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Physiological response of smolts from two strains of Atlantic salmon, Salmo salar L., to a temperature increase on their migratory route

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Physiological response of smolts from two strains of Atlantic salmon, *Salmo salar* L., to a temperature increase on their migratory route.

A dissertation submitted by

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in fulfillment of the requirements for the degree of

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Physiological response of smolts from two strains of Atlantic salmon, *Salmo salar* L., to a temperature increase on their migratory route.

by Benoît Bernard

Abstract

In Belgium, the population of Atlantic salmon disappeared in the 1940's. A restoration program in the Meuse Basin was launched in 1987. Thanks to many efforts, results are encouraging, but only few adult spawners have been captured yet. Environmental data show temperature differences, sometimes exceeding 5°C, between a tributary and a larger river during the downstream migration period. Since temperature is a primary cue in smolting, we investigated the potential effects of such thermal conditions on smoltification. We looked for differences of various physiological smoltification markers between two strains of fish and between early and late migrants. We also examined the transcriptional response of smoltification-related genes in two crucial organs, the liver and the gill. We then aimed at verifying our experimental results in the field by sampling smolts in two sites where temperature differences had been measured.

First, we compared two foreign strains, commonly used in Belgium for stocking, under simulated natural conditions based on the temperature and photoperiod of a tributary annually restocked. We observed strain-related differences of the influence of temperature and daylength on cortisol, GH and sodium plasma levels. Using Na^+/K^+ATPase activity as an indicator, both strains smoltified successfully and simultaneously under local conditions.

Then, we investigated the effect of a rapid temperature increase on hypo-osmoregulatory capacities in early and late migrants. The ability to hypo-osmoregulate was assessed by seawater challenges. We observed that a rapid temperature increase during the smoltification period similarly impaired osmoregulatory capacities in smolts of both strains (decreased NKA activity and increased plasma osmolality). After an early or late temperature increase, smolting indicators tend to be modified toward parr values within one week after the increase, suggesting that local temperature conditions may heavily compromise smolt survival chances at sea entry.

We also examined gene expression in the liver in response to a swift temperature increase. Results showed deleterious effects of temperature in all defined gene groups like endocrine regulation of smoltification, oxygen transport, iron metabolism, lipid and carbohydrate metabolism, immune response and cell cycle. Findings also revealed important changes occurring during smoltification. Differences between the strains are thought to be linked to water temperature and migration distances of the rivers of origin. Gene expression in the gill was also impaired after a temperature increase. Changes in the transcription of genes associated with the endocrine control of smoltification (*igf1r* and *igf2*) were identified and altered expression of genes linked to hypo-osmoregulation (*nkaα1b* and *nkcc1a*) was consistent with physiological markers. Data suggests dual roles in the smoltification and desmoltification process for GH and IGF1 and points to the implication of genes, previously unstudied (*nbc*) or with little data available (*igf2*), in the smoltification process. This study gives further insights on the molecular processes underlying smoltification and desmoltification in Atlantic salmon and possible responses to human-related water temperature increase.

Field work presented numerous challenges that had to be overcome. Environmental conditions and infrastructure-linked issues caused many setbacks and only limited data were obtained. However, differences emerged compared to laboratory conditions, notably early migrants seemed much less affected than late migrants three days after transfer into warmer water while early and late migrants were similarly affected under simulated natural conditions. Our results suggest a deleterious effect of human-linked temperature increase on migrating salmon.

All in all, a rapid temperature increase arising between two rivers seems to strongly influence the smoltification. Considering the response of early migrants in the field experiment, early migrating strains may have better chances to reach the sea with high capacity for hypo-osmoregulation. Effects of modelled temperature increase due to climate change, stocking management and strain selection were discussed in regard to our results. Early migrating strains and efforts to facilitate downstream migration may promote smolt survival under local conditions.

List of abbreviations

acox	Peroxisomal CoA peroxidase
ACTH	Adrenocorticotropic hormone
apoa1	Apolipoprotein A1
ATP	Adenosine tri phosphate
bax	Bcl-2-associated X protein
bclx	B-cell lymphoma-extra large
c3	Complement C3
cADN	Complementary deoxyribonucleic acid
calm1	Calmodulin
casp3	Caspase 3
cat	Catalase
cd36	Cluster of differentiation 36
cdkn1b	Cyclin-dependent kinase inhibitor 1B
CFTR	Cystic fibrosis transmembrane conductance regulator
cftr1	Cystic fibrosis transmembrane conductance regulator 1
cftr2	Cystic fibrosis transmembrane conductance regulator 2
CG	Cong
cox5b	Cytochrome C oxidase subunit 5B
CRF	Corticotropin-releasing factor (old)
CRH	Corticotropin-releasing hormone (new)
cs	Citrate synthase
DNA	Deoxyribonucleic acid
efiα	Elongation factor I alpha
est	Expressed sequence tag
fads6	delta-6 fatty acyl desaturase
fth1	Ferritin heavy subunit
FW	Freshwater
galk2	Galactokinase 2
GH	Growth Hormone
ghr1	Growth hormone receptor 1
ghrh	Growth hormone-releasing hormone
glyp	Glycogen phosphorylase, liver-like.
gpx7	Glutathione peroxidase 7
gr1	Glucocorticoid receptor 1
gr2	Glucocorticoid receptor 2
gst	Glutathione S-transferase
hatp6vb	V-type H+ATPase subunit b
hba	Hemoglobin subunit alpha
hbb	Hemoglobin subunit beta

HPI	Hypothalamic-pituitary-interrenal
HSP	Heat Shock Protein
hsp70	Heat shock protein 70
hsp7c	Heat shock cognate 7c
hsp90b1	Heat shock protein 90 beta 1
IGF1	Insulin-like growth factor-1
igf1r	Insulin-like growth factor receptor
IGF2	Insulin-like growth factor-2
igfbp	IGF binding protein
LA	Loire-Allier
lxr	Liver X receptor
lyg	Lysozyme G
lyz	Lyzozyme C
mr	Mineralocorticoid receptor
MS-222	Tricaine methane sulfonate
nbc	Na^+/HCO_3^--cotransporter
NKA	Na^+/K^+-ATPase
nkaα1a	Na^+/K^+-ATPase subunit 1a 'freshwater type'
nkaα1b	Na^+/K^+-ATPase subunit 1b 'seawater type'
NKCC	$Na^+/K^+/2Cl^-$ co-transporter
nkcc1a	$Na^+/K^+/2Cl^-$ co-transporter 1a
p53	Cellular tumor antigen P53
pcna	Proliferating cell nuclear antigen
PRL	Prolactin
prlr	Prolactin receptor
PrP	Prolactin-releasing peptide
pygl	glycogen phosphorylase L
ra51a	DNA repair protein RAD51 homolog a
RAS	Recirculating Aquaculture System
rhbg	Rhesus B glycoprotein, nonerythroid ammonia transporter
rhcg1	Rhesus C glycoprotein 1, nonerythroid ammonia transporter
rhcg2	Rhesus C glycoprotein 2, nonerythroid ammonia transporter
rl10	60S Ribosomal protein L10
RNA	Ribonucleic acid
s20	40S Ribosomal protein S20
sod1	Superoxide dismutase 1
SOR	Salmon olfactory receptor
SVR	Salmon vomeronasal receptor
SW	Seawater
T3	3,3',5-triiodo-L-thyronine

T4	Thyroxine
taldo1	Transaldolase 1
tf	Transferrin
thra1	Thyroid receptor alpha
thrb	Thyroid receptor beta
TSH	Thyroid stimulating hormone
β-actin	Beta-actin

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1 General Introduction

1.1 Study context

1.1.1 Salmon demographic history and the "Saumon Meuse" initiative

Until 1840, the Atlantic salmon (*Salmo salar*, Linnaeus 1758) was a common fish found in the Meuse basin up to Monthermé in France, more than 470km away from the North Sea. Close to the end of the 19th century, the number of captured salmon in the Rhine and Meuse common estuary fluctuated between 21.600 and 104.000 individuals per year (Philippart, 1987). In Belgium, from 1885 on, the amount of catches began dwindling despite serendipity catches of large adults until the 1920's (Figure 1). Primary causes were the construction of dams on the Meuse and its tributaries; industrial development causing chemical pollutions, high-yield commercial fishing and poaching. However, as early as 1880, a protection effort was undertaken by the Belgian and Dutch public authorities consisting of:

➔the construction of fish ladder prototypes since 1880,

➔the modification of the fishing legislation in 1883,

➔artificial restocking with fry from the last wild spawners of the Meuse between 1920 and 1925.

Figure 1: 14.6 kg Atlantic salmon caught in Lixhe in 1919.

Despite these protective actions, the salmon stock continued dwindling in Belgium and only 6 fish where caught in 1932. A few more catches were registered until 1942, that very year it is thought that the last specimen was caught near Visé. Between 1840 and 1950, anthropic use

of the Meuse river system caused the extinction of all the anadromous fish species present in this ecosystem; Atlantic salmon, sturgeon (*Acipenser sturio* L.), houting (*Coregonus oxyrhynchus* L.), allice shad (*Alosa alosa* Cuvier), twaite shad (*Alosa fallax* Cuvier), sea lamprey (*Petromyzon marinus* L.) and river lamprey (*Lampetra fluviatilis* L.) (Philippart, 1987; Philippart *et al*., 1988; Philippart & Vranken, 1983).

More than 40 years later, in 1983, 4 adult sea trouts (*Salmo trutta trutta*, Linnaeus 1758) were captured during an electrofishing campaign in the lower Berwinne, the first Belgian Meuse tributary next to the Netherlands border (Prignon *et al.,* 1999). This species is a sea-run form of the brown trout (*Salmo trutta fario*, Linnaeus 1758) and a close relative of the Atlantic salmon. Its resurgence, probably favoured by water quality improvement in the Meuse, was the starting point of a large and long-lasting attempt to restore the salmon population in the Meuse basin by means of appropriate restocking. A number of scientists from the University of Liège and the University of Namur rose to the challenge with the help of the Public Services of Wallonia. This initiative was proposed and selected within the framework of the European Year of the Environment 1987. The "Meuse Saumon 2000» initiative consisted primarily in rehabilitating the salmon to the Belgian rivers and reinforcing the sea trout population (Malbrouck *et al.,* 2007), this initiative's name was later changed to « Saumon Meuse » initiative.

Since 1988, annual restocking actions are carried out on various streams of the Meuse basin. Scottish, Irish and French strains have been used at various developmental stages (fry, parr, presmolt and smolt). Nowadays, the only non-native strain used originates from the Loire-Allier basin in France. Eventually, in 2002, these efforts bore fruits. Indeed, 11 adult salmon were caught in the fish ladder next to the dam in Lixhe (Malbrouck *et al.,* 2007). Two more were captured in 2003 on the same location in addition to the 2 salmon captured in the fish ladder next to the Dam in Berneau on the Berwinne. The project hit a major setback as no salmon were caught between 2004 and 2006. From 2007 on, adults are caught every year with an increasing tendency. Until October 2016, a total of 155 adults (139 in the Meuse, 14 in the lower Ourthe next to Liège and 2 in the lower Berwinne) were caught (Figure 2A). A noteworthy 59 adults where registered in 2015 in the Belgian Meuse basin. In addition, in the Roer, a German tributary of the Meuse, 8 salmon were also captured. All these fish measured between 45 and 100 cm, weighed between 1.1 and 7.5 kg and were mainly genetically characterized as the French strain Loire-Allier (Figure 2B). In 2006, the primary target of the "Saumon Meuse" initiative for the following years was to increase the restocking effort up to

50000 smolts and 200 000 parrs per year. These would be raised mainly from imported Loire-Allier eggs.

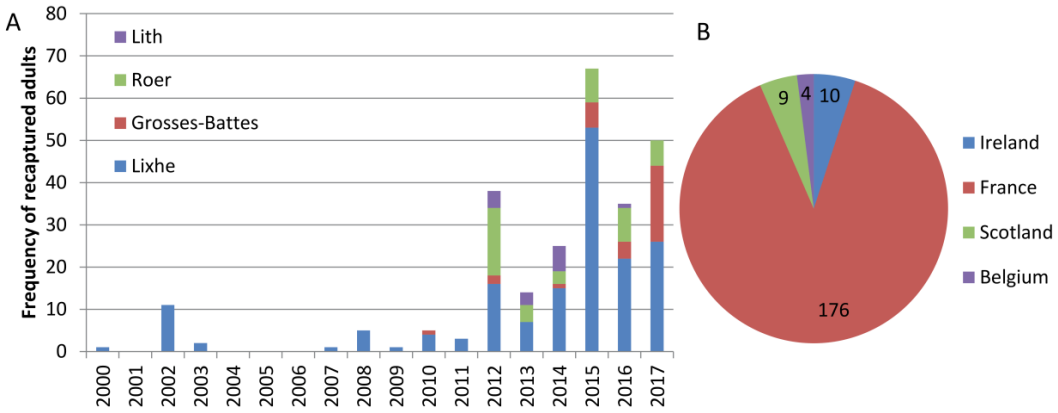

Figure 2: (A) Annual catch of adult salmon in the Meuse basin, Lith (The Netherland), Roer (Germany), Grosses-Battes (Belgium) and Lixhe (Belgium) and (B) best genetic characterization of the strain when available.

To further achieve this goal, a new dedicated aquaculture facility was established by the Walloon Region in Erezée in order to maximize the reproductive efficiency of spawners captured in the Meuse by artificial reproductive techniques, and then to increase the number of parrs and smolts released in the rivers (Figure 3). In collaboration with an older salmonid fish farm in Emptinne, figures shot up during the recent years, from 130.000 parrs in 2010 to 578.500 parrs in 2014. Since 2009, almost 2 million parrs have been raised in Erezée and Emptinne to be released in the rivers corresponding to twice the objective set by the Walloon government back in 2009.

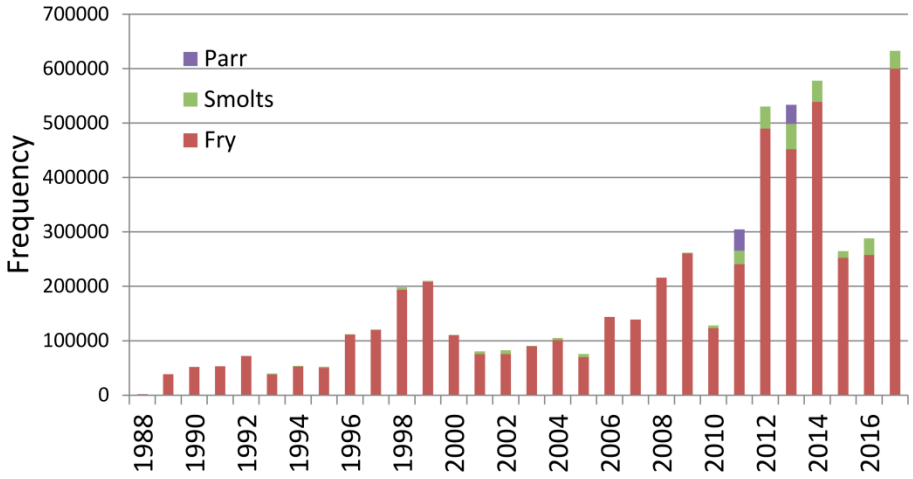

Figure 3: Annual restocking frequencies by life-stage

Over the years, the "Meuse Salmon" initiative also inspected aspects of the fish migration, focusing on smolt downstream migration with different capture and tagging techniques, launched a sperm cryopreservation program to increase the genetic pool of the newly bred salmon using sperm from spawners having completed their life-cycle in the Meuse basin and cartography suitable salmon habitats in various streams (Amblève, Ourthe and Samson).

On September 19th 2015, the Conservatorium of the Meuse Salmon (CoSMos) was inaugurated in Erezée next to the fish farm. It offers a didactic and touristic space showing the salmon's life-cycle, the milestone achievement of the "Saumon Meuse" initiative and the remaining obstacles to the salmon's rehabilitation in the Meuse basin.

1.1.2 Improvement of migrating conditions for fish populations

1.1.2.1 Obstacles clearing

In addition to the "Saumon Meuse" initiative, measures were taken by different services of the Walloon Region to ensure free route continuity for migrating species like the Atlantic salmon or the European eel (*Anguilla anguilla*, L.). Different types of adjustments for clearing a path over obstacles to migration were used, e.g. deflectors, predams, baffle fishway, eel-ramp or even bypass rivers (Figure 4).

Figure 4: Two examples of fish ladder in Belgium.

From 2007 to 2015, a total of 114 edifices were built on 63 different waterways scattered over 11 hydrographic basins. Most of these structures were built for multispecies purposes and not only for the Atlantic salmon, but we may still acknowledge the effort made for free movements of migratory fish. Another project of trapping adults and transporting them upstream of the Coo Falls is currently being studied. This would give access to numerous

favourable reproduction sites for the salmon on the Amblève. In the Namur district, all adjustments on the Lesse and Viroin basins should come to an end in 2017. In early 2016, there were still 2 obstacles on the Bocq where 22 of them have already been dealt with. Adjustments on the Samson stream should also start in 2017.

1.1.2.2 Water quality

An improvement of water quality was also noticed through regional waste water treatment (Philippart, 2014, REEW, 2017). The successful return of the salmon in the Meuse in the early 2000 was observed in a context of three decades of water quality improvement in the Meuse and its tributaries like the Ourthe and the Vesdre. The water quality of the Amblève should also improve as the catastrophic industrial pollution through the Warche has stopped. We should also see a major improvement of water quality of the Meuse in Liège through the opening of two water treatment plants close to Liège as well as other in Wallonia (Table 1) and the lower industrial pollutant discharge (Figure 5). From then on, the quality of surface water should have continued to improve to meet conditions set by the Water Framework Directive 2000/60/CE of the European Union which foresee the achievement in 2015 of a good ecological potential in terms of physico-chemical and biological characterictics, notably of fish. While there will be delays until 2021 or even 2027, the final date to achieve the goals set by the Water Framework Directive (REEW, 2017), the conditions for the salmon return should become more favourable in the coming years.

Table 1: Non-exhaustive list of the largest water treatment plants on the Meuse and its tributaries favouring water quality improvement for the return of the salmon opened in the last two decades. (www.spge.be).

Location	Capacity (EH)	Opening date	River/stream
Oupeye	401850	2007	Meuse
Andenne (Seille)	20000	2009	Meuse
Namur-Brumagne	93100	2011	Meuse
Sclessin	135000	2012	Meuse
Amay	54200	2013	Meuse
Grosses-Battes	53137	2002	Ourthe
Membach	25416	1998	Vesdre
Goffontaine	27000	2004	Vesdre
Wégnez	99000	2002	Vesdre

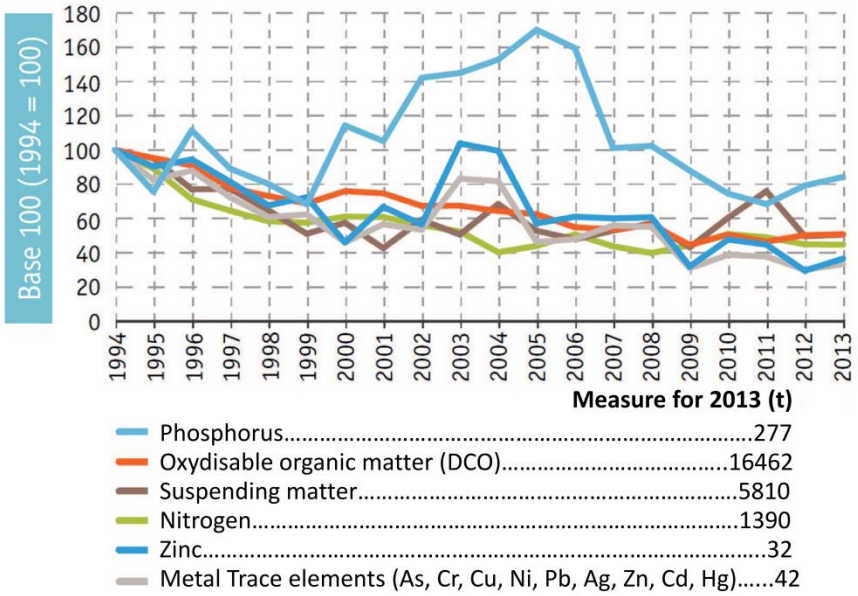

Figure 5: Industrial pollutant discharge in Wallonia in 2013. (SPW - DGO3 - DEMNA - DEE, 2017. Rapport sur l'état de l'environnement wallon 2017 (REEW, 2017). SPW Éditions: Jambes, Belgique.)

Despite all these efforts, results, in terms of adult salmon returns, are objectively poor. In the following section, we will discuss some issues potentially involved in the return of the salmon.

1.1.3 Influencing factors and bottlenecks for the salmon return

As a part of the ongoing restoration program of salmon in the Belgian Meuse basin, a preliminary data base has already been made comprising, environmental data (flow rate, temperature, degree*days,…) as well as stocking details on restocked juveniles (number, locations, strain, life-stages,…) and on adult returns (rates, sizes, strains, date,…). Over 15 years of data have been compiled from several sources, mainly from three universities (ULg-LDPH, UCL-ISV and UNamur-URBE) and numerous governmental agencies of the Public Services of Wallonia (Direction Générale "Agriculture, Ressources Naturelles et Environnement - DGO3 -Département de l'Environnement et de l'Eau - Direction des Eaux de Surface, Département de la Nature et des Forêts - Service de la Pêche, Département de la Police et des Contrôles - Réseau de Contrôle, Département de la Ruralité et des Cours d'Eau - Direction des Cours d'Eau Non-navigables et DGO2 - Département des Etudes et de l'Appui à la gestion - Direction de la Gestion Hydrologique Intégrée). This database already contains a huge amount of data that allow analysing some actions already undertaken for the rehabilitation of the Atlantic salmon but lack of encoding consistency of the rough files made it arduous to find usable items, especially over a large timespan. However, linear modelling

clearly showed an impact of the free migration way. The construction of a fish ladder next to the dam in Borgharen-Maastricht in 2007 finally cleared the way for migrating species, increasing numbers of adult spawners captured in Belgium (Figure 2A). The number of returning adults was also influenced by the amount of restocked parrs three years earlier. No correlation was found between individual returning date and the temperature, flow rate and sex variables but the strain seemed to influence both the size of returning adults and the period of year for capture (Ovidio *et al.*, 2016; Dierckx *et al.*, 2017).

1.1.3.1 Dams and hydroelectric plants

There are 15 dams on the Meuse in Belgium and 7 more in the Netherlands (Figure 6). All are equipped with fishways but numerous are not fit for large migratory fish like the Atlantic salmon. Indirect effect of dams on smolt survival have been underscored as they cause delays in migration (McCormick *et al.,* 2009, Stich *et al.,* 2015) which in turn influences survival rate at sea entry through desmoltifcation. Higher survival rates were measured through free-flowing reaches compared to reaches containing dams and an 8% increase in survival was registered after turbine shutdown at Howland Dam on the Penobscot River (Stich, 2014). Downstream of Namur, dams on the Meuse, and to a lesser extent on the Ourthe, are equipped with hydroelectric plants. Modality to run the turbines, their specifications and the hydrological regime during the migration period greatly impacts mortality rate (Philippart and Sonny, 2003; Philippart *et al.*, 2003). The official threshold in Belgium is fixed at 10% of mortality all species together for the Meuse downstream of Namur. Adequate measures to each case are necessary: special management to run the turbine (i.e. obligation of keeping a constant minimum overflow at dams during critical downstream migration periods), adjustments of bypass ways at hydropower plants, installations of behavioural or mechanical repulsion devices at the water inlet pipes, downstream migration outlets at dams and finally taking these protection measures into account in future building projects. Adequate management of flow and modality to run turbines may greatly favour migration. For example, while other factors may play a role, we might notice that from April to May 2001, water was flowing over the dam in Lixhe for 38 days out of 61 thus favouring downstream migration, resulting in 10 spawners captured the following year. On the contrary, in 2004, all the water flow was running through the turbines except for 48 h out of 61 days of migration and no adult was recaptured in 2005. The fact that fish stay in the Sea for only one year still needs to be clearly proved, though.

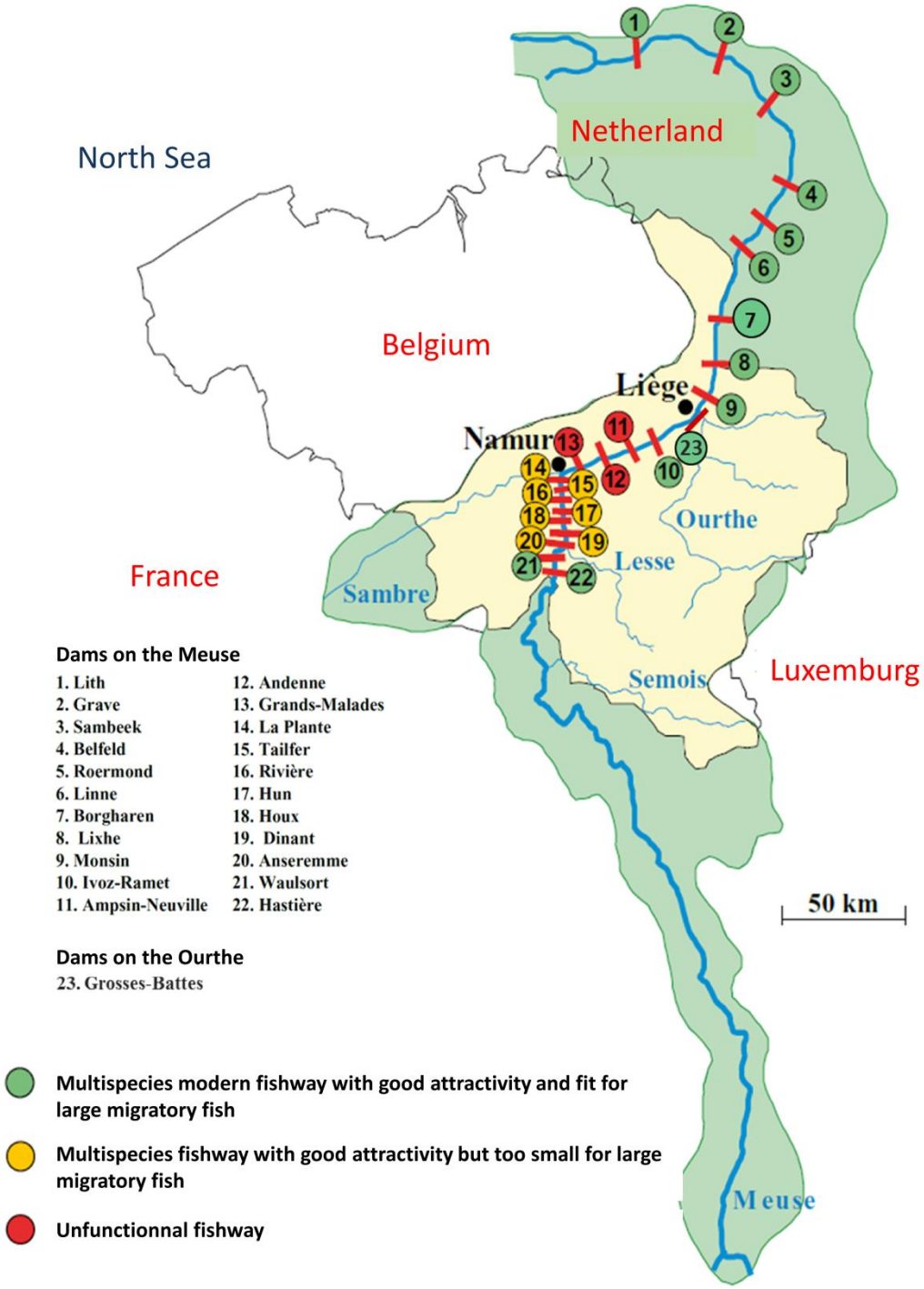

Figure 6: Dams on the Meuse and the Ourthe equipped with fishways.

In addition, behavioural repulsion may be an interesting solution as it showed promising results on cyprinids and salmon (Knudsen *et al.*, 1992; Sonny, 2006). Various mechanical systems exist to limit damage to fish at water entries of facilities; small mesh inlet grid, deflecting grid, inclined grid (Eicher) and rotating grid. Each requires specific water speed and maintenance and their use is usually site specific (Larinier, 2000; Therrien *et al.*, 2000 ; Boubée and Haro, 2003 ; Diana *et al.*, 2003).

Behavioural barriers have also been developed. Tests were made to use sound to repel fish, unfortunately, no multispecies devices could be developed due to species-specific frequencies. Light was also taken into consideration but the efficiency of such a device is strongly influenced by surrounding lights making it unsuitable in most cities (Larinier, 2000; Therrien *et al.*, 2000 ; Boubée and Haro, 2003 ; Diana *et al.*, 2003). Unfortunately, no clear conclusion could be drawn out of tests using mercury vapor lamps as an attraction for smolts in Belgium (Delforge *et al.*, 2003b). Electrical barriers may also be used to stop the progression of fish into an unwanted path. However, this method is suspected to have harmful effects, e.g. gametes impairment (Larinier, 2000; Therrien *et al.*, 2000 ; Hadderingh and Bruijs, 2002; Boubée and Haro, 2003). An air bubble curtain was also proposed but its efficiency is highly subjected to light, temperature, turbidity and flow rate which limit their use to a complementary method.

Bypass systems were studied to equip dams in Belgium. Migrating fish may then avoid injuries inflicted through the turbine blades. In the late 1990's, first tests were launched in Lixhe, the last Belgian dam upstream from the Dutch border, and one of the most dangerous for smolts in whole Wallonia with a theoretical mortality rate of 9% for smolts (Philippart *et al.*, 2003). In 1999, an experimental drain was added to the dam. It was placed so that the channel originally used for the turbine inlet trash evacuation could be used as a migration channel (Figure 7).

Construction details may be found in Prignon and Micha (1998). To evaluate its efficiency, tagged fish were released upstream of the dam. A trap at the end of the deviation helped numbering the fish successfully using the deviation channel. Figures were encouraging but needed improvement particularly because of the waste accumulation in the channel, blocking or impeding free movement of migrating fish (Prignon and Micha, 2000-2002; Delforge *et al.*, 2003a,b, 2004). In 2006, trappings were suspended and a chute was added ending directly downstream of the dam. Recently, in 2015 and 2016, smolts were equipped with RFID tags for an accurate following of their movement between the Ourthe and the Meuse and across the border in the Netherlands. This experiment showed a confused behavioural pattern with up-

and downstream swimming of the smolts between the Ourthe and the Meuse, especially upstream of the dams of Monsin and Lixhe (Ovidio *et al.*, 2016, Dierckx *et al.*, 2017).

Since 2012, migrating smolts are intercepted on the Ourthe and are directly transferred in the lower Berwinne downstream of the dam in Lixhe. Such a method is hardly without flaw, causing excessive stress through capture, transport and manipulation and its positive effect in term of adult returns still lacks proper investigation in Belgium.

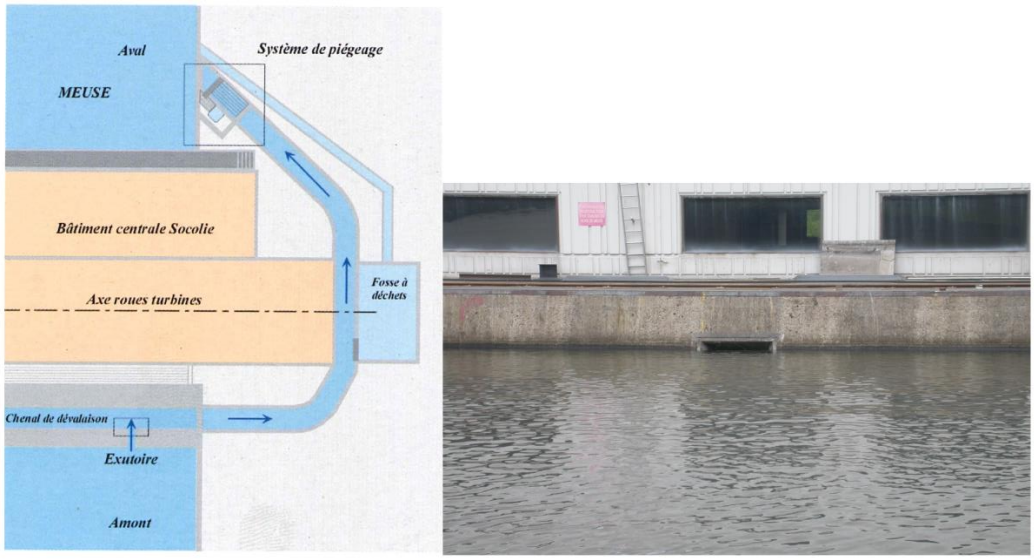

Figure 7: Drawing of the migration channel in Lixhe and picture of its entry.

In October 2017 the EC-Life « Life4Fish » initiative was launched which goals are to optimize the type and running of turbines in order to reduce their impact on migratory fish (Atlantic salmon and European eel) in the Meuse River.

To give access to the French part of the Meuse to migratory fish species, there will have to be a radical solution for overcoming the dams of Ampsin-Neuville, Andenne and Namur Grands Malades. A project was being studied for the dam of Ampsin-Neuville in 2016. Outdated fish ladder built in the 1970's on the mobile dams of La Plante, Tailfer, Rivière, Hun, Houx, Dinant and Anseremme should also be updated. Some structures should then also be built on the most attractive tributaries, like the Samson in Belgium and the Viroin, the Houille and the Semois in France.

1.1.3.2 Smolts deviation towards the Albert Canal

In 1939, the Albert Canal was opened in Belgium. Originally built to connect Walloon and Flemish ironworks with coal mining in the Limburg, this 130 km long channel stretches

between the autonomous harbour of Liège and the harbour in Anvers. To overcome a 56 m difference in level between those two cities, 6 locks were built in Wijnegem, Olen, Kwaadmechelen, Hasselt, Diepenbeek and Genk. A link with the city of Maastricht exists through 4 locks in Lanaye. To carry an average of 40 million tons of goods every year, a minimum flow of 40 m^3*s^{-1} to 80 m^3*s^{-1} is maintained during daytime (Figure 8). We may point out three potential issues deriving from the channel.

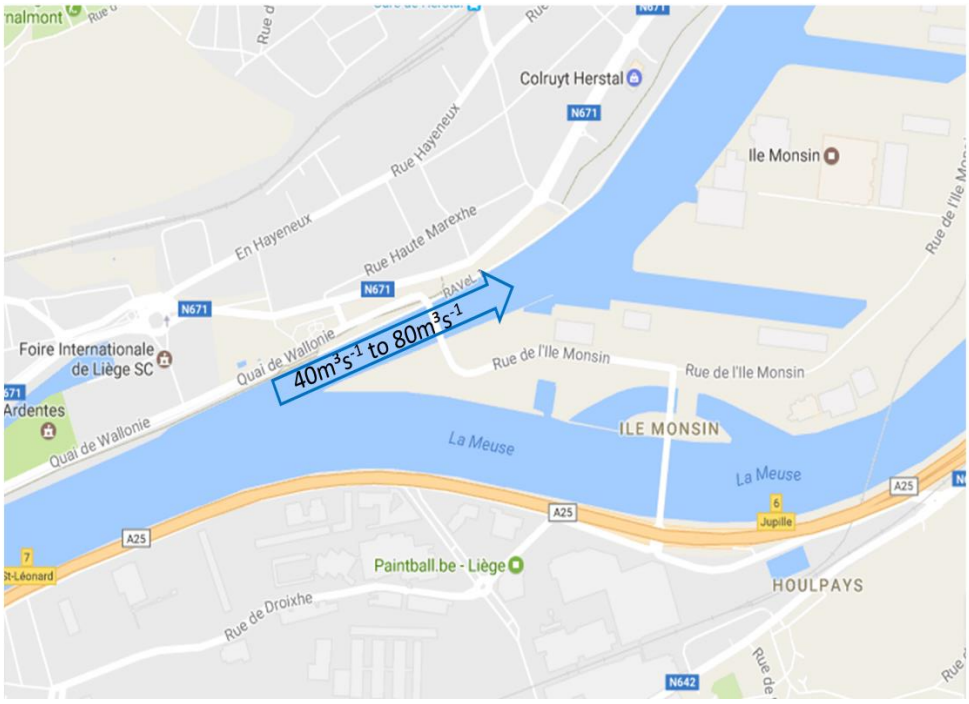

Figure 8: Albert Canal in Liège. (Google map, April 2017).

Firstly, to maintain a minimum flow, the flow in the main channel of the Meuse may be diverted towards the Albert Canal. In the early 2000's the comparison of capture figures from the trap on the Samson and in Lixhe showed that only very few smolts from the tributary reached Lixhe which is on their natural migration route. It appeared that the flow rate of the Albert Canal is sometimes equal or higher than that of the Meuse main channel. Whether smolt migration is passively entailed by current (Thorpe *et al.*, 1981; Tytler *et al.*, 1978) or if smolts actively swim (Davidsen *et al.*, 2005; Hansen and Jonsson, 1985) and seek high velocity areas (Jonsson *et al.*, 1991), the Albert Canal may constitute a more attractive route to them. Unfortunately, this would lead them through a series of locks delaying their arrival at sea. A longer stay in freshwater would expose smolts to riverine predators over a longer period (Zydlewski *et al.*, 2010) and may also hasten desmoltification (Muir *et al.*, 1994; McCormick *et al.*, 1999). During the downstream migration back in 2005, 14 smolts were captured in the Samson and the water cooling bypass from Tihange nuclear plant, equipped

with radio emitters and set free in the lower Ourthe, upstream from the Grosses-Battes dam. This telemetry survey showed that half the smolts entered the Albert Canal in case of a flow rate lower than 150-200 m^3/s in the Meuse in Liège. This study was repeated in 2006 with 12 smolts from which 9 favoured the Albert Canal (Philippart *et al.*, 2007).

Secondly, as the channel is used for inland navigation, flow inversion may happen, potentially confusing smolts during their downstream migration as they follow the flow (negative rheotactism), causing again delays in sea arrival.

Thirdly, low levels of dissolved oxygen ($<6mg*L^{-1}$) have been repeatedly measured (2002, 2003, 2008 and 2012) in the Meuse downstream of Liège during low water from June to October. This situation is linked to low flow at that period of the year for a natural reason (oceanic pluvial regime) but also due to the influence of human activities; the water intake for the Albert Canal and the low reoxygenation of the water through overflow at the Monsin dam mark the start of a uniform canalized reach of 13.5 km long towards Lixhe. A better management of water use on that stretch of the Meuse may help improve upstream migration conditions of salmonids between Lixhe and the confluence with the Ourthe when river hydrology is particularly low during the critical period of September to November.

1.1.3.3 Free migration and perspectives

In 1996, a Benelux decision stated the restoration of free fish migration from the Ourthe to the North Sea for 2002 as a primary target. In Wallonia, this was achieved lately in 2009 with the Grosses-Battes dam on the lower Ourthe being equipped with a fish ladder. Internationally speaking, the last Dutch dam equipped with a fish ladder was the one of Borgharen – Maastricht in late 2007. Since then, up to 2015, a sevenfold increase in the number of adults recaptured in the Belgian Meuse was registered. In comparison, from 2002 to 2007, only 16 salmon had been captured in Belgium. Furthermore, there are estimations of one in a hundred adults in the Meuse estuary reaching Belgium. However, a promising new management of the sluice gates of the anti-storm dams of the Delta Plan in the Meuse estuary (aka Haringvliet), which is hardly passable for returning adult salmon, will be established shortly. The Dutch government promised to open the gates almost totally from 2018 onwards, greatly easing the upstream migration of fish.

Concerning the rivers Ourthe, Vesdre and Amblève, there are still obstacles like the dams in Méry and Fêchereux that should be made passable. A fish ladder project should indeed be studied by the hydro-electrician on the latter site. These adjustments should then open the way

to the Ourthe upstream of Esneux, to the Amblève in Comblain-au-Pont and to the Aisne in Bomal.

1.1.3.4 Temperature shift and climate change

As previously said, anthropic activities have a considerable impact on river characteristics and it is easy to conceive that a lot of pollution sources may influence water quality of this river (Van Vliet *et al.*, 2008). One of the main consequences of industrialization and pollutant releases is an increase in temperature of certain rivers and streams as it is for the Meuse (Malbrouck *et al.*, 2007). For example, a study by Kirchmann (1985) on the impact of rejects of the power plant of Tihange, in Belgium, identified an increase of temperature linked to these rejects. As early as 1983, an increase of 2°C in mean temperature was measured downstream the plant compared to upstream. Indeed, annual temperature levels fluctuated between 1 and 25°C upstream and between 3 and 27.1°C downstream of the plant (Figure 9). Moreover, environmental data showed a difference regularly exceeding 4°C between the Meuse in Lixhe and the Ourthe in Méry

The power plant in Tihange is not the only cause for the increasing temperature in the Meuse. Other sources of organic and inorganic pollutants rejected punctually or in a diffuse manner may also spoil physico-chemical characteristics of the water, notably the temperature. These pollutants may come from industries, agriculture or from each of us. A study by the Conservatoire National du Saumon Sauvage in Chanteuges (Loire, France) showed that above a threshold temperature of 17°C, swimming speed of migrating smolts decreased by 80% and above 20°C, smolts exhibited positive rheotactic behaviour (Martin *et al.*, 2012). This same strain is used in Belgium for restocking actions and in May, temperature of the Meuse reaches regularly above 18°C which may then greatly influence the success of smolt migration.

In the Atlantic, milder and wetter winters have been predicted as a consequence of climate change, with more precipitation falling as rain and less as snow, decrease in ice-covered periods and frequent periods with extreme weather (IPCC, 2007). A decade ago, several reviews summarized likely effects of climate change on survival, developmental rate and disease resistance in migrating salmonids (Crozier *et al.*, 2008; Jonsson and Jonsson, 2009; McCormick *et al.*, 2009). Discussions were mainly focused on the freshwater environment as little is known about migration pathways in the ocean (Crozier *et al.*, 2008). Climate change may not have a direct lethal impact on smolts; however, by altering temperature and migration timespan, it will negatively affect smolt survival (McCormick *et al.*, 2009). Smolting is a size-dependent process and growth is strongly influenced by temperature. Decreased smolt age is

expected in populations where optimal growth temperature has not been exceeded. A northward shift of the populations has been suggested with extinction in the southern parts of the geographic range of the species (Jonsson and Jonsson, 2009). Many other consequences have been suggested, i.e. earlier migration, later spawning and sexual maturity and increased disease susceptibility and mortality (Jonsson and Jonsson, 2009). In the River Bush, Northern Ireland, large time series permitted to model seaward migration over decades and to point out earlier emigration of smolts (Kennedy and Crozier, 2010). Moreover, smolt migration was correlated with river temperature and potential thermal mismatch between fresh- and seawater was suggested for lower survival in early migrating fish (Kennedy and Crozier, 2010).

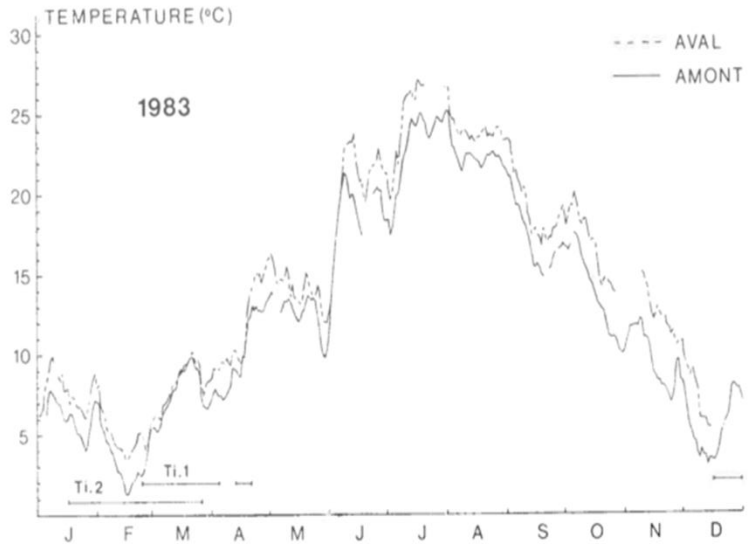

Figure 9: Mean daily temperature of the Meuse upstream (Live-sur-Meuse) and downstream (Ampsin-Neuville) of Tihange in 1983 (Kirchmann, 1985)

More recently, spatio-temporal variations of smolt migration in 67 rivers across the North Atlantic over five decades showed an earlier migration at the average rate of 2.5 days per decade (Otero *et al.*, 2014). These results match modifications in air, river and ocean temperature leading the authors to suggest that salmon population are responding to the current global climate changes (Otero *et al.*, 2014). Decreased migration size and up to 10 days earlier migration have been reported from 2000 to 2014 in the Simojoki River, Nothern Baltic Sea (Jokikokko *et al.*, 2016). Earlier migration was linked to increased air temperature at a nearby airport. Authors concluded that smolt emigration started when a suitable water temperature was reached independently of the date (Jokikokko *et al.*, 2016). Global climate

change will influence multiple ecosystems and cross-ecosystem studies are crucial for understanding how climate change will influence ecology of species. Based on global climate models, increased terrestrial primary production have been predicted which will in turn cause higher primary production in lakes. As a consequence, decreased occurrence of anadromy in Arctic char populations in Norway was predicted (Finstad and Hein, 2012). While earlier migration in response to climate change has been described by several authors, genetic data identifying the role of evolution in this timing alteration remains rare (Manhard *et al.*, 2017). First evidence that the trend toward earlier migration may reflect an adaptation to warming sea-surface temperatures was reported in pink salmon (*Oncorhynchus gorbuscha*) by monitoring allozyme alleles to differentiate early and late migrants over 14 generations (Manhard *et al.*, 2017). Other evidence point out that climate change related decrease in dissolved oxygen and increased temperature will result in vertical habitat contraction for the Atlantic salmon (Stehfest *et al.*, 2017). In addition, specific stock hypoxia tolerance thresholds and environmental conditions of an area would be most useful tools to estimate stocking densities (Stehfest *et al.*, 2017).

1.2 The Atlantic salmon

The Salmonidae family is divided into three sub-families, namely Coregoninae, Thymallinae and Salmoninae (Nelson, 1994). More recently, mitochondrial genome study provided evidence of the Coregoninae being the ancestral group in the Salmonidae family and the Thymallinae and Salmoninae two sister groups (Yasuike *et al.*, 2010). Within the Salmonidae, we distinguish 66 species dispatched among 11 genuses (Nelson, 1994). Salmonidae are principally present in holartic regions comprising North America, Europe and Asia, North of the tropic of Cancer (Nelson, 1994; Hutchings *et al.* 2002).

Most salmonids are of medium size (maximum 1,5m) cylindrical shape slightly laterally flattened (Scott *et al.*, 1973). The lateral line is well visible, the body is completely covered in cycloid-shaped scales with the exception of the head, an adipose fin is present between the dorsal and caudal fins and the three last caudal vertebrae are inverted (Kottelat and Freyhof, 2007). Salmonids may be differentiated using morphological traits like body shape, dentition, mouth position or fin size and life history traits like growth rate, maturity age or fecundity (Lindsey, 1981). Different salmonid species, characterized by different morphology, each of which specific to an ecological niche and thus a feeding regime, may cohabitate in the same environment. Those exploit prey from different trophic levels like zooplancton, zoobenthos or small fish (Lindsey, 1981).

Historically, in Carolus Linnaeus' *Systema Naturae*, the genus *Salmo* regrouped 4 species: brown trout (*Salmo fario*), Common trout (*Salmo trutta*), Seatrout (*Salmo eriox*) and the Atlantic salmon (*Salmo salar*). Nowadays, only two species are still regrouped in the *Salmo* genus, namely the common trout (*Salmo trutta*) and the Atlantic salmon. However, more than 60 varieties of common trout and 27 of Atlantic salmon are known of, differing by specific adaptations to habitats they may live in (Bruslé *et al.*, 2001; Kottelat and Freyhof, 2007). Most of the observed phenotypic variations between salmonids come from genetic variations, environmental signals and the interaction of both factors (Langerhans, 2008). Keeley *et al.* (2007) showed that genetic would have a stronger influence than environment in determining *Salmo* species' phenotype. This phenotypic variability was maintained through time because of important geographical isolation between populations (Jonsson and Jonsson, 2011).

Salmon have a particular life-cycle (Figure 10). During winter, when water is cold and more oxygenated, salmon spawn in the upper parts of river basin after completing their migration from the sea (Gueguen *et al.*, 1994; Malbrouck *et al.*, 2007). In spring, fry hatch and remain in the substrate until vitellus resorption (Malbrouck *et al.*, 2007). After an emergence phase, fry may then feed by themselves and colonize shallow river stretches with high flow velocity

(Malbrouck *et al.*, 2007; Tsukamoto *et al.*, 2013). They are now known as parr (McCormick *et al.*, 1998; Malbrouck *et al.*, 2007; Jonsson and Jonsson, 2011). These parr may choose two strategies; some will remain in freshwater and become sexually mature parr, so-called sneakers, and use a greater part of resources in reproduction, other will become smolts (Figure 11) through a complex process called smoltification and swim towards the sea (Hoar, 1988; McCormick *et al.*, 1998, 2013).

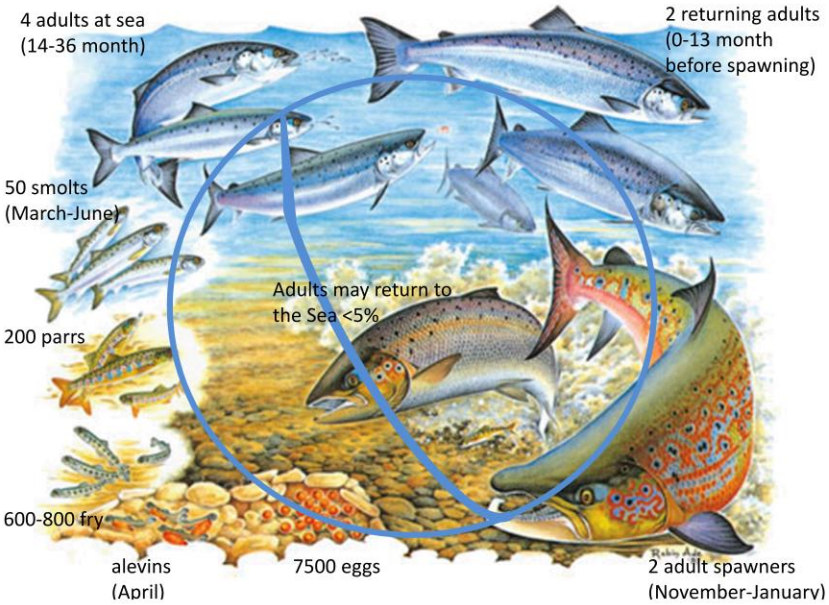

Figure 10: The Atlantic salmon's life cycle from egg to adult spawners (www.nasco.int modified according to Malbrouck *et al.*, 2007)

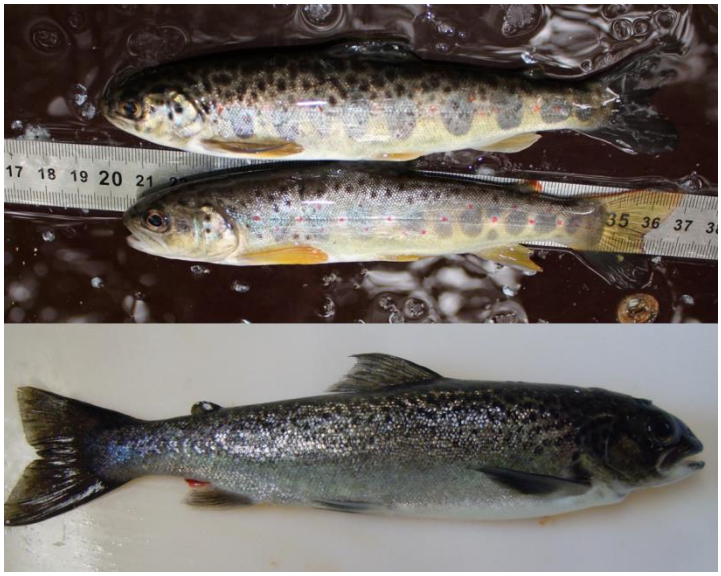

Figure 11: Atlantic salmon and brown trout parrs and Atlantic salmon smolt (top-down). (Pictures from Martine Fossion and Benoît Bernard)

Sneakers may stay in the river for years and sneak in between mature adults returning from the sea during spawning time (Jonsson and Jonsson, 2011). This strategy may also lead to hybrids with common trout (Gueguen *et al.,* 1994; Garcia-Vasquez *et al.,* 2002). The vast majority of parr will smoltify and migrate downstream towards the Atlantic Ocean. It's during smoltification that fish take an olfactory imprinting of the river which will help them come back to their natal river for spawning. This phenomenon is known as homing (McCormick *et al.,* 1998; Malbrouck *et al.,* 2007; Tsukamoto *et al.,* 2013). In seawater, salmon will continue their migration to feeding grounds close to Greenland and the Faroe Islands. Most of the energy intake is then used for growth (McCormick *et al.,* 1998). Finally, sexually mature adults will migrate back to freshwater spawning sites (Gueguen *et al.,* 1994; McCormick *et al.,* 1998; Jonsson and Jonsson, 2011).

The pressure through natural selection is very high on the salmon life cycle (Figure 10). From 7500 eggs, only 600 to 800 fry will emerge from the spawning nest. Parr will then compete for feeding grounds. Usually, they try to avoid this competition by changing the development site which in turn increases predation risk. Only 200 parr will survive this predation from which only 50 will undergo smoltification. After the downstream migration, only four smolts will enter the estuary. Finally, 2 adults will come back to the spawning site (Malbrouck *et al.,* 2007). It is important to state that this scenario is only true in a pristine medium which is seldom in Nature nowadays.

Some salmon populations or other salmonids called 'landlocked' or 'Ouananiche' have a life-cycle restricted to rivers and lakes. This particular life-cycle would originate from a single or double recolonization of some lakes as a consequence of an ice age; the way to the sea being blocked by ice. These populations evolved to gain some specific adaptations of non-salty environments (Tessier *et al.,* 2000). In general, these populations live in large lakes and swim upstream in rivers and streams to spawn (Tessier *et al.,* 2000; Hutchings *et al.,* 2002).

1.3 Migration

Migration is a common phenomenon in animal kingdom. They may be complex and vary among species but their ultimate goal is to maximize the species fitness (Jonsson and Jonsson, 2011). A migration is defined as a long-distance movement made by many individuals in an approximatively common direction and at the same time of the year (Endler 1977). Migrations are under genetic control (Northcote 1981; Svardson and Fagerstrom 1982; Jonsson 1982; Kallio-Nyberg *et al.* 2002), but may be modulated by environmental cues experienced by the fish such as temperature and water flow extremes (Jonsson and Jonsson, 2011).

In the case of migration in the aquatic environment, one should not consider a species capacity to migrate based only on its locomotion system (Hutchings *et al.*, 2002). Indeed, migrations will be mainly ruled and directed by environmental factors such as depth, flow, temperature and dissolved oxygen. Thus, migration may be defined as an adaptive strategy implicating a movement of a part or the whole population in time between distinct sites of a hypervolume with n dimensions, in which each dimension represents a biotic or abiotic factor of the ecological niche (Hutchings *et al.*, 2002). Disregarding inter-population variability, migrations are synchronized and predictable.

According to Jonsson and Jonsson (2011), migrations result from complex interactions from environmental biotic and abiotic factors with the genetic background, predefined to give migrating fish specific morphological, physiological and behavioural characteristics. Furthermore, migration may be passive, when individuals are transported by the flow, or active, when individuals start and steer their own movement.

1.3.1 Vertical migration

Vertical migrations are defined as synchronised diel movements among a water column (Brierly, 2004). During these migrations, fish swim up to the surface at night and dive into the depth during the day. Movement amplitude and general population distribution are different among species and may be very complex. Migration may also be influenced by the developmental stage of the fish and by factors such as turbidity, temperature and food quantity/availability (Johansson *et al.*, 2006; Oppedal *et al.*, 2007; Fore *et al.*, 2009). Such movements are thought to decrease predation risk and maximize yield linked to feeding search (Hutchings, 2002; Scheuerell and Schindler, 2003). However, this evolutionary strategy is energetically costly as energy allocated for movement cannot be used for

development or reproduction. In general, lower growth rate and fecundity in migrating species have been observed (Lampert, 1989).

1.3.2 Horizontal migration

Horizontal migrations are widespread in the aquatic world. They are an integral part of the life-cycle of numerous species and are essential to fulfil some primary needs like reproduction, growth and food search (Jonsson and Jonsson, 2011). Northcote (1978) defined 3 types of functional habitat used during migration; each species-specific and reflecting its needs. Horizontal migration may be summed up to all the movements between these 3 habitats (Figure 12).

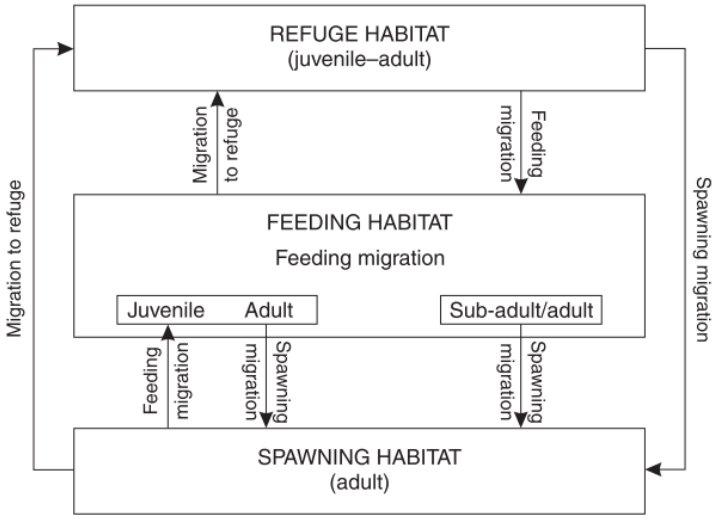

Figure 12: Schematic representation of horizontal migration across 3 types of functional habitats (Northcote, 1978)

More recently, a larger definition to horizontal migrations was given. There are 3 types of movements depending on the medium crossed, the direction of motion and the developmental stage at which migration takes place. Thus, we distinguish between anadromous, catadromous and potamodromous migrations (Yeh, 2002; Hutchings *et al.*, 2002; Tsukamoto *et al.*, 2013). We may add that in small streams, parr may swim between fresh- and brackish water in order to evade summer drought, thus exhibiting amphidromous behaviour (‘amphi’ means ‘both’ and ‘dromos’ means ‘running’ in Greek). Salmon are best known for their diadromous migration (‘dia’ is ‘between’ in Greek) between salt- and freshwater. Migratory species spending part of their life-cycle in freshwater and part in saltwater are known as eurybiotic (Yeh, 2002; Hutchings *et al.*, 2002). These species will undertake anadromous or

catadromous migrations and are capable of coping with environmental changes encountered along their migration route.

1.3.2.1 Anadromous migration

Anadromous ('ana' means ‘up’ in Greek) species have a life-cycle characterized by a high growth rate and maturation in seawater (Figure 13). Once sexually mature, adults will return to freshwater, swim upstream towards small streams and spawn. Juveniles will spend their first life-stages in freshwater and then migrate downstream towards the sea (Yeh, 2002; Hutchings *et al.*, 2002; Jonsson and Jonsson, 2011).

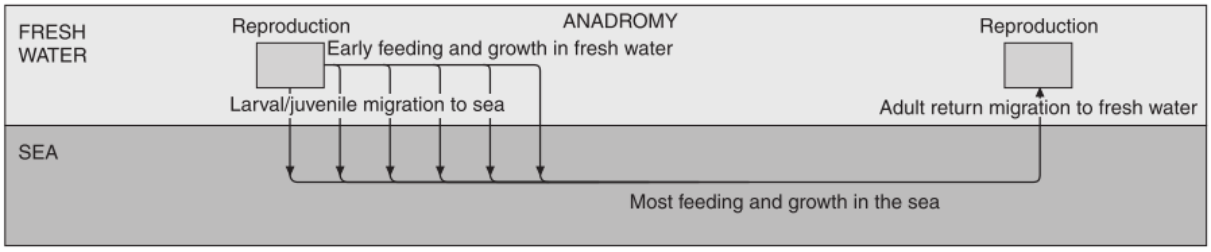

Figure 13: Schematic representation of anadromous species life-cycle (Hutchings *et al.*, 2002)

According to Yeh (2002), there are approximately 100 anadromous fish species. Among them, we find the Atlantic salmon, the brown trout and the European sturgeon (*Acipenser sturio* L.). Most populations of Atlantic salmon and brown trout are considered migratory (Jonsson and Jonsson, 2011). This migration is supposed to be adaptive, facultative and not compulsory step of the life-cycle of some salmonid populations (McDowall, 1988; Jonsson and Jonsson, 2001). Costs linked to physiological changes and increased mortality in seawater, especially during the first developmental stages may be an explanation for it (Thorpe, 1994; McCormick *et al.*, 1998; Yeh, 2002; Jonsson and Jonsson, 2009).

1.3.2.2 Catadromous migration

Catadromous ('cata' means ‘down’ in Greek) species reproduce and are born in saltwater but mainly develop in freshwater (Figure 14). Adults will start a downstream migration towards spawning site at sea (Yeh, 2002; Hutchings *et al.*, 2002; Jonsson and Jonsson, 2011).

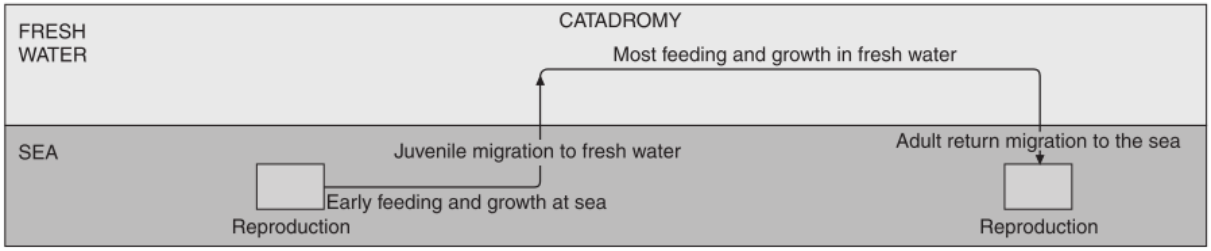

Figure 14: Schematic representation of catadromous species life-cycle (Hutchings *et al.*, 2002).

The best known group of catadromous species are eels including notably the European eel (*Anguilla anguilla* L.) which migrates across the Atlantic Ocean to the Sargasso Sea to reproduce and die (Tsukamoto *et al.*, 2013; Rigaud *et al.*, 2015).

1.3.2.3 Potamodromous migration

A migration between different areas in freshwater is called potamodromous migration as 'potamo' means 'river' in Greek (Hutchings *et al.*, 2002). Such holobiotic migrations are specific to species spending their entire life-cycle in the same environment (Yeh, 2002).

1.3.3 Smolt migration and influencing factors

Smolt migration pattern are not universal. Once the migration starts, most smolts will have entered the estuary within days or weeks but migration (or at least a downstream dispersal) may start a whole year preceding SW entry in some populations of long river systems (Rimmer *et al.*, 1983).

It was suggested that migration takes place only when fish are in the appropriate physiological state and simultaneously under the influence of environmental cues acting as 'releasers' (Baggerman, 1960). Factors influencing the downstream migration of smolts were subdivided into two categories by Byrne *et al.*, (2004). First, regulating factors act before the migration and affect physiological modifications linked to the smoltification and second, controlling factors have an effect during the migration by governing physical processes of migration; e.g. downstream swimming speed. Photoperiod and temperature influence preparatory modifications leading to a "migratory readiness". Environmental factors like water temperature, flow and turbidity then play a role as releasing factors for the initiation of smolt downstream migration (Jonsson and Jonsson, 2011; McCormick, 2013).

1.3.3.1 Photoperiod

Photoperiod modification is considered as a major cue for initiating migration (McCormick *et al.,* 1998; Jonsson and Jonsson, 2011; Melo *et al.,* 2014). In general, seaward migration starts in spring with increasing daylength (McCormick *et al.,* 1998). Rate of photoperiod change seems to be more important than daylength itself (Wedemeyer *et al.,* 1980). Fish held under constant light don't develop osmoregulatory mechanism typical for smoltification and conserve typical parr behaviour (McCormick *et al.,* 1987; Handeland *et al.,* 2013). On the other hand, increasing daylength during winter and spring had a stimulating effect (McCormick *et al.,* 1987).

1.3.3.2 Temperature

Temperature and more specifically accumulated thermal units or degree*days strongly influence the start of Atlantic salmon smolt migration (Figure 16; Zydlewski *et al.,* 2005). A highly accurate model of Atlantic salmon smolt migration was created using the spring water temperature curve on the river Imsa (Jonsson and Ruud-Hansen, 1985). A positive correlation was also found between the initiation of Arctic char smolt migration and water temperature (Jonsson and Antonsson, 2005) and earlier onset of smolt migration of Sockeye, Atlantic and Chinook salmon, in case of increased average temperature was reported (Foerster, 1937; Melnikova, 1970; Achord *et al.,* 2007; Otero *et al.,* 2014; Stich *et al.,* 2015, Jokikokko *et al.,* 2016). Migration over a shorter timeframe was also observed in warmer years compared to colder ones (Zydlewski *et al.,* 2005). Earlier sea arrival was recorded for fish with warmer thermal history (Stich *et al.,* 2015). Variations exist among salmonids, i.e. under natural conditions, Atlantic salmon smolts usually start their downstream migration before water temperature reaches 15°C (Jonsson and Jonsson, 2011), whereas the Pacific salmon is still smoltifying at this temperature (Wedemeyer *et al.,* 1980).

Temperature may also have deleterious effects on migration. Reduced swimming speed at 17°C and positive rheotaxis over 20°C were observed (Figure 15; Martin *et al.,* 2012). Furthermore, migration termination was induced at elevated number of degree*days (Zydlewski *et al.,* 2005). Other potential impacts of temperature changes, as a consequence of climate change, on smolt migration and survival have been previously described (see 1.1.3.4.).

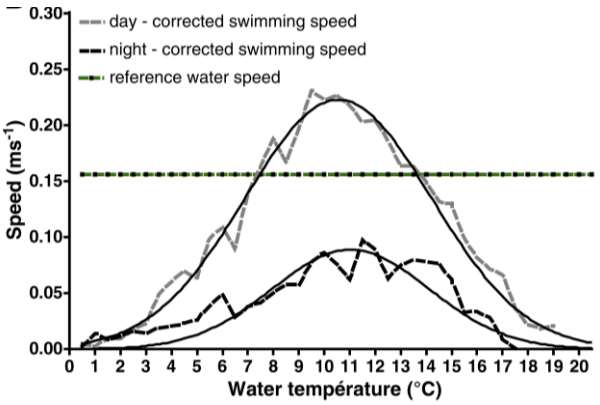

Figure 15: Relation between diurnal and nocturnal swimming speed and temperature after correction using constant water flow in the tank as reference. Non-linear Gaussian curve were used (diurnal: mean 10.55, SD=3.658, r2=0.91 and nocturnal: mean 11.04, SD=3.084, r2=0.48) (Martin *et al.*, 2012)

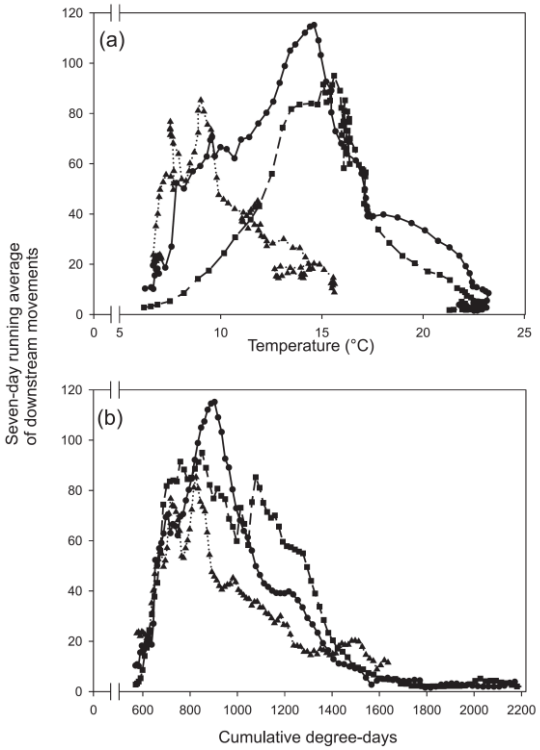

Figure 16: Seven-day running average of the number smolt swimming downstream under three temperature regime (circles, ambient; squares, advanced; triangles, delayed) in relation to (a) temperature and (b) cumulative degree*days since January 1 (Zydlewski *et al.*, 2005)

1.3.3.3 Flow and turbidity

Water temperature and flow, as well as their variations are major controlling factors of the smolt migration (Jonsson and Jonsson, 2009). High water flow appears to initiate Atlantic salmon smolt migration in some rivers (Österdahl, 1969; Hesthagen and Garnås, 1986; Hvidsten and Johnsen, 1993) or to stimulate migration of Chinook salmon smolts in March (Connor *et al.*, 2003). A positive correlation between the number of migrating smolts

(Atlantic salmon and Arctic char) and increased water level was reported in the Norwegian River Hals (Carlsen *et al.*, 2004). High water level, high turbidity and high water velocity may reduce predation risk and thus favour migration (Abrahams and Kattenfield 1997). Durif (2003) indicates that eel migration, in opposition with salmonids, is more based on the flood rhythm. Eels take advantage of increased flow rate, thus considerably limiting energy expenses. This hypothesis is still being debated by numerous authors who look at rainfall as a confusing factor (Van Ginneken *et al.*, 2007).

1.3.3.4 Predation

Predation may have various influences on migration and be a force driving adaptive behaviour (Jonsson and Jonsson, 2011). The abundance and relative effectiveness of predators may affect age at migration through negatively influencing growth because increased predation risk often suppresses foraging activity and thus diminishes food intake (Lester *et al.*, 2004). There is also evidence of compensatory post-smolt growth shortly after entering seawater as an adaptation to reduced predation on smolt from the Miramichi River (Friedland *et al.*, 2009). Synchronous smolt migration to form large schools could have the adaptive value to confound predator by sheer number (McCormick, 2013). Throughout the migration, a clear pattern of migration suppression at dawn and dusk was observed in the River Frome, England. To explain such behaviour, authors hypothesized an active decision and/or an adaptive strategy for avoiding feeding predators. Smolt may also benefit from increased invertebrate drift as a food resource at these times of day (Ibbotson *et al.*, 2006).

1.3.3.5 Strain influence and genetic basis

In Atlantic salmon, some authors proved a genetic relation between the timing of adult migration into freshwater and stock origin both among (Hansen & Jonsson 1991) and within (Stewart *et al*. 2002) river systems. After the introduction in the Connecticut River system (USA) of fry from three different sources, strain-specific differences in downstream migration timing have also been reported (Orciari and Leonard, 1996). Similarly, seaward migration timing differences were seen between stocks from different rivers after their relocation in restoration programmes (Nielsen *et al*., 2001) Comparing seaward migration timing after transferring eggs from a low catchment tributary to a high catchment tributary of a large river (River Tay, Scotland) and vice versa provided evidence of stock-specific timing and of a genetic influence for downstream migration in Atlantic salmon even at sub-catchment level (Stewart *et al.*, 2006).

Specific regions on three chromosomes of *Oncorhynchus mykiss* harboured 7 quantitative trait loci for migration-related traits (Hecht *et al.*, 2012). A phylogeny of the degree of anadromy and size at development of salinity tolerance has been created in the sub-family Salmoninae, showing increased anadromy in more recently evolved species and that there has been a heterochrony in the size (and age) of smolt development; trends towards smaller size at smoltification accompanied by earlier development of salinity tolerance (McCormick, 2013). However, a modification of expression of genes does not mean that the process is solely under genetic control (Ferguson, 2006), especially as there is evidence of phenotypic plasticity for migration trait as juveniles may leave the nursery area under poor nutritional conditions or intense predation (Forseth *et al.*, 1999; Jonsson and Jonnson, 2009). Recently, first evidence of epigenetic modifications influencing life history differences associated with migration-related traits between resident and anadromous *Oncorhynchus mykiss* have been reported (Baerwald *et al.*, 2016).

1.4 Smoltification

"Smolting is an adaptation not just to survive in seawater, but to thrive in seawater" (R.L. Saunders, personal communication to S.D. McCormick in McCormick, 2009).

Smoltification is a pre-adaptation to ocean life and regroups a large array of physiological, morphological and behavioural modifications which help smolts to migrate to and live in seawater (McCormick *et al.*, 1998). These modifications happen prior to and during the downstream migration to the ocean (Bœuf, 1993 ; McCormick *et al.*, 1998 ; Nilsen *et al.*, 2007). Hereafter, we provide a summary of changes happening during smoltification and silvering (Table 2).

Table 2: Comparison of principal modifications (non exhaustive list) during smoltification of the Atlantic salmon and silvering of the European eel (McCormick *et al*., 1998, McCormick, 2013, Durif *et al*., 2005, 2008)

Species	Atlantic salmon	European eel
Migration type	Anadromous	Catadromous
Morphological changes	-Silvering -Darkening of caudal and pectoral fins -Elongated body shape -Increased swim bladder size	-Silvering -Darkening of pectoral fins and dorsal face -Increased eye size -Increased swim bladder size
Behavioural changes	-Schooling -Negative rheotaxis -Near surface migration -Impregnation	-Schooling -Stop feeding -Increased locomotor activity
Physiological changes	-Increase in olfactory sensibility -Hypo-osmoregulation -Increase in lipid and carbohydrate catabolism -Increase in protein synthesis	-Increase of olfactory sensibility -Gonad development -Demineralisation

1.4.1 Morpho-anatomical and behavioural modifications

1.4.1.1 Morphological modifications

The principal morphological change in migrating species is the appearance of silver body-colour masking the dark vertical marks of parr in salmonids (Folmar and Dickhoff, 1980; Wedemeyer *et al.*, 1980; Fontaine, 1994; McCormick *et al.*, 1998; Durif *et al.*, 2005; Van Ginneken *et al.*, 2007). This colour alteration is a consequence of the deposition of guanine and hypoxanthine crystals in the dermis. These two purines are metabolic by-products of

protein synthesis. In parr, they are stocked in two slim and distinct skin layers, the first directly under the scales and the second adjacent to body muscles. These layers broaden and become more visible in smolts. During smoltification, there is also a drop in the relative fraction of hypoxanthine relative to guanine (Folmar *et al.*, 1980; McCormick *et al.*, 1998). The change in colour, from dark parr to light silvery smolt colours, functions as cryptic coloration in open water (Hoar, 1988). Reflective silvering (scales acting like tiny mirrors reflecting incoming light) is common to many pelagic fishes and is presumably adaptive for predator avoidance (McCormick *et al.,* 1998). In addition, the dark back of smolts and their white belly effectively camouflages them when seen respectively from above or underneath (Jonsson and Jonsson, 2011). In addition, it may play an essential role in osmotic balance in a hyper-osmotic environment (Hoar, 1988). Indeed, excretion of purine nitrogen is costly in the marine environment as it requires water. Therefore, the deposition of purine helps to reduce water loss. Fins also change colour; pelvic fins lighten while pectoral and caudal fins darken during smoltification (Wedemeyer *et al.,* 1980; Kottelat and Freyhof, 2007).

During smolting, the body becomes more streamlined with a pointy snout, probably better adapted to a migratory way of life (Webb, 1984; Hoar, 1988). A decreased condition factor (increased length relative to mass) has also been documented (Gorbman *et al.,* 1982; Hoar, 1988; Beeman *et al.*, 1995). But, it remains unclear if this decrease is part of an adaptive change (slimmer body for increased swimming performance in open water) or a consequence of decreased lipid reserves occasioned by the energetic demands of smoltification (Woo *et al.* 1978; McCormick *et al.,* 1998; Jonsson and Jonsson 2005). There is also an elongation of the caudal peduncle, suggested to improve pelagic swimming performances (Winans and Nishioka, 1987), a reduced skin mucus secretion and scale attachment (O'Byrne-Ring *et al.,* 2003). Morpho-anatomical differences during smoltification may exist between species as the emergence of teeth that has been described in coho salmon, *Oncorhynchus kisutch* (Gorbman *et al.,* 1982) has not been demonstrated in Atlantic salmon yet.

1.4.1.2 Behavioural modifications

As a pre-adaptive development for sea-life, smoltification is accompanied by a number of behavioural modifications. Parr typically exhibit a territorial behaviour, securing food in rivers with low nutritional resources. A decrease in agonistic behaviour and a switch from territorial to schooling behaviour occurs during smolting (Iwata 1995; Kiilerich *et al.*, 2011). This may decrease predation risks during the river and early marine migration and could also be an effective predator strategy for salmon in the open seawater (McCormick, 2009). There

is also evidence of smolts migrating in kin-structured groups (Olsén *et al.,* 2004). In opposition to ground-dwelling parr, smolt will move higher in the water column (Hoar, 1988). Increased negative rheotaxis (downstream migration) was observed. Smolts master their downstream movement, often swimming only a few hundred meters at a time then pausing. They may swim actively out of sloughs and backwaters (Davidsen *et al.,* 2005; Hansen and Jonsson, 1998) and be transported, head first, by the flow over sections of their journey (Thorpe *et al.,* 1981; Tytler *et al.,* 1978). Nearing rapids or in front of a small waterfall, smolts may swim in an upstream direction a number of times before clearing the obstacle, head or tail first, almost as if they were gauging water velocity (Jobling, 1995).

In association with osmoregulatory capacities development, smolts will develop a preference for saltwater (Hoar 1988; Iwata 1995; Bone, 1995; McCormick *et al.,* 1998). Population specific behavioural adaptations have also been documented, e.g. the abilities to pass through lakes and search out the outlet of a lake during downstream migration seem to be inherited (Aarestrup *et al.,* 1999). In lakes, smolt schools swim around close to the surface, exhibiting a searching behaviour before moving towards the outlet and migrating downstream with the current. This exploratory behaviour seems to be an adaptation for population passing through lakes on their downstream migration. Smolts originating from a stream population and relocated in a lake, could encounter difficulties in finding the outlet in low flow conditions, causing delay in their migration (Thorpe *et al.* 1981; Hansen and Jonsson 1985).

Swimming behaviour is influenced by environmental factors. In colder water, smolt will migrate at night and this movement can decrease under direct illumination of the moon or artificial light (Hansen and Jonsson, 1985). In the River Meuse, video-surveillance of the trap in Lixhe revealed 67% of smolts were migrating early during the night (8 pm-0 am) and 25 % before dawn (4:30 am-6:30 am) (Delforge *et al.,* 2003a). During a field survey on the Amblève (Belgium) with radio-tagged smolts and a detection antenna at the opening of a bypass, 98% of the detection were registered at night between 9 pm and 3 am (Ovidio *et al*., 2017). Similarly, in the River Imsa (Norway), smolts are active for 5 to7 hours a day especially from 8–9 pm to 1–3 am. Smolts become more day-active with increasing temperature and become mainly diurnal when temperature reaches above *ca* 13 °C (Thorpe *et al.,* 1994). Increasing swimming speed was also measured between early and late migrants and is believed to be a result of increased temperature. Similar results were noticed on the River Frome (England) with migration patterns showing downstream migration in the evening and at night early in the migration period and becoming increasingly diurnal later in the season (Ibbotson *et al.,* 2006). Temperature strongly influenced the number of migrants being

significantly higher at night than during the day when mean daily temperature was lower than 12 °C. No difference could be seen when water temperature rose above 12 °C. Results also showed a clear suppression of migration at dawn and dusk throughout the migration period which may be an active decision and/or an adaptive strategy to either take advantage of increased food (drifting invertebrates) and/or evade predation from actively feeding piscivores. In contrast to these studies, video-monitoring showed smolts migrated all day long in the sub-arctic river Tana with a peak from 7:00 to 10:00 h (Davidsen *et al.,* 2005). Here smolt migration ranged over early June to mid-July, the period of midnight sun. 55% of the day-to-day variation in numbers of migrating smolts was explained by the number of hours of sunshine and water level modifications. Only 4% of smolts were recorded migrating in the upper 30cm surface water layer while most smolts were recorded to migrate actively (head-first) in the lower part of the water column (Davidsen *et al.,* 2005).

1.4.1.3 Sight, buoyancy and olfactory imprinting

There are different changes occurring in the retina during smolting. Ultraviolet sensitive cones disappear and visual pigments switch from porphyropsin to rhodopsin (Alexander *et al.,* 1994; Dann *et al.,* 2003). The latter is characteristic of ocean fishes (Alexander *et al.,* 1994) and it has also been observed in silver eels (Archer *et al.,* 1995). Marine fishes are typically most sensitive to blue and green colours (ranging from 450 to 550 nm) according to rhodopsin being maximally blue-sensitive. Freshwater fishes, on the opposite, are more sensible to longer wavelengths, up to nearly 650nm, as is porphyropsin, most sensitive in the red spectrum (Lythgoe 1979; Levine *et al.,* 1980). Moreover, it's been hypothesized that the increased sensibility towards blue may be an explanation for the lower ability of smolts to maintain visual positioning at dusk in fresh water (Hasler and Scholz, 1983).

Another adaptation occurring during smolting is an augmentation in relative size of the swim bladder (Saunders, 1965). This organ composed of a rete mirabilis and a gas gland is responsible for depth control. Increased buoyancy may be advantageous during downstream migration and for feeding in the open ocean (Saunders, 1965). By means of comparison, such an adaptation has also been reported in the American eel (*Anguilla rostrata*) during silvering, where a 50% increase in guanine quantity was observed in the wall of the swim bladder translating in an increased swim bladder size (Fontaine, 1994; Kleckner *et al.,* 1981).

Homing, the ability of coming back to their natal stream as adults for spawning (Dittman and Quinn, 1996), is an outstanding feature displayed by salmonids and is achieved through olfactory imprinting during smoltification (Dittman *et al.,* 1996).

1.4.2 Physiological modifications

1.4.2.1 Ionic and acid-base homeostasis

Physiological changes include alterations in the gills, intestine and kidney that allow the fish to move from freshwater to seawater with minimal internal osmotic perturbations. In freshwater, teleost fish have a higher osmolality than the surrounding water, meaning they have to cope with the loss of ions by diffusion and gain of water by osmosis. In seawater, the opposite phenomenon happens and fish have to retain water and eliminate excess salt in their cells (Folmar *et al.,* 1980; McCormick *et al.,* 2009; McCormick, 2013). The development of hypo-osmoregulatory capacities during smoltification is considered by most scientists as a major acquisition (Strand *et al.,* 2011). The development of these homeostatic mechanisms is accompanied by biochemical and morphological changes in the gills and guts. There are two major types of gill cells: pavement cells and mitochondria-rich cells also called ionocytes or chloride cells (Lai *et al.,* 2015). The latter are crucial for osmoregulation and acid–base balance. Numerous authors have witnessed an increased expression of Na^+/K^+-adenosine triphosphatase (NKA) activity in chloride cells in the gills during smoltification (Wedemeyer *et al.* 1980; Hoar 1988; McCormick *et al.,* 1998; McCormick, 2013). NKA activity increases prior to the migration before decreasing while the migratory urge increases and finally increase again towards the end of smoltification (Spencer *et al.,* 2010).

There are three major transport proteins involved in salt secretion, NKA, $Na^+/K^+/2Cl^-$ co-transporter (NKCC) and cystic fibrosis transmembrane regulator (CFTR), a Cl^- channel (Figure 17) which have been specifically localized to ionocytes (McCormick *et al.,* 2003; Hiroi *et al.,* 2005). The basolateral membrane of these mitochondria-rich cells is endued with NKA (Jobling, 1995). NKCC is also found in the basolateral membrane while CFTR is located apically (McCormick, 2013). NKA generates a low Na^+ intra-cellular level by transporting 3 sodium ions out of the cell and pumping 2 potassium ions in. This creates an electric gradient across the cell membrane with a negative charge within the ionocyte. NKCC then uses this sodium gradient to transport Cl^- into the ionocyte which can then leave the cell, on a favourable electrical gradient, through apical CFTR (Marshall, 2002). Sodium, pumped into the paracellular space by NKA leaves the gills by a paracellular pathway using leaky tight junction and a favourable electrochemical gradient (McCormick *et al*., 2013).

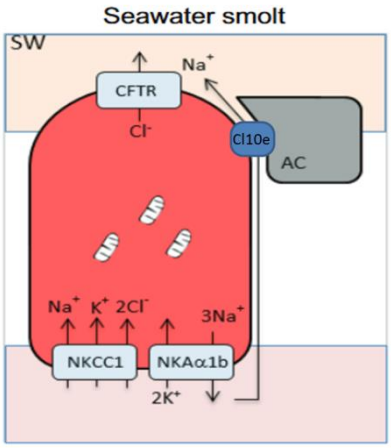

Figure 17: Scheme of the role of chloride cell in osmoregulation and ion transfer through these cells (McCormick, 2013 modified according to McCormick *et al.*, 2013)

Modification in the expression of transport proteins means differences in salinity tolerance and NKA activity is acknowledged as a signifier of the ionoregulatory capacity of fish (Bisbal and Specker 1991; Seidelin *et al.*, 1999; Mackie *et al.*, 2007). Its measurement may even be used to predict smolting in individual fish prior to visible morphological changes (Nielsen *et al.*, 2004). Various isoforms of gill NKA exists (Richards *et al.*, 2003) and are differentially expressed depending on the environment. NKA genes have distinct regulation by the endocrine system (Tipsmark and Madsen, 2009). NKAα1a shows high activity in freshwater but becomes almost undetectable in seawater. On the contrary, only small quantity of the NKAα1b isoform have been measured in freshwater, but increase ten times plus after seawater acclimation (McCormick *et al.*, 2009). These isoforms are localized in distinct chloride cells in gills, which activation depends on the osmotic environment the fish is in (Jobling, 1995; McCormick *et al.*, 2009).

Other proteins also play an important role in ionic and acid-base homeostasis. A family of ammonia-specific transporters, Rhesus glycoproteins, was discovered (see Wright and Wood, 2009 for review). These proteins seem to work as channels for the translocation of ammonia gas (NH_3) using a favourable partial pressure gradient (Knepper and Agre, 2004; Javelle *et al.*, 2007; Nawata *et al.*, 2010). They transport NH_3, NH_4^+ and there is evidence that they may also be capable of transporting CO_2 (Weinar and Verlander, 2010). In freshwater teleosts, about 20% of ammonia is excreted by the kidney and 80% by the gills (Smith, 1929; Zimmer *et al.*, 2014) but little is known about these proteins during smoltification. V-type H^+-ATPase, Na^+/H^+ exchanger are other proteins contributing to the excretion of $NH3^+H^+$. Simultaneously

they facilitate active Na^+ uptake in freshwater helping to maintain ionic and acid-base homeostasis (Wright and Wood, 2009).

Tipsmark *et al.* (2010) noted tissue specific expression of aquaporins during smolting. Intestinal expression of AQB-8b was stimulated during smoltification. Renal AQP-10 peaked in March and AQP-1a in April while AQP-1b and AQP-3 decreased. In gills, AQP-3 declined through smoltification and AQP-1a and -b peaked in April.

1.4.2.2 Swimming performance and metabolism

Enhanced swimming performances are a consequence of some morphological modifications (Winans and Nishioka, 1987, Hoar, 1988) which are even more improved by a switch in haemoglobin isoforms, increasing oxygen carrying-capacity of blood (Fyhn *et al.,* 1991; Koch, 1982). Furthermore, to accomplish their migration, smolts will need a great amount of energy. The liver develops augmented capacity for lipid catabolism and decreased capacity for lipid synthesis (Sheridan, 1989). Increased lipolysis, protein synthesis, carbohydrate and proteins catabolism and higher oxygen-consumption rate have been reported (Wedemeyer *et al.* 1980). Quantitative as well as qualitative changes of body lipids and blood proteins are known of. Increased breakdown and decreased synthesis result in declining glycogen and lipid in the tissue (Hoar, 1988). The lipid metabolism is mainly an energy source, e.g. β-oxidation of fatty acid stocked in adipose tissues (Verstregren, 2012), while carbohydrate metabolism has also another function, supplying oxidisable precursors for the tricarboxylic acid cycle (Guillaume *et al.,* 1999). Somatic energy density has also been found to decrease while water content increases which has been hypothesized to be a consequence of high energy demanding physiological changes concomitantly with decreased external feeding (Jonsson and Jonsson 2011). When comparing Atlantic salmon smolts and parrs of the same age, Jonsson and Jonsson (2003) found a 25% lower energy density, consequently to a lower lipid concentration, in smolts. This concentration was 3.8 g*100 g wet $mass^{-1}$ in smolts aged one year and two-times higher in parrs (7.9g*100 g wet $mass^{-1}$). Differences between species were also found as energy density was lower in brown trout smolts than in Atlantic salmon smolts (Jonsson and Jonsson 1998, 2005). Authors hypothesized a smaller energy need in brown trout smolts due to their shorter sea stay.

1.4.2.3 Endocrinology

For all these adaptations to take place, an accurate and complex endocrine control is set up prior to migration. The endocrine system has the ability to control and coordinate complex

developmental responses, internal rhythms and feedback from the internal state of the animal and sense the external environment (Gwinner, 1981; McCormick *et al.,* 2000). During smolting, an increase in plasma cortisol, growth hormone, insulin-like growth factor 1 and thyroxine levels is observed while prolactin concentration decreases (Figure 18, Björnsson *et al.,* 2011; Dickhoff *et al.,* 1997; McCormick, 2001; Prunet *et al*., 1989). Other hormones like melatonin (Handeland *et al.,* 2013), insulin (Plisetskaya *et al.,* 1988; Mayer *et al.,* 1994) and sex steroids (Nagahama *et al.,* 1982; Patiñio and Schreck, 1986; Sower *et al.,* 1992; Yamada *et al.,* 1993) have been reported to change during smoltification. Little research has been carried out on these, leaving their roles speculative (Björnsson *et al.,* 2011). We will now detail the roles of the major hormones playing a role in smoltification.

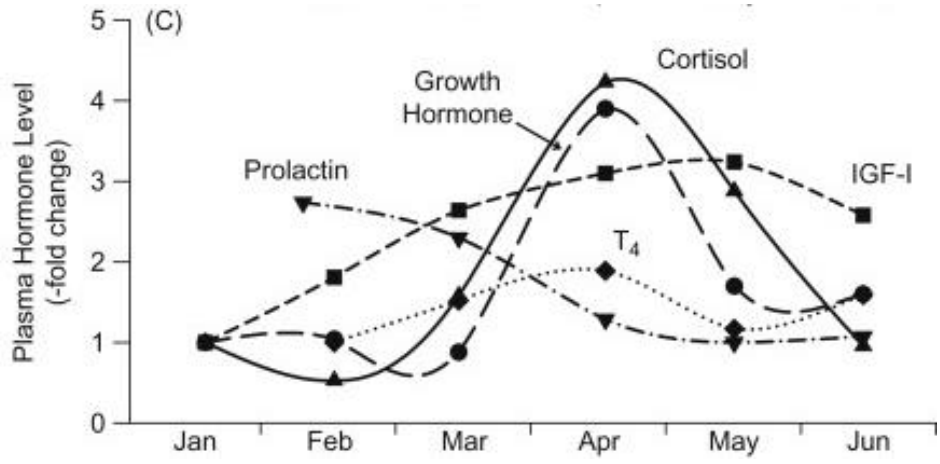

Figure 18 : Plasma hormone level (-fold change) during smoltification (McCormick, 2013 based on Prunet *et al.*, 1989 and McCormick *et al.*, 2009)

1.4.2.3.1 Cortisol and corticosteroid

In spring, cortisol plasma levels increase 10-fold in smolts but remain low in parrs when reared under the same conditions (McCormick *et al.,* 2007). This increase hasn't been observed in landlocked salmon while it was in anadromous smolts (Nilsen *et al.,* 2008). Cortisol is secreted by the interrenal gland under the influence of adrenocorticotropic hormone (ACTH), its primary secretagogue. The regulation of the hypothalamic–pituitary–interrenal (HPI) axis during smolting still lacks some explanatory mechanisms and there has been no measurement of circulating or pituitary secretion levels of ACTH during smoltification (McCormick, 2013). Minor cytological modifications have been reported in ACTH producing cells during smolt development (Nishioka *et al.*, 1982). There is evidence of increased abundance of corticotropin releasing hormone (CRH) neurons, at least partially as a

response to upregulation through thyroid hormones (Ebbesson *et al.,* 2011). Increased sensitivity of the interrenal to ACTH is a likely direct effect of GH, increasing cortisol production in response to any level of ACTH (Young, 1988). This would also explain how GH treatment is followed by higher circulating cortisol levels (Quinn *et al.,* 2003). In spring, increased number of branchial cortisol receptors was reported in Atlantic salmon parrs and smolts (Shrimpton and McCormick, 1998b) in opposition with coho salmon where a decrease has been observed (Shrimpton *et al.,* 1994). Furthermore, immunocytochemical and *in situ* hybridization approaches revealed more cortisol receptor in branchial ionocytes than in other gill cell types (Uchida *et al.,* 1998).

Two kinds of corticosteroids may be distinguished; glucocorticoid and mineralocorticoid with their associated receptors. Cortisol binds to glucocorticoid (GR) and aldosterone to mineralocorticoid receptors (MR). But, in teleosts, levels of aldosterone are very low with supposed minimal influence in hormonal regulation (Prunet *et al.,* 2006; Bury and Sturm, 2007). However, high levels of 11-desoxycorticosterone (DOC) have been measured which may bind on MR and be considered a mineralocorticoid in teleosts (Bury and Sturm, 2007; Kiilerich *et al.,* 2011; Prunet *et al.,* 2006; Stolte *et al.,* 2008). More recently, molecular data brought evidence of two GR and one MR in most teleosts (Takei and McCormick, 2013). This nomenclature is based on their similarity to mammalian receptor where corticosteroid affects the regulation of metabolism and ion regulation through these receptors using distinct regulatory pathways. This distinction does not apply to fish (Takei and McCormick, 2013).

Other differences do exist, gill GR transcription increases in masu (*Oncorhyncus masou*) and Atlantic salmon during smolt development but no modification of transcription of gill MR was observed (Mizuno *et al.,* 2001; Nilsen *et al.,* 2008). A number of studies have shown GR to be specific for cortisol whereas MR may bind to cortisol and DOC (Bury and Sturm, 2007; Stolte *et al.,* 2008). Using RU486, a specific GR blocking agent, it was shown that cortisol influenced gill permeability through GRs and MRs in Atlantic salmon (Kelly and Chasiotis, 2011; Kiilerich *et al.,* 2011; McCormick *et al.,* 2008; Tipsmark *et al.,* 2009). Current knowledge tend to show a major role of GRs in salt secretion whereas MRs would have a more limited role in the ionic equilibrium regulation and water content in most species (McCormick, 2013). Co-localisation of MR and GR in osmoregulatory organs implies an importance of the equilibrium of their expression in physiological responses (Kiilerich *et al.,* 2011). Furthermore, an implication of MR in behaviour and/or neuroendocrine responses has been suggested as there was high MR transcription in the brain of teleosts (McCormick, 2013).

Differential actions of GR and MR may also be implicated in the dual role of cortisol promoting both ion uptake and salt secretion (McCormick, 2013).

Many roles of cortisol were shown through exogenous treatment. Cortisol stimulates salinity tolerance and many underlying mechanisms involved in hypo-osmoregulation in gills and gut (McCormick, 2001), increases the number of ionocytes and the major transport proteins involved in salt secretion, NKα1b, NKCC1 and CFTR, (Pelis and McCormick, 2001; Kiilerich *et al.,* 2007a; McCormick *et al.,* 2008) and increases transcription of gill claudin 28e, a tight junction protein that increases after seawater acclimation of Atlantic salmon (Tipsmark *et al.,* 2009). These increased intestinal water uptake capacity and NKA activity in juvenile Atlantic and Chinook salmon have also been reported after cortisol treatment (Veillette *et al.,* 1995; Veillette and Young, 2004).

It also plays an important role in freshwater osmoregulation (Bisbal and Specker, 1991 ; Sakamoto and McCormick, 2006) increasing the surface of gill chloride cell, promoting active absorption of sodium and chloride and increasing NKAα1a transcription (Kelly and Chasiotis, 2011; Kiilerich *et al.,* 2011; McCormick *et al.,* 2008; 2009).

Cortisol also influences metabolism, development, immune functions and is secreted in a stressful situation (Bisbal and Specker, 1991; McCormick, 2009). Increased levels were measured when fish were transferred from freshwater to seawater and vice versa indicating once more the dual role of cortisol in ion uptake and salt secretion as well as in response to a stress (Björnsson *et al.,* 2010; McCormick, 2009; McCormick *et al,*.2009).

There is also evidence for the HPI axis playing a role in behavioural changes during smolt development. Increased downstream migratory behaviour was observed after the administration of corticotropin-releasing factor (CRF) in juvenile coho and chum salmon (Clements and Schreck, 2004; Ojima and Iwata, 2009, 2010). The fast response indicates a direct influence of this peptide but an action through cortisol cannot be excluded as it increased the salinity preference of juvenile coho salmon (Iwata *et al.,* 1990). ACTH increases fin darkening in Atlantic salmon (Langdon *et al.,* 1984). As this is a characteristic change during smoltification in this species, it suggests a role of the HPI axis in the morphological changes too.

1.4.2.3.2 Growth hormone and insulin-like growth factor 1

Growth Hormone (GH), aka somatotropin, secretion is regulated by somatotropin release-inhibiting factor (SRIF) and growth hormone releasing factor (Figure 19). In addition, GH stimulates IGF-1 secretion in the liver which in turn inhibits pituitary GH secretion

(Björnsson *et al.,* 1997). During smoltification, high plasma GH level is due to an increase in pituitary secretion early in smolt development and then through increased GH synthesis and secretion (Ágústsson *et al.,* 2001). Growth hormone and Insulin-like growth factor-1 play a crucial role in seawater acclimation in teleosts in addition to their role in growth in vertebrates (McCormick *et al.,* 2009). A number of studies on the Atlantic salmon showed that a GH treatment increases the size of fish and promotes saltwater tolerance (Saunders *et al.,* 1989; Bœuf, 1994; McCormick *et al.,* 1995) even promoting precocious acclimation to seawater (Bœuf, 1994). This enhanced tolerance is explained through the action of GH on ionocytes, influencing the development in type, size and number of chloride cells (Björnson *et al*., 1997; Folmar et Dickhoff, 1980; McCormick, 2009).

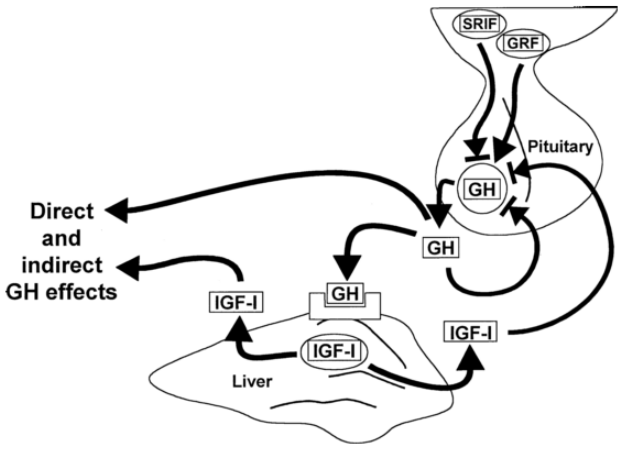

Figure 19 : GH regulation mechanism in salmon. Somatotropin Release-Inhibiting Factor. (SRIF) and Growth Hormone Releasing Factor regulate GH secretion. GH stimulates IGF-1 secretion in the liver which in turn may inhibit GH secretion in the pituitary. IGF-1 and GH also have direct and indirect effects in salinity tolerance (Björnsson *et al*., 1997)

GH is usually believed to be the major secretagogue for plasma IGF-1, primarily produced in the liver (McCormick, 2013). Other factors may also be involved and local production of IGF-1 is more than likely to be involved in smolting (McCormick, 2013). IGF-1 increases in parr and smolt have been reported in spring but levels were measured in smolt throughout (McCormick *et al.,* 2007) and some studies did not find increased IGF-1 levels during smolting (Nilsen *et al.*, 2008).

Increased transcription of branchial growth hormone receptors (Kiilerich *et al.*, 2007b) and elevated levels of circulating GH, may provide an explanation to the rise in IGF-1 production in the gills (Robertson and McCormick, 2012a). Pituitary GH transcript increased as well as hepatic GH receptors and local IGF-1 production which suggested a physiological basis explaining the modification in circulating GH and IGF-1 levels (Stefansson *et al.*, 2012). In

addition, increased transcription and/or protein abundance of the three primary transport proteins playing a role in hypo-osmoregulation (NKAα1b, NKCC1 and CFTR) made authors suggest that GH controls a differentiation program for salt-secreting ionocytes (Robertson and McCormick, 2012a; Tipsmark and Madsen, 2009). Brain and pituitary expression of GH and IGF-1 receptors tend to indicate an active role of these hormones in growth and differentiation of these tissues during the critical early marine phase (Stefansson *et al.,* 2012).

In the gill, increased transcription of both IGF-1 and IGF-1 receptor has been measured in anadromous Atlantic salmon smolts but not in a landlocked strain (Nilsen *et al.,* 2008). An increased number of mRNA coding for IGF-1 was also measured in the liver of smolts compared to parr in several salmonids (Seear *et al.,* 2010; Duguay *et al.,* 1994; Sakamoto *et al.,* 1995). Furthermore, a large number of IGF binding proteins (IGFBP) are expressed in salmonids and *igfbp6b1* and *igfbp6b2* increase in gill during smolting (Breves *et al.,* 2017). Local modulation of branchial IGF activity may also be regulated through transcriptional control of IGFBP (Breves *et al.,* 2017). Cortisol was found to amplify the transcription of GH and IGF-1 branchial receptors (Tipsmark and Madsen, 2009). Furthermore, GHR transcript continues to increase in postsmolts migrating through Fjords, but decreases offshore. These results suggest a major role of GH in seawater adaptation (Stefansson *et al.,* 2012). Gill IGF-1 production follows a similar pattern. However, branchial IGF-1 receptor expression increased from the river through the Fjord to the sea. These results led the authors to suggest an important role of IGF-1 in the adaptation to the marine environment (Stefansson *et al.,* 2012).

1.4.2.3.3 Interaction between GH, IGF-1 and cortisol

Increases in circulating levels and exogenous treatment with hormones indicate that salinity tolerance is under the positive control of cortisol, GH and IGF-1 (McCormick 2001). An increase in plasma levels of these hormones will translate in the proliferation and differentiation of branchial seawater-type chloride cells and modify intestinal osmoregulatory functions (Björnsson *et al.,* 1997; McCormick, 2009). Fish may then compensate osmotic water losses in a hyperosmotic environment through drinking seawater and then extruding monovalent ions by the gills and divalent ions by the kidney (Jobling, 1995; Dickhoff *et al.,* 1997; Jonsson and Jonsson, 2011).

Cortisol treated salmon showed an increase in activity of both NKA isoforms (McCormick, 2009). However, a cortisol treatment coupled with GH promotes only the seawater isoform and reduces the freshwater isoform (McCormick, 2009). In salmonids, GH and cortisol can individually affect hypoosmoregulation and even achieve similar salinity tolerance as smolts

when injected together (McCormick, 2001). Moreover, GH and cortisol independently upregulate the number of NKA and NKCC and together act additively or synergistically (Madsen, 1990; Pelis and McCormick, 2001). GH seems then to act like a switch on the role of cortisol, from ion uptake to salt secretion. On the contrary to gills where this interaction is well established, there are only few indications for it in the intestine and kidney. An increase in the intestinal transcription of claudin 25b was noticed after seawater exposure and GH treatment, but no preparatory upregulation of transcription of this gene was seen during smolting (Tipsmark *et al.*, 2010b).

More interactions between these hormones have been described. On one hand, cortisol increases the transcription of gill GH and IGF-1 receptors (Tipsmark and Madsen, 2009). On the other hand, GH increases the transcription of cortisol receptor in gills (Shrimpton *et al.*, 1995), thus increasing the responsiveness of branchial tissue to any level of endogenous cortisol (Shrimpton and McCormick, 1998a).

1.4.2.3.4 Thyroid hormones

Increases in cell size of thyroid tissue as well as an intensified activity during smoltification have been reported for a long time (Hoar, 1939; Folmar et Dickhoff, 1980; Fagan *et al.*, 2003; Björnsson *et al.*, 2010). Increased thyroid hormone production during smoltification has also been measured (Figure 20, Figure 21, Fagan *et al.*, 2003; Robertson and McCormick, 2012a). Under the action of the thyroid stimulating hormone (TSH), thyroglobulin is secreted by the thyroid gland system to form thyroxine (T4) and 3,3',5-triiodo-L-thyronine (T3). T4 is an inactive pro-hormone, that becomes active in the T3 shape, considered the active molecule for the receptor (Bœuf *et al.*, 1994). Thyroid hormones play a role in the most conspicuous change during smoltification, silvering (Hoar, 1988), which may be induced artificially. Thyroid hormones appear to influence both the ultraviolet sensitive cone-loss and the change of visual pigments in smolts (Allison *et al.*, 2004; Raine and Hawryshyn, 2009). These hormones also play an important role in the control of behavioural modifications, although the precise controlling mechanism has not been elucidated yet (Hoar, 1988; Ojima and Iwata, 2007). Indeed, landlocked freshwater salmon treated with thyroxine are less aggressive towards each other in comparison to a control group (Iwata, 1995). Wedemeyer *et al.* (1980) noticed a similar effect in other anadromous salmonids. T3 and T4 coupled to cortisol and GH will indirectly favour seeking behaviour and promote negative rheotaxis (Folmar and Dickhoff, 1980).

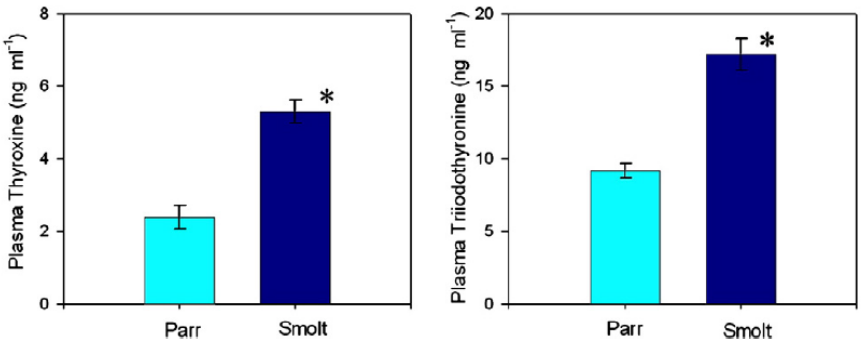

Figure 20 : Comparison of T4 and T3 plasma levels between parrs and smolts of Atlantic salmon. A star indicates a significant difference (p < 0,05, t-test) intervals are standard error of the mean (Robertson and McCormick, 2012a)

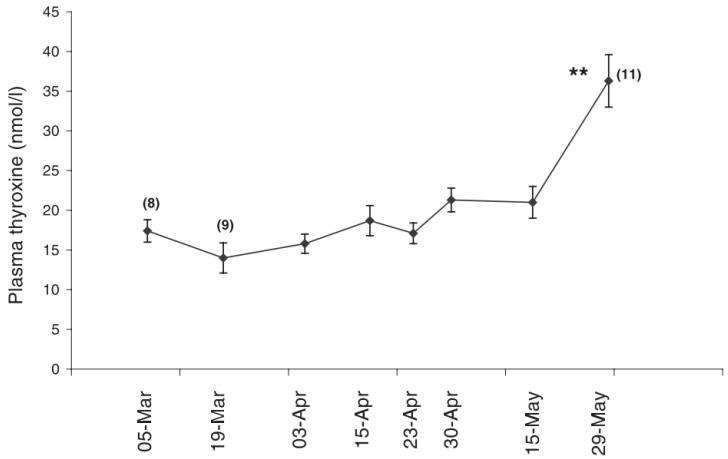

Figure 21: Plasma thyroxine levels (nmol*mL^{-1}) during smolting (Fagan *et al.*, 2003)

Last but not least, thyroid hormones may not have a direct effect on osmoregulation but when the conversion of T4 to T3 is interrupted, natural or GH-induced seawater acclimation lacks efficiency (increase in plasma ions), thus indicating an impaired regulation (McCormick, 2001). According to Ojima and Iwata (2007), a T4 treatment has no effect by itself but potentiates the cortisol and GH actions on gill NKA activity which concur with observations of increases in chloride cells as well as NKA activity during smoltification after a T4 treatment (McCormick, 2001). T3 treatment only increased ionocytes number without any effect on NKA activity. Thyroid hormones may then play a supporting role in seawater acclimation through interactions with the GH/IGF-1 axis and cortisol (McCormick, 2001). In post-smolts in seawater, an increase in thyroxine (T4) and triiodothyronine (T3) levels was observed (Stefansson *et al.,* 2012). The latter authors suggested an important activation of hepatic conversion of T4 to T3 in relation with the high metabolic activity and fast growth and development of the post-smolts.

1.4.2.3.5 Prolactin

In teleosts, prolactin (PRL) expression, synthesis, secretion and plasma level increase when exposed to freshwater and decrease in seawater (Hoar, 1988 Young *et al.,* 1989). Prolactin helps retaining chloride and sodium ions in osmoregulatory organs in freshwater (Pickford and Phillips, 1959; Evans, 2008; McCormick, 2009). In Salmonids, there are more PRL receptors in ionocytes and enterocytes of the intestine when in freshwater than seawater. In salmon, PRL reduces gill permeability (Hoar, 1988). There is a surge in PRL prior to smolt migration and then levels decrease sharply (Høgåsen, 1998; Prunet and Boeuf, 1989; Prunet *et al.,* 1989; Young *et al.,* 1989). This decrease is associated with the initiation of downstream motion (Høgåsen, 1998).

1.4.2.4 Gene expression of other metabolic functions during smoltification

Genes involved in smolting are regrouped on specific regions of the genome in *Oncorhynchus mykiss* (Nichols *et al.,* 2008). Parrs undergo numerous changes to become smolts but until recent years the molecular modifications driving this developmental stage still suffered a lack of understanding (Robertson and McCormick, 2012a). To investigate such a profound modification of the organism, the use of microarrays is helpful. It provides a look at transcriptional changes in a large number of genes. Different chips have been developed (3K, 17K and 44K) over the years by the Genomic Research on Atlantic Salmon Project (Rise *et al.,* 2004b; Taggart *et al.,* 2008; von Schalburg *et al.,* 2005b, 2008). These are spotted with roughly 80% of Atlantic salmon and 20% of Rainbow trout cDNA features (von Schalburg *et al.,* 2005b) and have been used in a wide range of studies to investigate the changing transcriptome in developing Atlantic salmon (Jantzen *et al.,* 2011), differential gene expression between parrs and smolts (Seear *et al.,* 2010; Robertson and McCormick, 2012a), the molecular differences between precociously mature male parrs and immature males and females (Aubin-Horth *et al.,* 2005), the effect of nonylphenol on gene expression in Atlantic salmon smolts (Robertson and McCormick, 2012b), ovary development in rainbow trout (von Schalburg *et al.,* 2005a, 2006) and response to *Piscirickettsia salmonis* infection (Rise *et al.,* 2004a) or infectious hematopoietic necrosis virus (IHNV) vaccination in rainbow trout (Purcell *et al.,* 2006).

Using more or less stringent method, 48 to 477 differentially expressed features have been determined during smolting in different organs (Seear *et al.,* 2010; Robertson and McCormick, 2012a). Seear *et al.,* (2010) reported most changes in gill (148 out of 259 altered features). This was also reported by Robertson and McCormick (2012a) with 172 changes out

of 477. Most genes are associated with general functions like growth, metabolism, oxygen transport or osmoregulation. There is also evidence of increased expression of genes involved in regulation of transcription, protein synthesis and folding, electron transport and sensory reception whereas expression of genes involved in proteolysis decreased (Robertson and McCormick, 2012a).

Compared to parrs, there is an increased number of mRNA coding for transferrin (transporting iron towards hepatic reserves) in the liver of Atlantic salmon smolts (Hardiman and Gannon, 1996, Seear *et al.*, 2010) although genes associated to transferrin are more active in smolts in seawater than in those that haven't reached seawater yet (Hagen-Larsen *et al.*, 2005). Changes in expression of ferritin, a protein responsible for intestinal iron absorption, have also been reported (Seear *et al.*, 2010; Robertson and McCormick, 2012a).

During smoltification, lots of changes occur in lipid and carbohydrate metabolism and electron and oxygen transport systems which help the salmon to gain essential capacities to accomplish their migration (Jonsson and Jonsson, 2011). In general there is an increased capacity for lipolysis and a decreased capacity for lipid synthesis (Sheridan, 1989). The expression of catalytic enzymes and some receptors (i.e. Peroxisome Proliferator-Activated Receptor (PPAR)) has been shown to change during the parr-to-smolt transformation. This family of nuclear receptors influences, through the retinoid-X-receptor (RXR), the expression of some target genes implicated in lipid and carbohydrate homeostasis regulation (Jump *et al.*, 2005). The Liver-X-receptor, a transcription factor, plays a role in fatty acid biosynthesis and in cholesterol catabolism (Carmona-Antoñanzas *et al.*, 2014). The expression of six enzymes involved in the oxidative phosphorylation, cytochrome b, cytochrome c oxidase, NADH dehydrogenase subunits 1, 4, and 4L, and ATP synthase F0 subunit 6, were upregulated (Seear *et al.*, 2010). Galactokinase 2 (Robertson and McCormick, 2012a) and the citrate synthase were also reported as increased (Hagen-Larsen *et al.*, 2005).

Modifications of metabolism are tuned by at least three different hormone axis, the growth hormone, thyroid hormone and corticosteroid hormone axis (Jonsson and Jonsson, 2011). The secretion of these hormones is modified during smoltification (McCormick *et al.*, 1998) and regulated by hypothalamic factors (Robertson and McCormick, 2012a). These hormones may influence transcription of other smoltification-related genes (Yada *et al.*, 1992; Sakamoto *et al.*, 1995).

Transcriptional changes related to osmoregulation are known for a long time with both gill NKA and vacuolar-type H^+-ATPase changing significantly, although this change is operated gradually across smoltification (Seidelin *et al.*, 2001). More recently, increased transcription

and/or protein abundance of the three primary transport proteins for hypo-osmoregulation (NKAα1b, NKCC1, and CFTR) were reported (Tipsmark and Madsen, 2009; McCormick *et al.*, 2013, Breves *et al.*, 2017). Expression of CFTR I and II was shown to decrease in post-smolts (Stefansson *et al.,* 2012). Moreover, while the expression of NKAα1a isoform is downregulated, the expression of NKAα1b is upregulated. mRNA abundance of NKAα1c remains unchanged (Nilsen *et al.*, 2007).

Modifications of opiate receptor distribution (Ebbesson *et al.,* 1996), retinal innervation of the preoptic nucleus (Ebbesson *et al.,* 2007) and elevated abundance of CRF neurons (Ebbesson *et al.,* 2011) have been reported. It's been hypothesized that these modifications are crucial to the revised photoperiod responsiveness, typical of smolt development (Ebbesson *et al.,* 2007; McCormick *et al.,* 2007). Imprinting is likely to be related to the upregulation of salmon olfactory receptor gene (SORB) and two salmon vomeronasal receptors (SVRA and SVRC) during smolting (Dukes *et al.,* 2004). A number of other genes may be involved as 88 features were differentially expressed in the olfactory rosettes of smolts compared to parr (Robertson and McCormick, 2012a).

An increased expression of genes for collagen formation in gills was also measured (Seear *et al.*, 2010). Considering that the growth rate of fish is dependent of the respiratory surface of the gills (Pauly, 1981), this increase would then be linked to the need of an increased respiratory surface to maintain a higher growth rate in smolts (Seear *et al.*, 2010).

Numerous other studies showed differentially expressed genes during smoltification, e.g. igM (Melingen and Wergeland, 2000), cytokine (Ingerslev *et al.,* 2006), aquaporins (Tipsmark *et al.,* 2010). However, there are contradictory reports, with results suggesting a drop in somatolactin mRNA expression (O'Keeffe *et al.,* 2008) or an increase (Ágústsson *et al.,* 2003).

1.4.3 Influencing factors

Numerous studies have dealt with the regulation of smoltification but it is still not fully understood. However, both environmental and endogenous factors play an important role in it. An endogenous rhythm controls the smolting process, itself directed by the nervous and endocrine systems. External factors then act as synchronizers (Folmar and Dickhoff, 1980; Wedemeyer *et al.,* 1980; Hoar, 1988; Duston and Saunders, 1990).

1.4.3.1 Photoperiod

Photoperiod and temperature are well acknowledged as major regulating factors. The length of day acts as a timer and up and down changes in photoperiod are major predictive, proximate factors indicating the season (Smith, 1985; Wootton, 1998). As photoperiod does not change from year to year in one place on a specific date, this factor is not responsible for annual differences in smoltification season. However, temperature is a rate controlling factor of development (Hoar, 1988; McCormick *et al.,* 2002) even limiting photoperiod responsiveness when too low (McCormick *et al.,* 2000) and may therefore be responsible for annual variation in population smoltification timing.

Increasing gill NKA activity was measured in pre-smolts reared under constant and increasing daylength but initial increase and final levels were lower under constant illumination (Olsen *et al.,* 1993). Other differences (increase in plasma cortisol and lower plasma chloride levels) were also measured, indicating better hypo-osmoregulatory capacities of smolts reared under increasing light regime. Increasing daylength in spring isn't the only influencing factor. Earlier and more marked increases in gill NKA, decrease body lipid and increase moisture in fish in the later winter and spring under reciprocal photoperiod compared to natural photoperiod suggest that long winter nights trigger pre-adaptations for the smoltification (Saunders and Henderson, 1978). Under hatchery conditions, precocious timing of physiological changes could be achieved under advanced photoperiod regimes in different species of salmonids (Hoar, 1988, Berge *et al.,* 1995; Johnsen *et al.,* 2000).

Accurate mechanisms modifying the sensitivity of migrating salmonids to photoperiod still lacks complete understanding (McCormick, 2009). Ebbesson *et al*. (2002) hypothesized that increased plasma levels of GH and cortisol may be related to the development of a network linking the retina to the hypothalamus. This increase would also be linked to the development of olfactory capacities and an extension of retina diameter (Ebbesson *et al.,* 2002). There is good evidence that changes in photoperiod are relayed through the photoreceptor organs-brain-pituitary axis as in Atlantic salmon (McCormick, 2013), melatonin secretion profiles, by the photosensory pineal gland, match the scotophase throughout the annual cycle (Komourdjian *et al.,* 1976). Furthermore, pinealectomy appears to delay the onset of smoltification suggesting an important role or melatonin in the the parr-to-smolt transformation (Porter *et al.,* 1998). This role was already suggested as melatonin implants in steelhead trout, increased abundance of chloride cell and NKA activity (Rourke, 1994). However, while melatonin secretion can be directly synchronized to photoperiod, it is still not

clear how photoperiodic information is translated to a neuroendocrine response leading to the development of hypo-osmoregulatory capacity (Handeland *et al.*, 2013; McCormick, 2013).

1.4.3.2 Temperature

Temperature has many directive influences on smolting. First of all, as smolting is a size-dependent process (McCormick *et al.*, 1998), temperature modifies, through its influence on juveniles' growth rate, the age and size at which parr will smoltify (Stefansson *et al.*, 2008; Jonsson and Jonsson, 2009). In Europe, 1+ smolts may be found in southern rivers and 4+ smolts in higher latitudes like Norway and Russia (Stefansson *et al.*, 2008). Life history traits, such as fecundity, longevity, maturity age or age at hatching are also influenced by temperature and modifications of temperature (Jonsson and Jonsson, 2009).

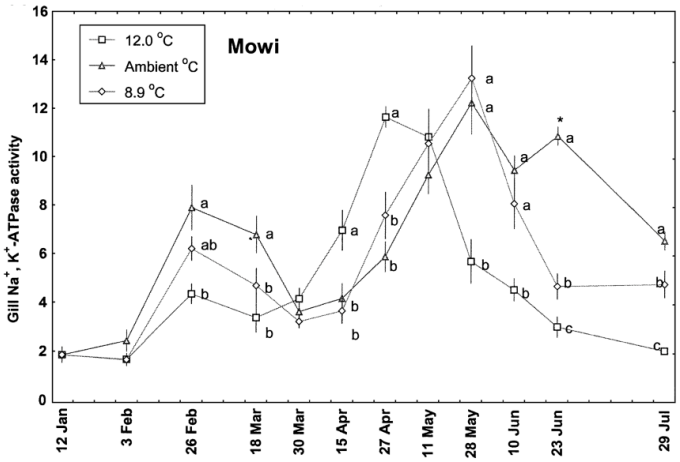

Figure 22: Gill NKA activity at three temperature levels (8.9°C, ambient and 12°C) from January 12 to July 29. Values are depicted as mean ± SE and letters indicate differences among temperature regimes (p<0.05) (Handeland *et al.*, 2004)

Initially, a threshold temperature of 10 °C or slightly above was thought to initiate smolting (Osterdahl, 1969; Solomon 1978, White 1939). More recent findings showed a certain amount of degree*days (average temperature (°C) * number of days) to be a much better indicator (Handeland *et al.*, 2004). Indeed, temperature experienced over time influences behavioural and physiological modifications related to the smolting process. When fish were reared at 12 °C or at 8.9 °C (Figure 22), maximum gill NKA activity was measured respectively in late April and in late May (Handeland *et al.*, 2004). Gill peak NKA activity was observed at 350 dd following the onset of the typical smolt-related increase in activity and the smolt window was defined as the period when NKA activity was >90 % of peak value (Handeland *et al.*, 2004). Similar models have been presented (Stefansson *et al.*, 1998; McCormick *et al.*, 1999).

Depending on the definition of the cut-off level, the smolt window was calculated to last between 300-400 dd (McCormick *et al*., 1999).

Increased temperature will stimulate smoltification (Wedemeyer *et al.,* 1980; Smith, 1985; McCormick *et al.,* 1998; Handeland *et al.,* 2014). Smolting may be advanced up to 5 weeks (McCormick *et al.,* 1996) or even up to 7 weeks (Solbakken *et al.,* 1994). An exposition to a temperature of (12.7 °C) stimulates Na^+/K^+-ATPase activity and advances smolting up to 4 weeks in comparison to smolts exposed to ambient (2.4-11.9 °C) temperature or 8.3 °C (Handeland *et al.,* 2004). On the contrary, low temperature will slow down the development of various adaptations. Low temperature will considerably decrease the response capacity of parrs to increased daylength and limit NKA activity (Figure 23) and key hormones levels like IGF-1, cortisol, GH and thyroid hormones (McCormick *et al.,* 2000). In addition, temperature is a rate controlling factor of development (Hoar, 1988; McCormick *et al.,* 2002) and may therefore be responsible for annual variation in population smoltification timing. High temperature may also have deleterious consequences on smoltification. Indeed, decreased NKA activity at higher number of degree*days was reported (Figure 24; Stefansson *et al*., 1998; McCormick *et al*., 1999). A strong increase in temperature may stimulate the opposite effect of smolting and induce modifications towards the parr state called desmoltification (Wedemeyer *et al.,* 1980; see 1.4.5.).

1.4.3.3 Life History traits

Smolting is a size-related developmental stage (McCormick *et al.,* 1998) and as such, it is influenced by age and growth rate which in turn depend on environmental conditions (Jonsson and Jonsson, 2011). A threshold size of 10 cm in autumn preceding smolting was already observed by Elson (1957).

Age at smolting in Atlantic salmon vary considerably among populations, rivers or year. It may even vary within river from 12 cm to 22 cm (Power 1969; Jensen and Johnsen 1986). Among populations, smolt size may depend on phenotypic plasticity (Jonsson, 1985) and inheritance of population variation to local growth and survival opportunities (Refstie *et al.* 1977, Lester *et al.,* 2004). Large variations (from 1 to 8 years) in smolt age are found in wild populations. Some populations have mean smolt age of less than 2 years to over 4 years (Power 1969; Metcalfe and Thorpe 1990; Metcalfe *et al.* 1990; Englund *et al.* 1999). Prehatch environmental conditions during embryonic development may also influence smolt age as a positive correlation between prehatch winter temperature and rainfall with growth and consequently a higher 1-year-old smolt proportion of the cohort (Jonsson *et al.,* 2005;

Strothotte *et al.,* 2005). In anadromous brown trout, smolt age increases with latitude (L'Abée-Lund *et al.*, 1989; Jonsson and L'Abée-Lund, 1993). However, this relationship is far less pronounced in Atlantic salmon (Metcalfe and Thorpe, 1990). Brown trout smolt age also decreases with increasing fresh- or seawater temperature (L'Abée-Lund *et al.* 1989) and increasing growth season length (Symons, 1979; L'Abée-Lund *et al.* 1989).

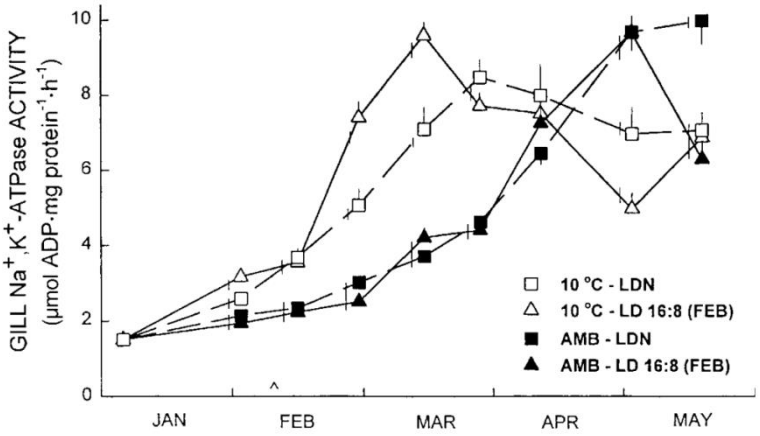

Figure 23: NKA activity from January to May in 4 experimental groups under different photoperiod and temperature conditions. LD 16:8, increased daylength with 16h of light and 8 of darkness; LDN, natural daylength; AMB, ambient temperature. Values are given as mean ± SE. Vertical lines indicate a significant difference from other groups at that time (P < 0.05, Kruskal-Wallis test) (McCormick *et al.*, 2000)

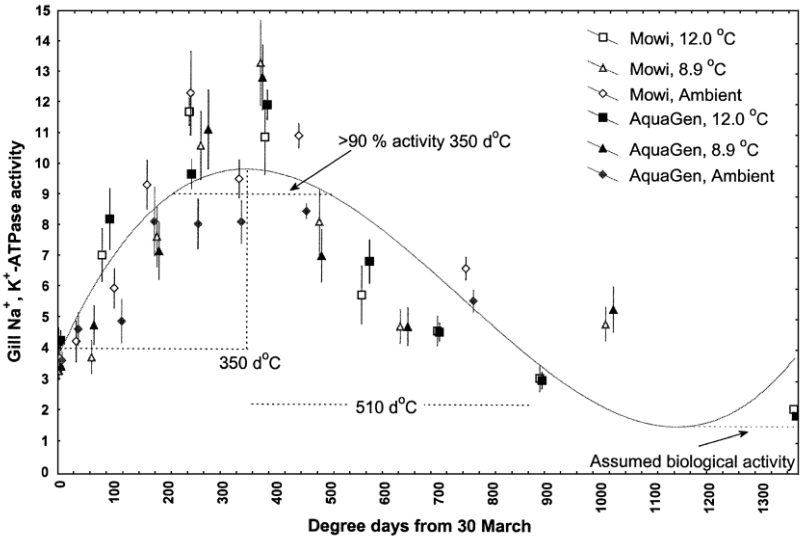

Figure 24: Relation between NKA activity (in all groups: two strains and three temperature regimes) and degree*days starting on March 30 (onset of the smolt-related increase in enzyme activity). On the figure, peak activity was reached after approximately 350degree*days (r^2=0.44). Transfer into seawater of the different groups occurred after 290 degree*days at 12.0 °C, 480 degree*days at 8.9 °C and 520 degree*days at ambient temperature (Handeland *et al.*, 2004)

Metcalfe and Thorpe (1990) thought up an index table of growth opportunity based on temperature and photoperiod records and found that this variable could explain 82% of the variance in smolt age of Atlantic salmon in North American and European rivers. However, this method did not improve prediction for brown trout smolt age (Jonsson and L'Abée-Lund, 1993). Predation, competition and food availability also influences parr growth (Jonsson and Jonsson, 2011).

1.4.3.4 Strain influence and genetic basis

There is a growing body of evidence that smolting, timing and propensity of smoltification in salmonids is under genetic control. Early smolting trait (smolting after one year in freshwater) was found to be dominant in the progeny over late smolting trait (smolting after one-two years in freshwater) when two Chinook salmon populations with smolting timing differences were crossed (Clarke *et al.,* 1994). When anadromous and non-anadromous forms of rainbow trout (Johnsson *et al.,* 1994) or sockeye salmon (Foote *et al.,* 1992) were crossed, offspring in both species exhibit intermediary freshwater and migratory forms in terms of hypo-osmoregulatory capacities. These results lead to hypothesize an additive control rather than a dominant/recessive gene alternative on the propensity of juveniles to smoltify. Several smoltification-related traits (silvering, condition factor and growth) were found to be associated to a particular region of the genome in rainbow trout (Nichols *et al.,* 2008). There is also evidence of heritability for age at smolting in Atlantic salmon and Chinook salmon (Refstie *et al.,* 1977; Clake *et al.,* 1992, 1994). Strain and cross-breeding between native and non-native fish may then influence smoltification timing. Furthermore, there is evidence of strain-specific timing of salinity development (Handeland *et al*., 2004).

1.4.4 Disrupting factors and desmoltification

Since the 17th Century, anthropic activity expansion considerably disrupted rivers. From the industrial bloom to leisure activities, overfishing and introduction of foreign species, rivers have constantly been used by Man (Willis *et al.,* 1980; Smith, 1985; Hutchings *et al.,* 2002; Malbrouck *et al.,* 2007). From there on, fish community composition has been heavily modified and various species have been observed to decline or become extinct (Philippart, 1987; Philippart *et al*., 1988; Philippart & Vranken, 1983).

Expansion of industrialisation produced lots of wastewaters leading to eutrophication and chemical pollutions. These wastewaters lead to an increase in temperature and to a higher biochemical demand in oxygen (Hutchings *et al.,* 2002). Coal-burning power plants produce

sulphur and nitrogen oxide which may result in acid rain. In eastern Canada (Watt, 1987; Lacroix, 1989) and southern Norway (Hesthagen, 1989; Hesthagen and Hansen, 1991) rivers and streams have poor buffering capacity as a consequence of local geology. Such conditions lead to increased vulnerability to all year round acidification. In turn, lower pH makes aluminium leaches in the surrounding watershed (Driscoll, 1984). Increased acidity also increases aluminium solubility and subsequently, the abundance of inorganic aluminium, the form most toxic to fish (Gensemer and Playle, 1999). Episodic acidification events may also have played a role in salmon decline in the north-eastern United States (National Academy of Science, 2004). Together, acid and aluminium caused accumulation of that metal on the surface and within the gill (Lacroix *et al.*, 1993; Wilkinson and Campbell, 1993; Teien *et al.*, 2004). Damages to the epithelium, increased gill permeability, decreased NKA activity (Staurnes *et al.*, 1993a, 1996; Kroglund and Staurnes, 1999; Magee *et al.*, 2003) and related active ion uptake (Booth *et al.*, 1988; McDonald *et al.*, 1991) lead to disrupted ion regulation (McCormick *et al.*, 2009).

In addition to acidity, heavy metals and organic pollutants may also impact the parr-to-smolt transformation. Indeed, heavy metals tend to block ion transfer through the gill membrane by inhibiting specific transporters (McCormick *et al.*, 1998). They may also diminish olfactory capacities and negatively impact the homing phenomenon through the imprinting phase. To a smaller scale, Roux (1984) showed that a localised organic pollution led to the migration of graylings (*Thymallus thymallus* L.) on a large portion of the Rhone. In Canada, the use of nonylphenol as a surfactant in pesticide for forest-spraying is correlated with historical declines in adult return rates of Atlantic salmon (Fairchild *et al.*, 1999). Fish treated with nonylphenol or estradiols exhibited reduced gill NKA activity and expression, decreased numbers of gill chloride cells causing lower seawater tolerance and an inhibition of smolt development (Madsen *et al.*, 1997; McCormick *et al.*, 2005).

The construction of hydroelectric plants and dams in a lot of occidental and continental regions also hinders migration of numerous species (Willis *et al.*, 1980; Smith, 1985; Malbrouck *et al.*, 2007). The installation of turbines to produce electricity leads to increased mortality rates (2 %-15 %) in migrating species (Coutant *et al.*, 2000). Through their morphology, eels are probably most heavily impacted by turbines and mortality rates may be up to 20% in large turbines and even up to 50-100 % in smaller ones (Durif, 2003). Most "fish-friendly" turbines and deterrent devices are still in development or require high funding to replace aging turbines (Čada, 2001). Grids may prevent fish from entering turbines however grids may inflict injuries to fish like ripping off scales and descaling was shown to impair

hypo-osmoregualtory capacity of smolts at seawater entry (Zydlewski *et al.,* 2010). Furthermore, dams may have multiple effects on migrating fish; they slow down water flow and may thus lead to an increase in temperature, force fish through turbines if joined with a hydropower plant or delay fish that have to find the entry of an adjacent bypass river or fishladder.

Sometimes, smolts are still in freshwater past the usual season for their seawater migration, e.g. if delayed through dams or under elevated spring temperature (McCormick *et al.,* 1999; McCormick *et al.,* 2009). Smolts may then exhibit signs of desmolting (Wedemeyer *et al.,* 1980). Smolts lose their silvery colour and gill NKA activity drops (Jonsson and Jonsson, 2011). A direct relationship between reduced gill NKA activity and degree*days has been found in wild Atlantic smolts (McCormick *et al.,* 1999). Moreover, at the end of the migration season, lower NKA activity was measured in smolts from warm, southern rivers (Connecticut River and Penobscot River, Maine), but not from colder northern rivers (Catamaran Brook, New Brunswick and Conne River, Newfoundland). Furthermore, a faster decrease in NKA activity was measured under higher temperature in the lab in both hatchery-reared and stream-reared fish. Degree*days are also correlated to the loss of salinity tolerance (Stefansson *et al*., 1998; McCormick *et al*., 1999) and migration termination (Zydlewski *et al.,* 2005). Modification of certain tissue composition back towards that of parr has also been observed in masu salmon desmolts with increased lipids in muscle, liver, intestines and gills (Li and Yamada, 1992). This high rate of lipids is also observed at the parr stage. In addition, an increase in triacylglycerol and a decrease in the phospholipid proportion have been observed in this study. Desmolting may be linked to sexual maturation, temperature, photoperiod and ion content of water but there are still discussions to how long it takes for smolts to desmoltify. Males have a lower minimal size for becoming sexually mature than females (Jonsson, 1989; Fängstam *et al.,* 1993) making them more incline to desmoltify. The timeframe during which smolts tolerate a transfer from freshwater to seawater depends on the temperature. At 10 °C and 12 °C, it lasts for 280–350 degree*days and up to 450 degree*days at 14 °C in Atlantic salmon (Stefansson *et al*., 1998). Photoperiod joined by ionic content of the water may have a stronger influence (Soivio *et al.,* 1988). For example, desmolting in the Atlantic salmon would be initiated by a salt concentration under 15 ‰; this threshold could be lower for Baltic populations (Mortensen and Damsgård, 1998). As in the smoltification, the endocrine system plays a role in desmoltification but it has not been elucidated in detail yet (Björnsson *et al.,* 2011). Some hormones may even play a role in both processes (Ágústsson *et al.,* 2001). The role of GH is somewhat ambiguous in that point as secondary increases in

late June have been measured, suggesting GH favours smoltification and plays a role in desmolting (Ágústsson *et al.*, 2001). Given inter-individual differences in desmoltification pace, some authors suggested that modifications linked to desmolting are not synchronized while they are during smolting (Stefansson *et al*., 1998). Furthermore, it is not a parr reversion as it was seen in some Pacific salmonids or at least, it was not until the end of their study. Atlantic salmon desmolts can resmoltify the following year (Eriksson 1984; Shrimpton *et al.*, 2000) even at a faster pace and to a greater extent (Wedemeyer *et al.*, 1980). The fact that larger anadromous salmonids begin their seaward migration earlier than smaller and younger Atlantic salmon and brown trout (Jonsson 1985; Jonsson *et al.*, 1990) may then be explained by a more conducive surface to volume ratio of large fish and better resistance to cold seawater (Finstad *et al.*, 1988). Some stocks may be unable to readapt to freshwater (Boeuf, 1993).

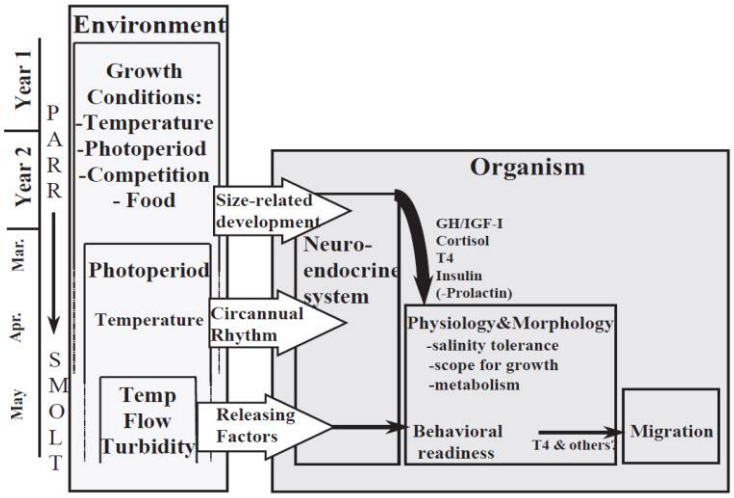

Figure 25 : Sum up of multiple interactions between environment and organism leading to the smoltification and migration in Atlantic salmon (McCormick *et al.*, 1998)

1.4.5 Smoltification in brief

To sum up (Figure 25 & Figure 26), environmental conditions (photoperiod, temperature, food availability, competition) influences Atlantic salmon parr growth. Once a critical threshold size is reached, smoltification may occur. Increased sensitivity of the light-brain-pituitary axis results in increased circulating levels of GH. GH increases liver IGF-1 secretion, TSH influences thyroid hormone secretion and ACTH stimulates interrenal cortisol secretion. In spring, these changes (and interactions between GH-IGF-1 axis with cortisol) will coordinate the development of physiological, morphological and behavioural preadaptation for sea-life, notably hypo-osmoregulatory capacity in gills, gut and kidney. They also

influence growth and metabolism with increased lipid and carbohydrate catabolism and increased protein synthesis. Thyroid hormones also influences metabolism as well as behavioural (schooling, negative rheotaxis) and morphological (silvering, darkened fins, imprinting) changes. Prolactin is believed to be inhibitory to smoltification. Temperature influences the pace of development of these changes. After these modifications have been induced, resulting in a migratory readiness, environmental factors (temperature, flow rate, turbidity…) then act as releasing factors and initiate seaward migration (McCormick *et al.*, 1998; Jonsson and Jonsson, 2011; McCormick, 2013).

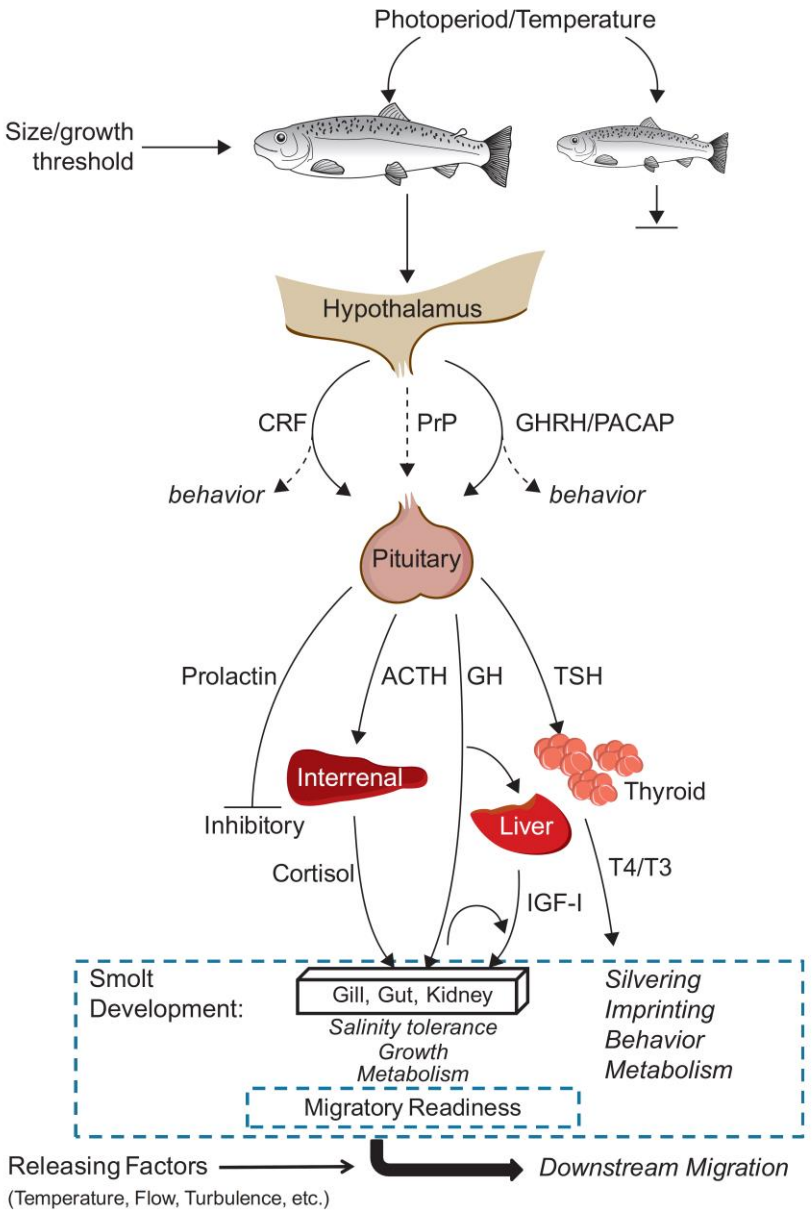

Figure 26 : Role of environmental factors and neuroendocrine system in smoltification and seaward migration in Atlantic salmon. CRF: corticotropin-releasing factor; PrP: prolactin-releasing peptide; GHRH: growth hormone-releasing hormone; PACAP: pituitary adenylate cyclase-activating peptide; ACTH: adrenocorticotropic hormone; TSH: thyroid stimulating hormone (McCormick, 2013)

2 Objectives and Scientific Strategy

2.1 Objectives

The present work is a paper-based thesis and each part of the result chapter will address a specific goal. The primary aim of this study was to investigate to what extent a temperature increase arising during seaward migration of the Atlantic salmon may affect the physiological status of smolts. In addition, we wanted to investigate whether different strains would react differently to such a change in environmental conditions. To achieve our primary goals, we developed a scientific strategy (Figure 27) with specific goals and corresponding hypotheses. Three experiments in controlled conditions were conducted and one in the field. Results should help us gain insights on the impact of anthropogenic use of waterways and climate change on anadromous salmonid species. Ultimately, in terms of restocking programs, the results of this research should help for a better understanding of the adaptability of allochtonous Atlantic salmon strains to the environmental conditions of a modified river system through human use.

➔Firstly, we wanted to investigate the potential differences in the endocrine and enzymatic mechanisms involved in smoltification of two non-native strains.

Therefore, our first hypothesis states that there are differences in the smoltification process between allochtonous strains reared under the same conditions.

We put this hypothesis to the test in our first experiment in Chapter 4.1 where we looked at specific smolting indicators found in literature and compared them between the two strains at several time points across smoltification. This also gave us a selection of the most useful markers to compare the strains in the following experiments.

➔Secondly, we intended to highlight the response to a rapid raise in temperature using endocrine and enzymatic markers as well as gene expression modulation.

Our hypothesis is that a temperature increase based on environmental data will impair smoltification with differences in response between the strains and between early and late migrants.

In our second experiment, we simulated the migration conditions of early and late migrants in the Meuse system from the cooler tributary through the main channel and end with a seawater challenge mimicking sea-entry. In Chapter 4.2, selected markers from the first experiment were used to assess the physiological response in early and late migrants to a temperature increase. In Chapter 4.3, we investigated the transcriptional response in the liver, a key organ for smoltification notably for its role in metabolism. A selection of genes based on literature

served the purpose of comparing the response in early and late migrants in both strains. In Chapter 4.4, the transcriptional response in the gills, the major organ for hypo-osmoregulation, was investigated in early and late migrants in one strain only (third experiment) because the second one was unavailable.

➔Thirdly, we set out to verify our laboratory findings under natural conditions by field samplings during downstream migration.

Our third hypothesis states that there are differences between smoltification under simulated natural and natural conditions.

Therefore, we intended to compare our laboratory results with field data. Our simulated conditions are based on environmental data from the river Meuse and one of its tributary, the river Ourthe. We sampled migrating smolts in both locations and compared several physiological indicators.

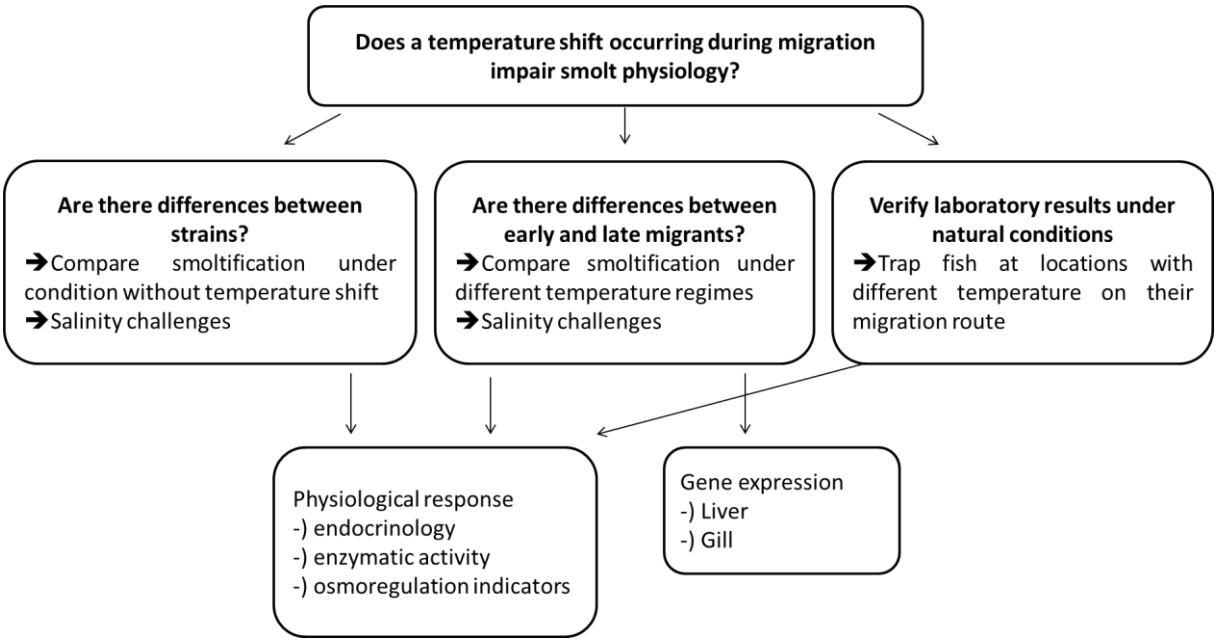

Figure 27: Scientific strategy to meet the thesis' objectives

3 Material and Methods

3.1 Fish origin

Two allochthonous salmon strains were compared. The first strain is originated from the Cong Hatchery on the River Cong rising near the Cong village, County Mayo in Ireland (Figure 28, Figure 29). It is an outflow of Lough Mask and runs for 2 km into Lough Corrib at Ashford Castle with an average flow rate of 37.6 m^3*s^{-1}. Strict fishing rules are in application to protect its Brown trout, Atlantic salmon and Ferox trout populations. Inland Fisheries Ireland (IFI) runs the hatchery which benefits from a large run of adults spawners returning to the hatchery. Recently, IFI announced the cessation of IFI fish farm activities and that they would only maintain one facility for research and necessary stocking. Considering high water quality and quantity, IFI identified Cong as the site with most potential. Cong is expected to benefit from an upgrade to become a modern hatchery research facility (www.fisheriesireland.ie).

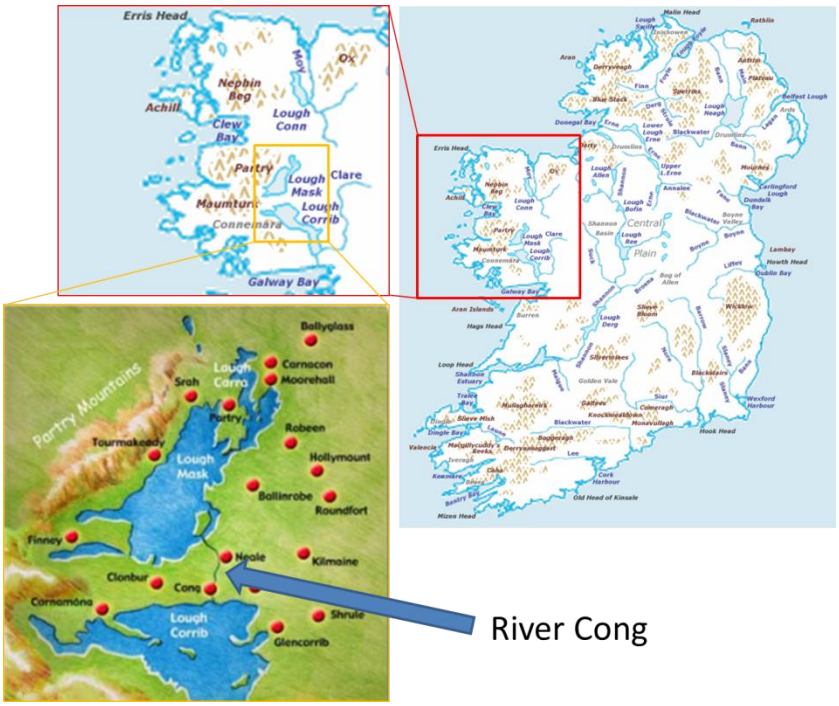

Figure 28: Location of the River Cong (County Mayo, Ireland) in between Lough Mask and Lough Corrib (www.fairhillhouse.com, www.wikipedia.org)

Figure 29: Pictures of the River Cong at the Quiet Man Museum beside the hatchey and at Monks Fishing House (www.congregation.ie)

The second strain comes from the 'Conservatoire National du Saumon Sauvage de Chanteuges" opened in 2001 in the Haute-Loire region, France. It was built close to the confluence of the rivers Desges (35.3 km long, 1.88 m^3*s^{-1} flow rate in Chanteuges) and Allier. The latter is one of the largest tributary of the Loire (1006 km long) with its 421 km long run and a flow rate of 144 m^3*s^{-1} in Cuffy at the confluence. Designed as the biggest European hatchery, it was meant to protect the last wild salmon population in Western Europe capable of spawning in area 1000 km upstream. The facility fulfils its dual role of production and research with state of the art technologies. It is capable of producing up 2 250 000 eggs, 600 000 parrs and 235 000 smolts (http://www.saumon-sauvage.org).

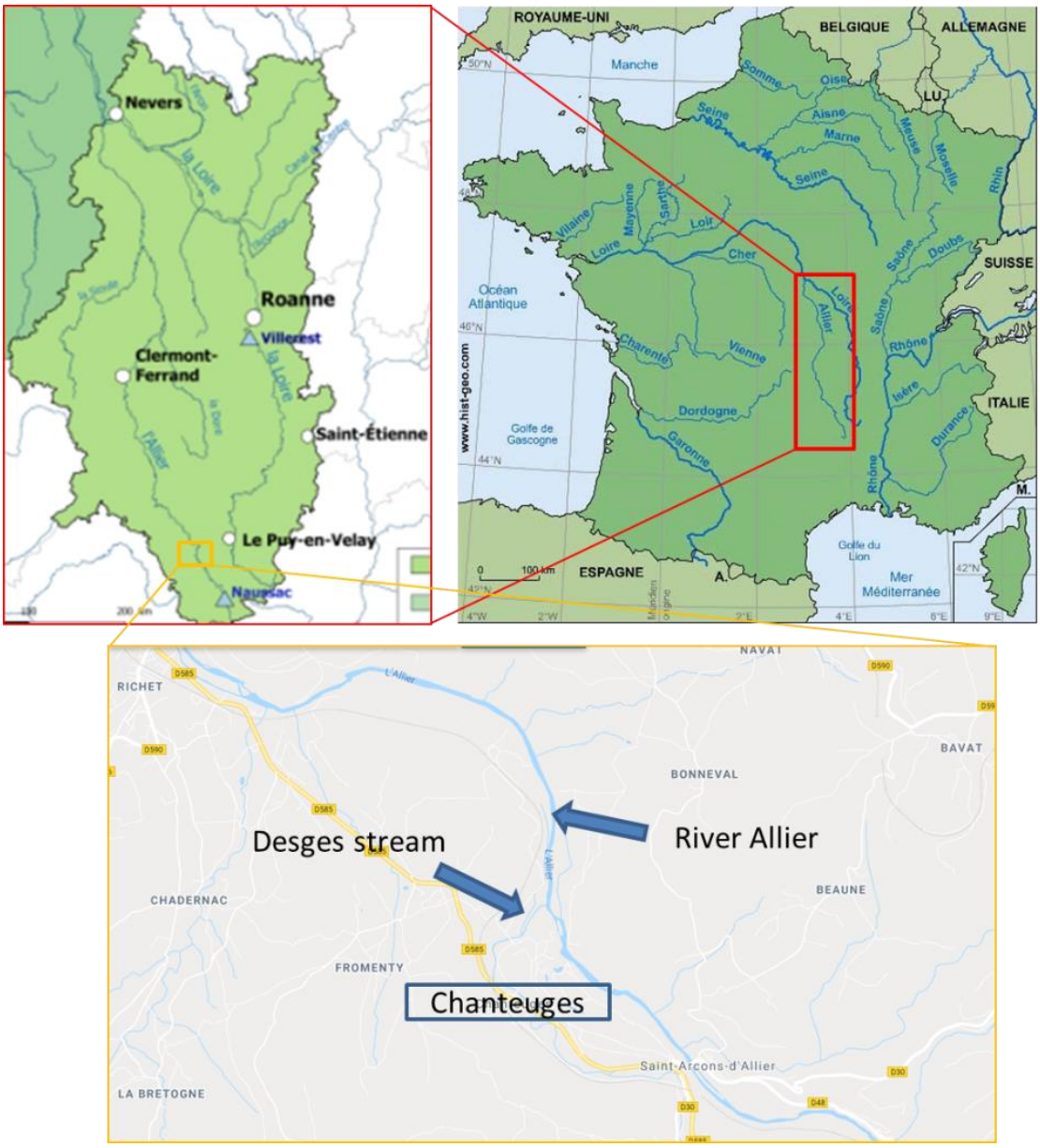

Figure 30: Location of the confluence of the Desges stream and River Allier in Chanteuges (Department of Haute-Loire, France) (www.grattepanche-mairie.fr, www.wikipedia.org, www.googlemap.fr)

Figure 31: Pictures of the Desges stream close to Besseyre-St.-Mary, River Allier next to the Blot cliff and River Loire close to Chinon (www.routard.com, www.eauvergnat.fr, www.wikipedia.org)

3.2 Study area

From a geographic point of view, the Meuse has its spring in Pouilly-en-Bassigny in France from where it flows for 492km in France, 194 km in Belgium and 239 km in the Netherlands to drain in the North Sea at Haringvliet. Downstream of Lixhe, at Eijsden, the Meuse crosses the Dutch border, runs through Maastricht and then runs along the border between the two countries for 47 km to Stevensweert. For that distance, it is called Grensmaas (Border Meuse). Geologically speaking, the Meuse runs through three major geological zones. From Pouilly-en-Bassigny to Charleville-Mézières, the Lotharingian Meuse flows mainly through consolidated sedimentary Mesozoic rocks. From Charleville-Mézières to Liège, the Meuse runs through the Paleozoic rock of the Ardennes Massif, hence its name of Ardennes Meuse. From Liège onwards, the lower reaches of the Meuse cross Dutch and Flemish lowlands consisting of Cenozoic unconsolidated sedimentary rocks (Nienhuis, 2008).

The geology hugely influences the hydrological conditions of the Meuse basin. Annual precipitation averages range from 800-900 $mm*year^{-1}$ the upper reaches, over 1000 $mm*year^{-1}$ in the Ardennes and 700-800 $mm*year^{-1}$ on the lower reaches. An annual discharge average at the outlet (Hollands Diep) is approximately 350 m^3*s^{-1}. This corresponds to a precipitation surplus of 400 $mm*year^{-1}$ (De Wit *et al.,* 2002). The Meuse exhibits a typical rainfall – evaporation regime of a temperate climate zone (Figure 32). This causes low flow in summer and high flow in winter. In Waulsort, close to the Belgian-French border, the range of discharge varies from 14 to 700 m^3*s^{-1} (average of 140 m^3*s^{-1}) over a year and from 10 to 3000 m^3*s^{-1} (average of 230 m^3*s^{-1}) in Borgharen, a representative measuring point in the Netherlands. Of the approximate 33000 km^2 of catchment area, 13500km^2 are in Belgium, 9000 km^2 in France, 6000 km^2 in the Netherlands, and despite not flowing through these countries, 4000 km^2 in Germany and 600 km^2 the Grand-Duchy of Luxembourg. In the southern part, the gradient of the Meuse is steep and erosive and tends to become rather

constant gradient (0,5 m*km^{-1}) from Neufchâteau to Maasbracht. The steepest slopes (up to 5 m*km^{-1}) are found in tributaries that spring in the Ardennes/Eiffel Massif (Amblève, Lesse, Ourthe, Semois, Vesdre, Viroin,…) (Nienhuis, 2008).

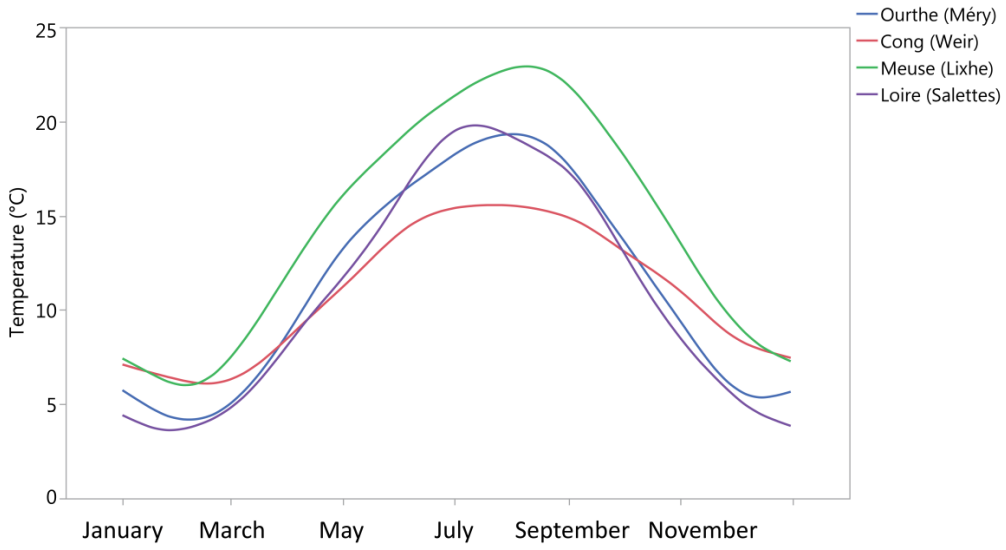

Figure 32: Mean (2013-2017) daily temperature of the river Ourthe in Méry, the river Meuse in Lixhe, the river Cong at the Weir and the river Loire in Salettes (DGO3, Département de la Police et des Contrôles - réseau de contrôle ; Laboratoire de démographie Piscicole et Hydrologie, University of Liège ; Fédération de pêche et de protection du milieu aquatique de Haute-Loire and Office of Public Works-Hydrometric Section)

The first part of the Meuse flows through a hilly landscape and benefits from wide floodplains which get inundated even during an average flood event, thus weakening the flood and reducing the risk of serious problems in the central and lower reaches of the Meuse. In addition, weirs and a lateral canal are also present. From Charleville-Mézières on, the Meuse flows through a steep valley flanked by the Ardennes Massif which does not allow a weakening of floods like in the upper part. A large amount of weirs regulate entirely the Meuse until downstream of the Dutch weir at Lith where tidal influences are present (Van Leussen *et al.*, 2000).

The Ourthe is a right tributary to the river Meuse (Figure 33). This 165 km long river is formed by the confluence in Engreux of the Western Ourthe and the Eastern Ourthe which spring are located next to Libramont-Chevigny and Gouvy respectively. The river will flow through several regions of Belgium, and cross the Calestienne, mainly formed of limestone rocks. The river exhibits a typical rainfall – evaporation regime of a temperate climate zone and is annually restocked with Atlantic salmon (Figure 32).

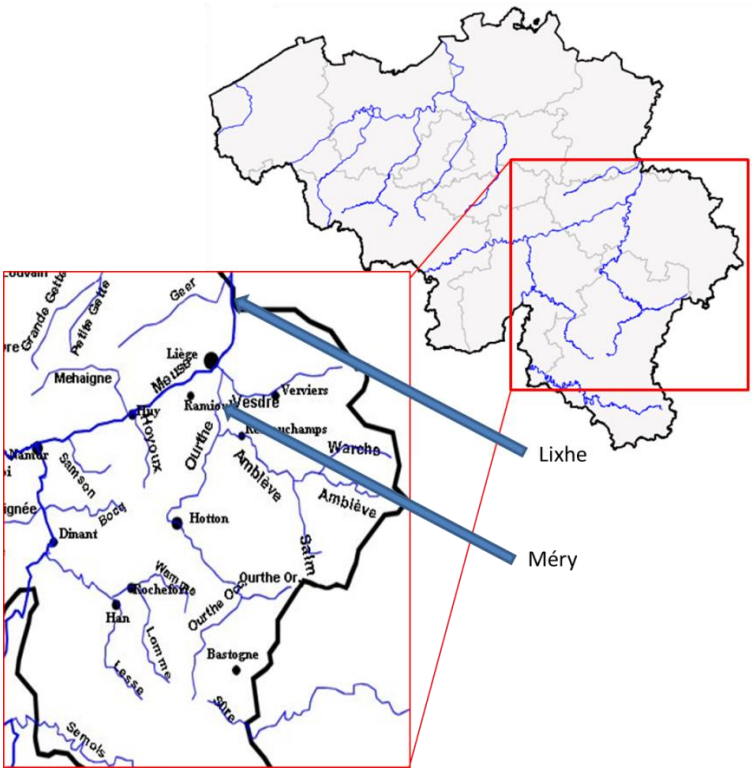

Figure 33: Location of Méry on the River Ourthe and Lixhe on the River Meuse in Belgium (www.excursions-scolaires.com, www.wikipedia.be)

3.3 Fish rearing and sampling sites

For each of our three laboratory experiments, a new batch of fish was raised (Table 3) to presmolts in the "Conservatoire du Saumon Mosan" hatchery where rearing conditions were as follows: simulated natural photoperiod based on Liège latitudes (50°37'59"N), Aisne river temperature and daily feeding with a fixed ration (5% of fish biomass after yolk sac resorption, 3% for fry and 1% for parr) provided with automatic feeders along the day. Details for rearing conditions for each laboratory experiment are given in the corresponding chapters. For the field experiment, annual fry restocking (Table 4) was carried out in the River Ourthe basin. Migrating fish were then caught in the following springs.

Several recirculating aquaculture systems (RAS) were used in our experiments. In our first investigation (Figure 34A), temperature was controlled by an electronic sensor for a given temperature ± 0.5 °C coupled to a plate heat exchanger. Water was pumped continuously in the tanks to create a circular flow. Supplemental aeration insured sufficiently high dissolved oxygen concentrations.

In our second experiment, three RAS were used (Figure 34B), each one for a different temperature regime. The filter units were all linked together into an external tank coupled to a plate heat exchanger. For each RAS, a temperature sensor (± 0.1 °C) was connected to 1000 W element and to a solenoid valve controlling the inlet of cooled water from the tank.

Three independent RAS were used for the third experiment (Figure 34C), each equipped with a 300 W heating element and a solenoid valve connected to a common water cooler.

In the field, migrating smolts entering the trap in Méry, were channelled towards a life-box (Figure 35). Details and frequency of the trapping and sampling are given in Capter 4.5. In Lixhe, we used a trap next to the hydropower plant and a circular tank next to the fish ladder (Figure 36). Details of the trap used in Lixhe can be found in Prignon and Micha, 1998 or in Capter 1.1.3.1. Sampling and frequency of trapping are given in Chapter 4.5.

Table 3: Number of fish used per experiment

	Number of fish used	Chapters
Laboratory experiment 1	720	Chapter 4.1
Laboratory experiment 2	1300	Chapter 4.2 & Chapter 4.3
Laboratory experiment 3	200	Chapter 4.4
Field experiment	349 (total over three years)	Chapter 4.5

Table 4: Fry restocking amount in the Ourthe basin from 2011 - 2015

Year	Restocked number
2011	72032
2012	187958
2013	231395
2014	294534

Figure 34: Recirculating aquaculture systems used for fish rearing during the first (A), the second (B) and the third experiment (C)

Figure 35: Trap location in Méry on the Rive Ourthe, (A) aerial vue, (B) side vue of the building beneath which the trap is located and (C) life-box at the end of the trap (www.trekearth.com)

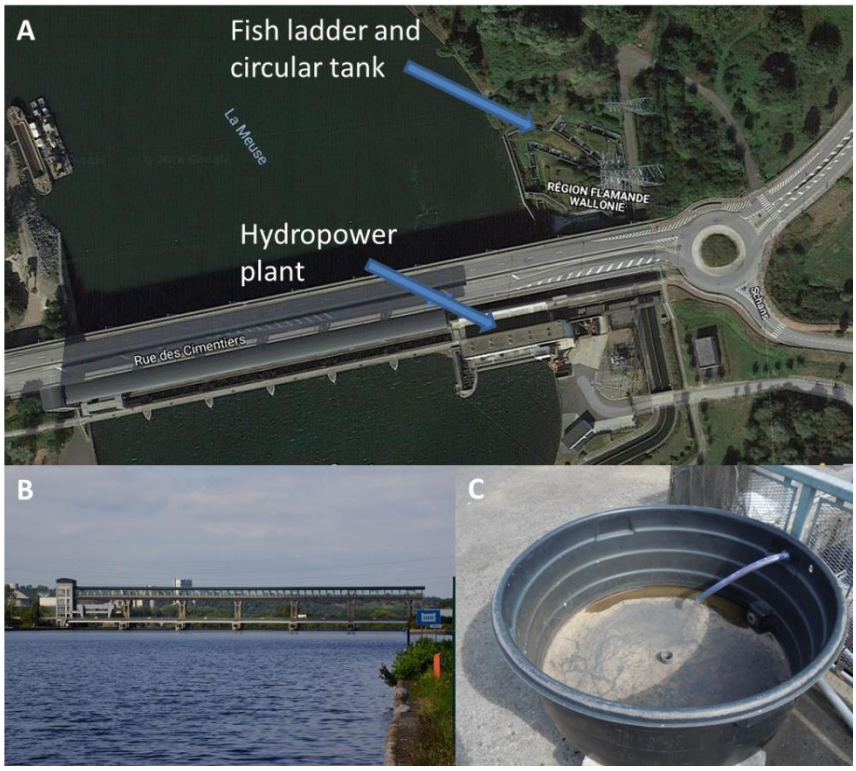

Figure 36: Tank location in Lixhe, (A) aerial vue of the hydropower plant, (B) front vue of the damn and (C) circular tank used in our study next to the fish ladder (www.googlemap.be, www.structurae.info)

4 Results

4.1 Influence of strain origin on osmoregulatory and endocrine parameters of two non-native strains of Atlantic salmon (*Salmo salar* L).

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Abstract

Non-native strains of Atlantic salmon are used in reinstatement trials where populations are extinct. Environmental cues like photoperiod and temperature are known to influence the smolting process and there is evidence of strain-, stock- or population-specific differences associated with seaward migration or smoltification. The objective of this study was to compare morphological, osmoregulatory and endocrine features between two strains, one originating from a cold and short river in Ireland (Cong) and another from a long and warm river in France (Loire-Allier), reared under Belgian conditions in order to highlight major differences in restocking adaptability. Comprehensive endocrine profiles, consistent with their interactive role of mediating changes associated with smolting, have been observed. Na^+/K^+ATPase activity (1.3-10.5 µmol ADP*mg prot.$^{-1}$*h^{-1}) and hormone plasma levels (e.g. 55-122 ng*mL^{-1} of cortisol and 4.5-6.4 ng*mL^{-1} of GH) were consistent with reported values. We observed strain-related differences of the influence of temperature and daylength on cortisol, GH and sodium plasma levels. These may be related to the respective environmental conditions prevailing in the river of origin, which have impacted the genetic background for smoltification. Using Na^+/K^+ATPase activity as an indicator, both strains smoltified successfully and simultaneously testifying a prevailing influence of environmental cues over genetic factors for smoltification.

Atlantic salmon, smoltification, repopulation, Na^+/K^+ATPase, hormone profile

1. Introduction

Despite their economic value and ecological and cultural interest, Atlantic salmon (*Salmo salar* Linnaeus, 1758) populations are declining across their entire distribution range (Hawkins, 2000; Jonsson and Jonsson, 2011). The causes of such decline are multiple (Julien and Bergeron 2006; Klemetsen *et al.*, 2003; Webb *et al.*, 2007), though essentially linked to anthropogenic activities (Jonsson and Jonsson, 2009). Following this population decline, many countries have set up compensatory and enhancement stockings for wild populations (Brown and Laland, 2001). Where populations are already extinct, the use of non-native strains in unavoidable. However, the salmon's particular life-cycle, including a switch from fresh- to seawater after a seaward migration, may impede the success of these initiatives. Therefore, a wise choice of strain is of prime importance to achieve good cost-effective management of the salmon population.

The transformation from stream-dwelling parr to seawater-tolerant smolts is a complex developmental process involving a wide array of physiological, morphological and behavioural modifications (Björnsson *et al.*, 2011; McCormick *et al.*, 1998; McCormick *et al.*, 2013). Smolts are at their peak preparedness, insuring the highest survival rate at sea entry, during a limited time frame (Hansen and Jonsson, 1989; McCormick *et al.*, 1999). This physiological smolt window coincides with an environmental condition range in which seasonal changes in environmental conditions in rivers, estuaries and sea coasts are appropriate for high smolt survival (Jonsson and Jonsson 2011; McCormick *et al.*, 1998).

Photoperiod and temperature are the primary environmental factors influencing the smolting process (Björnsson and Bradley, 2007; Jonsson and Jonsson, 2011; McCormick *et al.*, 1998). Increasing and decreasing photoperiods are reliable and predictive factors indicating the season (Wootton, 1998) and temperature affects the rate of development (McCormick *et al.*, 1999; McCormick *et al.*, 2002; Shrimpton *et al.*, 2000). These factors vary considerably across the salmon's geographic distribution. Compared to the southern part, changes in photoperiod are much more pronounced in the northernmost latitudes where total darkness and constant illumination alternate over a year. On the Loire-Allier axis, in southern France, the middle catchment river temperature may rise up to 30°C in July (Martin *et al.*, 2012). On the contrary, temperature stays low all spring and early summer with a mean summer temperature of 8.9°C in the Stryn River, Norway (Jonsson *et al.*, 2001).

There is also evidence of population and stock-specific differences in downstream migration timing (Orciari and Leonard, 1996; Stewart *et al.*, 2006) and strain-specific increases in salinity tolerance (Handeland *et al.*, 2004) under the same environmental cues which led to

the hypothesis of a genetic structuring of Atlantic salmon at the sub-catchment scale (Stewart *et al.*, 2006).

The Cong River in Ireland is only 2km long and joins the Lough Mask with the Lough Corrib. To reach the sea, smolts have to cross the Lough Corrib (44 km) and the Corrib River (6 km) for a total migration distance of 50 km. On the opposite, smolts from the Loire-Allier Axis have to cover over 900 km to reach the sea.

This study put in comparison two non-native strains, one from a long and southern river in France and one from a short and cold river in Ireland, reared under the same Belgian river conditions and aimed to highlight the importance of genetic pool for restocking adaptability by comparing some smoltification features.

2. Materials and methods

2.1 Fish rearing

The two strains of Atlantic salmon used during this study originated from the Cong Hatchery on the Cong River (CG) in Ireland and from the ”Conservatoire National du Saumon Sauvage” in Chanteuges on the Loire-Allier (LA) River in France. Fertilised eggs (F1) from recaptured wild spawners (F0) were directly imported and reared at the “Conservatoire du Saumon Mosan” hatchery (Public Service of Wallonia, Fisheries Services), located in Erezée (Belgium) along the Aisne River, a historical salmon river, until they reached the pre-smolt stage. Rearing conditions were as follows: simulated natural photoperiod based on Liège latitudes (50°37’59”N), Aisne river temperature and daily feeding with a fixed ration (5% of fish biomass after yolk sac resorption, 3% for fry and 1% for parr) provided with automatic feeders along the day. In early March, these pre-smolts, aged 0+ (mean body weight = 25.7 g for CG and 26.7 g for LA), were transferred from Erezée to a wet laboratory of the University of Namur. Fish were equally divided into three 500 L tanks per strain (N= 120 fish per tank). Throughout the study, fish were maintained in these tanks with a circular stream flow and supplemental aeration under the same simulated natural photoperiod as in Erezée. Water temperature during the experimental period was based on the mean value of a decade of data from the river Aisne (Table 1). Fish were daily fed (TroCo Supreme-21 Coppens International bv, Helmond, The Netherlands) a fixed ration of 1% of the biomass with automatic feeders along the day. Oxygen concentration and temperature were checked daily and water physico-chemical parameters (pH=7.4, $NH_4^+ < 0.06$ mg L^{-1}, $NO_2^- < 0.05$ mg L^{-1} and $NO_3^- < 10$ mg L^{-1}) were monitored weekly.

Table 1: Water temperature [°C] and daylength [h] on sampling dates.

Date	Temperature [°C]	Daylength [h]
07/03	6.5	11.4
14/03	7	11.8
21/03	7.2	12.3
28/03	7.5	12.7
04/04	8	13.2
11/04	8.5	13.6
18/04	10	14.0
25/04	10.5	14.4
02/05	11.5	14.8
09/05	12	15.2
16/05	13.2	15.6
23/05	14.5	15.9

2.2 Sampling

After two weeks of acclimation, samples were collected once a week from mid-March to end-May. Four fish were quickly dip-netted from each tank and directly anaesthetised with 120 mg L^{-1} of tricaine methanesulfonate (MS-222, Sigma). Blood was collected from the caudal vein into 1mL heparinized syringes. The needle was removed and the blood was expelled into a 1.5-mL Eppendorf tube, stored on ice for less than 30 min and then centrifuged at 3000g for 10 min. The supernatant was then divided into six parts and kept at -80 °C until subsequent analyses. The fish were then measured (total length and fork length) to the nearest 0.1 cm and weighed to the nearest 0.1 g allowing calculation of the condition factor $K= 100*(W/L^3)$. External morphological characteristics including silvering, presence of reddish dots and typical parr oval-shaped marks on the flanks were recorded. Finally, left-sided branchial arches 1 and 2 were cut out, immediately frozen in liquid nitrogen and stored at -80°C until assayed.

2.3 Plasma osmolality and ion concentrations

Plasma sodium and potassium concentrations were measured using a Philipps PU 9200 atomic absorption spectrophotometer (Pye Unicam, Cambridge, United Kingdom) with 0.25-, 0.5-, 0.75- and 1 ppm external standards for sodium and 0.5-, 1-, 2- and 3 ppm external standards for potassium. Plasma osmolality was measured using a Löser Type 6 freezing point

depression osmometer (Löser Messtechnik, Germany) with 0-, 300- and 900-milliosmole external standards.

2.4 Na^+/K^+ATPase activity

Gill Na^+/K^+ATPase (NKA) activity was measured according to the method described by McCormick (1993) with slight modifications. Gill filaments were homogenized in ice-cold phosphate buffer (50mM KH_2PO_4, pH 7.4) followed by centrifugation (3000g for 5 min) to remove large debris. NKA activity was determined with a kinetic assay linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), in presence and absence of the Na^+/K^+-ATPase specific inhibitor Ouabain. Ten-microliter samples were run in two sets of duplicates at 25 °C and read at a wavelength of 340 nm for 10 min. The first set contained assay mixture (TrisHCL 50 mM ; $MgCl_2$ 10 mM ; EGTA 0.5 mM ; pH 7,4 ; NaCl 500 mM ; KCl 125 mM ; PEP 30 mM ; NADH 3 mM ; PK 7U mL^{-1}; LDH 5U mL^{-1}; ATP 50 mM) and the other assay mixture was the same plus Ouabain (10 mM). This assay was run on an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, USA) with use of MARS software (BMG LABTECH GmbH, Germany).

Total protein concentration of the gill homogenate was measured in duplicate using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumen as the standard. This assay was run on a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany) with use of MARS software. NKA specific activity is expressed as micromoles of ADP per milligram of protein per hour (µmol ADP*mg^{-1} of protein*h^{-1}).

2.5 Hormone assays

Hormone assays were performed on pooled samples of 3 fish per tank. Plasma cortisol levels were measured by a direct competitive enzyme-linked immunosorbent assay (DRG Instruments GmbH, Germany, EIA-1887). The lower detection limit was 2.5 ng*mL^{-1} and the intra-assay variation coefficients was 5.63 %. Fish prolactin ELISA kit (CSB-E12695Fh, Cusabio, P.R. China) has been designed on Atlantic salmon amino acid sequence found on Uniprot (http://www.uniprot.org/uniprot/P48096). The lower detection limit was 0.5 ng*mL^{-1} and the intra-assay variation coefficients was <15 %. Fish GH (CSB-E12121Fh) and Fish IGF-1 (CSB-E12122Fh) ELISA kits (Cusabio, P.R. China) were used. Detection lower limits were 312.5 pg*mL^{-1} (intra-assay variation coefficient <15 %), 25 pg*mL^{-1} (intra-assay variation coefficient <15 %) respectively. These kits have been designed on salmonids amino acid sequences; (Sockeye salmon (*Oncorhynchus nerka*) for GH (http://www.uniprot.org/uniprot/Q91222) and Rainbow trout (*Oncorhynchus mykiss*) for IGF-

1 (http://www.uniprot.org/uniprot/Q02815)).). Furthermore, it has been documented that IGF-I amino-acid sequence has been well conserved during evolution and that a high degree of similarity is observed among the different vertebrate groups (Planas *et al*., 2000). Sequences among different fish species (e.g. barramundi (*Lates calcarifer*), coho salmon (*Oncorhynchus kisutch*), Southern Bluefin tuna (*Thunnus maccoyii*), tilapia (*Oreochromis mossambicus*) and seabream (*Pagrus auratus*)) were 83 % identical (Dyer *et al*., 2004). In addition to these theoretical concepts, parallelism was tested between the serially diluted plasma and standard curve. Results give further confidence that this assay is suitable for our species. These assays were run on a FLUOstar Omega microplate reader with use of MARS software. Thyroxine (T4) concentrations were measured by a direct radioimmunoassay (RIA). The T4 RIA had a detection limit of 0.4 ng*mL^{-1} and an intra-assay variability of 2.8 %. For the T4 RIA cross-reactivity with T3 was 3.5 %. All samples were measured in duplicate within a single assay.

2.6 Statistical analysis

All statistical analyses were performed with R version 3.3.3 and packages mgcv and ggplot2. We used a generalized additive model (GAM) with temperature (T), daylength (D), strain (S) and their interaction T*D, T*S, D*s and T*D*S as explanatory variables (Zuur *et al.*, 2009). LOESS smoothing was used to generate graphs (Hastie and Tibshirani, 1990; Wood, 2006). Validation tools for the GAM model were used as described by Zuur *et al.* (2009); qq-plot and the histograms to assess normality and the residuals versus fitted values to assess homogeneity. The best model was chosen by means of lowest generalized cross-validation (GCV) value (Wood, 2006; Zuur *et al.*, 2009) amongst models using only single effects, single effects and two-way interactions and single effects and all possible interactions. A summary of significant effects is presented in Table 2.

Table 2: Summary of significant effects of explanatory variables in the GAM models and adjusted R^2 of the respective models. T=temperature; D=daylength and S=strain.

	Cortisol	GH	Prolactin	IGF-1	T4	NKA	[Na^+]	[K^+]	Osmolality
T			$3.39E^{-2}$	$3.13E^{-2}$	2.3E-2	$5.46E^{-16}$		$1.99E^{-4}$	$4.04E^{-6}$
D		$1.24E^{-3}$				$8.19E^{-4}$	$3.56E^{-2}$		$7.71E^{-6}$
S		$3.43E^{-5}$					$2.41E^{-4}$		
T*S	$1.7E^{-3}$	$6.04E^{-5}$							
T*D				$4.14E^{-2}$		$<2E^{-16}$	$2.65E^{-2}$	$1.69E^{-4}$	
D*S	$4.1E^{-3}$	$3.87E^{-5}$					$4.55E^{-4}$		
R^2adjusted	0.67	0.46	0.05	0.02	0.15	0.53	0.22	0.05	0.09

3. Results

3.1 Growth, condition factor and morphologic criteria

Both strains exhibited a negative allometric growth type indicating a faster increase in length than in weight (Table 5). Allometric growth was slightly more pronounced in the LA population ($y = 2.69x-1.7$, $R^2=0.84$, N=108) than in the CG ($y = 2.73x-1.7$, $R^2=0.87$, N=120). Modelling of the condition factor K showed an influence of the strain ($p = 1.18\ E^{-4}$). Values range from 0.83 to 0.89 in CG smolts and from 0.84 to 0.94 in LA smolts with a higher average in LA smolts (0.91 vs 0.87). Silvering of the flanks progressively appeared as oval parr marks faded away across the smoltification season. Reddish dots were noticeable longer but eventually disappeared. In March, about 50% of the smolts still exhibited typical parr marks. On April 4th onwards, these percentages were reduced to around 30%, and from May 9th onwards, 97% of the sampled fish were fully silvered with no trace of parr marks. No difference between the strains was noticed.

3.2 Plasma osmolality and ion concentrations

Single-effect modelling showed significant influence of temperature ($4.04E^{-6}$) and daylength ($7.71E^{-6}$) on plasma osmolality (Figure 1a and b). It ranged from 280 mOsm up to 320 mOsm. Highest osmolality was measured at low and medium temperature and short and medium daylength. A complete model was used for sodium (Figure 1c and d). Two-way interactions P*S ($4.55E^{-4}$) and T*D ($2.66E^{-2}$) influenced sodium levels. Potassium was best modelled by single and two-way interactions (Figure 1e). T*D influenced potassium levels ($1.69E^{-2}$). Sodium level increased at low temperature and daylength values, reached a maximum at mid-range and decreased again at high values of these factors. Daylength also had an impact but it differed between the strains ($4.55E^{-4}$). At low values of temperature and daylength, interaction of these factors tended to influence a decrease in potassium level and an increase at high values. Sodium values ranged from 160.6 to 231.6 $mEqu*L^{-1}$ in CG smolts and from 136.9 to 219.6 $mEqu*L^{-1}$ in LA smolts. Potassium levels ranged from 3.3 to 6.3 $mEqu*L^{-1}$ in the CG strain and from 3.5 to 8.2 $mEqu*L^{-1}$ in the LA strain.

3.3 Gill Na^+/K^+-ATPase activity

Both lowest and peak values were similar in both strains (1.3-10.45 µmol ADP*g $prot^{-}1*h^{-1}$ in LA and 1.8-10.5 in CG) (Figure 1f). Modelling of NKA included single and two-way interactions and showed that T*D had a significant influence on NKA activity ($p<2E^{-16}$). NKA activity changes along smoltification with moderately high value at low temperature and short daylength, lowest value at mid-range temperature and daylength and highest values at high temperature and long daylength.

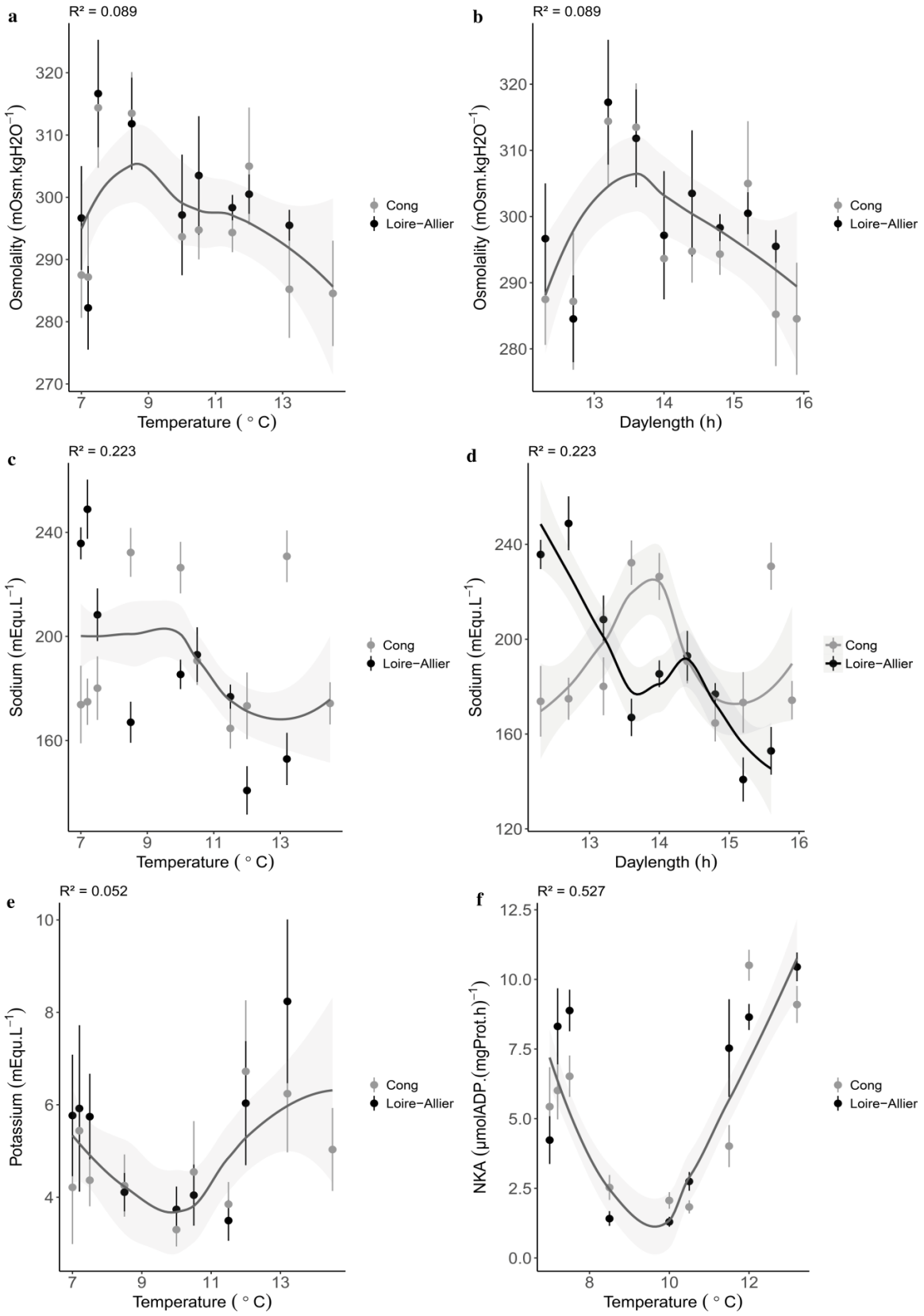

Figure 1: Plasma osmolality [mOsm*kgH_2O^{-1}] (a and b), sodium [mEqu*L^{-1}] (c and d) and potassium [mEqu*L^{-1}] (e) levels and NKA activity [µmolADP*mg protein^{-}1*h^{-1}] (f) across the study timeframe. When strain had no significant influence, a common profile is presented. When T*D was significant in the model, graphs are represented only with temperature for easier representation.

3.4 Plasma hormones

Cortisol modelling comprised single effects, two- and three-way interactions. D*S ($p=4.1E^{-3}$) and T*S ($p=1.7E^{-3}$) were significant, indicating a different influence of both environmental factor depending of the strain on cortisol plasma levels (Figure 2a and b).

Daylength and temperature influenced cortisol levels of LA smolts in a wave shape starting with a decrease in concentration with increasing daylength, reaching a low point at mid-range, then increasing towards a summit, and finally decreasing at highest temperature and longest daylength. In CG smolts, cortisol levels followed a similar shape but compressed horizontally. In addition, cortisol levels were continuously higher in CG smolts than in LA.

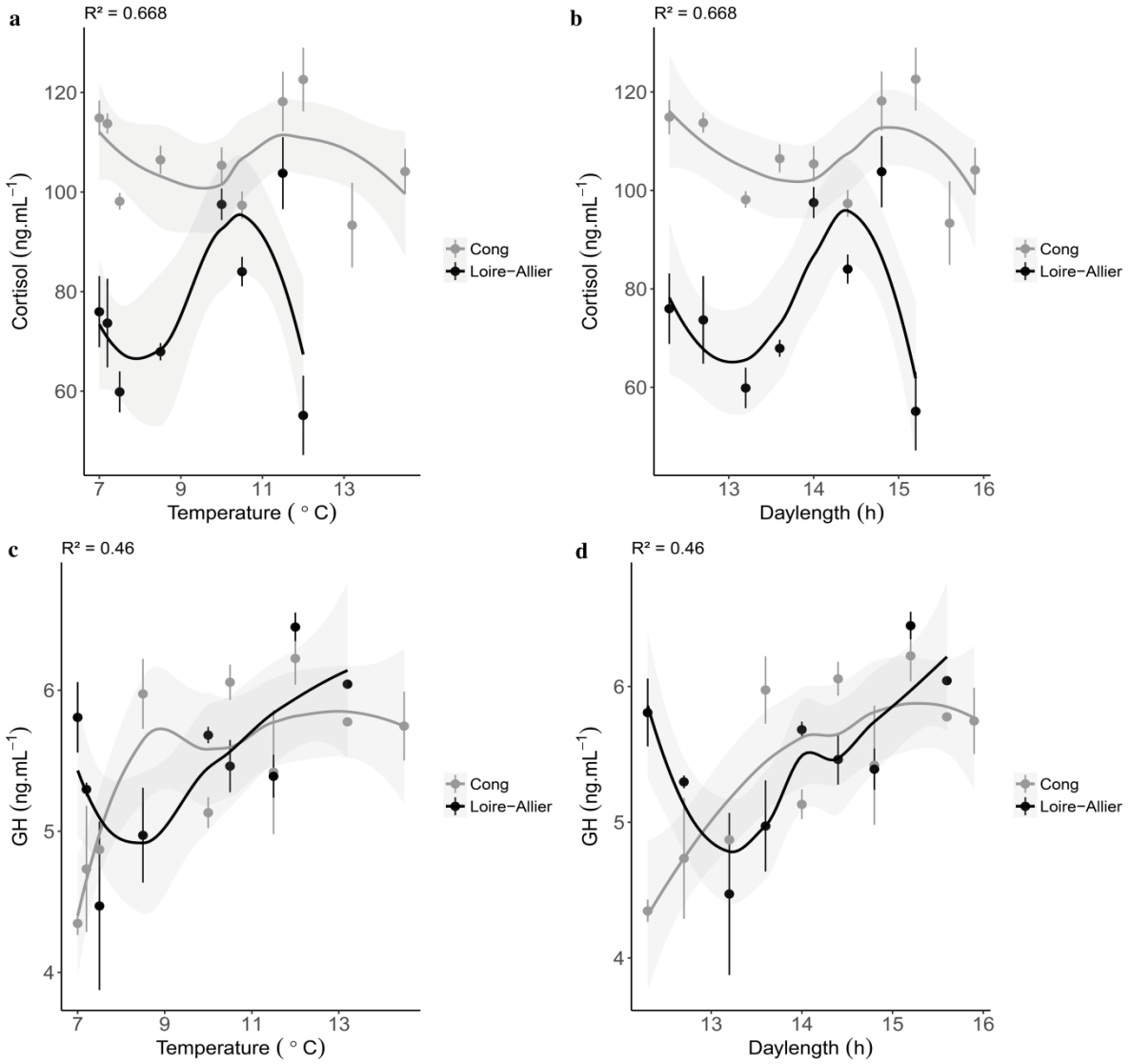

Figure 2: Cortisol (a and b) and GH (c and d) plasma levels [ng*mL^{-1}] across the study timeframe. When T*D was significant in the model, graphs are represented only with temperature for easier representation.

Levels ranged from 93 to 122 ng*mL^{-1} in CG and 55 to 104 ng*mL^{-1} in LA with an increase in mid-April and early May in LA. GH was best modelled without three-way interactions (Figure 2c and d). This model showed two-way effects of daylength*strain ($p=3.87E^{-5}$) and temperature*strain ($p=6.04E^{-5}$). In the CG strain, GH level was low at the onset of the experiment, increased with increasing temperature and daylength and then tended to reach a plateau at higher temperature and daylength. In the LA strain, GH level decreased at low temperature and short daylength, reached a low point and then increased at higher temperature and daylength without seemingly reaching a maximum level. Levels ranged from 4.3 to 6.2 ng*mL^{-1} in CG and from 4.5 to 6.4 ng*mL^{-1} in LA.

T4 (Figure 3a) and prolactin (Figure 3b) levels were best modelled using only single factors. No difference between the strains was observed. Temperature influenced T4 ($p=2.3E^{-2}$) and prolactin ($p=3.39E^{-2}$) levels. We also noticed a trend of daylength ($p=6.95E^{-2}$) to influence prolactin levels. T4 levels remained constant until medium temperature, increased towards a summit and finally decreased at the highest temperature.

Prolactin increased at low temperature, reached a maximum, then decreased and remained low until, at the highest temperature, when it tended to increase again. IGF-1 modelling included single effect and two-way interactions (Figure 3c). We also observed a significant influence of T*D ($4.14E^{-2}$) on IGF-1 plasma levels with a maximum reached at medium temperature and daylength. Levels ranged from 1.5 to 7.8 ng*mL^{-1} (T4), from 3.2 to 29.4 ng*mL^{-1} (prolactin) and from 31.6 to 57.8 ng*mL^{-1} (IGF-1).

4. Discussion

The experiment spanned the period of smoltification for both strains. This statement is supported by NKA values, low in mid-April, increasing in early May and culminating in mid-May for both tested strains. Values of gill NKA at these peak (10.45 and 10.5 µmol ADP.h^{-1}.g prot^{-1}) did not differ between strains and are consistent with reported smolting peak values elsewhere (Handeland *et al.*, 2004; McCormick *et al.*, 2000; Zydlewski *et al.*, 2010). So this result provides further evidence of a successful smoltification under our laboratory conditions. Furthermore, our results concerning morphological characteristics of the smolts revealed that from the 8th sampling (April 25th) onwards, 97% of the smolts from both strains were fully silvered with no traces of parr marks or reddish dots on the flanks.

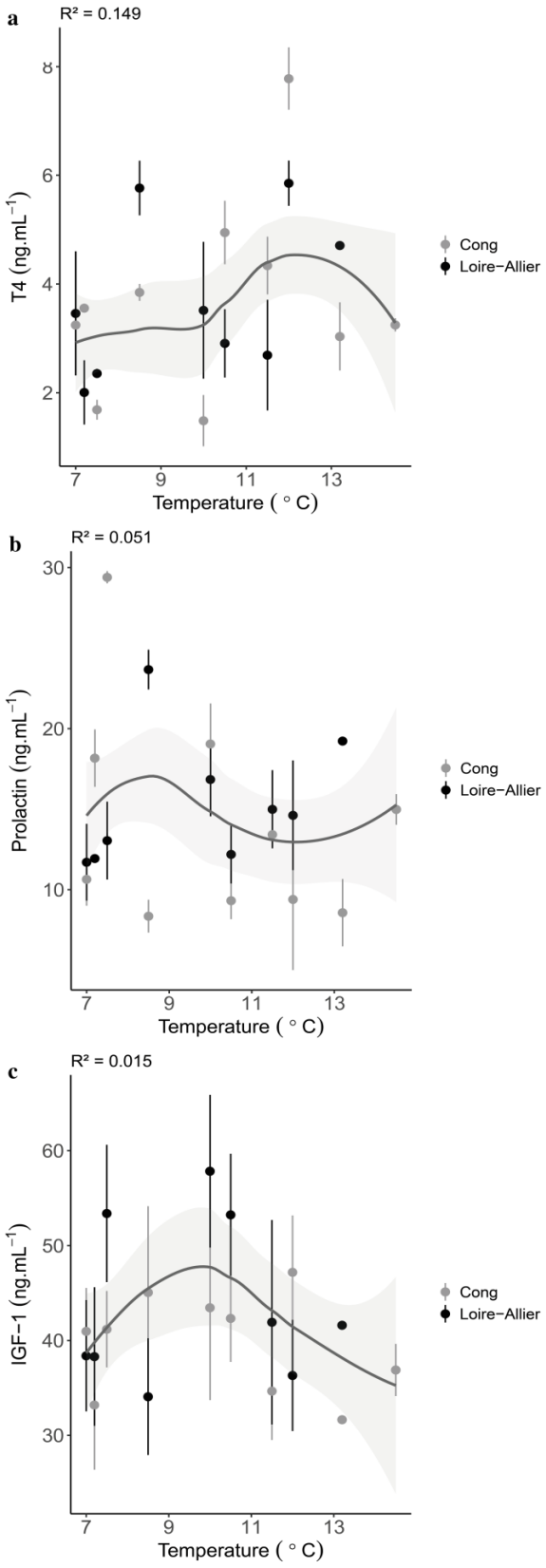

Figure 3: T4 (a), prolactin (b) and IGF-1 (c) plasma levels [ng*mL^{-1}] across the study timeframe. When strain had no significant influence, a common profile is presented. When T*D was significant in the model, graphs are represented only with temperature for easier representation.

In the present study, both strains were submitted to the same photoperiod, temperature and water quality conditions. Daylength alone only influenced osmolality. Photoperiod only differs modestly between our experimental conditions and those prevailing in the latitudes of Atlantic salmon origin (corresponding to Cong and Loire-Allier rivers). A tendency of daylength to influence prolactin plasma level ($p=6.95E^{-2}$) has been observed. Regularly an interaction with the strain or the temperature significantly influenced a smolting feature. Thus, a direct influence of daylength cannot be fully dismissed in this study as it is a primary factor influencing smolting (McCormick *et al.*, 1996; McCormick, 2009; Wedemeyer *et al.*, 1980). Temperature, by contrast, may have played a more important role as it strongly affects the rate of change during smoltification (McCormick *et al.*, 1999; Shrimpton *et al.*, 2000). Water temperature during spring is similar between the Loire-Allier and the Meuse water systems. Differences are much more pronounced when compared to the Cong River. This may explain higher and more stable cortisol levels observed in CG smolts indicating a lower welfare of this strain under these conditions. Similarly, increasing GH levels at the beginning of the study timeframe in Cong smolts, compared to decreasing values in Loire-Allier smolts, may indicate a timing gap between both strains. Increased temperature may advance smolting (McCormick *et al*., 1996) and temperature difference is higher between our conditions and Cong River than with the Loire-Allier River, thus favouring early development of smolts and earlier typical increase in GH during smoltification (Jonsson and Jonsson, 2011; McCormick *et al*., 1998). Extending the study timeframe may shed light on these differences and could show a decreasing level in Cong smolt earlier in the season.

Differences in sodium concentration between the strains at the beginning of the study timeframe may be a consequence of differences in cortisol and GH plasma level as these hormones strongly influence osmoregulation (McCormick *et al*., 1998; Jonsson and Jonsson, 2011). Furthermore, Saunders and Henderson (1970) demonstrated stable osmolality during smoltification whereas others showed an increase or a decrease in osmolality (Parry, 1960; Hickman and Trump, 1969). Sodium is an important part in osmolality and differences in plasma sodium level at the beginning of the study could then be related to a strain specific pattern.

Significant interaction of strain with temperature or daylength clearly indicates an influence of the genetic background of each strain. In other words, as rearing conditions were the same for both strains from the eyed-egg stage, resulting differences of temperature and daylength on the two strains, as seen for cortisol and GH plasma levels, should then be put in relation to a genetic basis for smoltification. These endocrine differences may be associated to the lower

condition factor and less allometric growth observed in CG compared to LA. Moreover, very few adult CG returners from the sea are observed since more than 20 years of the restocking program in the Belgian Meuse system (data obtained from X.Rollin, SPW-DGARNE-DNF-Fisheries Services), perhaps in relation to a lower downstream migration of CG smolts. This failure may indicate a lower welfare level of CG parrs and smolts than LA ones in the Meuse river conditions as evidenced by a higher cortisol response along the smolting period in the present study. Stock- and population-specific differences in migration timing (Orciari and Leonard 1996; Stewart *et al.* 2006) or strain-related differences in hypo-osmoregulatory capacities (Handeland *et al.*, 2004) already led Stewart *et al.* (2006) to hypothesize a genetic structuring at a sub-catchment level. Further evidence of the influence of genetics on smoltification may lead to important innovation in restocking and reinstating extinct populations of Atlantic salmon.

Despite these differences, NKA activity reached peak activity simultaneously in both strains. Environmental cues best explained NKA activity without any strain related difference. As a major factor indicating hypo-osmoregulatory capacities (Handeland *et al.*, 2004), NKA activity is largely acknowledged as a smolting marker. Our results would then not suggest the use of one particular strain but a higher condition factor and lower overall cortisol level may indicate a better fitness of LA smolts which may come into account for survival and return rates. However, seaward migration may still differ in timing in relation with migration distance (50km for Cong and 900km for Loir-Allier) of both strains and add evidence to Stewart *et al.* (2006) hypothesis.

In both strains, we observed a comprehensive scheme of hormonal interactions to achieve smolting as described in literature (Dickhoff *et al.*, 1997; Hoar 1988; McCormick, 2009). Although there is an initial divergence in GH levels between both strains and higher cortisol levels along the investigation in Cong smolt, an increase is noticed along the study. At that time, low prolactin levels were also observed which concurs with its inhibitory effect on smolting (Prunet *et al.*, 1989; Young *et al.*, 1989). This is consistent with their interactive effects in mediating environmentally induced changes in smolt development as high NKA activity was measured at the same time. Increasing T4 levels along the study also supports the involvement of thyroid hormones in promoting migration (Boeuf, 1993; Hoar, 1988; Ojima and Iwata, 2007) and in the control of osmoregulation during smolting (Hoar, 1988).

Extending the study time lap to involve desmoltification, loss of hypo-osmoregulatory capacities and readaptation to freshwater life, may add further proof of strain specific smolting windows. There is evidence of secondary peaks of prolactin and cortisol during the

period of decreasing levels of gill NKA activity in Atlantic or coho salmon along with other hormones like T4 and GH (Boeuf *et al.*, 1989; Prunet *et al.*, 1989, Young *et al.*, 1989). Increasing prolactin levels observed at the end of the study may then indicate the beginning of desmolting. However, the significance of these changes remains unclear (Høgåsen, 1998) and involvement of the endocrine system in the loss of smolt characteristics has not been elucidated in detail yet (Björnsson *et al.*, 2011).

Clearer differences between the strains may have been observed with more distant strains, e.g. from North Norway. Temperature encountered, length of winter, drastically different annual photoperiod regime may emphasize strain related differences glimpsed in our study. Strains from close water systems should definitively be preferred in a restocking program and may show better results (Jonsson and Jonsson, 2011). If geographically close strains are unavailable, a strain from another similar river system may be a good backup solution. The Loire-Allier and Meuse systems have roughly the same distance to the sea and similar temperature regime. Considering the high and constant cortisol level in CG smolts and more pronounced allometric growth in LA smolts, Loire-Allier originated fish may be better suited for the Belgium Meuse. The difficulty to obtain enough plasma from small-sized fish like smolts may lead to the use of more fish. Despite the fact that further measurements may show clear strain differences in hormone levels, this would be at the expense of fish casualties. Three individuals pooled together in each of the three tanks per strain should already depict a confident model.

The use of GAM modelling should be considered for further studies on salmon smoltification as it is particularly useful for modelling ecological datasets. It also helps modelling complex responses with multiple explanatory variables and may help to gain new insights in the control mode of complex biological processes like smoltification as illustrated with the various models used in this study.

5. Conclusion

In conclusion, the results showed that under foreign simulated environmental conditions, LA and CG smolts smoltified successfully and simultaneously despite strain-related divergent influences of temperature and photoperiod on growth parameters, cortisol and GH profiles. Various models had to be used for best modelling of smoltification features and might show complex interactions between environmental cues and/or between environmental and genetic factors.

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Addendum

Table 5 : Mean (SE in parentheses) condition factor and weight-length trend line equations in both strains at each sampling date and during the whole experiment.

	Strain					
	LA			CG		
Date	CF	WL	R^2	CF	WL	R^2
March 21	0.90 (0.01)	y=2.7x-1.8	0.93	0.88 (0.01)	y=3.1x-2.2	0.951
March 28	0.89 (0.01)	y=2.9x-1.9	0.92	0.86 (0.01)	y=2.9x-2.0	0.95
April 4	0.94 (0.03)	y=2.5x-1.5	0.62	0.89 (0.03)	y=2.0x-0.9	0.67
April 11	0.89 (0.02)	y=2.2x-1.1	0.68	0.88 (0.01)	y=2.8x-1.9	0.93
April 18	0.93 (0.04)	y=2.1x-1.0	0.76	0.83 (0.01)	y=2.7x-1.8	0.96
April 25	0.90 (0.02)	y=2.8x-1.8	0.67	0.89 (0.03)	y=2.8x-0.8	0.62
May 2	0.87 (0.01)	y=2.0x-0.8	0.64	0.85 (0.02)	y=2.9x-2.3	0.88
May 9	0.91 (0.02)	y=2.6x-1.5	0.76	0.87 (0.02)	y=2.7x-2.3	0.84
May 16	0.84 (0.02)	y=2.8x-3.1	0.89	0.84 (0.02)	y=2.1x-1.8	0.80
May 23				0.87 (0.02)	y=3.0x-2.3	0.90
All	0.91 (0.01)	y=2.69x-1.7	0.84	0.87 (0.01)	y=2.73x-1.7	0.87

(N = 12 fish per strain per date) CF: condition factor; WL: weight-length trend line equation; R^2: coefficient of determination.

In the previous chapter, we reported on some differences (plasma cortisol and GH levels) during smoltification between two allochtonous Atlantic salmon strains when reared under the same local conditions. Despite these differences, both strains smoltified successfully and simultaneously according to our indicators. Local temperature conditions were based on a tributary on the river Meuse, the river Ourthe. Data showed a temperature difference in Spring, sometimes even exceeding 5°C, between these two rivers. We will now investigate the potential influence of such a temperature increase on the smoltication process and look for differences in response between both strains. The development of hypo-osmoregulatory capacity is a major change occurring during smoltification and makes it possible for smolts to switch from fresh- to seawater with minimal internal fluctuations (McCormick *et al*., 1998). Therefor, we will challenge our fish with a transfer to saltwater to investigate the effect of our temperature treatment on the ability of smolts to acclimate to seawater. As the smolt migration lasts for several weeks in the Meuse basin (Dierckx *et al*., 2017), we will also check for differnces in response between early and late migrants (Figure 37).

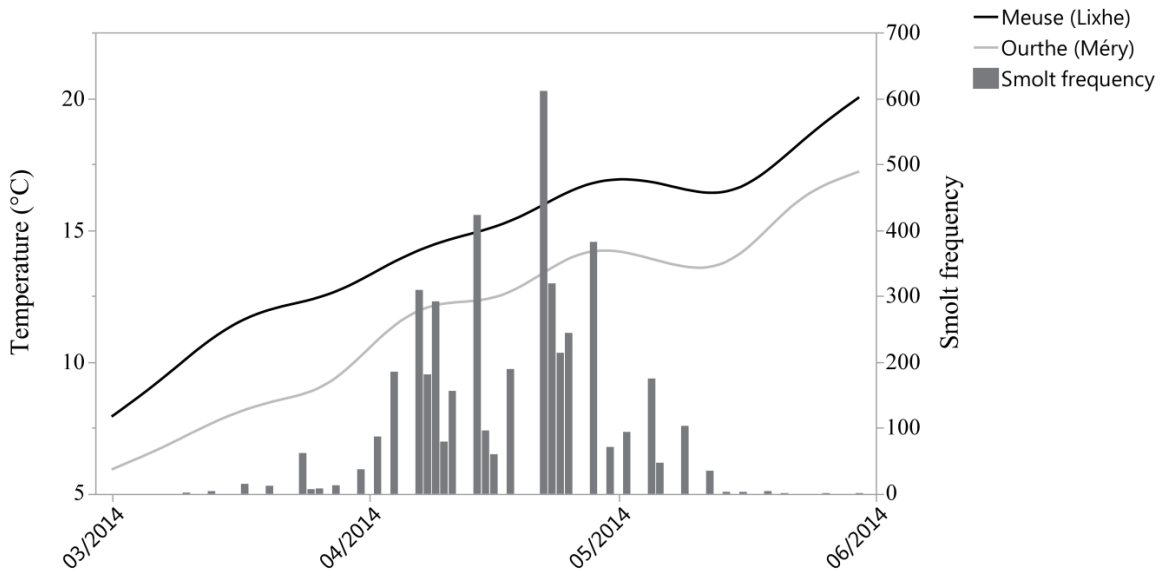

Figure 37: Temperature curves in Méry and Lixhe and smolts capture frequency in spring 2014. Arrows indicate transfer dates.

4.2 A temperature shift on the migratory route similarly impairs hypo-osmoregulatory capacities in two strains of Atlantic salmon (*Salmo salar* L.) smolts.

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In preparation

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Abstract

Temperature influences smoltification in Atlantic salmon and anthropogenic use of watersystems may cause temperature fluctuations between tributaries and large rivers. Based on local field data, we simulated the downstream route to investigate the impact of a 5°C temperature shift during smoltification on hypo-osmoregulatory capacity of smolts. Three temperature regimes were tested; control treatment without temperature shift, early treatment and late treatment. Fish were subjected to seawater challenge during and after downstream migration peak time. Two strains were used, Loire-Allier and Cong, to compare possible differences in response due to local adaptations to environmental conditions. Without temperature shift, differences between the strains were noticed in date of peak and maximum activity of gill Na^+/K^+ATPase (Loire-Allier = 8.1 µmol ADP $*mg^{-1}prot*h^{-1}$ vs Cong = 7.4 µmol ADP$*mg^{-1}prot*h^{-1}$) as well as in plasma sodium and potassium concentrations. In early and late temperature treatment, gill Na^+/K^+ATPase activity, plasma osmolality and ion concentrations were negatively influenced in both strains. After salinity challenge, the highest osmolality was measured in smolts subjected to the temperature shift. Predictably circulating levels of GH and IGF-1 changed over the smolting period but they did not explain the observed modifications in hypo-osmoregulatory abilities. Results show a negative impact of a temperature shift on hypo-osmoregulatory capacities of smolts regardless of the strain. As a one-week delay after the temperature treatment was necessary for a response to be measured, efforts to favour downstream migration may help limit the impact of a rapid temperature increase.

Keyword: Smoltification, osmoregulation, temperature, endocrine features, Atlantic salmon

1. Introduction

Smolting is a preparatory process allowing anadromous fish to survive the transition from fresh- to seawater. It involves numerous morphological, physiological and behavioural changes (McCormick *et al.,* 1998, McCormick, 2009, Stefansson *et al.,* 2008). Adjusting peak preparedness through smolting to river and ocean conditions for optimal smolt survival is the ultimate biological role of the environmental signals governing the smolting process (McCormick *et al.,* 2000). Adaptation to local conditions may then exist as strain- or population-specific traits in the development of hypo-osmoregulatory capacity or downstream migration timing have been reported (Aarestrup *et al*., 1999; Birnie-Gauvin *et al.,* 2018; Handeland *et al.,* 2004; Orciari and Leonard, 1996; Stewart *et al.,* 2006).

Photoperiod and temperature are known to be primary environmental factors influencing the smoltification (Jonsson & Jonsson, 2011, McCormick *et al.,* 1998). Temperature and more specifically accumulated thermal units or degree*days strongly influence the start of Atlantic salmon smolt migration (Zydlewski *et al.,* 2005) and the development of hypo-osmoregulatory capacity (Handeland *et al.,* 2004). In warmer years, earlier onset of migration was observed (Otero *et al.*, 2014; Zydlewski *et al.*, 2005) and earlier sea arrival was recorded for fish with warmer thermal history (Stich *et al.,* 2015). Temperature is known to influence the rate of development of morphological and physiological changes (McCormick *et al.,* 2000; 2002; Shrimpton *et al.,* 2000). Under increased temperature conditions, smolting may be advanced by several weeks (McCormick *et al.,* 1996; Solbakken *et al.,* 1994). Changes in circulating levels of cortisol, GH, IGF-1 and thyroid hormones will coordinate the development of physiological, morphological and behavioural preadaptation for sea-life (McCormick, 2013). Increases in circulating levels and exogenous treatment with hormones indicate that salinity tolerance is under the positive control of cortisol, GH and IGF-1 (McCormick 2001). Cortisol increases the number of ionocytes and the abundance and activity of the major transport proteins involved in salt secretion, NKA, NKCC and CFTR (Pelis and McCormick, 2001; Kiilerich *et al.,* 2007; McCormick *et al.,* 2008). GH also acts on ionocytes, influencing the development in type, size and number of chloride cells (McCormick et al, 1998, McCormick, 2009) thus promoting saltwater tolerance (Bœuf, 1993; McCormick *et al.,* 1995). Increased circulating levels and local production of IGF-1 also support this increased salinity tolerance and NKA activity (McCormick, 2001).

But swimming speed in migrating smolts was reduced by 80% at 17°C and positive rheotaxis was induced over 20°C (Martin *et al.,* 2012) showing that temperature may also have deleterious effects on smolts. Furthermore, at elevated number of degree*days, migration

termination was induced (Zydlewski *et al.*, 2005) as well as the loss of hypo-osmoregulatory capacity (Handeland *et al*., 2004). The loss of smolt characteristics is called desmoltification and endocrine control of this process has not been elucidated in details yet (Björnsson *et al.*, 2011).

Since 1987, a restocking plan has been set up to restore the extinct Atlantic salmon population in the Meuse basin by using foreign strains. However, results are poor in term of adult returns. It was hypothesised that temperature may negatively impact the salmon life-cycle through swift anthropogenic-linked increase in temperature (Martin *et al.*, 2012).

Anthropogenic use of rivers may ultimately lead to a temperature gap between the main channel and their tributaries (Kirchmann, 1985; Lair and Reyes-Marchant, 2000). The effects of such a temperature shift on the efficiency of the smoltification process and the success of downstream migration of salmon smolts are not well described. In spring, temperature difference between the River Meuse (Belgium) and one of its major tributaries, the River Ourthe, regularly exceeds 4°C (data provided by SPW-DGARNE - Département de la Police et des Contrôles - Direction des Contrôles and Laboratoire de Démographie des Poissons et d'Hydroécologie-University of Liège, Belgium).

We hypothesize that a temperature shift during migration negatively impacts hypo-osmoregulatory capacity of smolts, dramatically reducing their survival chances at sea-entry. In addition, we will investigate the response of different strains as they may perform differently due to adaptation to local conditions of their origin river.

2. Materials and methods

2.1 Fish origin

Two strains of Atlantic salmon were used in this study. The first strain (CG) originated from the Cong Hatchery on the Cong River in Ireland and the second one (LA) from the ‘Conservatoire National du Saumon Sauvage de Chanteuges” on the Loire-Allier River in France. Fertilised eggs (F1) from recaptured wild spawners (F0) were directly imported to and reared at the “Conservatoire du Saumon Mosan” hatchery (Public Service of Wallonia, Fisheries Service), located in Erezée (Belgium) until they reached the pre-smolt stage. On February 24, parrs (average bodymass of 23.1 g for CG smolts and 24.1 g for LA smolts) were transferred from Erezée to a wet laboratory in the University of Namur (Belgium).

2.2 Experimental design

Both allochthonous strains, LA and CG, were equally allocated into three recirculating water circuits. Each circuit was composed of six 120 L tanks, three per strain (N= 72 fish per tank).

Throughout the study, fish were maintained in these tanks with a circular stream flow and supplemental aeration under simulated natural photoperiod (Figure 1) based on Namur latitude (50°28'00"N). Water temperature management was based on a decade of field data collected on the Meuse and Ourthe Rivers (Belgium). Fish were daily fed (TroCo Supreme-21 Coppens International B.V., Helmond, The Netherlands) with a fixed ration (1% of fish biomass) provided with automatic feeders during the day. Oxygen concentration and temperature were checked daily and other water characteristics (pH=7.2, $NH_4^+ < 0.06$ mg*L^{-1}, $NO_{2-} < 0.05$ mg*L^{-1} and $NO_{3-} < 10$ mg*L^{-1}) were checked weekly. Each circuit followed a specific temperature regime (Figure 1).

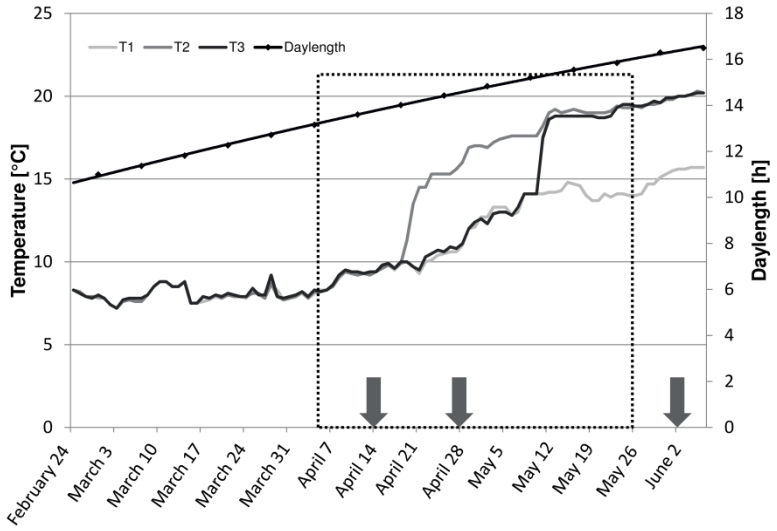

Figure 1: Applied temperature regimes and photoperiod for the three treatment groups along the study. T1: control conditions without temperature shift; T2: early treatment (temperature shift comleted on April 21) and T3: late treatment (temperature shift completed on May 12). Dashed square represents downstream migration period (from percentile 10 to 90 of migrating smolts) based nine years of field monitoring data (Dierckx *et al.*, 2017). Arrows indicate the start of each of the three 96 h salinity challenges.

Dates and temperature are based on field monitoring of downstream migration (Dierckx *et al*., 2017). The control treatment (T1) mimics the temperature conditions of the Ourthe River (slowly increasing over the study period). The early treatment (T2) mimics the conditions early migrants would encounter. A 5 °C increase was applied over the period from April 19 to April 21 and represents the passage from tributary into main channel. The late treatment (T3) mimics the conditions late migrants would encounter with a 5 °C increase over the period from May 9 to May 12. Temperature increases were completed within three days, corresponding to the time smolts need to cover the distance between the sampling points on the tributary and on the main channel using an average speed calculated on field data of smolt

migration monitoring between two Belgian rivers (Ovidio *et al.*, 2016). In the following work, we will refer to the treatment with the date the increase was completed on (early treatment = April 21 and late treatment = May 12). To assess hypo-osmoregulatory capacities of smolts at sea entrance, three times over the study timelap, eight smolts from each tank of the control treatment were challenged with a direct transfer into 35 ‰ salinity water (Staurnes *et al.*, 2001; Zydlewski *et al.*, 2010) for 96 h (Saunders and Henderson, 1970; Komourdjian *et al.*, 1976; Saunders *et al.*, 1985). To assess long-term effects of a temperature treatment, fish from the T2 and T3 groups were also used for the third seawater (SW) challenge. As we intend to understand if a rapid temperature increase during migration may negatively impact long-term survival at sea, a 96 h test was preferred over a 24 h test. All experiments were conducted in accordance with local ethic committee on animal experimentation of the University of Namur (KE13193) that agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

2.3 Sampling

After two weeks of acclimation, fish were sampled weekly at dawn, from early March to early June (13 sampling timepoints). Sampling procedure is based on McCormick *et al.*, 2013). Three fish were quickly dip-netted out of each tank, anaesthetised with tricaine methanesulfonate (120 mg*L^{-1}, pH 7.2) and blood was collected into 1 mL ammonium heparinized syringes from the caudal vein. The needle was removed and the blood was expelled into a 1.5 mL Eppendorf, stored on ice for less than 30 min and then centrifuged at 3000 g for 10 min. The supernatant was collected and stored at -80 °C until subsequent analyses. Total length and bodymass were then measured to the nearest 0.1 cm and 0.1 g, respectively. External morphological characteristics including silvering, presence of reddish dots and typical parr oval-shaped marks on the flanks were recorded. The first left and right-sided branchial arches were excised and immediately frozen in liquid nitrogen, then stored at -80 °C until assay. Samplings after seawater challenges were performed following the same protocol as previously stated.

2.4 Na^+/K^+ATPase

Gill Na^+/K^+ATPase (NKA) activity was measured according to the method described by McCormick (1993) with slight modifications as follows. Gill filaments were homogenized in ice-cold phosphate buffer (50 mM KH_2PO_4, pH 7.4) and followed by centrifugation (3000 g for 5 min) to remove large debris. NKA activity was determined with a kinetic assay linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), in the presence and absence of Na^+/K^+-ATPase specific inhibitor ouabain. Samples of 10 microliters

were run in two sets of duplicates at 25 °C and measured at a wavelength of 340 nm for 10 min. The first set contained assay mixture (TrisHCL 50 mM ; $MgCl_2$ 10 mM ; EGTA 0.5 mM ; pH 7.4 ; NaCl 500 mM ; KCl 125 mM ; PEP 30 mM ; NADH 3 mM ; 5 U pyruvate kinase mL^{-1}; 4 U lactate dehydrogenase mL^{-1}; ATP 50 mM) and the other assay mixture as before plus ouabain (10 mM). The absorbance was measured using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany) and the included data analysis software MARS (BMG LABTECH GmbH, Germany).

Total protein concentration of the gill homogenate was measured in duplicate using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. NKA activity is expressed as µmol ADP*mg^{-1} of protein*h^{-1}. This assay was run on a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany) with use of MARS data analysis software (BMG LABTECH GmbH, Germany).

2.5 Plasma ion and osmolality analysis

Plasma sodium and potassium concentrations were measured using a Philipps PU 9200 atomic absorption spectrophotometer (Pye Unicam, Cambridge, United Kingdom) with 0.25-, 0.5-, 0.75- and 1 ppm external standards for sodium and 0.5-, 1-, 2- and 3 ppm external standards for potassium. Plasma osmolality was measured using a Löser Type 6 freezing point depression osmometer (Löser Messtechnik, Germany) with 0-, 300- and 900-milliosmole external standards.

2.6 Hormone assays

At four timepoints, hormone assays were performed on pooled samples of 3 fish per tank. Fish GHand Fish IGF-1 ELISA kits (Cusabio, PRC) were used as per the manufacturer's instructions. Detection lower limits were 312.5 pg*mL^{-1} (intra-assay variation coefficient < 15 %), 25 pg*mL^{-1} (intra-assay variation coefficient < 15 %) respectively. Plasma ran out after a technical failure and cortisol levels could only be measured on two dates (April 28 and May 5) by a direct competitive enzyme-linked immunosorbent assay (DRG Instruments GmbH, Germany). The lower detection limit was 2.5 ng*mL^{-1}; and the intra- and inter- assay coefficients were 5.63 and 6.93 % respectively. These assays were run on a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany) with use of MARS data analysis software (BMG LABTECH GmbH, Germany). All samples were measured in duplicate within a single assay.

2.7 Statistical analysis

All statistical analyses were performed with R packages, version 3.3.3. Homogeneity of variances was tested using Levene's F-test and normality was tested using a Shapiro-Wilk W-

test. Data of the three salinity tests for the T1 circuit was analysed using a two-way analysis of variance (ANOVA) with strain and date as factors. Data from hormone levels and the third salinity test (3 circuits) was analysed using a three-way ANOVA with strain, circuit and date as factors. To investigate a significant effect, a Tukey post hoc test was used for pairwise comparisons. Where normality tests failed, data was log-transformed. NKA data was examined with a generalised linear model (GLM). Weight-length data was used to test growth allometry differences between strains using linear regression t-test to compare slopes. Statistically significant differences were accepted at $p < 0.05$. All data are given as means ± standard error of the mean (SEM).

3. Results

3.1 Plasma osmolality and ions concentrations

On April 14, differences were seen in plasma osmolality ($p < 0.001$), sodium ($p < 0.05$) and potassium ($p < 0.01$) levels between the two strains in the control treatment. Lower values were found in CG smolts (Figure 2A, B and C). No further differences between the strains were observed until the end of the study. Date influenced all three measured parameters ($p < 0.001$). An increase in plasma osmolality was measured after the first ($p < 0.01$) and third ($p < 0.01$) salinity challenges in both strains. Osmolality ranged from 269 to 334 $mOsm*kg^{-1}$ H_2O. Sodium levels increased after all three SW challenges ($p < 0.001$ in S1, $p < 0.001$ in S2 and $p < 0.05$ in S3). Potassium levels only increased after the second SW challenge ($p < 0.01$).

Date - Treatment two-way interaction had an effect on plasma osmolality ($p < 0.05$), sodium ($p < 0.05$) and potassium ($p < 0.01$) levels during the third salinity challenge (Figure 3A, B and C). Osmolality increased in all three treatments and the increase was more pronounced in the early and late treatment groups. The same observation was made for sodium levels with the strongest increase in the early treatment group. Potassium levels increased in the early treatment group and decreased in the late treatment group. Strain-Treatment two-way interaction influenced plasma sodium ($p < 0.01$) and potassium ($p < 0.001$) levels (Figure 4A and B) during the third salinity challenge. No differences were seen in LA smolts but CG smolts from the early and late treatment groups displayed increased sodium levels ($p < 0.01$) and they had increased potassium levels in the early treatment group ($p < 0.01$). No differences were seen between the late treatment group and the other two for CG smolts.

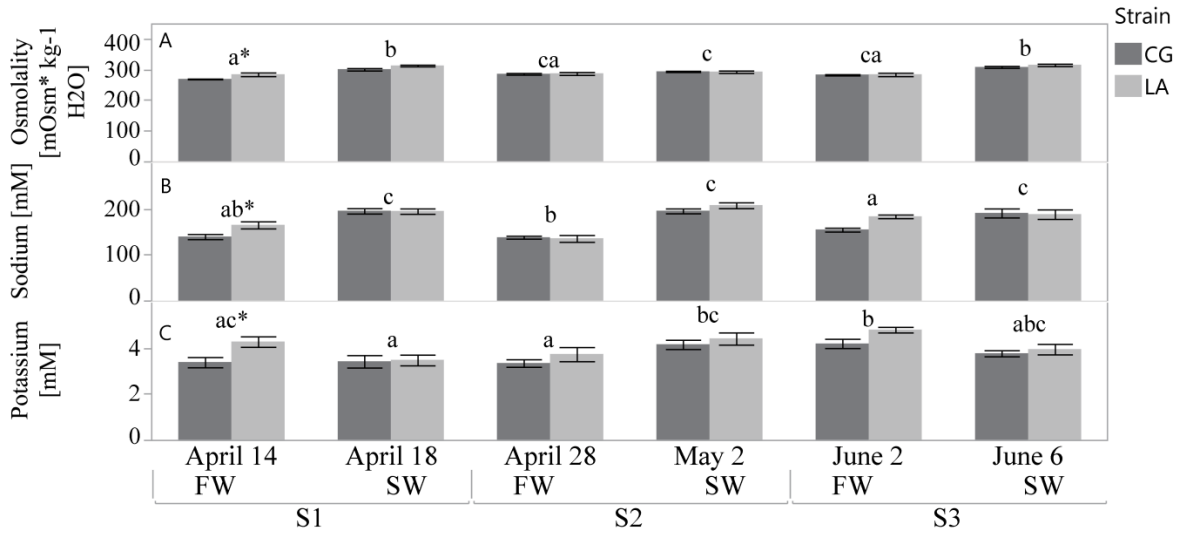

Figure 2: Plasma osmolality (A), sodium (B) and potassium (C) levels in Cong (CG) and Loire-Allier (LA) smolts under control conditions (T1) in freshwater (FW) early in the migration period (April 14), in the middle (April 28) and after (June 2). These dates were followed by a 96 h challenge in seawater (SW; April 18, May 2 and June 6). Dates were chosen based on field survey data of downstream migration (Dierckx *et al.*, 2017). N = 9 fish per strain per date. S1: first salinity challenge; S2: second salinity challenge; S3: third salinity challenge.The symbol * stands for a significant difference ($p < 0.05$) between the two strains on a given date. Different lower case letters were used to represent a significant difference ($p < 0.05$) between dates.

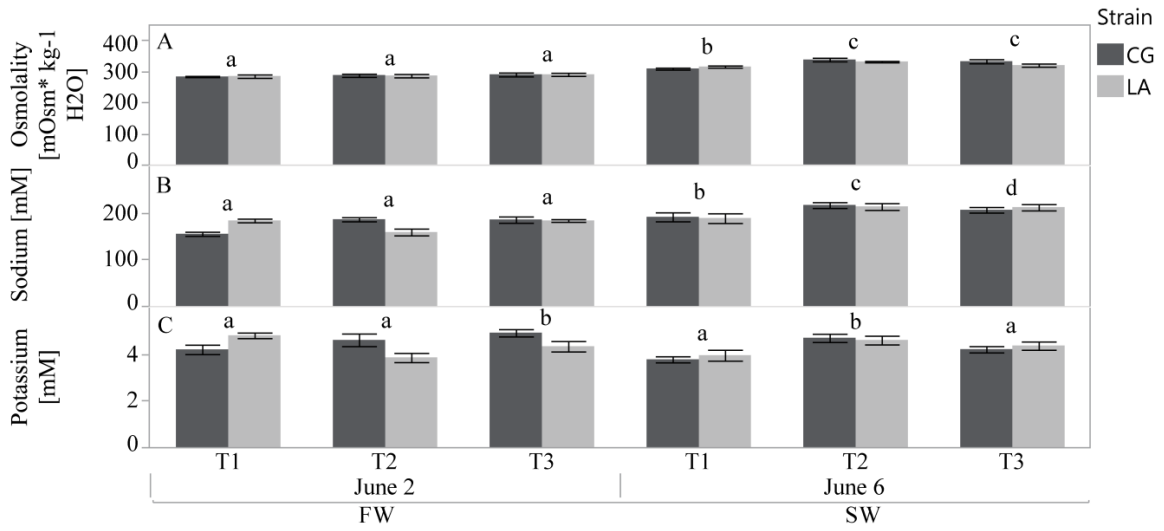

Figure 3: Plasma osmolality (A), sodium (B) and potassium (C) concentrations in Cong (CG) and Loire-Allier (LA) smolts before and after a 96 h salinity challenge in the control (T1), early treatment (T2) and late treatment (T3) groups. FW: freshwater; SW: seawater. Different lower case letters stand for a significant difference ($p < 0.05$) between the treatment groups and dates. N = 9 fish per strain per date per treatment. Data analysis revealed no differences between the strains.

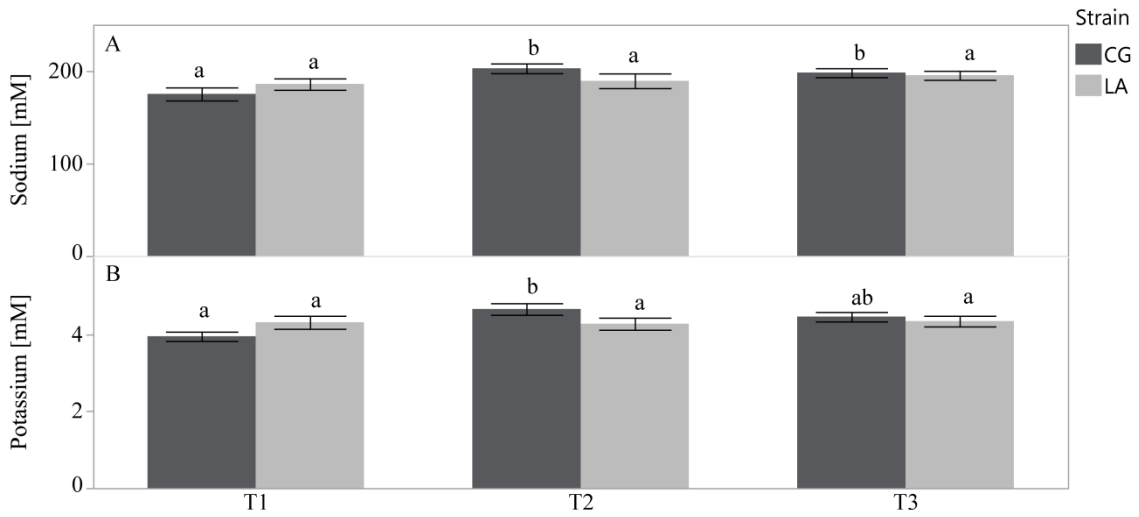

Figure 4: Representation of the Strain-Treatment two-way interaction on plasma sodium (A) and potassium (B) levels during the third salinity challenge (June 2-6). CG: Cong; LA: Loire-Allier; T1: control treatment; T2: early treatment; T3: late treatment. Different lower case letters stand for a significant difference ($p < 0.05$) between the strains and treatment groups. N = 36 fish per strain per treatment.

3.2 Gill Na^+/K^+-ATPase activity

Two-way interactions Date-Strain ($p < 0.001$) and Date-Treatment ($p < 0.001$) influenced NKA activity (Figure 5). In the control group, a first activity peak was measured on March 31 in both strains with lower ($p < 0.01$) values in CG than in LA smolts (7.6 vs 9.5 µmol ADP*mg^{-1}prot*h^{-1}). A second peak was measured in LA smolts (7.4 µmol ADPmg^{-1}prot.h^{-1}) on April 28 and in CG smolts (8.1 µmol ADPmg^{-}1*prot*h^{-1}) on May 5. Activity in the early and late treatment groups does not differ from the control group until temperature treatment is applied. In both strains, NKA activity sharply decreased ($p < 0.01$) one week after the temperature increase in the early (April 28) and late (May 19) treatment groups and remained low until the end of the study.

Results from the salinity challenges (Figure 6) indicate that NKA activity was influenced by the date ($p < 0.001$). For both strains in the control treatment, an increase of NKA activity was measured after the first ($p < 0.001$) and second ($p < 0.001$) salinity challenges but not after the third one. Date-Treatment interaction influenced NKA activity ($p < 0.001$) during the third challenge. Before the challenge, activity was lower in the early and late treatment groups compared to the control group. Activity did not change after the challenge in the control and early treatment groups. NKA activity increased in the late treatment group ($p < 0.01$). No differences between the strains were seen.

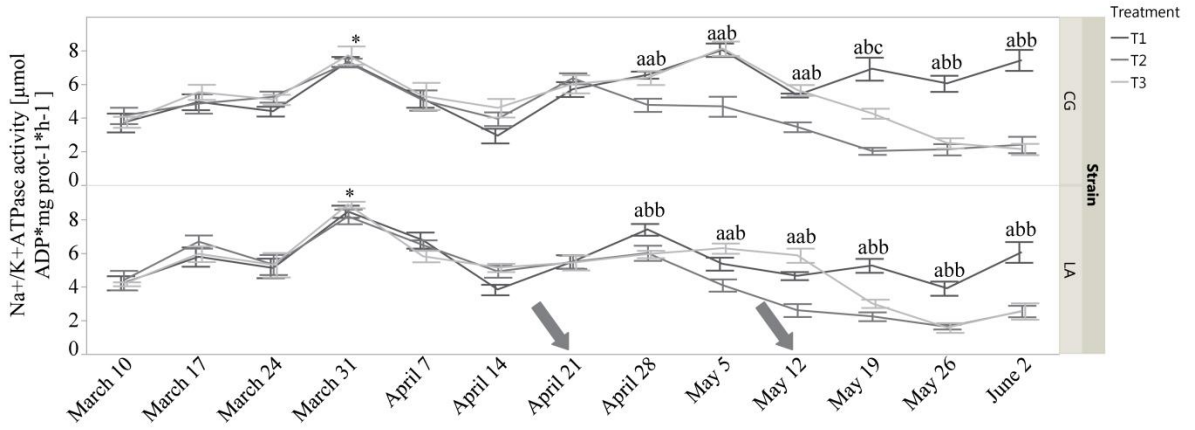

Figure 5: Variation of NKA activity levels in Cong (CG) and Loire-Allier (LA) smolts over the smoltification period in the control (T1), early treatment (T2) and late treatment (T3) groups. The symbol * stands for a significant difference ($p < 0.05$) between the two strains on a same date. Different lower case letters indicate a difference ($p < 0.05$) between the treatment groups for one strain on one date. N = 9 fish per strain per date per treatment. Arrows point the date of temperature treatment (April 21 for the early treatment and May 12 for the late treatment)

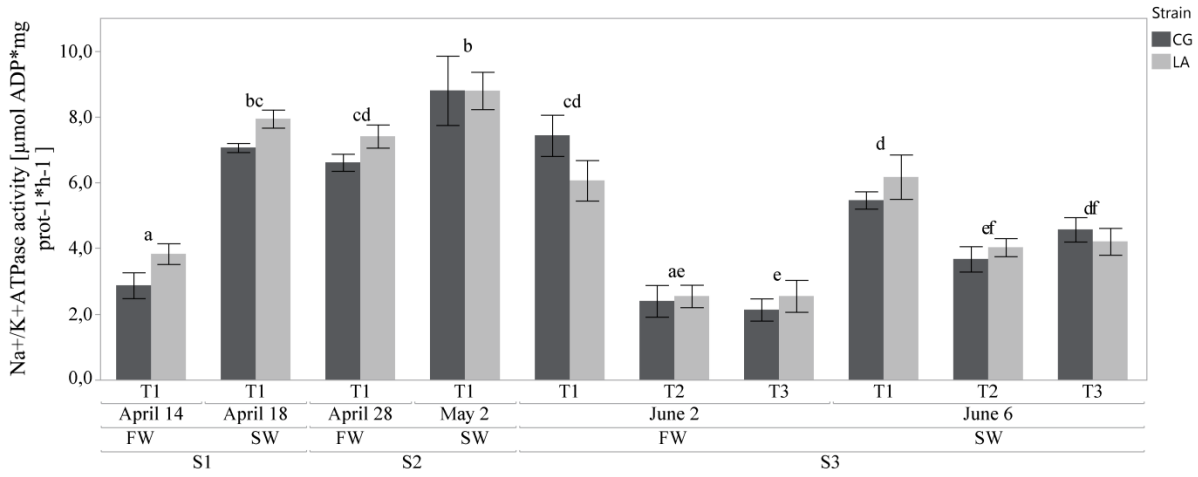

Figure 6: NKA activity in Cong (CG) and Loire-Allier (LA) smolts in freshwater (FW; April 14, April 28 and June 2) and after a 96 h challenge in seawater (SW; April 18, May 2 and June 6). The influence of different temperature treatment was also assessed during the third test. Different lower case letters stand for a significant difference ($p < 0.05$) between the dates and treatments. N = 6 fish per strain per date per treatment; T1: control treatment; T2: early treatment; T3: late treatment; S1: first salinity challenge; S2: second salinity challenge; S3: third salinity challenge. Data analysis revealed no differences between the strains.

3.3 Plasma hormone profiles

No influence of the strain was seen on plasma GH levels. One week after the early treatment (April 28), no differences were seen in plasma GH levels between the three treatment groups

(Figure 7A). Two weeks after the early treatment, levels decreased in the early treatment group ($p < 0.001$) and rose in the other two ($p < 0.001$). On June 2, levels decreased in the control and late treatment groups ($p < 0.001$) and were lower in early and late treatment groups compared to the control group. After a salinity challenge (June 6), levels did not change in the control group but increased in both early ($p < 0.001$) and late ($p < 0.01$) treatment groups. Mean values ranged from 2.8 ng*mL^{-1} to 11.1 ng*mL^{-1}across the study timeframe.

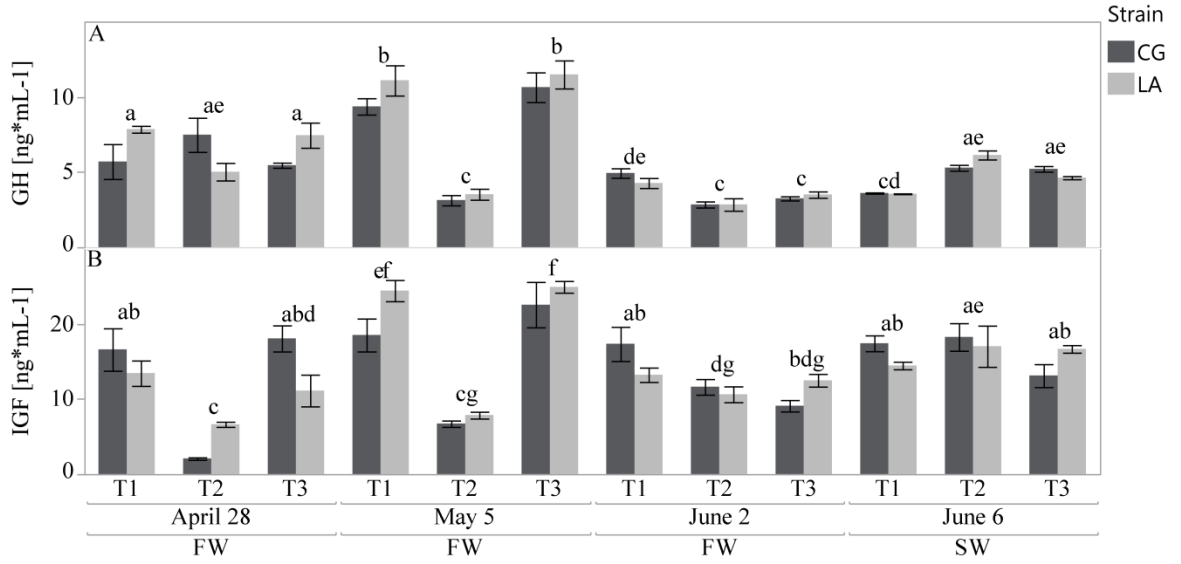

Figure 7: Plasma GH (A) and IGF-1 (B) levels in Cong (CG) and Loire-Allier (LA) smolts from the control (T1), early (T2) and late (T3) treatment groups one week after the early treatment (April 28), two weeks after the early treatment (May 5), after the downstream migration period (June 2) as defined by Dierckx *et al.* (2017) and after a 96 h salinity challenge (June 6). Different lower case letters stand for a difference ($p < 0.05$) between dates and treatments. FW: freshwater; SW: seawater; N=6 fish per strain per date per treatment. Data analysis revealed no differences between the strains.

One week after the early treatment (April 28), IGF-1 levels were lower ($p < 0.001$) in the early treatment group (Figure 7B). Two weeks after the early treatment, levels remained low in the early treatment group ($p < 0.001$) and rose in the control ($p < 0.01$) and late treatment groups ($p < 0.001$). On June 2, levels decreased in the control ($p < 0.001$) and late treatment groups ($p < 0.001$). After a salinity challenge (June 6), levels increased in the early treatment group ($p < 0.01$) but did not change in the control and late treatment group. Mean values ranged from 4.4 ng*mL^{-1} to 23.7 ng*mL^{-1} over the study period. Strain-Date interaction also influenced ($p < 0.05$) IGF-1 levels (Figure 8). An increased level of IGF-1 was observed in LA smolts on May 5 ($p < 0.001$) followed by a decrease on June 2 ($p < 0.001$). No changes

were seen in CG smolts for that period. IGF-1 levels in both strains increased after the SW challenge ($p < 0.01$ in CG and $p < 0.001$ in LA).

Cortisol level (Figure 9) was influenced by the treatment ($p < 0.01$) with lower levels in the early treatment group. Mean values were 61.7 ng*mL^{-1} in T1, 44.9 ng*mL^{-1} in T2 and 59.9 ng*mL^{-1} in T3.

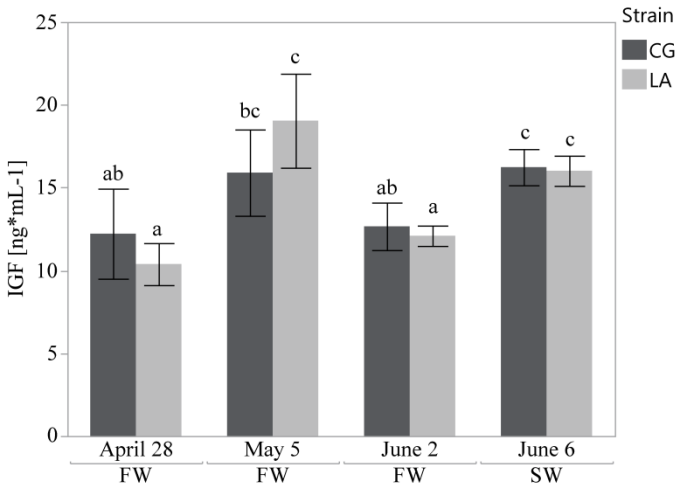

Figure 8: Representation of the Strain-Date two-way interaction on plasma IGF-1 levels in Cong (CG) and Loire-Allier (LA) smolts on three dates during the study peiod in freshwater (FW) and after a 96 h challenge in seawater (SW). Different lower case letters stand for a difference ($p < 0.05$) between strains and dates. N = 18 fish per strain per date.

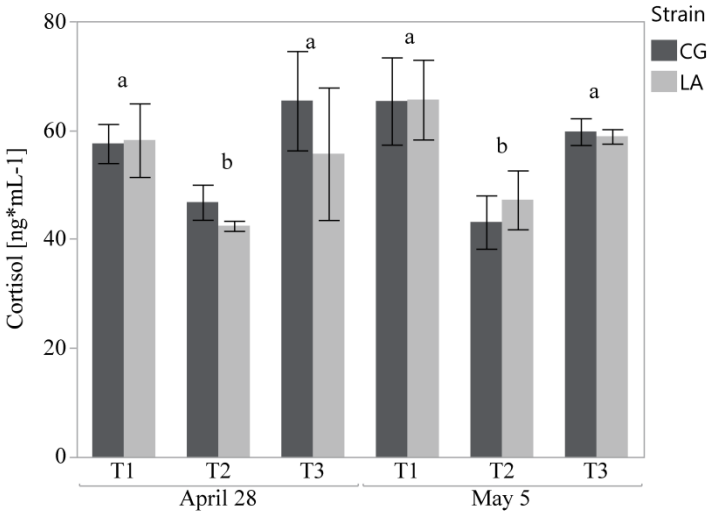

Figure 9: Plasma cortisol levels in Cong (CG) and Loire (LA) smolts from the control (T1), early (T2) and late (T3) treatment groups one week after the early treatment (April 28) and two weeks after the early treatment (May 5). Different lower letters stand for a difference ($p < 0.05$) between dates and treatments. N = 6 fish per strain per date per treatment. Data analysis revealed no differences between the strains.

3.4 Growth, condition factor and morphological criteria

Over the whole study period, both strains exhibited a negative allometric growth type indicating a faster increase in length than in mass (Table 1). No differences were seen between strains or treatments. Values of condition factor were comparable between strains and did not vary between treatments or dates. Silvering of the flanks progressively appeared as oval parr marks faded away across the smoltification season. Reddish dots were noticeable longer but eventually disappeared. In March, about 50 % of the smolts still exhibited typical parr marks. From early April onwards, that percentage was reduced to about 30%, and from early May onwards, 95% of the sampled fish were fully silvered without any trace of parr marks. No differences between strains were noticed.

Table 1: Mean (SE in parentheses) condition factor and weight-length trend line equations for the control, early and late treatment groups in both strains before any treatment (April 14) one week after the early treatment (April 28) one week after the late treatment (May 19) at the end of the study (June 2) and all dates together.

		Strain					
		LA			CG		
Treatment	Date	CF	WL	R^2	CF	WL	R^2
T1	April 14	0.79(0.02)	y=2.5x-4.0	0.93	0.84(0.01)	y=3.2x-5.4	0.95
	April 28	0.74(0.02)	y=2.6x-4.3	0.74	0.76(0.02)	y=3.1x-5.4	0.91
	May 19	0.77(0.02)	y=2.9x-4.8	0.65	0.78(0.03)	y=2.8x-4.6	0.67
	June 2	0.81(0.01)	y=2.8x-4.7	0.98	0.81(0.03)	y=2.8x-4.6	0.82
	All	0.79(0.02)	y=2.7x-4.4	0.92	0.79(0.03)	y=3.0x-5.1	0.87
T2	April 14	0.83(0.02)	y=2.8x-4.6	0.93	0.81(0.02)	y=3.1x-5.3	0.96
	April 28	0.77(0.01)	y=2.8x-4.6	0.80	0.78(0.01)	y=3.0x-5.0	0.99
	May 19	0.78(0.02)	y=2.8x-4.6	0.76	0.77(0.01)	y=2.8x-4.7	0.89
	June 2	0.85(0.03)	y=2.6x-4.2	0.80	0.79(0.02)	y=2.9x-4.2	0.80
	All	0.80(0.02)	y=2.9x-4.9	0.89	0.80(0.02)	y=3.0x-5.1	0.92
T3	April 14	0.83(0.01)	y=2.6x-4.1	0.92	0.87(0.02)	y=2.9x-4.8	0.84
	April 28	0.80(0.02)	y=2.6x-4.3	0.85	0.81(0.01)	y=3.1x-5.2	0.98
	May 19	0.78(0.02)	y=3.2x-5.6	0.72	0.76(0.02)	y=3.2x-5.6	0.92
	June 2	0.82(0.02)	y=3.3x-5.8	0.95	0.79(0.01)	y=3.1x-5.2	0.94
	All	0.83(0.02)	y=2.8x-4.6	0.90	0.82(0.02)	y=2.9x-5.0	0.92

(N = 9 fish per strain per date per treatment) CF: condition factor; WL: weight-length trend line equation; R^2: coefficient of determination; T1: control treatment; T2: early treatment; T3: late treatment.

4. Discussion

4.1 Osmoregulation profile during the downstream migration

The present experiment spanned the period from early smolting to desmolting as evidenced by NKA activity, seawater tolerance and silvering. In early spring, increasing gill NKA activity provided evidence that the fish were smolting under simulated temperature conditions of the Meuse River basin (Figure 5). NKA activity peaks (7.4 and 8.1 µmol ADP $mg^{-1}prot.h^{-1}$) were consistent with reported values at the peak of smolting in Atlantic salmon (Handeland *et al.*, 2004, Zydlewski *et al.*, 2010). NKA activity fluctuated slightly but remained high during the downstream migration period as defined by 9 years of field survey using the same two strains (Dierckx *et al.*, 2017). A decreasing trend in NKA values observed in late spring may indicate a switch over to desmoltification process. Increasing circulating levels of GH and IGF-1 in early May at a time of high NKA activity and decrease in early June gives further confidence that the study period spanned over the smolting season. Osmoregulatory ability of T1 smolts paralleled the profile of gill NKA activity. Salinity tolerance was low during the first challenge performed in mid-April (Figure 2) suggesting a not fully-achieved smoltification. During the second salinity test performed at the beginning of May, smolts osmoregulated effectively, maintaining the lowest osmolality during seawater challenge. During the third challenge applied between June 2 and 6, fish were probably already desmoltifying as evidenced by increased osmolality. According to previous field data (Dierckx *et al.*, 2017), the majority of smolts have already migrated towards the sea before the latter date in the temperature conditions of the Meuse River. Smolt developmental stage was further evidenced by allometric growth tending to a more elongated shape along the smoltification as extensively described in literature (Hoar, 1939a; Martin, 1949; Houston and Threadgold, 1963; Fessler and Wagner, 1969, Hoar 1988). A declining condition factor (McCormick *et al.*, 2000; Handeland *et al.*, 2004; Zydlewski *et al.*, 2010) was not observed despite a negative allometric growth. Daily feeding probably may have provided sufficient energy to cover the expenses of smolting and to enable a gain in bodymass. In our study, we measured two increases in NKA activity with an early peak on March 31. Two increases in NKA activity have already been observed (Handeland *et al.*, 2004). Prolactin plays an important role in osmoregulation in freshwater and is inhibitory to smoltification (McCormick, 2009). A peak in circulating levels has been documented early in the smolting season (Prunet and Boeuf,

1989; Prunet *et al.*, 1989). A surge in thyroid hormones associated with the initiation of smoltification has also been reported in several species (Hoar *et al.*, 1988; Björnsson and Bradley, 2007) and a role of thyroid hormones in osmoregulation has been suggested, albeit probably in interaction with other hormones involved in osmoregulation (Ojima and Iwata, 2007; McCormick, 2001). We may then speculate that increases in these hormones (unmonitored during our study) have influenced the increase of NKA activity. Furthermore, during smolting there is a switch between NKAα1a (freshwater isoform) and NKAα1b (seawater isoform) subunits (Tipsmark and Madsen, 2009). We may hypothesise that there is a higher proportion of NKAα1a during the first increase and NKAα1b during the second. Our test measuring NKA activity does not differentiate both forms of subunits but specific RNA expression may confirm our hypothesis.

4.2 Effect of early and late temperature shift on osmoregulation features

Temperature was the same in all three circuits from March 10 to April 19 when the temperature was increased over three days in the early treatment group. No difference was seen on April 21 between the treatment groups, but, one week after the temperature shift was completed, a marked decrease in NKA activity level in fish sampled under T2. Similarly, a decrease in NKA activity was measured one week after the temperature shift in the late treatment group. Such a decrease may indicate that these fish were undergoing desmoltification. Both strains reacted in the same way and displayed decreased hypo-osmoregulatory capacities one week after the temperature shift. NKA activity level decreased back to those of parr, namely 2-3 µmol ADP mg^{-1}*prot*h^{-1} (McCormick *et al.*, 2009) and osmolality increased after seawater exposure on June 6. On that date, plasma sodium and potassium levels were different between T1 and T2. Plasma sodium was also increased in T3 but to a lesser extent and plasma potassium was not different from the control. These differences may be explained by the later temperature shift meaning the salmon were still undergoing desmoltification in opposition to T2 fish which were believed to be already desmoltified. Temperature has a role in the timing of smolting by affecting the rate of development (McCormick *et al.*, 2002). Gill NKA activity is an acknowledged indicator of hypo-osmoregulatory capacity of the fish (Bisbal and Specker, 1991; Mackie *et al.*, 2007) but other osmoregulatory systems, developed chloride cells (Hoar, 1988), $Na^+/K^+/2Cl^-$-cotransporter (Boeuf, 1993) or more efficient glomerular filtration (McCormick and Saunders, 1987) exist and may still be, at least partially, functional, thus limiting plasma ion increase. As high variability was observed, we may also hypothesise inter-individual differences in desmoltification pace as reported by Stefansson *et al*. (1998) who suggested that desmolting

in Atlantic salmon is not a synchronised process, while smolting is. Exposing fish to 24 h salinity tests may have enabled more differences to be seen but a 96h exposure to seawater may be a more realistic challenge to investigate the hypo-osmoregulatory capacities under simulated natural river conditions. Nevertheless, a longer test might have shown long-term effects on fully or partially desmoltified fish only capable of coping with excess salinity for short time till exhaustion.

The smolting process is under endocrine control (Hoar, 1988; Bœuf, 1993; McCormick *et al.,* 1998; Ebbesson *et al.,* 2003; Stefansson *et al.,* 2008) and thus endocrine profiles may help to understand the observed features and how a temperature shift results in poor osmoregulation. Under T1 conditions, comprehensive endocrine profiles, consistent with their interactive role of mediating changes associated with smolting, have been observed. Cortisol and GH are known to increase during smoltification and to favour the development of hypo-osmoregulatory capacity of smolts through increasing NKA activity and chloride cell proliferation (Hoar, 1988; McCormick, 2001; McCormick, 2009). Downregulation in IGF-1 signaling at elevated temperature has been also reported in some salmonid fish including Atlantic salmon even if circulating IGF-1 was not affected (Hevrøy *et al.,* 2015). In the present study, plasma cortisol level was lower in smolts having experienced a temperature treatment (T2) already one week after the treatment. Two weeks after the treatment, we observed the same pattern. Higher cortisol level and an increase in plasma GH and IGF-1 in the control group (T1) or when the treatment had not been applied yet (T3) were observed on the date corresponding to high NKA activity and efficient osmoregulation as evidenced by the results of the second salinity test on smolts from the T1 group. An increase of GH values in fish of the T2 and T3 groups and of IGF-1 in the T2 group after the third salinity test may indicate an attempt to compensate for poor hypo-osmoregulation.

4.3 Strain-related differences to a temperature shift

The timing difference in smolting peak observed in the current study was probably related to the genetic background of the strains. Stewart *et al*. (2006) compared two populations originating from upper and lower catchment tributaries of Tay River in Scotland, and already noticed differences in downstream migration timing between these upstream and downstream populations. They concluded that a genetic population structuring may exist, even at a fine scale. Migration timing differences probably makes it possible for different populations to meet up simultaneously at sea under favourable conditions. Sea arrival is synchronized to occur at peak readiness (physiological window), under the best possible environmental conditions for smolt survival (ecological window) (McCormick *et al.,* 1998). Differences in

smolting peak in strains originating from different river systems and put under identical simulated local conditions, may reflect a genetic background of smoltification as environmental diversity is eliminated. To explain the earlier NKA activity peak under T1 conditions in LA smolts, we may hypothesise that LA smolts have to reach the sea earlier than CG smolts to have enough time to cover the additional distance to reach the feeding ground in the North Atlantic at the right time. While peak NKA activity was measured on the same date as peak plasma GH and IGF-1 levels in Cong smolts, hormone levels peaked one week after NKA maximum activity in LA smolts. Cortisol and GH are known to increase NKA activity (Tipsmark and Madsen, 2009; Takei and McCormick, 2013). Increased gill NKA activity during smolting is also partially supported by increases in circulating IGF-1 levels (McCormick, 2001). As sampling dates for hormones are limited, it might be speculated that we missed the actual peak in GH and IGF-1 levels in LA smolts. Cortisol was only measured on two dates which makes it possible that we failed to observe an increase leading to peak NKA activity.

It has been demonstrated that temperature influences the timing of smolt migration directly and/or indirectly (Stefansson *et al.,* 2008), but little information is available about the effects of a temperature shift on the smoltification process. According to the few available data, a threshold temperature may be needed for the smolt migration to take place (Jonsson and Ruud-Hansen, 1985) but elevated freshwater temperature may accelerate the loss of seawater tolerance, thus strongly linking temperature to hypo-osmoregulatory ability of Atlantic salmon smolts (Stefansson *et al.,* 1998; McCormick *et al.,* 1999). More recent findings indicate that degree*days are a better indicator for initiation and termination of downstream movement than the absolute temperature (Zydlewski *et al.,* 2005). Gill peak NKA activity was observed at 350 dd following the onset of the typical smolt-related increase in activity and the smolt window was defined as the period when NKA activity was >90 % of peak value (Handeland *et al*., 2004). Similar models have been presented (Stefansson *et al*., 1998; McCormick *et al*., 1999). Depending on the definition of the cut-off level, the smolt window was calculated to last between 300-400 dd (McCormick *et al*., 1999). Our results under T1 conditions are in accordance with these models. Peak activity was measured at 319 dd (April 28) for LA smolts and 408dd (May 5) for CG smolts after the first increase (March 24). As measurements were only carried out once a week, peak activity might have been missed, thus explaining the slightly out of range number of degree*days for CG smolts. The smolt window seems to be already over at 364 dd in the early treatment group and the late treatment was applied out of the smolt window (>600dd) but was still followed by a marked NKA activity

decrease. Results of both treatment groups indicate a deleterious effect of the treatment but do not support the degree*days model. Another explanation could be that the maximum threshold temperature was exceeded for these strains. The best swimming condition for LA smolts were determined to be between 7.5 and 13.5°C (Martin *et al.,* 2012). Moreover, the swimming speed was reduced by 80% in LA smolts at a water temperature of 17°C and a complete positive rheotaxis was observed from 20°C on (Martin *et al.,* 2012). A compromise of both threshold and cumulative temperatures may also be hypothesised with more or less dominance of one factor depending on the strain. On May 19, water temperature reached 20°C in the T2 and T3 circuits and NKA activity had already steeply decreased in LA smolts to similar values in both circuits, pointing out a stronger influence of a temperature threshold. On the same date, NKA activity in T3 CG smolts also decreased but did not reach values as low as in T2 which leads to hypothesise a more delayed impact of a temperature increase, supporting the degree*day hypothesis. In addition, thermal cues for migration may not be universal among salmon stocks (Stefansson *et al.,* 2008).

5. Conclusions

To summarize, this study confirmed that a temperature shift during downstream migration reduced hypo-osmoregulatory capacities of smolts. Both strains reacted in the same way for early and late temperature shifts despite differences in smoltification timing under conditions without temperature treatment. A one-week delay after the temperature treatment was necessary for a response to be measured, comforting the role of temperature in influencing the rate of changes occurring during smoltification. Given this delay, favouring downstream migration to help smolts reach the sea faster may help limit the impact of a rapid temperature increase.

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Previously we confirmed that a rapid increase in temperature during downstream migration reduces hypo-osmoregulatory capacities of smolts. Decreased NKA activity and increased plasma osmolality were measured in both strains as well as in early and late migrants one week after the raise in temperature. The response was monitored by means of markers found in literature, NKA activity and plasma osmolality and hormone levels.

While underlying molecular modifications driving the changes during smolting are still not fully understood, it is clear that transcriptional changes are involved in this process (Robertson & McCormick, 2012a). In this chapter we present the results from our study on the expression of smoltification-related genes in the liver using a high troughput RT-qPCR method. This organ plays an important role in metabolism (lipid, carbohydrate and iron) and endocrinology during smoltification but has only benefitted from few studies.

4.3 A temperature shift on the migratory route impairs gene expression in the liver during smoltification in two strains of Atlantic salmon (*Salmo salar* L).

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Abstract

Smoltification is a complex developmental process resulting in the ability of juvenile salmon to migrate to and live in seawater. Based on local field data, we used high throughput RT-qPCR chips to investigate the impact of a 5°C difference between tributary and river in the liver for its role in metabolism (lipid, carbohydrate and iron) and endocrinology. Smolts from two strains of Atlantic salmon (Loire-Allier, France and Cong, Ireland) were reared under three temperature regimes (no, early and late temperature increase). Usually upregulated genes during smolting were downregulated after a temperature increase, notably α-globin, ferritin and DNA repair protein *ra51a*. Difference between the strains, e.g. higher expression of genes involved in the carbohydrate catabolism (*taldo1*) and iron metabolism (*tf*) and lower expression of genes involved in *de novo* fatty acid synthesis (*lxr*) or hormonal regulation of smoltification (*igf1*) in Loire-Allier smolts, are thought to be linked to water temperature and migration distances of the origin rivers. All the selected genes from the 'Lipid and carbohydrate metabolism' group varied over the study period indicating that metabolism is an important but often neglected field of research during smoltification. This study gives further insights on the impact of human-related water temperature increase on molecular processes underlying smoltification and suggests reduced survival chances of smolts during migration.

Keywords: Smoltification, temperature, strain, gene expression, liver, high through-put RT-qPCR

1. Introduction

In spring, Atlantic salmon (*Salmo salar* Linnaeus, 1758) juveniles that have reached a size-related developmental stage will transform from stream-dwelling parr to seawater-tolerant smolts (McCormick *et al.,* 1998). It is a complex developmental process that involves a wide array of morphological (*e.g.* silvering of the flanks, accentuated streamline,…), behavioural (*e.g.* negative rheotaxis and schooling behaviour of the smolts,…) and physiological modifications (*e.g.* hypo-osmotic capacities, increased capacity for lipolysis…) that will ultimately result in the ability of juvenile salmon to migrate to and live in seawater (McCormick *et al*. 1998; Jonsson and Jonsson, 2011; McCormick, 2013). Migration over hundreds or thousands of kilometres from their native stream to the feeding grounds in the North Atlantic requires profound modifications of their metabolism. It was shown to increase during smolting, improving migration ability (Wedemeyer *et al.,* 1980 ; Jonsson and Jonsson, 2011) with higher liver capacity for lipolysis and decreased capacity for lipid synthesis (Sheridan, 1989).

Underlying molecular modifications driving the changes during smolting are still not fully understood, however, it is clear that transcriptional changes are involved in this process (Robertson & McCormick, 2012a). Several transcriptional changes have been identified in various organs (Seear *et al.,* 2010, Robertson and McCormick, 2012a). Focusing on the liver, production of α- and β-globins varies to meet increased demands in oxygen (Jonsson and Jonsson, 2011). Changes in expression of ferritin (Seear *et al.,* 2010; Robertson and McCormick, 2012a), a protein responsible for intestinal iron absorption, and transferrin (Seear *et al.,* 2010; Hardiman and Gannon, 1996), transporting iron towards hepatic reserves, have been reported. Cytokine, of which the expression seems to vary during smoltification (Ingerslev *et al.,* 2006), and transferrin are also involved in the immune response (Robertson and McCormick, 2012a). During smoltification, smolts are particularly sensitive to any stress (Jonsson and Jonsson, 2011). Heat shock proteins (HSP) are an important part of the cell protein folding mechanisms (Borges & Ramos, 2005) and play a crucial role in response to different stressors (Santoro, 2000; Morano, 2007) as well as in the immune response (Srivastava *et al.,* 1998). Hormones, like cortisol, GH, IGF-1 and prolactin are major mediators of changes during smolting (McCormick *et al.,* 1998, McCormick, 2013). Through the expression of hormones and their receptors in target organs, these hormones may in turn influence transcription of other smolting-related genes (Yada *et al.,* 1992; Sakamoto *et al.,* 1995).

Environmental cues, like temperature, strongly influence the smoltification process by modulating the pace of change (McCormick *et al.,* 2002). Increased temperature advances smoltification (McCormick *et al.,* 1996), limits smolts' saltwater tolerance timeframe (Handeland *et al.,* 2004) and migration duration (Zydlewski *et al.,* 2005). Elevated temperature conditions also reduce swimming speed or even stop migration by promoting positive rheotaxis (Martin *et al.,* 2012). It was hypothesized that temperature may negatively impact the salmon life-cycle through swift anthropogenic-linked increases in temperature (Martin *et al.,* 2012). Indeed, heavily modified rivers through anthropogenic use (industrial waste water, hot water from thermal plants, dams…) may ultimately lead to a temperature gap between main channel and tributaries. Under simulated conditions, this rapid temperature increase negatively impacted hypo-osmoregulatory capacities of early and late migrating smolts, causing a sharp decrease in gill Na^+/K^+ATPase activity and increased plasma sodium levels and osmolality after a seawater challenge (Bernard *et al.,* under review). Fish reared under conditions with a temperature increase also displayed reduced plasma GH and IGF-1 levels compared to the control group (Bernard *et al.,* under review).

Experiments also showed stock- and population-specific differences in downstream migration timing (Orciari & Leonard 1996; Stewart *et al.* 2006) and strain-specific increases in salinity tolerance (Handeland *et al.,* 2004) under the same environmental cues. The sensory-response system relies on a large number of genes which gives evolution the means to finely tune developmental and environmental responses (McCormick, 2009). This may result in differences as observed in various studies (Stewart *et al.,* 2006; Orciari *et al.,* 1996; Handeland *et al.,* 2004) which led to the hypothesis of a genetic structuring of Atlantic salmon at the sub-catchment scale (Stewart *et al.,* 2006). However, where salmon population is extinct, like in Belgium, the use of foreign strains in restocking programs is unavoidable and salmon from different origins may perform differently under local environmental conditions.

Compared to classic RT-qPCR, high throughput Fluidigm technology provides a useful tool to look at transcriptional changes in a large number of genes (up to 96) and samples (up to 88) in a single array.

Given the importance of the liver during smoltification for its role in lipid and carbohydrate metabolism and endocrine regulation, we used high throughput Fluidigm technology to investigate i) altered expression of selected genes during smoltification, ii) differences between two salmon strains and iii) the influence of an early or late temperature shift during smoltification in this organ. Genes of interest were chosen based on literature (Seear *et al.,* 2010; Robertson and McCormick, 2012a, 2012b; Song *et al.,* 2012; Verstergren, 2012, Olsvik

et al., 2013) or because of potential relevance in smoltification and are included in categories like stress response as well as DNA repair, cell cycle control, apoptosis, endocrinology and metabolism.

2. Materials and methods

2.1 Fish rearing

Two strains of Atlantic salmon were used in this study. The first strain (CG) originated from the Cong Hatchery on the Cong River in Ireland and the second one (LA) from the 'Conservatoire National du Saumon Sauvage de Chanteuges" on the Loire-Allier River in France. Fertilised eggs (F1) from recaptured wild spawners (F0) were directly imported to and reared at the "Conservatoire du Saumon Mosan" hatchery (Public Service of Wallonia, Fisheries Service), located in Erezée (Belgium) until they reached the pre-smolt stage. On February 24, parrs (average bodymass of 23.1 g for CG smolts and 24.1 g for LA smolts) were transferred from Erezée to a wet laboratory in the University of Namur (Belgium). Both allochthonous strains, LA and CG, were equally allocated into three recirculating water systems. Each system was composed of six 120 L tanks, three per strain (N= 72 fish per tank). Throughout the study, fish were maintained in these tanks with a circular stream flow and supplemental aeration under simulated natural photoperiod (Figure 1) based on Namur (Belgium) latitude (50°28'00"N). Water temperature management was based on a decade of field data collected on the rivers Meuse and Ourthe (Belgium). Dates and temperature are based on field monitoring of downstream migration (Dierckx *et al.,* 2017). The control treatment (T1) mimics the temperature conditions of the Ourthe River (slowly increasing over the study period). The early treatment (T2) mimics the conditions early migrants would encounter. On April 19, a rapid 5 °C increase was applied and represents the passage from tributary into main channel. The late treatment (T3) mimics the conditions late migrants would encounter with a 5 °C increase on May 9. Temperature increases were completed within three days, corresponding to the time smolts need to cover the distance between the sampling points on the tributary and on the main channel using an average speed calculated on field data of smolt migration monitoring between two Belgian rivers (Ovidio *et al.,* 2016). Fish were daily fed (TroCo Supreme-21 Coppens International B.V., Helmond, The Netherlands) with a fixed ration (1% of fish biomass per day) provided with automatic feeders.. Oxygen concentration and temperature were checked daily and other water characteristics (pH=7.2, $NH_4^+ < 0.06$ mg*L^{-1}, $NO_2^- < 0.05$ mg*L^{-1} and $NO_3^- < 10$ mg*L^{-1}) were checked weekly. All experiments were in accordance with local ethic committee on animal

experimentation of the University of Namur, Belgium (KE13193) that agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

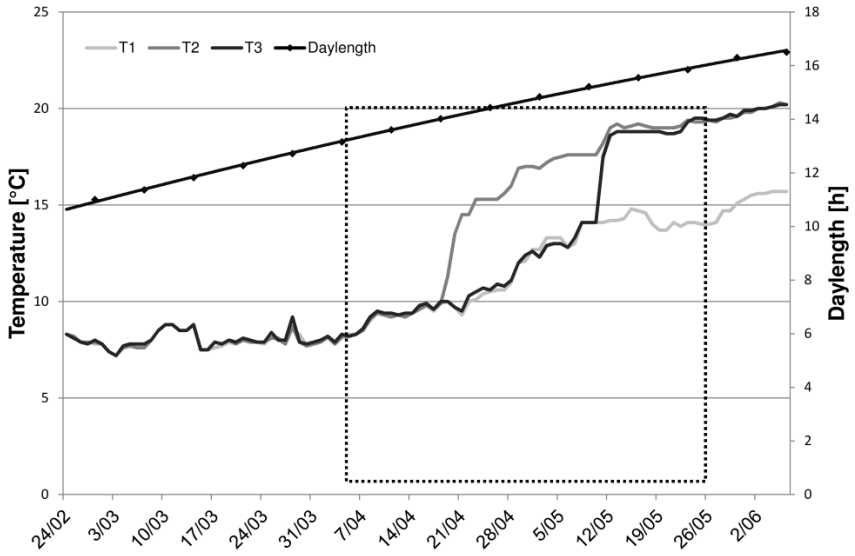

Figure 1: Applied temperature regimes and photoperiod for the three conditions along the study. T1: control condition without rapid temperature increase; T2: early treatment with a rapid temperature increase on April 19; T3: late treatment with a rapid temperature increase on May 12. Dashed square represents downstream migration period (from percentile 10 to 90 of migrating smolts) based on nine years of field monitoring data (Dierckx *et al.*, 2017).

2.2 Sampling

Four times over the study, three fish were quickly dip-netted out of each tank and directly anaesthetized with 120mg*L^{-1} MS-222 (pH 7.2) and then euthanized. The liver was excised and immediately frozen in liquid nitrogen, then stored at -80 °C until RNA isolation.

2.3 RNA isolation

Total RNA was extracted from tissue samples from 7 to 9 fish per strain per condition per date using Extract-All (Eurobio, Courtaboeuf, France) according to manufacturer's instructions. Tissue was homogenized using a SpeedMill Plus (Analytik Jena AG, Jena, Germany). RNA quantity and purity (DNA and solvent contamination) were measured with a Nanodrop 2000C UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA). Quality of the RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and Agilent RNA 6000 Nano serie II. RNA samples were stored at −80 °C until assay.

2.4 Primer design and checks

Some primers used in our array (Table 1) were designed using Primer3 (v.0.4.0) and Primer-BLAST (National Center for Biotechnology Information, NCBI) with a melting temperature

of 59–61 °C. We retrotranscribed 12 RNA samples including both salmon strains and the three conditions with RevertAid Minus First Strand, cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions. DNA matrix was made with equal proportion of cDNA from these samples. Serial dilution of this matrix was used in RT-qPCR (Power SYBR Green, PCR Master Mix, Thermo Fisher Scientific) using Stepone^{TM} Software v.2.1 for C_T calculation; cycling parameters were 2 min at 95 °C, 30 cycles of 95 °C for 30 s followed by 60 °C for 30 s and then 72 °C for 30 s and a final phase of 5 min at 72 °C. Primers with high yield (90 % < R < 110 %; R = $((10^{(-1/slope)}-1)*100)$) and efficiency (1.9 < E < 2.1; E = $(10^{(-slope)}-1)$) were selected. A melting curve was generated to confirm product specificity.

2.5 Gene expression analysis by Fluidigm BioMark system

The first step for qPCR was the reverse-transcription. Two microgram of RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega), as described by the manufacturer. The second step for high-throughput qPCR was a pre-amplification of cDNA with TaqMan® PreAmp Master Mix kit (Applied Biosystem) followed by an exonuclease I (NEB) treatment as recommended by the manufacturer's instructions. The third step was qPCR into the BioMark HD System after preparation and loading of Fluidigm 96x96 Dynamic Array. Two chips 96x96 were used in our experiment with 175 samples and 41 pair of primers in duplicate (Table 1). Among these 41 genes, 36 were grouped in 7 functional groups (Immune

Table 1: Groups, names, genes, accession numbers, primers sequences and references for primer sequences.

Group	Name	Gene	Accession	Forward Primer Reverse Primer	Primer source
Lipid / carbohydrate metabolism	Peroxisomal CoA peroxidase	*acox*	**DQ364432**	TCTCCGCAGTATGAACACACA TTGGGTCCTATGTCCCCTACC	Hecht, 2013
	Apolipoprotein A-I	*apoa1*	**NM_001123663**	TGGTCCTCGCACTAACCATC GCAGTCAACTTCACCTGAGCTA	
	delta-6 fatty acyl desaturase	*fads6*	**NM_001123575**	ATCTGGGAATATTGCTGGCCC TGATGCTGTCTGAGCCAAGTC	
	Liver X receptor	*lxr*	**FJ470290**	CGGCTACGTTAGGTTACAACG GCCTTCAGGCGAGAAGATGG	
	Cluster of differentiation 36	*cd36*	**NM_001124511**	GGATGAACTCCCTGCAT TGAGGCCAAAGTACTCGTCGA	
	Citrate synthase	*cs*	**A0A1S3MGM4**	CTACAAGATCGTGCCCCCAG CAAAACGCCCTTTTTGGGGA	
	Galactokinase 2	*galk2*	**B5X1Z3**	GGTTATGCTGTGCTCCCAAT TCATCCCAGACAGAGGAACC	
	Calmodulin	*calm1*	**NM_001139713**	CGACAAGGATGGTAACGGCT GTTGACAGTGAGTGTGTTGC	
	glycogen phosphorylase L	*pygl*	**A0A1S3SS35**	ACCTTTGCGTACACCAACCA CGTATCCGGTCCATGTCCTC	
	Transaldolase 1	*taldo1*	**NM_001146426**	GCCGCCTACCAGCATCT AGCTTGTCCATGGTGTTGGT	
Iron /oxygen	Transferrin	*tf*	**L20313**	CATCAAGAATGAACCCGACA ACGGACCTGACTGGAAGAGA	

transport /storage	Ferritin heavy subunit	*fth1*	**NM_001123657**	TCACTCACACCACCTCTTCG CTCACGTTCTTCGTGGGACT	
	Hemoglobin subunit alpha	*hba*	**NM_001123662**	AGGAAAGGCAGATGTCGTCG CCACGAGGTCGTCCATCAG	
	Hemoglobin subunit beta	*hbb*	**NM_001123666**	TACTGCCCTGAGTGTGATGC	
Stress response	Heat shock protein 90 beta 1	*hsp90b1*	**AF135117**	AAAGGAGTCCCGACCGTAGA GGGCTGGTGCTACAAGAGAG	
	Heat shock protein 70	*hsp70*	**BG933934**	CCCCTGTCCCTGGGTATTG CACCAGGCTGGTTGTCTGAGT	Olsvik *et al.*, 2013
Oxidative stress defence	Catalase	*cat*	**NM_001140302**	CATCCAGAAACGTTGGGTTC GAGGCACCTCTACGGGTGTA	Arukwe and Mortensen, 2011
	Cytochrome C oxidase subunit 5B	*cox5b*	**BT059830**	GGGGTGAAGTGGGGTTAGAC GCCTAGGCCTTTGGTACGTT	
	Glutathione peroxidase 7	*gpx7*	**NM_001140889**	GTGGGGAGTGGAAATCATGT ATTTGTTGAATGGGGAGCTG	Arukwe and Mortensen, 2011
	Superoxide dismutase 1	*sod1*	**NM_001123587**	AGCAGCTGACAGTGTGGCTA CGTTGTCTCCTTTTCCCAGA	Arukwe and Mortensen, 2011
	Glutathione S-transferase	*gst*	**DQ367889**	GCGTTGAGGACCTTCGTCTT TCGAGGTGGTTAGGAAGGTCT	
Immune response	Lysozyme G	*lyg*	**AM493682**	GACATCAACATGGGAGTTGGAG CCCACTGGTGTCAACCTTTGT	Myrnes *et al.*, 2013
	Complement C3	*c3*	**BI468074**	AGGGCATCAGTCACCAAGTG CCTCGTGGCTTGTTTTGTCC	
Cell cycle, apoptosis, proliferation, DNA repair	Bcl-2-associated X protein	*bax*	**EG801847**	ATTGGAAATGAGCTGGATGG GCCGACAGGCAAAGTAGAAG	Song *et al.*, 2012
	B-cell lymphoma-extra large	*bclx*	**NM_001141086**	GCCTGGACGCAGTGAAAGAG GGACGGCGTGATGTGTAGCT	Song *et al.*, 2012
	Caspase 3	*casp3*	**DQ008069**	TGCGATCAAAGTGTTCTCGAGTTT GGAAAGCAGCTGTTGTATCTGTTG	Takle *et al.*, 2006
	Cyclin-dependent kinase inhibitor 1B	*cdkn1b*	**BT045501**	GGAGGGAGTGTTTGGTTCAA GAGGCGGTCTGCTGTAAAAG	Song *et al.*, 2012
	Cellular tumor antigen P53	*p53*	**BT058777.1**	TGCGTGCTGCTTTCAGGT CGTCGGTTACAGGTGGTTG	
	DNA repair protein RAD51 homolog a	*ra51a*	**NM_001140555**	AGACAGGCTCCATCACAGAAA CACTCCCAACAAGTCCATACC	
Hormonal regulation	Insulin-like growth factor 1	*igf1*	**NM_001123623**	GATGTCTTCAAGAGTGCGATGTG CGCCGAAGTCAGGGTTAGG	Metzger *et al.*, 2013
	Insulin-like growth factor 2	*igf2*	**NM_001123647.1**	TGCCCACACTCAAACAGG CTTCCTCTGCCACACCTCA	
	Insulin-like growth factor I receptor	*igf1r*	**AY049954**	AGCCACCTGAGGTCACTACG CTCCCCAGCCATCTGAATAA	Tipsmark and Madsen, 2009
	Growth hormone receptor 1	*ghr1*	**AY462105**	TCCCAACATGCAGCTGTAGA TGTGGCACCTTGAAGAACAG	Tipsmark and Madsen, 2009
	Thyroid receptor alpha	*thra1*	**NM_001123628.1**	CGCCATCTTTGATTTGGG GGGGAATGTTGTGCTTGC	Spachmo and Aruwke, 2012
	Thyroid receptor beta	*thrb*	**AF302252.1**	GGAAACATGAGGCCATGC ACACGCGTACGTTGGGTT	Spachmo and Aruwke, 2012
	Mineralocorticoid receptor-like	*mr*	**XM_014209388**	ACGACGATGGAGCCGAAC ATGGCTTTGAGCAGGGATAG ACAACGACAGCCAGGAACTT	
Reference gene	Elongation factor I alpha	*efiα*	**AF321836**	AGAACCATTGAGAAGTTCGAGAAG GCACCCAGGCATACTTGAAAG	Tipsmark *et al.*, 2010
	Beta-actin	*β-actin*	**BG933897**	CCAAAGCCAACAGGGAGAAG AGGGACAACACTGCCTGGAT	Olsvik *et al.*, 2005
	40S Ribosomal protein S20	*s20*	**BG936672**	GCAGACCTTATCCGTGGAGCTA TGGTGATGCGCAGAGTCTTG	Olsvik *et al.*, 2005
	60S Ribosomal protein L10	*rl10*	**NM_001140680.1**	CCTTCCATGTCATCCGTATCAACAA AGACATGATCACCTGACCGATTC	
	EST : ssallna016029		**CA769178**	GGCTGTGCTAGGCTGGAGTT CGGTTCTGATGGCAGCACTT	Robertson and McCormick, 2012b

response, Oxidative stress defences, Cell cycle, apoptosis, proliferation and DNA repair, Hormonal regulation, Stress response, Iron/oxygen transport and storage, Lipid and carbohydrate metabolism) and 5 were initially considered as potential reference genes: EFIα, β-actin, 40S Ribosomal protein S20, 60S Ribosomal protein L10 and EST: ssallna016029 (Olsvik *et al.*, 2005; Robertson and McCormick, 2012b). β-actin was identified as not influenced by any factor ($p > 0.05$ ANOVA) and was selected as reference gene. Results were depicted as the expression of a target gene relative to a reference gene according to the ΔΔCT method (Livak and Schmittgen, 2001). Expression data were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6 Statistical analysis

All statistical analyses were performed with R 3.3.3 and Jmp 12. Data was tested for normality of distribution with Shapiro-Wilk W-test and homogeneity of variance by Levene f-test. Data was analysed by three-way ANOVA using date, strain and treatment as factors. Where normality tests failed, data was transformed (log or Box-Cox). Statistically significant differences were accepted at $p < 0.05$. All data are given as means ± standard error of the mean (SEM).

3. Results

The expression pattern of all the genes was influenced by at least one factor (Figure 2-7) with the exception of *ghr1*.

3.1 Lipid and carbohydrate metabolism

The date influenced ($p = 1.1e^{-8}$) the transcription of *lxr* (Figure 2) with higher expression in the beginning and lower expression at the end of the sampling period. Lower expression of *calm* ($p = 1.8e^{-6}$) and *lxr* ($p = 2.9e^{-11}$) was measured in the early treatment (T2) group. Transcription of other genes from the 'Carbohydrate and lipid metabolism group' was influenced by a two-way interaction Date*Treatment with lower RNA level of *acox*, *cd36*, *galk2* and *taldo1* in the T2 and T3 groups. Transcription of *cd36* ($p = 2.8e^{-3}$) and *lxr* ($p = 5.3e^{-3}$) was higher in CG smolts compared to LA smolts. RNA level of *taldo1* was higher in LA smolts on the first sampling date and transcription of *apoa1* ($p = 4.1e^{-2}$), *fads6* ($p = 8.1e^{-3}$) and cs ($p = 5.9e^{-3}$) were different between the strain on the last sampling date.

3.2 Iron and oxygen transport and storage

Expression of *hba* (Figure 3) decreased on the last sampling date in the late treatment group ($p = 8.5e^{-3}$). *hbb* expression was higher in the control group compared to the early treatment group on May 19 ($p = 1.1e^{-2}$) and decreased in the late treatment group in June ($p = 5.2e^{-3}$). Cong smolts in the early treatment had lower RNA levels of *hba* (T1 vs T2 $p = 9e^{-7}$ and T2 vs

T3 $p = 2.2e^{-4}$). Transcription of *tf* (Figure 4) decreased after the late treatment ($p = 1.1e^{-3}$) and expression of *fth1* decreased after the early on May 5 ($p = 3.2e^{-3}$) and late treatment on June 2 ($p = 2.6e^{-3}$). Higher *tf* RNA levels were measured in LA smolts on the two first sampling dates ($p = 2.8e^{-3}$ and $p = 2.6e^{-2}$).

3.3 Stress response

Expression of *hsp70* and *hsp90b1* (Figure 4) was not influenced by the treatment but higher RNA levels were measured in CG smolts compared to LA smolts on the last sampling date ($p = 1.5e^{-3}$for *hsp70* and $p = 2.7e^{-2}$ for *hsp90b1*).

3.4 Oxidative stress defence

The date influenced ($p = 2.1e^{-2}$) the transcription of *cox5b* (Figure 4) with higher expression in the beginning and lower expression at the end of the sampling period. Transcription of *cox5b* ($p = 3e^{-2}$) was higher in CG smolts compared to LA smolts. Lower expression of *cox5b* ($p = 2.5e^{-2}$) was measured in the early treatment (T2) group. Expression of *cat* (T1 vs T2 $p = 6.4e^{-3}$) and *sod1* (T1 vs T2 $p = 4.7e^{-3}$ and T2 vs T3 $p = 1.9e^{-2}$) was induced in the early treatment group on the first sampling after the temperature increase. Higher ($p = 3.1e^{-2}$) RNA levels of *sod1* between the control and the early treatment groups were also measured on May 19 (Figure 5). Higher transcription of *gst* were measured in LA smolts compared to CG smolts on April 28 ($p = 5.1.1e^{-4}$) and May 5 ($p = 1.7e^{-2}$) and of *gpx7* on April 28 ($p = 1.2e^{-3}$).

3.5 Immune response.

Transcription of *c3* (Figure 5) was higher in LA smolts compared to CG smolts on April 28 ($p = 7.1e^{-3}$) and May 5 ($p = 1.9e^{-3}$). RNA levels decreased in the early treatment group between May 5 and May 19 ($p = 1.1e^{-4}$) and in the late treatment group between May 19 and June 2 ($p = 3.2e^{-3}$). Expression of *lyg* was induced in the early treatment group one week after the temperature increase (T1 vs T2 $p = 1.2e^{-5}$ and T2 vs T3 $p = 1.2e^{-3}$).

3.6 Cell cycle, apoptosis, proliferation, DNA repair

Lower (p = 3e-2) expression of *p53* (Figure 6) was measured in the early treatment group as well as in the last sampling compared to the first in both strains ($p < 3.1e^{-2}$ for CG and $p < 1e^{-10}$ for LA). Expression *cdkn1b* increased in CG smolts between these two dates ($p < 2.3e^{-3}$) and was higher ($p < 1.1e^{-3}$) than in LA smolts on June 2. In the control group, transcription of *ra51a* decreased ($p < 8.4e^{-3}$) between April 28 and June 2 and decreased already on May 19 ($p < 1.6^{-5}$) in the late treatment group. Compared to the control group, lower transcription of *casp3* was also measured in the early treatment group from May 19 ($p < 2.8e^{-3}$) on and in the late treatment groups on June 2 ($p < 7.3e^{-4}$). Expression of *bclx* ($p < 8.4e^{-3}$) in LA smolts is lower on the last sampling date compared to April 28 ($p < 2.7e^{-2}$) and May 5 ($p < 8.4e^{-3}$).

Expression of *bax* decreased in LA smolts on May 19 ($p < 1.4e^{-2}$) and lower ($p < 7.6e^{-6}$) RNA level were found in LA smolt than in CG smolts on June 2.

3.7 Hormonal regulation

Single effects of the date were seen on the expression of *igf2* ($p = 5.6e^{-3}$) and *mr* ($p = 2.1e^{-5}$) with higher expression in the beginning and lower expression at the end of the sampling period (Figure 7). Transcription of *igf2* ($p = 2.5e^{-3}$) and *mr* ($p = 2e^{-2}$) was higher in CG smolts compared to LA smolts. Lower expression of *igf2* ($p = 7.3e^{-3}$) was measured in the early treatment (T2) group. In the control group, higher expression of *igf1* ($p=8.3e^{-3}$) was measured in CG compared to LA smolts. Transcription of *igf1r* decreased ($p=1.4e^{-2}$) on June 2 in LA smolts and was lower ($p=5.6e^{-5}$) than in CG smolts on that date. Transcription of *thra1* decreased ($p=1.8e^{-2}$) in LA smolts between May 5 and May 19.

4. Discussion

4.1 Experimental design

We used high throughput Fluidigm array to investigate the effects of an early or late temperature shift during the migration period on smoltification-related genes in the liver and compare the response in two strains. We might point out that our experimental design is less suited to find differences in the late treatment group (T3) as only two samplings were performed after the raise in temperature. In comparison, in the early treatment group the four samplings were made after the temperature.

Genes in our study were selected because they were shown in the literature to vary during smoltification (Seear *et al.,* 2010; Robertson and McCormick, 2012a, 2012b) and all of them were at least influenced by one factor, except *ghr1*. We selected genes in studies comparing two groups (parr and smolts or control and treated) but we compared smolts at four sampling points which may have limited the observable changes compared to studies looking at differences between two time points. A sampling point in early spring could add valuable information about gene expression during smoltification. As four out of five initial potential reference genes were influenced by at least one of the studied factors, they should be chosen wisely for future investigation depending on the organ and factor. The expression of β-actin has been found to increase three times in gills of smolts compared to parr and 40S Ribosomal protein S20 increased in the liver (Robertson and McCormick, 2012a). Olsvik *et al*. (2005) tested several potential reference genes for Atlantic salmon at different life stages and in various organs.

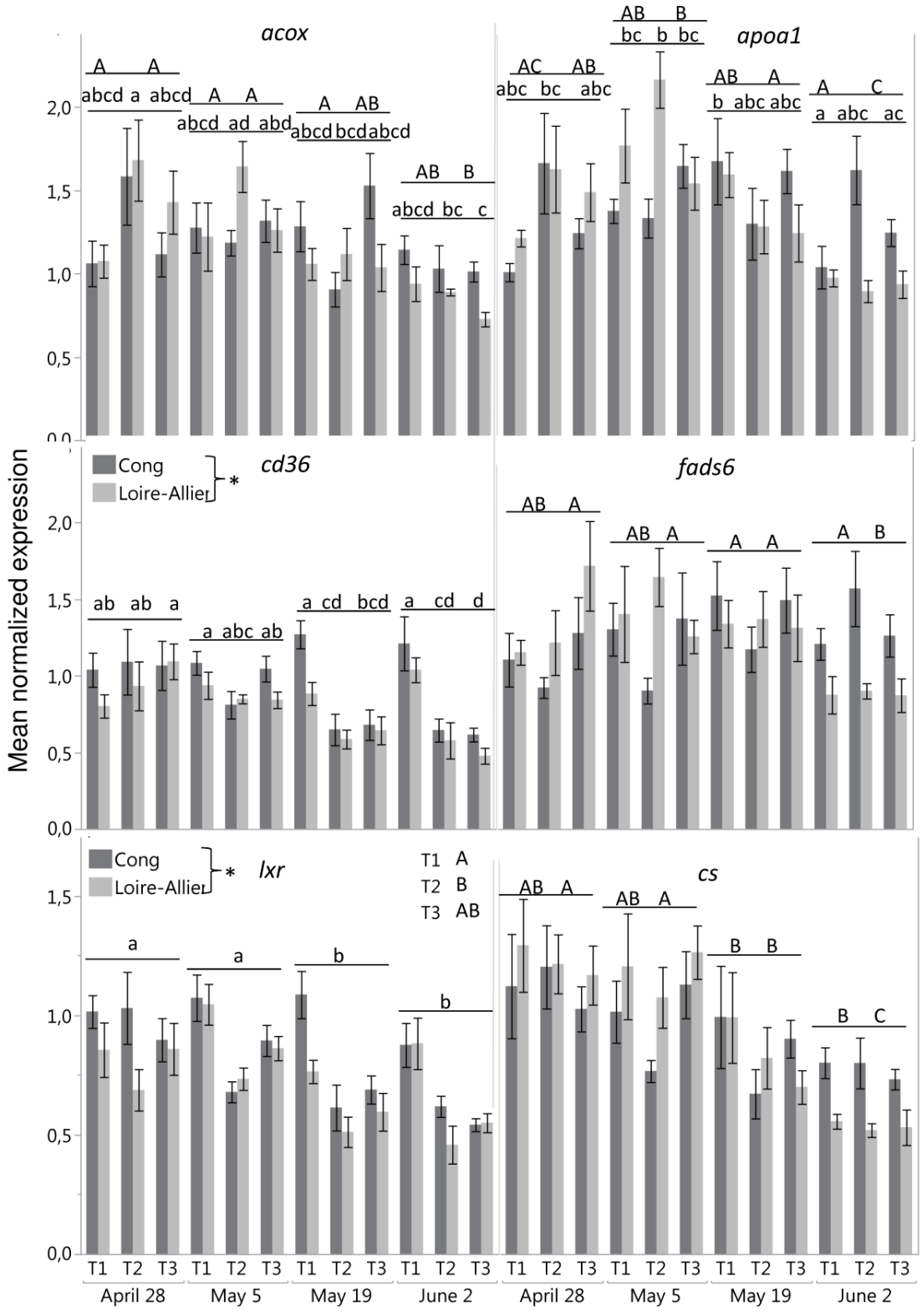

Figure 2: Mean normalized expression of genes in response to a temperature treatment in two strains during the smolting period. T1: control treatment, T2: early treatment, T3: late treatment, CG: Cong strain, LA: Loire-Allier strain. The symbol * represents a difference (p < 0.05) between the strains. Capital letters next to the treatments represent differences (p < 0.05) between the treatments. Capital letters over the histograms represent different Strain*Date interactions (p < 0.05) and lower case letters over the histograms represent diffrences (p < 0.05) between the date or date*treatment interactions.

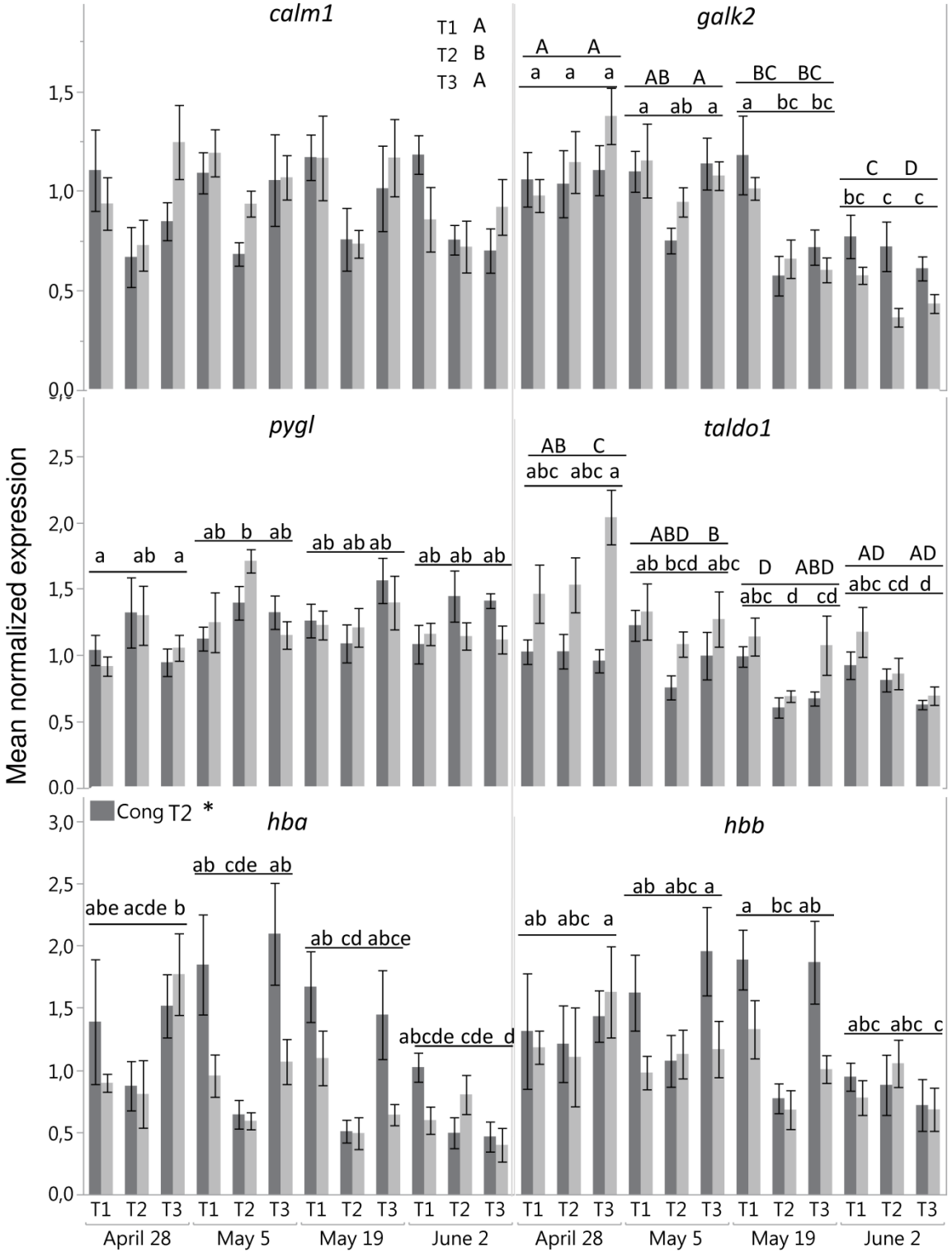

Figure 3: Mean normalized expression of genes in response to a temperature treatment in two strains during the smolting period. T1: control treatment, T2: early treatment, T3: late treatment, CG: Cong strain, LA: Loire-Allier strain. The symbol * represents a different ($p < 0.05$) strains*treatment interaction. Capital letters next to the treatments represent differences ($p < 0.05$) between the treatments. Capital letters over the histograms represent different Strain*Date interactions ($p < 0.05$) and lower case letters over the histograms represent diffrent ($p < 0.05$) date*treatment interactions.

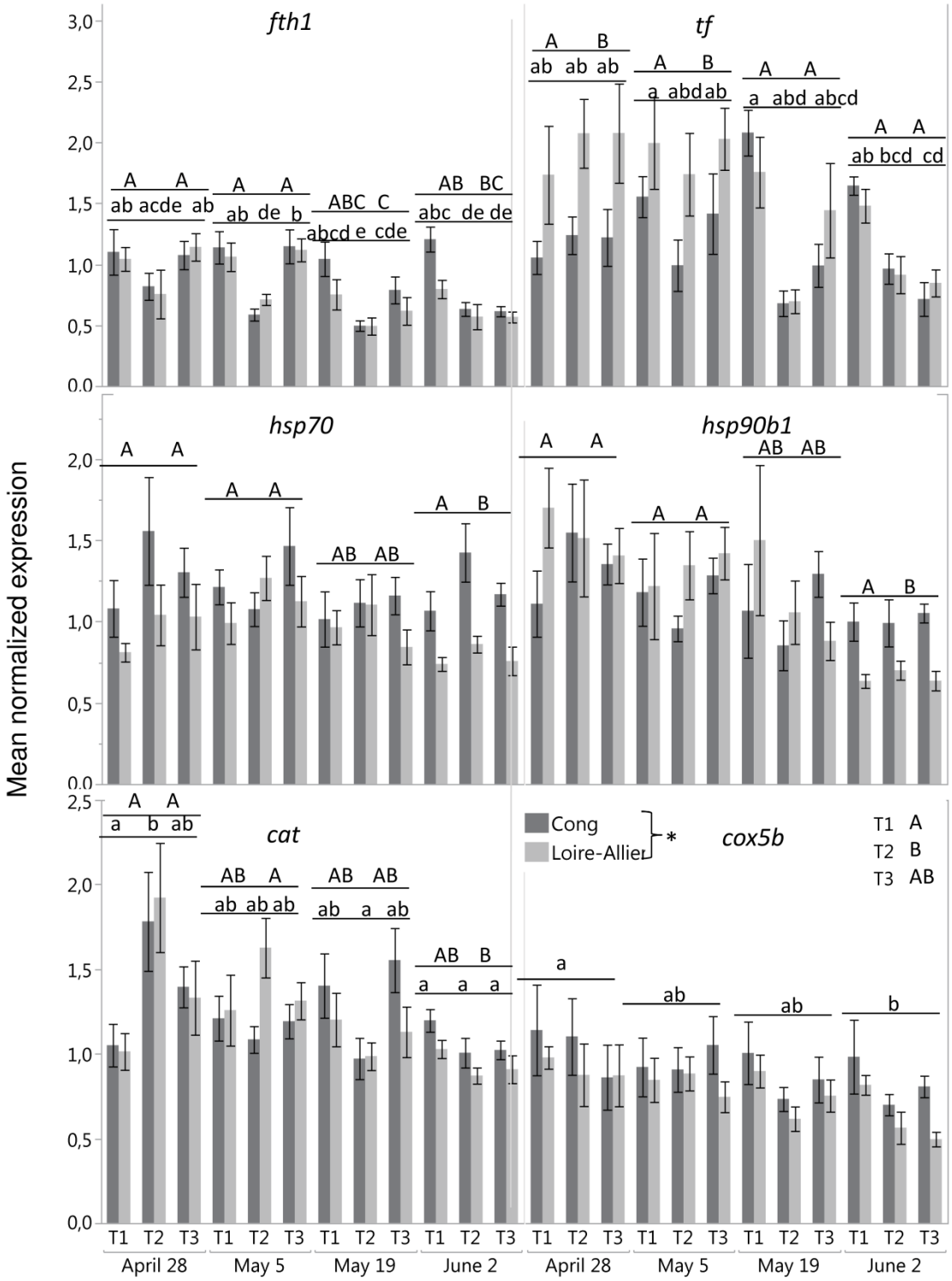

Figure 4: Mean normalized expression of genes in response to a temperature treatment in two strains during the smolting period. T1: control treatment, T2: early treatment, T3: late treatment, CG: Cong strain, LA: Loire-Allier strain. The symbol * represents a difference (p < 0.05) between the strains. Capital letters next to the treatments represent differences (p < 0.05) between the treatments. Capital letters over the histograms represent different Strain*Date interactions (p < 0.05) and lower case letters over the histograms represent diffrences (p < 0.05) between the date or date*treatment interactions.

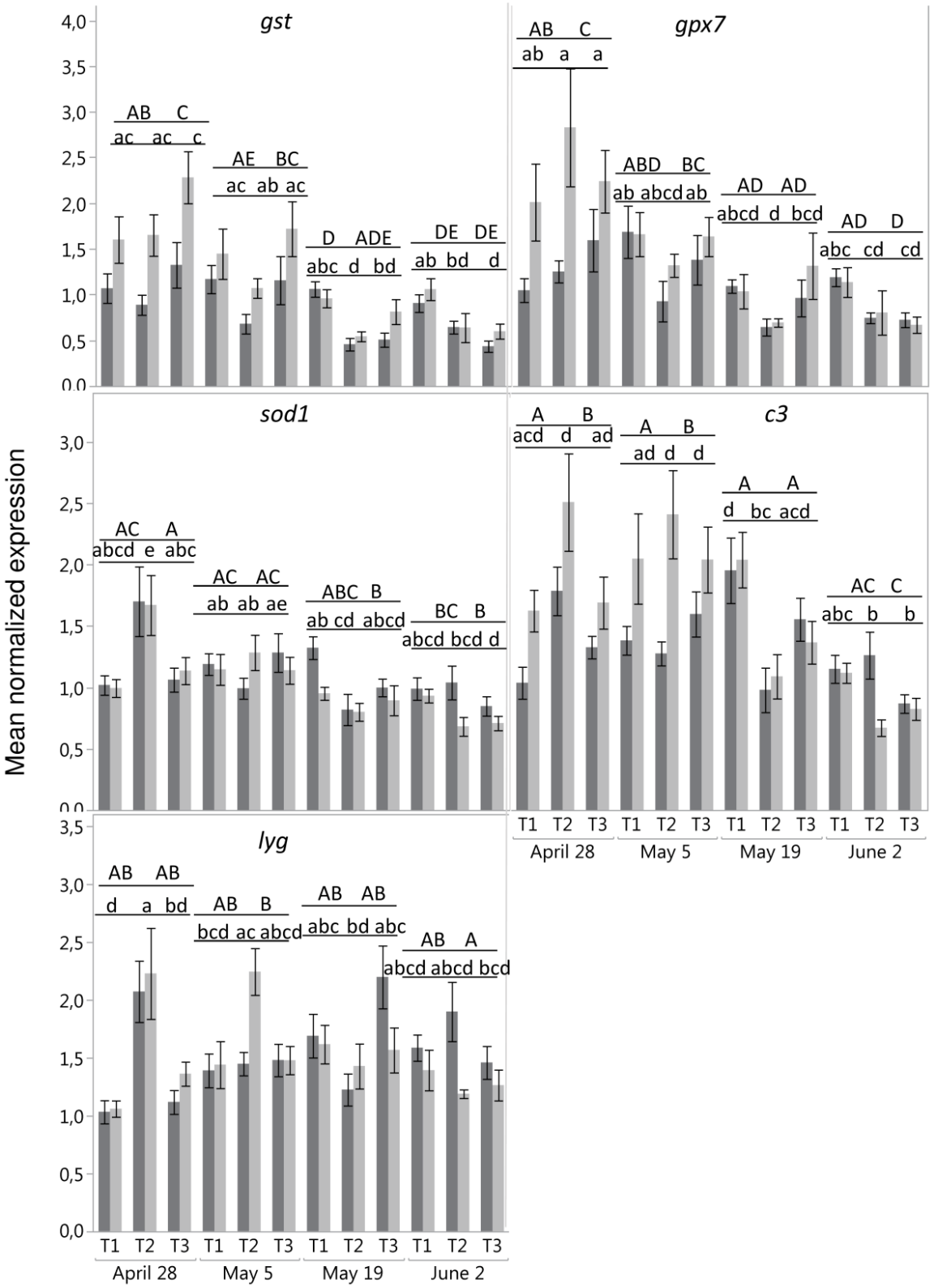

Figure 5: Mean normalized expression of genes in response to a temperature treatment in two strains during the smolting period. T1: control treatment, T2: early treatment, T3: late treatment, CG: Cong strain, LA: Loire-Allier strain. Capital letters over the histograms represent different Strain*Date interactions ($p < 0.05$) and lower case letters over the histograms represent diffrent ($p < 0.05$) date*treatment interactions.

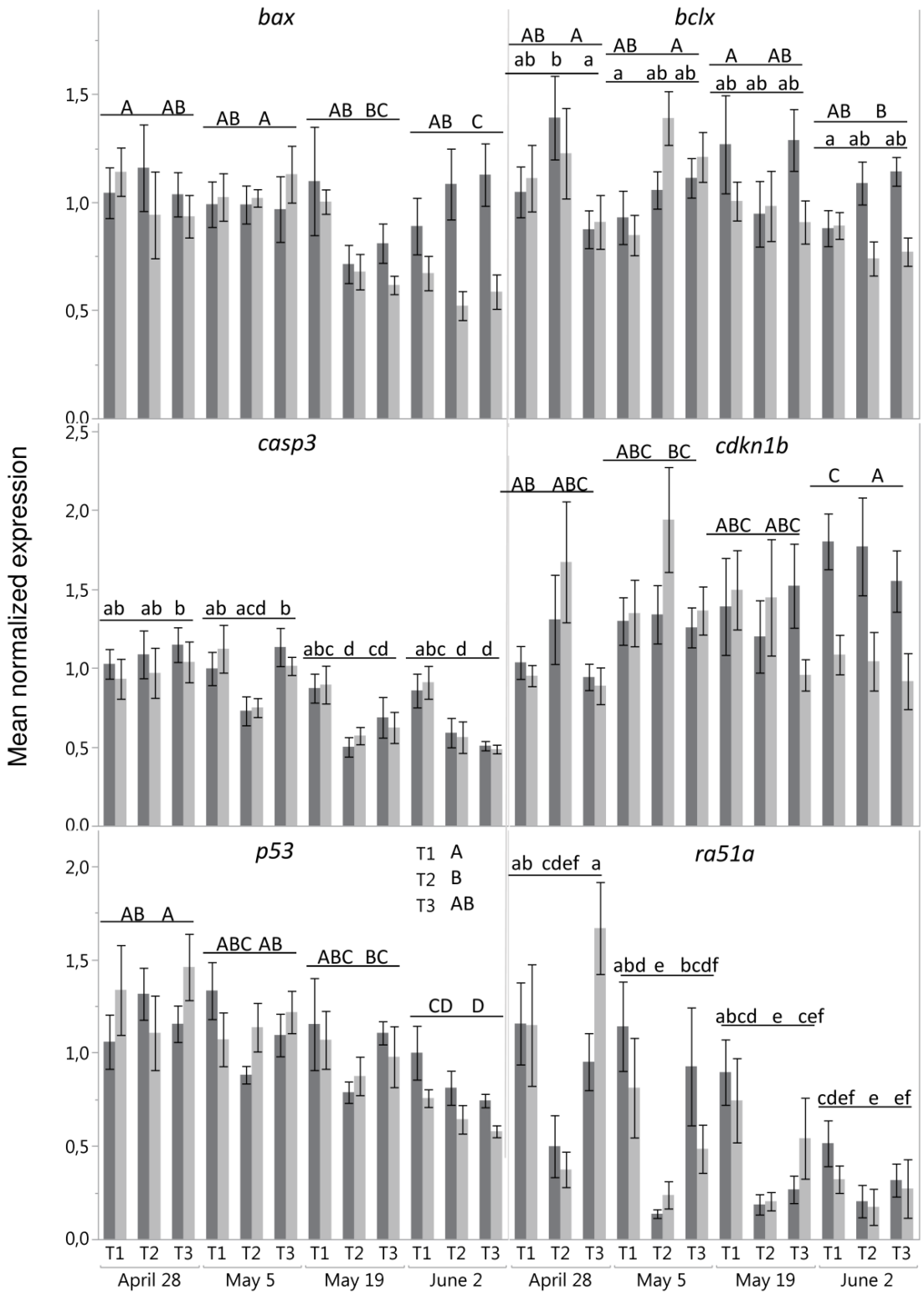

Figure 6: Mean normalized expression of genes in response to a temperature treatment in two strains during the smolting period. T1: control treatment, T2: early treatment, T3: late treatment, CG: Cong strain, LA: Loire-Allier strain. Capital letters next to the treatments represent differences ($p < 0.05$) between the treatments. Capital letters over the histograms represent different Strain*Date interactions ($p < 0.05$) and lower case letters over the histograms represent diffrent ($p < 0.05$) date*treatment interactions.

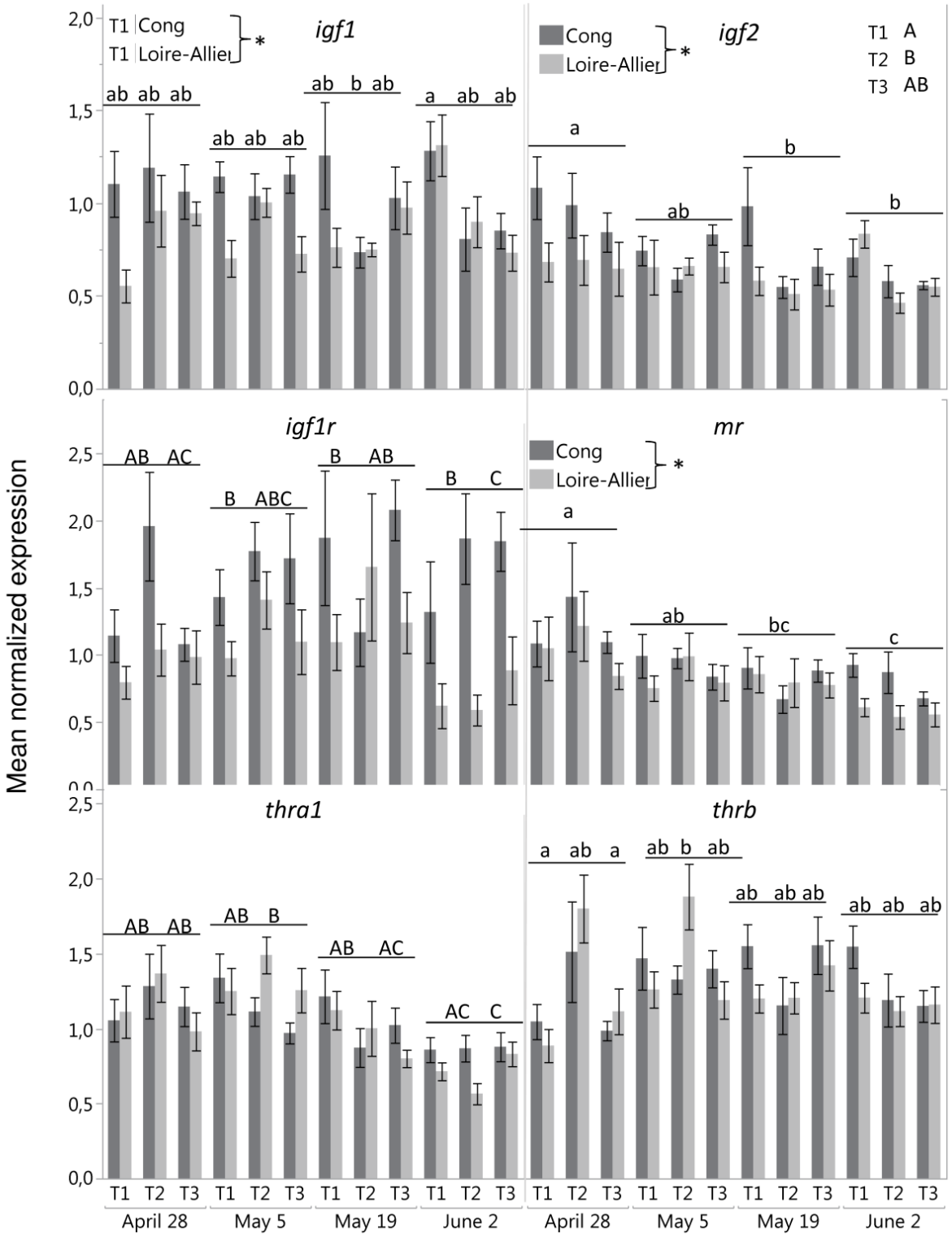

Figure 7: Mean normalized expression of genes in response to a temperature treatment in two strains during the smolting period. T1: control treatment, T2: early treatment, T3: late treatment, CG: Cong strain, LA: Loire-Allier strain. The symbol * represents a difference ($p < 0.05$) between the strains. Capital letters next to the treatments represent differences ($p < 0.05$) between the treatments. Capital letters over the histograms represent different Strain*Date interactions ($p < 0.05$) and lower case letters over the histograms represent diffrences ($p < 0.05$) between the date or date*treatment interactions.

4.2 Lipid and carbohydrate metabolism

Carbohydrate and lipid metabolism were affected by a temperature shift. RNA levels of calmodulin (activating phosphorylase kinase leading to glucose-1-phosphate being released from glycogen) only differed in the T2 condition but galactokinase 2 (contributing to the transformation of galactose into glucose-6-phosphate) and transaldolase (linking the pentose phosphate pathway to the glycolysis) were less expressed in both treatment groups after a temperature increase. This suggests a major impairment of smolts capability of sustained swimming efforts required during migration or to cover for the energetic demands of smoltification (McCormick *et al.*, 1998; Jonsson and Jonsson 2005).

Lower expression of *acox* (involved in fatty acid beta oxidation) and *cd36* (a fatty acid transporter also involved in the inflammatory response) after a temperature increase in the T2 and T3 groups, also point towards decrease capacity to sustain prolonged efforts. Expression of *cd36* may benefit from further investigation in the pyloric caeca as it is expressed at relatively high levels in post-smolts (Gu *et al.*, 2014). Considering increased capacity for lipolysis and decrease capacity for lipid synthesis during smolting (Sheridan, 1989), we were expecting higher expression of *lxr*, a transcription factor favouring *de novo* FA biosynthesis (Carmona-Antoñanzas *et al.*, 2014) in smolts from the T2 and T3 groups as increased temperature seems to initiate desmoltification. Desmoltification is not a parr reversion (Stefansson *et al.*, 1998) but it might still require large amounts of energy to enable all the changes required for re-adaptation to freshwater and thus explain lower *de novo* fatty acid synthesis.

Higher *taldo1*, and lower *lxr* and *cd36* expression in LA smolts may be comprehensible by higher demand in energy of muscles as they have to cover the longest migration route in the northeast Atlantic region with over 900 km to reach the sea (Martin *et al.*, 2012), compared to less than 50 km for Cong smolts. Decrease *apoa1* expression in the control group between the last two samplings and decreasing *galk2* in both strains toward the end of the study suggests the end of the smoltification.

The expression of all our ten selected genes from the ‘Lipid and carbohydrate metabolism’ group varied over the study period. This indicates that metabolism is an important field of research during smoltification.

4.3 Iron and oxygen transport and storage

Transferrin and ferritin expression were also reduced after a temperature shift when higher or stable expression has been repeatedly measured in smolts (Hardiman and Gannon, 1996; Seear *et al.*, 2010; Robertson and McCormick, 2012a). Decreased capacity to absorb and

transfer iron to the liver may explain decreased transcription of α- and β-globins. High levels of haemoglobin protein were reported in the blood of smolts (Sullivan *et al.*, 1985) and higher levels of mRNA of α- and β-globin genes were measured in smolts compared to parr (Seear *et al.*, 2010; Robertson and McCormick, 2012a), possibly to meet higher demands in oxygen during migration (Robertson and McCormick, 2012a) or as a preadaptation to compensate for lower oxygen tension in seawater than that experienced in freshwater by juveniles (Giles and Randall, 1980). Decreased expression, particularly in Cong smolts after an early treatment, may then limit the capacity of smolts to absorb enough oxygen and sustain long efforts. Higher transferrin expression in LA smolts on April 28 and May 5 may favour the iron metabolism in the liver for future oxygen transport to sustain their much longer migration.

4.4 Stress response

Both *hsp70* and *hsp90b1* were expected to be induced as a response to increased temperature but no influence of the treatment was seen despite a tendency ($p = 8.3e^{-2}$) for *hsp70*. Organ specific response may explain the lack of change as the expressions of *hsp7c*, *hsp70* and heat shock transcription factor *hsf1* were shown to increase in gills in response to increasing salinity in sea bream but only *hsp7c* transcription increased in the liver (Deane and Woo, 2004). Expression of *hsp70* was higher in Cong smolts than in Loire-Allier smolts on the last sampling. Heat shock proteins are an important part of the cell protein folding mechanisms (Borges & Ramos, 2005) and play a crucial role in response to different stressors (Santoro, 2000; Morano, 2007) including lethal heat shock, anoxia, heavy metals (Feige *et al.*, 1996; Parsell and Lindquist, 1994) and oxidative stress (Oksala *et al.*, 2014). The temperature regime between the River Meuse and the River Loire-Allier is similar (http://aquaphyc.environnement.wallonie.be; Martin *et al.*, 2012) but the difference is much more marked with the colder river Cong. Temperature reach up to 20°C in June, possibly inducing a stronger stress in Cong smolts and consequently the induction of *hsp70* and *hsp90b1*.

4.5 Oxidative stress defence

In case of oxidative stress, the cell antioxidant defences are activated in order to protect the cells against reactive oxygen species (ROS) damage (Di Giulio and Meyer, 2008). In the control group, no differences in RNA levels of genes involved in the protection of the cell from ROS damage were seen but *cat* and *sod1* were clearly induced in the early treatment group one week after the increase in temperature. This may indicate a response to stressful conditions for smolts. In the later samplings under T2 conditions, decreased RNA levels of *cox5b*, *gst*, *gpx7* and *sod1* suggest a reduced capacity of the organism to defend itself against

ROS which may cause damage to cell structures and DNA, lipid peroxidation and protein oxidation (Das and White, 2002; Valko *et al.,* 2007). Chronic thermal stress was shown to affect oxidative stress defences in liver cells, notably lowering *sod1* and *gpx7* expression (Olsvik *et al.,* 2013). Higher *cox5b* RNA levels were measured in Cong smolts and higher *gst* and *gpx7* expressions were measured in LA smolts at the beginning of the samplings. Both strains seem to be similarly impacted by a temperature increase.

4.6 Immune response.

The expression of *lyg* in smolts from the control group increased on May 19 compared to April 28. Increased *lyg* expression was already seen on April 28 in the early treatment group. Warmer water is prone to disease development which fits with the antibacterial role of *lyg* (Karplus and Post, 1996). As seen with a pollutant (Robertson and McCormick, 2012b), complement *c3* expression decreased in May in both groups previously exposed to a temperature shift. Ferritin is also known to respond to infection as seen in various fish species (Peatman *et al.,* 2007; Neves *et al.,* 2009) with increased expression of its heavy subunit, possibly starving the pathogen of required nutrient by sequestering iron. Decreased expression of *fth1* and *c3* under T2 conditions may render smolts prone to infectious diseases.

Transcription decrease of *c3*, *lyg* and *fth1* in the LA smolts may point towards a higher infection risk for that strain. However, we only looked at a few genes involved in the immunity and it probably doesn't depict a correct picture of the immune response capacity of the organism.

4.7 Cell cycle, apoptosis, proliferation, DNA repair

In our control group, only one change in expression was identified. Transcription of *ra51a* decreased between April 28 and June 2 maybe indicating the end of the smolting period. In response to a temperature shift, the expression of *p53* and *ra51a* decreased. Lower expression of genes involved in DNA damage and repair mechanisms (Song *et al.,* 2012), as well as the transcription of apoptosis related gene *casp3* was decreased at a time where huge cellular changes should happen (McCormick *et al.,* 1998, McCormick *et al.,* 2009). These decrease in expression coupled with decrease antioxidant defences may increase the risk of harmful DNA damage. Higher *cdkn1b* (involved in DNA repair mechanism) in CG smolts in June may have been induced by the temperature as it reaches up to 20°C.

4.8 Hormonal regulation

The expression of genes from the hormonal regulation of the smoltification seems to be minimally impaired by a temperature shift with lower *igf2* RNA levels in the early treatment group and intermediary levels in the late treatment group. There is scarce information about

the role of IGF2 during smoltification (Breves *et al.*, 2017) and in teleost in general (Hevrøy *et al.*, 2007). There is evidence of a role as an anabolic stimulatory agent (Hevrøy *et al.*, 2007) and it's been suggested to modulate local paracrine/autocrine regulation of tissue growth in teleosts (Wood *et al.*, 2005). No differences were seen in the expression of other genes from the 'Hormonal Regulation' group despite lower circulating levels of IGF1, GH and cortisol after a temperature shift (Bernard *et al.*, in prep) but smoltification is a month-long process and changes in hormone gene transcription are relatively small in magnitude (Robertson and McCormick, 2012a). We may then speculate that we might have seen more differences if a comparison with a date early in the smolting season (e.g.in presmolt in February) had been made. Sensitivity of receptors may then play a crucial role as it was hypothesized to favour increased circulating levels of thyroid hormone (Robertson and McCormick, 2012b) as neither pituitary transcription or circulating levels of thyroid stimulating hormone (TSH) are altered during smolt development (Larsen *et al.*, 2011).

Higher RNA levels of *igf1* in the control group and of *igf2* overall were measured in CG smolts. Smoltification-linked increase in salinity tolerance and gill NKA activity was shown to be partially supported by increases in plasma levels and local production of IGF1 (McCormick, 2001). There is only limited information about *igf2* during smoltification and no clear seasonal effect in the liver was measured (Breves *et al.*, 2017). However, a role in a 'transcriptional program underlying enhanced paracrine signalling in response to ionoregulatory demands' was hypothesized (Breves *et al.*, 2017). Yet, they did not measure the expression in different strains. From the migratory point of view, given the role of IGFs in promoting proliferation and differentiation of cells (Wilkinson *et al.*, 2004; McCormick, 2013), higher levels of *igf1* and *igf2* in Cong smolts may be explained by the necessity to acquire hypo-osmoregulatory capacities over a much shorter time as they are only separated from the sea by 50 km compared to LA smolts where changes may occur over a much longer period. Higher RNA level of *mr* was measured in Cong smolts. *mr* expression was reported to be stable in the gill during smoltification of Atlantic salmon (Nilsen *et al.*, 2008) and during seawater acclimation in rainbow trout but it increased in the intestine and kidney (Kiilerich *et al.*, 2011). As there seems to be an organ-specific expression of *mr* involved in hypo-osmoregulation, we might speculate a role of *mr* in the liver favouring smoltification. Transcription of *igf2* and *mr* decreased toward the end of the study in both strain as well as *igf1r* and *thra1* in LA smolts. Given the role of these genes in smoltification, these changes suggest the end of the process.

5. Conclusion

High throughput RT-qPCR chips are a useful tool to investigate the effects of a potential perturbation using a relatively large array of genes. A rapid temperature increase occurring during smoltification greatly impacts the transcriptional pattern in the liver of smolts. Lowered transcription of usually upregulated genes during this process probably reduces survival chances of smolts. Strain-specific differences in gene expression during smoltification have also been identified and are thought to be linked to temperature conditions of rivers of origin and migration distances. This study gives further insights on the impact of human-related water temperature increase on molecular processes underlying smoltification.

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In the previous chapter we investigated the transcriptional response induced by a swift increase in temperature on several functional groups of genes (lipid and carbohydrate metabolism, iron and oxygen transport,…) in the liver for its important role during smoltification. Results confirmed that the expression of smoltification-related genes was affected by a temperature increase (decreased RNA levels of usually upregulated genes) and suggest that smolt survival chances during their migration are strongly reduced. The acquisition of hypo-osmoregulatory capacity is a crucial change occurring during smoltification (McCormick *et al*., 1998). Therefor, we will now focus on the gene expression in the gills, a key organ for osmoregulation. More specifically, we will focus on genes involved in the hormonal control of smoltification, acid/base equilibrium and osmoregulation.

Initially, this approach was supposed to be done on the same samples as for the gene expression in the liver. However, gill samples were thawed due to a human error, causing excessive RNA degradation. We decided to renew this experiment however, only the Loire-Allier strain was available at that time.

4.4 Impact of rapid temperature increase on gill gene expression during smoltification of Atlantic salmon (*Salmo salar* L.)

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Abstract

Smoltification is a complex developmental process resulting in the ability of juvenile Atlantic salmon (*Salmo salar*) to migrate to and live in seawater. Exposure to changing environmental factors like temperature may disrupt smoltification. Based on local field data, we used q-RT-PCR coupled with osmoregulatory (Na^+/K^+-ATPase activity) and endocrine parameters (plasma cortisol and GH levels) to investigate the impact of a 5°C difference between tributary and river. Transcriptional responses were examined in the gill at four time points (early May – mid-June) in smolts reared under three temperature regimes (no, early and late temperature increase). Out of 20 genes, the expression of 6 was influenced by the temperature exposure and 11 changed over the smoltification season. Usually upregulated genes during smolting were downregulated after a temperature shift, notably *nkaα1b*, *nkcc1a* and *igf1r*. Temperature exposure also reduced gill Na^+/K^+-ATPase activity, plasma GH and cortisol levels which points toward hypo-osmoregulation impairment and reduced survival chances of smolts. Changes in mRNA abundance of genes involved in the hormonal regulation of smoltification in early June probably indicate the start of desmoltification. This study gives further insights on the molecular processes underlying smoltification and desmoltification in Atlantic salmon and possible responses to human-related water temperature increase. Data suggests dual roles in the smoltification and desmoltification process for GH and IGF1 and points to the implication of genes, previously unstudied (*nbc*) or with little data available (*igf2*), in the smoltification process.

Keywords: Smoltification, temperature, gene expression, gill, q-RT-PCR

1. Introduction

In spring, Atlantic salmon (*Salmo salar* Linnaeus, 1758) juveniles, that have reached a size-related developmental stage, will transform from stream-dwelling parr to seawater-tolerant smolts (McCormick *et al.*, 1998). Gills have benefitted of extensive research as the primary place of one of the main physiological change occurring during smoltification, the development of hypo-osmotic capacities, reducing internal osmotic perturbations when switching from freshwater (FW) to seawater (SW) (Jonsson and Jonsson, 2011; McCormick *et al.*, 1998; McCormick *et al.*, 2013). Changes occurring during smoltification are the results of external cues and internal rhythm mediated through the endocrine system (Gwinner, 1981). Changes in circulating levels of cortisol, GH, IGF1, prolactin and thyroid hormones will coordinate the development of physiological, morphological and behavioural preadaptation for sea-life (McCormick, 2013). Increases in circulating levels and exogenous treatment with hormones indicate that salinity tolerance is under the positive control of cortisol, GH and IGF1 (McCormick 2001, Tipsmark and Madsen, 2009) influencing the development of saltwater type ionocytes and the abundance and activity of the major transport proteins involved in salt secretion, Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$-cotransporter (NKCC) and cystic fibrosis transmembrane conductance regulator (CFTR) (Kiilerich *et al.*, 2007a; McCormick *et al.*, 2008; Pelis and McCormick, 2001; Tipsmark and Madsen, 2009). While it is clear that transcriptional changes are involved in this process, the underlying molecular modifications driving these modifications are still not fully understood (Robertson & McCormick, 2012a). Several studies focusing on the parr–smolt transformation (Robertson and McCormick, 2012a; Seear *et al.*, 2010; Tipsmark and Madsen, 2009) or the responses to a pollutant (Robertson and McCormick, 2012b) have identified altered gene expression in various organs. Through the expression of hormones and their receptors in target organs, these hormones may influence transcription of other smolting-related genes (Sakamoto *et al.*, 1995; Yada *et al.*, 1992).

Environmental cues like temperature strongly influences the smoltification by modulating the pace of change (McCormick *et al.*, 2002). Smolting may be advanced by several weeks under increased temperature conditions (McCormick *et al.*, 1996; Sobakken *et al.*, 1994). Increased temperature may have deleterious effects on smoltification like limiting the saltwater tolerance timeframe of smolts (Handeland *et al.*, 2004), the migration duration (Zydlewski *et al.*, 2005) and reducing the swimming speed of smolt or even stopping the migration by promoting positive rheotaxis (Martin *et al.*, 2012).

It was hypothesised that temperature may negatively impact the salmon life-cycle through swift anthropogenic-linked increases in temperature (Martin *et al.*, 2012). In heavily modified rivers, human use (industrial waste water, dams…) may ultimately lead to a temperature gap between main channel and tributaries. Under simulated conditions, this rapid temperature increase caused a sharp decrease in gill Na^+/K^+-ATPase activity and increased plasma sodium levels and osmolality after a SW challenge, generally impairing hypo-osmoregulatory capacities (Bernard *et al.*, submitted). The loss of smolt characteristics is called desmoltification and endocrine control of this process has not been elucidated in details yet (Björnsson *et al.*, 2011).

Based on local field data on the River Meuse (Belgium) and a tributary, the River Ourthe, where a temperature gap frequently exceeds 4°C during migration, we investigate gene expression alteration in smolt gills in response to a rapid temperature increase during their migration. Furthermore, we looked at differences in response depending on when the temperature treatment was applied (early migrant vs late migrants). We used a selection of genes grouped into four categories; endocrine control of smolting, hydro-mineral and acid base balance, ammonia excretion and stress indicators.

2. Materials and methods

2.1 Fish rearing

We used a strain of Atlantic salmon from the 'Conservatoire National du Saumon Sauvage de Chanteuges" on the Loire-Allier basin in France. Fertilised eggs (F1) from recaptured wild spawners (F0) were directly imported to and reared at the "Conservatoire du Saumon Mosan" hatchery (Public Service of Wallonia, Fisheries Service), located in Erezée (Belgium) until they reached the pre-smolt stage. On March 2, smolts (average bodymass of 20g) were transferred from Erezée to a wet laboratory in the University of Namur (Belgium). They were equally allocated into three recirculating water systems. Each system was composed of three 120 L tanks (N= 25 fish per tank). Throughout the study, fish were maintained in these tanks with a circular stream flow and supplemental aeration under simulated natural photoperiod based on Namur (Belgium) latitude (50°28'00"N). Fish were daily fed (TroCo Supreme-21 Coppens International B.V., Helmond, The Netherlands) with a fixed ration (1% of fish biomass). Oxygen concentration and temperature were checked daily and other water characteristics (pH=7.2, NH_4^+ < 0.06 mg l^{-1}, NO_2^-< 0.05 mg l^{-1} and NO_3^-< 10 mg l^{-1}) were checked weekly. Water temperature management was based on a decade of field data collected on the Meuse and Ourthe Rivers (Belgium). Each system followed a specific

temperature profile (Figure 1). The control treatment (T1) mimics the temperature conditions of the Ourthe River (slowly increasing over the study period). The early treatment (T2) mimics the conditions early migrants would encounter. On May 1, a rapid 5°C increase was applied and represents the passage from tributary into main channel. The late treatment (T3) mimics the conditions late migrants would encounter with a 5°C increase on May 15. Temperature increases were completed within three days, corresponding to the time smolts need to cover the distance between the sampling points on the tributary and on the main channel using an average speed calculated on field data of smolt migration monitoring between two Belgian rivers (Ovidio *et al.,* 2016). All experiments were in accordance with local ethic committee on animal experimentation of the University of Namur, Belgium (KE13193).

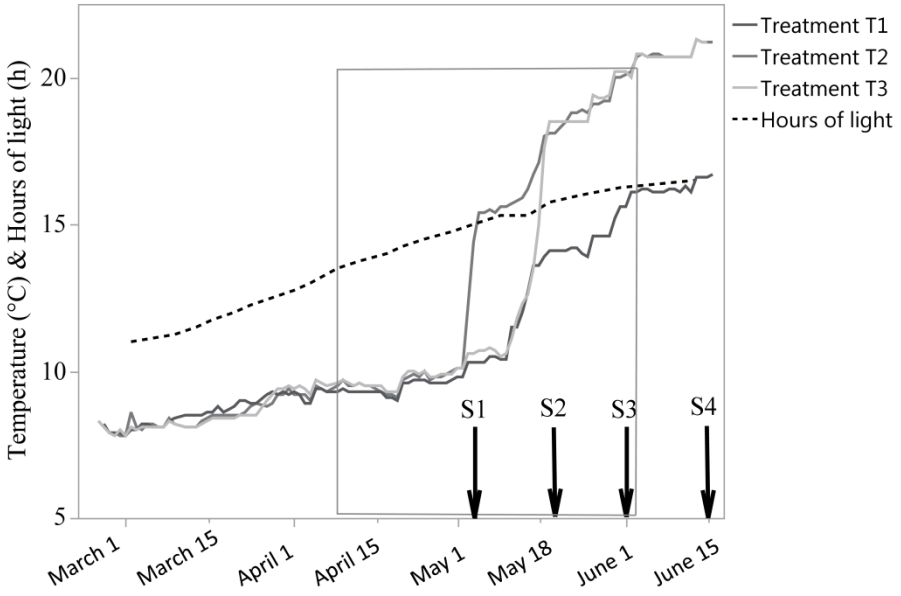

Figure 1: Applied temperature (°C) regimes and hours of light per day for the three conditions along the study. T1: control condition without temperature treatment; T2: early treatment with a rapid temperature increase on May 1; T3: late treatment with a rapid temperature increase on May 15. Sampling dates (S1, S2, S3 and S4) are pointed by an arrow. The square represents downstream migration period (from percentile 10 to 90 of migrating smolts) based on nine years of field monitoring data (Dierckx *et al.*, 2017).

2.2 Sampling

Four times over the study, three fish were quickly dip-netted out of each tank and directly anaesthetized with 120 mg L^{-1} MS-222 (pH 7.2). Blood was collected into 1 mL heparinized syringes from the caudal vein. The needle was removed and the blood was expelled into a 1.5-mL Eppendorf, stored on ice for less than 30 min and then centrifuged at 3000 g for 10 min.

The supernatant was collected and stored at -80 °C until subsequent analyses. Fish were then euthanized and the first branchial arches on both sides were excised. One was immediately frozen in liquid nitrogen for enzymatic assay and the other was plunged in 1mL Extract-All (Eurobio, Courtaboeuf, France) for RNA isolation and then stored at -80°C.

2.3 Na^+/K^+-ATPase activity and hormone levels

NKA activity was determined with a kinetic assay linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), in the presence and absence of Na^+/K^+-ATPase specific inhibitor ouabain as described by McCormick (1993). Total protein concentration of the gill homogenate was measured in duplicate using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. NKA activity is expressed as µmol ADP*mg^{-1} of protein*h^{-1}. Fish GH ELISA kit (Cusabio, PRC) and Cortisol ELISA kit (DRG Instruments GmbH, Germany) were used as per the manufacturer's instructions to measure plasma levels. Detection lower limits were 312.5 pg*mL^{-1} (intra-assay variation coefficient <15 %) and 2.5 ng*mL^{-1} (intra-assay coefficients 5.63) respectively. These assays were run on a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany) with use the included MARS data analysis software (BMG LABTECH GmbH, Germany). All samples were measured in duplicate within a single assay.

2.4 RNA isolation

Total RNA was extracted from tissue samples from 7 fish per condition per date using Extract-All (Eurobio, Courtaboeuf, France) according to manufacturer's instructions. Tissue was homogenized using a Bullet Blender Storm 24 (Next Advance, NY, USA). We used an Ambion DNA removal kit (Thermo-Fisher Scientific, Wilmington, DE, USA) to take out genomic DNA. RNA quantity and purity (DNA and solvent contamination) were measured with a Nanodrop 2000C UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA). Quality of the RNA was assessed on agarose gel (1%).

2.5 Primer checks, reverse transcription and quantitative real-time PCR

Some primers used in our assay (Table 1 & 2) for q-RT-PCR were designed using Primer3 (v.0.4.0) and Primer-BLAST (National Center for Biotechnology Information, NCBI). First strand cDNA was synthesized with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, Wilmington, DE, USA) according to manufacturer's instructions. Gene expression was measured by quantitative real-time PCR (Power SYBR Green, PCR Master Mix, Thermo Fisher Scientific, Wilmington, DE, USA) using StepOne™ Software v.2.1 (Applied Biosystem, Foster City, Ca, USA) for C_T calculation. Reactions were setup in a 10 µl final reaction volume with 5 µM of each primer, 2.5 µL cDNA and 5 µL of 2× SYBR

Green PCR Master Mix. Standard curves were prepared from serial dilutions of pooled gill cDNA and included on each plate. Cycling parameters were as follows: 2 min at 95 °C, 40 cycles of 95 °C for 10 s followed by 60 °C for 30 s and a final cycle of 95 °C for 15 s followed by 60 °C for 1 min and 95 °C for 15 s. Primers with high yield (90% < R < 110%; R = $((10^{(-1/slope)}-1)*100)$) and efficiency (1.9 < E < 2.1; E = $(10^{(-slope)}-1)$) were selected. A melting curve was generated to confirm product specificity. Geometric mean of two reference genes *ef1α* and 40S Ribosomal protein *s20* was used to normalize target genes after verification that levels did not vary across groups ($p > 0.05$ ANOVA).

2.6 Statistical analysis

All statistical analyses were performed with R 3.3.3 and Jmp 12. Data was tested for normality of distribution with Shapiro-Wilk W-test and homogeneity of variance by Levene f-test. NKA activity and hormone measurements were then analysed by two-way ANOVA using date and treatment as factors. Gene expression data was also analysed by two-way ANOVA. Where normality tests failed, data was transformed (log or Box-Cox). Normality of distribution could not be found for *cftr1* despite several transformation attemps (log, Box-Cox, square-root…). Homogeneity of variance was respected for *cftr1* and data was analysed by ANOVA as it is robust to non-normal distribution. Statistically significant differences were accepted at $p<0.05$. All data are given as means ± standard error of the mean (SEM).

Table 1: Genes classified in functional groups.

Hydro-mineral & acid-base balance	Ammonia transport	Stress indicators	Hormonal regulation	Reference genes
nka1αa	*rhbg*	*hsp70*	*igf1*	*efiα*
nka1αb	*rhcg1*	*lyz*	*igf2*	*s20*
nkcc1a	*rhcg2*		*igf1r*	
cftr1			*ghr1*	
cftr2			*gr1*	
nbc			*gr2*	
hatp6vb			*mr*	
			prlr	

Table 2: Genes, accession and forward and reverse primer sequences.

Gene	Accession	Forward/Reverse primer sequences
nkaα1a[a]	**CK878443**	CCAGGATCACTCAATGTCACTCT/ GCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA
nkaα1b[a]	**CK879688**	GCTACATCTCAACCAACAACATT/ TGCAGCTGAGTGCACCAT ACAC
hatp6vb	**NM_001124597**	GATGGGATGAACAGCATTGCT/ GGCCAGCGGCAGAAAAG
nkcc1a	**NM_001123683**	GATGATCTGCGGCCATGTTC/ TCTGGTCATTGGACAGCTCTTTG
cftr1[b]	**AF155237**	CCTTCTCCAATATGGTTGAAGAGGCAAG/ GAGGCACTTGGATGAGTCAGCAG
cftr2[b]	**AF161070**	GCCTTATTTCTTCTATTTGTATGCACTT/ GCCACCATGAAAAACTAAAGAGTACCTCAG
nbc[c]	**AAN52239**	TGGACCTGTTCTGGGTAGCAA/ AGCACTGGGTCTCCATCTTCAG
rhbg[d]	**EF051113**	CGACAACGACTTTTACTACCGC/ GACGAAGCCCTGCATGAGAG
rhcg1[d]	**DQ431244**	CATCCTCAGCCTCATACATGC/ TGAATGACAGACGGAGCCAATC
rhcg2	**EF051115**	CAACATCACCAGCGACATAGA/ CGAAGGAAGCAATGAGGAAG
hsp70[e]	**BG933934**	CCCCTGTCCCTGGGTATTG/ CACCAGGCTGGTTGTCTGAGT
lyz[f]	**AF179305**	GACATCAACATGGGAGTTGGAG/ CCCACTGGTGTCAACCTTTGT
igf1[g]	**NM_001123623**	GATGTCTTCAAGAGTGCGATGTG/ CGCCGAAGTCAGGGTTAGG
igf2	**NM_001123647**	TGCCCACACTCAAACAGG/ CTTCCTCTGCCACACCTCA
igf1r[h]	**AY049954**	AGCCACCTGAGGTCACTACG/ CTCCCCAGCCATCTGAATAA
ghr1[h]	**AY462105**	TCCCAACATGCAGCTGTAGA/ TGTGGCACCTTGAAGAACAG
gr1[i]	**AF209873**	ACGACGATGGAGCCGAAC/ ATGGCTTTGAGCAGGGATAG
gr2[j]	**AY495372**	TGGTGGGCTGCTGGATTTCTGC/ CTCCCTGTCTCCCTCTGTCA
mr	**XM_014209388**	CATCAAGAATGAACCCGACA/ ACGGACCTGACTGGAAGAGA
prlr[i]	**AF229197**	CTCGAGTCCAAGAGCCAGTC/ CCACACTTCTCCATCAGCAA
efiα[b]	**AF321836**	AGAACCATTGAGAAGTTCGAGAAG/ GCACCCAGGCATACTTGAAAG
s20[k]	**BG936672**	GCAGACCTTATCCGTGGAGCTA/ TGGTGATGCGCAGAGTCTTG

[a] Stefansson *et al.,* 2007 ; [b] Kiilerich *et al.,* 2007a ;[c] Perry *et al.,* 2003; [d] Nawata *et al.,* 2007; [e] Olsvic *et al.,* 2013 ; [f] Yada *et al.,* 2012 ; [g] Metzger *et al.,* 2013 ; [h] Tipsmark and Madsen, 2009; [i] Kiilerich *et al.,* 2007b; [j] Kiilerich *et al.,* 2011; [k] Olsvic *et al.,* 2005

3. Results

3.1 Physiological parameters

Two-way interaction Date*Treatment strongly influenced gill NKA activity ($p = 2.2E^{-5}$; Figure 2). Fish from the control treatment exhibit moderately high NKA activity (5.7 µmol ADP*mg prot.$^{-1}$*h^{-1}) in early May which is climbing mid-May to 7.5 µmol ADP* mg prot.$^{-1}$*h^{-1} ($p = 1.8E^{-2}$). Decreasing activity ($p = 9.7E^{-3}$) was measured on June 1 (6.3 µmol ADP*mg prot.$^{-1}$*h^{-1}) and continued decreasing to the lowest activity registered mid-June (4.4 µmol ADP*mg prot.$^{-1}$*h^{-1}). On the first sampling after the temperature increase in the early treatment group, NKA activity is lower than under control conditions ($p = 5.7E^{-3}$). NKA

activity remains low until the end of the experiment with levels down to 3 µmol ADP*mg prot.$^{-1}$*h^{-1}. On the first sampling date, smolts from the late treatment group have not been treated yet and NKA activity is as elevated as in smolts from the control group. After the late treatment, NKA activity dwindles ($p = 5.5E^{-3}$) and remains low until the end of the experiment.

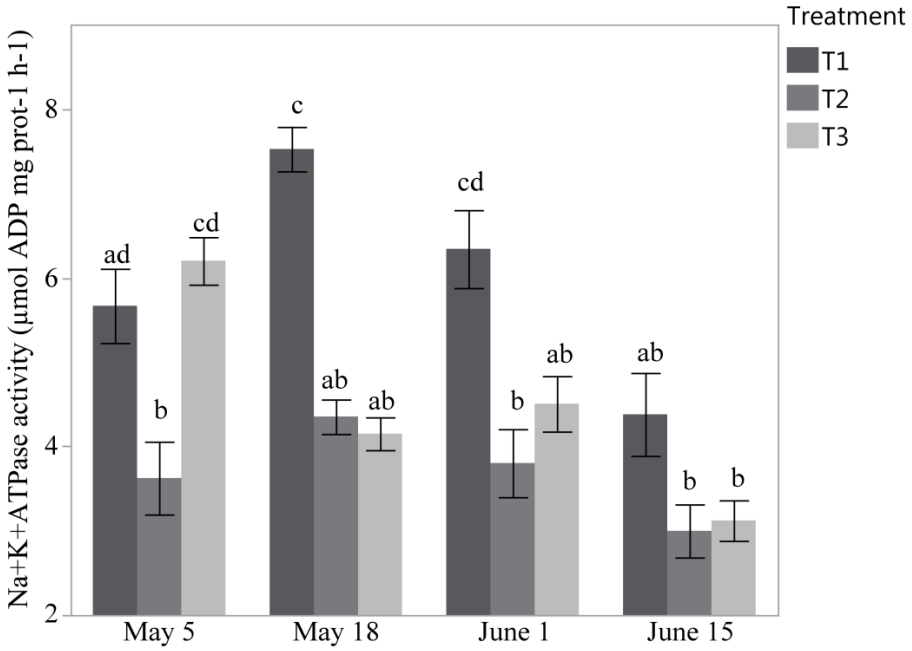

Figure 2: Mean gill Na^+/K^+-ATPase activity (µmol ADP*mg prot.$^{-1}$*h^{-1}) in smolts from the control (T1), early (T2) and late (T3) treatment groups at sampling dates. N = 6 fish per date per treatment. Letters represent statistical difference at $p < 0.05$.

Two-way interaction Date*Treatment strongly influenced circulating levels of GH ($p = 6.1E^{-6}$) and cortisol ($p = 1.9E^{-6}$). GH profile (Figure 3A) under control conditions (T1) shows an increase from early to mid-May ($p = 1.9E^{-4}$) followed by a decrease in early June ($p = 1.6E^{-5}$) and a secondary rise mid-June ($p = 5.6E^{-4}$). Smolts having experienced a temperature shift prior to the first sampling date exhibit low circulating levels (2.3 - 3.3 ng*mL^{-1}) of GH across the whole experiment. Before the temperature increase in the late treatment group (T3), GH plasma levels are not different from the other groups. On the sampling date after the late thermal treatment (May 18), GH levels reached intermediary values (5.4 ng*mL^{-1}) between control smolts (7.2 ng*mL^{-1}) and early treatment (2.3 ng*mL^{-1}) smolts. On the last sampling date, GH levels are similar in both treated groups (T2 and T3).

On the first sampling date, three days after the early treatment, no differences in cortisol level were seen between the groups (Figure 3B). Plasma cortisol level then decreases ($p = 6.6E^{-3}$) and remains low in T2 smolts until the end of the experiment. Similarly, on the sampling date

after the late treatment, cortisol levels are not different in control and late treatment smolts with values over 40 ng*mL^{-1}. However, on the third sampling date levels sharply decreased ($p = 8.0E^{-7}$). A less steep decrease ($p = 7.5E^{-4}$) was measured in smolts from the control group. On the last sampling, no differences in cortisol levels were measured between the groups.

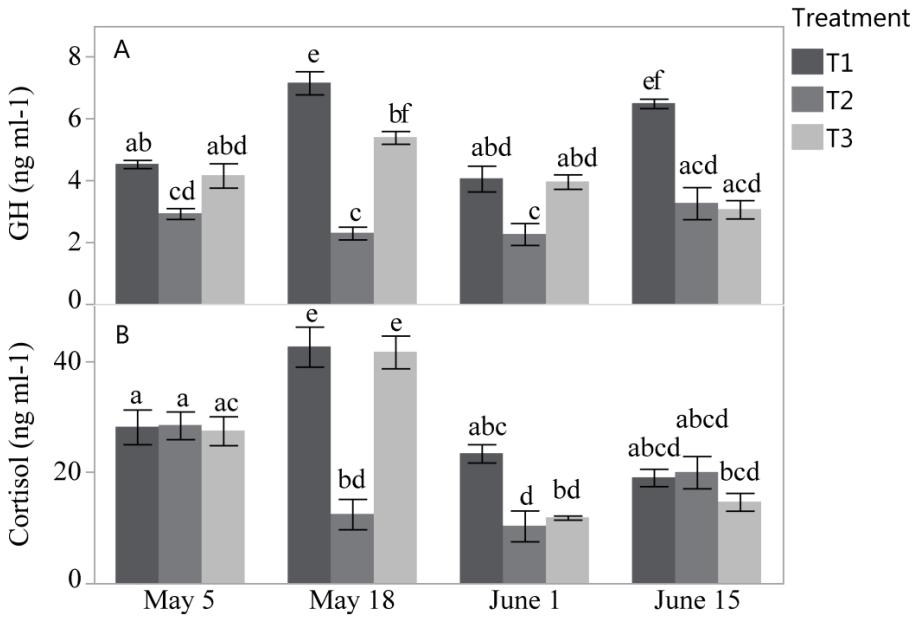

Figure 3: Mean plasma growth hormone (A) and cortisol (B) level in smolts from the control (T1), early (T2) and late (T3) treatment groups at sampling dates. N = 6 fish per date per treatment. Letters represent statistical difference at p < 0.05.

3.2 Genes differentially expressed by q-RT-PCR

The expression pattern of 14 genes out of 20 varied over the sampling period (Figure 4) including all the functional groups with the exception of the 'ammonia transport' group. The transcription of 11 genes was influenced by the date (*gr1*, *ghr1*, *mr*, *prlr*, *igf1r*, *igf1*, *igf2*, *cftr2*, *nkaα1b*, *nbc* and *hsp70*) and 6 by the temperature treatment (*igf1r*, *igf2*, *nkaα1b*, *nkcc1a*, *hatp6vb* and *lyz*). No two-way interaction Date*Treatment was found to influence the expression of genes. For the gene expression influenced by the date, most differences were seen between the second (May 18) and the third sampling (June 1) with 6 genes differentially expressed. Transcription of 5 out of 8 genes from the 'hormonal regulation' group was modified between these sampling dates. mRNA abundance of *cftr2* and *nkaα1b* (Figure 5) varied between the two first sampling dates (May 5 and May 18). The expression of *nbc* changed between May 18 and June 15. mRNA abundance of all the genes from the 'ammonia

transport' group was stable over the study. Higher expression of *hsp70* was measured on the last two sampling dates.

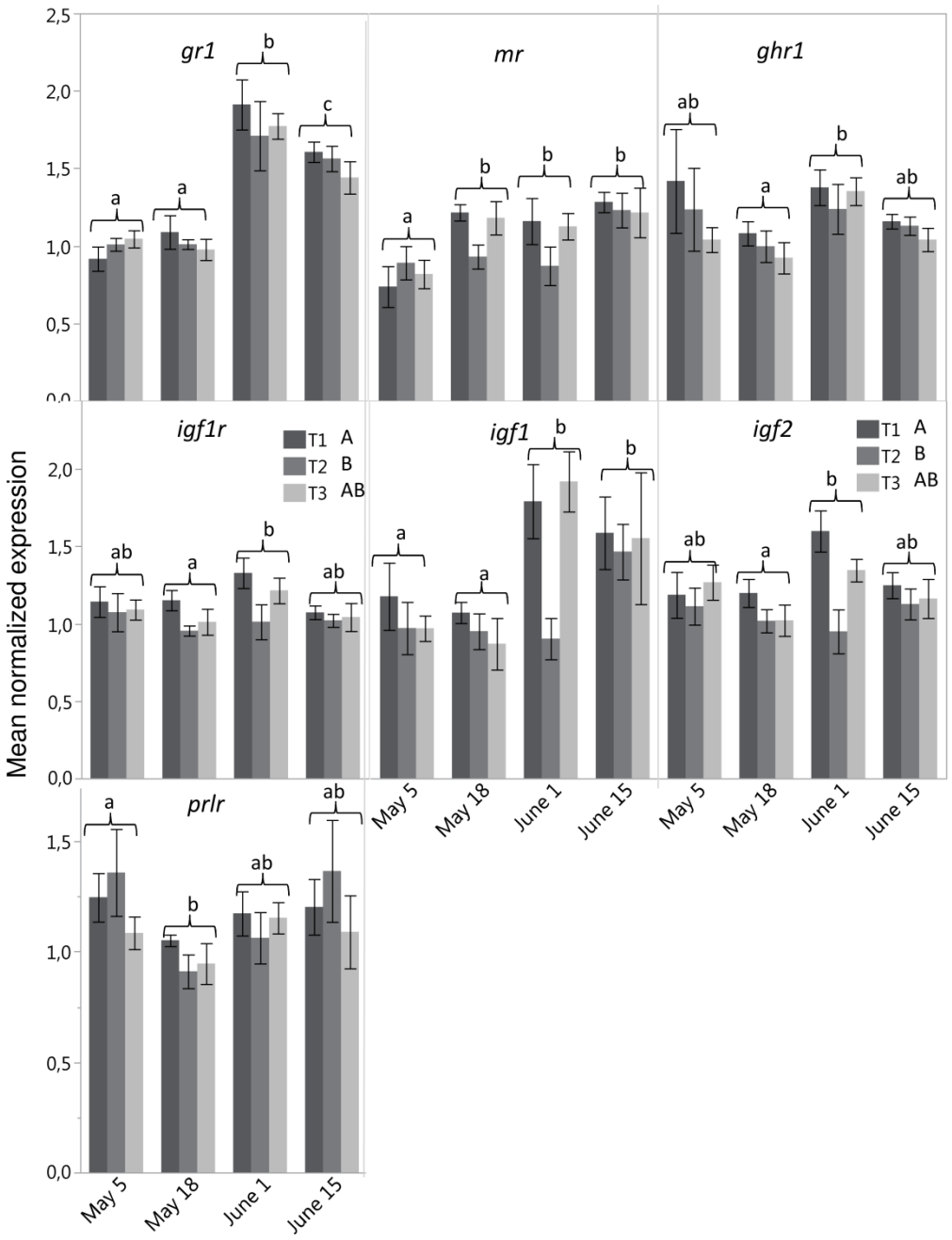

Figure 4: Genes differentially expressed between the sampling dates and temperature treatment. T1: control treatment, T2: early treatment, T3: late treatment. Capital letters represent significant ($p < 0.05$) differences between treatments; lower-case letters represent significant ($p < 0.05$) differences between the dates.

For the gene expression influenced by the treatment, all the identified genes were expressed differently between the control (T1) and the early treatment (T2) groups. The strongest effect was seen in *nkaα1b*, *nkcc1a*, and *lyz*. The expression of *hatp6vb* was also differentially

modulated between the control (T1) and the late treatment groups (T3). Transcription of *lyz* was also different between the early (T2) and late (T3) treatment groups. Expression in the late treatment (T3) group for the other genes was not different from that in the control (T1) or early treatment (T2) groups. For all the identified genes, transcription was always downregulated under conditions with a temperature increase except for *lyz*.

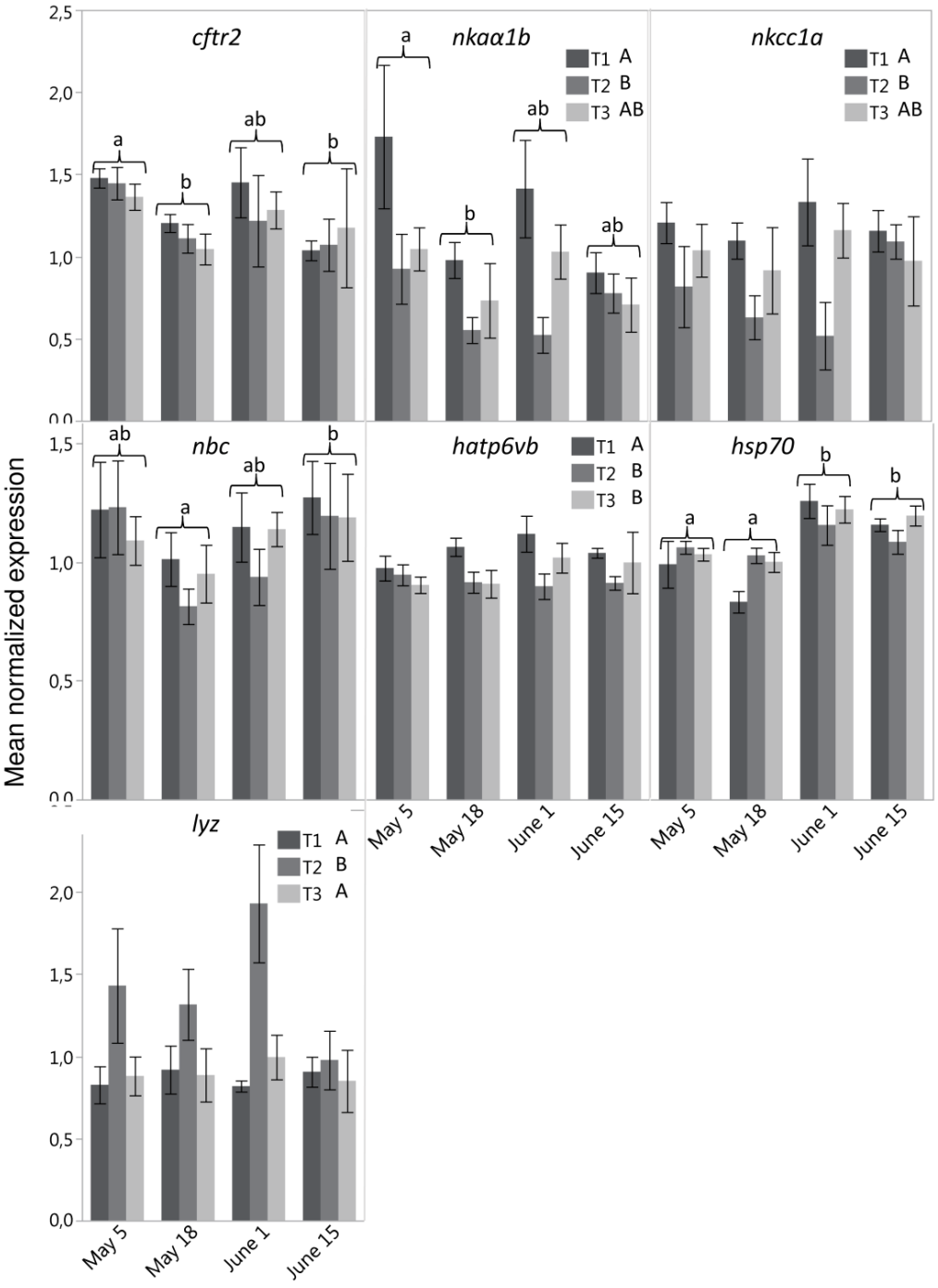

Figure 5: Genes differentially expressed between the sampling dates and temperature treatment. T1: control treatment, T2: early treatment, T3: late treatment. Capital letters represent significant ($p < 0.05$) differences between treatments; lower-case letters represent significant ($p < 0.05$) differences between the dates.

4. Discussion

Temperature is a crucial factor in smoltification (Handeland *et al.,* 2004; Jonsson and Jonsson, 2011; McCormick, 2013) and a rapid temperature increase mimicking temperature conditions between a tributary and the main river negatively impacted hypo-osmoregulatory capacities of smolts (Bernard *et al.,* in prep). Therefore, we investigated the effect of such a temperature increase on endocrine (cortisol and GH) and osmoregulatory (NKA activity) parameters and on the expression on a selection of genes in smolt gills. In our experiment, control smolts (T1) successfully smoltified as evidenced by Na^+/K^+-ATPase activity peaking mid-May with values consistent with literature and decreasing to typical parr level at the end of the study (Breves *et al*., 2017; Zydlewski *et al.,* 2010).

Hormonal profiles concur with that observation; plasma cortisol and GH levels reached maximum levels on the same date as peak NKA activity, then decrease in early June. However, in the early (T2) and late (T3) treatment groups, NKA activity and circulating levels of GH sharply decreased on the sampling date following the temperature increase. This indicates a rapid impairment of the smoltification process. Cortisol level reacted slower as a decrease was only visible on the second sampling date following the temperature increase. Delayed changes in cortisol level may point towards a response to stressful environmental conditions as evidenced by high expression of *lyz,* another stress indicator, in the early treatment group. Heat shock proteins are an important part of the cell protein folding mechanisms (Borges & Ramos, 2005) and play a crucial role in response to different stressors (Morano, 2007; Santoro, 2000) including lethal heat shock, anoxia, heavy metals and oxidative stress (Feige *et al.,* 1996; Oksala *et al.,* 2014; Parsell and Lindquist, 1996). Higher expression of *hsp70* as a response to increased temperature was expected but no differences were seen between our treatment groups. A 5°C increase over 3 days may not trigger a stress response requiring more *hsp70*. High RNA levels of *hsp70* in June may be explained by the temperature being out of the species thermal preferendum or at least for that strain. In June, temperature reaches above 20°C in the early and late treatment groups and negative effects of temperature above 18°C on swimming speed and activity of smolts from the same strain have been reported (Martin *et al.,* 2012). Heat shock proteins react to a variety of stressors (Morano, 2007; Santoro, 2000) and while temperature in the control group reaches only 16°C in June, desmolting may be a stressful process.

Temperature influenced genes in all the functional groups investigated in our study, with the exception of the 'ammonia transport' group, showing an overall important effect of temperature during smolting. All the genes differentially expressed in response to a

temperature increase were measured between the control group (T1) and the early treatment group (T2). Comparatively, only one (*hatp6vb*) was differentially expressed between the control and late treatment (T3) groups showing a stronger influence of temperature on the smolting process early in the season and a higher sensitivity of these genes to temperature.

Higher abundance and activity of specific isozymes of ion transport proteins as a preadaptation to SW have been reported (Hiroi *et al.,* 2005; McCormick *et al.,* 2009; Tipsmark *et al.,* 2002). Smolts from the early treatment group exhibit decreased NKA activity and reduced *nkaα1b* and *nkcc1a* expression. Decreased expression of ion transport proteins after a temperature increase is likely to contribute to the explanation of reduced hypo-osmoregulatory capacity observed in smolts after a temperature increase (Bernard *et al*., in prep). *nkaα1b* is coding for the seawater isoform of the NKA and was downregulated on May 18 but in the control group, highest NKA activity was measured on this date. Decreased RNA level but unaffected NKA activity has been reported previously (Seidelin *et al.,* 2001). The link between transcript and protein activity or abundance is indirect as further evidenced by decreasing *nkcc1* transcript levels along the migration route while NKCC protein abundance did not change (Stefansson *et al.,* 2012). Decreased expression of *cftr2* in smolts compared to parr (Robertson and McCormick, 2012a) coincide with lowest *cftr2* expression measured at smolting peak (May 18) in our study. Intermediary *cftr2* expression in June suggests desmoltification. Differences between transcript level and protein abundance or activity have been previously discussed but further investigation could clarify the role of CFTR2 in desmoltification. No differences in expression were seen for *cftr1* in our study despite a two-fold increase reported in smolts compared to parr (Robertson and McCormick, 2012a). However, salmon juveniles were already smolts on the first sampling date (May 5) which may explain the lack of change.

Little is known about acid-base equilibrium mechanisms during smolting and this topic may benefit from future research. To our knowledge, this is the first study looking at $Na^+/HCO3^-$ cotransporter (*nbc*) during smoltification of Atlantic salmon. *nbc* transcription is influenced by the date and decreased expression at smolting peak (May 18) suggesting a role in SW acclimation. It may function in efflux or influx mode and roles for systemic acid-base or intracellular pH regulation have been proposed (Perry *et al.,* 2003). V-type H^+-ATPase subunit B expression was reported to increase in the early phase of smoltification, probably to compensate the effect of increased ionic efflux while in freshwater (Seidelin *et al.,* 2001). At smolting peak, transcript levels were low to favour the complete transformation of gill into a hypo-osmoregulatory organ (Seidelin *et al.,* 2001). During desmoltification, fish readapt to

freshwater and ionic efflux should be reduced, which is in accordance with low levels of *hatp6vb* in smolts from the early and late treatment groups. Under normal physiological conditions, over 80% of whole-body ammonia is excreted at the gills (Smith, 1929; Zimmer *et al.,* 2014). In teleosts, several key transporters (Rh proteins, V-type H^+-ATPase, Na^+/H^+exchanger) play a role in excreting ammonia and at the same time, favouring active Na^+ uptake (Nakada *et al.,* 2007; Nawata *et al.,* 2007). There is evidence that Rh glycoproteins transport NH_3, NH_4^+ and may also function as CO_2 transporters (Weinar and Verlander, 2010) and contribute to systemic ionic and acid–base homeostasis (Wright and Wood, 2009). Ammonia excretion increases during smolting and was correlated with decreasing condition factor due to increased activity and decreased after meal size was increased (Wiggs *et al.,* 1989). We did not record activity but swimming activity in our strain was shown to decrease strongly above 18°C (Martin *et al.,* 2012). On June 1, water is already above 20°C in the early and late treatment groups and smolts from the control group have passed the smolting peak. Reduced activity and sufficient feeding during the study could explain stable transcript levels of *rhbg*, *rhcg1* and *rhcg2*. Further, differences may have been seen if sampling dates started earlier in the smoltification period. Ammonia-excretion mechanisms and rates during smolting still lack complete understanding and would benefit from future research as it is linked to osmotic balance.

Considering the important role of the endocrine system in mediating changes associated to smoltification (Jonsson and Jonsson, 2011; McCormick *et al.,* 1998; McCormick, 2013), it was anticipated to see changes in plasma levels and transcription of several genes. Hormonal regulation of the smoltification seems to be impaired by a temperature shift with decreased *igf1r* and *igf2* RNA levels in smolts from the early treatment group and intermediary values for *igf2* in the late treatment group. Smoltification-linked increase in salinity tolerance and gill NKA activity was shown to be partially supported by increases in plasma levels and local production of IGF1 (McCormick, 2001). In addition to decreased transcription seen in our study, lower circulating levels of IGF1 after a temperature shift have been measured (Bernard *et al.,* in prep). There is scarce information about the role of IGF2 during smoltification (Breves *et al.,* 2017) and in teleost in general (Hevrøy *et al.,* 2007). There is evidence of a role as an anabolic stimulatory agent (Hevrøy *et al.,* 2007) and it's been suggested to modulate local paracrine/autocrine regulation of tissue growth in teleosts (Wood *et al.,* 2005) or to be involved in a 'transcriptional program underlying enhanced paracrine signalling in response to ionoregulatory demands' (Breves *et al.,* 2017). Increased transcription of *mr* has been measured during acclimation of Rainbow trout from SW to FW (Kiilerich *et al.,* 2011).

Cortisol plays a dual role in osmoregulation, favouring ion uptake in freshwater and excretion in saltwater (McCormick, 2013; Tipsmark and Madsen, 2009).An increase in GR1 and GR2 expression was also noticed (Kiilerich *et al.,* 2011). Increases in *mr* and *gr1* transcript levels observed late in the smolting season suggest that it is part of the process of desmoltification and acclimation to FW. Smolting peak was defined on May 18 and increased transcription of *gr1* was seen from June 1 on. *mr* transcription already increased on May 18 but we could have failed to identify accurately the smoltification peak as no samplings were done between May 5 and May 18. We could also hypothesize an organ specific response as *mr* increased in the intestine for FW to SW acclimation and remained stable from SW to FW but increased in both cases in the kidneys (Kiilerich *et al.,* 2011). *prlr* expression was downregulated on May 18 corresponding with peak NKA activity, GH and cortisol plasma levels. This is consistent with the inhibitory role of prolactin in smoltification and salt secretion differentiation of ionocytes (Björnsson *et al.,* 2011; Tipsmark and Madsen, 2009). Increased expression of *igf1r, igf1* and *igf2* on June 1 may indicate a role of IGFs in desmoltification as it is two weeks after the smoltification peak. In addition, upregulated expression of *ghr1* on June 1 may indicate an implication of GH in desmoltification too. The role of GH is somewhat ambiguous (Björnsson *et al.,* 2011) as it seems to favour differentiation towards SW-type ionocytes (Tipsmark and Madsen, 2009) but secondary increases in late June have been reported (Ágústsson *et al.,* 2001). A second increase in plasma GH level mid-June was also observed in our study. This points towards a complex regulation of desmoltification with several mediators playing a role in both smolting and desmolting. Previously unknown interactions or mediators may be involved as the role in smolting of certain IGF binding proteins was only recently reported (Breves *et al.,* 2017).

5. Conclusion

A rapid temperature increase during smoltification greatly impacts the gill transcriptional pattern and other physiological parameters in smolts and consequently probably reduces survival chances of smolts at sea-entry. Data suggests dual roles in the smoltification and desmoltification process for GH and IGF1 and points to the implication of genes, previously unstudied (*nbc*) or with little data available (*igf2*), in the smoltification process. This study gives further insights on the molecular processes underlying smoltification and desmoltification in Atlantic salmon and possible responses to human-related water temperature increase.

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In the previous chapters, we investigated the effect of a rapid temperature increase on smolts (endocrine and enzymatic indicators, gene expression…). We reported about potential deleterious effects which could negatively influence smolt survival chances. These studies were performed under simulated natural conditions in a wet lab. While hatchery and laboratory structures offer convenient study possibilities where it is possible to mimic some environmental factors (e.g. temperature, photoperiod), they lack of structural complexity and numerous secondary factors (e.g. diurnal light variation) (Björnsson *et al.,* 2011). Effective smoltification occurs under simulated conditions (Björnsson *et al*., 2011) but there is also clear evidence of differences between hatchery-reared and wild smolts (McCormick *et al.,* 2003). Our simulations for the previous experiments are based on environmental data (temperature and phtoperiod), but as it is impossible to simulate all the environmental cues influencing smoltification, corroborating laboratory results with field studies may be of valuable interest especially in the light of restocking actions and costs. In this chapter we will present the results from three years of field sampling of smolts at two sites where the temperature data used in our simulations was measured.

4.5 Temperature differences between rivers influence Atlantic salmon (*Salmo salar* L.) smolts physiology during downstream migration.

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Abstract

Temperature is a crucial factor for smoltification and migration (timing, survival). Climate change and anthropogenic use of water system may result in a temperature gap between tributaries and main channel. There is a growing body of evidence showing differences between fish under hatchery conditions and natural conditions. We intended to study the effect of a rapid temperature increase on the physiology of migrating smolts in the River Meuse basin, Belgium. Results would then be compared with those of a study inquiring the same question under simulated natural conditions. Migrating fish were sampled in a tributary and in the main channel between which a 3.5-4 °C difference is regularly measured in spring. Early migrants were almost not affected by an increased temperature, for the duration of the study at least. Late migrants were the most affected and displayed reduced Na^+/K^+-ATPase activity (3.7 µmol ADP*mg^{-1} of protein*h^{-1}) and circulating GH levels (5.5 ng*mL^{-1}). Results suggest a complex role of some endocrine features (GH and IGF-1) in the desmoltification process. Differences emerged from the comparison of results under natural and simulated natural conditions, notably in Na^+/K^+-ATPase activity rate (maximum 10.7µmol ADP*mg^{-1} of protein*h^{-1} vs 8µmol ADP*mg^{-1} of protein*h^{-1}) and plasma GH level (maximum 18.1 ng*mL^{-1} vs 10 ng*mL^{-1}), both higher in wild smolts. Our results tend to indicate a deleterious effect of human-linked temperature increase on migrating salmon. Differences between results under simulated natural conditions and natural conditions suggest the necessity to confirm laboratory findings under natural conditions.

Keywords: smoltification, temperature, Na^+/K^+-ATPase, wild

1. Introduction

Smolting is the biological process making it possible for salmonid juveniles to switch from freshwater to seawater as well as accomplishing the migration between both environments (McCormick *et al.*, 1998; Jonsson and Jonsson, 2011; McCormick, 2013). Water temperature influences the smolting process in many ways; impacting hypo-osmoregulatory capacities development (Handeland *et al.*, 2004; 2014), limiting the influence of photoperiod (McCormick *et al.*, 2000), or even advancing smoltification for several weeks (McCormick *et al.*, 1996). Water temperature also influences the smolt downstream migration timing and termination (Zydlewski *et al.*, 2005; Kennedy and Crozier, 2010; Otero *et al.*, 2014) and modulates swimming speed and activity (Martin *et al.*, 2012). Climate change and anthropogenic use of water systems cause an increase in water temperature (Kirchmann, 1985; IPCC, 2014). Heavily modified rivers, through anthropogenic use (industrial waste water, hot water from thermal plants, dams…), may ultimately lead to a temperature gap between the main channel and their tributaries. It was hypothesised that temperature may negatively impact the salmon life-cycle through swift anthropogenic-linked increase in temperature (Martin *et al.*, 2012). A clear decrease in hypo-osmoregulatory capacities of smolts was reported under simulated natural conditions with a 5°C temperature increase (Bernard *et al.*, submitted). While hatchery and laboratory structures offer convenient study possibilities where it is possible to mimic some environmental factors (e.g. temperature, photoperiod), they lack of structural complexity and numerous secondary factors (e.g. diurnal light variation) (Björnsson *et al.*, 2011). Effective smoltification happens under simulated conditions, though (Björnsson *et al.*, 2011). But, there is clear evidence of differences between hatchery-reared and wild smolts (McCormick *et al.*, 2003). As it is impossible to simulate all the environmental cues influencing smoltification, corroborating laboratory results with field studies may be of valuable interest especially in the light of restocking actions and costs.

Under natural conditions, the effects of a temperature shift on the smoltification process and the success of downstream migration of salmon smolts are largely unknown. Based on local field data, the present study aimed to assess the physiological response of smolts to the temperature difference between a river and one of its tributaries on the seaward migration way. Hormonal, osmotic and enzymatic smoltification indicators were measured to monitor the response. Results will then be compared to those of a sister study performed under controlled conditions in a laboratory (see Chapter 4.2).

2. Material and method

2.1 Fish rearing

In Belgium, salmon has been extinct since the 1940's. A strain, originating from the 'Conservatoire National du Saumon Sauvage de Chanteuges" on the Loire-Allier water system (France), is used for restocking. Both rivers are similar in length (>900km) and temperature, regularly exceeding 20°C during smolt migration in May (http://aqualim.environnement.wallonie.be/, http://aquaphyc.environnement.wallonie.be/, Martin *et al.,* 2012). Fertilised eggs (F1) from recaptured wild spawners (F0) were directly imported to and reared at the CoSMos (Conservatoire du Saumon Mosan) hatchery in Erezée (Public Services of Wallonia, Fisheries Service), along the River Aisne, a historical salmon river. Rearing conditions were as follows: simulated natural photoperiod based on Liège latitudes (50°37'59"N), River Aisne temperature and daily feeding with a fixed ration (5 % of fish biomass after yolk sac resorption, 3 % for fry and 1 % for parr) provided with automatic feeders along the day. Once they reached the 0+ parr stage, they were then restocked in the River Ourthe, a tributary of the larger River Meuse. Annual restockings were carried out and samplings were done on migrating fish the following springs. For details about the fish rearing for the experiment under simulated conditions, see Chapter 4.2.

2.2 Sampling

In 2013, a trap, installed for migration monitoring in Méry on the River Ourthe as part of a restocking program, was checked twice a week for fish sampling. A second trap, in Lixhe on the River Meuse, was checked on the same dates. The latter was built in the bypass system of the hydropower plant in Lixhe on the border with the Netherlands (Prignon and Micha, 1998). However, this trap proved inefficient due to large debris blocking the way every day. The trap was unusable for the following years because of turbine servicing. We therefor switched to an alternative method. A fish ladder is located next to the hydropower plant with a large 2 m in diameter circular tank with central evacuation directly supplied with Meuse water. In 2014 (Figure 1), in addition to the sampling in Méry, 20 smolts were transferred to the tank in Lixhe and into a 1 m^3 cage in the stream in Méry as a control for transport, handling and captive condition. Transfer was done by road, in a 100 L transport tank with continuous aeration in water obtained at the site. Fish were transferred three times over the migration period to investigate differences between early, mid-time and late migrants. Fish were intended to be sampled three and seven days after the transfer. The period of three days corresponds to the time smolts need to cover the distance between the two sampling points

using an average speed calculated on field data of smolt migration monitoring between two Belgian rivers (Ovidio *et al.*, 2016). High mortality after the first transfer only permitted a single sampling three days after transfer. A flooding event in early May turned the control cage in Méry upside down releasing the fish into the stream. The transfer to the cage and to the tank was renewed for a single sampling three days later. In 2015, unfavourable river conditions did not allow a sufficient number of fish to be regularly captured in Méry to transfer fish to Lixhe. Number of fish used each year in the experiment is given in Table 6.

Table 6: Number of fish used for our experiment per year and location

	Méry	Méry Cage	Lixhe
2013	84	0	0
2014	70	80	80
2015	35	0	0

Table 7: Temperature (°C) on transfer dates (bold letters) and sampling dates.

	April 14	April 17	**April 28**	May 2	May 5	**May 9**	May 12
Lixhe	16,2	15,1	17,6	17,8	17,8	17,6	16
Méry	11,6	11,4	14,9	14,7	14,5	14	12
Difference	4,6	3,7	2,7	3,1	3,3	3,6	4

Sampling was as follow, seven fish were quickly dip-netted out of the trap and directly anaesthetised with 120 mg*L^{-1} of tricaine methanesulfonate (MS-222, Sigma-Aldrich). Blood was collected into 1 mL heparinized syringes from the caudal vasculature. The needle was removed and the blood was expelled into a 1.5 mL Eppendorf, stored on ice for less than 30 min and then centrifuged at 3000 g for 10 min. The supernatant was then subdivided and kept on ice. Left- and right-sided first branchial arches were cut out and immediately frozen in liquid nitrogen. Samples were then stored at -80 °C until subsequent analyses. The sampling procedure in the experiment under simulated conditions is described in Chapter 4.2.

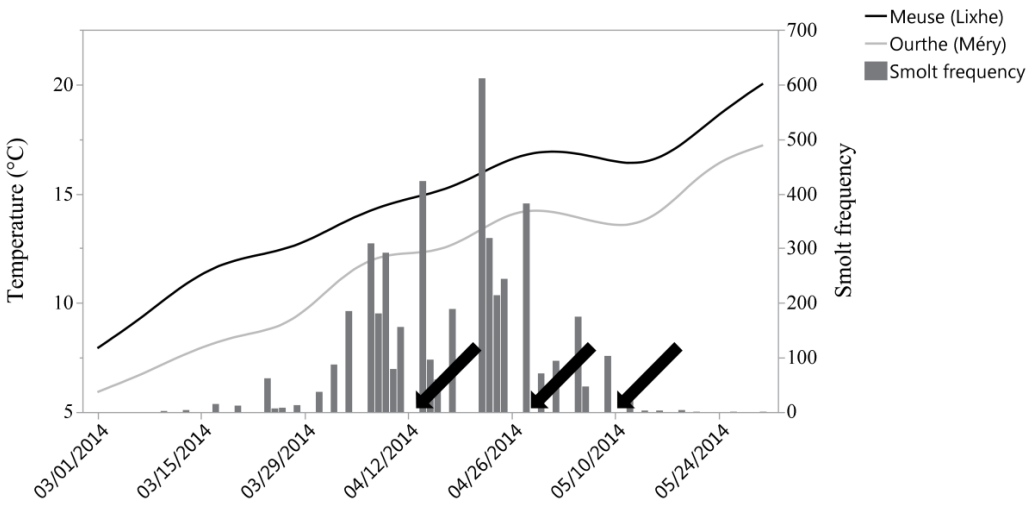

Figure 1: Temperature curves in Méry and Lixhe and smolts capture frequency in spring 2014. Arrows indicate transfer dates.

2.3 Na^+/K^+ATPase

Gill Na^+/K^+ATPase (NKA) activity was measured according to the method described by McCormick (1993). NKA activity was determined with a kinetic assay linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), in presence and absence of the Na^+/K^+-ATPase specific inhibitor ouabain. Samples were run in duplicates and the assay was run on an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, USA) with use of the included MARS data analysis software (BMG LABTECH GmbH, Germany).

Total protein concentration of the gill homogenate was measured in duplicate using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. This assay was run on a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany) with use of MARS software. NKA specific activity is expressed as micromoles of ADP per milligram of protein per hour (µmol ADP*mg^{-1} of protein*h^{-1}).

2.4 Plasma ion concentration and osmolality

Plasma sodium and potassium concentrations were measured using a Philipps PU 9200 atomic absorption spectrophotometer (Pye Unicam, Cambridge, United Kingdom) with 0.25-, 0.5-, 0.75- and 1 ppm external standards for sodium and 0.5-, 1-, 2- and 3 ppm external standards for potassium. Plasma osmolality was measured using a Löser Type 6 freezing point depression osmometer (Löser Messtechnik, Germany) with 0-, 300- and 900-milliosmole external standards.

2.5 Hormone assay

Hormone assays were performed using Fish GH (CSB-E12121Fh) and Fish IGF-1 (CSB-E12122Fh) ELISA kits (Cusabio, P.R. China) as per the manufacturer's instructions. These kits were validated for use with Atlantic salmon by a test of parallelism on serial diluted samples. Detection lower limits were 312.5 pg*mL^{-1} (intra-assay variation coefficient <15 %), 25 pg*mL^{-1} (intra-assay variation coefficient <15 %) respectively. These assays were run on a FLUOstar Omega microplate reader with use of MARS software. All samples were measured in duplicate within a single assay.

2.6 Statistical analysis

All statistical analyses were performed with R packages, version 3.3.3 and jmp 12. The homogeneity of variances was tested using Levene's F-test and normality was tested using a Shapiro-Wilk W-test (Zar, 1996). For all variables in accordance with the requirements for parametric tests (Zar, 1996), data was analysed using a two-way analysis of variance (ANOVA) with date and location (Méry, Méry Cage and Lixhe) as factors. In case of significant ANOVAs, we performed a Tukey's HSD test for unequal n (Zar, 1996). As temperature conditions vary between field and laboratory conditions, degree*days were used as a common variable. In case of non-normality of distribution, and ineffective log-transformation, data was analysed by a Kruskal-Wallis ANOVA by ranks (Zar, 1996). Statistical significance was accepted at $p<0.05$. All data are given as means ± standard error of the mean (SEM).

3. Results

3.1 NKA activity

Two-way interaction Date-Location ($p = 8.7E^{-5}$) influenced NKA activity (Figure 2A). In early migrants, activity increased in smolts three days after transfer (Lixhe vs Mery $p = 3.6E^{-2}$ and Lixhe vs Mery Cage $p = 2.1E^{-2}$). Mid-time migrants don't seem to be affected by a temperature increase and late migrants displayed decreased NKA activity three days post transfer (Lixhe vs Mery $p = 2.3E^{-2}$ and Lixhe vs Mery Cage $p = 4.7E^{-3}$). Measured activity ranged from 3.7 up to 10.7 µmol ADP*mg $prot^{-1}$*h^{-1}. Prolonged exposure to warmer water influenced NKA activity ($p = 2.3E^{-2}$) with reduced values 7 days post-transfer (Figure 3A). We also noticed a trend of Date-Location interaction ($p = 6.7E^{-2}$) which could influence NKA activity.

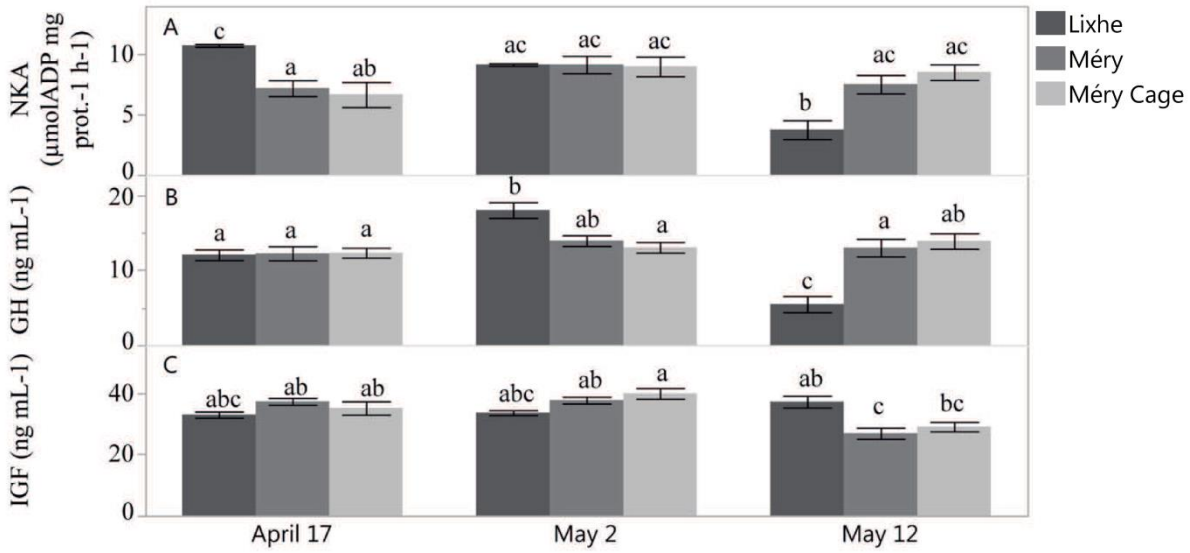

Figure 2: NKA activity (A), plasma GH (B) and IGF-1 (C) levels 3days after transfers in Méry, Méry Cage and Lixhe. Letters indicate a difference ($p < 0.05$) between dates and locations.

3.2 Hormones

Two-way interaction Date-Location ($p = 1.5E^{-6}$) strongly influenced plasma GH levels (Figure 2B). In Lixhe, plasma level rose ($p = 5.3E^{-3}$) between the first and second transfer (April 17 and May 2) and then decreased on the third one (May 12; $p < 1E^{-8}$). No effect of a temperature increase on circulating GH level was seen in early migrants. Higher levels ($p = 2.9E^{-2}$) in Lixhe than in Méry Cage were noticed in mid-time migrants 3 days after transfer. A temperature increase also influenced ($p = 1.6E^{-3}$) GH levels 7 days post transfer (Figure 3B; Lixhe vs Méry $p = 4.4E^{-3}$ and Lixhe vs Méry Cage $p = 3.2E^{-3}$). Late migrants displayed decreased GH levels (Figure 2B; Lixhe vs Méry $p = 9.4E^{-5}$ and Lixhe vs Méry Cage $p = 1.6E^{-5}$). GH levels ranged from 5.5 to 18.1 ng*mL^{-1}. Two-way interaction Date-Location ($p = 9E^{-4}$) influenced plasma IGF-1 levels (Figure 2C). Early and mid-time migrants do not seem to be affected by a temperature increase. In late migrants, while IGF-1 levels in smolts from Lixhe do not change compared to earlier samplings, levels have decreased in Méry ($p = 7.1E^{-3}$) and in Méry Cage ($p = 7.1E^{-3}$). Values ranged from 26.8 to 38.5 ng*mL^{-1}. One week exposure to warmer water doesn't seem to influence IGF-1 levels in mid-time migrants.

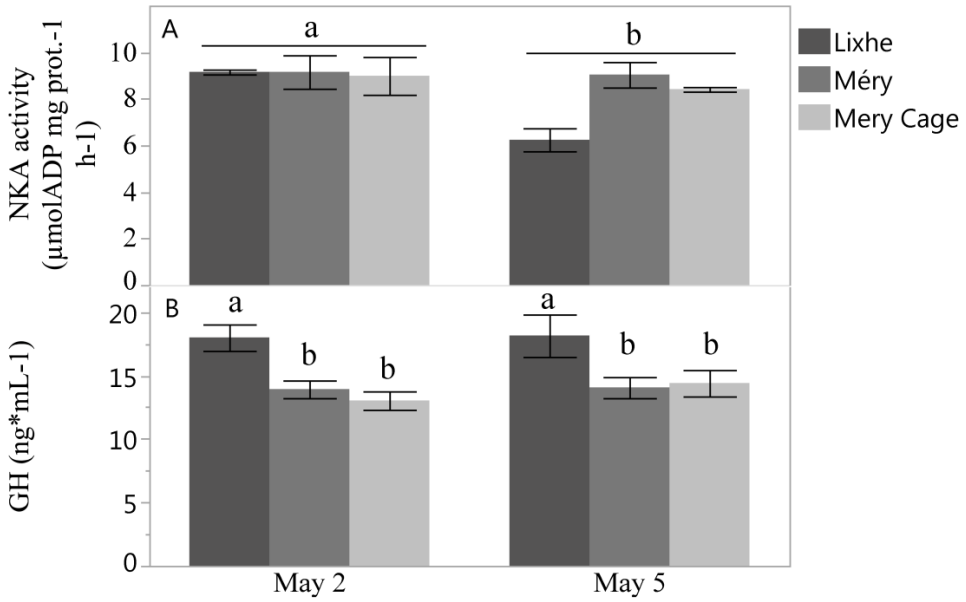

Figure 3: Gill NKA activity (A) 3 and 7 days after the second transfer and plasma GH levels (B) in the three locations after the second transfer. Letters indicate a difference ($p < 0.05$) between dates and locations.

3.3 Plasma osmolality and ions concentration

Using data from three days after transfer to warmer water (Figure 4A and B), we found that the date ($p = 7.4E^{-3}$) and location ($p = 2.6E^{-2}$) influenced sodium plasma levels. Highest sodium levels were measured after the second transfer on May 2 (April 17 vs May 2 $p = 1.1E^{-2}$ and May 2 vs May 12 $p = 2.1E^{-2}$). Sodium levels were the highest in Méry (Figure 4B) and lowest in Méry Cage ($p = 3.9E^{-2}$). Levels vary from 203 to 243 mM. Location also influenced plasma osmolality ($p= 1.8E^{-2}$) with decreased osmolality in Lixhe compared to Méry ($p = 1.4E^{-2}$; Figure 4C). Three days after any transfer, plasma potassium level did not vary. In mid-time smolts (Figure 5A), data from three and seven days post transfer showed an influence of the location on Na^+ level (Figure 5B; $p = 5.4E^{-3}$) with lower levels in Lixhe (Méry vs Lixhe $p = 3.9E^{-3}$ and Méry Cage vs Lixhe $p = 3.4E^{-2}$). Concentration ranges from 212 to 237 mM. Plasma osmolality (Figure 5C) was also influenced by the location (Figure 5C; $p = 4.1E^{-3}$). Osmolality ranges from 302 to 340 mOsm*kg $H2O^{-1}$ with lowest values in Lixhe (Lixhe vs Méry p = 3.4E-3 and Lixhe vs Méry Cage $p = 3.8E^{-2}$). Two-way interaction Date-Location influenced K^+ levels with increased values in Lixhe on May 5 ($p = 5E^{-3}$). Levels ranged from 3.2 to 5.1 mM.

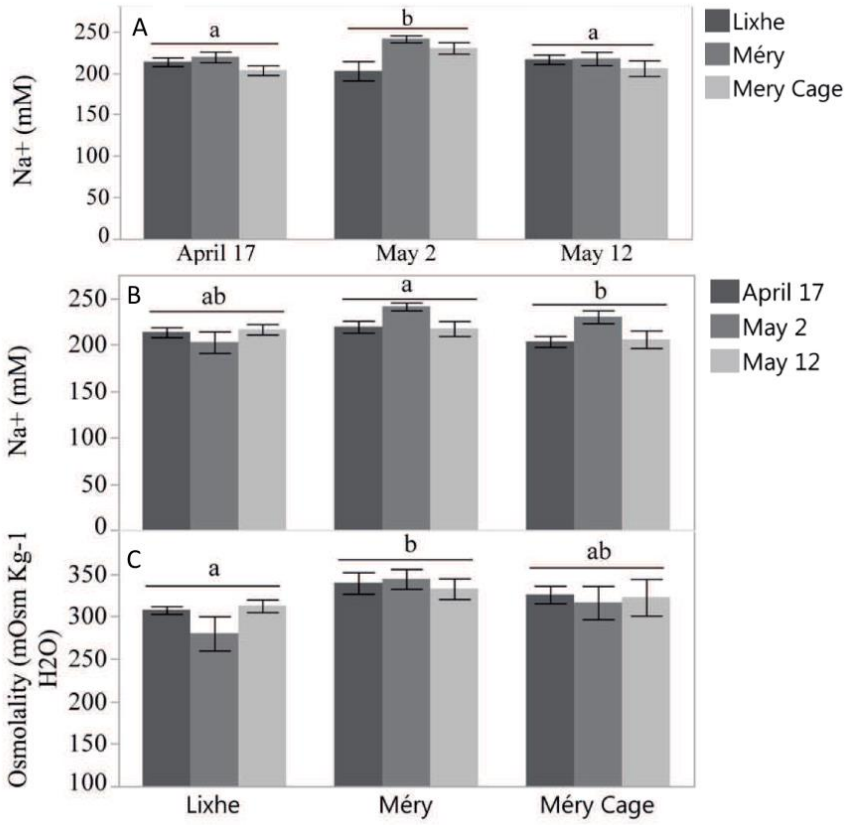

Figure 4: Plasma Na^+ (A) levels 3 days after transfers and plasma Na^+ (B) levels and plasma osmolality (C) in different sampling locations. Letters indicate difference between dates and locations ($p < 0.05$).

Comparison between simulated natural conditions and natural conditions

We compared results from samplings in Méry (natural conditions) with samplings in a laboratory based on field-data from Méry (simulated natural conditions) along the smolting period. Rearing conditions strongly influenced ($p < 1E^{-4}$) four out of six indicators (Figure 6A, C, D and F). Gill NKA activity, circulating levels of GH, Na^+ plasma level and plasma osmolality were higher in field samples. Circulating levels of IGF-1 and K^+ plasma level were not different between our conditions. NKA activity, IGF-1, GH, plasma sodium and osmolality are strongly influenced ($p < 1E^{-4}$) by degree*days (Figure 6A-E). Plasma potassium level (Figure 6F) is also influenced but to a lesser extent ($p = 2.5E^{-2}$).

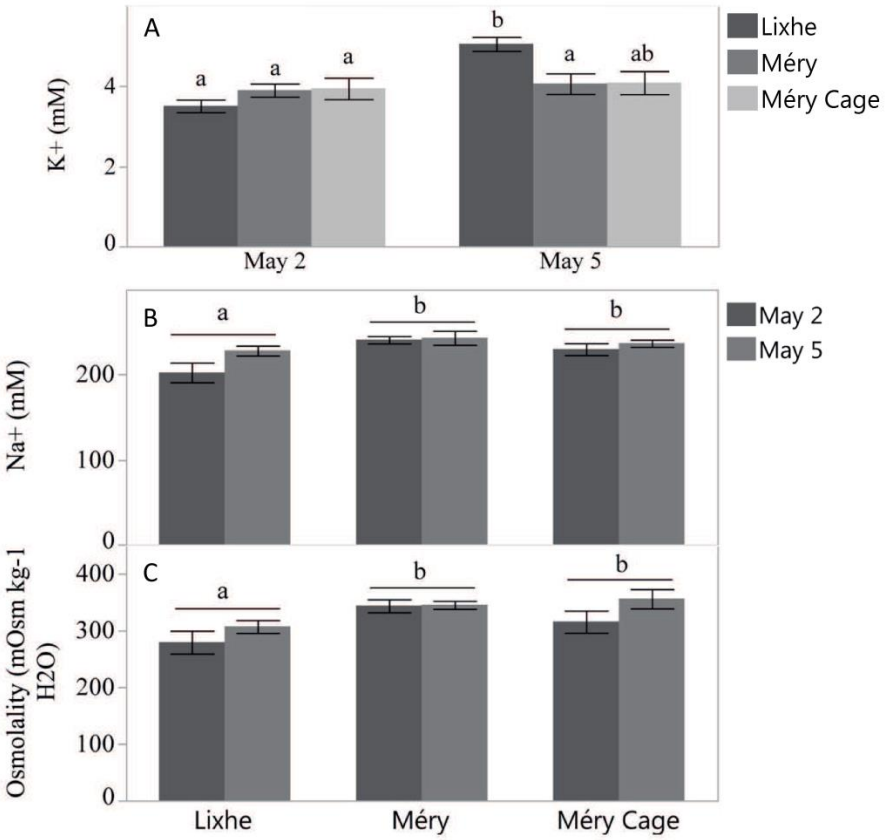

Figure 5: Comparison of plasma K^+ (A) three and seven days after transfer in warmer water and Na^+ (B) levels and plasma osmolality (C) in different locations. Letters indicate difference between dates and locations ($p < 0.05$).

4. Discussion

4.1 Field sampling

After three years of survey, 2014 was the only year when smolts could be successfully sampled before and after a temperature shift. Our three transfers from Méry to Lixhe covered the period of migration defined by field-monitoring (Figure 1). Early migrants don't seem to be affected by a temperature shift, at least not after three days and according to our indicators. The only influence registered was an increase in NKA activity in Lixhe after the first transfer. Increased temperature is known to hasten smolting (McCormick *et al.*, 1996; Handeland *et al.*, 2004; 2014) and may advance it even by several weeks (McCormick *et al.*, 1996). As increasing NKA activity is an acknowledged smolting indicator (McCormick *et al.*, 1998, Jonsson and Jonsson, 2011, McCormick, 2013), temperature seem to play a role in speeding up smolting as predicted. It was hypothesized that a number of accumulated thermal units or degree*days were necessary to acquire salinity tolerance but exceeding a certain amount, it also diminishes hypo-osmoregulatory capacities (Handeland *et al.*, 2004). This could explain

the decreased activity 7 days after the second transfer and 3 days after the third transfer; late migrants have been submitted to a higher number of degree*days and therefore less time in warmer water is needed to exceed the threshold.

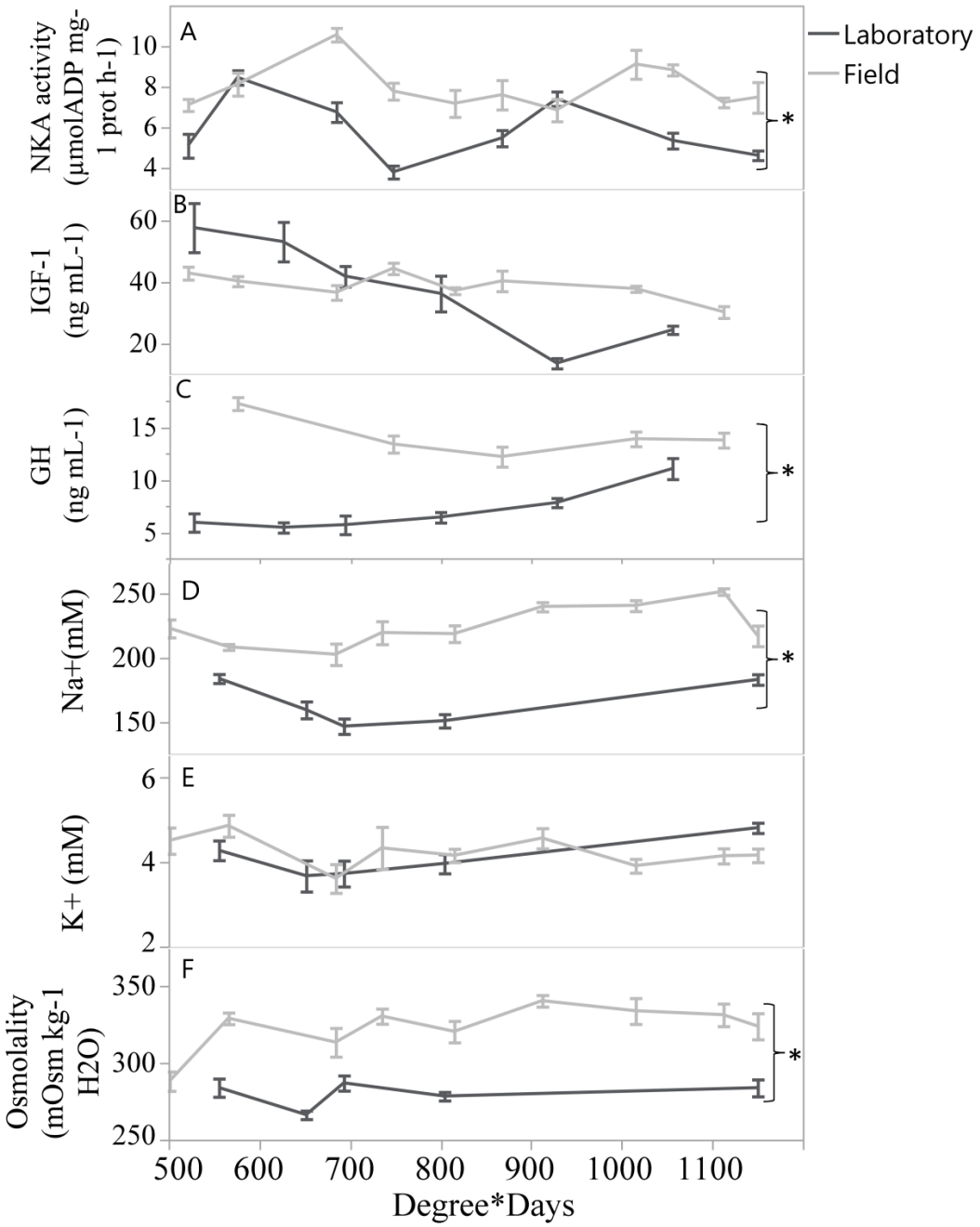

Figure 6: Comparison of laboratory and field results for NKA activity (A), plasma IGF-1 (B), GH (C), Na^+ (D), K^+ (E) and plasma osmolality (F). * is for a difference between laboratory and field results ($p<0.05$).

While an amount of temperature is also needed to initiate downstream migration, again, a higher amount will favour migration termination (Zydlewski *et al.*, 2005). Furthermore, high temperature (> 17 °C) caused decreased swimming speed and even positive rheotaxis above a

threshold temperature (> 20 °C) in the same strain as we used (Martin *et al.,* 2012). Smolts from the third transfer reacted with decreased NKA activity already 3 days after transfer while an effect on NKA activity was only measured 7 days after the second transfer. Temperature (Table 7) in Lixhe on the transfer dates (April 28 and May 9) was the same and it even decreased on the last sampling date (May 12). This would advocate in favor of the degree*days hypothesis (Stefansson *et al.,* 1998; McCormick *et al.,* 1999) rather than a threshold temperature. However, temperature in our experiment did not rise above 20°C which might be a threshold temperature (Martin *et al*., 2012). Further, the maximum difference between Méry and Lixhe was 4.4 °C which might not be enough to trigger a response. Whether accumulated thermal units or threshold temperature causes decreased NKA activity (and thus the start of desmoltification) or migration issues (Handeland *et al.,* 2004; Zydlewski *et al.,* 2005; Martin *et al.,* 2012) our results seem consistent with literature and tend to point toward a negative effect of thermal conditions in our river system.

Salinity tolerance is under the positive control of cortisol, GH and IGF-1 (McCormick 2001). Cortisol and GH may increase NKA transcription, abundance and activity (McCormick, 2001; Tipmark and Madsen, 2009; Kiilerich *et al.*, 2007; McCormick *et al.,* 2008). GH and cortisol independently upregulate the number of NKA and together act additively or synergistically (Madsen, 1990; Pelis and McCormick, 2001). Increased NKA activity in early migrants after a temperature shift may be primarily due to increased cortisol, maybe as a response to thermal stress which would explain increased NKA activity despite the lack of response of GH and IGF-1. Cortisol wasn't measured in our assay as fish where manipulated and kept in a cage or tank and thus it may reflect the response to many stressors. After the second transfer, GH levels where higher in Lixhe 3 and 7 days post transfer but longer exposure to warmer temperature resulted in decreased NKA activity (7 days post transfer). Late migrants exhibit decreased levels of GH and NKA activity suggesting that desmoltification had started. IGF-1 plays an important role in smolting (McCormick, 2001; 2013) and a decrease could point to the upcoming end of smoltification. Decreased levels were expected in Lixhe in late migrants; however, higher levels were measured. This suggests a complex role of IGF-1, influencing both smoltification and desmoltification. Few indications of the role of IGF-1 in desmoltification exist and the role of the endocrine system in desmoltification is still a misunderstood process (Björnsson *et al.,* 2011). Given inter-individual differences in desmoltification pace, some authors even suggested that desmoltification in Atlantic salmon is not a synchronised process, while smolting is (Stefansson *et al.,* 1998). Considering that NKA activity did not rise after the second transfer compared to the first, elevated levels of GH 3

and 7 days after the transfer could also be associated with desmoltification. Secondary GH increases in June have been measured; suggesting GH favours smoltification and plays a role in desmolting (Ágústsson *et al.,* 2001; Bernard *et al.,* submitted). Increased transcription of IGF-1 receptor and GH receptor in the gill after the smolting peak suggests complex regulation of desmoltification yet to be understood (Bernard *et al.,* submitted). Previously unknown interactions or mediators may be involved as the role in smolting of certain IGF binding proteins was only recently reported (Breves *et al.,* 2017).

Temperature effect on plasma ions and osmolality seem dependent on the duration of exposure to warmer water. We didn't read any change in K^+ after 3 days and no clear pattern was seen in sodium and osmolality responses until we analysed measurements 7 days post transfer. In Lixhe, lower Na^+ level and osmolality have been measured. This change may reflect changes in the homeostatic mechanisms of smolts with increased capacity for sodium extrusion in Lixhe. This is consistent with our results of NKA activity showing higher activity in Lixhe than Méry at the same date. However, it is more likely than this loss of ion is related to stressful conditions for the fish (McDonald and Milligan, 1992; Carey and McCormick, 1998).

4.2 Natural versus simulated conditions

Important differences emerged from the comparison between results obtained under natural and simulated natural conditions. Higher NKA activity and circulating GH levels have already been measured in smolts in the wild compared to hatchery reared smolts (McCormick *et al.,* 2003). Levels of IGF-1 were not found to be different in our study while they were in literature (McCormick *et al.,* 2003). However, given stock-specific differences in migration timing (Stewart *et al.,* 2006; Birnie-Gauvin *et al.,* 2018) and salinity tolerance development (Handeland *et al.,* 2004), variations in endocrinology may also exist and IGF-1 was not always found to increase during smolting (Nilsen *et al.,* 2008). Differences in daily temperature fluctuation, changes in weather conditions, illumination intensity, lunar cycle,… exist between hatchery and natural rearing conditions (McCormick *et al.,* 1989). Hatchery-reared fish do smoltify (Björnsson *et al.,* 2011) but variations in response may arise from such condition differences. Over the recent years, a temperature difference of 5 °C has been registered several times between the two rivers in our study and such a difference was applied under simulated conditions with minimum daily variations. However, an average temperature difference of 3.5-4 °C was measured between the two rivers during the study period in 2014 with nictemeral and day-to-day differences of over 2 °C. Highly impaired hypo-osmoregulatory capacities under simulated natural conditions while early migrant seems to

cope well under natural conditions may be explained by such complexity differences. As temperature differences were seen on a same date between our rearing conditions, we decided to compare results at equal degree*days as it was shown to be a good indicator for salinity tolerance development (Handeland *et al.,* 2004). Variation in all our indicators with increasing degree*days reflect the ongoing smoltification process across the study period.

Handling and keeping fish caged could have influenced our results. Descaling was often seen on fish kept in a metallic cage and experimental descaling was shown to impair smoltification (Zydlewski *et al.,* 2010). However, with the exception of Na^+ levels 3 days after the second transfer, no differences were seen between Méry and Méry Cage giving further confidence that measured differences between the two rivers resulted from the increased temperature.

5. Conclusion

Field results tend to confirm that an increase in temperature occurring during downstream migration influences the physiological status of smolts under natural conditions and suggest a deleterious effect of human-linked temperature increase on migrating salmon. However, given the differences (e.g. circulating GH and NKA activity) that emerged from the comparison of field and laboratory results, they also point out the necessity to validate laboratory findings in the field and if possible over a large timespan. This is especially true with organisms with a complex life-cycle like migrating salmonids as only a limited amount of conditions can be simulated in a laboratory.

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5 General Discussion

5.1 Experimental conditions and results

In this work, we carried out three experiments in a wet laboratory. Simulated conditions in these experiments were based on the same data set. However, differences in the results emerged between the experiments. Cortisol plasma levels were markedly higher (60 – 120 ng*mL^{-1}) in the first experiment than in the second (40 - 70 ng*mL^{-1}) and third one (10 - 40 ng*mL^{-1}). Levels in the first experiment tend to indicate a stress condition instead of basal levels. A plausible explanation for this difference lies in the timing of sampling. In the first experiment, samplings were performed at dawn while they were postponed by 1h30 to 2h after dawn in the other experiments. By examining the daily endocrine profile in smolts, Ebbesson *et al*. (2008) found that cortisol levels peaked at dusk and dawn with levels up to 145 ng*mL^{-1}. In addition, when the control unit turned on the lights, the transition from night to day is not as smooth as in natural dawn conditions and rapid change from dark to light could induce a stress for fish. However, with the exception of CG smolts in the first experiment, levels measured in our different experiments are in the range found in literature (35-80ng*ml^{-1} in Ebbesson *et al*., 2008; 20-60 ng*ml^{-1} in McCormick *et al*., 2000; 10-65 ng*ml^{-1} in Nilsen *et al*., 2008).

While NKA activity levels were similar in our three laboratory experiments (2 - 10.5 vs 1.5 - 8.5 vs 3 - 8 µmol ADP*mg prot^{-1}*h^{-1}), activity profile over the smoltification period differed. Only one increase was measured in our first experiment and no differences were seen between our strains while two increases were measured in the second experiment with peak activity on different dates for both strains. Sampling period and statistical analysis in the first experiment may have contributed to the observation of a single increase. Earlier sampling points could have shown low activity levels and modify the smoothing curve with two increases. Modification of the date of peak, i.e. May 5 (second experiment) and May 18 (third experiment) in the LA strain may be a consequence of the rearing facility. Each experiment has been performed in a different facility with different size and colour for the tanks. In addition, the temperature and light control units were upgraded between the first and second experiment and three newly built RAS were used in the third experiment. Technical specifications may have caused small differences in the daily temperature curves, flow rate, light managing,… which all put together may have influenced the results. Furthermore, for each experiment a new batch of smolt was reared which may also influence the timing of peak activity as there is evidence that smolt timing is at least partially hereditary (Clake *et al.,* 1992, 1994) and the spawners were not the same for each batch. In the third laboratory experiment,

sampling dates are separated by two weeks. We may then hypothesise that we failed to identify the correct date of smolting peak which may have been closer to May 5.

5.2 Strain selection and stocking management

In our study, we intended to compare the smoltification process between two allochtonous strains reared under the same conditions. In our first experiment, we reported some differences in cortisol and GH levels. Differences in GH plasma levels were seen in the beginning of the experiment but not at the smolting peak. High levels of cortisol have been measured over the whole sampling period and reasons for that have been discussed (see 5.1). High levels similar to those measured in LA smolts (50-70 $ng*mL^{-1}$) have been reported in literature (35-80$ng*ml^{-1}$ in Ebbesson *et al*., 2008; 20-60 $ng*ml^{-1}$ in McCormick *et al*., 2000; 10-65 $ng*ml^{-1}$ in Nilsen *et al*., 2008). The even higher levels found in Cong smolts (> 100 $ng*mL^{-1}$) are thought to be linked to more stressful conditions for that strain due to a higher temperature difference between local and origin rivers. Despite these differences, peak NKA activity was measured on the same date. In our second experiment, smolting peak based on NKA activity differed by one week between the two strains. Samplings were performed once a week and it may be possible that we failed to recognise the actual NKA activity peak. Reasons for differences between the experiments have also been discussed previously (see 5.1). Even if the two strains had close smolting peak dates, they might not be both similarly suited to local rivers. The overlapping of physiological smolt window and ecological smolt window is crucial (McCormick *et al*., 1998) but migration timing differences have been observed (Stewart *et al*., 2006). Smolts may then start their migration early or late and arrive at sea at an unfavourable timing. Furthermore, after a rapid increase in temperature, both strains reacted similarly with decreased capacity for hypo-osmoregulation (lower NKA activity and increased osmolality after a salinity test). Liver gene expression showed few differences between the strains, e.g. higher *igf1* expression in CG smolts. No differences were measured in circulating levels but the link between transcript and protein activity or abundance is indirect. Higher expression of *gst* and *gpx7* in April in LA smolts may indicate that smolts were experiencing oxidative stress as the cellular antioxidant defence systems are normally activated in case of an oxidative stress (Di Giulio and Meyer, 2008). However we did not measure any ROS in smolts and cannot confirm wether they were experiencing such a stress or not. Similarly, higher *c3* expression in LA smolts could be a reaction to an infection; UV treatment of the water should have decreased that risk to a minimum, though. However, higher *gst* and *gpx7* transcription may also suggest a stronger response capacity and thus a

better anti-oxidant defense that LA smolt have evolved to sustain prolonged efforts in potentially stressful environment during the migration over several hundreds of kilometer to reach the sea. Similarly, due to warmer water in spring, which may be more prone to disease development against which LA smolts have evolved a higher *c3* expression during smolting than the colder water adapted Cong smolts. Given the similarities between the Meuse and Loire-Allier in terms of migration distance and temperature, this might give an advantage to the LA smolts to complete their migration under Belgian conditions. We might have seen more differences if we could have tested both strains in the field.

From a larger perspective on strain selection, the neuroendocrine system is a key mediator of environmental and developmental information in most complex organisms (Scharrer and Scharrer 1963). Hormones are able to control and coordinate complex responses throughout the body. Notably, it's through the endocrine system that temperature and photoperiod influence physiological changes linked to smoltification and downstream migration (Björnsson and Bradley, 2007; McCormick, 2009). This makes hormones primary targets of selection and they are probably involved in a lot of evolutive adaptations (Gould, 1977; West-Eberhard 2003). Furthermore, the large numbers of genes that are involved in the sensory response capacity of the endocrine system display a lot of targets for evolution to adjust precisely developmental and environmental responses (McCormick, 2009). Populations may then have evolved local adaptations in the form of differences in hormonal secretion pattern, plasma level or migration timing. Such differences and gene expression differences under the same conditions between two strains as seen in our experiments tend to confirm this hypothesis but suggest complex hormonal interaction, yet to be understood. Other strain specific traits have been reported (Orciari and Leonard, 1996; Aarestrup *et al.*, 1999; Stewart *et al.,* 2006; Birnie-Gauvin *et al*., 2018). However, as hormones move throughout the body and control a variety of responses, selection for one trait may cause the promotion of another trait possibly reducing overall fitness i.e., a negative or antagonistic pleiotropy (McCormick, 2009). Two hypothesizes were examined by Hau (2007) to work out whether hormones control responses in several tissues that are always linked (evolutionary constraint hypothesis), or alternatively can evolve tissue-specific responses (evolutionary potential hypothesis). Later, McCormick (2009) discussed this method with other hormones with broad regulatory capacities like cortisol. This, and other developmental constraints, may limit the capacity of selection to alter endocrine control of performance (McCormick 2009). In our experiments, absence of differences in some hormones between our two strains may then be explainable.

Restocking bears many difficulties and success depends on numerous factors, time, site and technique of release, strain, size and age of the fish, as well as rearing techniques (reviewed by Jonsson and Jonsson, 2009b). New management practices, like adaptive management, have been shown to be useful for population and habitats restoration or rehabilitation (Jonsson and Jonsson, 2009b). Such an approach attempts to deal with the uncertainties of working with natural systems, and helps to accommodate for unforeseen events and may therefore be a relevant tool to address the managing of freshwater barriers (Birnie-Gauvin *et al.,* 2017). However, important gaps in knowledge are an issue (Jonsson and Jonsson, 2009b). Furthermore, adaptive management may not be suitable in every case and even recently, virtually no results were found on that topic (Birnie-Gauvin *et al.,* 2017). A step-by-step guide for implementing such management on freshwater barriers was therefore proposed. To enhance results, successes and failures should be reported so that other may benefit from the experience (Birnie-Gauvin *et al.,* 2017). Effective management also requires the best understanding of influencing factors in order to take adequate measures (Lenders *et al.*, 2016). For example, recent discoveries showed that the salmon decline in Western Europe goes back to the early middle-age with the improvement of watermills and their geographic expansion (Lenders *et al.,* 2016) and did not start with the industrialisation during the 19th century.

Where wild populations are extant, data advices against enhancement with hatchery reared fish even from closely located catchments (McGinnity *et al.,* 2007). There is also clear evidence of differences between hatchery-reared and wild smolts in the endocrine and physiological development of smolts (McCormick *et al.,* 2003). Some have been reviewed (Jonsson and Jonsson, 2009b) and wild fish should be favoured above hatchery-reared fish. Wild Atlantic salmon parrs are more competitive to occupy shelter even outnumbered by four to one by hatchery-reared fish (Orpwood *et al.,* 2004). Experiments carried out in the River Imsa showed that the average survival rate of hatchery-reared smolts was only half that of wild fish (Jonsson *et al*., 1991) and hatchery-reared fish usually have lower rates than wild fish (Jonsson *et al.,* 2003). If wild fish can't be used and if suitable grounds for fry stocking are unavailable, good results may still be possible with later release. Substantial physiological smolt development was observed 10-20 days after release of hatchery-reared fish in the river or imprint ponds with almost identical changes than in fish released 2 years earlier in the river (McCormick *et al.,* 2003). Recently, three common stocking techniques have been tested in Denmark and resulted in half-year fish being most cost effective (Birnie-Gauvin *et al.*, 2018). Reducing rearing density was also shown to increase migration success after release (Larsen *et al.,* 2016). Environmental enrichment (physical structure and substrate) has been

successfully tested to increase survival chances of hatchery-reared fish (Näslund and Johnsson, 2016). Such 'trained' fish ought to be favoured for restocking (Arlinghaus, 2017). Location of restocking is important too. Survivorship was higher in smolts released at the river mouth (30%) compared with smolts released in the river (12%) (Thorstad *et al.,* 2012) and hatchery-reared smolts from an upper release site had lower detection rate compared to a lower release site (McCormick *et al.,* 2014). Further, smolt stocking at sea and in the fjord resulted in recovery rates of adult salmons which were up to three times better than those resulting from river stockings (Hvidsten and Mokkelgjerd, 1987). Early released hatchery-reared smolts were more likely to initiate migration than late released smolts; the latter moved quicker though (Nyqvist *et al.,* 2017). However, during migration within the Winoosky River (USA) and hydropower dams, hatchery and wild smolts performed similarly (Nyqvist *et al.,* 2017) showing the importance of free-migration. However, salmon is extinct in Belgium since several decades offering an opportunity to try various strains even from catchment with different environmental conditions without risking poor quality hybrids with wild fish (McGinnity *et al.,* 2007). Inherited migration traits for early timing of a strain or fish from upper populations in a catchment (Orciari and Leonard, 1996; Aarestrup *et al.*, 1999; Stewart *et al.,* 2006; Birnie-Gauvin *et al.,* 2018) could be used for restocking in Belgium. Even if they are submitted to a temperature shift, they would benefit from more time to reach the estuary before the number of degree*days surpasses the threshold initiating the desmoltification process (Handeland *et al.,* 2004; Zydlewski *et al.,* 2005).

Interesting data about desmoltification were obtained through samplings in mid-June in our third laboratory experiment. Increases in plasma cortisol, GH and IGF-1 are well described during the smoltification (McCormick, 2013) but the endocrine control of desmoltification has not been elucidated in details yet (Björnsson *et al*., 2011). An increase in plasma GH was measured in mid-June. Secondary increases in late June have been reported (Ágústsson *et al.,* 2001). Increases in RNA levels of *gr1*, *gh*r1, *igf1r*, *igf1* and *igf2* in the gills in early June suggest that these receptors and hormones also play a role in desmoltification. Cortisol plays a dual role in osmoregulation, favouring ion uptake in freshwater and excretion in saltwater (McCormick, 2013; Tipsmark and Madsen, 2009). No increase in circulating cortisol was measured in June but sensibility of receptors may play a role or we might have measured such an increase on a later sampling date. Hormones can have many different effects. The role of increased plasma levels of GH and IGF-1 in spring on osmoregulation has been extensively studied. Secondary increases in GH, *ghr1*, *igf1r* and *igf1* may be associated with osmoregulation or with their role in growth. In June, river temperature is favourable for the

development of food resources (e.g. macroinvertebrate) for fish and the increases that we observed may mark the initiation of a period of high growth rate.

Definite survey goals and precise methodology should then be clearly stated in advance. In Belgium, an ongoing salmon restoration program for 30 years generated a large data set but it lacks clear structure due to different studies, squads, laboratories and hand writing data logging. Professional use of biostatistics and bioinformatics on the whole data set may prove helpful and shed light on unnoticed influencing factors and give valuable advice for future data selection to log and upgrading the restocking policy. New tendencies and helpful insights in the physiology of the salmon may be gained with field study following smolts and post-smolts to the sea as well as actual survival rate calculation over the entire seaward migration. In the case of the Meuse basin spread across several borders, it would necessitate teaming up with neighbouring countries in a common large-scale international effort but this would most probably be a hard-fought battle. During our field study, we met numerous challenges which mainly resulted in setbacks. However, salmonid smolt migrations are monitored in many countries and studies have been published with results over large distances (>100km) or of post-smolts in the estuary or even in the open sea (Moore *et al.,* 2012; Stefansson *et al.,* 2012; McCormick *et al.,* 2014; Stich *et al.,* 2015; Birnie-Gauvin *et al.,* 2018) showing the potential realisation of such surveys.

5.3 Temperature effect

The reality of global climate change is now unequivocal, with many of the changes occurring at a rate or intensity unprecedented over decades to millennia (IPCC, 2014). Species and particularly aquatic species such as fish, are thus facing multiple modifications in their environment, and might migrate, adapt or become extinct. The intensity of changes and species specific adaptive capacities will greatly influence the fate of a species (Butt *et al.,* 2016; Ofori *et al.,* 2017). A physiological readiness requiring environmental cues (e.g. photoperiod, temperature) and endocrine factors precedes the onset of smolt migration which is then triggered by releasing factors (e.g. flow, temperature) (McCormick *et al.,* 1998; Jonsson and Jonsson, 2011; McCormick, 2013). In Atlantic salmon, photoperiod is the most decisive factor for the timing of smolt development as it indicates the time of year (McCormick, 2013). Temperature plays a role in pace of change and may limit the influence of photoperiod on this process (McCormick *et al.,* 2000; 2002) or limit the timeframe for salinity tolerance (Handeland *et al.,* 2004) and migration timing (Zydlewski *et al.,* 2005). In a context of climate change with modelled forthcoming increases in temperature (IPCC, 2014),

relative importance of the different factors will play a crucial role (Björnsson *et al.*, 2011). Temperature strongly influences the timespan of a 'physiological smolt window', during which smolts are capable of good survival after sea-entry, and an 'ecological smolt window' during which environmental conditions are favourable for smolt survival (McCormick *et al.,* 1998; McCormick *et al.,* 2009). In the case of temperature being the primary factor for migration initiation, smolts may react appropriately to increased temperature. Earlier migration in response to climate-change driven increased temperature have been reported (Otero *et al.,* 2014; Jokikokko *et al.,* 2016). However, if photoperiod and water flow are the primary releasing factors, smolts may only have a limited capacity for earlier migration leading to population extinction. Differences between populations are not to be excluded. Increased temperature may influence the occurrence of anadromy in some salmonid species to adapt to new conditions like it was modelled in Arctic char (Finstad and Hein, 2012). Traits for earlier migration may also become more frequent as an adaptation to warming sea-surface temperatures. First evidence therefor was reported recently in pink salmon after the monitoring of allozyme alleles, designed to differentiate early and late migrants, over 14 generations (Manhard *et al.,* 2017).

However, earlier migration may not guarantee survival. Matching completion of smolting with optimal survival conditions in fresh- and seawater is the ultimate biological role of the environmental cues governing the smoltification (McCormick *et al.,* 2000). If freshwater and coastal environment respond differently to increased temperature, there might arise a mismatch of the timing of migration that used to favour high survival of smolt between both environments (Björnsson *et al.,* 2011). These optimal conditions are not only related to season, so that the fish enter the ocean in early summer when near-coast food supplies have increased, but are also related to temperature. In the river, in spring, increased temperatures often signify increased water flow, which, in turn, makes downstream migration faster and diminishes predation risk in rivers and estuaries (Jonsson and Jonsson, 2011). Further, food availability usually increases with higher river temperatures (Jonsson and Jonsson, 2011). The decoupling of freshwater and marine habitats was modelled for the Australian grayling (*Prototroctes maraena G.),* with particular references to climate change and dam removal (Lin *et al.,* 2017). Decreased marine habitat suitability for larvae was predicted as a consequence of warming sea-water in the eastern and south-eastern distribution range of this species.

We set out to investigate the effect of a temperature increase based on environmental data on the smoltification in two strains. Our results indicate a negative effect of such a temperature

increase on hypo-osmoregulatory capacities with low NKA activity and increased plasma osmolality after a seawater challenge. Both strains were similarly influenced as well as early and late migrants. Transcription of osmoregulatory related genes in the gills are consistent with this observation (decreased *nkaα1b* and *nkcc1*). Only few genes involved in the endocrine control of smoltification were affected by a temperature increase. The transcription of *igf2* decreased in the liver and gill where lower *igf1r* RNA level were also measured. Transcription of *igf2* also changed during the sampling period in both organs. Very few information about its role during smoltification is known but our results suggest it is involved in the acquisition of hypo-osmoregulatory capacities. Expression changes in other genes (*nbc* in the gill and *taldo1* in the liver) with little information about their roles during smoltification propose them as primary target genes for future research. In addition, the impact of a temperature treatment on genes from the carbohydrate and lipid metabolism suggests that the capacity to sustain prolonged efforts to accomplish their migration is affected. Nevertheless, returning adult salmons are caught each year in the Meuse indicating that some smolts perform well enough to successfully complete their migration to the sea. Differences between our laboratory and the natural system may provide a partial explanation. Our simulated conditions lack the complexity of a natural system with day-to-day and nictemeral variations especially in terms of temperature. Smoltification did not seem to be impaired in early migrants during our field experiment which is consistent with the hypothesis of degree*days influencing the smoltification process (Stefansson *et al*., 1998; McCormick *et al*., 1999; Handeland *et al*., 2004). In our field experiment, we measured mean daily temperature differences of 3.5-4°C while in the wet laboratory a 5°C increase caused an impairment of smoltification in early migrants. This suggests that there might be a threshold temperature difference, that once crossed, causes an impairment of smoltification. We investigated the influence of a temperature increase between a tributary and a river but temperature differences were also measured between tributaries of a larger basin (Figure 38). Within a limited timeframe, migrating smolts will encounter multiple temperature shifts which may worsen migration conditions. In our laboratory experiment, temperature increases were completed within three days, corresponding to the time smolts need to cover the distance between the sampling points on the tributary and on the main channel using an average speed calculated on field data of smolt migration monitoring between two Belgian rivers (Ovidio *et al*., 2016). However, field surveys with radio-tagged smolts showed complex behaviour when entering the larger river with up and downstream movements (Ovidio *et al*., 2016). This suggests that the temperature increase may take more than three days in the wild. Temperature differences

may get amplified in the future with continuous anthropic activity and predicted temperature increase due to climate change (Björnsson *et al.*, 2011; IPCC, 2014). In case the difference of temperature was too high between tributary and river, we may hypothesise that fish would remain in the colder tributary, but such behaviour may cause excessive delay for sea arrival and further compromise smolt survival.

Figure 38: Mean daily temperature (2003-2015) in the River Meuse, the River Ourthe (a tributary from the previous) and the River Aisne (a tributary of the previous)

5.4 Migration issues

Temperature issues will be difficult to solve and climate change may accentuate difficulties for migrating salmonids and fish in general, especially with predicted temperature increase (IPCC, 2014). The physiological and ecological smolt windows (McCormick *et al.*, 1998) will be strongly influenced. In addition to climate change linked issues, delays due to dams and their cumulative effect will become an even more serious issue to remedy (Figure 39; McCormick *et al.*, 2009).

Effort to improve migration ways will then be of prime importance to limit negative effects of temperature on fish population and help them reach the estuary within their physiological window. In a study following the migration of radio-tagged smolts past three hydropower dams equipped with fishways, unusually high spill levels positively influenced passage rates. However, only 10% of the smolts successfully past the three dams, showing that simply building a fish bypass is by no means synonymous to providing fish passage (Nyqvist *et al.*, 2017). Furthermore, on the Penobscot River, the increasing number of past dams (from 2 to 9) reduced estuary survival by 40% (Stich, 2014). These results underline the necessity to take other remedial measures into consideration (Nyqvist *et al.*, 2017).

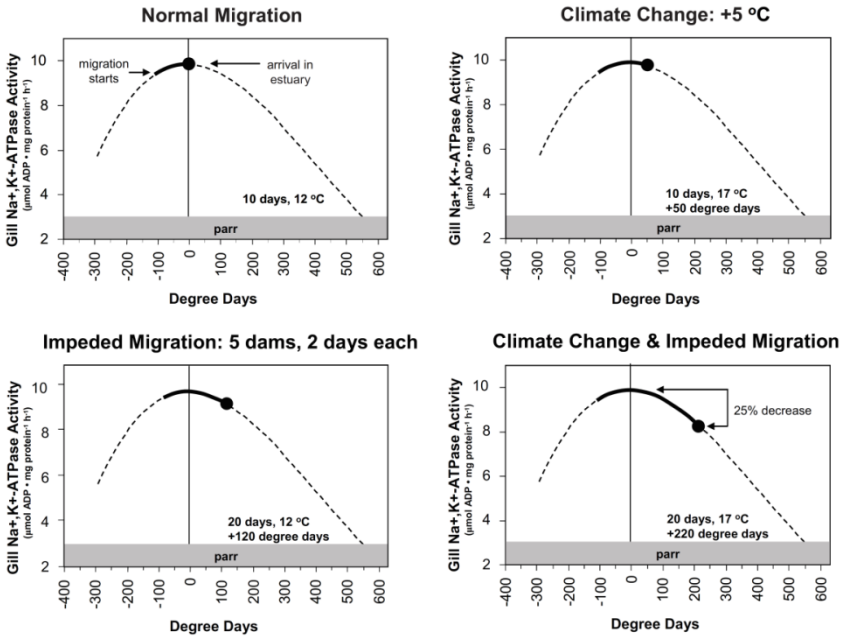

Figure 39: Migration profiles under normal conditions, a 5°C increase due to climate change, delays through dams and both effect cumulated on the timing of sea-arrival and gill Na^+/K^+ATPase activity (McCormick *et al.*, 2009).

Opening the migration way by totally removing obstacles (undamming) could benefit the salmon. After dam removal, improved spawning grounds as well as other habitats resulted in increased biodiversity and enhanced fish passages (Bednarek, 2001). Several examples (Table 8) may be found in the USA (Bednarek, 2001), notably on the Kennebec (Dadswell, 1996) or Penobscot Rivers (Stich, 2014) and in Canada.

Following their example, other countries like the bordering Grand-Duchy of Luxembourg have started to remove dams on their river as part of a recently launched initiative for salmon population restoration. Dam removal may be controversial because of contaminated sediment release if no pre-removal is carried out (Chatterjee, 1997). Nevertheless, it should be considered as an important alternative to river restoration (Bednarek, 2001). The Meuse is bound to commercial navigation and simply removing dams is impossible. However, in regard to the removing of large obstacles, the new management of the sluice gates of the anti-storm dams in the Meuse estuary (a.k.a. Haringvliet), which is hardly passable for returning adult salmon, will be established shortly. The Dutch government promised to open the gates almost totally from 2018 onwards, greatly facilitating fish upstream migration. Making the upstream migration easier to increase the number of spawners reaching Belgium is an important step, however, in order to increase return rates, it is important that as many smolts as possible reach the estuary during a timeframe in which they are capable of surviving the

change of environment (McCormick *et al.,* 1999). Decreased mortality (25%) was observed in fish with the highest gill NKA activity through the estuary compared to fish with lowest activity (Stich *et al.,* 2015). A similar observation was reported by McCormick *et al*. (2014). Trapping and truck transport may also be considered for juveniles and adults. On the River Klarälven, adult spawners were transported past eight hydropower plants (Hagelin *et al.,* 2016) and released upstream to resume their migration. Care should then be taken to match release with discharge as the probability of becoming fallbacks increased with high mean daily discharge.

Table 8: Example of dam removal and some ecological impact (Bednarek, 2001)

Location	Removal status	Ecological impact of removal addressed by study	Reference
Chipola River, Florida, USA	Removed December 1987	Improved fish passage; increased flow fluctuations; Number of fish species increased; Improved water quality	Hill and others 1993, Estes and others 1993
Kennebec River, Maine, USA	Removed July 1999	Sediment changes (improved spawning habitat); Improved fish passage	Dadswell 1996
Elwha River, Washington, USA	Not yet removed	Change in coastal sediment transport; Return of native species	DOI 1995
Similkameen River, Oregon, USA	Not yet removed	Improved fish passage	Winter 1990
Hudson River, New York, USA	Breached in 1973 (not intentionally removed)	Released PCBs	Shuman 1995, Chatterjee 1997
Yahara River, Wisconsin, USA	Removed 1993	Change in community composition; Loss of reservoir species	ASCE 1997, Born and others 1998
Clearwater River, Idaho, USA	Removed 1963	Improved sediment movement	Winter 1990
Clearwater River, Idaho, USA	Removed 1973	Improved sediment movement	Winter 1990
Snake River, USA	Not yet removed	Improve fish passage	Wik 1995
Muskegon River, Michigan, USA	Removed 1969	Sediment release	Simons and Simons 1991
Oklawaha River, Florida, USA	Not yet removed	Improved mammal and waterfowl habitat;	Kaufman 1992, Shuman 1995
AuSable River, Michigan, USA	Removed 1991	Temperature changes	Pawloski and Cook 1993
Pine River, Michigan, USA	Undergoing removal	Improved sediment and fish movement	American Rivers and others 1999
Mad River, California, USA	Removed 1969	Reservoir silted in; improved fish passage	Winter 1990
Milwaukee River, Wisconsin, USA	Removed May 1988	Sediment release; Improved organism movement	Nelson and Pajak 1990; Staggs and others 1995, Kanehl and others 1997
Clearwater River, Idaho, USA	Removed 1963	Improved fish passage and habitat for chinook salmon	Shuman 1995

6 General Conclusions and Perspectives

6.1 Conclusions

To sum up, the salmon disappeared in Belgium some 70 years ago. Since 1987, rebuilding a population in the Meuse Basin has been a priority for authorities and scientists. Several strains were used at different life stages for stocking actions and considerable efforts have been made in terms of obstacles management to achieve free migration ways from spawning area to the sea and vice versa. This initiative met success with adults captured during their spawning migration in Belgium but in very few numbers, considering the amount of effort. Field data shows a temperature difference up to 5°C between a major tributary and the main channel during smolt migration. As temperature is a primary cue in smolting, we decided to investigate the potential impact of such a temperature difference during smolt sea-run. We looked for differences between two strains and between early and late migrants on a variety of physiological smoltification markers as well as on the expression of smoltification related genes in the liver and gills.

We showed that a temperature shift based on environmental data impaired hypo-osmoregulatory capacities of smolts both under laboratory conditions and in the wild. Both strains reacted similarly to a swift increase in temperature; some differences in physiological markers were measured under control conditions without temperature increase, though. Smolting was impaired within one week after the increase, suggesting a rapid and harmful effect on migrating smolts, drastically decreasing their survival chances at sea entry or even compromising their migration to the sea. Early and late migrants were affected in the same way by a change in thermal conditions. We also examined gene expression in the liver in response to a swift temperature increase. Results showed potentially deleterious effects of a temperature shift on the expression of genes involved in the endocrine control of smoltification, oxygen transport, iron metabolism, lipid and carbohydrate metabolism, immune response and cell cycle and DNA repair mechanisms. Findings also revealed important changes occurring during smoltification and differences between strains which were associated to characteristics of rivers of origin. Gene expression in the gill was also impaired after a temperature increase. Changes in the transcription of genes associated to the endocrine control of smoltification and hypo-osmoregulation were consistent with physiological markers. New insights were gained in the molecular mechanisms underlying desmoltification (with secondary increases of circulating GH level and gr1, *ghr1*, *igf1*, *igf1r* transcription). We also reported on new expression data of genes with no or little information available (*nbc*, *taldo1*, *igf2*).

Field work presented numerous challenges; environmental conditions (flood events) and infrastructure-linked issues caused many setbacks and only limited data was obtained. However, differences emerged compared to laboratory conditions, notably early migrants seemed much less affected than late migrants three days after transfer into warmer water while early and late migrants were similarly affected under laboratory conditions. These results point toward the importance to verify findings under natural conditions especially when it comes to animals with complex life-cycles or complex processes as smoltification.

To conclude, a rapid temperature increase arising between two rivers strongly influences the smoltification. Considering the response of early migrants in the field experiment, early migrating strains may have better chances to reach the sea with high capacity for hypo-osmoregulation. Given the modelled temperature increase due to climate change, efforts to facilitate downstream migration are likely to improve smolts survival chances.

6.2 Perspectives

Smoltification is a complex process and despite the fact that it has been studied for several decades, it still bears secrets. The regulation of the hypothalamic–pituitary–interrenal (HPI) axis during smolting still lacks some explanatory mechanisms and there has been no measurement of circulating or pituitary secretion levels of ACTH during smoltification (McCormick, 2013). Only recently, melatonin implants in relation with photoperiod were used in Atlantic salmon (Handeland *et al.,* 2013) or the roles of IGF binding proteins were investigated (Breves *et al.,* 2017). Insulin (Plisetskaya *et al.,* 1988; Mayer *et al.,* 1994) and sex steroids (Nagahama *et al.,* 1982; Patiñio and Schreck, 1986; Sower *et al.,* 1992; Yamada *et al.,* 1993) have been reported to change during smoltification but little research has been carried out on these, leaving their roles speculative (Björnsson *et al.,* 2011).

The loss of smolt characteristics like hypo-osmoregulatory capacity usually happens when fish did not reach the sea within the timeframe set by the ‘physiological smolt window’ and is known as desmoltification. As in the smoltification, the endocrine system plays a role in desmoltification but its role has not been elucidated in details yet (Björnsson *et al.,* 2011). Our experiments seem to indicate a dual role of at least GH and IGF-1 in smoltification and desmoltification. Given its inhibitory role to the development of salinity tolerance (Madsen and Bern 1992) and generally to smolt development (Thorpe, 1982; Young *et al.,* 1989; Björnsson *et al.,* 2011), prolactin pattern (circulating levels, expression, …) may be interesting during the desmoltification process. Prolactin controls the ionic balance and water volume inside the cells. It decreases osmotic permeability to water and increases Na^+ and Cl^-

reabsorption through the epithelium in freshwater (Nagahama *et al.,* 1975) and new mechanistic insights of the role of prolactin in the development, function, and dysfunction of osmoregulatory systems across the vertebrate lineage were reviewed recently (Breves *et al.,* 2014). Further investigation may finally reveal the role of the endocrine system and the mechanisms involved in desmoltification. Inter-individual pace of modification (Stefansson *et al*., 1998) also asks for the definition of factors (e.g. environmental cues) triggering and controlling this process.

NAK activity represents, through its indication of acquired euryhalinity, an interesting indicator of smolting status. A bimodal pattern has been found in, our experiments. Two peaks of NAK activity have already been observed in literature (Handeland *et al.,* 2004; Kiilerich *et al*., 2011; Spencer *et al*., 2010). During smoltification there is a shift between α-subunits isoforms in the NAK which is under differential endocrine control (Tipsmark and Madsen, 2009). Furthermore, salinity challenges tend to show that salinity tolerance follows a similar pattern in the Loire-Allier strain (unpublished results, personal communication of Xavier Rollin, PhD). This early increase in activity may be caused by the prolactin or thyroxine surge reported early in the smoltification (Björnsson *et al*., 2011; Ojima and Iwata, 2007). The control of this increase in activity and the biological significance of early salinity tolerance still need to be investigated.

In a time when invasive species are news topic, investigating the impact of piscivorous predators may give us insight on the proportion of smolts actually reaching the estuary. The status of some species as invasive is still arguable; but the great cormorant (*Phalacrocorax carbo* L.), the wels catfish (*Silurus glanis* L.) and the asp (*Leuciscus aspius* L.) may represent new or recent threat smolts hadn't to cope with previously during their seaward journey. While still at the stage of experimental technology, specific tags changing transmission signal when in contact with gastric acid are being developed (Personal communication with Mark Hambrook, Miramichi Salmon Association, NB, Canada). These will open new research possibilities to assess accurately predation rate of invasive or endemic predators like the striped bass (*Morone saxatilis* W.) in the Miramichi River and Gulf of St. Lawrence, Canada. While no difference of predation was seen between hatchery and wild smolt in the estuary (Hvidsten and Lund, 1988), predation rate may vary between the rivers; cod predation was estimated at 20% in the estuary of the River Orkla, Norway (Hvidsten and Lund, 1988) and up to 24.8% in the River Surna, Norway (Hvidsten and Mokkelgjerd, 1987). Predation rate assessment in a large river basin may point out best suited tributaries for stocking actions.

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