, HW2 (50% HW1, 50% SSC 2X) 2 x 30 min, HW3 (SSC 2X, 1% Tween 20) 2 x 30 min, HW4 (SSC 0.2X, 0.1% Tween 20) 2 x 2 min. Sections were soaked in TNE buffer (10 mM Tris HCL pH 7.6, 500 mM NaCl, 1 mM EDTA) at 37°C for 30 min then treated with RNaseA (Sigma-Aldrich, 20 µg/ml in TNE buffer) for 30 min at 37°C. Sections were washed in TNE buffer, 2 x 10 min at 37°C followed by SSC 0.2X 0.1% Tween 20, 2 x 30 min at 55°C. Sections were washed in PBST for 10 min with agitation at RT and incubated in PBST with 2% H2O2 for 30 min in order to inactivate endogenous peroxidases, followed by washes in PBST, 3 x 10 min with agitation. Blocking was performed using 1% Blocking Reagent (Roche Diagnostics) in Maleic acid buffer (MAB) for 2 h with agitation. Sections were incubated in blocking/MAB buffer with anti-digoxigenin peroxidase-conjugated antibody (1/250, Roche Diagnostics) overnight at 4°C, and then washed in PBST for 2 h with agitation. Tyramide revelation was carried out using green FITC conjugated tyramide (Sigma-Aldrich, 1/200 in PBST with 0.01% H2O2) for 30 min in darkness at RT. After washing in PBST for 5 x 20 min, sections were let overnight in PBST at 4°C in darkness. Cell nuclei were stained using DAPI staining (Sigma-Aldrich, 1/1000 in PBST) for 20 min at RT. Sections were mounted in Vectashield H-1000 Mounting Medium (Vector, Eurobio/Abcys, CA, USA). Confocal images were obtained using a confocal microscope (Zeiss LSM710, Oberkochen, Germany). Channels were acquired sequentially to avoid signal crossover between the different filters. Images were processed using the ZEN software (version 2009, Zeiss). Z-plan and Z-projection images were obtained using Image J software (Fiji software; http://rsbweb.nih.gov/ij/).

Data availability: Data generated or analysed during this study are included in this article (and its Supplementary Information file).

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Author Contributions

M.S.F and J.R. performed fish monitoring. M.S.F., G.M., A.G.L., S.D., P.M. performed tissue sampling. M.S.F, G.M and A.G.L performed qPCR analyses. G.M. did phylogeny and synteny analyses. M.S.F, R.F and F.A.W performed FISH. M.S.F., G.M., K.R. and S.D. wrote the main manuscript. M.S.F. and G.M created the figures. S.D and P.M. supervised the work. All authors reviewed the manuscript.

Supplementary Information for

Functional divergence of thyrotropin beta-subunit paralogs in the Atlantic salmon gives new insight into smoltification

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Supplementary Figures

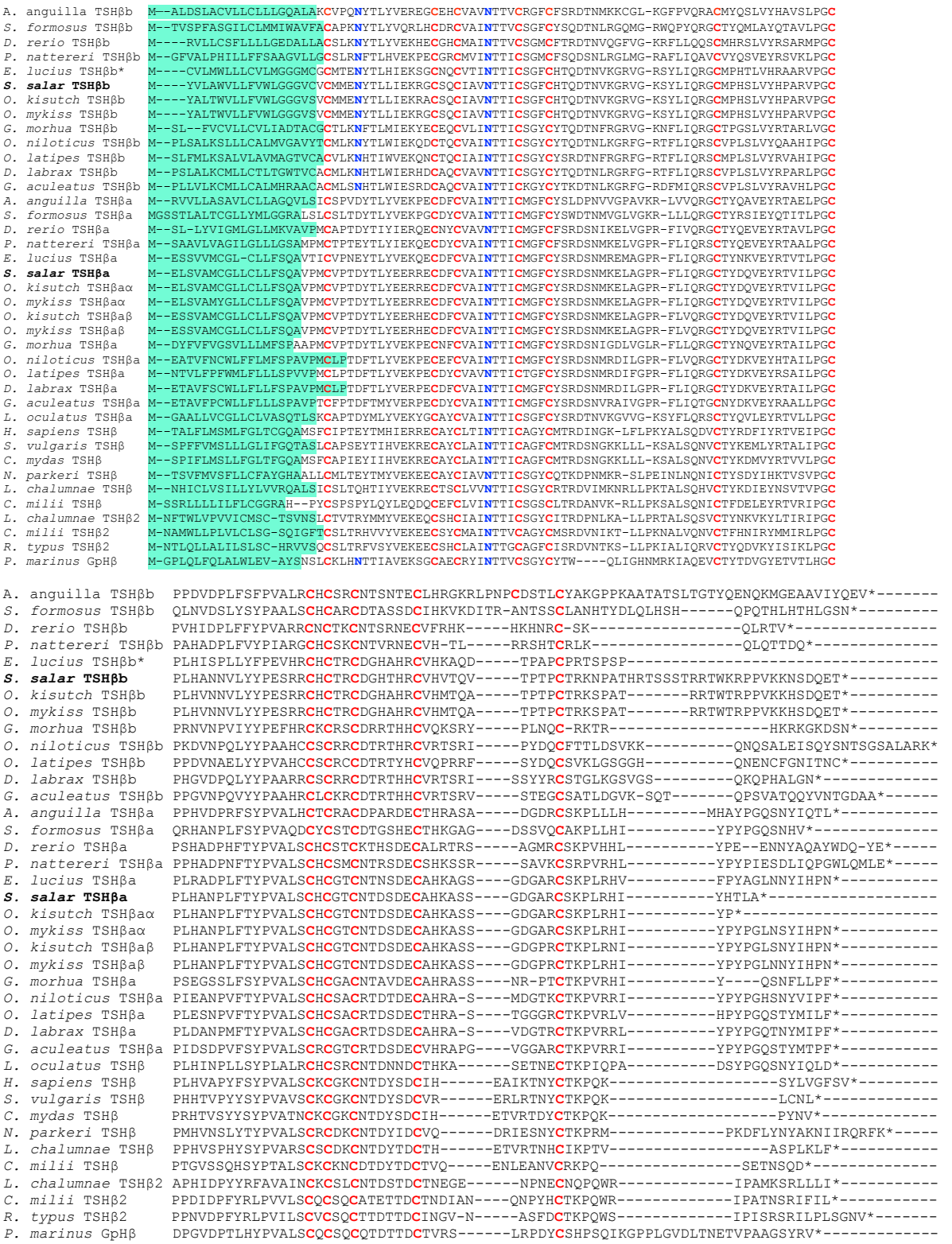

Figure S1. Alignment of TSHβ sequences. The alignment displays amino-acid sequences of gnathostome TSHβ and of lamprey glycoprotein hormone β-subunit, used for phylogeny analysis (Fig. 1). The amino-acid sequences were deduced from genomic sequences, alignment was performed and signal peptides predicted, using CLC Main Workbench 8.

Sequence references are provided in Supplementary Table S1. Signal peptide sequences are highlighted in green, cysteine residues are in red, and conserved potential N-glycosylation sites in blue. *: indicates partial sequence.

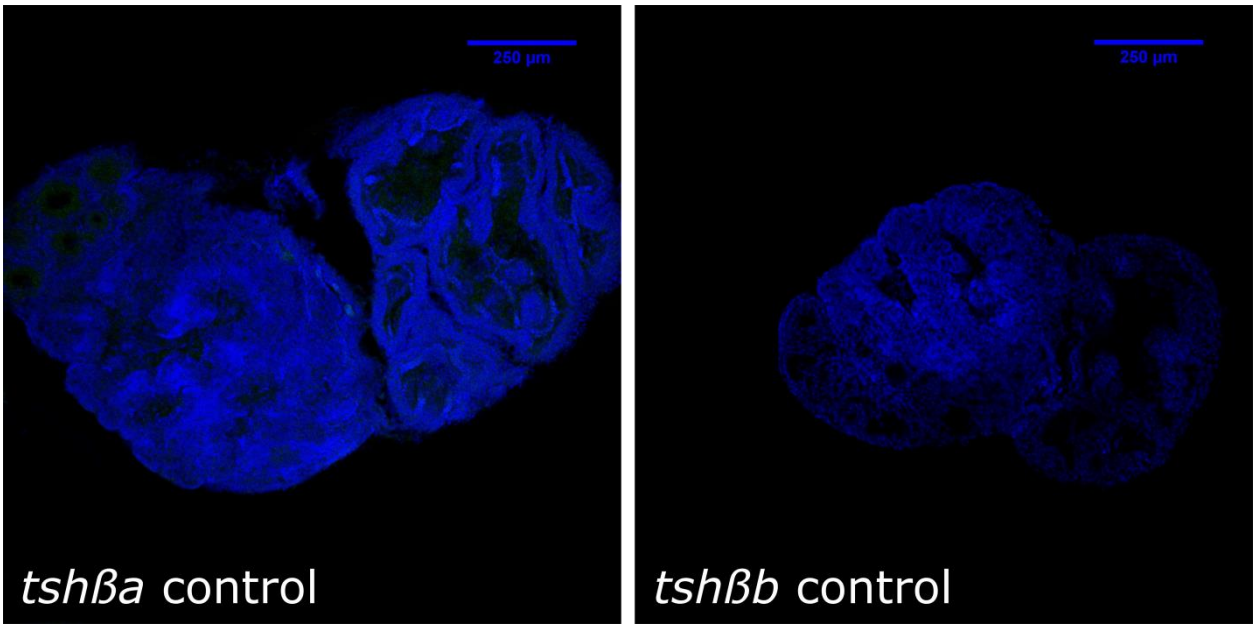

Figure S2. Control fluorescent *in situ* hybridization. Fluorescent *in situ* hybridization (FISH) with sense probes of Atlantic salmon *tshβa* and *tshβb* was performed on 70 µm parasagittal sections of pituitaries of smolts sampled in April 2017. Photos: Confocal z-plan images. No *tshβa* and *tshβb* labelling was observed (green, FITC); cell nuclei are labelled in blue (DAPI).

Supplementary Tables

Scientific name	Common name	Gene	Sequence reference
Anguilla anguilla	*European eel*	*tshβa*	X73493
		tshβb	LM655248
Callorhinchus milii	*elephant shark*	*tshβ*	LOC103183879
		tshβ2	LOC103179889
Chelonia mydas	*green sea turtle*	*tshβ*	LOC102944542
Danio rerio	*zebrafish*	*tshβa*	LOC353223
		tshβb	LOC100001596
Dicentrarchus labrax	*European sea bass*	*tshβa*	HG916837
		tshβb	HG916841
Esox lucius	*northern pike*	*tshβa*	LOC105007569
		tshβb	GATF01036761.1 (tsa-eluc-ngs-13528670)
Gadus morhua	*Atlantic cod*	*tshβa*	CAEA01180393 (WGS)
		tshβb	CAEA01110666 (WGS)
Gasterosteus aculeatus	*three-spined stickleback*	*thsβb*	AANH01002636
		tshβa	AANH01007474
Homo sapiens	*Human*	*tshβ*	LOC7252
Latimeria chalumnae	*coelacanth*	*tshβ*	LOC102363810
		tshβ2	LOC102362870
Lepisosteus oculatus	*spotted gar*	*tshβ*	LOC102690058
Nanorana parkeri	*Tibetan frog*	*tshβ*	LOC108799084
Oncorhynchus kisutch	*coho salmon*	*tshβaα*	LOC109890149
		tshβaβ	LOC109899110
		tshβb	LOC109882835
Oncorhynchus mykiss	*rainbowtrout*	*tshβaα*	LOC100136289
		tshβaβ	LOC110494700
		tshβb	LOC110531299
Oreochromis niloticus	*Nile tilapia*	*tshβa*	LOC100534562
		tshβb	LOC102082579
Oryzias latipes	*Japanese medaka*	*tshβa*	LOC101171658
		tshβb	LOC105354380
Petromyzon marinus	*sea lamprey*	*gphβ*	AY730276.1
Pygocentrus nattereri	*red piranha*	*tshβa*	LOC108427544
		tshβb	LOC108424941
Rhincodon typus	*whale shark*	*tshbβ2*	LOC109924889
Salmo salar	*Atlantic salmon*	*tshβaα*	LOC100136355
		tshβaβ	LOC106566072
		tshβb	LOC106572976
Scleropages formosus	*Asian arowana*	*tshβa*	LOC108918388
		tshβb	LOC108939147
Sturnus vulgaris	*common starling*	*tshβ*	LOC106851066

Table S1. References of sequences used for alignment (Figure S1) and phylogeny (Figure 1). The table provides species names, gene names and gene sequence references.

		Spotted gar (*Lepisosteus oculatus*)	Pike (*Esox lucius*)			Atlantic Salmon (*Salmo salar*)				Rainbow trout (*Oncorhynchus mykiss*)			
Gene	Gene description	LG3	LG17	NW_017858932	LG12	ssa22	ssa12	ssa15	ssa13	17	7	9	16
ap4b1	adaptor related protein complex 4 beta 1 subunit	102684786			105030751			106572995				110532865	
rplp2	ribosomal protein lateral stalk subunit P2	102692126		105008159	105009991	100194651	106566032	106572979		110494710	110527753	110532870	
kcna10	potassium voltage-gated channel subfamily A member 10	102689659		105008161		106582604	106566035	106572980	106566322	110494706	110527755	110531301	110492706
ngf	nerve growth factor	102692738		105007566	105028952	106582728	106566077		106566305		110527108		
tspan2	tetraspanin 2	102689859		105007567	105028940	106582727	106566039	106572998		110494702	110527760	110531298	
slc25a22	mitochondrial glutamate carrier 1-like	102692936		105007568	105028941	106582726	106566040			110494701	110527759		
tshβ1	thyroid stimulating hormone beta	102690058		105007569	join(484522.486383)	100136355	106566072	106572976		110494700	100136289	110531299	
slc16a1	solute carrier family 16 member 1	102684984	105023676		105028950	106582732	106566043		106566331	110494698	110527763		110492722

Table S2. References of genes used for synteny. The table provides gene name description and GenBank identification number for *tshβ* and its neighbouring genes used for the synteny analysis (Figure 2). Chromosome or scaffold numbers are highlighted in grey. Each gene is single in the spotted gar (used as a reference) while they are represented by up to 2 paralogs in the pike and up to 4 paralogs in the Atlantic salmon and rainbow trout.

Gene Name	Primer name	Purpose	Primer Sequence	Annealing Temperature	Primer Reference
tshβa	tshβa-CL-F	PCR	GTCTCCTTTGCCTGCTCTTC	55°C	This paper
	tshβa-CL-R	PCR	GTCTCCTTTGCCTGCTCTTC	55°C	This paper
tshβb	tshβb-CL-F	PCR	CTGGTCGCTGTGTGGATAGG	55°C	This paper
	tshβb-CL-R	PCR	CGCTGTGTGGATAGGCAGTT	55°C	This paper
tshβa	tshβa-F	qPCR	CTCCTTTGCCTGCTCTTCAG	60°C	This paper
	tshβa-R	qPCR	GGCCAGCTCCTTCATGTTAC	60°C	This paper
tshβb	tshβb-F	qPCR	TTGCCGTCAACACCACCAT	62°C	This paper
	tshβb-R	qPCR	GGGATGATAGACCAGGGAGTG	62°C	This paper
β-actin	actin-F	qPCR	CCAAAGCCAACAGGGAGAAG	60°C	56
	actin-R	qPCR	AGGGACAACACTGCCTGGAT	60°C	56

Table S3. Primer sequences. The table provides the sequences of Forward and Reverse primers used for PCR of Atlantic salmon *tshβa* and *tshβb* (cloning and FISH) and for qPCR of Atlantic salmon *tshβa*, *tshβb* and *β-actin*.

Chapter 2

Manuscript in Preparation

Environmental modulation of thyrotropin beta subunit paralogs and downstream migratory behaviour during Long-River Atlantic salmon smoltification metamorphosis.

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Abstract

In preperation

Introduction

The Atlantic salmon (*Salmo salar*), like other salmonids, has a complex life cycle with the reproduction occurring in the upper part of the rivers, while the majority of feeding and growth phase taking place in the ocean. A crucial life history transition, called smoltification (or parr-smolt transformation) initiates downstream migration and pre-adapts the juvenile salmon to seawater entry (Boeuf, 1993). The parr-smolt transformation of salmonid species has captivated scientists for decades. Fuelled by the drastic decline of wild populations and economic importance of farmed salmon, the research into the metamorphic of smoltification has been robust (reviews: (Wedemeyer, Saunders and Clarke, 1980; Hoar, 1988; Boeuf, 1993; Björnsson, Stefansson and McCormick, 2011; Rousseau *et al.*, 2011; McCormick, 2012). Despite this, the discussion of to what extent smoltification is regulated by exogenous or endogenous factors remains unclear.

Smoltification can be considered a metamorphosis due to multiple morphological, physiological, and behavioural changes that occur. These changes are considered to be managed by the endocrine system and synchronized with environmental factors (for reviews: (Rousseau *et al.*, 2011; Björnsson, Einarsdottir and Power, 2012). In amphibians, extensive research has demonstrated that larval metamorphosis is triggered mainly by thyroid hormones (TH, thyroxine T4 and triiodothyronine T3). In Atlantic salmon, a histological study by Hoar first reported an activation of thyroid follicles during smoltification (Hoar, 1939). Since then, surges of thyroxine (T4) during the period of smolitfication has been shown in a variety of salmonid species (coho salmon, *Oncorhynchus kisutch*: Dickhoff, *et al*., 1978; *Larsen et al*., 2011; masu salmon, *Oncorhynchus masou*: Nishikawa, Kazuyoshi, 1979; Atlantic salmon: Boeuf and Prunet, 1985; Prunet *et al.*, 1989); even though changes in T4 plasma levels were relatively limited as compared to other hormones such as cortisol, insulin-like growth factor-1, and growth hormone (for reviews: (Björnsson, Einarsdottir and Power, 2012; McCormick, 2012). Experimental hormonal treatments suggested that TH may

be responsible for smoltification-related changes such increased skin silvering, inversion of rheotaxis and swimming behaviour yet concrete evidence is still lacking (Iwata, 1995).

In amphibians, TH production is stimulated by a pituitary hormone, thyrotropin (or thyroid-stimulating hormone, TSH). While many studies have addressed TH in salmonids, little is known about salmonid thyrotropin. TSH is comprised of two subunits, a common alpha subunit shared with the gonadotropins, luteinizing hormone and follicle-stimulating hormone, and a beta subunit (TSHβ) conferring hormone specificity. Our recent work discovered the presence and functional divergence of the duplicated thyrotropin beta subunit paralog in Atlantic salmon during the period of smoltification (tshßa/tshßb; Fleming et al., in review). The beta subunit paralog, *tshßb*, displayed a dramatic peak of pituitary expression during the period of smoltification concomitant to the inversion of rheotaxis (Fleming et al., in review). This was the first time pituitary expression of *tsh* was related with smoltification. Localization of tshßb expression cells in the pituitary revealed a distinct location on the dorsal region of the pituitary near the point of attachment to the hypothalamus. This region could be related to the pars tuberalis in mammals and birds which produces a particular TSH that is responsible for seasonal life history traits such as reproduction and migration which can be stimulated *via* long day photoperiod exposure (Nakao *et al.*, 2008; for review: Nakane and Yoshimura, 2014).

Prior to and during smoltification, environmental conditions act through the endocrine system to relay vital information for the timing of physiological and behavioural changes (Hoar, 1976, 1988; Björnsson, Stefansson and McCormick, 2011). The synchronization of physiological changes with environmental conditions is crucial for successfully smolitfication and is often discussed through the idea of physiological and environmental “windows of opportunity” or “smolt windows” (Berglund, 1991; McCormick *et al.*, 1998). These temporal timing windows are used to describe the period in which both the environment and the physiology of the fish are in the correct state to make a successful transition to the sea (Boeuf and Prunet, 1985; McCormick *et al.*, 1998). Therefore departure from the rearing grounds, especially for long river populations may be a crucial factor for the success of downstream migration and transition to the sea (for review: (McCormick, 2012).

Photoperiod and temperature are two of the main environmental factors proposed to regulate the smoltification metamorphosis (for reviews: Boeuf, 1993; Rousseau *et al.*, 2011; McCormick, 2012). For Atlantic salmon, the vast knowledge of the effect of photoperiod and temperature on the physiological development during smoltification comes from salmon aquaculture research. Using artificial photoperiod and temperature schemes, early smoltification can be trigged out of season in juvenile salmon as measured by increase of plasma GH (Björnsson *et al.*, 2000), plasma cortisol (Sundell *et al.*, 2003) and increased NKA activity (Berge *et al.*, 1995) similar to that of naturally occurring smolts. Interestingly, some endocrine factors could not be manipulated by photoperiod and temperature such as plasma IGF-1 and plasma T3 (McCormick *et al.*, 2002).

With regards to the initiation of migration, mixed and non-conclusive evidence has accumulated for the exogenous regulation of downstream migration. Photoperiod, temperature, lunar cycle and social interactions have all been reported to contribute to the timing of downstream migration; it has been suggested that migration timing is an adaptation to the area's specific prevailing environmental conditions and regulated by endogenous factors (for reviews: Boeuf, 1993; Rousseau *et al.*, 2011; McCormick, 2012). For the Loire-Allier, inversion of rheotaxis and increased swimming behaviour has been reported to commence when water temperatures ranged from 7.5-13°C (Martin *et al.*, 2012a) yet more studies are needed to understand the endocrine regulation of this behaviour.

This study will further explore the tshßb paralog during smoltification by investigating the modulation of pituitary expression by increased water temperature and constant short day photoperiod. In addition, we will investigate the effect these environmental conditions on body weight, length and condition factor and the timing of rheotaxis change indicating downstream migration. Investigating these factors will enhance our knowledge on the smoltification metamorphosis and bring new insights to the timing of downstream migration.

Materials and Methods

Fish

The study was carried on juvenile Atlantic salmon (*Salmo salar*) of the Loire-Allier population raised indoor in six tanks under three experimental conditions at the Conservatoire National du Saumon Sauvage (CNSS), Chanteuges, France (Agreement N° B43 056 005; according to the ARRETE N° DDCSPP/CS/2016/40). CNSS breeds wild returning adult Atlantic salmon genitors caught at the Vichy dam, 620km from the Loire estuary in part of an on-going conservation project to augment the endangered Loire-Allier population. The research project was performed in accordance with guidelines and regulations according to the protocol approved by Cuvier Ethic Committee France.

The three experimental conditions were as follows: Natural photoperiod with natural water temperature (NN), natural photoperiod with increased water temperature (N+5) and constant short day photoperiod with natural water temperature (SN). Each condition was replicated in two tanks; one tank was dedicated to sampling and the other tank was dedicated to video monitoring. No fish were sampled from the video tanks in order to avoid perturbation of behaviour from repeated sampling. Tanks (3 m diameter; depth range 0.5 m) were supplied with UV filtered natural running water from the Desges River (tributary of the Allier). Increased water temperature was achieved through a heat exchange system with that of the local well water and sought to have a difference of +5°C to that of natural water temperature. Water temperature was measured hourly using probes (Johnson control, Colombes, France; TS 9101: accuracy ± 0.2°C). An anti-clockwise flow was achieved by a tangentially oriented water inlet at the periphery of the tank and a central drain as previously described (Martin et al., 2012).

Photoperiod regime mimicked the natural photoperiod by using an outside light sensor that controlled the light above each tank. For the winter photoperiod conditions, the light regime was kept constant at 8L:16D throughout the experiment representing the LD regime of winter solstice. Each tank had a LedBulb (D 14-75W E27 827 A67; Philips, Amsterdam, Netherlands) 3 m above the water surface. Fish were fed automatically with a custom fish

diet (Turbot label Rouge, Le Gouessant, Lamballe France) in excess five times a day at equal intervals during daylight hours.

Behaviour

Fish swimming behaviour was observed using a video recording system mounted above each tank. Video was captured continuously throughout the experiment and was used to analyse the time in which fish changed from positive (facing the streaming) to negative rheotaxis (facing downstream). Fish rheotaxis was measured 3 times a day at 9:00, 13:00 and 17:00 during the months of March and April, the period of time in which rheotaxis inversion occurs for the Loire-Allier population (Martin et al., 2012). During each measurement, a line was drawn in each tank and the number of fish and their orientation when crossing the line was recorded over a 10 second period. The total numbers of fish movements were summed number of fish exhibiting negative rheotaxis for each day and gave a representation of total daily movements within the tanks.

Biometric parameters

Fish were anaesthetized with an overdose of ms222 (0.4 ml/l; Sigma-Aldrich, St Louis, MI, USA) and biometric measurements were taken. Total body length, fork length and total body weight were recorded and condition factor (K) was calculated [$k=(W/L^3)*100$] using Fork length for L.

Tissue collection

After body measurements were taken the fish were killed by decapitation. For qPCR analyses of pituitary *tshβa* and *tshβb* expression profiles throughout smoltification, 8 fish (mixed sex) of each condition were sampled at frequent intervals from December 2015 to June 2016; individual pituitaries were collected in RNALater and stored at -20°C until RNA extraction.

RNA extraction and cDNA synthesis.

Total RNA was extracted by homogenizing tissues in TRIzol (Thermo-Fisher) according to the manufacturer's protocol, using TissueLyser II (Qiagen). After a chloroform separation step, RNA was precipitated in ice cold isopropanol with 1 µl of glycoblue (Ambion). Total RNA was treated with DNase I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's

instructions. RNA concentration was measured using Nanopore 2000c/2000 (Thermo-Fisher).

Reverse transcription was performed using 75 ng random hexamer primers (Invitrogen) and SuperScriptIII First Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocol. For pituitaries, 250 ng of total RNA were used.

Quantitative RT-PCR.

Specific quantitative real-time PCR (qPCR) primers for tshßa and tshßb were designed and confirmed by Fleming *et al*. (in review)

Quantitative PCR assays were performed using LightCycler 480 II (Roche Diagnostics) and LightCycler FastStart Master plus SYBR Green I kit (Roche Diagnostics). For each sample date, pools of cDNA from 8 pituitaries were used. Each reaction contained: 4 µl of diluted cDNA template, 2 µl of SYBR green master mix and 1µl of specific primers (500 nM final concentrations). The following thermal cycling steps were used for each qPCR run: initial denaturation 94°C for 10 min followed by 41 cycles of 10 s of denaturation at 95°C, 5 s of annealing temperature (60°C or 62°C) and 6 s of elongation at 72°C (Table S1). The program ended by slowly increasing temperatures (0.1°C/s) from 68-95°C for amplification specificity controlled by melting curve analysis. Relative quantification was performed using standard curves created for each gene with serial dilutions of pooled pituitary cDNA. One dilution of the cDNA pool was added in each run as a calibrator. All samples were analysed in duplicates and each qPCR run contained a negative control using water in substitute for template cDNA. Calculations of sample concentrations were made using the Roche LightCycler 1.2 manufacturer's software.

Statistics

In preparation

Results

Water temperature

Due to the nature of the heat exchange system, the difference in water temperature between natural and warm water conditions varied throughout the experiment. From December 21 to May 1, the temperature difference between the tanks varied from 1.61°-4.20°C with an average difference of 2.68°C. Daily average waters began to significantly increase during the second week of March and increased steadily until the end of the Experiment. Temperatures between the two conditions had less difference as the natural temperature increased. By the first week April only a slight difference between the tanks was achieved and by mid-April the water temperature in each condition was nearly identical (Figure 1).

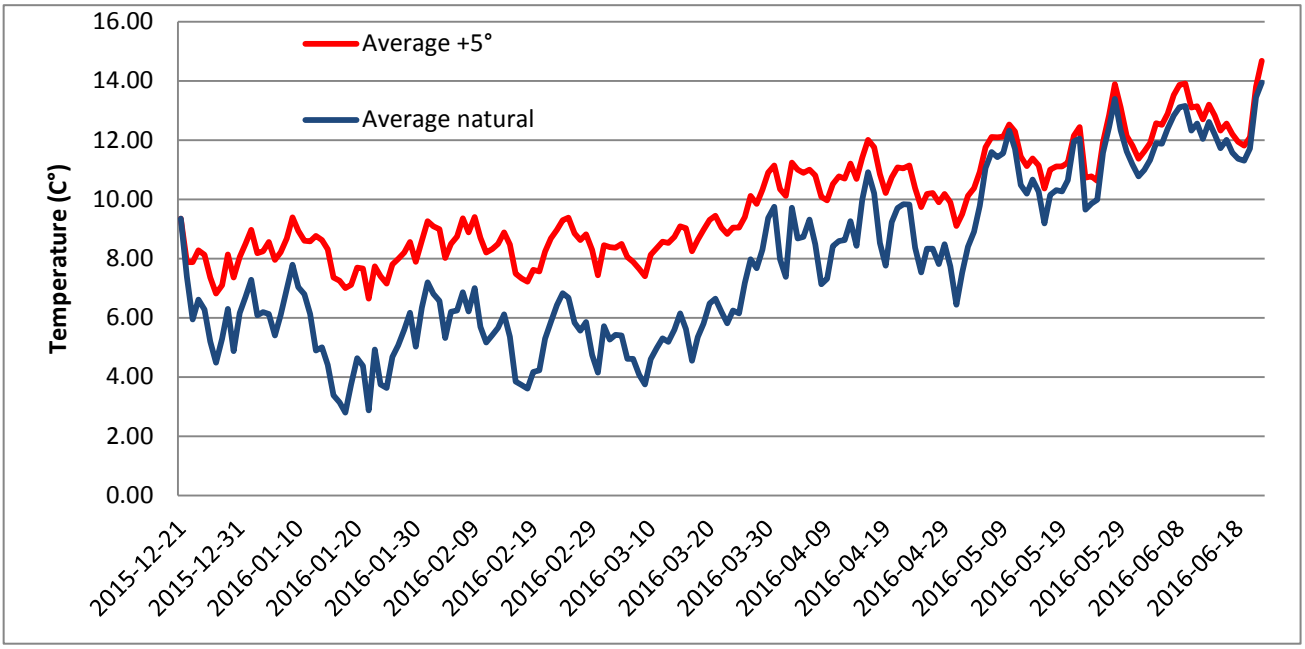

Figure 1: Water temperature in Natural temperature (blue) and increased water temperature (red) tanks through the experimental period

Growth rate,

Growth, in both length and weight was significantly greater in fish raised in warm water and began to diverge near the end of January. Warm water fish remained significantly larger and heavier throughout the experiment and on average were roughly 20-30% (both length and weight) larger than controls from February to May. At the end of the experiment, increased water temperature fish were on average 10% larger than controls (Figure 2).

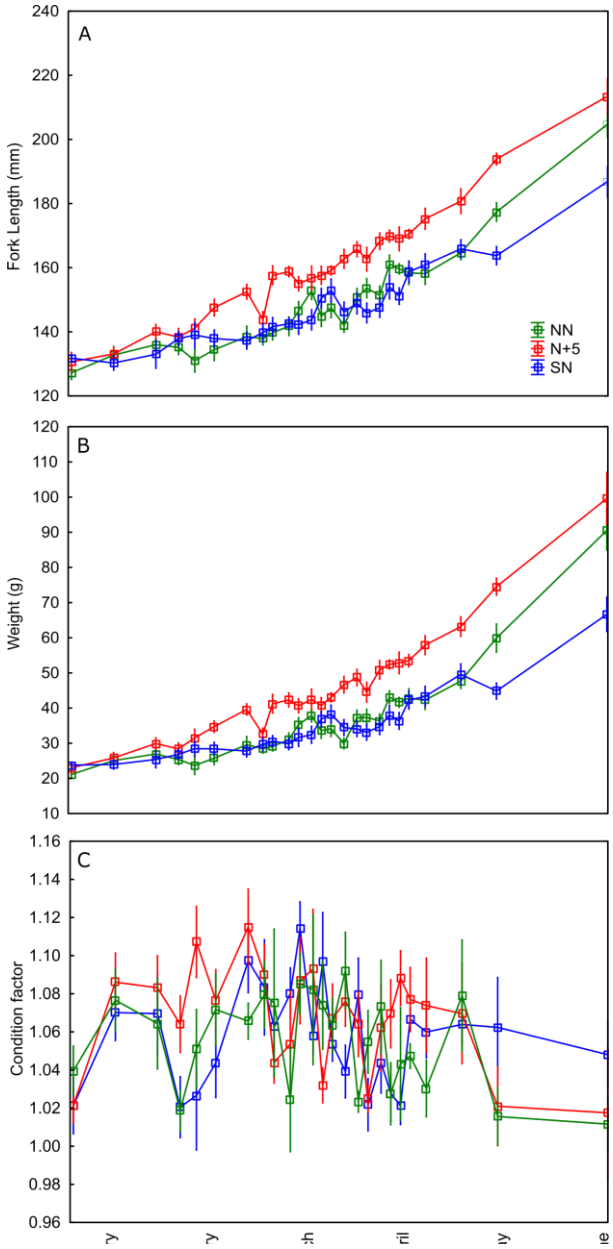

Figure 2: Loire-Allier Atlantic salmon fork length, weight and condition factor (K). Salmon were raised at the Conservatoire national du salmon sauvage (CNSS: 45.07°90' North, 3.53°20' East) in natural photoperiod and temperature (NN: Green), natural photoperiod with increased water temperature (N+5: red) and constant short day photoperiod with natural water temperature (SN: blue)

Fish raised in natural and winter photoperiod conditions shared similar body length and weight from first sampling until May. A significant divergence between the two conditions happened after May where the constant short day photoperiod fish were significantly shorter and lighter than the natural condition fish. In May and June, constant short day photoperiod fish were approximately 25-30% smaller (both length and weight) than the natural condition fish (Figure 2).

Condition factor for all three conditions increased significantly from the December to January. Condition factor in all conditions remained generally constant with no significant differences between conditions from February to the end of April. In May, both natural and increased water condition fish had a significant drop in condition factor whereas constant short day photoperiod remained high at similar levels to that of previous months (Figure 2).

Swimming behaviour

Pattern of activity

During the winter and up to the first change in rheotaxis, all fish faced the current (positive rheotaxis) and did not exhibit any net movement. Some downstream drifting occurred during periods of high flow but during these events all fish remained facing the current (data not shown). In early spring, around the end of March, fish began to exhibit a change in rheotaxis behaviour during daylight hours facing downstream (negative rheotaxis) for periods of time before returning again to positive rheotaxis. By the first week of April, in all conditions, the majority of fish exhibited negative rheotaxis behaviour during daylight hours with the majority of movements being observed during 17:00 recording. Daily accumulated negative rheotaxis movements were recorded for each condition and are displayed in Figure 3. These results reveals a distinct change to negative rheotaxis during the first week of April disregarding the prevailing photoperiod and temperature conditions. Periods of high flow brought murky water into the tanks and block vision in the tanks. During these times, behaviour analyses were not possible (light grey regions; Fig 3)

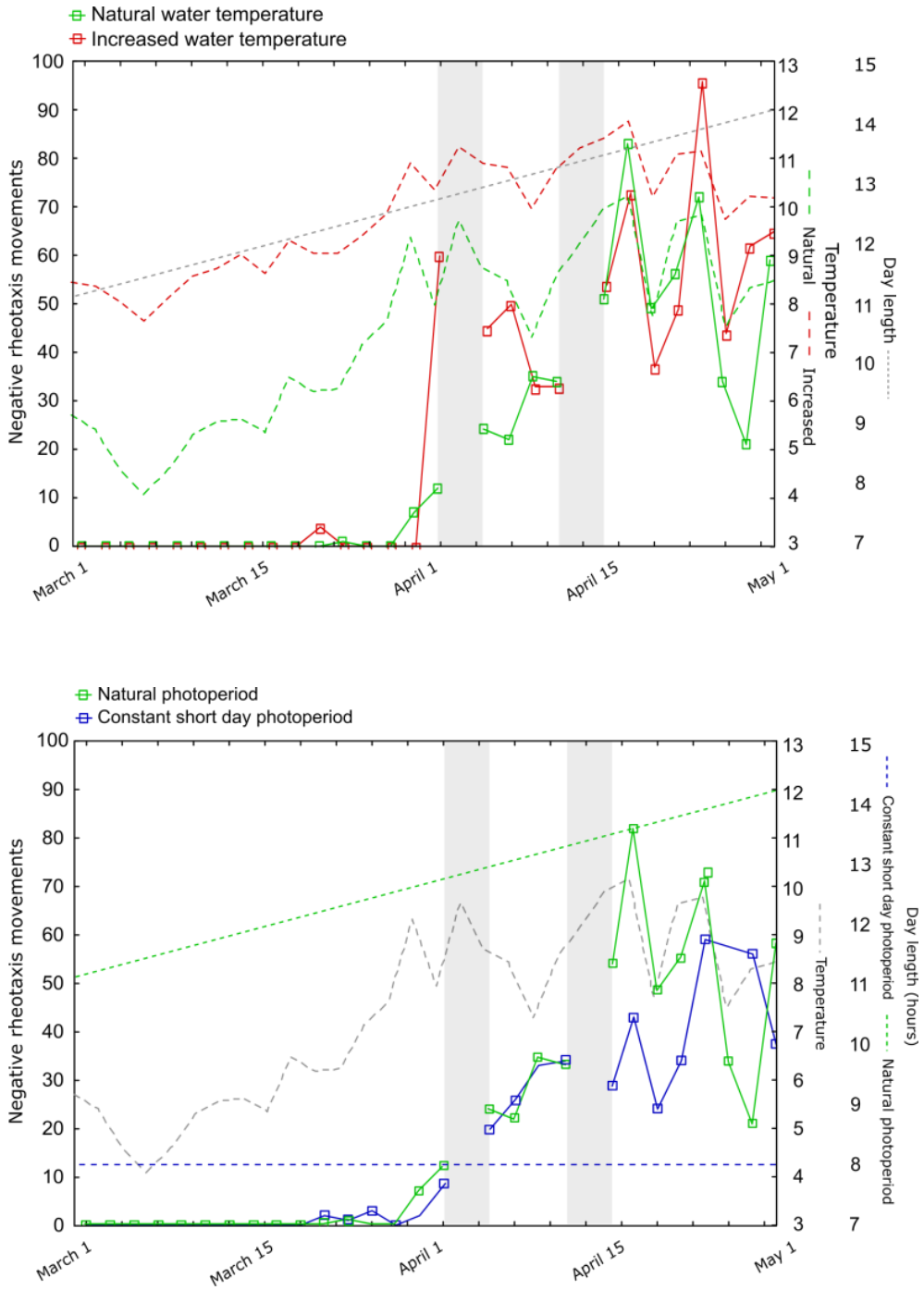

Figure 3: Total daily negative rheotaxis movments of fish raised in Natural (green), increased water temperature (red) and constant short day photoerpiod (blue). Dash line in in figures represents water temperature. Dotted line in bottom figure represents photoperiod (day length)

Effect of increased water temperature

Increased water temperature had no effect on the time at which the fish changed from positive to negative rheotaxis (Figure 3A). During the first week of April fish from both

control and warm water conditions began to change rheotaxis and move downstream. The numbers of fish movements were initially higher in the warm water conditions however after mid-April; both conditions had similar negative rheotaxis movements which fluctuated with that of water temperature. Drops in daily mean water temperature decreased the number of negative rheotaxis movements while an increased daily mean water temperature increased negative rheotaxis movements.

Effect of short day photoperiod

Constant short day photoperiod had no effect on the time at which the fish changed from positive to negative rheotaxis (Figure 3B). During the first week of April from both control and constant short-day photoperiod conditions began to change rheotaxis and move downstream. The number of fish movements were initially the same. Slightly less negative rheotaxis movements were recorded in the constant short day photoperiod condition during mid-April however increased again by the end of April. Like that of the other conditions, negative rheotaxis movements in the constant short-day photoperiod condition also fluctuated with that of daily mean water temperature.

Tsh beta paralog profiles

Pattern of expression

An increase of tshßb pituitary expression was observed in all three conditions (Figure 4). In controls, tshßb expression began to increase in the middle of March reaching peak expression during the mid of April. Expression began to drop during the start of May and returned to basal levels by mid-June. Tshßa expression levels remained constant throughout the whole experiment in all conditions.

Effect of increased water temperature

Tshßb expression in warm water conditions began to increase during the mid of March reaching peak expression during the mid of April. Peak expression lasted till the starts of May were it then dropped faster than controls, reaching basal levels by the first week of May. Expression continued to drop reaching levels lower than initial levels by the mid of June.

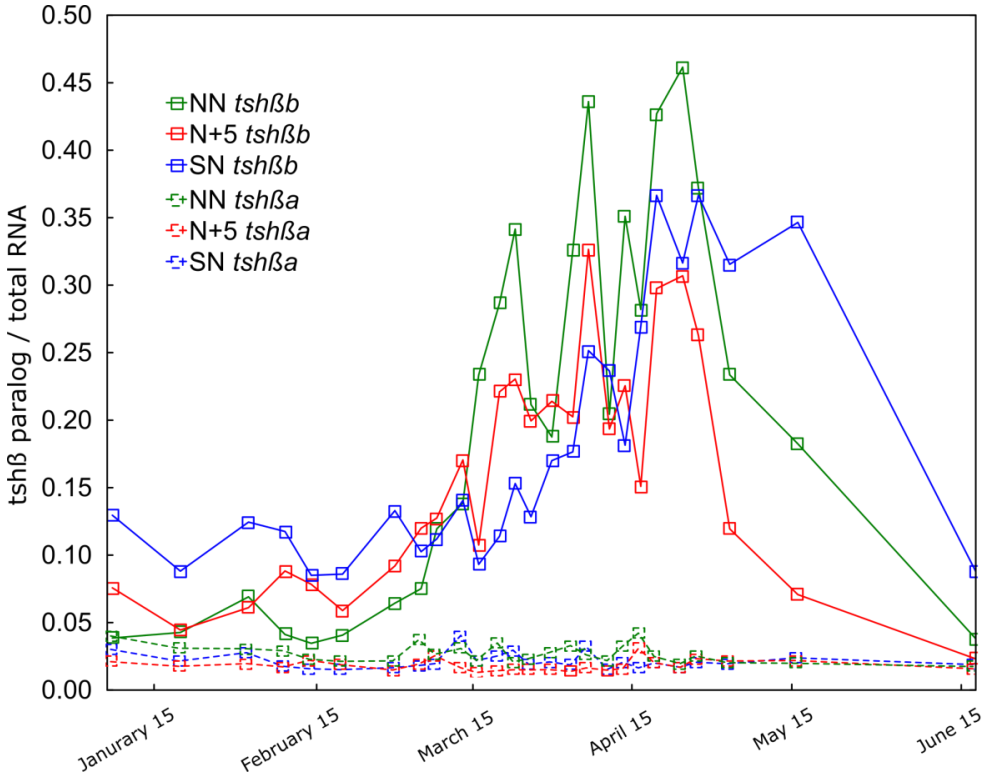

Figure 4: Atlantic salmon pitutiary *tshßa* and *tshßb* expression during smoltification. Salmon were raised at the Conservatoire national du salmon sauvage (CNSS: 45.07°90' North, 3.53°20' East) in natural photoperiod and temperature (NN: Green), natural photoperiod with increased water temperature (N+5: red) and constant short day photoperiod with natural water temperature (SN: blue)

Effect of constant short day photoperiod

Tshßb expression in short-day photoperiod began to increase during the first week of April reaching peak expression at the end of April. Peak expression was later than that of controls and expression did not begin to decrease until the mid of May returning to basal levels by June.

Discussion

The present study investigated the effect of warm water temperatures and constant short day photoperiod on the body growth, inversion of rheotaxis and pituitary expression of *tshßb* during the period of smoltification. The result of the current study suggests an endogenous regulation of both a change of rheotaxis and *tshßb* pituitary expression through the period of smoltification. The lack of significant effect of both short day photoperiod and warm water temperature suggest the timing of rheotaxis inversion to be a trait that is regulated by endogenous and genetic factors. An alternate hypothesis could be that the

timing of rheotaxis inversion depends on conditions before juvenile fish begin to smoltify, during the autumn and winter months prior to smoltification.

Nevertheless, the timing of migration onset is extremely important to the success of downstream migration and transition to the sea the results of this study emphasizes the importance of using local strains for the augmentation of declining fish populations during conservation restocking programs.

Length of photoperiod does not affect daytime behaviour

Photoperiod had no effect on the body growth of the fish during the smoltification period. Up to May, controls and short day photoperiod fish had nearly identical body growth suggests that photoperiod does not play a role in the growth of fish during this period. This is in agreement with McCormick et al. (2002) who observed no difference in growth rates between control and constant short day photoperiod and displayed similar body growth in tanks raised in the same water temperature in Atlantic salmon reared at the White River hatchery in Vermont USA.

Interestingly, the authors conclude that constant short day photoperiod reared fish remained at a stage of incomplete smoltification measured by lower NKA activity and no decrease in condition factor when compared to controls. In the current study, NKA activity was not measured but we report a similar tendency in condition factor with constant short day photoperiod not declining like that of increased water temperature condition and controls. The parameters used in the current study as indication of smoltification were rheotaxis inversion and pituitary expression peak of *tshßb*. In this case, smolitfication was similar between controls and constant short-day photoperiod showing both a change in rheotaxis and tshßb peak expression, albeit slightly delayed. The completion and success of smolitfication would only be known after successful transfer to sea hence the varying results of smoltification parameters by environmental factors exaggerates the complexity of this metamorphic event and the multiple components involved.

The investigation of tshßb is new and the relationship to smoltification has not been clearly established. The dramatic peak of activity indicates a significant role and previous research indicates a relationship to downstream migration. The current study supports this idea as we

see report similar timings of increase *tshßb* expression and the same timings of rheotaxis change. More studies will be needed to confirm the relationship between the two but the current study supports the integral role of tshßb in the smoltification process. No modulation of both the timing of tshßb expression increase and change in rheotaxis by environmental factors suggesting a strong endogenous regulation of these smoltification characteristics. Our results suggest that *tshßb* is similar to that of plasma IGF-1 and plasma T3 which have shown to be unaffected by varying environmental conditions (McCormick et al., 2002). On the other hand, other mechanisms of smoltification such as NKA and plasma GH have been shown to be modulated by photoperiod and water temperature again exemplifying the multiple regulatory mechanisms involved in the smoltification metamorphosis.

Warmer water does not cause earlier rheotaxis inversion

The current experimental design used a heat exchange system to increase the water temperature in order to mimic possible future warmer river conditions and foreshadow the consequences of so on the endangered Loire-Allier Atlantic salmon population. The Loire-Allier River has risen 2°C in the past two decades (Moatar and Gailhard, 2006) and thus a +5°C difference between controls and increased water temperature conditions would have represented a realistic case of future conditions. Albeit, the temperature difference achieved in this experiment is still an accurate representation of the coming decades and displays an effect on growth rate however shows no little to any effect on the change of rheotaxis and migration onset behaviour.

Martin et al., (2012) observed a significant increase of swimming behaviour related to rheotaxis inversion at 7.5C for Loire-Allier population (Martin *et al.*, 2012a). In the current study, the water temperature in the increased water temperature tanks remained above 7.5C for the majority of the experiment without a change in swimming behaviour or rheotaxis inversion until the first weeks of April. Therefore, the timing of rheotaxis inversion appears unrelated to water temperature.

The significant increase in body size due to warm water is in agreement with past studies and clearly shows that growth is direct response to prevailing water temperatures (REFS). No change to rheotaxis change or tshßb expression was observed in increased water

temperature condition suggesting that *tshßb* expression is not regulated by body growth signalling pathways.

Implication for conservation

For the Loire-Allier population, smolts must reach the oceans before water temperatures reach 20°C as swimming speed for this population severely decreases above this temperature (Martin *et al.*, 2012a). In addition, warm water has lower oxygen carrying capacity further decreasing migratory ability of Atlantic salmon smolts (for review: Jonsson and Jonsson, 2009). Thus, if river water temperature continues to rise in the coming decades, the period of time before the water temperature meets this critical threshold decreases, lowering the period of time in which salmon have for successful migration to the sea. Interestingly, the increased growth rate could be advantageous for the future as larger fish migrate faster allowing for long river fish, such as the Loire-Allier population to complete longer migrations in a shorter time (Brett, 1965; Brett and Glass, 1973). This may partially compensate for the fact that increased water temperature did not affect the time in which fish changed rheotaxis in the water which may otherwise cause a mismatch between the physiological and environmental smolt window.

In conclusion, in the future when river temperatures are warmer, salmon may have increased risk of incomplete migration due to the fact that departure from the rearing grounds does not seem to be related to water temperature. The increased body size may compensate for this and allow for migration to be complete successfully. More importantly, these results suggest a strong endogenous regulation to the timing of migration onset and exaggerates the importance of using local strains for the augmentation of declining Atlantic salmon populations, especially true for the endangered Loire-Allier population.

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Discussion & Perspectives

A novel TSH in Atlantic salmon

The current thesis reports the discovery and characterisation of a novel TSH in Atlantic salmon which peaks during the smoltification metamorphosis. TSH is a pituitary glycoprotein composed of a α-subunit common to gonadotropins and a β-subunit conferring hormone specificity. In this thesis, we report the presence of duplicated TSH β-subunit paralogs (*tshßa* and *tshßb*) in Atlantic salmon. Phylogeny and synteny analyses allowed us to infer that they originated from teleost-specific whole genome duplication (3R) and not from the salmonid specific genome duplication (4R). During the period of smoltification, the paralog *tshßb* pituitary expression dramatically peaks and for the first time shows a relationship between pituitary *tsh* expression with the smoltification metamorphosis. The expression peak of *tshßb* was concomitant with the inversion of rheotaxis behaviour which is a characteristic of smoltification indicating the time of downstream migration onset. Therefore the discovery of tshßb expands our knowledge on the complex process of smoltification and suggests a relationship to downstream migratory behaviour.

Previous research on TSH were unable to relate pituitary expression to the smoltification process as they did not report large change in pituitary mRNA levels in both Altnatic salmon (Martin *et al.*, 1999; Robertson and McCormick, 2012) and coho salmon (Larsen *et al*., 2011). These results suggest that previous research was in fact investigating the *tshßa* paralog. The results of our study are in agreement with previous reports as pituitary tshßa expression remained constant throughout smoltification. The peak of tshßb has only been investigated in the Loire-Allier Atlantic salmon population and therefore must be confirmed in other Atlantic salmon populations. Additionally, phylogeny analyses showed the presence of multiple *tshßb* paralogs among various salmonid species including additional paralogs originating from the salmonid specific genome duplication event (4R) in rainbow trout and coho salmon (Maugars *et al.*, 2014). Therefore, the discovery of the functional *tshß* paralogs in Atlantic salmon opens the possibility of investigating novel roles of *tshß* paralogs among various salmonid species.

Localization of *tshß* paralog expression in the Atlantic salmon pituitary revealed a distinct cell population on the dorsal region of the PPD expressing *tshß*, different to the cells expressing *tshßa,* which localized to the anterior portion of the pituitary on the boarder of

rostral pars distalis (RPD) and pars proximal distalis (PPD). The location of *tshßb* expression is comparable to the location of pars tuberalis TSH discovered in mammals and birds. TSH produced by PT cells plays a key role in the seasonal regulation of major steps of life cycles, including reproduction, migration and hibernation (for reviews: Hanon *et al.*, 2008; Hazlerigg and Loudon, 2008; Dardente, Hazlerigg and Ebling, 2014; Nakane and Yoshimura, 2014). In our study during Atlantic salmon smoltification, the peak of *tshßb* was concomitant to the inversion of rheotaxis in spring and may suggest similarities of the TSH pathway in seasonal life history events between vertebrate lineages.

Local action of TSHb in the hypothalamus

In mammals, photoperiodic regulation of PT-TSH production is mediated *via* melatonin production from the pineal gland. In contrast, birds PT-TSH production is regulated through deep brain photoreceptors and is not affected by melatonin levels. Despite the differences in the integration of photoperiodic information between mammals and birds, PT-TSH exerts a similar action on neighbouring hypothalamus. PT-TSH stimulates the expression of DIO2 in the hypothalamus which catalyses the conversion of T4 into the more biologically active T3, thus leading to the activation of TH-regulated brain functions (for reviews: Hanon *et al.*, 2008; Hazlerigg and Loudon, 2008; Dardente, Hazlerigg and Ebling, 2014; Nakane and Yoshimura, 2014). In a recent study, a salmonid specific 4R-issued DIO2 paralog (*dio2b*) has been identified in the Atlantic salmon, the expression of which increases in circumventricular brain areas of cell proliferation, during experimental photoperiod-induced smoltification. The authors proposed a specific role of DIO2b in promotion of TH-dependent brain development during smoltification (Lorgen *et al.*, 2015). Thus, in-line of PT-TSH role and action mechanism in amniotes, we propose that TSHb produced by dorsal PPD cells in Atlantic salmon may stimulate brain DIO2b expression and promote TH-activated brain functions related to smoltification.

Studies are needed to confirm the tshßb/dio2b pathway and could be done by further investigating the brain distribution of tsh receptor (*tshr*) paralog expression. Three tsh receptors have been characterized in Atlantic salmon and more could be present due to the 4R in salmonid species (Maugars *et al.*, 2014). In European eel (*Anguilla anguilla*), tsh receptor paralog (called *tshrb* by the authors) showed expression in various parts of the brain (Maugars *et al.*, 2014). In the current study, brain distribution investigated by qPCR

showed expression of salmon *tshßb* in multiple regions of the brain while *tshßa* was only expressed in the pituitary. Additional experiments are needed to confirm these expressions but suggest potential roles in other parts of the brain aside from the hypothalamus. Investigating, the co-localization of *tshr* and *dio2* expressing cells in the hypothalamus and other brain regions could help better understand the tshßb/dio2b pathway and potentially discover other pathways between paralogs.. The investigation into the tshr paralogs and their co-localization with dio2 paralogs would help characterize multiple pathways of tsh/dio2 and lead to potential roles in the regulation of seasonal life history events.

Furthermore, tissue distribution of *tshßb* paralog showed expression throughout peripheral tissues such as the gills, whereas *tshßa* was only expressed in the pituitary. It could be hypothesized that a similar mechanism of tshßb activation of dio2 may take place throughout the body to promote local TH-activated peripheral tissue functions during smoltification or other developmental processes. This is supported by the fact that dio2 and dio3 paralogs are observed throughout the tissues of flatfish during larval metamorphosis (Itoh *et al.*, 2010; Alves *et al.*, 2017). These results lend support to the peripheral regulation model of thyroid function proposed by Eales and Brown (1993) who propose thyroid hormones to be regulated peripherally in teleosts (for review: Eales and Brown, 1993). Further experiments are needed to confirm these peripheral expressions observed in this study but suggest potential roles of tshßb in various peripheral tissues. Investigating the co-localization of *tshr* and *dio2* expressing cells among various tissues would help decipher activation of local TH depend processes (Figure 11). Additionally, *tshßb* expression profiles in the brain and peripheral tissues through the period of smoltification would be interesting as to date only the expression profile for pituitary have been investigated.

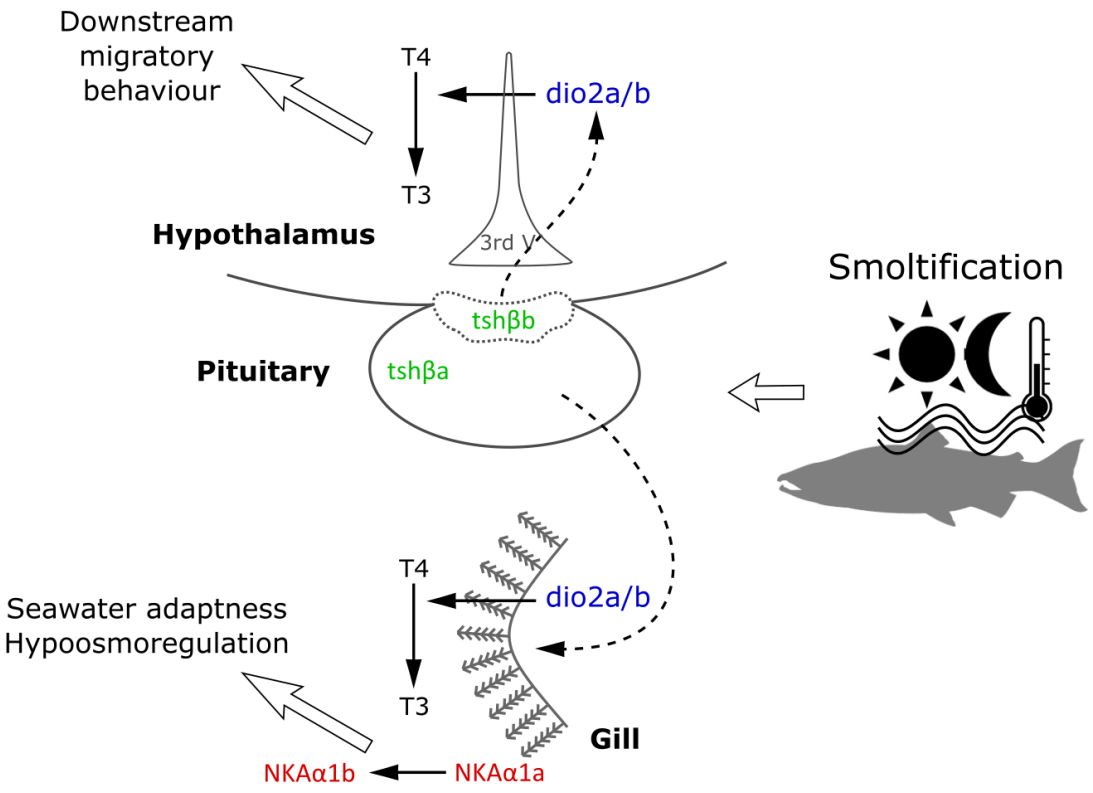

Figure 11: Proposed mechanims of *tshß*/dio2 paralogs in both the hypothalamus and perifpheral tissues such as the gills during the smoltification metamorphosis

Modulation of pituitary *tshßb* peak by environmental factors

Constant short day photoperiod throughout smoltification had a minor effect on the expression of *tshßb* in our study. A slight delay in the peak expression of *tshßb* was observed and the drop was also delayed. In a previous experiment, McCormick et al. (2002) fount that constant short day photoperiod were able to delay the increase of gill NKA activity and plasma levels of GH without affecting the levels of plasma IGF-1 (McCormick *et al.*, 2002). The slight modulation of the peak of pituitary *tshßb* expression by constant short day photoperiod will need to be confirmed in future studies. The minor effect of drastically different photoperiod regimes suggests that the peak of *tshßb* expression is not particularly regulated *via* photoperiod cues; rather a genetic/developmental aspect may be slightly modulated by photoperiodic conditions.

The teleost pineal gland is known to be involved in the synchronization of a number of physiological events and is responsible for the synthesis and release of melatonin (for review: Korf, 1994). The duration of nocturnal melatonin surge in Atlantic salmon is dictated by photoperiod while the amplitude of the surge depends on water temperature (for review: Falcón *et al.*, 2010). Pinealectomised Atlantic salmon undergo delayed smoltification as compared to controls and authors suggest melatonin to be an important factor in the regulation of certain smoltification processes (Porter *et al.*, 1998). In order to determine if *tshßb* production is regulated *via* melatonin I assayed samples from a previous experiment that took place at CNSS which investigated the effect of melatonin implants in juvenile Loire-Allier Atlantic salmon. I tested *via* qPCR the *tshßa* and *tshßb* paralog expression levels in melatonin treated fish and saw a dramatic peak of *tshßb* expression similar to that of control fish (Figure 12). This suggests that *tshßb* production does not rely on melatonin signalling and may be modulated by other photoperiodic integration mechanism, possibly through the eyes and deep brain receptors similar to that of birds (for review: Dawson *et al.*, 2001).

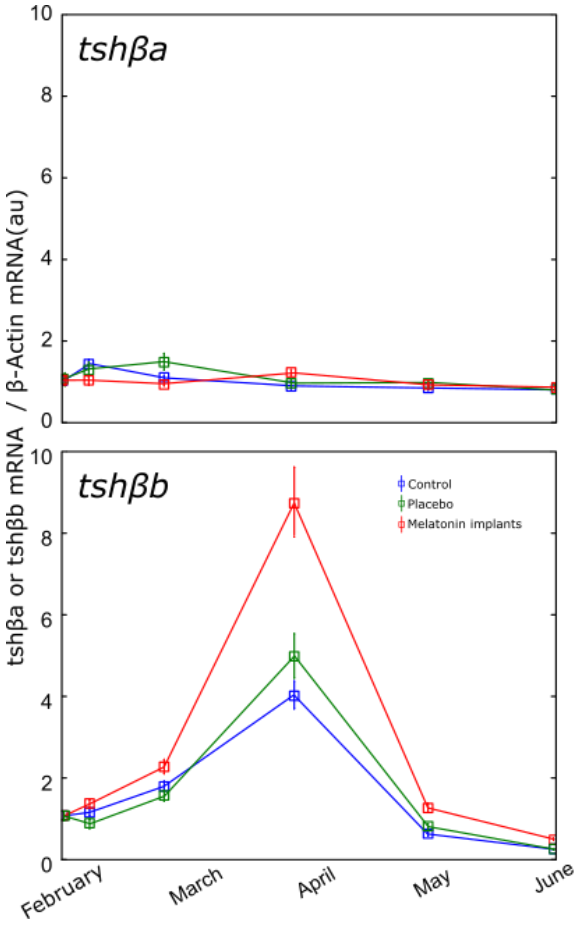

Figure 12: *tshβa and tshβb* pituitary expression in control (blue) placebo (green) and melatonin implanted Atlantic salmon during the period of smoltification

Interestingly, in the same melatonin experiment, no inversion of rheotaxis was observed in the melatonin treated fish during daytime. The melatonin implants caused the fish to remain with positive rheotaxis in the water corresponding to the night-time behaviour observed in control fish (data not shown). Similarly, putting naturally smolting Atlantic salmon in constant darkness stopped diurnal downstream migratory behaviour which was restored after returning salmon back to natural photoperiod (Martin *et al.*, 2012). Therefore, it seems that melatonin has the ability to block day time behaviour (negative rheotaxis) by simulating constant darkness *via* high melatonin levels; however the peak of *tshβb* expression remains similar to that of controls. It may be that the expression of *tshβb* only affects day time behaviour is smoltifying salmon and that the behaviour mechanism of melatonin is able to override the photoperiodic information relayed through the light-brain-pituitary axis. The

relationship between endocrine and behavioural mechanisms of smoltification is something I will continue to investigate after my PhD.

Similarly to photoperiod, increased water temperature did not cause a significant effect on the *tshßb* expression. Although the fish were subjected to increased water temperature for months before the peak of *tshßb* expression both conditions peaked during the first weeks of April. The expression decreased slightly sooner than that of controls. The similar peaks between controls and increased water temperature suggest that *tshßb* expression is not predominantly regulated *via* thermal cues. In previous studies, increased water temperature has been shown to advance many aspects of smolting including silvering, body growth, swim speed and behaviour (Johnston and Saunders, 1981; Muir *et al.*, 1994; McCormick *et al.*, 2002; Zydlewski, Haro and Mccormick, 2005) . The peak of *tshßb* however is unaffected by prevailing water temperatures further suggesting the endogenous regulation of *tshßb*.

Interestingly, other endogenous regulated process such as body weight and length were significantly affected by increased water temperature. Despite a 20-30% increase in body size in increased water temperature reared fish only minor differences of the time of rheotaxis inversion and peak *tshßb* expression were observed as compared to controls. Therefore, this suggests that the induction of *tshßb* expression and rheotaxis inversion does not rely on endogenous body growth signalling pathways.

Inversion of rheotaxis is predetermined

Concomitant to the increase of *tshßb* expression was the inversion from positive to negative rheotaxis. This behaviour change indicates the timing of migration onset and is a crucial component to the smoltification process (Hoar, 1976; Boeuf, 1993). The time in which salmon deport from the rearing grounds is a determining factor in the survival of smolts (Boeuf, 1993; McCormick *et al.*, 1998) and therefore is an important consideration for conservation programs. Completing migration and arriving at the estuary within the physiological and environmental smolt windows depends on the time in which salmon depart from the rearing grounds. Particularly true for long river populations such as the Loire-Allier were barriers, weirs and other migratory obstacles can cause significant delays to downstream migration.

The change in rheotaxis in the current experiment happened during the first weeks of April in all experimental conditions. Neither increased water temperature nor constant short day photoperiod disrupted the time in which salmon began to change from positive to negative rheotaxis during day light hours as compared to controls. During this period, fish would keep positive rheotaxis during morning hours and begin to change to negative rheotaxis towards the end of the day. Downstream movements increased significantly during the dusk hours. This is in agreement with Martin *et al*., (2012) who saw a rapid change switch from positive to negative rheotaxis during the end of March or start of April depending on the year (Martin *et al.*, 2012). Nocturnal behaviour remained the same throughout the experiment represented by persistent positive rheotaxis with little to no movements. Similar behaviour for the Loire-Allier population has been reported with only slight movements at night through the period of smoltification across many years of study (Martin *et al.*, 2012).

Downstream migratory strategies of smolts have been investigated for many years and reports include nocturnal (Thorpe *et al.*, 1981; Aarestrup, Nielsen and Koed, 2002), diurnal (Davidsen *et al.*, 2005) or a mix of the two movements (McCormick *et al.*, 1998; Ibbotson *et al.*, 2006). The current study reports little nocturnal movements throughout the experiment with a rapid change to diurnal migration during the first weeks of April for the Loire-Allier population. Interestingly, the time in which this inversion of rheotaxis takes places seems to be only slightly modified by environmental factors. The timing of rheotaxis and the migratory strategy used may be a heritable trait, genetically specific for the specific river. The specific timing of such crucial events may be genetically coded in a way which disregards the variations in environmental factors which would protect crucial life history events from yearly fluctuations in river temperature. Long term increases in water temperature however may pose a serious threat as timing of migration onset appears to be predetermined.

In order to compare Loire-Allier Atlantic salmon population with that of others, common garden experiments could be done to investigate if whether inversion of rheotaxis and peak expression of *tshßb* are endogenously and genetically regulated. The Loire-Allier is highly protected through the “Plan Loire Grandeur Nature” and thus transferring salmon to CNSS is forbidden; however, transferring Loire-Allier salmon eggs to more northern latitudes and raising them to the smolt stage under foreign photoperiodic and thermal regimes would reveal if indeed rheotaxis inversion and *tshßb* expression are regulated internally. It could be

hypothesized that these smoltification characterises are determined during the autumn and winter months prior to the period of smoltification and common garden experiments would help answer these questions.

To date, the most recent published genome is that of an Atlantic salmon from the AquaGen strain, a commercially farmed strain of Atlantic salmon that has been selectively bread for multiple generations (Lien *et al.*, 2016). As an approach to investigate the uniqueness of the Loire-Allier population, the genomes of a wild smolt from the Loire-Allier caught in 2017 and one caught in 1894 (provided by the collections at MNHN) are being sequenced and assembled by Future genomics in Leiden, Netherlands in collaboration with CNSS and MNHN. This research will compare both the current and ancient Loire-Allier genome to that of the published genome and could reveal a level of genomic divergence allowing to characterize the uniqueness of the Loire-Allier population. As an example, this research could be used to investigate the promoter regions of Atlantic salmon *tsh* paralogs which may explain the differences in pituitary expression during smoltification. A similar mechanism was reported in stickleback (*Gasterosteus aculeatus*) by Kitano et al (2010) in which *cis*-regulatory changes in tshß paralogous genes were suggested to account for the difference in mRNA expression and behaviour between marine and freshwater populations (Kitano *et al.*, 2010). Of course, multiple different candidate genes may account for the differences in gene expression and behaviour between various Atlantic salmon populations.

Implications for conservation in the frame of global climate change

Persistent increasing of river water temperature has occurred in the Loire-Allier for the last 25 years. Since the late 1970s the Loire-Allier has raised approximately 2˚C during the late winter and spring months and the trends indicate further warming in the coming decades (Moatar and Gailhard, 2006). The current study mimics possible future conditions by subjecting fish to increased water temperature throughout the entire smoltification process. Reports have hypothesized that warming rivers in the frame of global climate change will cause earlier migration onset for various salmonid species (for review: Jonsson and Jonsson, 2009) however the results of this study provides arguments against this hypothesis.

The current study saw no advancement of rheotaxis change and thus suggests an alarming consequence of global climate and anthropogenic changes. In addition to global climate

change, there are many situations along the Loire-Allier which cause the occurrence of local warming which will further hinder the downstream migratory ability of smolts. In the Haut-Allier region, water is often drawn from streams and tributaries of the Loire-Allier for agriculture purposes causing a reduction in water flow further increasing river water temperatures. During downstream migration, smolts are subjected to many occurrences of local pockets of warm water from nuclear power plant cooling station run offs and other city activities and influence which can threaten the migratory ability of smolts. Thus, no change in departure time from rearing grounds in a warmer future will significantly reduce the period of time smolts have to migrate down river before water temperatures reach 20 degrees or above, the temperature at which downstream swimming halts for the Loire-Allier population (Martin *et al.*, 2012b). Furthermore, along with warm water come decreased oxygen availability and increase pathogens compounding the negative consequences of increased water temperature for the Loire-Allier Atlantic salmon (for review: Jonsson and Jonsson, 2009).

A possible compensatory effect of increased water temperature for the wild population could be that the increased water temperature is shown to have a significant effect on the rate of body growth. The current study saw a 20-30% increase in body size in the increased water temperature fish which would result in faster migrating smolts as swim speed has been shown to increase with body size (Brett, 1965; Brett and Glass, 1973). Therefore the high plasticity in body growth regulated by prevailing water temperatures could partially compensate for the consistent timing of rheotaxis inversion and migration onset.

Although increased growth rate in warmer waters may contribute to faster migration, the situation for the Loire-Allier looks alarming. As the last long river migrating population of Atlantic salmon in Europe, the Loire-Allier salmon should be given special attention to ensure this population prevails. Multiple global and anthropogenic related threats are putting enormous pressure on this salmon population to adapt faster than they are physiologically capable of. In order to help, conservation strategies could be set in place which would facilitate successful downstream migration and transition to the sea. First, attention must be put into restoring river continuity and increase the ease of access to the upper portions of the Loire-Allier. The pristine spawning grounds of the region should be used as much as possible before environmental and anthropogenic factors force salmon out

of the area. Large progress was recently made in the restoration of river continuity as the Poutès dam began reconstruction in the spring of 2018 and the new dam designed for the location will drastically improve the bypass methods allowing for better access to the Haut-Allier region.

Secondly, conservational efforts should allocate resources to the restoration of rearing grounds lower down river than the Haut-Allier region. Lower spawning grounds have been modified or destroyed by human presence and restoration to these locations would reduce the overall migratory distance for Loire-Allier Atlantic salmon. As global climate change continues, focus must be made to provide sufficient spawning locations lower than the Haut-Loire region to ensure downstream migration will be successful before physiological thresholds of water temperature are reached halting migration; especially in the light of the results of this thesis which demonstrates a strong endogenous regulation of migration onset for the Loire-Allier population. Providing suitable rearing grounds for migrating salmon downriver of the Haut-Allier may allow for the prevalence of this special population in a warmer future.

The current thesis provides novel knowledge to the smoltification metamorphosis of Atlantic salmon. Functional divergence of TSH in Atlantic salmon provides for the first time, *tshß* expression with that of the smoltification metamorphosis. Similarities to PT-TSH in mammals and birds helps conserve the idea that this is an ancestral mechanism regulating seasonal life history events, in this case the inversion of rheotaxis. Dominantly regulated by endogenous factors and only slightly modified by prevailing environmental conditions suggests genetic regulation of *tshßb* expression and migration onset for the Loire-Allier population. In the frame of global climate change these results suggest focus should be put on restoring downriver rearing grounds to facilitate shorter migration distances and help the special Loire-Allier population prevail in a warming future.

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Résumé: Contrôle environnemental et neuroendocrine de la smoltification chez le saumon Atlantique, *Salmo salar*, de longue rivière

La smoltification est un évènement métamorphique chez le saumon qui initie la migration de dévalaison et pré-adapte le juvénile à l'entrée en mer. Cette thèse a pour objectif d'étudier les régulations endocrines et environnementales de la smoltification chez le saumon Atlantique de la longue rivière Loire-Allier, population qui est en danger. Nous montrons la présence et la divergence fonctionnelle de deux paralogues de la sous-unité ß (*tshßa* & *tshßb*) de la thyrotropine (TSH) chez le saumon Atlantique et observons un pic d'expression de *tshßb* dans l'hypophyse à la smoltification, pic concomitant à l'initiation de la migration de dévalaison. Ce résultat est le premier à mettre en relation l'expression hypophysaire de TSH avec la smoltification et le comportement migratoire de dévalaison. L'exposition expérimentale à une photopériode constante de jours courts ou à une température augmentée n'affecte pas nettement le pic de *tshßb* ni l'initiation de la migration de dévalaison, ce qui met en évidence l'importance de contrôles endogènes. Cette étude apporte de nouvelles connaissances fondamentales sur le cycle de vie du saumon Atlantique avec la découverte de nouveaux acteurs dans le processus de smoltification et avec des implications dans le domaine de la conservation.

Mots-clés: *Salmo salar*, smoltification, migration de dévalaison, hormone thyréotrope, photopériode, température

Abstract: Environmental and Neuroendocrine control of smoltification in long-river (Loire-Allier) Atlantic salmon, *Salmo salar*

Smoltification is a metamorphic event in salmon, which initiates downstream migration and pre-adapts juvenile for seawater entry. The PhD aimed at investigating endocrine and environmental regulation of smoltification in the endangered long-river Loire-Allier Atlantic salmon. We report the presence and functional divergence of thyrotropin ß-subunit paralogs (*tshßa* & *tshßb*) in Atlantic salmon and showed a peak pituitary expression of *tshßb* at smoltification which was concomitant with the initiation of downstream migration. This is the first time pituitary TSH expression is related to smoltification and downstream migratory behavior. Experimental exposure to constant short-day photoperiod or to increased temperature did not markedly affect the peak of *tshßb* nor the initiation of downstream migration, highlighting the importance of endogenous controls. This study brings new insights to the life cycle of Atlantic salmon with the discovery of novel components of the smoltification process, and with implications for conservation.

Key-words: *Salmo salar*, smoltification, downstream migration, thyrotropin, photoperiod, temperature