THE PHYSIOLOGY OF SMOLTIFICATION AND SEAWATER ADAPTATION IN RAINBOW TROUT

THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

AQUACULTURE

by

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UNITED KINGDOM

June 2019

Declaration

I, the undersigned, hereby declare that this thesis has been composed entirely by me and has not been submitted for any other degree. The work presented in this thesis, except where specifically acknowledged, is the result of my own investigations.

Word count: Approximately 49,000 words

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This is to certify that this thesis for the degree of Doctor of Philosophy entitled "The Physiology of Smoltification and Seawater Adaptation in Rainbow Trout" submitted to the University of Stirling (UK), is an original work carried out by Bernat Morro Cortès under our supervision.

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Acknowledgements

My biggest thanks go to Dr. Amaya Albalat. Her constant guidance and supervision while always seeking my opinion and allowing me the liberty to make my own decisions are what have made this thesis possible and this time so enjoyable. Never have I met a supervisor who works harder for her students. Thanks go to Prof. Simon MacKenzie, my secondary supervisor, as well. Not only did he offer advice, encouragement and support, but he also gave me my first job in research and offered me the opportunity to take on this PhD. Without a doubt, I would not be where I am now without him. I look forward to continuing work with both of them.

I am very grateful to all the members of our Fish Behaviour and Welfare group and good friends – Dr. Sonia Rey, Dr. Shankar Mandal, Dr. Benjamin Clokie, Ahmed Ahmed, Chris Payne, Savitree Ritchuay, and Maureen Ellis. I would also like to express my gratitude to other members of staff at the University of Stirling. Jacquie Ireland and Dr. John Taggart for their help in the molecular lab from when I was still an MSc. student; James Dick and staff of the Nutrition Analytical Service for letting me use their equipment and installations on several occasions; Dr. John Taylor and Dr. Mikey Clarkson for interesting chats about salmonid aquaculture and for always being happy to offer a hand; and to Mónica Betancor for her guidance on how to interpret lipidomics data. I would also like to acknowledge the mass spectrometry gurus, Dr. Mary K. Doherty and Prof. Phillip D. Whitfield from the University of the Highlands and Islands, and apologise for my weekly pestering emails about getting remote access to their computers.

From the University of Bergen, huge thanks to Prof. Sigurd O. Handeland, whom I consider another supervisor of my PhD, and Dr. Pablo Balseiro. No one works harder than these two. Working alongside them was a real privilege. Also a big thanks to the other members of NORCE – Dr. Tom O. Nilsen, Prof. Lars O.E. Ebbesson, Cindy Pedrosa, Valentina Rossi, Nauoel Gharbi, Enrique Pinto and Patrik Tang. Although for some reason I never felt truly at home during my long stays in Bergen, whenever I spent time with them I always cheered up. I would also like to thank Dr. Harald Sveier, for providing the animals and installations for experimentation though Lerøy Seafood Group ASA, and for being involved in the funding and planning of some of the experiments in this PhD project, Prof. Munetaka Shimizu and his students, for performing measurements of circulating IGF-I and IGFBP1b, and Dr. Marnix Gorissen, for being in charge of cortisol measurements.

Although they still do not fully understand what I have been doing these past three years, my family also deserve a thank you. They have always understood that my professional life and career should come first and they respected my decisions, even if it meant seeing me only a few times every year.

Similarly, thanks go to Francesc Bibiloni for our surreal everyday conversations that make me laugh to tears.

Finally, I wholeheartedly blame my friends Carolina Fernández, Lewis Warren, Irene M. Martín, Dr. Xingkun Jin, Dr. Michael McGowan, Dr. Andre Van, Filitsa Vasilaki, Samuel Pountney, Jadwiga Sokolowska, and Kristina Ulrich for not letting me finish this PhD thesis much sooner. It were our long lunch breaks and frequent get-togethers that kept me from losing what is left of my sanity. Big thanks to all of them.

Peer-reviewed publications

1. Morro B, Balseiro P, Albalat A, Pedrosa C, MacKenzie S, Nakamura S, Shimizu M, Nilsen TO, Sveier H, Ebbesson LOE, Handeland SO (2019) Effects of different photoperiod regimes on the smoltification and seawater adaptation of seawater-farmed rainbow trout (*Oncorhynchus mykiss*): Insights from Na^+, K^+–ATPase activity and transcription of osmoregulation and growth regulation genes. Aquaculture 507:282-292.
2. Morro B, Balseiro P, Albalat A, MacKenzie S, Pedrosa C, Nakamura S, Shimizu M, Nilsen TO, Sveier H, Gorissen M, Ebbesson LOE, Handeland SO (Submitted to Aquaculture). Effects of temperature and photoperiod on rainbow trout (*Oncorhynchus mykiss*) smoltification and haematopoiesis.
3. Morro B, Doherty KM, Balseiro P, Handeland SO, Mackenzie S, Sveier H, Albalat A (Submitted to PLOS ONE). Plasma proteome profiling of freshwater and seawater life stages of rainbow trout (*Oncorhynchus mykiss*).

Non peer-reviewed publications

1. “Rising profile: Proteomics as a tool for biomarker discovery in aquaculture”. Fish Farmer Magazine, Institute of Aquaculture Special Issue. May 2018. Page 37.

Scientific conferences and meetings

1. Morro B, Albalat A, Handeland SO, Sveier H, Nilsen TO, MacKenzie S. Biomarker discovery using MALDI-TOF MS in salmonids. Proteomics Methods Forum, 30th June – 1st July 2016, Warwick, UK (Oral presentation).

2. Morro B, MacKenzie S, Albalat A. Molecular changes during steelhead trout (*Oncorhynchus mykiss*) smoltification and seawater adaptation. Lunch time seminar, 14th of March 2018, Stirling, UK (Oral presentation).
3. Morro B, Doherty MK, Balseiro P, Handeland SO, Pedrosa C, Samanta S, Woodward J, Nilsen TO, Ebbesson LOE, MacKenzie S, Albalat A. Plasma proteome during smoltification and seawater adaptation of steelhead trout (*Oncorhynchus mykiss*). 5th PhD Research Conference, 17th April 2018, Stirling, UK (Oral presentation).
4. Morro B, Doherty MK, Balseiro P, Handeland SO, Pedrosa C, Nilsen TO, Ebbesson LOE, MacKenzie S, Albalat A. Mass Spectrometry-Based Plasma Proteomics for Steelhead Trout (*Oncorhynchus mykiss*) Aquaculture. CtrlAQUA Annual Meeting, 8th – 9th May 2018, Svolvær, Norway (Oral presentation).
5. Morro B, Doherty MK, Balseiro P, Handeland SO, Samanta S, Woodward J, Nilsen TO, Ebbesson LOE, MacKenzie S, Albalat A. Changes in Plasma Proteome and Peptidome during Smoltification and Seawater Adaptation of Steelhead Trout (*Oncorhynchus mykiss*). 13th International Congress on the Biology of Fish, 15th – 19th July 2018, Calgary, Canada (Oral presentation).
6. Morro B, Doherty MK, Balseiro P, MacKenzie S, Handeland SO, Nilsen TO, Ebbesson LOE, Albalat A. Plasma and liver proteome of an unwanted phenotype of seawater-transferred rainbow trout (*Oncorhynchus mykiss*). Marine Alliance for Science and Technology Scotland (MASTS) Annual Science Meeting, 30th October – 2nd November 2018, Glasgow, UK (Oral presentation).

Trainings and workshops

1. ScotPil Personal Licence training course, 4th April 2016, Glasgow, UK.
2. Teaching Assistant course, 22nd October 2018, Stirling, UK.
3. Early career researcher Grant workshop, 2 November 2018, Glasgow, UK.

Grants

1. SASG9: Plasma proteome of an unwanted phenotype of steelhead trout (*Oncorhynchus mykiss*). MASTS, 2017. Amount: £1200. Principal investigator: Bernat Morro.
2. SG506: Liver lipidome of an unwanted phenotype of seawater-farmed rainbow trout (*Oncorhynchus mykiss*). MASTS, 2019. Amount: £500. Principal investigator: Bernat Morro.

List of abbreviations

µg	Micrograms
µl	Microliters
µm	Micrometers
1-D SDS-PAGE	1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
ACN	Acetonitrile
ACTH	Adrenocorticotropin hormone
ADP	Adenosine diphosphate
Ambic	Ammonium bicarbonate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APP	Advanced phase photoperiod
ATP	Adenosine triphosphate
AWERB	Animal Welfare and Ethical Review Body
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
ACN	Acetonitrile
cDNA	Complementary DNA
CE	Cholesterol esters
CFTR	Cystic fibrosis transmembrane regulator
CL	Cardiolipins
cm	Centimeter
CTSL	Cathepsin L
Da	Dalton
DAP	Differentially abundant protein
df	Degrees of freedom
DG	Diglycerides
DNA	Deoxyribonucleic acid
DPP	Delayed phase photoperiod
e.g.	*Exempli gratia* (for example)
ED_{50}	Half-maximal displacement
EF1α	Elongation factor 1α
EP	Enriched plasma
F	F value
FC	Fold change
FDR	False discovery rate
FW	Freshwater
GeLC-MS/MS	Gel electrophoresis liquid chromatography tandem mass spectrometry
GH	Growth hormone
GHr1	Growth hormone receptor 1
GMP	Granulocyte-monocyte progenitor
GO	Gene ontology
h	Hours
HSP90	Heat shock protein 90

HT	High temperature
i.e.	*Id est* (in other words)
IAA	Iodoacetamide
IGFBP	Insulin-like growth factor binding protein
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL1β	Interleukin 1β
IL-4/13	Interleukin 4/13
init	Initial values
kDa	Kilo Dalton
kg	Kilogram
kV	Kilovolt
L	Litre
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LD	Light/dark
LED	Light-emitting diode
LH	Luteinizing hormone
LL	Continuous light
Ln	Natural logarithm
M	Molar
m/z	Mass-to-charge
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight
MASTS	Marine Alliance for Science and Technology Scotland
mg	Miligrams
min	Minutes
mM	Millimolar
mRNA	Messenger RNA
MS	Mass spectrometry
NAD^+	Micotinamide adenine dinucleotide
NARA	Norwegian animal research authority
NKA	Na^+, K^+–atpase
NCBI	National Center for Biotechnology Information
NKAα1a	Na^+,K^+–atpase α-subunit isoform 1a
NKAα1b	Na^+,K^+–atpase α-subunit isoform 1b
NKCC1a	Na^+,K^+, $2Cl^-$ cotransporter 1a
nm	Nanometers
NT	Natural temperature
p	p-value
PCA	Phosphatidylcholines
PDI	Protein disulfide-isomerase
PIA	Phosphatidylinositol
ppm	Parts per million
PS	Phosphatidylserines
q	q-value
r	Correlation coefficient

Rf	Relative mobility
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
s.d.	Standard deviation
s.e.	Standard error
SGR-L	Specific growth rate in length
SGR-W	Specific growth rate in weight
SM	Sphingomyelin
SNP	Simulated natural photoperiod
SVM	Support vector machine
SW	Seawater
T3	Tri-iodothyronine
T4	Tetra-iodothyronine
TC	Temperature convergence
TFA	Trifluoroacetic acid
TG	Triglycerides
TGC-L	Thermal growth coefficient in length
TGC-W	Thermal growth coefficient in weight
TH	Thyroid hormone
Tmax	Time when Ymax
TMT	Tandem mass tag
TR-FIA	Time-resolved fluoro-immunoassay
v/v	Volume/volume
WP	Whole plasma
xg	Centrifugal force
Ymax	Maximum value

Abstract

Seawater-transferred rainbow trout (*Oncorhynchus mykiss*) has become an attractive aquaculture product in recent years. Industrial interest is mainly due to its resistance to infectious pancreatic necrosis and its adaptability to brackish water, which allows farming in otherwise unexploited locations. However, most practices for the aquaculture of this species have been imported from Atlantic salmon (*Salmo salar*) without evidence data supporting their suitability for the species. These include protocols to induce their preadaptation to seawater (smoltification). As a result, following seawater transfer, large numbers of fish die (around 10%) or become growth-stunted (GS; 10-60%). Therefore, species-specific rearing protocols and seawater-readiness biomarkers are needed.

In the present PhD thesis, the effects of different photoperiod and temperature protocols for rearing in freshwater were assessed on the development of smoltification traits and subsequent seawater performance. This was achieved by using an array of molecular tools to measure osmoregulation-, growth- and haematopoiesis-related genes, proteins and hormones. Moreover, the discovery of potential seawater-readiness biomarkers and the study of smoltification, seawater adaptation and GS fish development were performed using several mass spectrometry proteomic and lipidomic approaches.

Results suggest that winter light signals are inadvisable for the species, while all tested summer signals produced similar good results. Moreover, increased temperature protocols not only failed to improve smoltification and growth but potentially compromised the immune system of the fish. Overall, continuous light seems an advisable light regime, irrespective of temperature. Moreover, new promising potential biomarkers for seawater-readiness were identified using proteomics, while also suggesting a previously unknown role of these proteins in smoltification and seawater adaptation. Finally, GS development was shown to be related to low insulin-like growth factor 1 levels following seawater transfer. Moreover, other related factors to the phenotype were higher stress levels, possibly caused by bullying by bigger fish, and hepatic anomalies related to oxidative stress.

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Chapter 1: General introduction

1.1. General background

Over 600 different animal species are currently produced in aquaculture systems, including finfish (*e.g.* catfish, trout, carp, tilapia, salmon), crustaceans (*e.g.* shrimp, prawn, crabs, freshwater crayfish), and molluscs (*e.g.* mussels, oysters and clams) (Troell *et al.*, 2014). In Northern Europe, salmonids are the main cultured fish species. Worldwide, Norway is the biggest salmonid producer with over 1.3 million tonnes of Atlantic salmon (*Salmo salar*) in 2016 (Food and Agriculture Organization of the United Nations, 2019), followed by Chile at over 600,000 tonnes/year, and Scotland at over 170,000 tonnes/year.

While still small compared to Atlantic salmon production, over the last three decades there has been an increase in the aquaculture production of seawater-transferred rainbow trout (*Oncorhynchus mykiss*), which reached over 84,000 tonnes in Norway alone in 2016 (Figure 1.1) (Food and Agriculture Organization of the United Nations, 2019). The situation in Scotland is similar, with several aquaculture companies now increasing their production. Industrial interest in seawater-transferred rainbow trout is based on their resistance to infectious pancreatic necrosis (Okamoto *et al.*, 1993; Ozaki *et al.*, 2001) and due to their preference for brackish water (0.5 to 30‰ salinity) compared to Atlantic salmon (Altinok and Grizzle, 2001). This preference for brackish water allows producers to grow rainbow trout in locations that are less prone to sea lice (*Lepeophtheirus salmonis*) infestations and that would not be as suitable for Atlantic salmon production, thus making use of otherwise unexploited locations.

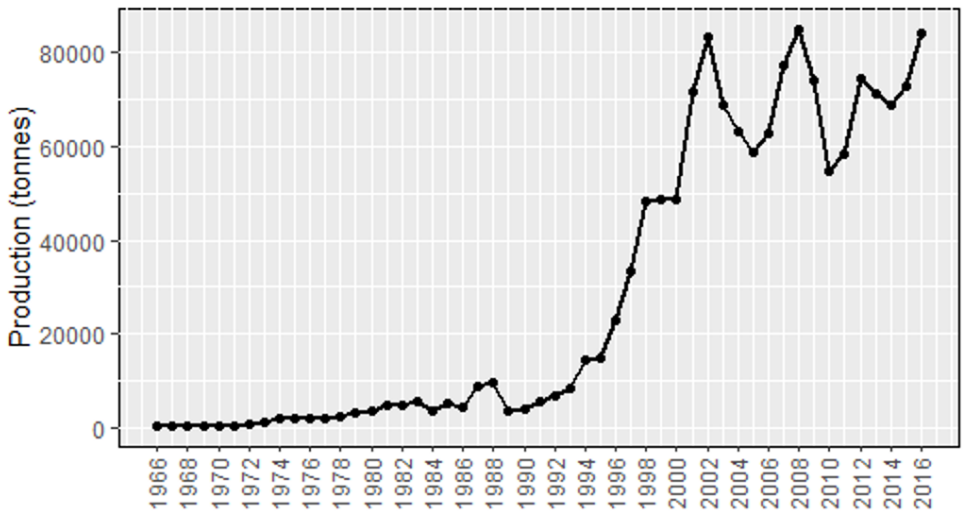


Figure 1.1. Seawater-transferred rainbow trout production in Norway. Source of data: Food and Agriculture Organization of the United Nations (2019).

Having said this, the industry is faced with a major challenge: independently of the location, following seawater transfer, fish experience high mortality rates (around 10%) and a significant portion (between 10 and 60%) become growth-stunted (GS). GS rainbow trout exhibit little to no growth following transfer to seawater and become extremely lean. Moreover, they often show injuries, acute fin damage, and high abundance of melanin in their skin, indicating that they are under severe stress (Ellis, *et al.*, 2008; Ellis, *et al.*, 2012). Therefore, this is not only problematic in economic terms but also when it comes to animal welfare. The problem occurs throughout the year but more notably during the summer months, forcing some companies to cease their production during this season, and therefore abandoning one third of their potential production per year.

Before transfer to seawater, salmonids need to develop hypo-osmoregulatory competence in freshwater, as well as a series of other adaptations that are grouped under the term smoltification, which are aimed at maximizing performance and survival in seawater. In salmonids, the smoltification process is controlled by changes in day length and temperature, although developmental responses vary between species and strains. While in Atlantic salmon these mechanisms are well described and the knowledge is implemented into a successful all-season smolt production (Stead and Laird, 2002), in rainbow trout there is limited knowledge on the environmental control of smoltification and appropriate tools to assess smolt status are lacking (Berejikian *et al.*, 2016). An example of this knowledge gap in rainbow trout production is illustrated in the common practice of using continuous light (LL) and to regard seawater tolerance only as size-dependent, despite this being a production strategy that gives reduced smolt quality in Atlantic salmon (Farmer, 1994; Stead and Laird, 2002). Hence, the development of GS fish is likely related to a lack of understanding of vital intrinsic (*e.g.* endocrine control) and extrinsic (environmental control) factors governing smolt development in rainbow trout and leading to suboptimal timing of seawater transfer.

1.2. Anadromy and Salmonids

Migration is a long-distance movement driven by the need to obtain energy for maintenance, growth, and reproduction. Migratory animals take advantage of seasonally predictable patterns of resource availability and predator abundance within or among habitats and they travel accordingly to meet their energy demands while seeking maximal survival, although there is often a trade-off between the two (Fleming and Reynolds, 2004; Quinn *et al.*, 2011). For fish, most of the species do not migrate or these migrations occur within the same water type. However, in less than 1% of fish species, which are known as diadromous, migrations involve crossing the boundary from freshwater to seawater, or vice versa (Quinn *et al.*, 2016). Generally, in lower latitudes, where productivity in freshwater

ecosystems tends to exceed that of seawater, these fish are catadromous, spawning in seawater and migrating to freshwater streams to grow. Contrarily, in higher latitudes freshwater ecosystems are less productive than the ocean and diadromous fish tend to be anadromous, spawning in freshwater and growing primarily at sea (Gross *et al.*, 1988).

Anadromy is widespread over several fish families including salmonids, lampreys, sturgeons, clupeids, osmerids, and basses (McDowall, 2008; Potter *et al.*, 2015). But the most studied exponent are salmonids (salmons, trouts and charrs), which provide an economic and cultural benefit to humans and to their ecosystem by transporting nutrients between the ocean and freshwater habitats (Gende *et al.*, 2002).

The salmonid family is composed of entirely freshwater-resident species such as graylings (*Thymallus* spp.), obligate anadromous species like the pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*), and species that can present both life histories, like rainbow/steelhead trout (Quinn and Myers, 2004). Having both life-histories in a species is understood as a strategy that spreads mortality over space and time, which decreases population fluctuations and makes the species more resilient to environmental changes (Moore *et al.*, 2014).

While anadromous fish grow more than their freshwater-resident counterparts, environments with the greatest food availability tend to be characterised by a larger density of predators (Lima and Dill, 1990). Consequently, anadromous fish face a mortality rate that may exceed in 95% that of freshwater-residents (Hendry *et al.*, 2004). However, their bigger size gives them a reproductive advantage (Fleming and Reynolds, 2004; Quinn *et al.*, 2011).

O. mykiss is a species with dual life history in terms of migration. The freshwater-resident phenotype of the species is called rainbow trout while the anadromous phenotype is known as steelhead trout (Pearse *et al.* 2009). These two phenotypes usually cohabit in sympatry and can originate from the same cohort (Christie et al., 2011; Kendall et al., 2015). The proportion of each phenotype is influenced by genotype (Nichols et al., 2008; Hecht et al., 2013), individual condition (size, growth rate, energy storage) (McMillan et al., 2012) and environmental factors (Sloat et al., 2014). Regardless, efforts to produce a strain with a single phenotype, either selecting the sea-run (Sharpe et al., 2007; Christie et al., 2011; Sloat and Reeves, 2014) or the freshwater-resident (Thrower and Joyce, 2005; Hayes et al., 2012), have failed.

O. mykiss were first introduced to Europe in 1870, supposedly from a freshwater-resident population in the San Francisco Bay area (Stankovic *et al.*, 2015). They were mostly released in lakes for recreational fishing and used for freshwater aquaculture (Savini *et al.*, 2010). As a result, these fish are rarely anadromous and they are only referred to as rainbow trout in Europe. Regardless, the

steelhead phenotype is still present in some of these introduced populations since, if seawater can be reached, some individuals will naturally migrate (case of Lake Constance, Germany; Stankovic *et al.*, 2015). Therefore, European rainbow trout still had the potential to be reared in seawater. After generations of selection for seawater migrants, this is what some countries including Norway or Scotland are currently doing (Food and Agriculture Organization of the United Nations, 2019).

1.3. Smoltification

Salmonids spawn in freshwater. Therein, the first stage of their life cycle occurs, that is, from the hatching of the eggs, through alevins living off their yolk-sac, to fry feeding actively. During their fry stage, parr marks (dark vertical bars or ovals found along the side of the fish) will appear on their skin and the fish will enter their parr stage, which lasts from months to years depending on the species (Björnsson *et al.*, 2011). At this point, freshwater-residents will keep growing and mature in freshwater while migrating salmonids will develop a series of preadaptations to life in seawater, collectively known as smoltification, and become smolts. After migrating to the ocean, smolts start their post-smolt (or adult) stage during which they grow for a number of years before returning to their natal freshwater streams to reproduce. While some species like the sockeye salmon (*O. nerka*) die after spawning, others like Atlantic salmon and wild migrating rainbow trout or steelhead trout will usually survive (Auer *et al.*, 2018).

Smoltification takes place upon reaching a threshold size, typically between 10 and 15 cm (McCormick and Saunders, 1987; Kendall *et al.*, 2015). At this point environmental cues, such as changes in photoperiod and water temperature, trigger an endocrine reprogramming (*i.e.* changes in the cytology of the tissue) of mainly the pituitary, thyroid and inter-renal tissues of parr fish (Prunet, P., Boeuf, Bolton and Young, 1989). In turn, these endocrine tissues orchestrate a series of nearly simultaneous, yet often independent, changes that preadapt anadromous salmonids to life in seawater and are collectively grouped under the term smoltification (Hoar, 1988; Björnsson *et al.*, 2011). Most seawater adaptation changes, but not all, are reversible and last during a short period of time (*i.e.* smolt window). If fish do not reach seawater within the smolt window, seawater adaptation changes are lost and fish readapt to freshwater (desmoltification) (Stefansson *et al.*, 1998).

For steelhead trout, the smolt window typically lasts for 3-4 months (Wagner, 1974a; Dickhoff *et al.*, 1978; Negus, 2003) and migration occurs in midspring after the first year of life (Hayes, *et al.*, 2008; Satterthwaite *et al.*, 2009), although it can also happen at age 2, 3 or above (Sogard *et al.*, 2012).

A majority of the work studying smoltification has been done in Atlantic salmon, coho salmon (*O. kisutch*), Chinook salmon (*O. tshawytscha*), brown trout (*Salmo trutta*) and steelhead trout. Therefore,

the knowledge on smoltification comes from a generalization over a collage of species. However, for the most part, smoltification control (intrinsic and extrinsic) and smoltification changes are consistent across species (Høgåsen, 1998; Stefansson *et al.*, 2008; Rousseau *et al.*, 2012).

1.3.1. Intrinsic factors. Endocrine control of smoltification

While environmental factors set the smoltification in motion, controlling its timing and magnitude, the endocrine system is the effector as it orchestrates all the seawater adaptation changes that take place. In this sense, the key endocrine organ is the pituitary gland and smoltification cannot occur in hypophysectomised parr (Nishioka *et al.*, 1982). In turn, the pituitary is responsible for controlling the function of other endocrine organs. It exerts influence over the thyroid through the pituitary hormone thyrotropin, promoting the production of thyroid hormones (TH), over the inter-renal tissue through the pituitary hormone adrenocorticotropin (ACTH), which promotes the release of corticosteroids (mainly cortisol), and over other tissues through the pituitary growth hormone (GH), which acts on tissues directly or indirectly via the hepatic production of insulin-like growth factor 1 (IGF-I) (Rousseau *et al.*, 2012).

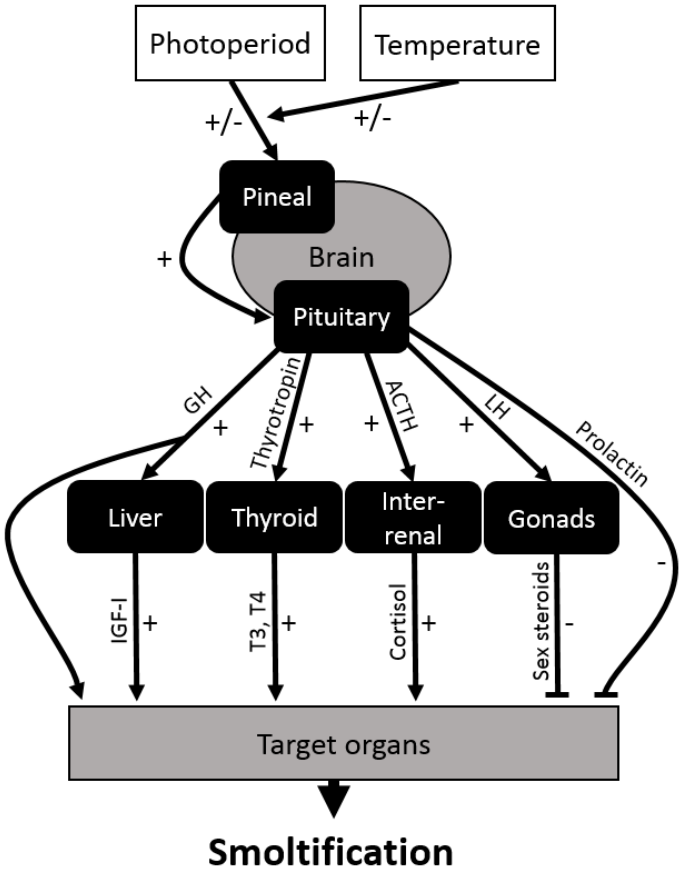


Figure 1.2. Environmental and endocrine control of smoltification.

1.3.1.1. The thyrotropic axis

THs are pituitary hormones and crucial regulators of metabolism, differentiation and development in vertebrates (Ávila-Mendoza *et al.*, 2016). They are involved in most metabolic pathways influencing changes in protein, lipid, carbohydrate and vitamin metabolism and are key for the normal function of processes like growth, metabolic balance or cognition (Zhang and Lazar, 2000; Smith *et al.*, 2002). Tetra-iodothyronine (T4) is the predominant hormone secreted. T4 has few direct actions and acts mainly as a precursor for tri-iodothyronine (T3), which is biologically more active (Power *et al.*, 2001). The conversion of T4 to T3 occurs in the peripheral tissue by the enzymatic removal of one iodide unit (Eales and Brown, 1993). These hormones circulate in plasma attached to proteins like albumin, transthyretin, thyroxine-binding globulin or lipoproteins (Babin, 1992; Power *et al.*, 2001). In the case of migrating salmonids, T3 and tetra-iodothyronine (T4) have been demonstrated to be deeply involved in the regulation of smoltification (Iwata, 1995).

Circulating TH levels increase at the start of the smolt window (Boeuf and Prunet, 1985; Hoar, 1988) and their function in the regulation of smoltification is mainly related to the development of migratory behaviour and changes in energy reserve metabolism, but also to changes in skin coloration and in the composition of muscle, visual pigments and hemoglobin isoforms (Høgåsen, 1998; Stefansson *et al.*, 2008; Rousseau *et al.*, 2012).

1.3.1.2. The somatotropic axis

The somatotropic axis, including GH, IGF-I, IGF-II, their associated carrier proteins and their receptors, is classically involved in the regulation of growth and metabolism in vertebrates. GH is protein hormone that has an important role in growth control, mainly promoting body growth by stimulating the liver and other tissues to secrete IGF-I. In turn, IGF-I is protein hormone functionally and structurally related to insulin but with higher growth-promoting activity (Renaville *et al.*, 2002). In fish, the mechanism of somatotropic action on growth through GH directly, or by the activation of GH receptor (GHr) and the subsequent production of hepatic IGF-I has been studied in multiple groups (Beckman, 2011). In turn, the activity of IGF-I (and IGF-II) is regulated by a family of six IGF binding proteins (IGFBPs), which either either inhibit or enhance the growth-promoting actions of IGF-I and IGF-II (Shimizu *et al.*, 2011a). IGFBP1 is one of the most studied in the family. It is an inhibitor of growth which is expressed under catabolic conditons. Similarly, IGFBP2, 4 and 6 are considered growth inhibitors (Rajaram *et al.*, 1997, Duan et al., 1999; Zhou et al., 2008), while IGFBP3 and 5 potentiate the effects of IGF-I and II (Rajaram *et al.*, 1997, Shimizu *et al.*, 2011b).

When it comes to the smoltification process, circulating levels of both GH and IGF-I, and the abundance of GHr, increase at the start of the smolt window (Mori *et al.*, 2001; Kiilerich *et al.*, 2007;

Shimomura *et al.*, 2012). GH and IGF-I are mainly involved in the development of seawater tolerance, migratory behaviour and smolt morphology (Boeuf *et al.*, 1994; Shrimpton and McCormick, 1998a; Rousseau *et al.*, 2012).

1.3.1.3. The corticotropic axis

The corticotropic axis, otherwise known as the stress axis, is formed by ACTH and corticosteroids like cortisol. Its main role is to allow the adaptation of an organism to bodily and environmental challenges by inducing behavioural and physiological changes that improve the ability of the organism to regain homeostasis (Tsigos and Chrousos, 2002). Cortisol is the hormonal end product of the axis and it plays a crucial role in the adaptation to challenges by binding to glucocorticoid receptors that are present in almost every tissue of the body. As a result, cortisol mediates many metabolic processes including energy mobilization towards brain and muscles, modulation of the immune system, increasing glucose utilization, or increasing blood flow and respiration (McEwen and Seeman, 1999; Fries *et al.*, 2009). Because of its activation under conditions of homeostatic challenge, this axis is classically involved in the response to stress, and cortisol is the most widely used stress marker in vertebrates (Tsigos and Chrousos, 2002), including fish (Sloman *et al.*, 2001; Ellis *et al.*, 2012).

Similarly to TH, GH and IGF-I, circulating levels of cortisol increase during smoltification (Langhorne and Simpson, 1986; Shrimpton and McCormick, 1998b). The main implications of this hormone in smoltification include the development of seawater tolerance, migratory behaviour and changes in energy reserve metabolism (Richman *et al.*, 1985; Sheridan, 1986; Madsen 1990a)

1.3.1.4. The gonadotropic axis

The gonadotropic axis is classically linked to sexual maturation and is involved in development of the reproductive system, ageing, and immunity in vertebrates (Veldhuis, 2008). It is regulated by the pituitary luteinizing (LH) and Follicle-stimulating hormones, which promote the release of sex steroids from the gonads. For fish, these steroids are mainly estradiol in males and females and 11-Ketotestosterone in males (Rousseau *et al.*, 2012).

In a smoltification context, the seawater adaptation process follows its natural course when no increase in sex steroids occurs (Patino *et al.*, 1986; Parhar and Iwata, 1996). However, the release of sex steroids from mature gonads has been shown to inhibit seawater adaptation changes such as the development of migratory behaviour, or the smoltification process altogether (Munakata *et al.*, 2001; Madsen *et al.*, 2004). Hence, sexually mature fish either do not start the smoltification process or abort the development of related traits. Therefore, sexually mature freshwater salmonids do not migrate to seawater (Foote *et al.*, 1994; Thorpe and Metcalfe, 1998; Nichols *et al.*, 2008).

1.3.1.5. The pituitary-prolactin axis

Prolactin is produced mainly in the pituitary gland. Its secretion is controlled by dopamine and affected by several factors, like stress (Torner and Neumann, 2002) or sex steroids (Palm *et al.*, 2001). Prolactin is a multifunctional hormone involved in processes such as membrane lactation, reproduction, immune response, angiogenesis or osmoregulation in vertebrates (Bole-Feysot *et al.*, 1998; Freeman *et al.*, 2000). In fish it has been studied mainly in relation to osmoregulation due to its ion intake promoting effects (McCormick, 2001; Mancera and McCormick, 2007).

Regarding the smoltification process, prolactin is considered a freshwater adaptation hormone, thus working against smoltification (Madsen, Steffen S. and Bern, 1992; McCormick, 2001). In this sense, prolactin is antagonistic to GH and IGF-I; it interacts with cortisol to revert seawater tolerance adaptation (Madsen, Steffen S. and Bern, 1992; Seidelin and Madsen, 1997; Sakamoto and McCormick, 2006). Prolactin circulating levels have been observed to increase towards the end of the smolt window (Prunet and Boeuf, 1989; Young *et al.*, 1989), likely as part of the desmoltification process.

1.3.2. Extrinsic factors. Environmental control of smoltification

Though endogenous circannual rhythms influence smoltification of salmonids (Wagner, 1974b; Björnsson and Bradley, 2007), exogenous factors are the main drivers of smoltification. Hoar (1988) proposed that the environmental factors that influence smoltification are photoperiod, temperature, salinity, lunar phases, turbidity and flow rate. Nowadays, photoperiod is considered the most important factor (McCormick and Björnsson, 1994; Handeland and Stefansson, 2001; Taylor *et al.*, 2005), while temperature is less important but still has considerable influence (Handeland *et al.*, 1998; McCormick and Moriyama, 2000; McCormick *et al.*, 2002).

1.3.2.1. Photoperiod

Photoperiod is commonly manipulated in settings for aquaculture production of salmonids. For salmonids that migrate to seawater in spring or summer, including steelhead trout (Zaugg and Wagner, 1973a; Wagner, 1974a), long days after a period of short days (advanced photoperiod; summer signal) advance the onset of smoltification, while a shift from long to short days delays it (delayed photoperiod, winter signal) (Barron, 1986; Björnsson *et al.*, 2011; Hayes, S. A. *et al.*, 2012). For Atlantic salmon, this can be accomplished even in out-of-season periods of the year, allowing full year production (Handeland and Stefansson, 2001). However, this is not yet possible for rainbow trout, as the effects of photoperiod on their smoltification are still unclear.

Moreover, photoperiod is known to affect maturation rates and growth in Atlantic salmon and rainbow trout (Davies and Bromage, 2002; Berrill *et al.*, 2003; Taylor *et al.*, 2005). While freshwater maturation occurs more frequently after a long summer signal (8 months) following a winter signal (1 month), growth rate is consistently highest at LL.

Experimental work has shown the photoperiod control of smoltification to be regulated by the pineal gland through the production and release of melatonin (Porter *et al.*, 1998; Iigo *et al.*, 2005). The pineal gland is closely connected to the pituitary through catecholaminergic and nonapeptidergic neurons in what is described as the light-brain-pituitary axis (Holmqvist and Ekström, 1995). At the start of the smoltification process, a structural reorganization of this axis occurs, which has been shown to take place just prior to the increment of circulating TH and GH levels (Holmqvist and Ekström, 1995; Ebbesson *et al.*, 2003; Ebbesson *et al.*, 2007).

1.3.2.2. Water temperature

Temperature manipulation can also be used to control the smoltification process. The onset of smoltification can be advanced by an increase in water temperature, which is at least partly due to an increase in metabolism and growth rate that makes the fish reach their threshold size for smoltification sooner (Feldhaus, 2006; Kammerer and Heppell, 2013; Doctor *et al.*, 2014), but also because temperature acts as a rate-controlling factor on the physiological responses to changes in photoperiod (Handeland *et al.*, 2004; Handeland *et al.*, 2013).

It is likely that both temperature and fish size affect many factors related to smolt survival, therefore conditioning the timing of migration and ocean entry (Björnsson *et al.*, 2011; Handeland *et al.*, 2013). If done correctly, an early temperature increment can result in an advance of the onset of smoltification (Staurnes *et al.*, 1994; Solbakken *et al.*, 1994; Handeland *et al.*, 2004). However, overly high temperatures (over 13-15^{o}C) can shorten the smolt window (Björnsson *et al.*, 2011) or inhibit the smoltification process altogether (Zaugg and Wagner, 1973a; Ewing *et al.*, 1979; Handeland *et al.*, 2000), while temperatures that are too cold (2^{o}C) can also inhibit the development of seawater adaptation traits (McCormick and Moriyama, 2000). Deleterious effects of temperature on smoltification have been related to low circulating T4 levels (McCormick and Moriyama, 2000).

Contrarily to these results, some studies show that steelhead trout are more likely to migrate when they do not find optimal conditions in freshwater (Sloat *et al.*, 2014): a higher proportion of wild migrants was found in warm streams, with temperatures that went as high as 19.2^{o}C on average, while sites where temperatures were around 15^{o}C produced mostly freshwater-residents (Sogard *et al.*, 2012).

1.3.3. Seawater adaptation changes

These changes can be biochemical, physiological, morphological, and behavioural in nature.

1.3.3.1. Biochemical and physiological changes

1.3.3.1.1. Osmoregulation

1.3.3.1.1.1. Gill

Gills are respiratory organs that extract dissolved oxygen from water and excrete carbon dioxide. However, they are also critical for ionic exchange and osmotic regulation. This osmoregulatory function is carried out by two types of specialized chloride cells, each present in different parts of the gill, proliferating at different stages and having differential roles due to their differential Na^+,K^+-ATPase (NKA) pump repertoire (Richards *et al.*, 2003; Katoh *et al.*, 2008; McCormick *et al.*, 2009). Hence, lamellar chloride cells are found mainly in the lamellae of gills, are more abundant during the parr stage, and mainly contain the freshwater pump NKA α-subunit isoform 1a (NKAα1a), thus contributing towards freshwater tolerance (Figure 1.3). On the other hand, filamental chloride cells are found mainly in the gill filament, increase in abundance during smoltification and seawater adaptation, and mainly contain seawater pumps NKAα1b and Na^+,K^+,$2Cl^-$ cotransporter 1a (NKCC1a), thus being responsible for seawater tolerance (Nilsen *et al.*, 2007; Flores and Shrimpton, 2012; McCormick *et al.*, 2013).

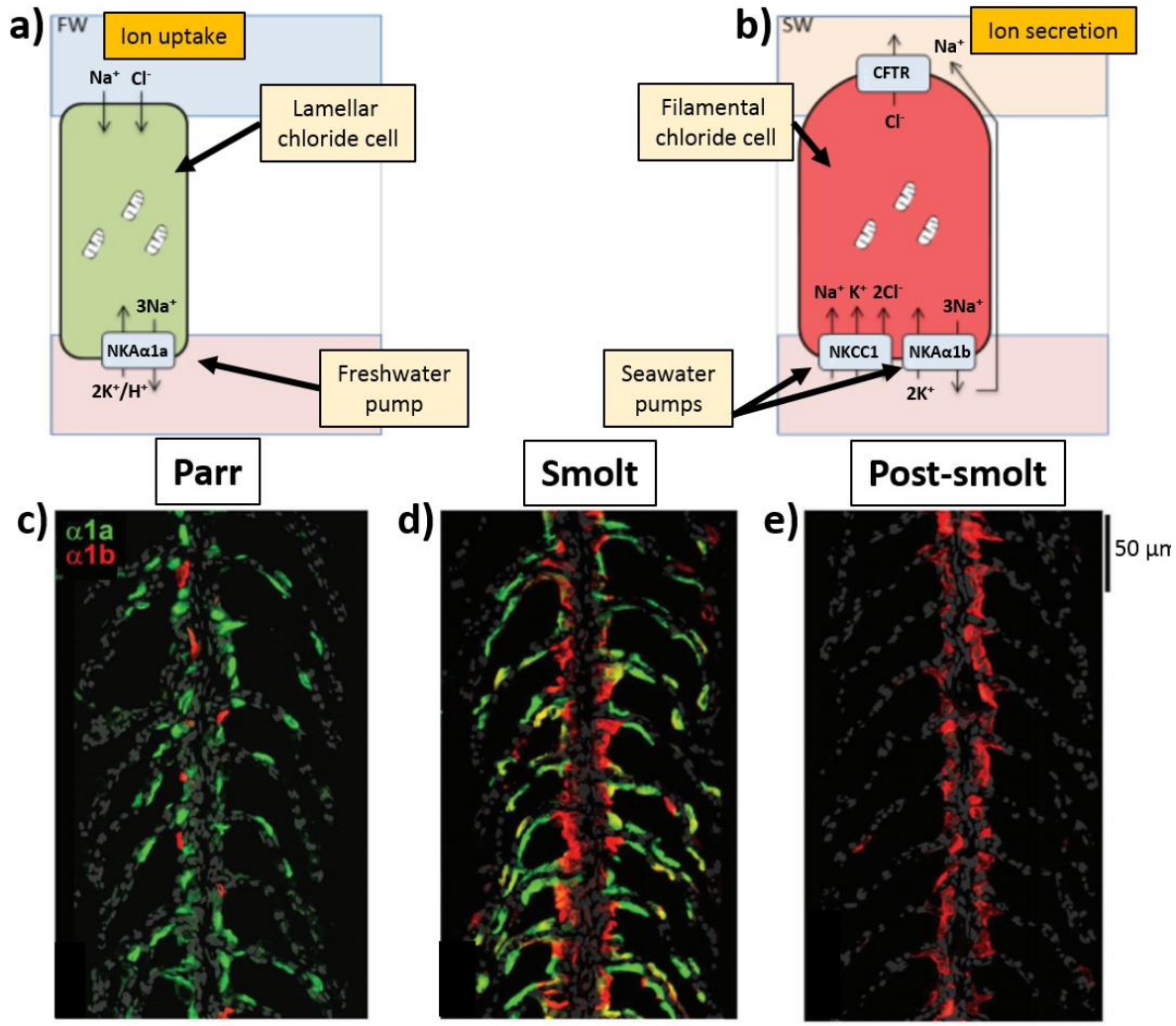


Figure 1.3. Chloride cells and pumps involved in salmonid osmoregulation. Mechanism of lamellar chloride cell (a) and filamental chloride cell (b), and in situ hybridization to show the location of NKAα1a (green) and NKAα1b (red) RNA in gills of parr (c), smolt (d) and post-smolt (e) Atlantic salmon. Yellow cells express both kinds of pump. FW: Freshwater, SW: Seawater, CFTR: cystic fibrosis transmembrane regulator. Adapted from McCormick *et al.* (2013).

This increase in seawater pumps of gill tissue during smoltification is associated with an increase in the overall NKA activity of the gill, which creates an osmotic gradient that results in ion excretion, thus making the fish hypo-osmotic in seawater (Zaugg and Wagner, 1973a; McCormick, 1995). Therefore, NKA activity Is correlated to seawater performance and survival. After increasing during the smoltification process, NKA activity remains high during a variable number of months, depending on the species (Wagner, 1974a; Dickhoff *et al.*, 1978; Negus, 2003), before decreasing again if the fish did

not successfully enter seawater (desmoltification). This period of high NKA activity and seawater tolerance is used to determine the smolt window.

While experimental work indicates that development of gill NKA activity is not regulated by TH (Madsen, 1990b; Boeuf *et al.*, 1994), treatment of parr salmonids with GH (Wedemeyer *et al.*, 1980; Boeuf *et al.*, 1994; Shrimpton and McCormick, 1998a), IGF-I (McCormick *et al.*, 1991), and cortisol (Richman *et al.*, 1985; Madsen, 1990a) significantly increased their gill NKA activity. On the other hand, prolactin prevents the formation of filamental chloride cells and increases the number of lamellar chloride cells, thus negatively affecting NKA activity and hypo-osmoregulatory capacity (Madsen, Steffen S. and Bern, 1992; Seidelin and Madsen, 1997; McCormick, 2001). Furthermore, sex steroids inhibit an increase in NKA activity (Madsen *et al.*, 1997; Madsen *et al.*, 2004), likely by preventing TH production (Ikuta *et al.*, 1985; Ikuta *et al.*, 1987).

1.3.3.1.1.2. Intestine, urinary bladder and kidney

Other key osmoregulatory organs include the intestine, the kidney and the urinary bladder. While freshwater fish receive hydration through passive gradient, due to their hyper-osmotic ion concentration in respect to their environment, seawater fish need active water intake and excrete excessive ions. In this case, water is absorbed through the gut, which experiences an increase in net fluid absorption during the smolt and post-smolt stages (Collie and Bern, 1982), mainly in the posterior intestine (Veillette *et al.*, 1993; Veillette and Young, 2005). This phenomenon occurs due to an increase in the intestinal NKA activity of the anterior intestine and a decrease in transepithelial resistance in the posterior intestine (Sundell *et al.*, 2003). In turn, this increase in water absorption causes the accumulation of ions in blood that need to be filtrated in the kidney, which undergoes changes in glomeruli (increased juxtaglomerular proliferation) and increases its glomerular filtration rate (Ford, 1958; Mizuno *et al.*, 2001). This is accompanied by a reduction of electrolyte absorption in the urinary bladder and a reduction of urine production to store water (Loretz *et al.*, 1982). The resulting urine is more concentrated in seawater than in freshwater (Boeuf, 1993).

1.3.3.1.2. Energy reserve metabolism

As a general trend, the metabolism of proteins, lipids and carbohydrates is altered to sustain the increased metabolic demands of smoltification and as an adaptation to the composition and availability of marine prey (Stefansson *et al.*, 2008). However, during the post-smolt phase, energy procurement is enough to replenish and enlarge these energy reserves (Stefansson *et al.*, 2003; Björnsson *et al.*, 2011).

Smoltification involves a depletion of whole body lipids, and especially from muscle and liver (Sheridan, 1989). These lipids are depleted due to high lipolytic rates and low lipid synthesis, shown to be caused by increased levels of the THs; T4 (Sheridan, 1986) and T3 (Farbridge and Leatherland,

1988), and cortisol (Sheridan, 1986). Mobilized lipids are mainly triglycerides (TG) and cholesterol, which are needed to supply energy during a period of high metabolic requirements (Sheridan *et al.*, 1985; Sheridan, Woo *et al.*, 1985; Rousseau *et al.*, 2012).

Similarly for protein reserves, protein catabolism is increased, leading to a depletion of whole body, muscle and liver protein (Fessler and Wagner, 1969; Nordgarden *et al.*, 2002).

Also for carbohydrates, liver glycogen is decreased due a decrease in liver glycogen synthesis and an increase in glycogen phosphorylase activity (Sweeting *et al.*, 1985; Sheridan, Woo *et al.*, 1985). This energy reserve is translocated from liver to muscle (Hemre *et al.*, 2002).

1.3.3.1.3. Hemoglobins

The ratios of hemoglobin isoforms are altered, with positively charged fractions of hemoglobin increasing in abundance while those that are negatively charged do not change, making the hemoglobin system more complex and increasing the overall hemoglobin concentration (Sullivan, C. V. *et al.*, 1985; Zaugg and McLain, 1986; Seear *et al.*, 2010). These changes are triggered by T3 while dietary administration of propylthiouracil, a hormone used to treat hyperthyroidism, prevents them (Sullivan, Dickhoff *et al.* 1985, Hoar 1988). In turn, this causes a major increase in the oxygen carrying capacity of blood (Fyhn *et al.*, 1991), which is needed due to the dramatic increment in oxygen consumption that occurs by action of GH during smoltification (Maxime *et al.*, 1989; Seddiki *et al.*, 1996; Robertson and McCormick, 2012), likely to prepare the fish for higher oxygen demands during migration.

1.3.3.1.4. Muscle composition and performance

During smoltification, T4 induces a reorganization of red muscle, resulting in a decrease in myosin heavy chain abundance (Martinez, I. *et al.*, 1993; Coughlin *et al.*, 2001). Physiologically, this affects the swimming kinetics of the fish, as parr have faster twitching muscles which they use to maintain a faster frequency tailbeat than smolts (Coughlin *et al.*, 2001). These differences in muscle composition and swimming behaviour are likely related to the downstream position that smolts adopt, thus taking advantage of the current to aid them in their downstream migration while saving energy.

1.3.3.1.5. Visual pigment composition

Salmonid rod photoreceptors contain either rhodopsin or porphyropsin. During smoltification rhodopsin photoreceptors become dominant as a preadaptation to the shorter wavelengths characteristic of oceanic environments (Bridges and Delisle, 1974; Temple *et al.*, 2006; Temple *et al.*, 2008). At the same time, ultraviolet-sensitive cones are lost in the smolt retina due to apoptosis (Dann *et al.*, 2003; Allison *et al.*, 2003; Allison, Dann *et al.*, 2006) triggered by increasing levels of T4 (Veldhoen *et al.*, 2006; Allison, Veldhoen *et al.*, 2006). Interestingly, these cones are regenerated upon

the return of the post-smolts to freshwater for spawning (Browman and Hawryshyn, 1994; Allison, Dann *et al.*, 2006) in a process also controlled by T4 (Browman and Hawryshyn, 1994).

1.3.3.2. Morphological changes

While parr rainbow trout tend to be of dark green and red tones and present parr marks, smolts have a silvery body colour (Figure 1.4), which functions as cryptic coloration in open waters (Hoar, 1988). This is caused by the accumulation of the metabolic by-products guanine and hypoxanthine in the skin and scales of smolts (Kazuhiro *et al.*, 1994) in a process triggered by elevated T4 and GH levels (Miwa and Inui, 1983; Miwa and Inui, 1985).

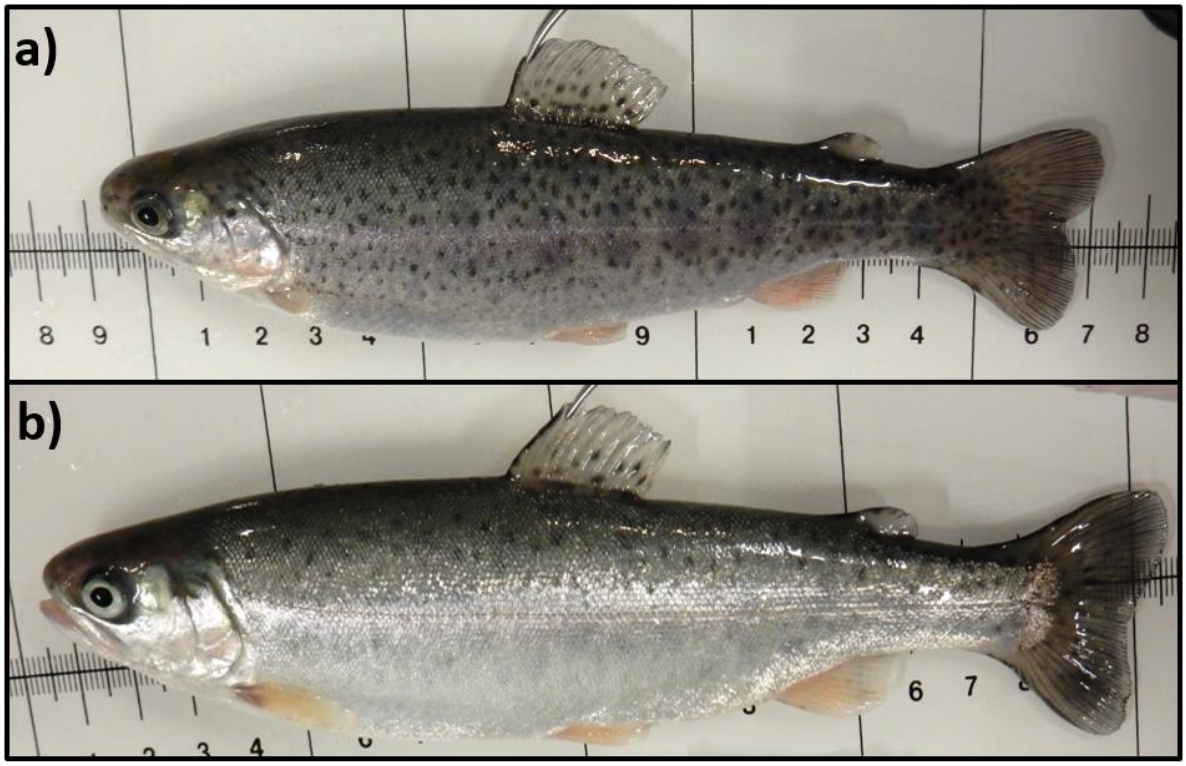


Figure 1.4. Morphology of rainbow trout parr (a) and smolt (b).

Changes in body shape include an elongation of the caudal peduncle of smolts and a transition from a rounded parr to a streamlined smolt, which causes a decrease in condition factor (Winans and Nishioka, 1987) and is likely controlled by GH (Wedemeyer *et al.*, 1980; McCormick, 2009). Both of these changes improve swimming performance.

1.3.3.3. Behavioural changes

Behavioural changes during smoltification are related to seawards migration, the timing of which varies among species. As a general trend, it occurs earlier (as soon as early spring) for salmonids of latitudes closer to the equator, and progressively later (as late as August) for salmonids of higher latitudes (Utrilla and Lobón-Cerviá, 1999; Antonsson and Gudjonsson, 2002).

While parr tend to swim against currents, smolts swim downstream (Martin, P. *et al.*, 2012) and experience increased salinity preference (Hoar, 1988). During their migration, the bottom-dwelling, aggressive and territorial parr progressively become pelagic, gregarious and migratory smolts (Thorpe, 1994), in a process that is induced by increased levels of TH (Iwata, 1995), GH (Iwata *et al.*, 1990; Ojima and Iwata, 2009; Ojima and Iwata, 2010) and cortisol (Munakata *et al.*, 2007; Ojima *et al.*, 2007), while sex steroids prevent it (Berglund *et al.*, 1994; Munakata *et al.*, 2001). The reduction in aggression allows school formation (only during the day in rivers, Riley *et al.*, 2014). This is possible because the difficulty in finding food, which is of low nutritional content in rivers, is less of a problem in seawater (Godin *et al.*, 1974; Iwata, 1995). And it is an advantageous strategy as fish become exposed to a higher risk of predation during their migration and once in seawater, being in a school can act as a defence mechanism (McCormick *et al.*, 1998).

1.3.4. Desmoltification

Smoltification is an example of programmed rheostasis; a process that adjusts the homeostasis of an organism to future conditions (Mrosovsky, 1990). In the case of smoltification, fish become adapted to seawater while still in freshwater. However, fish might be unable to reach seawater, in which case seawater adaptation changes become detrimental. Therefore, if after the smolt window fish have not entered seawater, the desmoltification process begins.

Desmoltification is an alternative life history event to partially or completely revert seawater adaptation changes and readapt the salmonids to freshwater, although some exceptions like pink and chum salmon, as well as some strains of Atlantic salmon, seem unable to do so (Boeuf, 1993; Rottiers, 1994). This process, like smoltification, is affected by several environmental factors, such as photoperiod (Kurokawa, 1990; Duston and Saunders, 1990), temperature (Zaugg and Mclain, 1976; Duston *et al.*, 1991), or salinity (Mortensen and Damsgård, 1998). Moreover, it has been suggested that the lack of migration movement accelerates the process (Soivio *et al.*, 1988; Høgåsen, 1998).

Hence, desmolted salmonids adopt an upstream orientation and revert to aggressive, territorial behaviour (Schmitz, 1992; Ojima and Iwata, 2007). They also lose their hypo-osmoregulatory capacity (McCormick *et al.*, 1997) and their silvery coloration while increasing their condition factor and their fat deposits in muscle and liver back to normal parr levels (Lundqvist and Eriksson, 1985; Li, H. and Yamada, 1992). However, they do not lose the characteristic feeding habits and growth potential of a smolt (Duston *et al.*, 1991; Høgåsen, 1998).

Desmoltification seems to be induced by a decrease in GH production (Ágústsson *et al.*, 2001), while other hormones are likely to be implicated such as cortisol (Young *et al.*, 1989), T4 and T3 (Boeuf *et*

al., 1989; Prunet, P., Boeuf and Young, 1989), prolactin (Young *et al.*, 1989) or sex steroids released after sexual maturation (Fängstam, 1994). However, the endocrinology of the process has still not been elucidated in detail (Høgåsen, 1998; Björnsson *et al.*, 2011).

1.3.5. Emerging technologies to study smoltification

Untargeted –omics technologies have the potential to unravel the underlying mechanisms that lead to a process (Beale *et al.*, 2016; Raposo de Magalhães *et al.*, 2018; Karczewski and Snyder, 2018). They provide a holistic view of this process by measuring the abundance of large numbers of posteriorly annotated biomolecules (*e.g.* genes, transcripts, proteins, metabolites, lipids). This information can then be used to infer the involved pathways.

Currently, and especially after the publication of several salmonid genomes (*e.g.* Atlantic salmon (Davidson *et al.*, 2010) and rainbow trout genomes (Berthelot *et al.*, 2014)), a great body of genomic resources are available for salmonid molecular work (*e.g.* PCR, genomics, transcriptomics, proteomics). They provide a scaffold sequence for BLAST (*i.e.* database search) of DNA, RNA or protein sequences. Once identified, genes, transcripts and proteins can be annotated. These genomic resources can be accessed in salmonid specific databases like SalmoBase (Samy *et al.*, 2017) or in multispecies databases like NCBI (ncbi.nlm.nih.gov), Mascot (matrixscience.com) or UniProt (uniprot.org).

However, while as previously described there has been a great amount of research done on the topic of smoltification, –omics studies on this topic are still relatively rare. Mainly genomic and transcriptomic studies have been used in relation to the smoltification process. Findings include the repression of the immune system in smolts (Boulet *et al.*, 2012; Johansson *et al.*, 2016; Healy *et al.*, 2018), gene expression changes associated to seawater transfer (Norman *et al.*, 2013; Norman *et al.*, 2014; Johansson *et al.*, 2016), and elevated transcription of *nkaα1b* in gill (Healy *et al.*, 2018), epigenetic modifications (Baerwald *et al.*, 2016), quantitative trait loci (Hecht *et al.*, 2012), and gene expression patterns (Hecht *et al.*, 2014; Sutherland *et al.*, 2014; Hale *et al.*, 2016) associated with the likelihood of steelhead trout to migrate to seawater. Moreover, some also provide confirmation of physiological and biochemical changes described by previous studies (Seear *et al.*, 2010; Robertson and McCormick, 2012; Norman *et al.*, 2014).

The –omics technology that studies proteins is proteomics, which is often used for clinical and animal research. Modern tools to study the proteome of a sample are based on mass spectrometry (MS) platforms. An example is liquid chromatography tandem MS (LC-MS/MS), which is one of the preferred pipelines used due to identify proteins in a complex sample, due to its high versatility and

high protein identification potential. This is achieved thanks to the physical separation capabilities of liquid chromatography coupled with the ionization capabilities of MS (Geromanos *et al.*, 2009). Other examples of MS platforms used for proteomics include 1- and 2-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protein separation followed by MS, capillary electrophoresis–mass spectrometry, which separates proteins in a liquid sample using capillary electrophoresis, and matrix assisted laser desorption/ionization time of flight (MALDI-TOF) MS, which uses a laser to ionize peptides and proteins by excitation of a matrix that is added to the sample. Different MS platforms have advantages and disadvantages when compared (*e.g.* LC-MS/MS excels at protein identification whereas MALDI-TOF is fast and expensive in comparison; Volmer *et al.*, 2007). However, proteomic approaches also have common limitations, which include their limited proteome coverage of low-abundant proteins when not coupled with protein depletion/enrichment steps (Liumbruno *et al.*, 2010), their poor detection of membrane proteins (Tan *et al.*, 2008) and the fact that post-translational modifications are rarely detected unless specifically targeting them (Betzen *et al.*¸ 2015). Moreover, the physico-chemical properties of the proteins coupled with the chosen extraction method strongly bias which proteins can be detected (Encheva *et al.*, 2006).

Proteomic studies have successfully gained a deeper understanding of processes and conditions such as embryological development (Link *et al.*, 2006; Papakostas *et al.*, 2010), starvation (Martin *et al.*, 2001; Martin *et al.*, 2003), response to stressors (Mendelsohn *et al.*, 2009; Zhang, W. *et al.*, 2012), disease (Zhang, A. -H *et al.*, 2013; Ruprecht and Lemeer, 2014), and infection (Chongsatja *et al.*, 2007; Somboonwiwat *et al.*, 2010; Medina-Gali *et al.*, 2019), amongst many others. However, in a smoltification context only targeted, top-down studies of key protein hormones such as IGF-I, GH, insulin and their receptors have been carried out (Beckman *et al.*, 2004a; Mancera and McCormick, 2007; Shimomura *et al.*, 2012). In this sense, an untargeted, bottom-up protein approach (*i.e.* proteomics) would have the potential to identify proteins previously unknown to be related to the smoltification process.

1.4. Growth-stunted phenotype

In aquaculture, GS fish are a recurring problem in seawater-transferred rainbow trout, similarly to other salmonids (Folmar *et al.*, 1982; Vindas *et al.*, 2016), which represent between a 10% and a 60% of the summer production. Moreover, around 10% of the fish transferred to seawater during this season die, which are very likely also GS fish. GS fish experience reduced growth, decreased condition factor, and often present lesions such as fin damage (Figure 1.5). Therefore, they do not only represent a financial problem but also a fish welfare issue (Ellis et al., 2008).

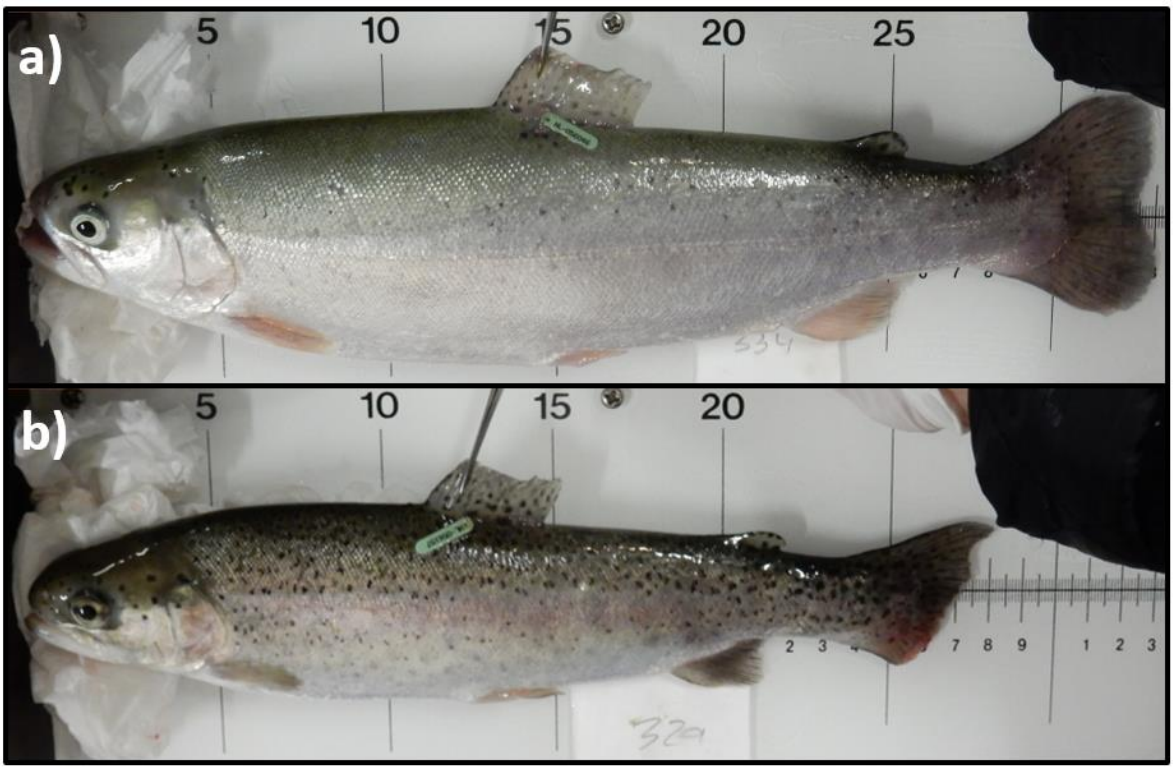


Figure 1.5. Morphology of robust (a) and GS (b) rainbow trout post-smolts.

Currently, there is still no published literature investigating the specific issue of GS seawater-transferred rainbow trout. However, a similar phenotype has been described in freshwater rainbow trout (Sloman *et al.*, 2000a; Sloman *et al.*, 2000b). These studies linked the phenotype to subordinate behaviour (Gilmour *et al.*, 2005). Findings report that the characteristics of the phenotype include reduced food intake (Abbott and Dill, 1989; DiBattista *et al.*, 2006), higher standard metabolic rate (Sloman *et al.*, 2000a), increased protein catabolism (Mommsen *et al.*, 1999; DiBattista *et al.*, 2006), increased lipid metabolism (Kostyniuk *et al.*, 2018), increased mobilization of stored glycogen and gluconeogenic potential (Gilmour *et al.*, 2012), and higher plasma glucose levels (Peters *et al.*, 1988).

While some or all of the above-mentioned characteristics of the freshwater phenotype are likely to affect seawater GS rainbow trout, the problem at hand occurs only after a forceful seawater transfer and in aquaculture production conditions instead of lab conditions. Therefore, more factors involving smoltification traits are likely to be relevant to seawater GS development. In this sense, it is possible that the problem relates to the dual life history of the species; the GS fish originating from rainbow trout that would have been natural freshwater-resident individuals. In this case, optimizing smoltification protocols, as well as discovering and monitoring biomarkers for the early detection of GS fish, could prove a successful strategy to increase the sustainability, profitability and welfare of seawater rainbow trout farming.

In cases like this, with many possible explanations for a physiological alteration but still very little known, a first characterisation of the phenotype can be the way forward, providing a holistic view of the underlying mechanisms that lead to its development (Beale *et al.*, 2016; Raposo de Magalhães *et al.*, 2018; Karczewski and Snyder, 2018). Therefore, –omics approaches like proteomics and lipidomics are an optimal strategy.

Shotgun lipidomic approaches allow for rapid and sensitive identification and quantification of individual lipid species and their composition. Similarly to proteomics, they offer great potential to unravel mechanisms related to lipid metabolism, which are likely to be relevant in GS due to the starvation and stress related characteristics of the phenotype (Abbott and Dill, 1989; DiBattista *et al.*, 2006; Kostyniuk *et al.*, 2018).

In this sense, the liver, due to its central role in energy storage and mobilization, is the sample type that is more likely to reflect differences in physiology and metabolism (Burra, 2013). Both proteomic and lipidomic studies have been used on liver to assess the effects of differential feeding (Martin *et al.*, 2001; Jové *et al.*, 2014; Skorve *et al.*, 2015), stress (Wu *et al.*, 2016), and disease (Martel *et al.*, 2012; Lee *et al.*, 2017), among others. In this sense, a multi–omics approach targeting liver has high chances of unravelling mechanisms involved in GS development.

1.5. Biomarkers

Biological markers (biomarkers) are molecules or characteristics that can be objectively measured and evaluated in biological material as an indicator of normal biological processes, pathological processes or pharmacological responses to a therapeutic intervention (Frank and Hargreaves, 2003). Biomarkers are used commonly in human and animal medicine to identify disease or propensity to it (Hye *et al.*, 2006; Hanash *et al.*, 2008; Geyer *et al.*, 2017) and in ecological studies to estimate age (Allain and Lorance, 2000; Fablet and Le Josse, 2005) and life history events based on otoliths (Jónsdóttir *et al.*, 2006; Elsdon *et al.*, 2008) or to assign personality traits (Dadda *et al.*, 2010; Ariyomo *et al.*, 2013). In aquaculture, they are commonly used to determine gender (Palaiokostas *et al.*, 2015; Robledo *et al.*, 2018) or the presence of desirable attributes such as fast growth (Beckman, 2011; Salem *et al.*, 2012; Tsai *et al.*, 2015) or disease resistance (Villanueva *et al.*, 2011; Vallejo *et al.*, 2017), which can then be used for selective breeding.

1.5.1. Biomarkers for salmonid aquaculture

In the case of anadromous salmonid aquaculture, the most interesting biomarkers are for the identification of robust smolts. Robust smolts quickly adapt to seawater and grow to harvesting size, thus maximizing production profitability. In this sense, seawater adaptation is highly dependent on the correct monitorization of the smolt window and on transferring the fish to seawater during the optimum time within this window (Handeland and Stefansson, 2001; Handeland *et al.*, 2013). Correct growth depends on the well-being of the fish, which in turn is dependent on rearing conditions. However, growth potential can also be genetically determined (Beckman, 2011; Salem *et al.*, 2012; Tsai *et al.*, 2015). Finally, disease and mortality are some of the main threats to production (Asche *et al.*, 2009; Dale *et al.*, 2009; Kristoffersen *et al.*, 2009). Hence, the capacity to fight infection is a critical factor. Therefore, biomarkers of a robust smolt are mainly related to seawater tolerance, growth potential, and immune capacity.

1.5.1.1. Seawater tolerance

Seawater tolerance biomarkers are needed to predict the optimal time for seawater transfer. In this sense, the development of smoltification traits can be used as an indirect measure of seawater tolerance. The easiest biomarkers of smoltification would be the occurrence of seawater adaptation changes in coloration and body shape, thus they are often used as smolt markers since they can be visually assessed. However, they are not reliable, as often fish present this morphological changes before fully developing hypo-osmoregulatory competence (Staley and Ewing, 1992). Another potential seawater tolerance biomarker, in this case a physiological smoltification change, would be the change in composition of visual pigments. However, it would not be reliable either, as Temple *et al.* (2006) demonstrated that these pigments change not only because of the smoltification process but also seasonally in fish that stay in freshwater. Other biomarkers could be hormonal, such as TH levels, which increase during smoltification but several studies have failed to determine a relationship between the hormone levels and seawater tolerance (Madsen, 1990b; Boeuf *et al.*, 1994; Rousseau *et al.*, 2012). Similarly, cortisol cannot be used either because it has been shown to increase during desmoltification to interact with prolactin (Høgåsen, 1998).

On the other hand, circulating GH and IGF-I levels are more promising options due to their implication in the development of seawater tolerance. Particularly IGF-I has been singled out as a reliable robust smolt biomarker (Beckman *et al.*, 1999). However, fish farmers do not rely on this biomarker to measure hypo-osmoregulatory capacity. Regardless, it is an interesting candidate for seawater-readiness but its suitability for rainbow trout has not been investigated yet.

Currently, the most frequently used biomarker test for seawater tolerance and smoltification in both fish farms and salmonid research (Handeland *et al.*, 2013; Elsner and Shrimpton, 2018; McGowan, 2018) is the direct measurement of NKA activity in gill tissue using a kinetic assay measured by spectrophotometry (McCormick, 1993) that is based on the enzymatic production of NAD^+ in the reaction:

$$\mathrm{ATP} \underset{\mathrm{NKA}}{\longleftrightarrow} \mathrm{ADP} + \mathrm{Pi}$$

$$\mathrm{ADP} + \mathrm{phosphoenolpyruvate} \xrightarrow[\mathrm{pyruvate\ kinase}]{} \mathrm{pyruvate} + \mathrm{ATP}$$

$$\mathrm{pyruvte} + \mathrm{NADH} \xrightarrow[\mathrm{lactate\ dehydrogenase}]{} \mathrm{lactate} + \mathrm{NAD}^+$$

Moreover, the transcription of NKA-related pumps *nkaα1a*, *nkaα1b* and *nkcc1a* is increasingly used in research and production as a biomarker of seawater tolerance (Flores and Shrimpton, 2012; McCormick *et al.*, 2013; McGowan, 2018). However, both NKA activity and measurement of NKA-related gene transcription have been reported to have low accuracy, being respectively of 60% and 57% for Atlantic salmon (McGowan, 2018). Therefore, there is a need for new, more accurate biomarkers of seawater tolerance.

1.5.1.2. Growth potential

Due to the lack of literature on the GS seawater-transferred rainbow trout, it is hard to pinpoint biomarkers that could be used for the early detection of the GS phenotype. However, Beckmann *et al.* (1999) indicated that in Chinook salmon circulating IGF-I could be a good biomarker for growth, as behaviourally dysfunctional fish had lower levels of the hormone shortly after release in seawater, and these levels were correlated with growth and survival. Interestingly, IGF-I levels before seawater transfer correlate with growth rate in seawater for some species, while they do not for others (Pierce *et al.*, 2005; Picha *et al.*, 2008; Beckman, 2011). Moreover, some IGFBPs, and particularly IGFBP1b, are negatively correlated with growth rate and may be useful as a negative marker of growth for some salmonids (Shimizu *et al.*, 2006; Kawaguchi *et al.*, 2013; Kaneko *et al.*, 2019).

Both IGF-I and IGFBP1b are promising candidate biomarkers for growth but their suitability for seawater-transferred rainbow trout remains untested.

1.5.1.3. Immune capacity

Organisms are constantly exposed to immune challenges. If their defences against these pathogens are low their risk of becoming infected increases, which can cause weakening or even death. Therefore, a robust smolt would have a strong immune capacity. Generally, this capacity is associated to a fast and effective immune response upon the detection of a pathogen, carried out by cells of both the innate and the adaptive immune systems. The innate immune system non-specifically recognizes

and attacks non-self agents with specialized cells such as macrophages and releasing molecules that promote inflammation and the further recruitment of these specialized cells towards the site of infection (Janeway, 1998). In turn, some of these molecules induce the proliferation and differentiation of cells from the adaptive immune system (Iwasaki and Medzhitov, 2010), which uses lymphocytes to recognize and attack a broad range of agents, thanks to their structural diversity generated by gene rearrangement, conversion, and hypermutation (Takano *et al.*, 2010; Attaf *et al.*, 2015).

Therefore, immune cells are the effectors of the immune system that determine the capacity of an organism to fight infection, and their abundance is often used as a measurement of immune capacity (Haney *et al.*, 1992; Zmistowski *et al.*, 2012; Albalat *et al.*, 2019). In turn, the proliferation of innate and adaptive immune cells is dependent on haematopoiesis (Iwasaki and Akashi, 2007; Orkin and Zon, 2008), thus constituting an indirect measure of immune capacity (Baldridge *et al.*, 2010; Martin *et al.*, 2012). Hence, haematopoiesis regulators like Pu.1, which controls lymphoid cell production (DeKoter and Singh, 2000; Ribas *et al.*, 2008), interleukin 1β (IL1β), a pro-inflammatory cytokine that promotes the proliferation and maturation of lymphocytes (Pleguezuelos *et al.*, 2000; Reis *et al.*, 2012), IL-4/13, involved in the stimulation of B lymphocyte proliferation and activation of macrophages (Martinez *et al.*, 2009; Takizawa *et al.*, 2011; Sequeida *et al.*, 2017), and GATA3, which regulates the development of the T lymphocyte lineage and differentiation of T helper type 2 cells (Kumari *et al.*, 2009), can all be used as biomarkers of immune capacity.

1.5.2. Emerging technologies for biomarker discovery

The general lack of reliable biomarkers in salmonid aquaculture, and especially for seawater-transferred rainbow trout, are highlighted by the problems currently faced by producers (Vindas *et al.*, 2016; McGowan, 2018). This has elicited a rise in genomic work aimed at choosing targets for selective breeding (Villanueva *et al.*, 2011; Salem *et al.*, 2012; Vallejo *et al.*, 2017). Moreover, one study based on the meta-analysis of transcriptomic data in gill tissue elaborated a list of 37 candidate gene biomarkers for smoltification (Houde *et al.*, 2018). These genes are mainly related to immunity (*e.g. T cell receptor alpha*, s*erine/threonine-protein kinase plk2*), ion regulation (*e.g. nkaα1a*, *nkaα1b and nkcc1*), metabolism (*e.g. NADH dehydrogenase 1 beta subcomplex subunit 2* and *4*), and oxygen transport (*e.g. hemoglobin subunit α*).

In this sense, proteomics, and especially matrix assisted laser desorption/ionization time of flight MALDI-TOF MS, is one of the preferred platforms for identifying novel biomarkers (Karpova *et al.*, 2010; Ng *et al.*, 2014; Hajduk *et al.*, 2016). These high-throughput MS technology, which measures hundreds to thousands of molecules in each run, combined with the classification capabilities of

machine learning, is currently generating some of the most reliable clinical biomarkers (Timm *et al.*, 2008; Pyatnitskiy *et al.*, 2011; Lawton *et al.*, 2014). However, it has not yet been used for biomarker discovery in fish.

As a target biofluid, blood plasma is easily accessible in fish and it contains the most complex and informative proteome of an organism (Anderson and Anderson, 2002; Jacobs *et al.*, 2005; Pernemalm and Lehtiö, 2014). Plasma protein studies have succeeded in discovering biomarkers for disease (Hye *et al.*, 2006; Hanash *et al.*, 2008; Geyer *et al.*, 2017), growth (Beckman, Fairgrieve *et al.*, 2004; Beckman, 2011), and stress (Fast *et al.*, 2008; O'Loughlin *et al.*, 2014), amongst many others. Therefore, it is highly likely that more protein biomarkers for the smoltification and seawater adaptation processes may be discovered in blood plasma.

1.6. Objectives

In order to improve the profitability, sustainability and welfare of seawater-transferred rainbow trout aquaculture, a better understanding of the intrinsic and extrinsic factors driving rainbow trout smoltification, optimised photoperiod and temperature production protocols capable of producing robust smolts, reliable biomarker tools to assess their smolt status, and a better understanding of the processes driving GS fish development are all needed. Therefore, this PhD thesis will address four specific objectives:

a) Determine an optimised protocol for production of robust rainbow trout smolts. This will be achieved by testing the effects of different photoperiod and temperature regimes on fish smoltification and seawater adaptation.
b) Determine the main intrinsic factors implicated in smoltification and seawater adaptation. This will be carried out to further our understanding of these processes in rainbow trout and it will be studied using both targeted approaches, by measuring the seasonal changes of molecules known to be implicated in the smoltification and seawater adaptation of salmonids, and an untargeted proteomic approach.
c) Test known, and discover new candidate robust smolt biomarkers in rainbow trout. This will achieved using untargeted proteomic approaches.
d) Determine the underlying mechanisms driving the development of the GS phenotype in rainbow trout. This will be investigated with the measurement of a range of molecules believed to be involved in seawater adaptation and growth, and by untargeted proteomic and lipidomic approaches.

Chapter 2. Effects of different photoperiod regimes on the smoltification and seawater adaptation of seawater-farmed rainbow trout

2.1. Introduction

Anadromous salmonids migrate to seawater in order to meet their energy demands for maintenance, growth and reproduction. However, juveniles do not have the biological traits needed for life in seawater and therefore require to go through a series of simultaneous, yet often independent, changes that prepare salmonids to life in seawater, collectively known as smoltification (Hoar, 1988; Björnsson *et al.*, 2011). These changes are biochemical in nature, such as the alteration of the haemoglobin isoforms, which increases oxygen carrying-capacity of blood (Fyhn *et al.*, 1991); physiological, such as the increase of gill NKA activity, which is the main enzyme involved in ion absorption and secretion (Mancera and McCormick, 2007; McCormick, 2001); morphological, such as the transition from dark, rounded parr to silvery, streamlined smolts; and behavioural, including the shift from bottom-dwelling, aggressive and territorial parr to pelagic, schooling and downstream migrating smolts (Riley *et al.*, 2014). Once anadromous fish reach a threshold size (Kendall *et al.*, 2015), smoltification is triggered by environmental cues, such as changes in photoperiod, water temperature and salinity, which in turn alter the pituitary, thyroid and inter-renal tissues (Prunet, P., Boeuf, Bolton and Young, 1989). These tissues are key orchestrators of the seawater adaptation changes. Most seawater-adaptation changes are reversible and last during a short period of time (smolt window). If fish do not reach seawater within the smolt window changes are lost (desmoltification) (Stefansson *et al.*, 1998).

Over the last three decades there has been an increase in the aquaculture production of the sea-run phenotype of rainbow trout, which reached over 84,000 tonnes in Norway alone in 2016 (Food and Agriculture Organization of the United Nations, 2019). Industrial interest of this phenotype is based on its resistance to infectious pancreatic necrosis (Okamoto *et al.*, 1993; Ozaki *et al.*, 2001) and its preference for brackish water compared to Atlantic salmon (Altinok and Grizzle, 2001). This preference for brackish seawater gives the possibility to grow rainbow trout in locations that are not as suitable for Atlantic salmon production and that are less prone to sea lice infestations. However, there is limited knowledge on the environmental control of rainbow trout smoltification and there is a need for appropriate tools to assess rainbow trout smolt status. In fact, the aquaculture industry has

reported that rearing protocols developed for the all-season production of Atlantic salmon might not be suitable for other salmonids. Reported issues include high mortality and fish that experience sub-optimal growth (GS phenotype) after seawater transfer, especially in summer post-smolts, similarly to growth-stunted Atlantic salmon (Stephen and Ribble, 1995; Stien *et al.*, 2013; Vindas *et al.*, 2016). These problems are likely related to a current lack of understanding of how vital intrinsic (*e.g.* critical size, genetically determined phenotypic plasticity) and extrinsic (*e.g.* temperature, light) factors impact smoltification in rainbow trout, leading to suboptimal rearing conditions and/or mismatched timing of seawater transfer, as reported for other salmonids (Folmar *et al.*, 1982). Currently, for rainbow trout, seawater tolerance is regarded only as size dependent and no studies have analysed if phenotype plasticity is linked to particular genotypes. Regarding extrinsic factors, smoltification is currently induced for all year production by rearing the rainbow trout under LL photoperiod without much supporting evidence for such practice while the impact of other potentially related factors such as temperature and salinity are unknown.

Photoperiod is known to play a major role in the smoltification of anadromous salmonids, with short days (winter signal) followed by increasingly longer days (summer signal) acting as a *zeitgeber* that indicates the proximity to the summer season and the necessity to migrate, thus triggering smoltification (Zaugg and Wagner, 1973a; Brauer, 1982; Saunders *et al.*, 1985). The manipulation of this environmental factor is the most common tool for the all year production of anadromous salmonid aquaculture (Handeland and Stefansson, 2001). Extensive literature on the manipulation of photoperiod to induce smoltification is available for Atlantic salmon (McCormick and Moriyama, 2000; Stefansson *et al.*, 2007; Handeland *et al.*, 2013) and this knowledge is applied commercially by using dynamic photoperiod regimes optimised for each of the four harvest times in a year (Good *et al.*, 2016). On the other hand, for rainbow trout it is unclear whether the photoperiod that is currently being used in commercial farms, LL, is the most appropriate to produce smolts, although there is evidence that long day photoperiods (light/dark (LD) 18:6) stimulate growth in freshwater rainbow trout through the action of IGF-I (Taylor *et al.*, 2005).

Optimizing rearing conditions for rainbow trout, as well as identifying and implementing novel markers for the evaluation of the smolt status and the early detection of fish that will grow sub-optimally when transferred to seawater is crucial towards the improvement of both fish welfare and production. In this sense, a significant correlation between IGF-I abundance in blood plasma and growth has been previously reported for several fish species (Beckman *et al.*, 2001). However, the regulation of plasma IGF-I through the transcription of *igf-I*, which is highest in liver, has been studied mainly in relation to fish growth and information on the effects of photoperiod on its regulation is incomplete. Similarly, the transcription of other key growth-regulating genes in liver, such as *igfbp1b*,

that results in a protein that likely inhibits IGF-I from interacting with its receptor, and *ghr1*, which translates into the transmembrane receptor that activates the pathway that results in IGF-I production by the liver (Reindl and Sheridan, 2012) have not been studied in rainbow trout in relation to smoltification and photoperiod. Moreover, in a smoltification context, *cathepsin L* (*ctsl*) might prove an interesting marker, since it is a lysosomal endopeptidase involved in the turnover of cells and tissues, which is critical during the smoltification process (Björnsson *et al.*, 2012). The analysis of these growth-related factors is interesting not only from a mechanistic perspective but also within the context of this research, as they could be good candidates as growth-predictor markers.

From a smoltification perspective, *nkaα1a*, expressed in lamellar chloride cells in the gills, and *nkaα1b* and *nkcc1a*, found primarily in filamental chloride cells in the gills, have been reported in rainbow trout (Richards *et al.*, 2003; Katoh *et al.*, 2008; McCormick *et al.*, 2009). Studies in Atlantic salmon and rainbow trout showed that their transcription complements NKA activity, with *nkaα1b* and *nkcc1a* increasing in response to a seawater challenge while *nkaα1a* decreases, suggesting that the first two play a role in seawater tolerance while the third one is needed in freshwater (Nilsen *et al.*, 2007; Flores and Shrimpton, 2012; McCormick *et al.*, 2013). This is currently being exploited by the Atlantic salmon industry, as the transcription of these genes is increasingly replacing the analysis of NKA activity as quick smoltification markers (Nilsen *et al.*, 2007). However, their suitability as smoltification markers for rainbow trout is currently unknown.

Therefore, the objectives of this study are (1) to test the effect of different photoperiod regimes on the smoltification of rainbow trout through the measurement of the NKA activity over a five month period in freshwater, (2) to evaluate the suitability of gill *nkaα1a*, *nkaα1b*, *nkcc1a* transcription during this period as smoltification markers complementing NKA activity, (3) to analyse IGF-I abundance in plasma as a growth predictor in fish undergoing different photoperiod regimes and (4) to measure the transcription of key genes from the somatotropic axis in liver, *igf-I*, *igfbp1b*, *ghr1* and *ctsl*, to understand their role on growth regulation, the effect that different photoperiods have on them and evaluate their suitability together with plasma IGF-I as growth proxies.

2.2. Materials and methods

2.2.1. Fish and rearing conditions

Juvenile rainbow trout (AquaGen) with an initial weight of 78 ± 16.7 g were used in this experiment. Fish were fed *ad libitum* using a standard commercial dry diet (Skretting AS) from automatic feeders according to temperature and fish size. Fish were kept indoors in a flow through system using tanks

equipped with timer-controlled LED lights in a trout facility from Lerøy Vest AS (Bjørsvik, Hordaland, Norway). The fish were kept at ambient temperature, water flow at 0.4 L/kg/min and O_2 was above 80% saturation in the outlet.

2.2.2. Experimental design

Prior to the freshwater experimentation phase, fish were kept in 2 x 2 m rearing tanks (2,500 litres) under natural temperature and LL photoperiod for 2 weeks. On 18th February 2016 (mid-February), 160 fish were individually Carlin tagged for recording individual growth rates during both the freshwater and seawater phase of the experiment. Fish were randomly distributed into eight tanks, resulting in 20 tagged and 90 untagged fish per tank. After two weeks of acclimation the photoperiod regimes were initiated. The experimental design included four different photoperiod treatments from mid-February until mid-July, as shown in Figure 2.1a: LL (18 weeks of LD24:0), Advanced Phase Photoperiod (APP ; 6 weeks of LD12:12 followed by 12 weeks of LD24:0), Delayed Phase Photoperiod (DPP; 4 weeks of LD24:0 followed by 6 weeks at LD12:12 and 8 weeks at LD24:0) and Simulated Natural Photoperiod (SNP; starting at LD12:12 and increasing light time by 45 min every week until reaching LD24:0). Weight and length were recorded in tagged fish once per month during the freshwater phase (February to July). On the 5th of July 2016, the remaining non-tagged fish from all experimental groups were individually tagged and, along with previously tagged fish, length and weight were recorded before being randomly distributed into four replicate tanks supplied with seawater and kept at LL photoperiod (continuous light) in a common garden experiment to strengthen growth studies. Weight and length were recorded once more at the end-point sampling on the 14th of September. Water temperature was recorded once per day. During the freshwater phase it was measured on-site. For the seawater phase, it was retrieved from the records of a Marine Harvest facility (Stord, Hordaland, Norway) (Figure 2.1b).

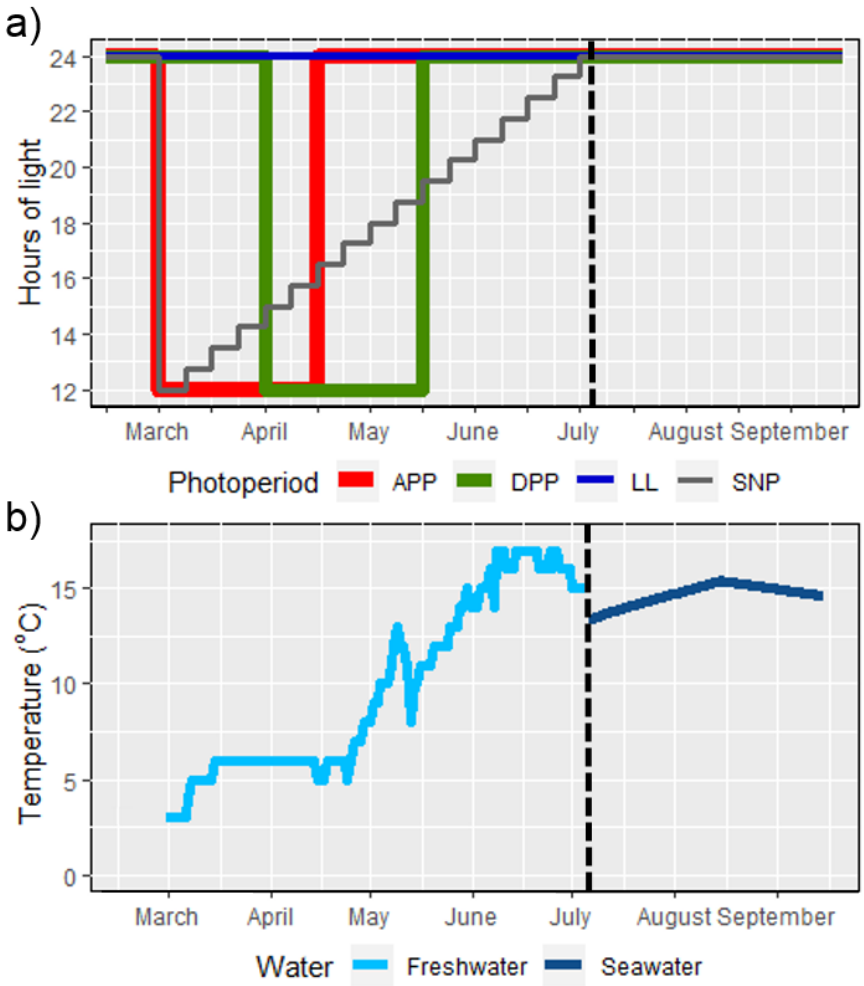


Figure 2.1. Photoperiod treatment and temperature during the experiment. Schematic representation of the number of hours of light for each of the four different photoperiod treatments (a) and water temperature during the experimentation period. Dashed lines indicate seawater transfer.

Experimental work was ethically reviewed, approved and registered by the Norwegian Animal Research Authority (NARA) and by the Animal Welfare and Ethical Review Body (AWERB 088), University of Stirling, UK.

2.2.3. Sampling

On the 18th of February 2016, ten fish per tank were sampled to secure a common biological starting point prior to experimental photoperiod treatments being initiated. Lethal samplings of six fish per tank (12 per group) were conducted every two weeks during the freshwater phase. Samplings took place on 3rd of March, 17th of March, 31st of March, 13th of April, 27th of April, 11th of May, 25th of May,

9th of June, 22nd of June and 5th of July. A final lethal sampling was done 9 weeks after seawater transfer (14th of September).

Fish were quickly dip-netted out of the tanks and euthanized by a lethal overdose of isoeugenol (AQUI-S). For each fish, weight and length were recorded. Blood was extracted using heparinised syringes and centrifuged at 3,000 x g for 5 min to obtain plasma, which was frozen at -80°C. The first gill arch from each side of the fish were dissected out and preserved at -80°C; one in SEI buffer (Sucrose 250 mM, Na_2EDTA 10 mM, Imidazole 50 mM (all Sigma-Aldrich)) and the other one in RNAlater (ThermoFisher Scientific). Liver samples were also preserved in RNAlater according to manufacturer's guidelines (overnight at 4°C and frozen at -80°C).

2.2.4. Gill NKA activity

Between March and July (freshwater phase) gill NKA activity of all fish sampled (12 per group) were analysed. For the seawater phase (final sampling), only the 50 fish above the third quartile in length (31.7 cm) with the highest condition factor and the 50 fish below the first quartile in length (29.0 cm) with the lowest condition factor were analysed.

NKA activity was measured according to McCormick's methodology, which couples the hydrolysis of ATP to the enzymatic production of NAD^+ through the involvement of the enzymes pyruvate kinase and lactate dehydrogenase, and uses the NKA inhibitor ouabain to trace the baseline (McCormick, 1993). Kinetic assay readings were carried out at 340 nm for 10 min (60 cycles) at 25°C in a Sunrise-basic (Tecan) spectrophotometer. Total amount of protein in the homogenate was analysed using a bicinchoninic acid (BCA) assay run in triplicate. NKA values were determined as the ouabain sensitive fraction of the ATP hydrolysis, expressed as µmol ADP mg $protein^{-1}$ $hour^{-1}$.

2.2.5. Real-time polymerase chain reaction (RT-PCR)

All freshwater samples were analysed for gill *nkaα1a*, *nkaα1b* and *nkcc1a* and, with the exception of the samples from the first sampling in February, for liver *igf-I*, *igfbp1b*, *ghr1* and *ctsl* mRNA abundance. For the seawater phase only the 50 fish above the third quartile (31.7 cm) in length with the highest condition factor and the 50 fish below the first quartile in length (29.0 cm) with the lowest condition factor were analysed for liver *igf-I*, *igfbp1b*, *ghr1* and *ctsl* mRNA abundance.

Before total RNA isolation of samples, 20-25 mg of tissue was homogenized in RLT buffer (Qiagen) with zirconium oxide beads (1.4 µm) using a homogenizer (5,000 rpm, 15 min) (Precellys 24, Bertin Technologies). Subsequent total RNA isolation was carried out using the Qiasymphony RNA kit in the QIAsymphony SP automatic system following manufacturer instructions (Qiagen).

Total RNA concentration and purity was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Purity was confirmed with 260/280 and 260/230 ratios above 1.8. A selected number of samples were assessed for RNA integrity on RNA 6000 Nano LabChip® kit using the Agilent 2100 Bioanalyzer (Agilent Technologies). Integrity was confirmed with RIN values higher than 8.

Complementary DNA (cDNA) was reversely transcribed using 1.5 µg (gill) or 1.4 µg (liver) of total RNA using oligo(dT_{20}) primer and the Superscript III kit (Thermo Fisher Scientific) using a MicrolabSTARlet Liquid Handling Workstation (Hamilton Robotics).

RT-PCR was carried out in a CFX-96 RT-PCR detection system platform (Bio-Rad) using the following PCR conditions: 3 min at 95°C, 34 cycles of 15 seconds at 95°C and 1 min at 60°C and a melting curve step at the end (10 seconds at 95°C, 5 seconds at 65-95°C with increments of 0.5°C and 5 seconds at 95°C). For each assay, triplicate two-fold cDNA dilution series from pooled samples (1:5-1:160) were used to determine amplification efficiencies. Samples were run in 25 µl duplicates using iTaq universal SYBR green supermix (Bio-Rad), 0.20 µM of each primer and 5 µl of diluted cDNA (dilution 1:50 for gill and 1:30 for liver). Each plate included a negative control as well as a common pooled sample used for the intercalibration of assays among plates. The relative transcription levels of the genes were normalized following the efficiency corrected method (Pfaffl *et al.*, 2004) using *elongation factor 1α* (*ef1α*) as an endogenous reference gene (Olsvik *et al.*, 2005). Primers used in this study are summarized in Table 2.1.

Table 2.1. Primers used for RT-PCR analysis and accession numbers of the gene sequences (GenBank).

Gene name	Primer sequence (5'>3')	Accession number	Reference
nkaα1a	CCAGGATCACTCAATGTCACTCT CAAAGGCAAATGGGTTTAATATCAT	XM_021573245	(Nilsen *et al.*, 2007)
nkaα1b	GCTACATCTCAACCAACAACATTACAC TGCAGCTGAGTGCACCAT	XM_021570999	(Nilsen *et al.*, 2007)
nkcc1a	GATGATCTGCGGCCATGTTC CTGGTCATTGGACAGTTCTTTG	XM_021601694	(Nilsen *et al.*, 2007)
igf-I	TGCGGAGAGAGAGGCTTTTA AGCACTCGTCCACAATACCA	M81904	(Rolland et al., 2015)
igfbp1b	AGTTCACCAACTTCTACCTACC GACGACTCACACTGCTTGGC	AF403539	(Gabillard et al, 2006)
ghr1	CGTCCTCATCCTTCCAGTTTTA GTTCTGTGAGGTTCTGGAAAAC	AF403539	(Gabillard et al., 2006)
ctsl	CAACTACCTGCAGGCACCTA ACATGATCCCTGGTCCTTGAC	AF358668	(Rolland et al., 2015)
efα1	CCCCTCCAGGATGTCTACAAA CACACGGCCCACGGGTACT	AF498320	(Genge *et al.*, 2013)

2.2.6. Time-resolved fluoro-immunoassay (TR-FIA) for plasma IGF-I

Circulating IGF-I levels were measured in plasma collected from 58 randomly selected tagged fish (n=16 SNP; n=16 APP, n=17 LL and n=9 DPP) at the beginning (July) and at the end (September) of the seawater period.

TR-FIA protocol was used to measure plasma IGF-I concentration (Small and Peterson, 2005). Prior to the assay, plasma IGF-I was dissociated from the binding protein with acid-ethanol (Shimizu *et al.*, 2000). Briefly, 96-well DELFIA pre-coated goat anti-rabbit IgG Microtitration plates (Perkin Elmer) were washed with 200 µl DELFIA wash buffer before each well received 20 µl anti-barramundi IGF-I rabbit antiserum (GroPep; diluted 1:8000) and 100 µl of standard-recombinant salmon IGF-I (GroPep) or 20 µl extracted sample (Cleveland *et al.*, 2018). Standards and samples were diluted in Assay Buffer (Perkin Elmer). Plates were incubated overnight with shaking (600 rpm at 4^{o}C). Europium labelled (0.05 ng μl^{-1}) IGF-I was added to each well and the plate incubated overnight under agitation (600 rpm at 4^{o}C). The plate was washed six times with 200 µl Washing Buffer (Perkin Elmer) before adding 200 µl DELFIA enhancement solution (PerkinElmer) to each well. After shaking at 600 rpm for 10 min at RT, time-resolved fluorescence was measured by a fluorometer (ARVO X4; PerkinElmer) with excitation and read wavelengths at 340 and 615 nm, respectively. Parallel displacement of dilutions of extracted plasma from rainbow trout with the standard was confirmed (Figure 2.2). The half-maximal displacement (ED_{50}) occurred at 0.77 ± 0.02 ng/ml (mean ± SEM, n = 4). The ED_{80} and ED_{20} were 2.08 ± 0.05 ng/ml (n = 4) and 0.29 ± 0.01 ng/ml (n = 4), respectively. The minimum detection limit of the assay, defined as the mean count of the zero standard minus two standard deviations, was 0.06 ± 0.03 ng/ml (n = 4). The intra- and inter-assay coefficients of variation were 5.4 ± 0.2% (n = 4) and 9.8 ± 0.9% (n = 4), respectively.

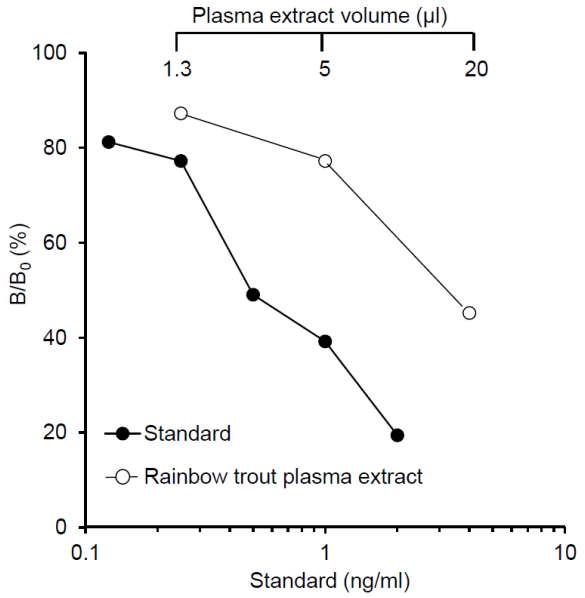


Figure 2.2. Displacement of Eu-labeled salmon IGF-I with IGF-I standard and plasma extract from rainbow trout. B represents binding in the presence of competitor and B0 represented binding in its absence.

2.2.7. Growth calculations

Specific growth rate in length (SGR-L) was calculated using the formula:

$$100 \times \frac{Ln\,(Length_{Final}) - Ln\,(Length_{Initial})}{Number\ of\ days}$$

Similarly for specific growth rate in weight (SGR-W):

$$100 \times \frac{Ln\,(Weight_{Final}) - Ln\,(Weight_{Initial})}{Number\ of\ days}$$

The condition factor was calculated with Fulton's formula:

$$100 \times \frac{Weight}{Length^{3}}$$

2.2.8. Data analysis and representation

Statistical tests were performed using R statistical software. Data representation was carried out using R package ggplot2 (Wickham, 2009).

One-way ANOVA was performed at each time point to find differences among treatments (effect of photoperiod) and also performed on the whole time-series for each treatment (effect of time). Data was transformed by either natural logarithm or square root to satisfy the normal distribution and homogeneity of variance assumptions, tested with the Shapiro and Bartlett tests, respectively. Significant comparisons ($p < 0.05$) were followed by Tukey's posthoc test to identify different treatments.

Linear relationship among variables was determined by linear regression using the QR method. Significance values ($p < 0.05$) were obtained by testing the null hypothesis: the slope of the least squares linear fit to the data is equal to 0.

Quadratic model fit: Measurements for any particular photoperiod treatment that followed a clear parabolic trend were fitted to a quadratic model. Estimated parameters were: initial value, *init*, maximum value, *Ymax*, and time when *Ymax* occurred, *Tmax*. These were estimated using the formula:

$$a \times Time^2 + b \times Time + c, \text{ where:}$$

$$a = (c - Ymax)/(Tmax^2),$$

$$b = -2 \times a \times Tmax,$$

$$c = init$$

Calculated estimates of a particular measurement were considered different between treatments (photoperiod effect) if the estimates did not overlap (value ± s.e.) between two treatments.

2.3. Results

2.3.1. Fish growth

2.3.1.1. Freshwater

When analysing tagged fish, it is possible to visualize the growth trajectory of each fish (Figure 2.3a) and to calculate specific growth rate (*i.e.* SGR-L and SGR-W). SGR-L was low during the first three months, after which it increased steadily until June, before decreasing again until the end of the experiment (Figure 2.3b). Though no significant differences were found for either length or weight (Figure 2.3a, Figure 2.4a) differences were present in both SGR-L and SGR-W. The overall SGR-L during the freshwater phase (February to July) was significantly lower ($p < 0.01$, df: 3, F: 4.53) in DPP compared to the other three treatments (data not shown). Month per month, the SGR-L of fish kept

at DPP was significantly lower than for fish kept at APP in April-May and May-June, for fish kept at SNP in April-May and May-June, and finally compared to fish kept at LL but only in May-June (Figure 2.3b). A very similar result was found for SGR-W (Figure 2.4b). Results related to length were given priority over those related to weight for simplicity in further analysis but since the two were so similar (*i.e.* highly correlated, $p < 0.001$, slope= 23.86), the results for one can be extrapolated to the other.

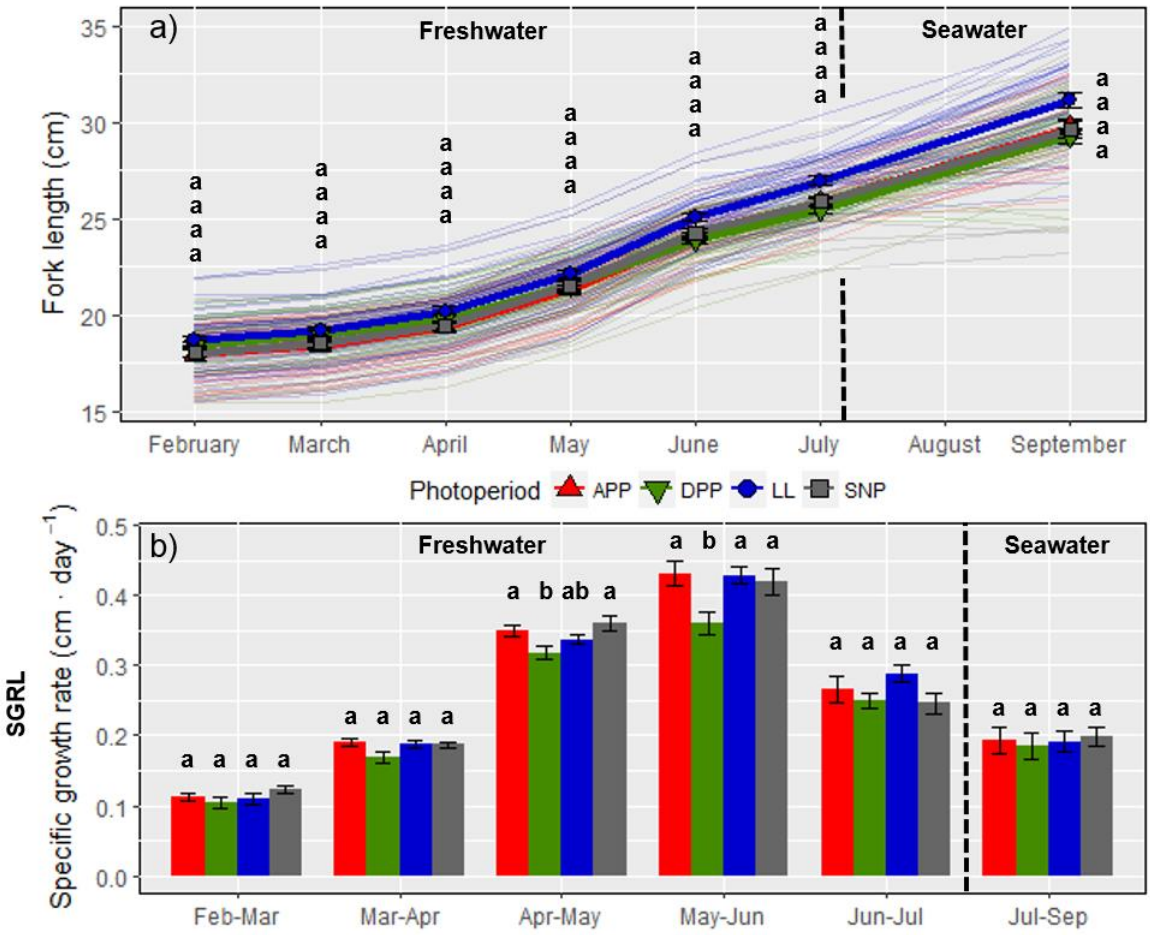


Figure 2.3. Fork length (cm, ± 1 s.e.) of juvenile rainbow trout reared in freshwater under four different photoperiod treatments and transferred to seawater on the 5th of July. Mean fork length (points and thick lines) and individual growth trajectory (thin lines) of each tagged fish, measured once per month (a) and mean SGR-L between samplings (b). Samplings took place on 24th of February, 18th of March, 14th of April, 12th of May, 10th of June, 5th of July and 14th of September. Error bars indicate s.e. The dashed line indicates seawater transfer. Different letters indicate statistical differences within a time point ($p < 0.05$).

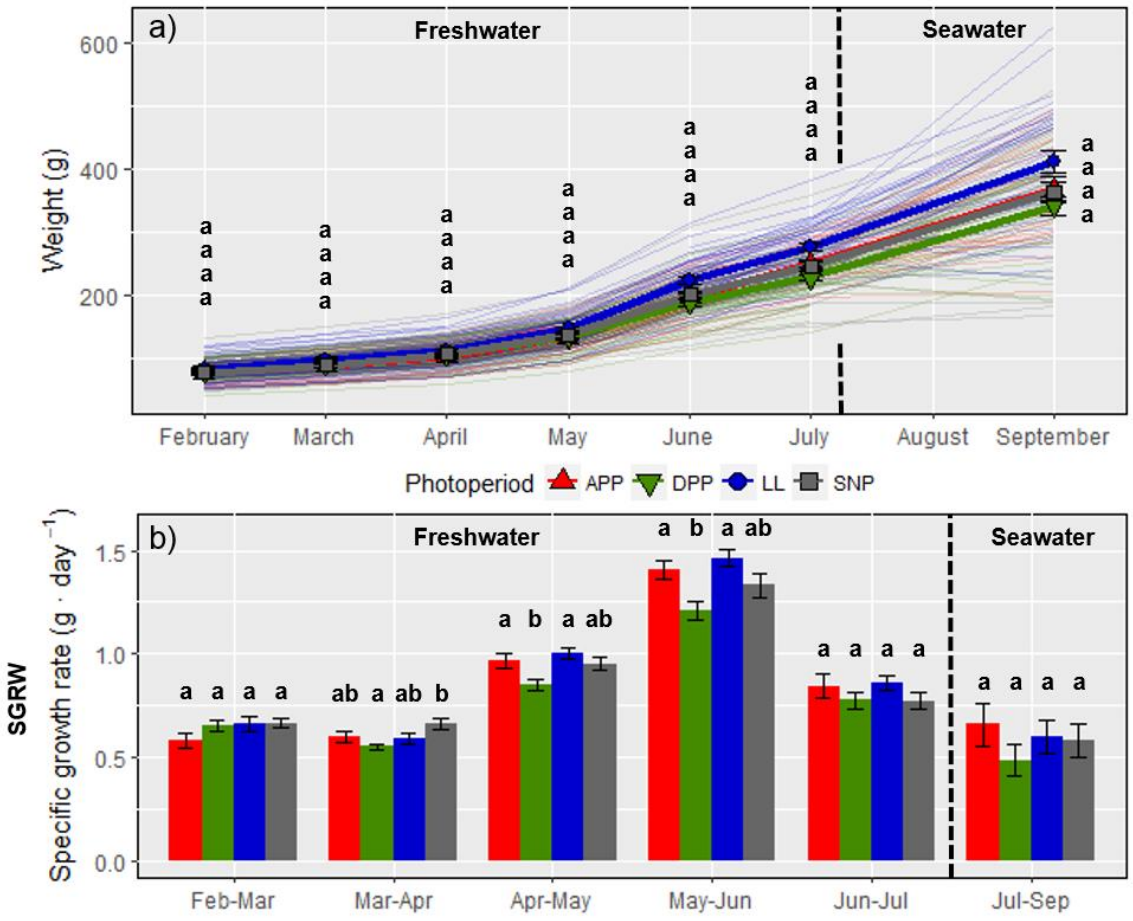


Figure 2.4. Weight (g, ± 1 s.e.) of juvenile rainbow trout reared in freshwater under four different photoperiod treatments and transferred to seawater on the 5th of July. Mean weight (points and thick lines) and individual weight increase (thin lines) of each tagged fish, measured once per month (a) and mean SGR-W between samplings (b). Samplings took place on 24th of February, 18th of March, 14th of April, 12th of May, 10th of June, 5th of July and 14th of September. The dashed line indicates seawater transfer. Error bars indicate s.e. Different letters indicate statistical differences within a time point ($p < 0.05$).

2.3.1.2. Seawater

After nine weeks in seawater at LL, no differences in length (Figure 2.3a), SGR-L (Figure 2.3b), weight (Figure 2.4a) or SGR-W (Figure 2.4b) were found among fish that had been reared in different photoperiod treatments during their freshwater phase.

There was a significant correlation between the overall freshwater SGR-L (February to July) and seawater SGR-L ($p < 0.01$, slope= 0.07). However, the worst performing fish in seawater (SGR-L below

0.10 cm*day^{-1}) were not consistently the fish with the worst freshwater SGR-L (0.25 ± 0.010 cm*day^{-1}) in the total population (0.27 ± 0.004 cm*day^{-1}).

2.3.2. The effect of different photoperiod regimes on NKA activity

2.3.2.1. Freshwater

Fish from all photoperiod treatments experienced a significant increase in gill NKA activity from mid-March to April, followed by a plateau from April until mid-May/June which was followed by a sharp decrease (Figure 2.5a). However, while NKA activity in fish kept at LL, SNP and DPP peaked in mid-May and started to decrease in June, NKA activity in fish kept at APP peaked two weeks later (June) and decreased also later in mid-June. At the final sampling point in July, NKA activity in fish from all treatments reached comparable low values, similar to those recorded in March (full statistical analysis available in Table 2.2). Significant differences among treatments were only found in early June when NKA activity in fish kept at APP was significantly higher than in fish kept at DPP (Tukey test, $p < 0.001$).

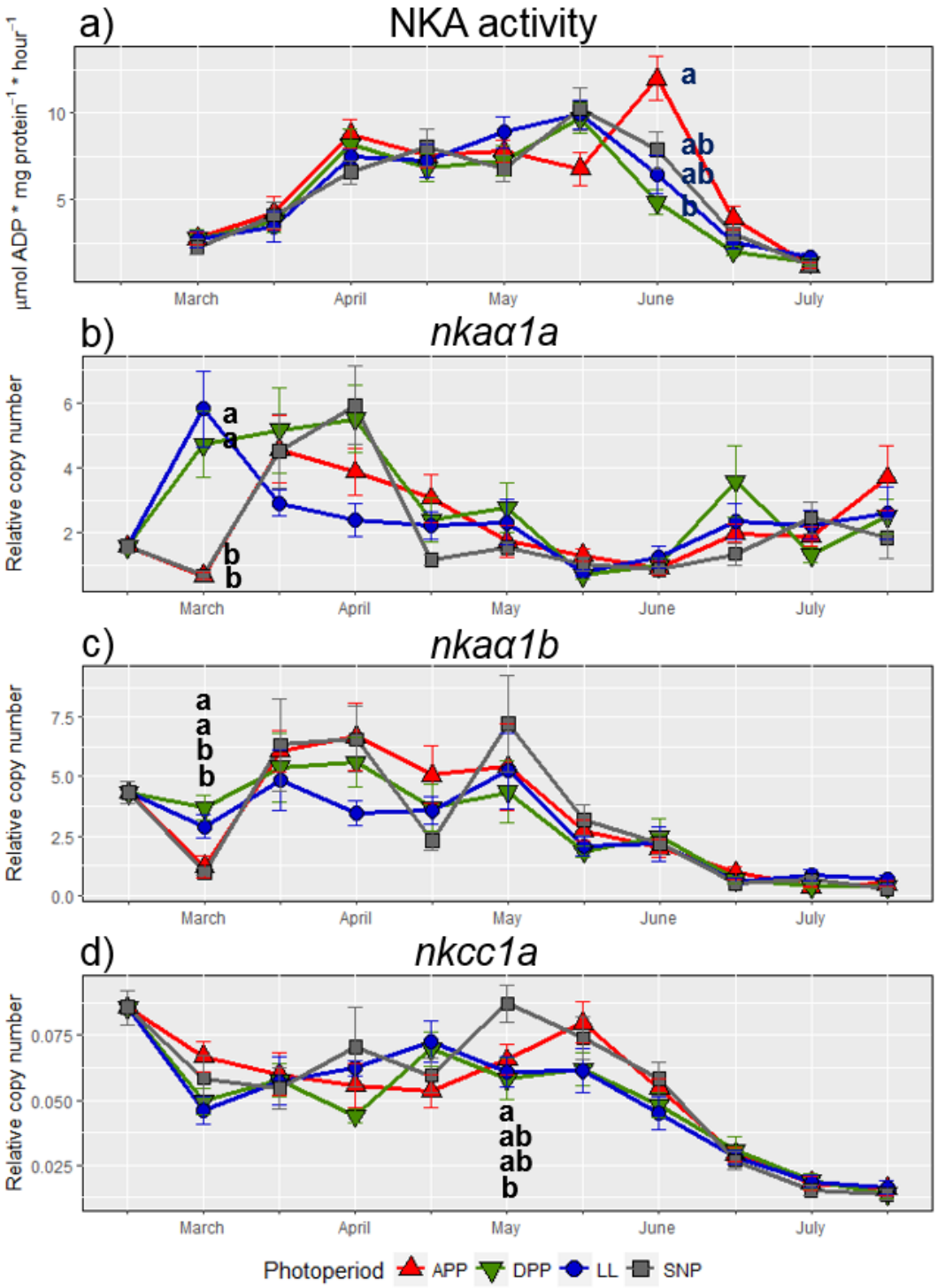


Figure 2.5. Gill NKA activity (a) and gill relative gene transcription of related genes, *nkaα1a* (b), *nkaα1b* (c) and *nkcc1a* (d) of juvenile rainbow trout in freshwater under four photoperiod treatments. Error bars indicate s.e. Different letters indicate statistical differences with a time point ($p < 0.05$). Samplings took place on 3rd of March, 17th of March, 31st of March, 13th of April, 27th of April, 11th of May, 25th of May, 9th of June, 22nd of June and 5th of July. The dashed line indicates seawater transfer.

Table 2.2. Tukey's test results for the differences among sampling points for each treatment. Different letters indicate significant differences within a time point.

	APP				DPP				LL				SNP			
	nkcc1a	*nka1a*	*nka1b*	NKA	*nkcc1a*	*nka1a*	*nka1b*	NKA	*nkcc1a*	*nka1a*	*nka1b*	NKA	*nkcc1a*	*nka1a*	*nka1b*	NKA
Mid-Feb	b	abc	cd	-	c	bcd	ab	-	c	bc	b	-	a	ac	c	-
March	ab	ab	ab	be	ab	ab	ab	abe	ab	a	ab	a	a	a	ab	bd
Mid-March	ab	c	c	ab	ac	a	ab	ab	ac	ab	ab	a	ab	bc	c	ab
April	a	c	c	cd	ab	a	a	c	ac	abc	ab	b	a	b	c	ac
Mid-April	ac	ac	cd	cd	ac	abcd	ab	cd	ac	abc	ab	b	a	ac	acd	c
May	ab	abc	cd	cd	a	abcd	ab	cd	ac	abc	ab	b	a	abc	c	ac
Mid-May	ab	abc	cd	ac	ac	c	b	c	ac	c	ab	b	a	a	cd	c
June	a	b	ad	d	ab	cd	b	ad	ab	c	ac	b	a	a	ad	c
Mid-June	cd	abc	ab	ab	bd	abd	c	be	bd	abc	d	a	bc	ac	be	bd
July	d	abc	b	e	d	bcd	c	e	d	abc	cd	a	c	abc	be	d
Mid-July	d	c	b	-	d	abd	c	-	d	abc	d	-	c	a	e	-

2.3.2.2. Seawater

The NKA activity in seawater (September) showed no differences among fish reared in any of the freshwater photoperiod treatments (Data not shown; APP: 2.7 ± 0.29, DPP: 3.2 ± 0.53, LL: 2.8 ± 0.30, SNP: 2.5 ± 0.31 µmol ADP mg protein^{-1} hour^{-1}).

2.3.3. Transcription of *nkaα1a*, *nkaα1b* and *nkcc1a* complement NKA activity results

Freshwater transcription of *nkaα1b* and *nkcc1a*, changed in a similar fashion to NKA activity while the transcription of *nkaα1a* changed in an inversely manner with highest values recorded from March to April instead of May to June (Figure 2.4b-d). Taking into consideration the whole freshwater period, all three genes, *nkaα1a*, *nkaα1b* and *nkcc1a*, correlated significantly with NKA activity (data not shown, $p < 0.001$, slope= -0.15; $p < 0.01$, slope= 0.13; $p < 0.001$, slope= 0.002; respectively).

The relative gill *nkaα1a* mRNA abundance was highest at the start of the trial, decreasing after mid-April and reaching minimum values in mid-May and June (Figure 2.4b). In March, the transcription of this gene in fish kept at LD24:0 (LL, DPP) was significantly higher than in those kept at a photoperiod that changed to LD12:12 (SNP, APP) ($p < 0.001$, df: 3, F: 18.72).

For all the different treatments, *nkaα1b* increased in mid-March and decreased in mid-May, earlier than NKA activity in both cases (Figure 2.4c). Similarly to *nkaα1a*, fish kept at LL (LL, DPP) was higher than those at LD12:12 (SNP, APP) in March ($p < 0.001$, df: 3, F: 11.56).

The transcription of *nkcc1a* was stable (no statistical differences on time) until it decreased at the same time as the NKA activity, in mid-June (Figure 2.4d). After decreasing, its values were significantly lower than in February (Figure 2.4d; Table 2.2). Transcription of *nkcc1a* in fish kept at SNP was significantly higher than in fish kept at DPP in May (Tukey test, $p < 0.05$).

2.3.4. *igf-I, igfbp1b, ghr1* and *ctsl* transcription and plasma IGF-I abundance in response to different photoperiod regimes

2.3.4.1. Freshwater

In freshwater, the transcription of *igf-I*, *ghr1* and *ctsl* followed a similar trend as gill NKA enzyme activity while *igfbp1b* showed less variation over time (Figure 2.6). Independently of photoperiod treatment, *igf-I* increased between March-April and then decreased in mid-June. This trend was also observed in *ghr1*, which increased sharply in mid-May before decreasing again in mid-June and in *ctsl*, which slowly increased until mid-May and decreased in June.

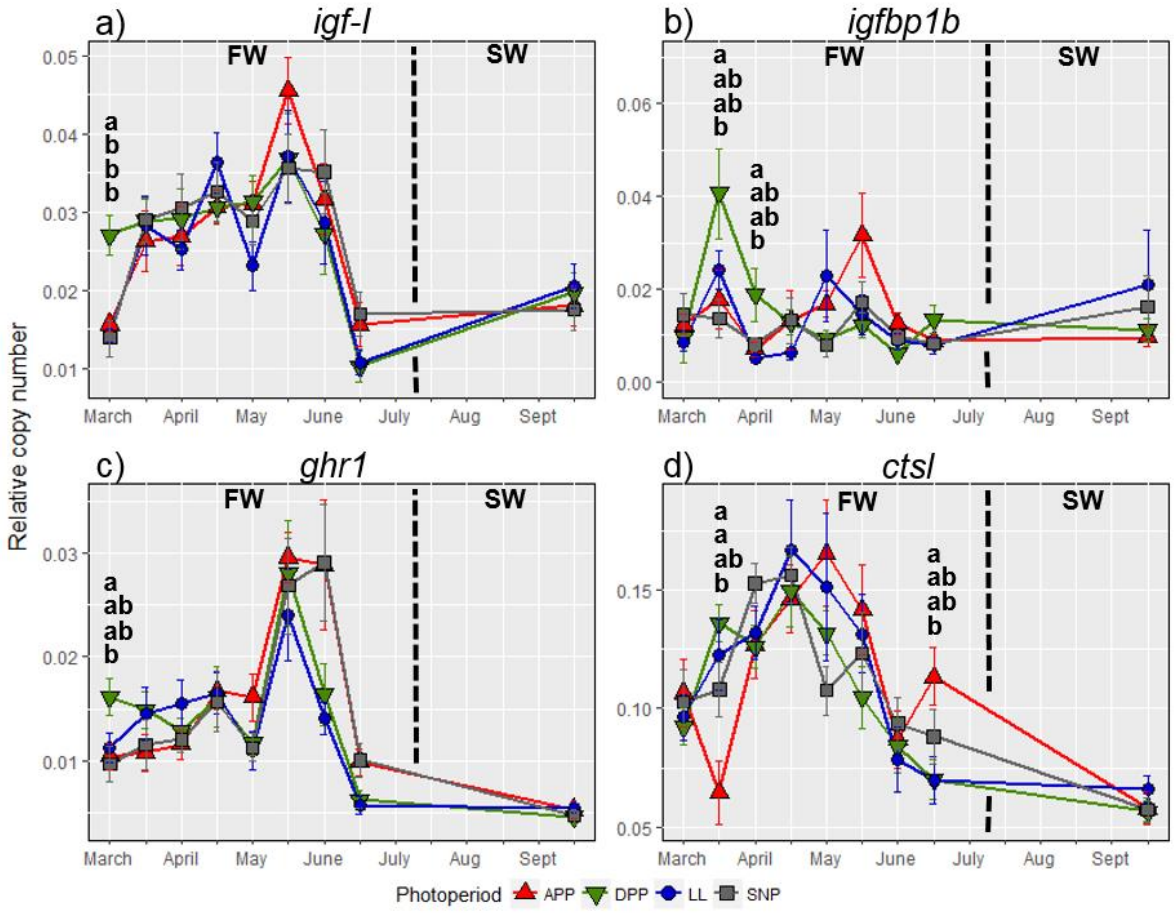


Figure 2.6. Liver transcription of genes of the somatotropic axis, *igf-I* (a), *igfbp1b* (b), *ghr1* (c), and *ctsl* (d), of juvenile rainbow trout reared in freshwater under four different photoperiod treatments and transferred to seawater on the 5th of July. Error bars indicate s.e. Samplings took place on 3rd of March, 17th of March, 31st of March, 13th of April, 27th of April, 11th of May, 25th of May, 9th of June, 22nd of June, 5th of July and 14th of September. Different letters indicate statistical differences within a time point ($p < 0.05$). No letters indicate lack of significant differences. The dashed line indicates seawater transfer.

Statistical differences among groups were found mostly at the beginning of the experiment, with *igf-I* in March being higher in fish kept at DPP compared to APP, LL and SNP. Similarly *ghr1* in March was higher in fish kept at DPP compared to those at SNP. During the two following months *igfbp1b* was higher in fish kept at DPP compared to those kept at SNP. Finally, *ctsl* was higher in fish kept at DPP and LL compared to those kept at APP in mid-March and in mid-June *ctsl* was higher in fish kept APP compared to those at LL.

Plasma IGF-I levels in July, were significantly higher in APP compared to the other treatments ($p < 0.001$, df: 3, F: 6.68) (Figure 2.7a).

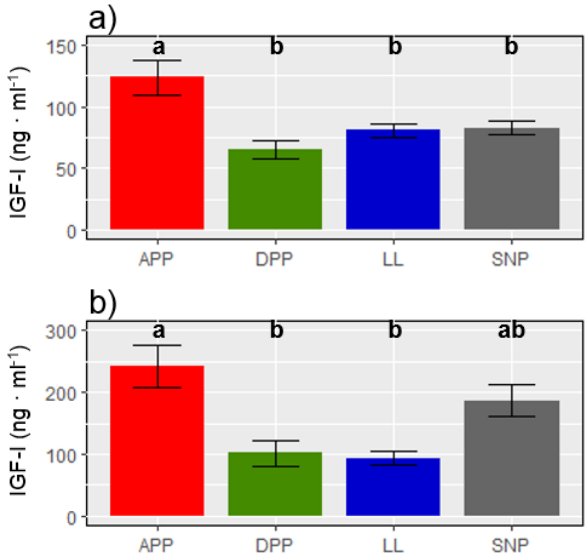


Figure 2.7. Circulating IGF-I in blood plasma of juvenile rainbow trout reared in freshwater under four different photoperiod, sampled before seawater transfer (5th of July) (a) and after two months in seawater (14th September) (b). Error bars indicate s.e. Different letters indicate statistical differences within a time point ($p < 0.05$).

2.3.4.2. Seawater

In the seawater phase, no significant differences among fish reared in any of the freshwater photoperiod treatments were found for any of the genes analysed (Figure 2.6).

In September, plasma IGF-I levels were significantly higher in fish that had been kept at APP during freshwater phase compared to fish that had been kept at DPP and LL (Figure 2.7b) ($p < 0.001$, df: 3, F: 6.98).

2.3.5. Quadratic model fit

Freshwater SGR-L over time for each photoperiod treatment was fitted to a quadratic model (Figure 2.8a, Table 2.3). According to the estimated model there were no differences among treatments for the initial values (*init)*. However, the maximum value (*Ymax)*, was lower in DPP (0.33 ± 0.008 cm * day^{-1}) compared to the other treatments (ranging from 0.356 to 0.379 cm * day^{-1}). Moreover, the day when the maximum value occurred (*Tmax*) was later for LL (at day 155 ± 2.5) than for SNP (at day 147 ± 2.1).

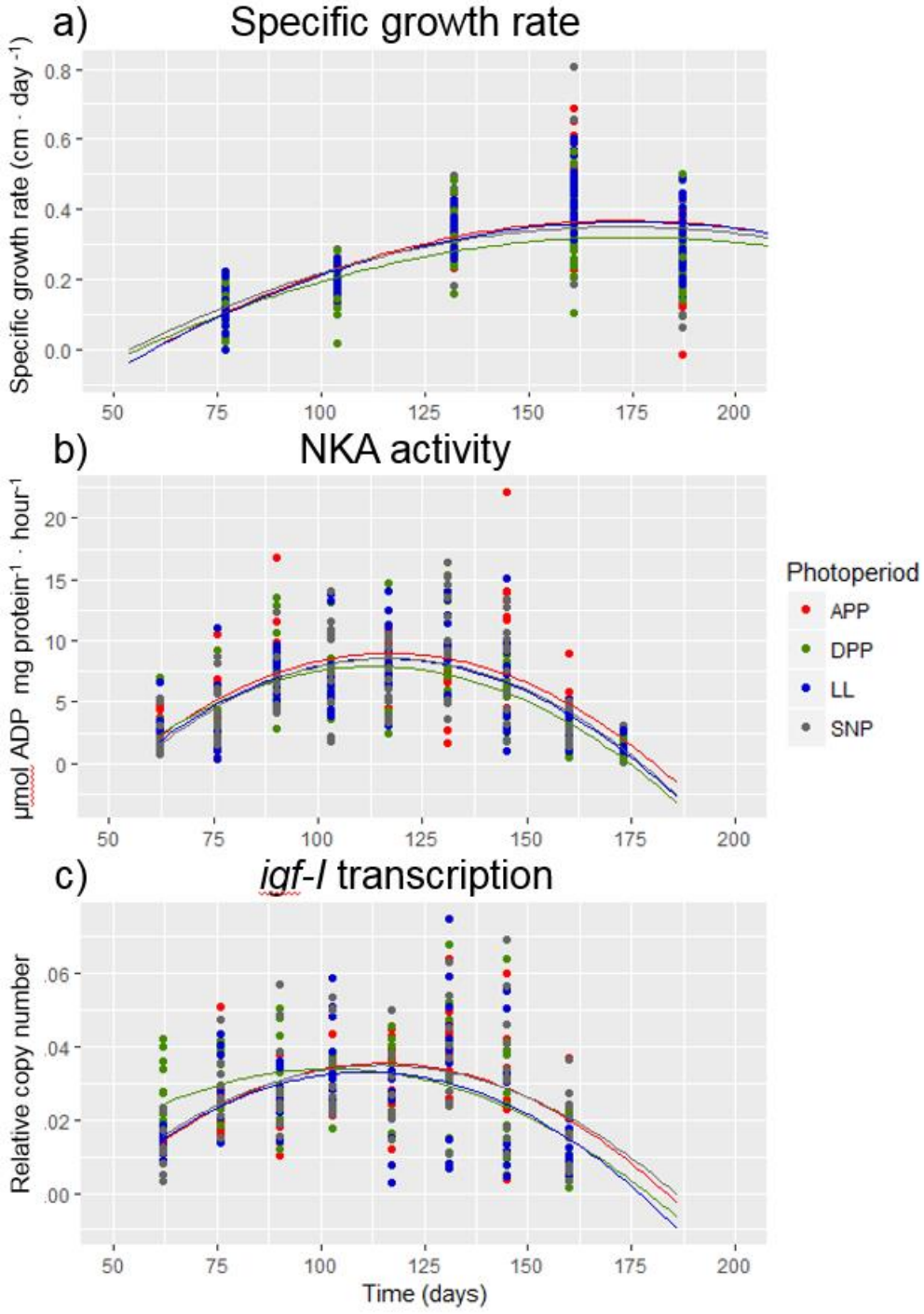


Figure 2.8. Quadratic model fit to SGR-L (a), gill NKA activity (b) and liver *igf-I* gene transcription (c) during the freshwater phase of juvenile rainbow trout under four different photoperiod treatments. Samplings for SGR-L took place on 24th of February (day 55), 18th of March (day 78), 14th of April (day 105), 12th of May (day 133), 10th of June (day 162), 5th of July (day 188). Samplings for NKA activity and *igf-I* transcription took place on 3rd of March (day 63), 17th of March (day 77), 31st of March (day 91), 13th of April (day 104), 27th of April (day 118), 11th of May (day 132), 25th of May (day 146), 9th of June (day 161), 22nd of June (day 174).

Table 2.3. Estimates of a quadratic model fit to SGR-L (a), gill NKA activity (b), liver *igf-I* gene expression (c), and *ctsl* gene expression (d) of juvenile rainbow trout in freshwater under four different photoperiod regimes. *init* is the initial value, *Ymax*, the maximum value and *Tmax* is the day when *Ymax* occurred.

		APP		DPP		LL		SNP	
		Estimate	**s.e.**	**Estimate**	**s.e.**	**Estimate**	**s.e.**	**Estimate**	**s.e.**
SGR-L									
	init	-0.806	0.083	-0.691	0.073	-0.755	0.067	-0.838	0.0829
	tmax	152.515	2.753	152.127	2.533	155.372	2.478	147.772	2.069
	ymax	0.370	0.009	0.327	0.008	0.366	0.007	0.365	0.009
NKA									
	init	-21.772	3.677	-19.393	3.058	-22.669	3.079	-23.431	3.268
	tmax	117.148	1.970	113.501	1.78	116.016	1.592	116.925	1.673
	ymax	9.000	0.478	7.924	0.387	8.557	0.403	8.568	0.426
Igf-I									
	init	-0.065	0.017	-0.028	0.017	-0.061	0.019	-0.056	0.018
	tmax	115.110	2.681	102.720	3.990	111.016	2.97	114.577	3.100
	ymax	0.035	0.002	0.034	0.002	0.033	0.002	0.035	0.002
ctsl									
	init	-0.117	0.079	-0.090	0.049	-0.179	0.081	-0.054	0.059
	tmax	114.178	4.869	101.347	3.234	104.683	3.582	103.440	4.619
	ymax	0.141	0.009	0.136	0.006	0.150	0.009	0.136	0.007

Similarly, freshwater NKA activity over time for each photoperiod treatment was also fitted to a quadratic model (Figure 2.8b, Table 2.3). According to the estimated model there were no differences among treatments for the initial values (*init)* and the day when the maximum value occurred (*Tmax*). However, the maximum value (*Ymax)*, was higher in APP (9.0 ± 0.48 µmol ADP mg protein^{-1} hour^{-1}) compared to DPP (7.9 ± 0.39 µmol ADP mg protein^{-1} hour^{-1}).

The transcription of *igf-I* and *ctsl* during the freshwater period followed a parabolic trend similar to NKA activity data, thus each treatment was fitted to a quadratic model. Estimates for *igf-I* indicate that *Tmax* occurs earlier for DPP (at day 102.7 ± 3.99) than for any of the other treatments (ranging from 108.1 to 117.79 days) (Figure 2.8c, Table 2.3). Similarly, for *ctsl* transcription *Tmax* occurs earlier in DPP (at day 101.4 ± 3.23) than in APP (at day 114.2 ± 4.87) (Table 2.3).

2.3.6. Relationship between plasma IGF-I, liver *igf-I*, *igfbp1b*, *ghr*, *ctsl* and growth in seawater

Tagged fish were used to study the relationship between growth in seawater (assessed through the SGR-L between the time of transfer in July and the end-point sampling after two months in September) plasma IGF-I (July and September) and liver gene transcription (September).

The relationship of SGR-L in September with the plasma IGF-I in July was not significant (Figure 2.9a, $p < 0.05$, slope= 96.67). However, SGR-L in September was significantly correlated with plasma IGF-I in September (Figure 2.9b, $p < 0.01$, slope= 431.21). Moreover, a significant correlation between the liver *igf-I* transcription in September and the SGR-L in seawater was found (Figure 2.9c, $p < 0.001$, slope= 0.08). Correlations of SGR-L with *igfbp1b*, *ghr1* and *ctsl* were not significant.

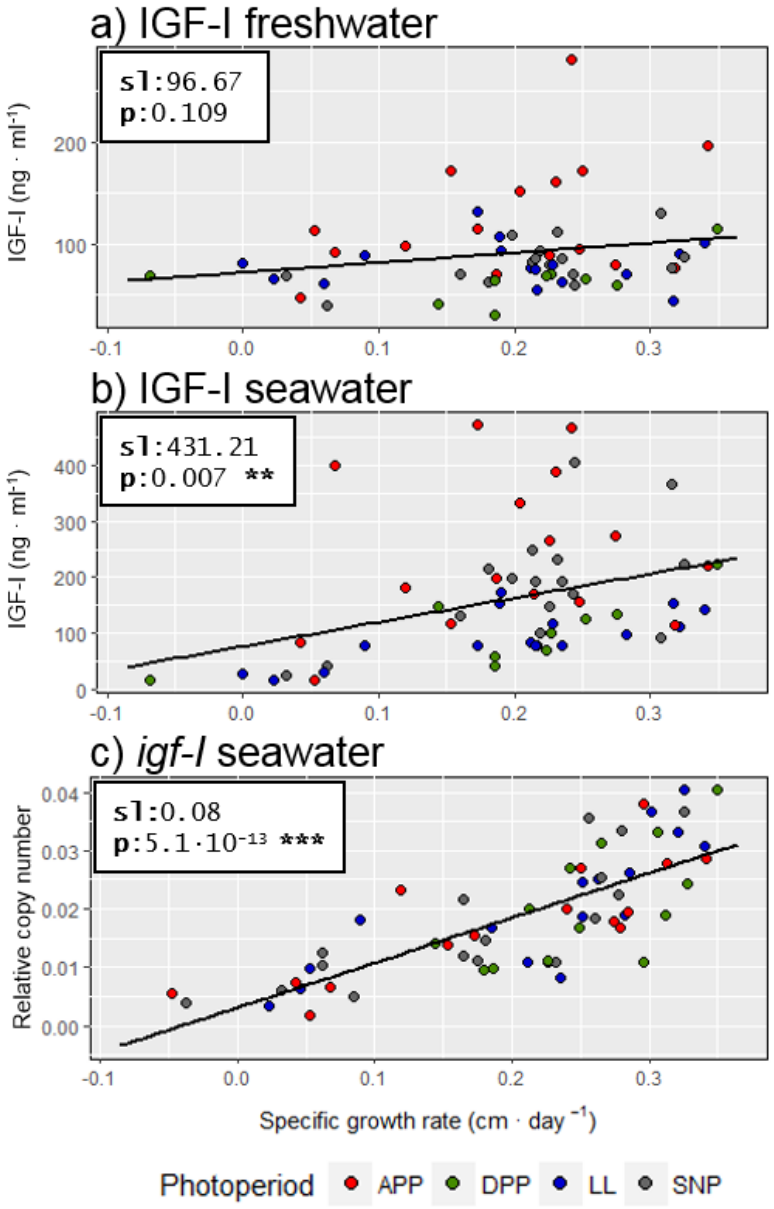


Figure 2.9. Relationship between SGR-L and plasma IGF-I or liver *igf-I* transcription of rainbow trout sampled in seawater on the 14th of September. Relationship between the SGR-L between July and September with plasma IGF-I prior seawater transfer (July) (a), plasma IGF-I in seawater (September) (b) and liver *igf-I* transcription in seawater (September) (c).

A significant correlation was also found for the plasma IGF-I in July and the plasma IGF-I in September ($p < 0.001$, slope= 0.17). However, the correlation between plasma IGF-I and liver *igf-I* in September was not significant (p: 0.16, slope: $2.7*10^{-5}$).

2.4. Discussion

In salmon aquaculture it is common to use different photoperiod regimes to produce robust smolts throughout the year while ensuring optimal growth and welfare of fish (Handeland and Stefansson, 2001). In contrast, the preferred photoperiod protocol for production of rainbow trout smolts is LL, despite little evidence to support that LL is indeed the best suited photoperiod to induce smoltification related traits in this species. This study aimed at evaluating the effect of different photoperiods on smoltification through the use of both traditional and well established smolt assessment tools, such as NKA activity measurement, and less established molecular tools, like the transcription of osmoregulatory genes through RT-PCR. According to results from this study, there is no reason to disregard LL as a suitable photoperiod regime for rainbow trout smoltification. Moreover, since a growth-stunted phenotype of fish has been reported in rainbow trout once transferred to seawater, liver transcription of *igf-I*, *igfbp1b*, *ghr1* and *ctsl*, and the abundance of circulating IGF-I in plasma were measured and evaluated in relation to both photoperiod treatment received in freshwater and somatic growth during the seawater phase. This experiment was performed on the winter to summer period, since a majority of the problems encountered by the industry occur in summer post-smolts. In this study, a strong correlation between growth in seawater and IGF-I (both circulating in plasma and in liver transcription) was found, highlighting that low levels of this hormone (among others that were not tested; a hormonal dysregulation) is one of the factors involved in the development of the GS phenotype.

Growth was lower in fish kept at DPP compared to fish kept at the other photoperiod treatments during spring in freshwater and after 9 weeks in seawater. However, in contrast to the results obtained in similar studies with Atlantic salmon (Handeland and Stefansson, 2001), differences in the smoltification process in response to the different photoperiod treatments were generally mild, with few significant differences in NKA activity. In the present study, different photoperiods only caused a minor desynchronization of the biological clock of rainbow trout in spring. In fact, rainbow trout can smolt in total darkness (Wagner, 1974b), hinting that light regime is not their main *zeitgeber* for smoltification. Although all four treatments generated a smolt window in April, its duration was slightly longer for APP and shorter for DPP than in the rest of the treatments, particularly in June. In this context, a longer smolt window could have an impact to fish farmers, allowing them to be less

constrained by time in something as crucial as the seawater transfer of the fish. The difference between these two treatments was further shown by the NKA quadratic model, which showed that the overall activity of NKA was higher in APP than in DPP, suggesting that fish at APP become more seawater-ready. Nonetheless, the differences in the duration of the smolt window in the different treatments was small and, in mid-June, all treatments converged at low NKA activities, suggesting that this drop in hypo-osmoregulatory capacity (desmoltification) is induced by other factors that were not considered in this study, such as water temperature or simply a biological clock that is poorly affected by exogenous stimuli.

Although it was tightly related to the NKA activity during the whole freshwater period, the transcription of *nkaα1a*, *nkaα1b* and *nkcc1a* offered further detail into the smoltification process. In this context, the transcription of both *nkaα1a* and *nkaα1b* in March allowed for the detection of a transient transcriptional response of SNP and APP to the switch from LD12:12 to LL, which the NKA activity did not show. This response may decrease the osmoregulation capacity of the fish, which are likely the effects of an transient response to photoperiod, as it has been previously observed on the immune response (Leonardi and Klempau, 2003; Valenzuela *et al.*, 2008). Nonetheless, this effect was only transitory and transcription recovered in the two week period between samplings. During the smolt window, even though NKA activity stayed relatively stable at high values, there were changes that affected the NKA pumps at the gene expression level. The transcription of *nkaα1a* suggests that fish would have experienced their highest freshwater preference up to April, after which it decreased, reaching a minimum in mid-May, towards the end of the smolt window. On the other hand, *nkaα1b* and *nkcc1a* stayed relatively stable at high values until there was a significant decrease in mid-June for both, suggesting that the fish were able to respond optimally to a seawater challenge until mid-June. During the desmoltification phase, the transcription of *nkaα1b* and *nkcc1a* decreased accordingly to the NKA activity, while *nkaα1a* rose back, showing that fish were abandoning their migratory instinct and readapting to a life in freshwater. At this point, the transcription of the two seawater genes was significantly lower than at the start of the experiment in February, while *nkaα1a* transcription was at similar levels. This could not be appreciated from the NKA activity, and it suggests that the osmotic stress of a sudden seawater challenge would be stronger for desmolted than for parr rainbow trout. Indeed, this would explain the mechanism behind the results of Wagner (1974b), who showed that parr steelhead trout in December can have a higher survival rate to a direct seawater transfer than desmolts in June, and it provides further evidence that the desmoltification process is not simply the reversal to the parr state.

In mid-June rainbow trout from all treatments had desmolted, as their NKA activity and transcription of both *nkaα1b* and *nkcc1a* decreased to low levels while the transcription of *nkaα1a* increased. Since

fish lose the characteristics of a smolt, it has been suggested that transferring Atlantic salmon to seawater at this point would be suboptimal (Arnesen *et al.*, 2003). Regardless, it was decided to proceed with the seawater allocation in order to understand the effects of a sub-optimal seawater transfer on rainbow trout. This approach offers the possibility to research the mechanism of the GS fish development. In fact, although mortality upon seawater transfer was negligible, approx. 14% of fish experienced very little to no growth (SGR-L below 0.10 %*day^{-1}) during the summer months in seawater (July to September). These results are therefore in line with previous similar studies (Wagner, 1974a) and with the frequencies reported by Norwegian fish farmers. These two phenotypes might be linked to the two classes that are found in all migration studies in rainbow trout: sea-run and freshwater-resident phenotypes are always present, even in the same cohort (Christie *et al.*, 2011; Kendall *et al.*, 2015). Although the proportion of each phenotype is influenced by genotype (Nichols *et al.*, 2008; Hecht *et al.*, 2013), individual condition (size, growth rate, energy storage) (McMillan *et al.*, 2012) and environmental factors (Sloat *et al.*, 2014), the current knowledge is not enough to completely explain their smoltification patterns (Kendall *et al.*, 2015). Efforts to produce a strain with a single phenotype, either selecting the sea-run (Sharpe *et al.*, 2007; Christie *et al.*, 2011; Sloat and Reeves, 2014) or the freshwater-resident (Thrower and Joyce, 2005; Hayes *et al.*, 2012), have proved unsuccessful. Therefore, it is likely that the fish that performed poorly in seawater would have been natural freshwater-residents, while the others would have been the sea-run phenotype. Another explanation could be related to fish personality, with several studies not related to seawater transfer showing that rainbow trout exhibit dominant and subordinate behaviour (DiBattista *et al.*, 2006; Kostyniuk *et al.*, 2018). The latter experience stress and reduced growth, although this has not been shown in aquaculture production setups, with bigger tanks and bigger numbers of fish per tank (which might difficult the establishment of dominance) and longer experiment durations. However, in the present study, no behavioural tests were performed on the fish.

The GH-IGF-I axis is involved in the control of both growth and smoltification (Shrimpton *et al.*, 2000; Mancera and McCormick, 2007; Shimomura *et al.*, 2012). The mechanism of the GH-IGF-I axis on growth, through the activation of GHr by GH and the subsequent production of hepatic IGF-I, the activity of which is regulated by IGFBP1b (Shimizu *et al.*, 2011a), among others, has been studied in multiple fish groups (Beckman, 2011). On the other hand, in relation to smoltification, it has been proposed that GH, through GHr and IGF-I stimulation, is related to an increase in gill salt secretion capacity through the increment of the number of seawater chloride cells (Mancera and McCormick, 2007; Reinecke, 2010). In these cells, the isoforms NKAα1b and NKCC1a are involved in osmoregulation, increasing the NKA activity and seawater tolerance (McCormick, 2001; Poppinga *et al.*, 2007). On the other hand, *ctsl* is lysosomal endopeptidase which is involved in the initiation of

protein degradation (Joseph *et al.*, 1988; Lysenko *et al.*, 2017), relevant during turnover of cells and tissues during smoltification. The present study showed that the photoperiod treatment has relatively little effect on the regulation of the transcription of *igf-I*, *igfbp1b, ghr1*, and *ctsl* in liver. In March, due to reasons that cannot be traced back but are likely not related to the photoperiod treatment, DPP showed an increased transcription of *igf-I* and *ghr1*. Interestingly, this growth-enhancing condition might be quickly stopped by an increase of the transcript of *igfbp1b* in mid-March and April, which would decrease the effect of IGF-I, therefore the growth of DPP fish was not higher than in the other treatments and the transcription of the measured genes was soon returned to normal levels. Regarding *ctsl*, it transiently decreased in mid-March for the two treatments that changed to LD12:12, SNP and APP, the latter one being more affected. This could be related to the transient stress response to photoperiod switching that was also seen for *nkaα1a* and *nkaα1b* and, in the same way, the transcription recovered in the two week period until the next sampling. Months after, in mid-June, *ctsl* transcription of APP was higher than that of LL. However, the SGR-L was not bigger in APP than in LL during the June-July or July-September periods, which puts into question the validity of the transcription of this gene as a growth proxy. When modelled, only DPP treatment showed differences on the transcription of *igf-I* and *ctsl*, as seen through the model fit, since it started decreasing earlier than other treatments, which is consistent with the lower SGR-L recorded in this group. In any case, regardless of the photoperiod, the relationship between *igf-I*, *ghr1* and *ctsl* and growth seemed clear: their trend is consistent with the SGR-L in tagged fish, with high gene transcriptions corresponding to periods of high SGR-L, which is especially clear in May and June, although with a phase delay. When compared to *igf-I* transcription, SGR-L has a phase delay of about 40 days, as would be expected when comparing a quick process (gene transcription) with a slow one (growth). It is likely that the liver transcription of *igf-I*, *ghr1* and *ctsl*, among other genes, are responsible for promoting the growth of rainbow trout in the medium to long term (Beckman *et al.*, 2004a; Picha *et al.*, 2008; Beckman, 2011). This suggests that the smoltification process anticipates a period of high growth during the post-smolt phase, triggering the earlier transcription of growth-promoting genes. During desmoltification, the transcription of these genes is down-regulated as the fish would not become post-smolts and this period of intense growth would no longer occur. Finally, although both *igf-I* transcription and NKA activity increased in mid-March, *igf-I* transcription reached the high plateau of the smolt window sooner than *nkaα1b* or NKA activity, suggesting that indeed IGF-I drives the increase in NKA activity (McCormick, 2001; Poppinga *et al.*, 2007). Moreover, NKA activity and *igf-I* transcription seemed to follow a very similar trend (*Tmax* for NKA activity: 111.72 to 119.12, *Tmax* for *igf-1* transcription: 98.73 to 117.79 days) (Sakamoto *et al.*, 1995; Shimomura *et al.*, 2012).

Unlike the case of the gene transcription of *igf-I*, *ghr*, *ctsl* and *igfbp1b*, the photoperiod treatment did affect the circulating IGF-I in plasma at the end of the freshwater phase (it was not measured prior to this point), when APP had significantly higher abundances than the other treatments, and in seawater in September, when it was higher in APP compared to DPP and LL. Similarly, Taylor *et al.* (2005) reported higher growth and plasma IGF-I levels in the LD18:6 treatment. It is possible that the rhythmicity of the APP provides the adequate *zeitgeber* for rainbow trout development, while DPP does the opposite. However, this difference due to the photoperiod was not found in the liver *igf-I* transcription. Assessing the relationship between plasma IGF-I and SGR-L in seawater (July-September), the data does not allow to accept plasma IGF-I in July as a reliable predictor of the growth to come during the next two months in seawater. However, overall SGR-L in freshwater was a predictor of SGR-L in seawater, as previously observed (Johnsson *et al.*, 1997).Moreover, both the plasma IGF-I and the liver *igf-I* transcription had significant correlations with SGR-L in tagged fish showing that they are involved in the regulation of growth in seawater for rainbow trout.

In conclusion, the effects of photoperiod on rainbow trout in winter-spring are relatively small when compared with the effects on other salmonids, as seen from the minor differences in NKA activity, osmoregulatory genes and the studied transcription of growth-related genes among treatments. To exemplify this, after 16 weeks of photoperiod treatment, Atlantic salmon kept at SNP showed an NKA activity of around 10 µmol ADP mg protein^{-1} hour^{-1} while those fish kept at LL presented values of around 4 (Handeland and Stefansson, 2001). While given the general lack of differences between APP, LL and SNP it is difficult to pinpoint the most appropriate photoperiod regime for rainbow trout, APP induced a longer smolt window and fish showed higher values of plasma IGF-I. On the other hand, DPP was consistently the worst treatment, as shown by the shorter length of the smolt window, reduced NKA activity, earlier decrease in the transcription of *igf-I* and *ctsl*, lower abundance of plasma IGF-I and lower SGR-L and SGR-W. From a growth perspective, although *igf-I*, *ghr1* and *ctsl* transcription were, mostly, not significantly affected by the light regime, they showed a clear increase during the smolt window, suggesting that during this phase the endocrine system of anadromous salmonids is preparing for the imminent period of high growth in seawater. Finally, while plasma IGF-I and liver *igf-I* transcription in seawater were clearly related to the regulation of growth in seawater, IGF-I prior seawater transfer was not a reliable predictor of growth. Since the present work seems to indicate that the photoperiod is not a strong inducer of smoltification for the species, future work should investigate the effect of other environmental cues, like temperature and salinity as smoltification inducers in rainbow trout.

Chapter 3: Effects of temperature and photoperiod on rainbow trout smoltification and haematopoiesis

3.1. Introduction

Most freshwater fish would not be able to cope with the osmotic stress intrinsic to seawater, quickly dehydrating and dying, and so are bound to freshwater ecosystems (Quinn *et al.*, 2016). However, anadromous salmonids have developed a strategy to minimize osmotic counter effects, thus allowing them to live in both freshwater, ideal for safe spawning, hatching and early growth stages, and seawater environments, rich in energy resources for fast growth (Lima and Dill, 1990; Hendry *et al.*, 2004).

Anadromous salmonids develop the attributes necessary for life in seawater during a process known as smoltification. Once anadromous salmonids reach a threshold size (Kendall *et al.*, 2015), smoltification is triggered by environmental cues, such as changes in photoperiod, water temperature and salinity, which in turn alter the pituitary, thyroid and inter-renal tissues (Prunet and Young, 1989). These tissues are key orchestrators of many biochemical (*e.g.* haemoglobin; Fyhn *et al.*, 1991), physiological (*e.g.* intake metabolism; Björnsson *et al.*, 2011), morphological (*e.g.* dark and rounded to silvery and streamlined; Winans and Nishioka, 1987) and behavioural changes (*e.g.* bottom-dwelling, aggressive and territorial to pelagic, schooling and downstream migrating, McCormick *et al.*, 1998; Riley *et al.*, 2014), all designed to improve seawater performance and survival. Among these changes is the development of hypo-osmoregulatory capacity. This is possible because salmonids have different gill cell types with a repertoire of NKA pumps. Each of these pumps have differences in function, peak in expression at different times and are predominantly found in one of the gill cell types (Nilsen *et al.*, 2007; Flores and Shrimpton, 2012). Thus, NKAα1a is a freshwater pump present in lamellar chloride cells, the function of which is to generate a hyper-osmotic gradient that results in ion uptake, and is highest during the parr stage of the fish. On the other hand, the seawater pumps NKAα1b and NKCC1a are found primarily in filamental chloride cells, their function is to generate a hypo-osmotic inner environment that results in ion secretion, and peak during the smolt stage and in seawater (McCormick *et al.*, 2013). All smoltification changes are reversible and last during what is known as the smolt window (time of maximal seawater-readiness). If the fish do not reach seawater during the smolt window, these changes are lost in the process known as desmoltification (Stefansson *et al.*, 1998).

Both photoperiod and temperature have been investigated in aquaculture management in a variety of farmed and potential farmed fish species (Deacon and Hecht, 1996; Allan and Burnell, 2013). In the case of salmonids, the artificial manipulation of these environmental factors has been shown to be a useful strategy for the production of out-of-season smolts, as well as for optimizing the induction of in-season smoltification and maximizing growth in aquaculture production systems (Solbakken *et al.*, 1994; Jørgensen *et al.*, 2007; Handeland *et al.*, 2013). Consequently, photoperiod is routinely manipulated according to the time of the year in order to obtain optimal smoltification rates in Atlantic salmon and Arctic char (*Salvelinus alpinus*) production (Stead and Laird, 2002; Johnston, 2008) while temperature is less often controlled due to heating costs.

In recent years, there has been an increase in the aquaculture production of the sea-run phenotype of rainbow trout, which reached over 84,000 tonnes in Norway alone in 2016 (Food and Agriculture Organization of the United Nations, 2019). Industrial interest on this phenotype arises from the need to diversify production and the possibility to expand on-growing production sites to low salinity locations, which are more suitable for rainbow trout smolts than for Atlantic salmon (Altinok and Grizzle, 2001) and are less prone to salmon louse infestations. However, our understanding of the environmental control of rainbow trout smoltification is insufficient and there is a need for appropriate tools to assess rainbow trout seawater-readiness. In the absence of a rainbow trout specific all-season production protocol, most hatcheries are rearing rainbow trout at LL and natural temperature while seawater-readiness is regarded exclusively as size-dependent. Under these conditions, high mortalities and high numbers of fish that experience sub-optimal growth (GS phenotype) upon seawater transfer, especially in summer post-smolts, have been reported by the industry. While in Chapter 2 LL was compared to other photoperiod regimes and shown to be an appropriate option for rainbow trout smoltification, the effect of temperature and its interaction with photoperiod remain untested.

In order to reduce the incidence of GS fish, optimizing rearing conditions for rainbow trout smoltification, as well as identifying and implementing markers of seawater-readiness and markers for the early detection of GS fish are all crucial, thus improving both fish welfare and production. In this sense, IGF-I is a peptide hormone that promotes growth of fish (Wood *et al.*, 2005). The activity of circulating IGF-I is stabilized and regulated by the presence of multiple IGFBPs. In salmonids and other teleost species, plasma IGF-I is positively correlated with growth rate and can be used as a positive marker of growth (Picha *et al.*, 2008; Beckman, 2011). On the one hand, IGFBP1b, one of three major circulating forms of IGFBP which is induced under catabolic conditions, is negatively correlated with growth rate and may be useful as a negative marker of growth in salmonids (Shimizu *et al.*, 2006; Kawaguchi *et al.*, 2013; Kaneko *et al.*, 2019).

Another factor to take into consideration due to its potential to significantly compromise fish welfare and production are pathogen infections. While a wide variety of measures can be taken to prevent them (Sommerset *et al.*, 2005; Sharifuzzaman and Austin, 2009; Oliva-Teles, 2012), disease is one of the main threats to production (Asche *et al.*, 2009; Dale *et al.*, 2009; Kristoffersen *et al.*, 2009). Therefore, immune capacity is a key factor to take into consideration when optimising new production protocols. Since blood cells constitute the main line of defence against pathogens and non-self particles, haematopoiesis can be used as a proxy of immune capacity (Baldridge *et al.*, 2010; Martin *et al.*, 2012). A number of transcription factors related to haematopoiesis control such as the transcription factor Pu.1 (also known as SPI1) are well known. Low concentrations of Pu.1 are essential for the differentiation of granulocyte-monocyte progenitor (GMP) myeloid cells (*i.e.* mast cells, eosinophils, neutrophils, macrophages and dendritic cells) (Ribas *et al.*, 2008; Orkin and Zon, 2008). In turn, cells from this myeloid lineage produce more Pu.1, increasing its abundance and shifting the haematopoiesis towards the productions of lymphoid cells. IL1β is a pro-inflammatory cytokine produced by immune cells like lymphocytes, monocytes and macrophages upon detection of signs of an infection or injury. IL1β enhances cell-mediated immunity by promoting the proliferation and maturation of lymphocytes (Pleguezuelos *et al.*, 2000; Reis *et al.*, 2012). IL-4/13 is the ancestral family cytokine related to both mammalian IL-4 and IL-13, involved in the stimulation of B lymphocyte proliferation and activation of macrophages (Martinez *et al.*, 2009; Takizawa *et al.*, 2011; Sequeida *et al.*, 2017). Finally, the transcription factor GATA3 is essential for the development of the T lymphocyte lineage and differentiation of T helper type 2 cells (Kumari *et al.*, 2009). Understanding the interplay of these key transcription factors and expression of cytokines in response to any changes in environmental conditions during smoltification is vital to assess the impact on the immune capacity of fish.

The objectives of this study are (1) to test the effect of different photoperiod and temperature regimes on the smoltification of rainbow trout through the measurement of the gill NKA activity, and gill *nkaα1a*, *nkaα1b*, and *nkcc1a* transcription over a three month period in freshwater, (2) to identify the optimal regime in terms of growth and its regulation (circulating IGF-I levels) and (3) to evaluate the effect of such regimes on the immune capacity of the fish as a function of the transcription of haematopoiesis related genes *pu.1*, *il1β*, *il4/13* and *gata3* in head kidney.

3.2. Materials and methods

3.2.1. Samples

Juvenile rainbow trout (AquaGen) of 1+ years with an initial weight of 75.0 ± 15.1 g were used in this experiment (n=716). Fish were fed *ad libitum* using a standard commercial dry diet (Skretting AS) from automatic feeders according to fish size. Fish were kept indoors in a flow through system using tanks equipped with timer-controlled LED lights in a trout facility from Lerøy Vest AS (Bjørsvik, Hordaland, Norway). The fish were kept at ambient temperature except for the high temperature group, where it was raised for a period as part of the experimental setup, water flow of 0.4 L/kg/min and O_2 was above 80% saturation in the outlet.

3.2.2. Experimental design

Prior to the freshwater experimentation phase, fish were kept in 2 x 2 m rearing tanks (2,500 L) under natural temperature and LL photoperiod for 2 weeks. On the 8th of March 2017, n=160 fish were individually Carlin tagged for recording of individual growth rates during both the freshwater and seawater phase of the experiment. Fish were randomly distributed into eight tanks, resulting in 20 tagged and 67 untagged fish per tank. After two weeks of acclimation, the photoperiod and temperature regimes were initiated. The experimental design included a factorial design of two temperature treatments (high temperature (HT) or natural temperature (NT)) and two photoperiod treatments (LL or SNP) resulting in a total of four treatments: HT+LL, HT+SNP, NT+LL and NT+SNP. High temperature water was achieved using heat interchange with fjord seawater and homogenised in a series of head tanks with degassers before pumping into the experimental tanks. Due to this heat interchange mechanism, fjord water temperature determined the reachable water temperature for HT. Temperature of the HT group was set to 8^{o}C with minor deviations from the 8th of March until the natural water temperature converged at 8^{o}C (temperature convergence, TC) on the 8th of May (Figure 3.1a). Fish at NT received water at ambient temperature for the duration of the experiment (8th of March to 13th of September 2017). After TC point, both NT and HT groups received water at natural temperature. LL photoperiod was constant light for the duration of the experiment, while SNP started at LD12:12 and increased light time by 45 min every week (Figure 3.1b). During the freshwater phase (8th of March to 2nd of June), weight and length were recorded in tagged fish on 8th March, 5th May and 2nd June. In June, the remaining non-tagged fish from all experimental groups were individually tagged and length and weight recorded before being randomly distributed into four replicate tanks supplied with seawater and kept at LL in a common garden experiment to strengthen growth studies. Weight and length were recorded once more at the end-point sampling on the 13th of September.

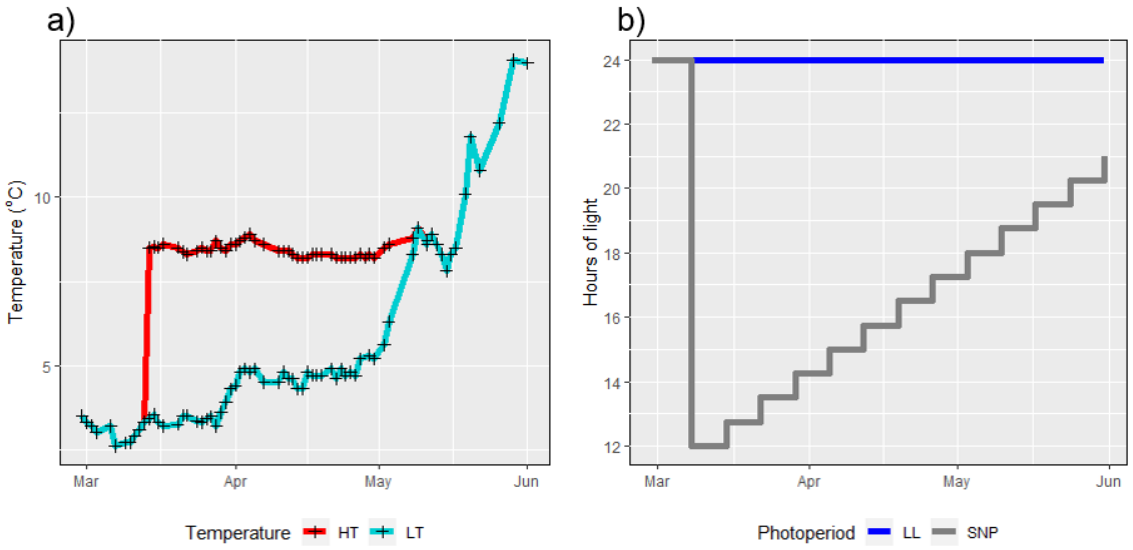


Figure 3.1. Representation of the temperature and light regimes during the freshwater experimentation phase. Mean temperature of high temperature tanks and natural temperature tanks (a) and number of hours of light for each of the four different photoperiod treatments (b). Symbol + indicates when the temperature was measured.

Experimental work was ethically reviewed, approved, and registered by the NARA and by the AWERB (088), University of Stirling, UK.

3.2.3. Sampling

Lethal samplings of six fish per tank (n=12 per group) were conducted every two weeks during the freshwater phase. Samplings took place on 23rd of March, 6th of April, 20th of April, 4th of May, 18th of May and 31st of May. A final lethal sampling was done 15 weeks after seawater transfer (13th of September).

Fish were quickly dip-netted out of the tanks and euthanized by a lethal overdose of isoeugenol (AQUI-S). For each fish, weight and length were recorded. Blood was extracted using heparinised syringes and centrifuged at 3,000xg for 5 min to obtain plasma, which was frozen at -80°C. The first gill arch from each side of the fish were dissected out and preserved at -80°C; one in SEI buffer (Sucrose 250 mM, Na_2EDTA 10 mM, Imidazole 50 mM (all Sigma-Aldrich)) and the other one in RNAlater (ThermoFisher Scientific). Head kidney samples were also preserved in RNAlater according to manufacturer's guidelines (overnight at 4°C and frozen at -80°C).

3.2.4. Gill NKA activity

Gill NKA activity of all freshwater fish (n=12 per group) was analysed. For the seawater phase (final sampling), only the 50 fish above the third quartile in length (34.0 cm) with the highest condition factor and the 50 fish below the first quartile in length (31.5 cm) with the lowest condition factor were analysed.

NKA activity was measured as described in Chapter 2.

3.2.5. RNA isolation and cDNA synthesis

RNA isolation was carried out on gill and head kidney freshwater samples. Before total RNA isolation of samples, 20-25 mg of tissue was homogenized in RLT Plus buffer (Qiagen) with stainless steel beads (5 mm) (Qiagen) using a homogenizer (5,000 rpm, 15 min) (Precellys 24, Bertin Technologies). Subsequent total RNA isolation was carried out using the Qiasymphony RNA kit in the QIAsymphony SP automatic system following manufacturer instructions (Qiagen).

Total RNA concentration and purity was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Purity was confirmed with 260/280 and 260/230 ratios above 1.8. A selected number of samples were assessed for RNA integrity on RNA 6000 Nano LabChip® kit using the Agilent 2100 Bioanalyzer (Agilent Technologies). Integrity was confirmed with RIN values higher than 8.

Complementary DNA was reversely transcribed using 1.5 µg (gill) or 2.5 µg (head kidney) of total RNA using oligo(dT_{20}) primer and the Superscript III kit (Fisher Scientific) using a MicrolabSTARlet Liquid Handling Workstation (Hamilton Robotics).

3.2.6. Transcription assay

Osmoregulation related gene transcription (*nkaα1a*, *nkaα1b* and *nkcc1a*) was measured in gill samples and haematopoiesis related gene transcription (*pu.1*, *il1β*, *il4/13* and *gata3*) in head kidney samples, using RT-PCR with minor differences between gene sets. RT-PCR was carried out in a CFX-96 Real-Time PCR detection system platform (Bio-Rad) using the following PCR conditions: 3 min at 95°C, 34 cycles of 15 seconds at 95°C and 1 min at 60°C and a melting curve step at the end (10 seconds at 95°C, 5 seconds at 65-95°C with increments of 0.5°C and 5 seconds at 95°C). For each assay, triplicate two-fold cDNA dilution series from pooled samples (1:5-1:160) were used to determine both amplification efficiencies for each oligo pair and optimal dilution for cDNA template. Samples were run in 12.5 µl duplicates using iTaq universal SYBR green supermix (Bio-Rad), 0.20 µM of each primer and 2.5 µl of diluted cDNA (dilution 1:50 for gill and 1:75 for head kidney). Each plate included a negative control

as well as a common sample (pooled sampled) used for the intercalibration of assays among plates. The relative transcription levels of the genes were normalized following the efficiency corrected method (Pfaffl, 2001) using *ef1α* as an endogenous reference gene (Olsvik *et al.*, 2005). Primers used in this study are summarized in the Table 3.1.

Table 3.1. Primers used for RT-PCR analysis and accession numbers of the gene sequences (GenBank).

Gene name	Primer sequence (5'>3')	Accession number	Reference
nkaα1a	CCAGGATCACTCAATGTCACTCT CAAAGGCAAATGGGTTTAATATCAT	XM_021573245	(Nilsen *et al.*, 2007)
nkaα1b	GCTACATCTCAACCAACAACATTACAC TGCAGCTGAGTGCACCAT	XM_021570999	(Nilsen *et al.*, 2007)
nkcc1a	GATGATCTGCGGCCATGTTC CTGGTCATTGGACAGTTCTTTG	XM_021601694	(Nilsen *et al.*, 2007)
pu.1	GTCTGAGAGACCACATTGC TCTTGTTGCCCAATTCTCC	NM_001124513	Present work
il1β	CGTCACTGACTCTGAGAACAAGT TGGCGTGCAGCTCCATAG	AJ223954	(Løvoll *et al.*, 2007)
il4/13	ATCCTTCTCCTCTCTGTTGC GAGTGTGTGTGTATTGTCCTG	AB574337	(Deshmukh *et al.*, 2013)
gata3	GCGCACAAACAGAGATTTGA TCCAAGGTCGTATCCAGTCC	NM_001195792	(Martin, E. *et al.*, 2012)
efα1	CCCCTCCAGGATGTCTACAAA CACACGGCCCACGGGTACT	AF498320	(Genge *et al.*, 2013)

3.2.7. TR-FIA for plasma IGF-I and IGFBP1b

Circulating IGF-I and IGFBP1b levels were measured in plasma collected from 32 randomly selected tagged fish (n= 8) at the beginning (June) and at the end (September) of the seawater period.

TR-FIA protocol was used to measure plasma IGF-I concentration (Small and Peterson, 2005). Prior to the assay, serum IGF-I was dissociated from the binding protein with acid-ethanol (Shimizu *et al.*, 2000). Briefly, 96-well DELFIA pre-coated goat anti-rabbit IgG Microtitration plates (Perkin Elmer) were washed with 200 µl DELFIA wash buffer before each well received 20 µl anti-barramundi IGF-I rabbit antiserum (GroPep; diluted 1:8,000) and 100 µl of standard-recombinant IGF-I (GroPep) or 20 µl plasma (Cleveland *et al.*, 2018). Standards and samples were diluted in standard assay buffer. Plates were incubated overnight with shaking (600 rpm at 4°C). After centrifugation (1 min at 3,000xg), europium labelled (0.05 ng μl^{-1}) IGF-I was added to each well and the plate incubated overnight under agitation (600 rpm at 4°C). Plates were washed six times with 200 µl washing buffer before adding 200 µl DELFIA enhancement solution (PerkinElmer) to each well. After shaking at 600 rpm for 10 min at

RT, time-resolved fluorescence was measured by a fluorometer (ARVO X4; PerkinElmer) with excitation, and emission and read wavelengths at 340 and 615 nm, respectively.

Serum IGFBP1b levels were quantified by TR-FIA, which has been validated for rainbow trout, as described in Fukuda *et al.* (2015). Briefly, a competitive method was employed by following a procedure for DELFIA immunoassays (PerkinElmer). Serum samples were first incubated overnight at 4°C with antiserum against purified salmon IGFBP1b (Shimizu *et al.*, 2006), in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). Biotinylated salmon IGFBP1b was added to each well and incubated overnight at 4°C. After washing with DELFIA Wash Buffer (PerkinElmer), each well received Eu-labelled streptavidin (PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured using a SPARK multimode microplate reader (Tecan).

3.2.8. Growth calculations

SGR-L was calculated using the formula and results are expressed in % day^{-1}:

$$100 \times \frac{Ln\,(Length_{Final}) - Ln\,(Length_{Initial})}{Number\ of\ days}$$

Similarly for SGR-W:

$$100 \times \frac{Ln\,(Weight_{Final}) - Ln\,(Weight_{Initial})}{Number\ of\ days}$$

Thermal growth coefficient in length (TGC-L) was calculated using the formula (Iwama and Tautz, 1981; Jobling, 2003):

$$1000 \times \left\{\frac{\left(\sqrt[3]{Length_{Final}} - \sqrt[3]{Length_{Final}}\right)}{Degree\ days}\right\}$$

Similarly for thermal growth coefficient in weight (TGC-W):

$$1000 \times \left\{\frac{\left(\sqrt[3]{Weight_{Final}} - \sqrt[3]{Weight_{Final}}\right)}{Degree\ days}\right\}$$

The condition factor was calculated with Fulton's formula:

$$100 \times \frac{Weight}{Length^{3}}$$

3.2.9. Data analysis and representation

Statistical tests were performed using R statistical software. Data representation was carried out using R package ggplot2 (Wickham, 2009).

Two-way ANOVA was performed at each time point to find differences among treatments (effect of temperature and photoperiod). One-way ANOVA was used for each treatment on the whole time-series to find differences among time points (effect of time). Similarly, one-way ANOVA was used to test differences among treatments for IGF-I and IGFBP1b plasma levels. Data was transformed by either natural logarithm or square root to satisfy the normal distribution and homogeneity of variance assumptions, tested with the Shapiro and Bartlett tests, respectively. Significant comparisons ($p < 0.05$) were followed by Tukey's posthoc test to identify different treatments.

3.3. Results

3.3.1. Mortality and Fish growth

Overall mortality during the 15 weeks in seawater was of 6.74% (25 fish. HT+LL: 7, HT+SNP: 6, NT+LL: 6, NT+SNP: 6).

When analysing tagged fish, it is possible to visualize the growth trajectory of each fish and to calculate thermal growth coefficient (TGC-L and TGC-W) and specific growth rate (*i.e.* SGR-L and SGR-W). Fish at HT became significantly larger in length by the time the second sampling took place, in May (Figure 3.2a, temperature effect, $p < 0.001$, df: 1, F: 74.90), even though similar TGR were observed for all groups during the March-May period (Figure 3.2b). The differences in length stayed significant until the next sampling in June (temperature effect, $p < 0.001$, df: 1, F: 41.32, interaction, $p < 0.05$, df: 1, F: 4.9). However, after May, an increase in the TGC of the NT groups was observed which progressively reduced the length difference between HT and NT groups. This compensatory growth of NT was maintained during the three months in seawater (Figure 3.2c), after which the differences in length between HT and NT groups had been reduced. Despite the fish kept at HT still being larger, this difference in length was only significant between fish kept at NT+SNP and at HT+LL (temperature effect, $p < 0.001$, df: 1, F: 11.50; Tukey test, $p < 0.01$).

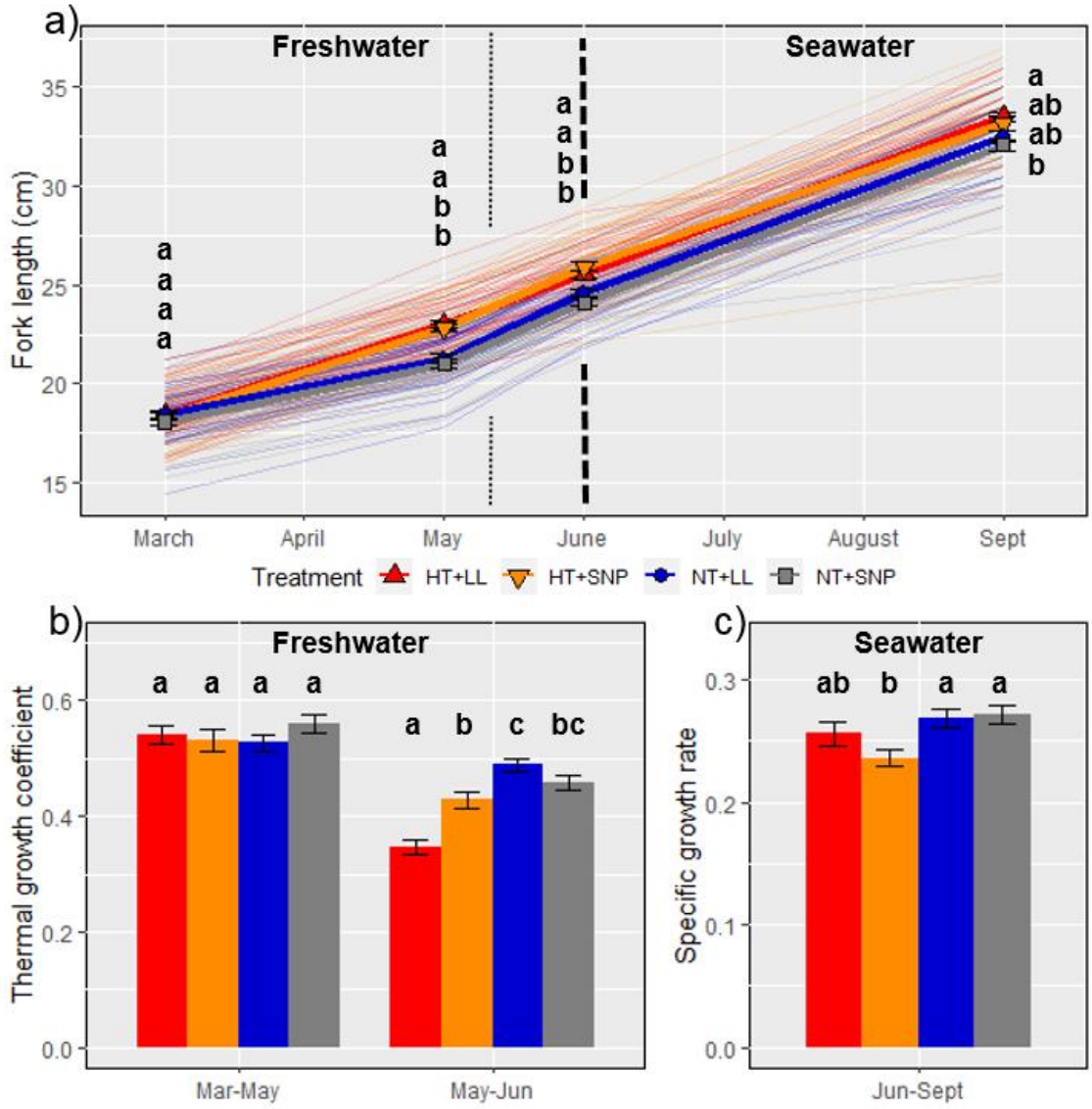


Figure 3.2. Fork length (cm, ± 1 s.e.) of juvenile rainbow trout reared in freshwater under four different photoperiod and temperature treatments and transferred to seawater on the 2nd of June. Mean fork length (points and thick lines) and individual growth trajectory (thin lines) of each tagged fish (a), mean TGC-L between measurements in freshwater (b), and mean SGR-L between measurements in seawater (c). Measurements took place on 8th March, 5th May, 2nd June and 13th of September. Error bars indicate s.e. Dashed line indicates seawater transfer. Dotted line indicates TC point. Different letters indicate statistical differences ($p < 0.05$) in a time point. Lack of letters indicates lack of significant differences.

Very similar results were obtained when performing the same tests on fish weights (Figure 3.3).

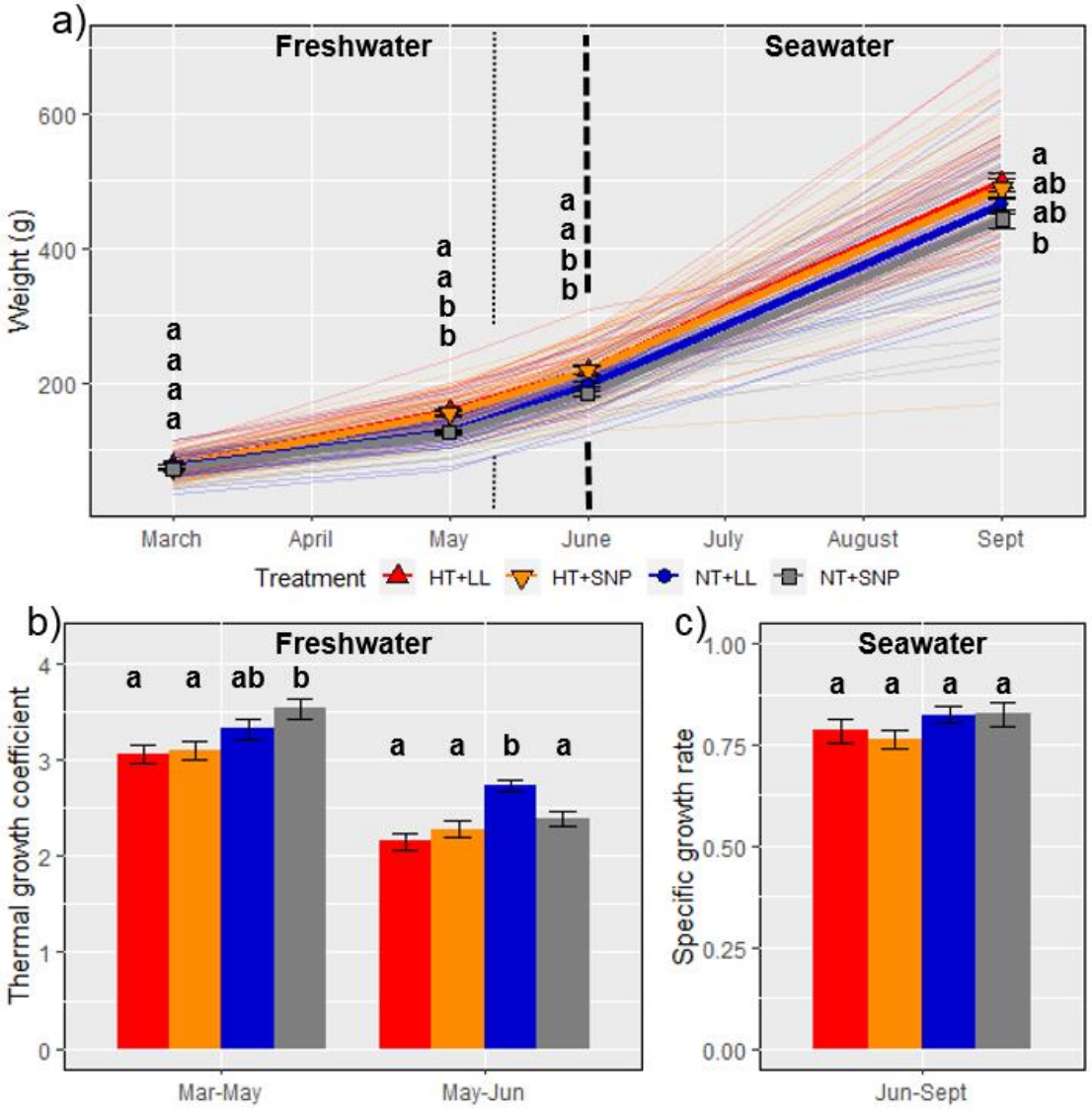


Figure 3.3. Weight (g, ± 1 s.e.) of juvenile rainbow trout reared in freshwater under four different photoperiod and temperature treatments and transferred to seawater on the 2nd of June. Mean weight (points and thick lines) and individual growth trajectory (thin lines) of each tagged fish (a), mean TGC-W between measurements in freshwater (b), and mean SGR-W between measurements in seawater (c). Measurements took place on 8th March, 5th May, 2nd June and 13th of September. Error bars indicate s.e. Dashed line indicates seawater transfer. Dotted line indicates TC point. Different letters indicate statistical differences ($p < 0.05$) in a time point. Lack of letters indicates lack of significant differences.

3.3.2. Osmoregulation

The measurement of NKA activity was used to assess how the different treatments affected osmoregulatory capacity during the experiment (March to June in freshwater and a final assessment in seawater in September). No clear increase of NKA activity during the freshwater stage was detected for either of the NT treatments, both lacking significant differences between sampling time points during the freshwater stage (Figure 3.4; Table 3.2). HT+LL peaked in May before decreasing again but this treatment too had no significant differences during the freshwater stage. The treatment with the clearest increase in NKA activity was HT+SNP, which was highest in mid-April and was significantly different from the values in June ($p < 0.001$, df: 6, F: 7.48; Tukey test, $p < 0.001$). NKA activity in seawater was consistently lower than in freshwater. However, no significant differences at any specific sampling were found among treatments, neither in freshwater (Figure 3.4a) or seawater (HT+LL: 2.79 ± 0.36, HT+SNP: 2.37 ± 0.46, NT+LL: 2.43 ± 0.25, NT+SNP: 2.09 ± 0.22 µmol ADP mg protein^{-1} hour^{-1})(Table 3.2). In seawater, no significant differences related to fish length were found either (50 fish above the third quartile in length with the highest condition factor and 50 fish below the first quartile in length with the lowest condition).

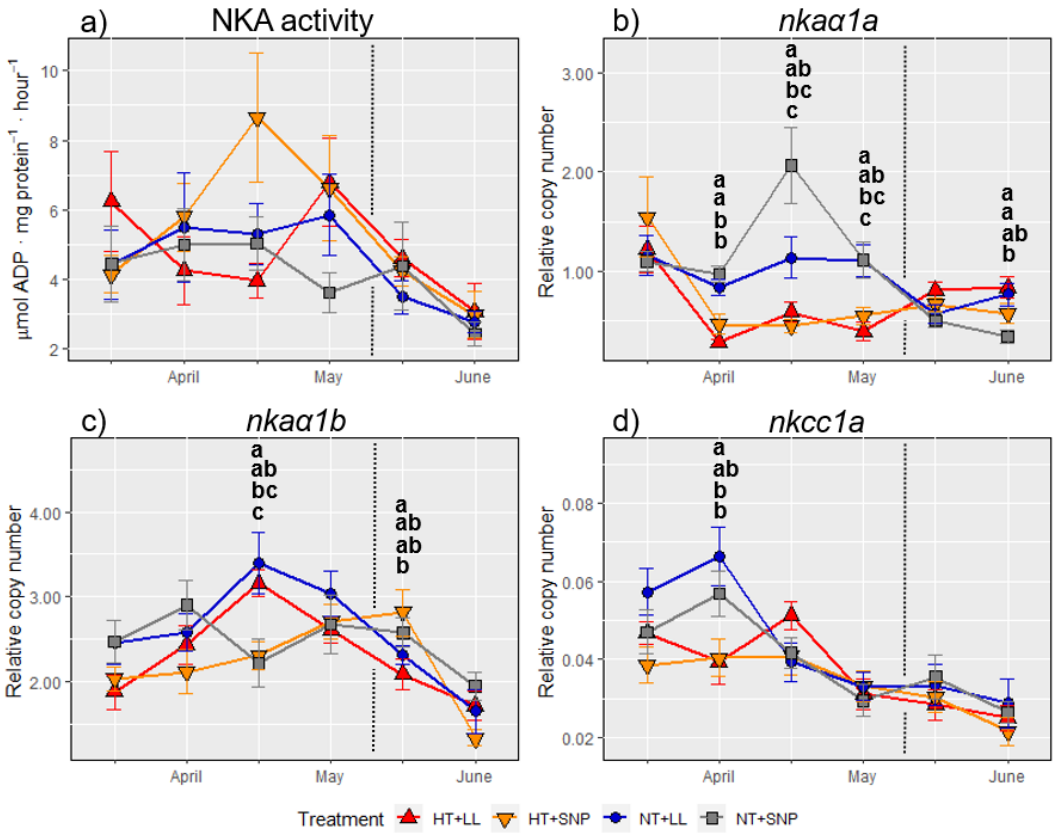


Figure 3.4. Gill NKA activity (a) and gill transcription of related genes, *nkaα1a* (b), *nkaα1b* (c) and *nkcc1a* (d) of juvenile rainbow trout in freshwater under four photoperiod and temperature treatments. Error bars indicate s.e. Different letters indicate statistical differences (p < 0.05). Samplings took place on 23rd of March, 6th of April, 20th of April, 4th of May, 18th of May and 31st of May. Dotted line indicates temperature convergence point. Different letters indicate statistical differences (p < 0.05) in a time point. Lack of letters indicates lack of significant differences.

Table 3.2. Tukey's test results for the differences among sampling points for each treatment. Different letters indicate significant differences among time points.

	Na^+,K^+–ATPase activity			
	HT+LL	**HT+SNP**	**NT+LL**	**NT+SNP**
Mid-March	a	abc	ab	ab
April	ab	ab	a	a
Mid-April	ab	a	a	a
May	a	ab	a	ab
Mid-May	ab	ab	ab	ab
June	ab	bc	ab	ab
September	b	c	b	b

While the transcription of the freshwater isoform *nkaα1a* remained relatively stable until May for NT groups, fish kept at HT decreased *nkaα1a* transcription after mid-March and remained low until May, thus originating significant differences between HT and NT groups in April (temperature effect, $p < 0.001$, df: 1, F: 47.71), mid-April (temperature effect, $p < 0.001$, df: 1, F: 31.96 and interaction: $p < 0.05$, df: 1, F: 4.34), and in May (temperature effect, $p < 0.001$, df: 1, F: 23.98). Afterwards, in mid-May, the transcription for both temperature groups converged at similar levels, coinciding with TC. Then, in June, new differences originated, this time driven by photoperiod, with HT+LL (Tukey test, $p < 0.01$) and NT+LL (Tukey test, $p < 0.01$) being significantly higher than NT+SNP (photoperiod effect, $p < 0.001$, df: 1, F: 15.33) (Figure 3.4b).

The transcription of the seawater isoform *nkaα1b* seemed to be mainly driven by photoperiod. LL groups experienced an increase in mid-April and then quickly decreased, while in SNP groups *nkaα1b* experienced a slower increase, peaking in mid-May. All groups were at similar transcription levels before seawater transfer in June. Significant differences were present during the LL peak in mid-April (photoperiod effect, $p < 0.001$, df: 1, F: 16.35) and during the SNP peak in mid-May (photoperiod effect, $p < 0.05$, df: 1, F: 6.73) (Figure 3.4c).

The transcription of *nkcc1a* was overall comparable between groups during the whole freshwater period, with the exception of samples taken in April, when groups at NT presented higher levels, this difference being significant between NT+LL and both HT+LL (Tukey test, $p < 0.05$) and HT+SNP (Tukey test, $p < 0.05$)(temperature effect, $p < 0.01$, df: 1, F: 12.83). As a general trend, the transcription of this gene tended to decrease progressively with time (Figure 3.4d).

3.3.3. Plasma IGF-I and IGFBP1b abundance

Neither the IGF-I levels measured prior seawater transfer nor after 15 weeks in seawater showed significant differences among fish reared in the different freshwater treatments (Figure 3.5a). But there was an overall effect of seawater transfer on plasma IGF-I levels, which were lower in fish in seawater ($p < 0.001$, df: 1, F: 23.81). No significant correlation was found between plasma IGF-I level in September and SGR during June-September (SGR-L: p: 0.41, r= -0.15, slope= 64.58; SGR-W: p: 0.12, r= -0.28, slope= 51.90).

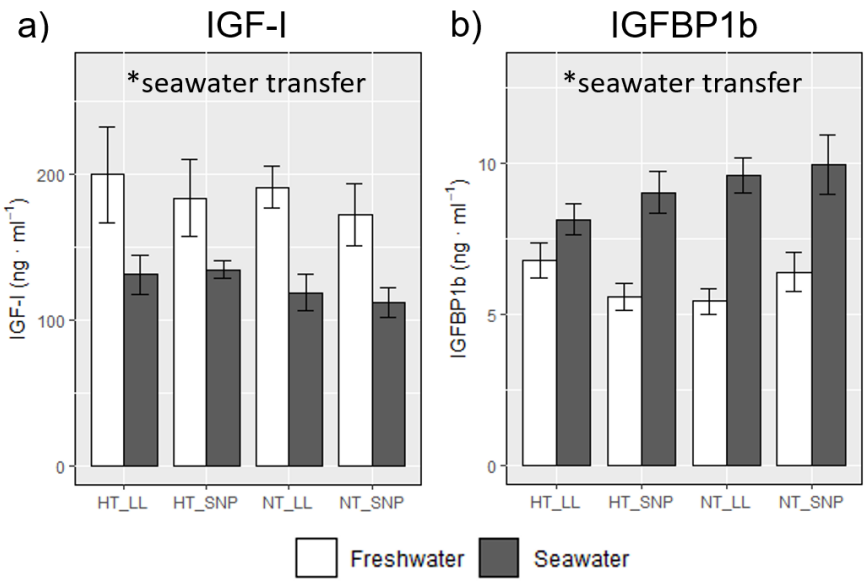


Figure 3.5. Circulating plasma IGF-I (a) and IGFBP1b (b) levels of juvenile rainbow trout reared in freshwater under four photoperiod and temperature treatments. Error bars indicate s.e. (*n* = 8). Asterisks (*) indicate an overall (*i.e.* treatment-independent) effect of seawater transfer. Samplings took place on May 31st (white bars) and September 13th (black bars).

Similarly for IGFBP1b levels, there were no significant differences among fish reared in the different freshwater treatments, either in June or September (Figure 3.5b). However, plasma IGFBP1b levels were significantly higher in fish in seawater than in freshwater ($p < 0.001$, df: 1, F: 51.52). Moreover, there was a negative correlation between plasma IGFBP1b level in September and SGR-L during June-September (SGR-L: $p < 0.05$, r= -0.45, slope= -12.80; SGR-W: $p < 0.05$, r= -0.42, slope= -5.20).

3.3.4. Haematopoiesis

Overall, temperature strongly affected the transcription of the tested haematopoiesis genes in head kidney, especially for *pu.1* and *il4/13*. With few exceptions towards the end of the freshwater phase, after the TC point, NT treatments presented invariably the highest transcription levels, often finding significant differences between HT and NT treatments. As a general trend, transcription levels decreased as the temperature increased and the differences between HT and NT treatments became progressively smaller as their temperature drew nearer (Figure 3.6).

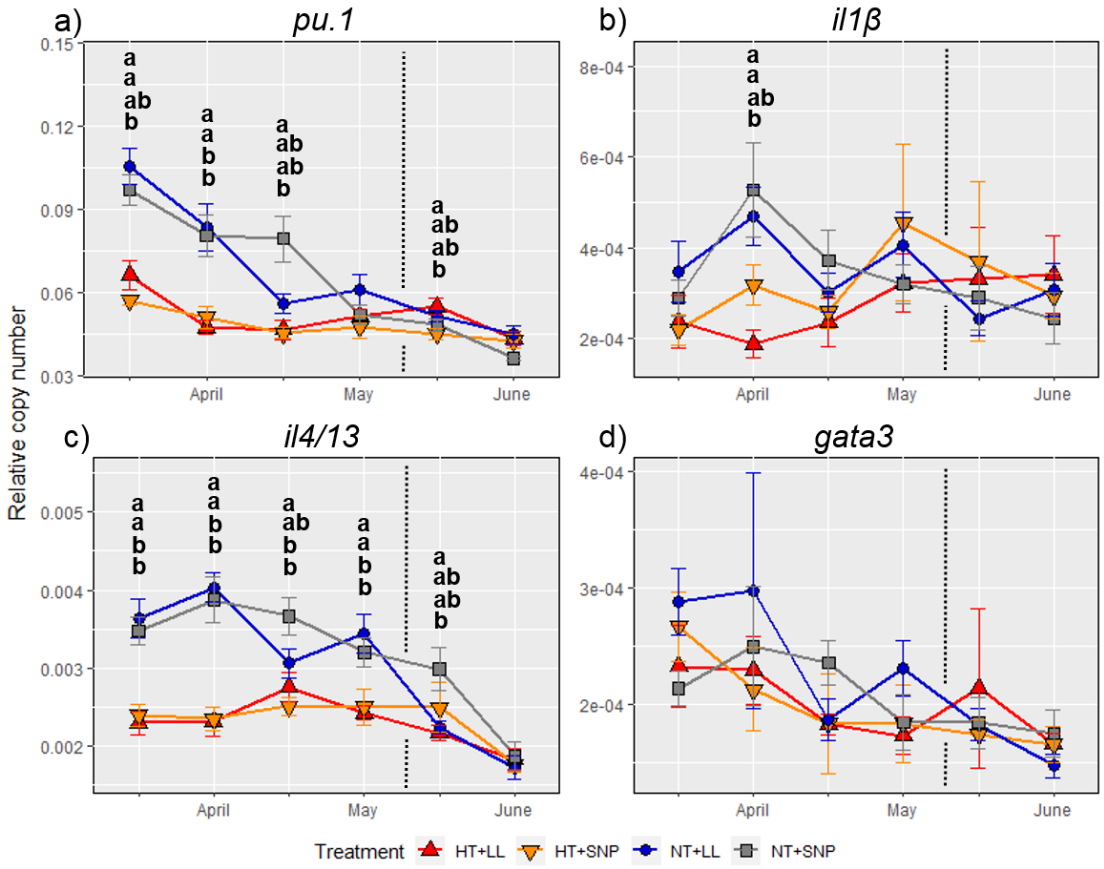


Figure 3.6. Head kidney transcription of genes of the related to haematopoiesis, *pu.1* (a), *il1β* (b), *il4/13* (c), and *gata3* (d), of juvenile rainbow trout reared in freshwater under four photoperiod and temperature treatments. Error bars indicate s.e. Samplings took place on 23rd of March, 6th of April, 20th of April, 4th of May, 18th of May and 31st of May. Dotted line indicates temperature convergence point. Different letters indicate statistical differences ($p < 0.05$) in a time point. Lack of letters indicates lack of significant differences. No letters indicate lack of significant differences.

The transcription of *pu.1* was clearly affected by temperature, being higher at lower temperatures. Significant differences due to temperature were present in mid-March (temperature effect, $p < 0.001$, df: 1, F: 70.65), April (temperature effect, $p < 0.001$, df: 1, F: 38.87), and mid-April (temperature effect, $p < 0.001$, df: 1, F: 20.15; photoperiod effect, $p < 0.001$, df: 1, F: 4.33), each progressively smaller as they approached the TC point. In mid-May, with the temperature treatment effectively removed, treatments at LL showed the highest transcription values (photoperiod effect, $p < 0.001$, df: 1, F: 7.96), finding significant differences between HT+LL and HT+SNP (Tukey test, $p < 0.05$) (Figure 3.6a).

The transcription of *il1β* was for the most part stable in time and with small differences due to treatment. Only the April sampling showed significant differences, with treatments at NT having the highest values (temperature effect, $p < 0.001$, df: 1, F: 17.30) (Figure 3.6b).

The transcription of *il4/13* was similar to that of *pu.1*, showing higher values in NT when the temperature treatment was in effect and reducing the differences between HT and NT as TC approached. Significant differences due to temperature were present in mid-March (temperature effect, $p < 0.001$, df: 1, F: 46.61) April (temperature effect, $p < 0.001$, df: 1, F: 59.27), mid-April (temperature effect, $p < 0.001$, df: 1, F: 15.78, photoperiod effect, $p < 0.05$, df: 1, F: 4.79), and in May (temperature effect, $p < 0.001$, df: 1, F: 19.12). Then, in mid-May after TC, treatments at SNP registered the highest transcription values (photoperiod effect, $p < 0.05$, df: 1, F: 5.26), with NT+SNP being significantly higher than HT+LL (Tukey test, $p < 0.05$) (Figure 3.6c).

Finally, no significant differences among treatments were found for the transcription of *gata3*. However, a downwards trend was observed in all treatments, with the highest values found in mid-March and April and the lowest in June (Figure 3.6d).

3.4. Discussion

In salmon aquaculture, it is common practice to control the photoperiod to match circannual rhythms. Even controlling temperature regimes is possible in some farms, though rarely done due to the obvious high costs of heating large volumes of water. This is done in order to produce robust smolts throughout the year while ensuring optimal growth and welfare (Stead and Laird, 2002; Johnston, 2008; Handeland *et al.*, 2013). However, for rainbow trout smoltification and seawater growth and survival, the effects of temperature and its interaction with photoperiod had not yet been tested. This study aimed at evaluating the effect of different temperature and photoperiod combinations on the induction of smoltification related traits using both traditional and well-established smolt assessment tools, such as NKA activity measurement, and less established molecular tools, like the transcription of osmoregulatory genes *nkaα1a*, *nkaα1b* and *nkcc1a* through RT-PCR. Moreover, the transcription of haematopoiesis related genes *pu.1*, *il1β*, *il4/13* and *gata3* in head kidney was measured to anticipate how, in the event of an infection during the freshwater phase, the fish would be prepared to respond. This experiment was performed on the winter to summer period, since the majority of the problems encountered by seawater-transferred rainbow trout producers occur when transferring 1+ smolts to seawater in summer.

Fish size (length and weight) was increased by HT, as was expected (Ege and Krogh, 1914; Pauly, 1979). However, when comparing TGC to avoid the effect of temperature on growth, HT seemed less advisable, as no differences between groups were found in the March to May period, while the temperature treatment was in effect. Then, in early May (8th of May), natural water temperature reached 8^{o}C and NT and HT started receiving water at the same temperature. Here, NT experienced a strong compensatory growth, growing faster than those fish in HT groups during the May to June period and slightly faster during the June to September period in seawater (similar to Solbakken *et al.*, 1994). As a result, the length and weight differences that the fish at HT had achieved in May were reduced 4 months later (1 more month in freshwater, 3 in seawater), although the fish at HT were still slightly bigger (3.17% more length, 8.48% more weight) and fish kept at NT+SNP were still significantly smaller than those kept at HT+LL. The effects of the phenomenon of compensatory growth in fish, mainly following a fasting period but also due to suboptimal temperature, crowding, or other stressful conditions like hypoxia, have been well-studied and do not affect fish survival, if carried out within reasonable levels (Ribeiro and Tsuzuki, 2010; Won and Borski, 2013; Remen *et al.*, 2014). In fact, its effects can even be positive, with fish that underwent compensatory growth reaching a bigger final size than the control group (Hayward *et al.*, 1997), thus making it a very interesting phenomenon from a production perspective. Nonetheless, the mechanism by which this compensatory growth in response to suboptimal temperature occurred remains unknown, as no changes in circulating IGF-I levels were found. This is consistent with the IGF-I abundance in plasma, abundance in white muscle and liver transcription during refeeding experiments (Montserrat *et al.*, 2007a; Montserrat *et al.*, 2007b) but also unlike muscle transcription and plasma abundance in other refeeding experiments (Chauvigné *et al.*, 2003; Picha *et al.*, 2006), making the role of this hormone in compensatory growth unclear. On another note, it is interesting to see that temperature was not the only driver of growth. The interaction between temperature and photoperiod also had a slight effect on growth, as seen on TGC-L in May-June (HT+SNP higher than HT+LL), and on TGC-W (NT+LL higher than NT+SNP). It is also remarkable to see the spread of individual growth trajectories for length did not dramatically increase after seawater transfer and that no fish grew less than 0.10 % day^{-1}, unlike what was seen in a previous study in Chapter 2, likely because in that case the fish had clearly desmolted. However, while seawater mortality was negligible in the previous study, 6.93% of the fish transferred to seawater died in the present study which, had they survived, might have become GS. Overall, in terms of growth, it is unclear whether LL or SNP would be better on either the scenario of typical winter-spring temperatures of a northern region or in the scenario of a warmer year, a warmer region or artificially increased temperature.

NKA activity in fish kept at treatments presented peaks in activity at different times, which are normally used to pinpoint the smolt window. Fish at NT did not seem to have a clear smolt window, while according to this test the most suitable seawater transfer time for HT+SNP would be in mid-April and for HT+LL in May. However, the activity levels in the first sampling date were already within smoltification levels (Madsen and Naamansen, 1989; Ewing *et al.*, 1994), pointing at the possibility that the increase in NKA activity took place previously. It is interesting to note that the NKA activity levels in seawater were lower than in freshwater, showing that the levels in freshwater are above those needed for efficient hypo-osmoregulatory activity, consistently with what was shown in Chapter 2. Regardless, the data shows that a timely seawater transfer was possible until mid-May. When it actually took place, two weeks later in June, the fish appeared to have just started their desmoltification. Nonetheless, their performance in seawater was good (no severely GS fish, mortality of 6.93%). As for the effect of the treatments, no differences between them were found for NKA at any point, unlike what has been observed for Atlantic salmon (Solbakken *et al.*, 1994; Handeland *et al.*, 1998). This is showing again the challenges associated with dysregulating the biological clock of this species by altering environmental factors; first for photoperiod in Chapter 2 and now for temperature. Hence, since the lack of significant differences in NKA activity does not allow for the identification of the most suited treatment, attention needs to be shifted to the transcription of NKA related genes. Interestingly, the data shows that they are regulated by different environmental factors: while the transcription of *nkaα1a* is strongly regulated by temperature and *nkcc1a* is mildly responsive to it, *nkaα1b* seems to be driven by photoperiod. According to this, high temperature treatment decreased the osmoregulatory capacity of the fish in both freshwater and seawater, as they negatively affected the transcription of both a freshwater and a seawater osmoregulatory gene. In any case, the downregulation of *nkcc1a* was only transient, whereas that of *nkaα1a* lasted for months, hinting that the fish would be more likely to migrate at HT (Sogard *et al.*, 2012). On the other hand, looking at *nkaα1b*, a long day photoperiod in the absence of a winter signal stimulates hypo-osmoregulatory capacity in mid-April, while an increasing photoperiod after a strong winter signal would stimulate it one month later, in a smaller magnitude, and is consistent with the lower hyper-osmoregulatory capacity of the same groups (HT+SNP ad NT+SNP) in June, after the increased temperature treatment was over. Admittedly, this effect of photoperiod on *nkaα1b* was not seen in Chapter 2, which started earlier on in the year. Further experimentation involving seawater transfer at different times, when significant differences for the transcription of these genes are present, followed by short and long term reassessment of the performance of the fish in seawater are needed to validate gene transcription of NKA related genes as seawater-readiness markers. It is hard to identify the most suitable treatment to induce hypo-osmoregulatory competence in rainbow trout.

However, the elevated transcription of *nkaα1b* in mid-April and *nkcc1a* in April seems to suggest that the seawater transfer during the first half of April of fish kept at LL would be the best strategy at NT, while at HT it would also be LL in mid-April.

No differences in plasma IGF-I levels were detected among treatments, either before or after seawater transfer. Similarly to Chapter 2, an advanced photoperiod increased plasma IGF-I levels but LL and SNP had similar levels. Water temperature is known to affect circulating IGF-I levels (Gabillard *et al.*, 2005; Beckman, 2011) but its effect might not last for several weeks. Beckman *et al.* (2004b) reported in post-smolt coho salmon that a rapid drop in water temperature affected plasma IGF-I levels for four weeks but fish appeared to be acclimatized after six. In the present study, fish were first reared in different water temperatures for about six weeks, with temperature differences being progressively smaller until TC, followed by nearly four weeks of no temperature differences among treatments. It is possible that an effect, if any, of water temperature disappeared during that time. Furthermore, a lack of correlation between plasma IGF-I and growth rate was unexpected (Beckman *et al.*, 2004b; Kawaguchi *et al.*, 2013; Kaneko *et al.*, 2019). One possible reason for this is the time interval to calculate growth rate. Most studies reported positive correlations between plasma IGF-I level and growth rate for one to two weeks (Beckman, 2011). In the present study, on the other hand, growth rate was calculated during 15 weeks. If growth rate of the experimental fish changed during the last weeks, this would explain the overall lack of relationship between plasma IGF-I levels in September and growth rate during June-September. To illustrate this, this relationship was significant for a 10-week seawater period (July-September), as shown in Chapter 2. Moreover, decreased IGF-I levels after 15 weeks in seawater contrasted with the results in the previous study, where plasma IGF-I levels were higher in fish in seawater than in freshwater. However, growth rates of fish during this phase in the present study (approximately 0.25 %/day) were comparable or even higher than those in the previous study (approximately 0.20%/day). The reason for the opposite response of plasma IGF-I is currently unknown but the result suggests that plasma IGF-I levels did not affect overall growth performance. In regards to IGFBP1b, the present study reports a negative correlation between plasma levels and SGR in rainbow trout during their 15-week seawater period, which is consistent with previous literature (Shimizu *et al.*, 2006; Kawaguchi *et al.*, 2013; Kaneko *et al.*, 2019). In juvenile chum salmon acclimated in seawater, the correlation coefficient (*r*) was high at -0.76 (Kaneko *et al.*, 2019), while it was relatively low in rainbow trout in the present study (r = -0.42). As discussed earlier for plasma IGF-I, this may be due to the long time-interval between length measurements to calculate SGR. Although more frequent sampling is necessary to validate the utility of plasma IGFBP1b as an index of growth retardation, this is the first report on a significant negative relationship between plasma IGFBP1b and SGR in rainbow trout.

Before TC, the transcription of haematopoiesis related genes was independent of the photoperiod treatment and unequivocally affected by temperature, with higher temperatures causing lower transcription levels of both adaptive and innate immune related genes. The exception was *gata3*, involved in the regulation of T lymphocytes, stimulation of phagocytosis and antibody production by B cells (Tort *et al.*, 2003; Kumari *et al.*, 2009), which did not respond to temperature variations. On the other hand, the key regulator of the development of the GMP myeloid cell lineage (*i.e.* mast cells, eosinophils, neutrophils, macrophages and dendritic cells; Orkin and Zon, 2008), *pu.1*, was strongly affected by temperature. Macrophages and dendritic cells play critical roles in antigen presentation during inflammatory responses, while eosinophils, basophils, and mast cells are involved in a variety of allergic and innate immune responses (Galli, 2000; Iwasaki and Akashi, 2007). However, as the temperature of fish kept at NT and at HT drew nearer, the differences decreased and did not seem to have further effects beyond the TC point. A pattern that can also be seen for the transcription of *il4/13*, which was the most affected gene by temperature. Through a lower *il4/13* transcription, high temperatures would result in less proliferation of B lymphocyte and immunoglobulins and lower activation of macrophages (Martinez *et al.*, 2009; Takizawa *et al.*, 2011; Sequeida *et al.*, 2017). Therefore, this could potentially affect the core components of the adaptive immune system: antibodies and memory B cells (Nutt and Kee, 2007), as well as the function associated to macrophages. Finally, *il1β* was slightly higher at NT during the first part of the freshwater experiment, being associated with a very mild and transient difference of both B and T lymphocyte abundance and activity between temperature groups (Pleguezuelos *et al.*, 2000; Reis *et al.*, 2012). More importantly, the general lack of big fluctuations over time in *il1β* transcription confirms that no infections occurred during the freshwater experimentation phase. It is interesting to see how, although the photoperiod treatment could have an effect on *pu.1* and *il4/13,* as seen in mid-May, and even on *nkaα1a* as seen in June, the effect of temperature before TC overrides any possible effects of photoperiod. While a higher transcription of haematopoiesis related genes is likely to be indicative of more robust fish against disease because they would be able to quickly produce large amounts of defence molecules (Iwasaki and Akashi, 2007; Baldridge *et al.*, 2010; Martin *et al.*, 2012), thus making NT more desirable than HT for rainbow trout production in this sense. Whether this higher resource investment in immune cells at NT is good for the fish or whether the temperature differences in these genes make any difference to response during an immune challenge is still a matter of debate.

In conclusion, while a larger length and heavier weight were achieved in freshwater by fish kept at HT, soon after TC this size advantage was quickly lost due to the compensatory growth that fish kept at NT experienced. While the temperature treatment could continue during more months, requiring temperatures above 8^{o}C and potentially maintaining the length and weight differences of fish at HT

for longer, high temperatures can negatively affect NKA activity (Handeland *et al.*, 2000). In fact, it has been suggested that smolt development is inhibited for steelhead trout at a temperature between 10°C and 15°C (Adams *et al.*, 1973). In this sense, data suggests LL photoperiod regimes would be more suitable than SNP for both NT and HT scenarios in terms of the development of osmocompetence in seawater if the fish were to be transferred in April. In terms of transcription of haematopoiesis genes, HT resulted in lower transcription levels, especially for *pu.1* and *il4/13*, thus decreasing the proliferation of lymphoid cells and macrophages (Pleguezuelos *et al.*, 2000; Iwasaki and Akashi, 2007; Sequeida *et al.*, 2017). This could affect the immune response of fish kept in these conditions, especially the adaptive immune response, potentially resulting in a lower abundance of crucial immune cells, reducing the value of prophylactic measures, and at the same time providing more suitable conditions for the proliferation of pathogens (Austin and Austin, 2012). As shown in other studies, temperature (and temperature choice) can be a crucial determinant of fish survival upon pathogen infection (Boltana *et al.*, 2013). Overall, the apparent disadvantages of using increased temperature treatments to induce smoltification on rainbow trout surpass the unclear advantages of it, therefore discouraging the use of water heating strategies and alerting farms found in warmer climates of the possible implications that their conditions might have on rainbow trout.

Chapter 4: Plasma proteome profiling of freshwater and seawater life stages of rainbow trout

4.1. Introduction

Migratory animals take advantage of seasonally predictable patterns of resource availability and predator abundance and migrate accordingly aiming at maximal survival and to meet their energy demands, though there is often a trade-off between the two (Fleming and Reynolds, 2004; Quinn *et al.*, 2011). For fish, most of these migrations occur within the same water type, while less than 1% of fish species cross the boundary between freshwater and seawater (Quinn *et al.*, 2016). This life strategy, known as anadromy, is widespread among several fish families, the most studied of which are the salmonids (salmons, trouts and charrs).

Juvenile anadromous salmonids, called parr, lack the biological traits needed for life in seawater. Upon reaching a threshold size (Kendall *et al.*, 2015), environmental cues, such as changes in photoperiod, water temperature and salinity, trigger hormonal alterations involving their pituitary, thyroid and inter-renal tissues (Prunet *et al.*, 1989). In turn, these tissues orchestrate a series of simultaneous, yet often independent, changes that preadapt anadromous salmonids to life in seawater (Hoar, 1988; Björnsson *et al.*, 2011). These changes are biochemical in nature, such as the increase of gill NKA activity, which is the main enzyme involved in ion absorption and secretion, and seawater tolerance (McCormick, 2001; Mancera and McCormick, 2007); morphological, such as the transition from dark, rounded parr to a silvery, streamlined phenotype (Winans and Nishioka, 1987); and behavioural, including the shift from bottom-dwelling, aggressive and territorial parr to pelagic, schooling and downstream migrating (Riley *et al.*, 2014). All of these changes are collectively grouped under the term smoltification and the resulting phenotype is known as a smolt. Then, after successfully reaching the ocean, they enter the post-smolt stage.

Extensive literature dating back to the 1950s is available on smoltification, its relationship with salmonid migration, ways to induce it in commercially valuable species, and subsequent seawater performance (Fontaine and Hatey, 1950; Kobayashi and Yuki, 1954; Hoar, 1988). A vast majority of these studies have a clear emphasis on the endocrinology of the process (Prunet *et al.*, 1989; Ebbesson *et al.*, 2008; Björnsson *et al.*, 2011) or on treatments to induce an increase in NKA activity levels (Zaugg and Wagner, 1973b; McCormick *et al.*, 2009; Handeland *et al.*, 2013). However, in the last two decades, and especially after the publication of the Atlantic salmon (Davidson *et al.*, 2010) and rainbow trout genomes (Berthelot *et al.*, 2014), an increasing number of DNA and RNA-based studies

have been published on smoltification. Findings include gene expression changes after seawater transfer (Norman *et al.*, 2013; Norman *et al.*, 2014; Johansson *et al.*, 2016), epigenetic modifications (Baerwald *et al.*, 2016), quantitative trait loci (Hecht *et al.*, 2012), and gene expression patterns (Hecht *et al.*, 2014; Sutherland *et al.*, 2014; Hale *et al.*, 2016) associated with the likelihood of steelhead trout to migrate to seawater. At the protein level, except for targeted, top-down studies of key protein hormones such as IGF-I, GH, insulin and their receptors (Beckman *et al.*, 2004a; Mancera and McCormick, 2007; Shimomura *et al.*, 2012), very little research has been done on smoltification. In this sense, an untargeted, bottom-up protein approach (*i.e.* proteomics) would have the potential to identify proteins previously unknown to be related to the smoltification process, which could be used as biomarkers in the future (Björnsson and Bradley, 2007). However, so far to our knowledge no proteomic work has been published in relation to the smoltification process.

Shotgun proteomics has been used for the study of proteins in complex biological samples (Zhu *et al.*, 2010). To this end, LC-MS/MS is one of the preferred pipelines used due to its high versatility and high protein identification potential in complex samples. This is achieved due to the physical separation capabilities of liquid chromatography coupled with the ionization capabilities of MS (Geromanos *et al.*, 2009). However, LC-MS/MS alone has not sufficient capability to comprehensively analyse complex samples with a high dynamic range such as blood plasma (Liumbruno *et al.*, 2010). To increase the coverage of the plasma proteome, samples can be fractionated prior to LC-MS/MS, thus dividing the original sample in less complex subsamples. Several studies have increased plasma proteome coverage by physically separating the proteins present according to their size by 1-D SDS-PAGE, cutting the gels at specific intervals, protease digesting each gel fraction independently, and analysing each protein digest one by one (Barnea *et al.*, 2005; Fang *et al.*, 2010). This method is known as gel electrophoresis LC-MS/MS (GeLC-MS/MS). However, even after this fractionation, low-abundant proteins might not be detected. Though low in abundance, these proteins can be highly relevant (Corthals *et al.*, 2000). In order to detect this group of molecules, a further high-abundant protein depleting step or low-abundant protein enrichment step is needed. Among them, Bio-Rad's ProteoMiner™ enrichment has been shown to significantly improve proteome coverage in blood plasma (Bandow, 2010; De Bock *et al.*, 2010; Millioni *et al.*, 2011).

Blood plasma is a key biofluid for the transport of proteins and peptides to and from tissues, thus containing other tissue proteomes as subsets, making it the single, most complex (with proteins that differ in over 10 orders of magnitude in abundance; from milligrams to pictograms per millilitre; Liumbruno *et al.*, 2010) and informative proteome (Anderson and Anderson, 2002; Jacobs *et al.*, 2005; Pernemalm and Lehtiö, 2014). Plasma protein studies have succeeded in discovering biomarkers for disease (Hye *et al.*, 2006; Hanash *et al.*, 2008; Geyer *et al.*, 2017), growth (Beckman, Fairgrieve *et al.*,

2004; Beckman, 2011), stress (Fast *et al.*, 2008; O'Loughlin *et al.*, 2014), exposure to water contaminants (Hiramatsu *et al.*, 2006; Palermo *et al.*, 2008), or doping (Barton *et al.*, 2009), amongst many others. Therefore, it is highly likely that more protein biomarkers for the smoltification and seawater adaptation processes may be discovered in blood plasma.

The aim of this study is to characterise proteome changes in blood plasma of rainbow trout, associated to the smoltification process. Its objectives are to 1) provide an in-depth characterization of the plasma proteome of rainbow trout, 2) make this plasma proteome publicly available, and 3) discover new candidate biomarkers to complement current seawater-readiness evaluation tests in rainbow trout.

4.2. Materials and methods

4.2.1. Ethics

Experimental work was ethically reviewed, approved and registered by the NARA, by the AWERB (088) at the University of Stirling (UK) and by the ethical review body of the University of the Highlands and Islands.

4.2.2. Fish and rearing conditions

Juvenile rainbow trout (AquaGen) with an initial weight of 78 ± 16.7 g were used in this experiment. Fish were fed *ad libitum* using a standard commercial dry diet (Skretting AS) from automatic feeders according to temperature and fish size. Fish were kept indoors in tanks equipped with timer-controlled LED lights in a rainbow trout facility from Lerøy Vest AS (Bjørsvik, Hordaland, Norway). The fish were kept at natural temperature, water flow at 0.4 L/kg/min and O_2 was above 80% saturation in the outlet.

The present experiment was carried out on a subset of samples generated in Chapter 2.

4.2.3. Sampling

Lethal samplings were conducted in freshwater on the 3rd of March and 11th of May and in seawater on the 14th of September 2016.

Fish were quickly dip-netted out of the tanks and euthanized by lethal overdose of isoeugenol (AQUI-S). For each fish, weight and length were recorded. Blood was extracted using heparinised syringes and centrifuged at 3,500xg for 10 min to obtain plasma, which was frozen at -80^{o}C. The first gill arch was dissected out and preserved at -80^{o}C in SEI buffer (Sucrose 250mM, Na_2EDTA 10mM, Imidazole 50mM (all Sigma-Aldrich)).

4.2.4. Gill NKA activity

NKA activity was measured as described in Chapter 2.

4.2.5. Sample pools for proteomic analysis

Three pools of plasma were made using equal amounts of protein per sample: Parr pool, Smolt pool and Post-smolt pool (Table 4.1; name of pools is capitalized hereafter while name of developmental stage is not). The Parr pool was made using 17 fish sampled in March (3rd March) that presented NKA values below 4 µmol ADP mg protein^{-1} hour^{-1}. The Smolt pool using 18 fish sampled during the smolt window (11th May) that presented NKA values above 6 µmol ADP mg protein^{-1} hour^{-1}, a value that is considered indicative of osmocompetence in seawater for rainbow trout and therefore of fish having entered their smolt phase (Madsen and Naamansen, 1989; Ewing *et al.*, 1994). Finally, the Post-smolt pool was made using 12 fish sampled at the end-point sampling in seawater (14th September), 9 weeks after seawater transfer, that presented a condition factor above 1.50 g cm^{-3}, thus avoiding the selection of GS fish, which are a phenotype that commonly appears after seawater transfer, characterized by high mortalities, stunted growth and a decrease in condition factor.

Table 4.1. Measurements in fish used for plasma pools (values ± s.e.).

Pool	Sampling	Length (cm)	Weight (g)	Fulton index	NKA activity
Parr	3rd March	18.5 ± 0.39^{c}	85.9 ± 5.36^{c}	1.3 ± 0.02^{b}	1.9 ± 0.26^{b}
Smolt	11th May	22.2 ± 0.35^{b}	147.6 ± 6.37^{b}	1.3 ± 0.02^{b}	9.9 ± 0.60^{a}
Post-smolt	14th September	30.2 ± 0.40^{a}	434.5 ± 17.08^{a}	1.6 ± 0.02^{a}	3.0 ± 0.71^{b}

Fulton index is measured in g cm^{-3}. NKA activity is measured in µmol ADP mg protein^{-1} hour^{-1}. Different letters indicate significant differences.

Admittedly, sample pooling is a controversial strategy when used to draw biological conclusions, with studies advocating for (Weinkauf *et al.*, 2006; Neubauer *et al.*, 2006; Zhang, Wuyan *et al.*, 2007) and against it (Karp, Natasha A. *et al.*, 2005; Horgan, 2007; Karp, Natasha A. and Lilley, 2007). A limitation of pooling is that proteins detectable in only a few samples are seldom not detectable in pools due to a dilution effect (Zolg, 2006; Diz *et al.*, 2009) but in the present study, a low-abundant protein enrichment step addressed this problem. Moreover, there is the concern that sample pooling may significantly reduce statistical power (Diz *et al.*, 2009). However, studies dedicated to studying the effects of sample pooling in proteomics conclude that pooling designs have statistical power almost matching that of separately analysed samples (Karp, Natasha A. *et al.*, 2005; Karp, Natasha A. and

Lilley, 2007; Karp, N. A. and Lilley, 2009; Diz *et al.*, 2009). Indeed, they showed that for a majority of the proteins, protein expression in a pool matches the mean expression of the individual biological replicates in it. It is an experimental design which allows to perform experiments when biological material per sample is limited (Shih *et al.*, 2004; Martínez-Fernández *et al.*, 2008), and when sample processing and analysis are excessively time-consuming and expensive (Zhang, Wuyan *et al.*, 2007), as was the case here. In this circumstances, rather than using a small number of individually analysed biological replicates, resulting in low power and preventing the detection of significant differences, a pooled design was more appropriate (Karp, Natasha A. *et al.*, 2005; Karp, Natasha A. and Lilley, 2007; Karp, N. A. and Lilley, 2009; Diz *et al.*, 2009). Furthermore, sample pooling is an especially appropriate strategy when interest lies not on the individual but on characteristics of a population, such as in biomarker studies or when performing a broad characterization of a type of sample, since pooling makes dominant differences and similarities between groups easily detectable due to an inherent reduction in biological variation (Kendziorski *et al.*, 2005; Karp, Natasha A. and Lilley, 2007).

An overview of the full methodological workflow used in the present experiment from this point onwards is provided in Figure 4.1.

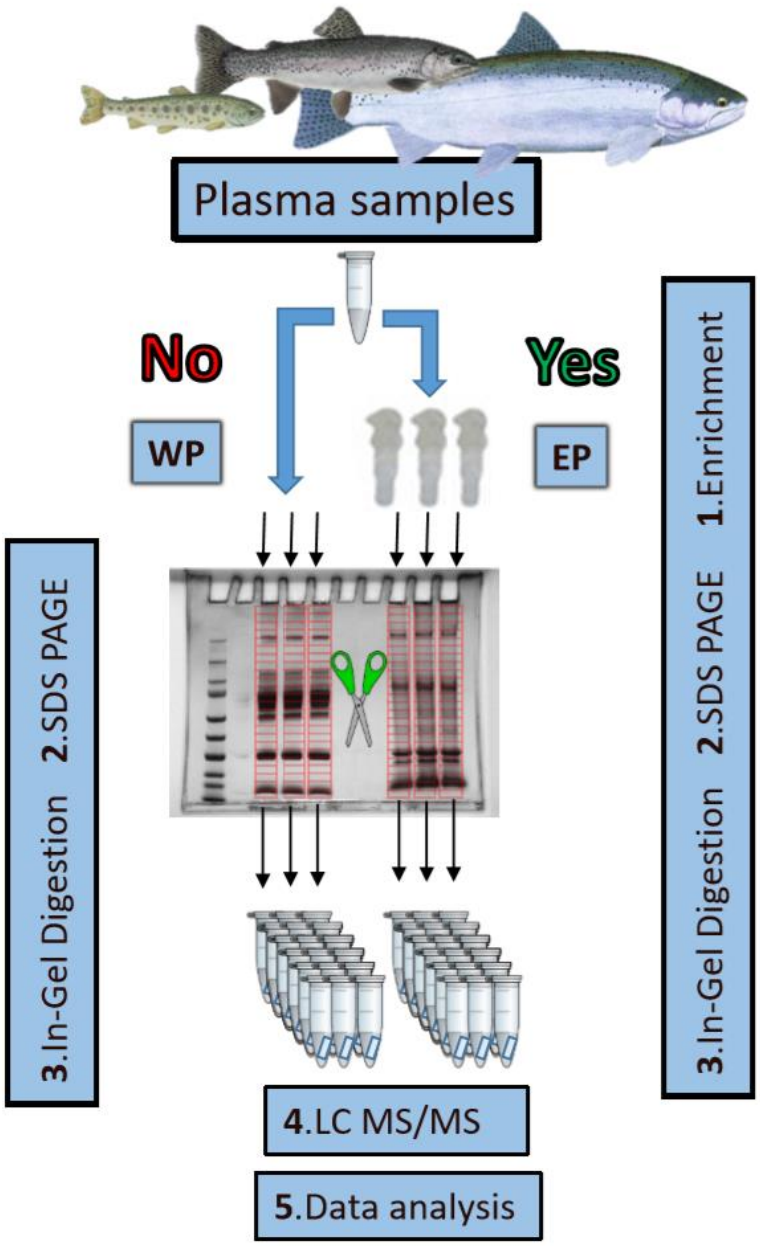


Figure 4.1. Methodological workflow. Scissors indicate fractionation and red divisions indicate gel cuts (done equally for each lane).

4.2.6. Low-abundance proteins enrichment

After measuring protein concentration by BCA using a plate reader (Synergy HT, BioTek), enriched plasma (EP) was prepared using ProteoMiner™ Protein Enrichment Small-Capacity Kit (Bio-Rad) in triplicates on each of the pools (10 mg of protein) following the manufacturer's instructions and increasing the sample binding time to 3 h.

4.2.7. Analysis of proteins by GeLC-MS/MS

4.2.7.1. 1-D SDS-PAGE analysis

For each plasma pool, 10 µg of plasma (EP or whole plasma (WP)) were analysed in triplicates in order to expand protein identifications and to account for technical noise during data analysis (Diz *et al.*, 2009; Paulo, 2013). Samples were mixed in reducing buffer (13.1 mM Tris—pH 6.8, 2.63% v/v Glycerol, 0.42% v/v sodium dodecyl sulfate (SDS), 0.243% v/v bromophenol blue and 163.5 mM dithiothreitol (DTT)), heated up to 95˚C for 5 min and centrifuged at 2,000xg for 30 s. Reduced lysates were loaded into a 1-D SDS polyacrylamide gel (4–15%, Mini-PROTEAN TGX, BIO-RAD) with a protein ladder reference (5µl, BenchMark, 10-220kDa, ThermoFisher Scientific). Gels were run using a Mini PROTEAN Tetra Cell System (Bio-Rad) at 200 V (400 mA) for around 50 min. Protein bands were stained with SimplyBlue Safestain (Thermo Fisher Scientific) following the manufacturer's instructions and destained overnight in MilliQ water (Millipore, Merck). 1-D SDS-PAGE pictures were taken using an inGenius LHR Gel Imaging System (SynGene) and band densitometry data was obtained using GeneTools software version 4.3.8 (SynGene). Profile height (*i.e.* band intensity) to relative mobility (R_f) data was imported into R and plotted using ggplot2 package (Wickham, 2009).

4.2.7.2. In-gel digestion

On the next day, each gel lane was cut in 24 gel plugs of 3-4 mm in a laminar flow fume cabinet and fractions were stored in separate 1.5 ml tubes. Destain solution (100 µl) (50% 100mM ammonium bicarbonate (Ambic, Sigma-Aldrich) and 50% acetonitrile (ACN, Fisher Chemical)) was added to each plug and incubated at 37^{o}C for 10 min in a thermoblock. The liquid was discarded and replaced with another 100 µl of destain solution and incubated again for 10 min. After discarding the liquid, 50 µl of 10 mM DTT was added to each sample to reduce proteins. After a 30 min incubation at 37^{o}C, the liquid was discarded and the plugs were alkylated with 50 µl of 55 mM iodoacetamide (IAA, GE Healthcare) for 30 min at 37^{o}C. After discarding the liquid, 50 µl of 100% ACN was added to each tube and incubated for 15 min at 37^{o}C. Then, the ACN was removed and the gels plugs were air dried at RT for 10 min before adding 50 µl of trypsin (Roche, 0.01 mg/ml in 10% acetic acid and 45 mM Ambic) and incubating it at 37^{o}C. After 30 min, an extra 20 µl of 50 mM Ambic was added to each tube and left overnight. Then, 70 µl of 100% ACN was added to each tube and incubated at RT for 15 min with shaking. The liquid was transferred to new tubes, while 50 µl of 97.5% ACN and 2.5% formic acid was added to each gel plug and incubated at RT for 15 min with shaking to extract any remaining peptides left in them. This liquid was added to the corresponding tube and the gel plugs were discarded. Finally, the trypsin digests were dried using a vacuum drier (Savant DNA SpeedVac 110, Thermo Scientific).

4.2.7.3. LC-MS/MS analysis

Tryptic digests were analysed with a LTQ-Orbitrap XL LC−MSn mass spectrometer (Thermo) equipped with a nanospray source and coupled to an Ultra High Pressure Liquid Chromatographer system (Waters nanoAcquity). Initially, 5 µL of sample resuspended in ultrapure water were loaded, desalted and concentrated in a BEH C18 trapping columns (Waters) with the instrument operated in positive ion mode. The peptides were then separated on a BEH C18 nanocolumn (1.7 µm, 75 µm × 250 mm, Waters) at a flow rate of 300 nL/min using an ACN/water gradient; 1% ACN for 1 min, followed by 0−62.5% ACN over 21 min, 62.5− 85% ACN for 1.5 min, 85% ACN for 2 min and 100% ACN for 15 min.

MS spectra were collected using data-dependent acquisition in the m/z range 400−2,000 using a precursor ion resolution of 30,000, following which individual precursor ions (top 5) were automatically fragmented using collision induced dissociation with a relative collision energy of 35%. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 s and exclusion duration of 180 s.

4.2.7.4. LC-MS/MS data analysis and protein identification

Mass spectrometry data was analysed using Progenesis QIP (Nonlinear Dynamics). WP and EP datasets were analysed independently following the 'fractionation experiment' analysis. Two pairwise comparisons were performed for WP datasets (*i.e.* Parr (WP) vs. Smolt (WP), Smolt (WP) vs. Post-smolt (WP)) and two more for EP datasets (*i.e.* Parr (EP) vs. Smolt (EP), Smolt (EP) vs. Post-smolt (EP)). The initial search parameters allowed for a single trypsin missed cleavage, carbamidomethyl fixed modification of cysteine residues, oxidation of methionine (variable), acetylation of N-terminal peptides, a precursor mass tolerance of 10 parts per million (ppm), charge of deconvoluted ions of over 1, a fragment mass tolerance of ±0.5 Da, and FDR of 0.01.

After normalization, the Hi3 (Top3) method was used for protein quantification (Li *et al.*, 2009); therefore a minimum of 3 peptides was required for quantification. Moreover, only those proteins identified based on at least one unique peptide were quantified. Statistical differences were tested by ANOVA in Progenesis QIP. To be considered differentially abundant proteins (DAPs), a q-value below 0.05 ($q < 0.05$) and a fold change (FC) bigger than 2 was required. However, since the sole purpose of EP was to improve the coverage of WP, proteins quantified in WP were not statistically tested in EP.

Peptide sequences were matched to a database search against the *Oncorhynchus mykiss* SwissProt database, which was downloaded from MASCOT [downloaded in August 2018] and loaded into Progenesis QIP. Those identified as 'uncharacterised' in the rainbow trout genome were sequentially blasted against the Atlantic salmon, zebrafish (*Danio rerio*), and human (*Homo sapiens*) SwissProt

databases, in this order of preference. Only homologies of E-value lower than 0.01 were accepted as valid.

4.2.8. Gene ontology (GO) analysis

GO of biological process, cellular component and molecular function were performed to compare the dataset of unique WP proteins with the dataset of unique EP proteins in order to identify possible differences in protein affinity between the two methods. Analysis was performed using STRAP v. 1.5. (Bhatia *et al.*, 2009).

4.2.9. Further data analysis and representation

Data representation was carried out using Microsoft Excel 2013 or R statistical software and R package ggplot2.

One-way ANOVA was performed to test for differences in morphometric measures and NKA activity among developmental stages. Data was transformed by either natural logarithm or square root to satisfy the normal distribution and homogeneity of variance assumptions, tested with the Shapiro and Bartlett tests, respectively. Significant comparisons ($p < 0.05$) were followed by Tukey's posthoc test to identify different developmental stages.

Linear relationship among variables was determined by linear regression using the QR method. Significance values ($p < 0.05$) were obtained by testing the null hypothesis: the slope of the least squares linear fit to the data is equal to 0.

Principal component analysis (PCA) and data representation were carried out using R and 'ggbiplot' package (Vu, 2011). Ellipses show 68% of Normal probability for each group.

Mean abundance per condition was plotted for DAPs in heatmap form scaling abundance by protein (*i.e.* by row).

4.3. Results

4.3.1. Characterisation of rainbow trout plasma according to GeLC-MS/MS alone or in combination with protein enrichment technology

4.3.1.1. Detected proteins

A total of 48,196 peptides were detected in WP, which were mapped onto 2,784 rainbow trout proteins. Of these, 1,495 met the requirements for reliable identification and quantification (*i.e.* quantified proteins; identified based on at least 3 peptides and at least 1 unique peptide)(Appendix 4.1). Similarly, for EP, 48,921 peptides were detected, which were mapped onto 1,892 rainbow trout proteins and 1,292 were quantified (Appendix 4.2). Interestingly, the number of unique peptides found for a particular protein was higher for more abundant proteins (Figure 4.2) and significantly correlated with the protein mass and length (Figure 4.3).

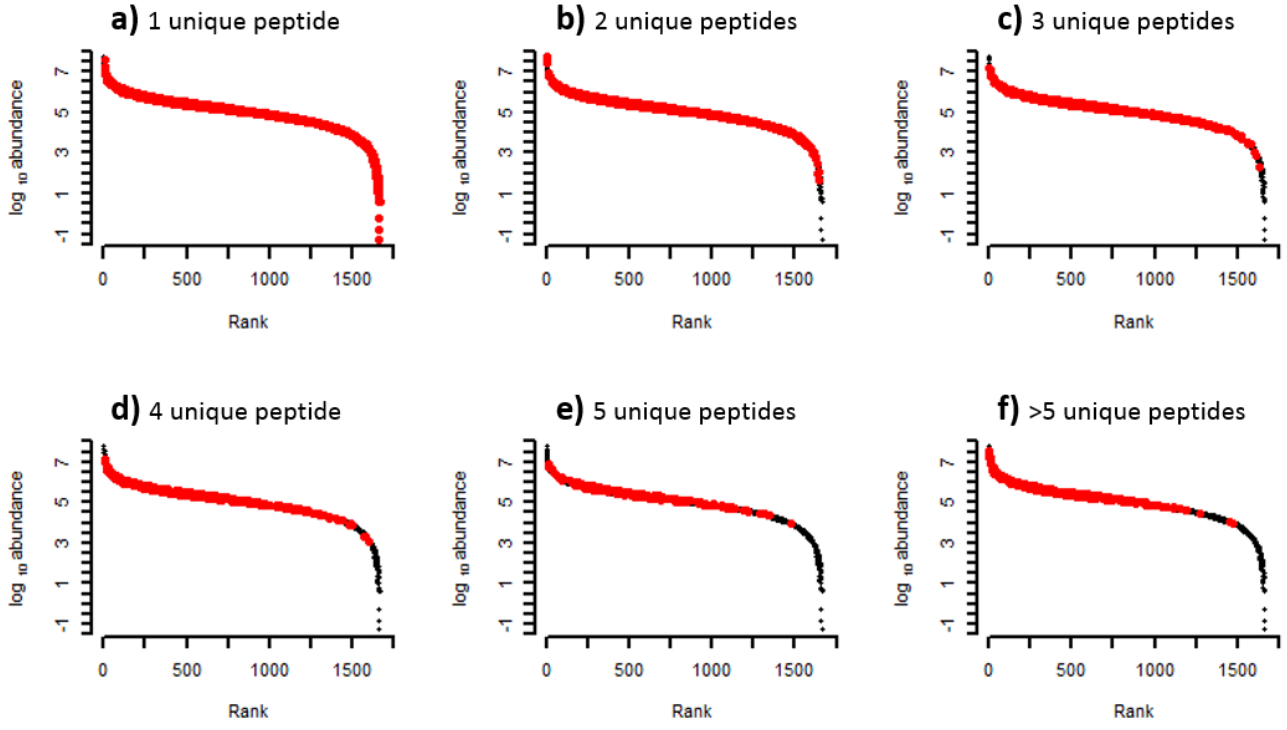


Figure 4.2. Detected unique peptides. Proteins for which 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), or more than 5 (f) unique peptides were detected. Proteins are arranged according to their dynamic range. Red points indicate proteins that meet the criteria of the corresponding panel.

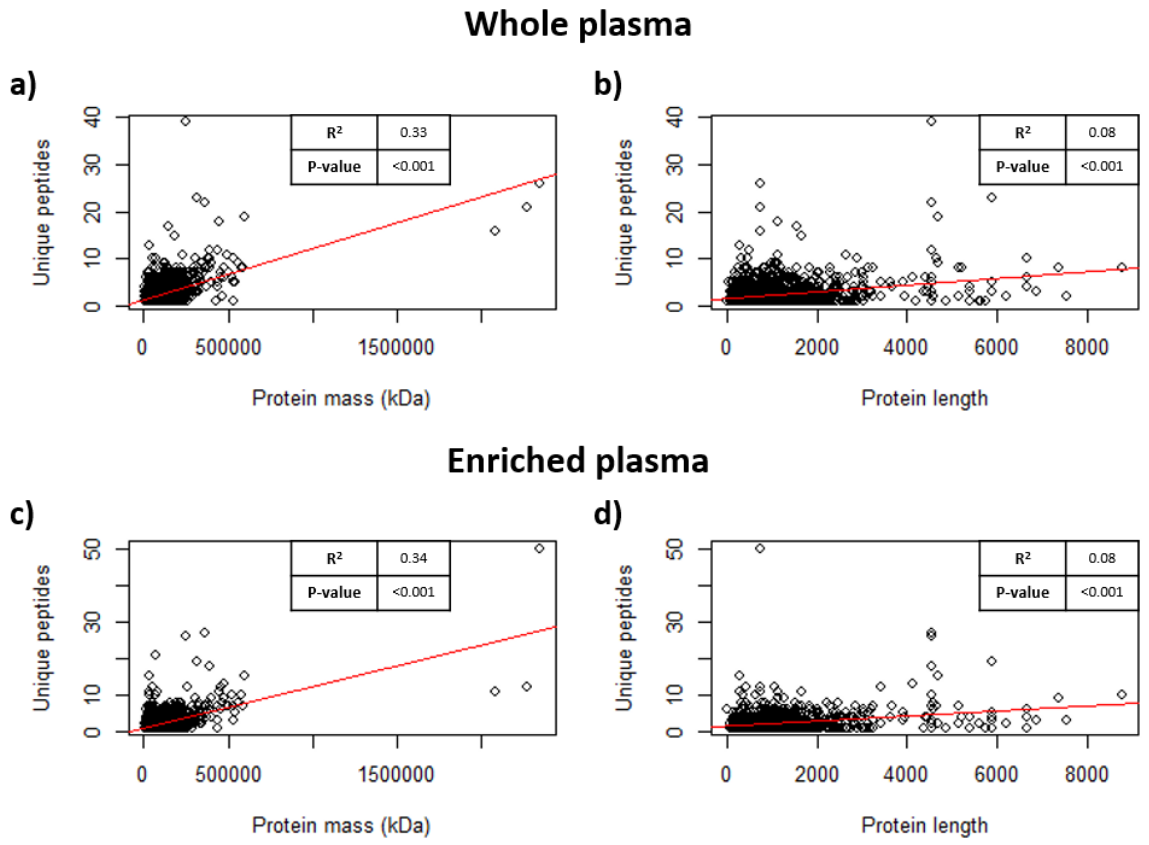


Figure 4.3. Number of detected unique peptides and protein mass and length. Correlation between the number of unique peptides with protein mass (a) and amino acid length (b) in WP and in EP (c, d).

Comparison of WP and EP datasets revealed that 965 proteins were detected by both strategies, these being a majority (52.96%). Another 530 (29.01%) were only found in WP samples and 327 (17.95%) were only detected after enrichment - only in EP (Figure 4.4a). These differences between WP and EP were already apparent when inspecting the protein profiles by 1-D SDS-PAGE, with EP missing some of the very intense bands of WP and having bands that could not be visualized in WP (Figure 4.5). For both WP and EP, samples corresponding to the three tested developmental conditions followed a similar trend of dynamic ranges, indicating that the quantitative distribution of the quantified proteins was comparable in Parr, Smolt and Post-smolt pools. For both WP and EP, the quantified proteins presented abundances that covered 9 orders of magnitude (Figure 4.4b and c).

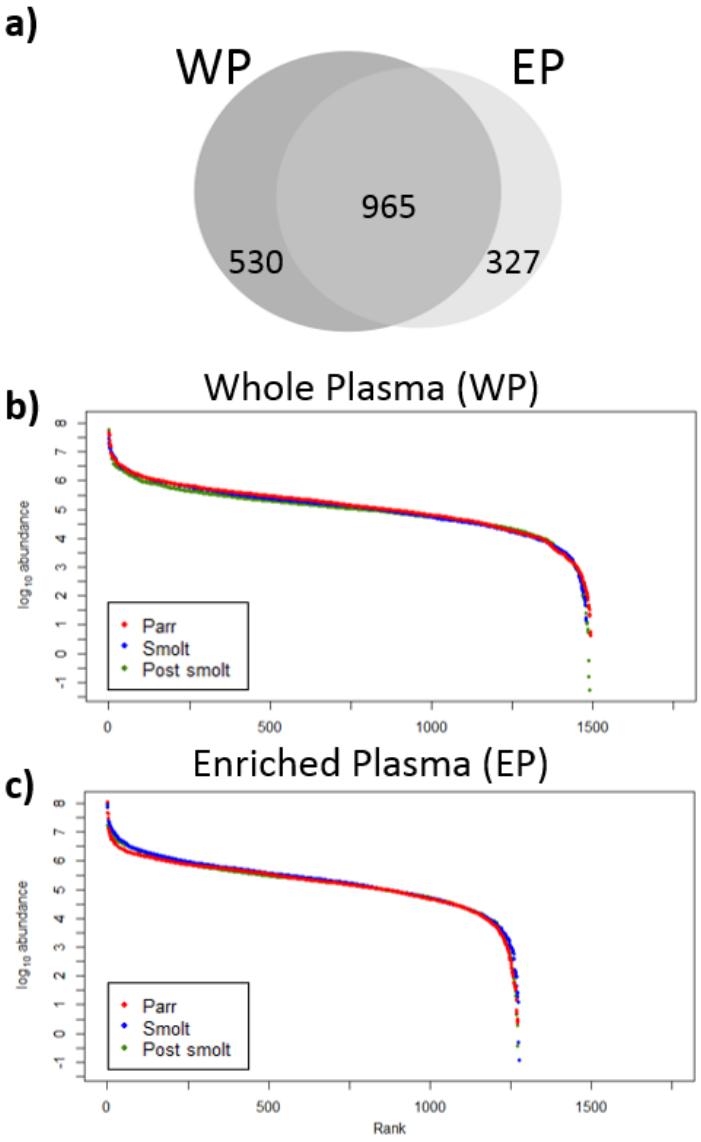


Figure 4.4. Quantified proteins in WP and EP. Venn diagram of proteins quantified in WP and EP (a). Dynamic range of proteins quantified in WP (b) and in EP (c). Rank indicates order of proteins in each condition, from most to least abundant.

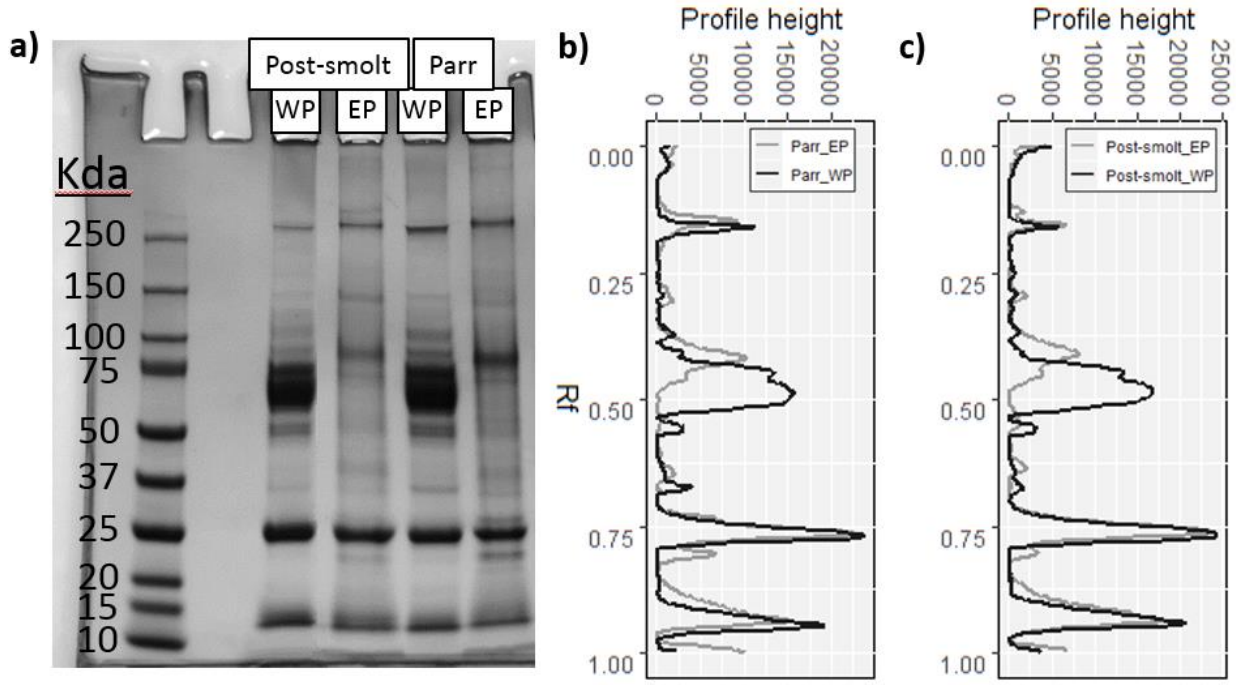


Figure 4.5. One-dimensional SDS PAGE of Post-smolt and Parr WP and EP. Gel image (a), densitometry of Post-smolt pool WP and EP (b) and densitometry of Parr pool WP and EP (c).

To facilitate comparison with other published or future studies: 2,784 proteins would have been identified based on a minimum of one peptide, 2,534 on a minimum of two peptides, 1,733 based on a minimum of one unique peptide and 885 on a minimum of two unique peptides.

4.3.1.2. Enrichment correlations

To test which protein physicochemical properties control the change in protein abundance due to enrichment (increase of low-abundant proteins and decrease of high-abundant proteins), correlations between the FC in abundance of a specific protein from WP to EP with the protein's mass, length and abundance in WP were tested.

Results indicate that neither protein mass (Parr: $r^2 = 1*10^{-4}$, p-value = 0.74; Smolt: $r^2 = 1*10^{-4}$, p-value = 0.76; Post-smolt: $r^2 = 3*10^{-4}$, p-value = 0.62) nor length (Parr: $r^2 = 1*10^{-4}$, p-value = 0.75; Smolt: $r^2 = 1*10^{-4}$, p-value = 0.76; Post-smolt: $r^2 = 3*10^{-4}$, p-value = 0.62) were correlated with enrichment. However, a pronounced significant correlation was present between the FC of enrichment and the original abundance of the protein in WP (Figure 4.6, Parr: $r^2 = 0.46$, p-value < 0.001; Smolt: $r^2 = 0.42$, p-value < 0.001; Post-smolt: $r^2 = 0.43$, p-value < 0.001). This demonstrated that the low-abundant protein enrichment worked satisfactorily, with the abundance/detectability of low-abundant proteins

being increased by up to almost 3 million times and that of high-abundant proteins decreased over 200,000 times.

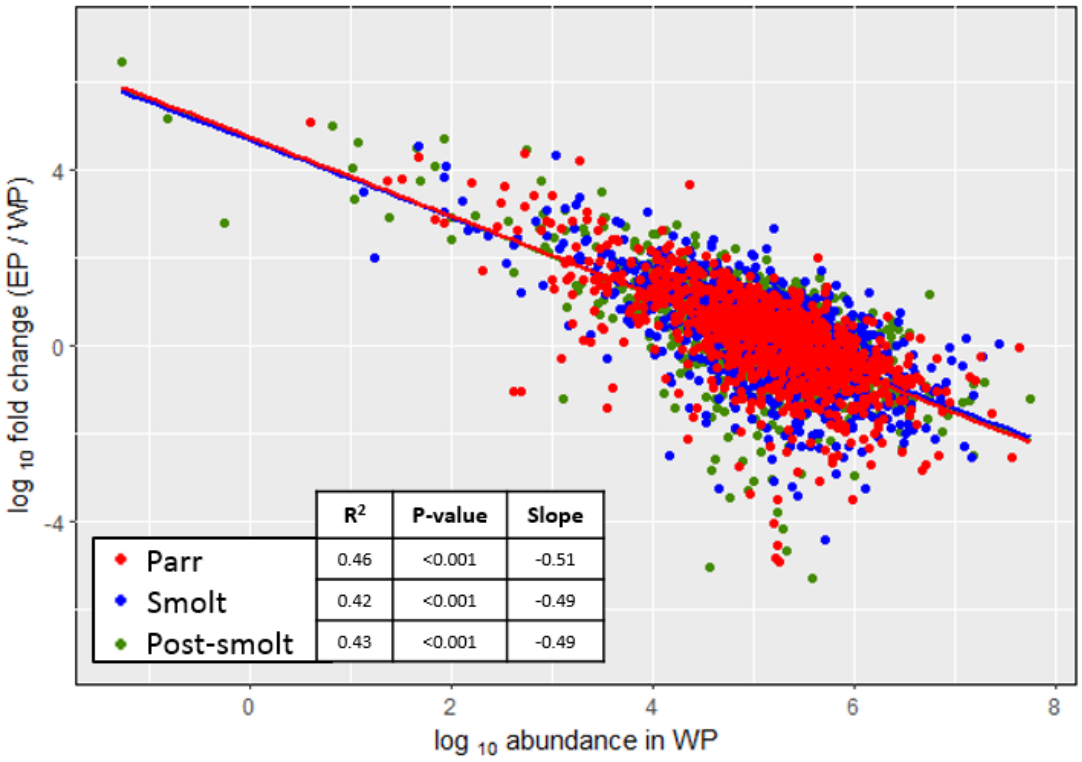


Figure 4.6. Effect of enrichment. Correlation between the FC from WP to EP and original abundance in WP for each protein. Points indicate individual samples and lines indicate linear regression fit.

4.3.1.3. GO of WP and EP unique proteins

General GO analysis revealed no differences between proteins quantified exclusively in WP or in EP pools regarding biological process, cellular component or molecular function, as there were no exclusive categories to either WP or EP (Figure 4.7).

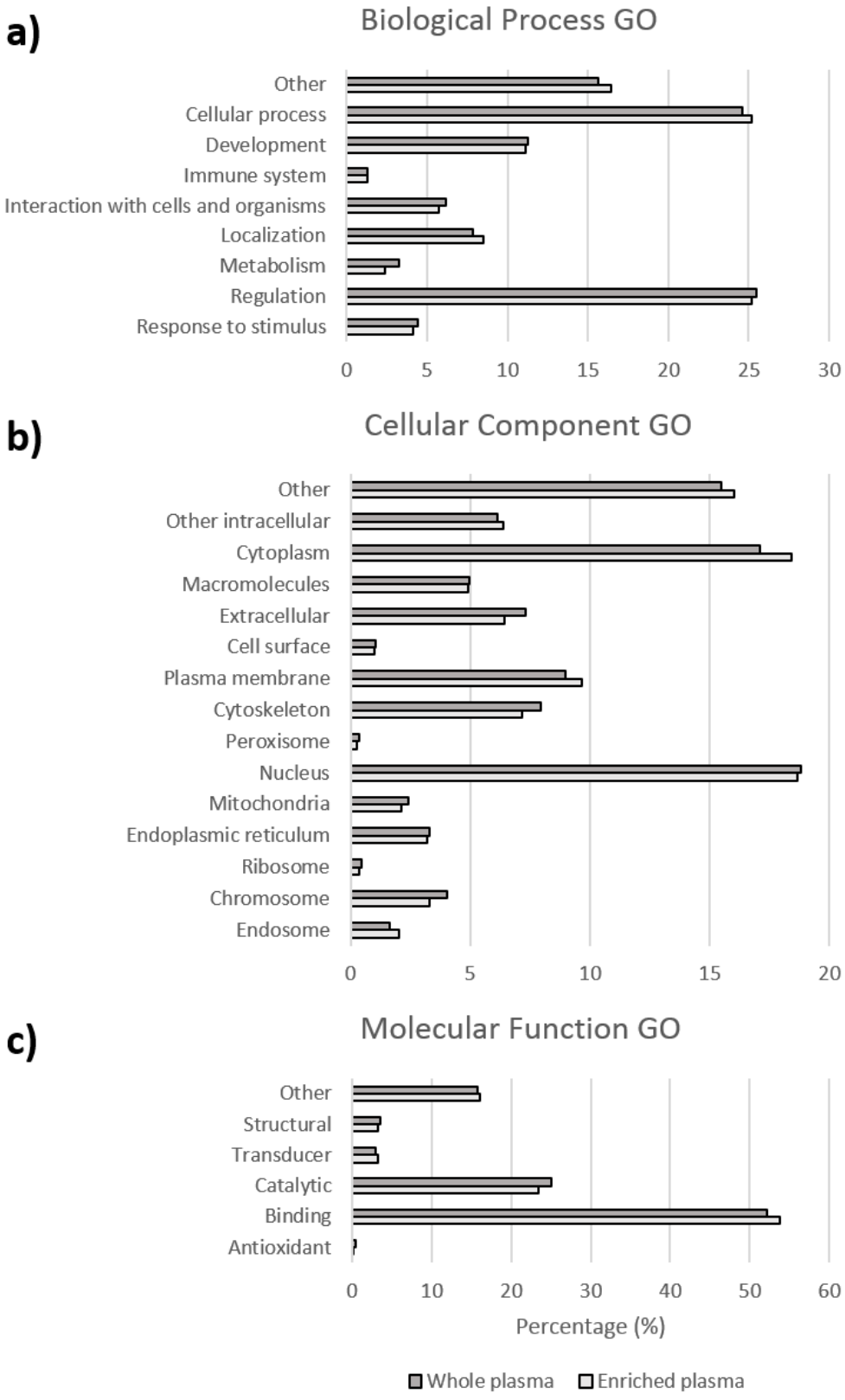


Figure 4.7. GO terms associated to proteins quantified in whole plasma and enriched plasma. Biological GO (a), cellular component GO (b) and molecular function GO (c).

4.3.1.4. PCA

PCA was used to visualize the relationship between replicates, the dissimilarity among developmental conditions, and the effects of the enrichment strategy. The first two components of the PCA explained 30.2% of the variation between samples. A clear distinction between the three tested developmental conditions was possible in WP samples, mainly due to PC2, but not in EP samples (Figure 4.8). The distinction between WP and EP was very clear, mainly driven by PC1.

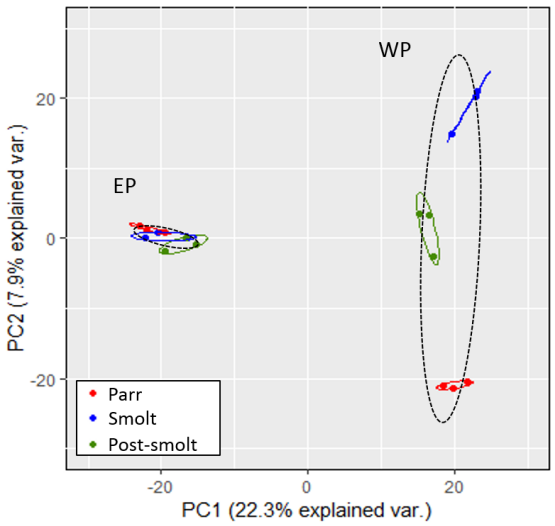
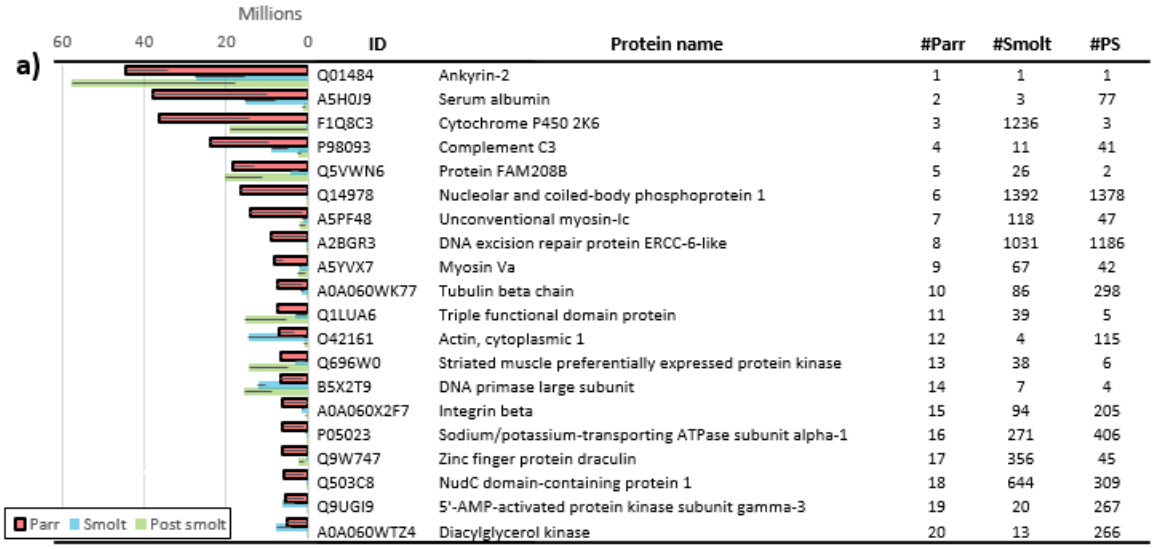


Figure 4.8. PCA of quantified parr, smolt and post-smolt rainbow trout plasma proteins. Points indicate individual replicates. Ellipses show 68% Normal probability for each group (colours) or enrichment (dashed).

4.3.2. Characterisation of rainbow trout plasma according to developmental stage

4.3.2.1. Most abundant proteins

Across all life stages, the most abundant proteins were ankyrin-2, DNA primase large subunit, actin, serum albumin, apolipoproteins, haemoglobin subunits, hemopexin-like proteins and complement C3. However, with the exception of Ankyrin-2, which was invariantly the most abundant protein in all three tested conditions, and DNA primase large subunit, which was consistently among the top 20 in all three conditions, the list of top 20 most abundant proteins was highly variable among Parr, Smolt and Post-smolt pools (Figure 4.9). Nevertheless, only one protein, P04114 (Apolipoprotein B-100), presented significant differences between conditions. Since Proteominer^{TM} was used to study low abundant proteins, EP was not considered in this section.



a)

ID	Protein name	#Parr	#Smolt	#PS
Q01484	Ankyrin-2	1	1	1
A5H0J9	Serum albumin	2	3	77
F1Q8C3	Cytochrome P450 2K6	3	1236	3
P98093	Complement C3	4	11	41
Q5VWN6	Protein FAM208B	5	26	2
Q14978	Nucleolar and coiled-body phosphoprotein 1	6	1392	1378
A5PF48	Unconventional myosin-Ic	7	118	47
A2BGR3	DNA excision repair protein ERCC-6-like	8	1031	1186
A5YVX7	Myosin Va	9	67	42
A0A060WK77	Tubulin beta chain	10	86	298
Q1LUA6	Triple functional domain protein	11	39	5
O42161	Actin, cytoplasmic 1	12	4	115
Q696W0	Striated muscle preferentially expressed protein kinase	13	38	6
B5X2T9	DNA primase large subunit	14	7	4
A0A060X2F7	Integrin beta	15	94	205
P05023	Sodium/potassium-transporting ATPase subunit alpha-1	16	271	406
Q9W747	Zinc finger protein draculin	17	356	45
Q503C8	NudC domain-containing protein 1	18	644	309
Q9UGI9	5'-AMP-activated protein kinase subunit gamma-3	19	20	267
A0A060WTZ4	Diacylglycerol kinase	20	13	266

b)

ID	Protein name	#Parr	#Smolt	#PS
Q01484	Ankyrin-2	1	1	1
P04114	Apolipoprotein B-100	164	2	29
A5H0J9	Serum albumin	2	3	77
Q7ZVI7	Actin, cytoplasmic 1	12	4	115
O57523	Apolipoprotein A-I-1	25	5	36
P79825	Hemopexin-like protein	22	6	15
B5X2T9	DNA primase large subunit	14	7	4
Q04206	Transcription factor p65	1044	8	596
Q8TDY2	RB1-inducible coiled-coil protein 1	271	9	46
Q2YDQ5	F-box/LRR-repeat protein 5	251	10	88
P98093	Complement C3	4	11	41
Q6NWE1	PHD finger protein 10	38	12	157
A0A060WTZ4	Diacylglycerol kinase	20	13	266
D5H440	Serum albumin 1 protein	70	14	240
Q9UPA5	Protein bassoon	214	15	9
A8K4G0	CMRF35-like molecule 7	42	16	30
Q8WXI9	Transcriptional repressor p66-beta	599	17	613
A0A060XBF4	Phospholipase	216	18	110
A5PF61	Protein leg1a	476	19	93
Q9UGI9	5'-AMP-activated protein kinase subunit gamma-3	19	20	267

c)

ID	Protein name	#Parr	#Smolt	#PS
Q01484	Ankyrin-2	1	1	1
Q5VWN6	Protein FAM208B	5	26	2
F1Q8C3	Cytochrome P450 2K6	3	1236	3
B5X2T9	DNA primase large subunit	14	7	4
Q1LUA6	Triple functional domain protein	11	39	5
Q696W0	Striated muscle preferentially expressed protein kinase	13	38	6
A0A0R4IB49	Si:ch211-166e11.5	59	164	7
A0A060WMK5	Non-specific serine/threonine protein kinase	1054	811	8
Q9UPA5	Protein bassoon	214	15	9
Q1LVF0	Laminin subunit gamma-1	389	523	10
A5PLE7	G-protein coupled receptor 183-A	1176	447	11
P55058	Phospholipid transfer protein	1435	398	12
C1BG67	Hemoglobin subunit beta-4	23	248	13
A0A060WN48	Adenylate cyclase	133	284	14
P79825	Hemopexin-like protein	22	6	15
Q7SXN5	Dynamin-1-like protein	33	869	16
Q9IAT6	Delta-like protein C	829	109	17
Q9NYC9	Dynein heavy chain 9, axonemal	1112	1167	18
A1L1R5	Protein tyrosine phosphatase domain-containing protein 1	558	421	19
Q9PT14	Precerebellin-like protein	536	1011	20

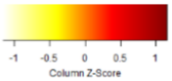
Figure 4.9. Most abundant proteins in rainbow trout plasma. Top 20 most abundant proteins in Parr (a), Smolt (b) and Post-smolt pools (c) of rainbow trout whole plasma. 'ID' indicates the UniProt accession numbers of the proteins. '#' columns indicate the rank in terms of abundance of each protein in each of the three tested developmental stages. '*' indicates significant differences. Error bars indicate s.e.

4.3.2.2. Differentially abundant proteins

For WP, statistical analysis revealed 7 differentially abundant proteins (q-value < 0.05, FC > 2) when comparing Parr vs. Smolt pools and 8 between the Smolt vs. Post-smolt pools comparison (Figure 4.10a). Of these, 1 protein, Q502K3 (Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit), was significantly higher in the Smolt pool in both comparisons.

a)

Parr	Smolt	Post smolt	ID	Protein name
A (7.04·10¹)	B (0)	AB (2.00·10⁴)	Q6DHR3	Ras-GEF domain-containing family member 1B-A
A (3.33·10²)	B (0)	AB (6.80·10¹)	P56945	Breast cancer anti-estrogen resistance protein 1
A (3.16·10⁴)	B (0)	AB (1.89·10⁴)	Q96Q89	Kinesin-like protein KIF20B
B (9.49·10⁵)	A (1.89·10⁷)	AB (2.79·10⁶)	P04114	Apolipoprotein B-100
B (7.11·10⁴)	A (2.51·10⁵)	AB (9.03·10⁴)	Q9H7U1	Serine-rich coiled-coil domain-containing protein 2
B (1.14·10³)	A (1.44·10⁶)	AB (4.65·10⁵)	Q9DE25	Forkhead box C1-A
B (1.32·10⁴)	A (1.03·10⁶)	B (2.25·10⁴)	Q502K3	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat sub. c
AB (6.58·10⁴)	A (5.53·10³)	B (0)	Q13796	Protein Shroom2
AB (2.18·10⁵)	A (1.20·10⁴)	B (0)	Q9PTH3	Insulin-like growth factor-binding protein 2-A
AB (6.12·10²)	A (1.11·10⁴)	B (0)	Q6PBM7	Protein FAM76B
AB (7.57·10³)	B (0)	A (8.31·10²)	Q90421	LIM/homeobox protein Lhx3
AB (1.28·10³)	B (0)	A (9.12·10³)	Q3YL68	Beta-1,4-galactosyltransferase 5
AB (1.36·10³)	B (0)	A (4.52·10³)	O94842	TOX high mobility group box family member 4
AB (2.79·10³)	B (0)	A (2.91·10³)	P35670	Copper-transporting ATPase 2



b)

Parr	Smolt	Post smolt	ID	Protein name
A (6.64·10⁵)	B (5.84·10⁴)	A (1.28·10⁵)	O94876	Transmembrane and coiled-coil domains protein 1
A (1.07·10⁵)	B (2.91·10⁴)	AB (8.68·10⁴)	A0A060XR35	AMP deaminase
A (1.84·10⁶)	B (2.45·10⁵)	AB (1.00·10⁶)	A2BGM5	Forkhead box protein N4
B (1.59·10⁵)	A (9.22·10⁵)	AB (1.88·10⁵)	A0A060WSL4	Stathmin
B (1.76·10⁵)	A (2.15·10⁶)	AB (5.52·10⁴)	Q6ZSC3	RNA-binding protein 43
B (1.32·10⁶)	A (4.97·10⁶)	AB (6.13·10⁵)	P10523	S-arrestin
B (1.31·10⁵)	A (2.09·10⁶)	AB (9.59·10⁵)	Q6NYB4	Putative nucleotidyltransferase MAB21L1
B (1.66·10⁴)	A (1.88·10⁵)	AB (1.60·10⁵)	Q9W740	Noggin-2
B (2.77·10⁵)	A (4.65·10⁶)	AB (4.57·10⁶)	P02751	Fibronectin
B (0)	A (8.47·10³)	AB (2.33·10⁵)	O60645	Exocyst complex component 3
AB (3.57·10⁴)	A (2.84·10³)	B (0)	Q14500	ATP-sensitive inward rectifier potassium channel 12
A (3.38·10⁵)	A (2.97·10⁵)	B (0)	Q7Z4G4	tRNA (guanine(10)-N2)-methyltransferase homolog)

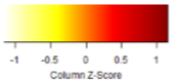


Figure 4.10. DAPs in WP (a) and EP (b). Protein abundance heatmaps indicate significant differences ($p < 0.05$, on the left of each cell with different letters) and mean protein abundance (in brackets in each cell). "ID" indicates UniProt accession number of the proteins. On the top right, colour scale of the heatmaps.

For EP, 10 proteins were found to be differentially abundant between the Parr vs. Smolt pools comparison, while 3 were identified for the Smolt vs. Post-smolt pools comparison (Figure 4.10). Of them, 1 protein, O94876 (Transmembrane and coiled-coil domains protein 1), was significantly lower in Smolt pool in both comparisons.

4.4. Discussion

Anadromous salmonids are subject to a series of adaptations needed to fulfil their unique life cycle, collectively known as smoltification (Hoar, 1988; Björnsson *et al.*, 2011). Due to these adaptations, after having been freshwater fish during their early life stages (parr and smolt phases), anadromous salmonids can fully adapt to life in seawater during their adult stage (post-smolt phase). To gain further insight into the smoltification and seawater adaptation processes, pools of plasma from parr, smolt and post-smolt rainbow trout were analysed by GeLC-MS/MS alone or in combination with protein enrichment technology. Results were then compared seeking to identify proteins related to the changes that take place from one developmental condition to another. Due to the integral role of blood in the transport of molecules to and from tissues, proteins from a variety of functions and target tissues were identified (Anderson and Anderson, 2002; Jacobs *et al.*, 2005; Pernemalm and Lehtiö, 2014). Therefore, being a highly informative biofluid, blood plasma can be used to obtain an overview of the overall physiological state of the fish (Adkins *et al.*, 2005; Simpson *et al.*, 2009).

In total, 1,822 proteins were reliably identified and quantified (based on a minimum of three peptides and one unique peptide) in rainbow trout blood plasma. Similar approaches in rainbow trout and other fish species reported a variety of protein identification results in plasma. Nynca *et al.* (2017) reported 119 proteins in adult freshwater rainbow trout plasma (identification based on a minimum of two unique peptides). Comparison with this list of proteins revealed that 83 of them were also quantified in the present study. In zebrafish, 3,024 proteins were reported (identified based a minimum of one peptide; Medina-Gali *et al.*, 2019), 939 in three-spined sticklebacks (*Gasterosteus aculeatus*) (identification based on a minimum of one peptide; Kültz *et al.*, 2015), and 717 in Atlantic cod (*Gadus morhua*) (identification based on a minimum of one peptide; Enerstvedt *et al.*, 2018). Interestingly, regarding other tissues and biofluids of rainbow trout, 3,241 proteins were reported in head kidney (identification based on a minimum of two peptides; Kumar *et al.*, 2018), 2,542 in spleen (identification based on a minimum of two peptides; Kumar *et al.*, 2018), 59 in ovarian fluid (identification based on a minimum of two unique peptides; Nynca *et al.*, 2015) and 152 in seminal plasma (Nynca *et al.*, 2014). Differences in the number of protein identifications can have both biological (fish tissues and fluids used) and technical origin (sample processing, fractionation strategy, MS-instrument type, data acquisition parameters, data trimming and database identification version and parameters). However, it is relevant to note that the present study represents an in-depth characterisation of the plasma proteome of rainbow trout across three developmental stages of the fish, having performed an enrichment step and extensive fractionation (each 1-D SDS-PAGE lane divided into 24 fractions), thus greatly increasing coverage.

ProteoMiner™ is a commercially available bead-based technology that relies on combinatorial peptide ligand libraries. Each bead is designed to specifically capture a protein up to saturation. Therefore, very abundant proteins will quickly saturate their beads and all remaining unbound protein is washed away, thus decreasing their relative abundance while increasing the concentration of low-abundant proteins (Murphy and Dowling, 2018). Due to this, peptides that would normally be below the limit of detection of the MS or peptides that would be masked by the peptides derived from highly-abundant proteins become detectable. In the present study, WP and EP datasets were similar, with 965 proteins (52.96% of the total) being quantified by both methods and presenting very similar dynamic ranges. Nonetheless, the treatment with ProteoMiner™ allowed an increase in the number of quantified proteins (317, 17.95%). Enrichment was independent of the sample type (very similar linear regression fits between the three plasma pools), protein mass, protein length, biological function GO, molecular function GO and cellular component GO. The only tested variable significantly correlated with the magnitude of enrichment was the protein abundance in the original WP sample, the abundance/detectability of low-abundant proteins being greatly increased and that of high-abundant proteins decreased. This demonstrated that ProteoMiner™ is a valid strategy to increase proteome coverage in rainbow trout plasma. It is relevant to note that the method was more efficient at increasing the detection of low-abundant proteins than at lowering it for high-abundant ones. This was expected, as not only the relative abundance of low-abundant proteins was increased but the abundance of high-abundant proteins was reduced, which was taking up a significant portion of the available analytical space. Therefore allowing a higher proportion of the proteins present to be detected. Another effect of the enrichment was that the three developmental conditions became indistinguishable by PCA, whereas in WP they were clearly classified into three separate groups (*i.e.* Parr, Smolt and Post-smolt pools). Therefore, both high- and low-abundant proteins were important for the distinction of the three developmental conditions in the PCA space.

In terms of the most abundant proteins there was large variability depending on the developmental stage. However, two proteins detected to be present in the top 20 of the three developmental stages were ankyrin-2 and DNA primase large subunit. In fact, ankyrin-2 was the most abundant protein in all three conditions and is a structural protein playing an essential role in the localization and retention of ion transporters and ion channels in several cell types (Cunha and Mohler, 2009). Ankyrin-2 deficiency has been associated with blood, cardiac and neurological disorders due to its implication in the correct functioning of calcium channels and transporters. Furthermore, it is a relatively highly abundant protein in human serum (Tanaka *et al.*, 2006) and its high occurrence in rainbow trout plasma is likely originating from erythrocytes, where Ankyrin-2 is crucial for correct functioning and mechanical stability (Mohandas and Evans, 1994; Yasunaga *et al.*, 2012). The second most abundant

common protein was DNA primase large subunit, which is a ubiquitous protein that can be found in any tissue that undergoes DNA replication. DNA primase is a polymerase that synthesizes small RNA primers for the formation of Okazaki fragments during discontinuous DNA replication (Kuchta and Stengel, 2010). Other commonly found highly abundant proteins identified in rainbow trout plasma in this study were actin, serum albumin, apolipoproteins, haemoglobin subunits, hemopexin-like proteins and complement C3, which have been reported to be high in fish plasma (Li *et al.*, 2016). Although differences in the top 20 most abundant proteins were observed, only one of the proteins was significantly different among conditions. Thus the top abundant proteins are useful characterisers of rainbow trout plasma but are not critical toward understanding differences at distinct developmental stages.

As expected from their extremely different phenotypes and life strategies, significant differences were present in the plasma proteome of Parr, Smolt and Post-smolt pools. In total, taking into consideration both WP and EP datasets, 26 DAPs were present. While some of these proteins are poorly characterised (*i.e.* Protein FAM76B), have associated functions that are unlikely to be found in adult fish (proteins related to embryogenesis like forkhead box protein N4; Chi *et al.*, 2008) or functions that are too general (*i.e.* forkhead box C1-A; Li *et al.*, 2015), others have well-understood and specific roles.

Seventeen DAPs were identified when comparing the Parr and Smolt pools. Their functions indicate preadaptations of smolts to seawater life, such as countermeasures against hyper-osmotic stress, shown by serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C, a protein related the preservation of cell shape under hyper-osmotic stress (Watanabe *et al.*, 2018). Retinal changes, shown by S-arrestin, a major retinal protein specific to rod photoreceptors (Renninger *et al.*, 2011; Murthy *et al.*, 2014; Sullivan *et al.*, 2017), are likely to be linked to a signal transduction pathway related to previously documented changes in the retina of salmonids during smoltification, leading to the dominance of rhodopsin over porphyropsin as a visual pigment and to the loss of ultraviolet-sensitive cones as an adaptation to oceanic life (Dann *et al.*, 2003; Ebbesson *et al.*, 2007). Moreover, several proteins that are lower in smolts are related to a series of previously described changes related to a lower resource investment towards some nonessential processes during smoltification. One of them is sexual maturation (kinesin-like protein KIF20B; Wang, Xueying *et al.*, 2018), which is delayed in migrators (Foote *et al.*, 1994; Thorpe and Metcalfe, 1998; Nichols *et al.*, 2008). Another is the repression of some immune pathways (Johansson *et al.*, 2016), exemplified here by a decrease in mast cell proliferation (ras-GEF domain-containing family member 1B-A; Tam *et al.*, 2004; Epting *et al.*, 2007). A third one is indicated by AMP deaminase (Fischer *et al.*, 2007) and is related to a reorganization of red muscle, resulting in a decrease in myosin heavy chain abundance and in lower performing muscles for smolts (Martinez, I. *et al.*, 1993; Coughlin *et al.*, 2001), as parr have faster

twitching muscles used to maintain a faster frequency tailbeat, whereas smolts tend to swim with the current towards the river mouth. Finally, changes in energy reserve metabolism result in depletion of lipids, proteins and carbohydrates in the smolt whole body (Rousseau *et al.*, 2012), which is shown here by a higher mobilization of lipids (apolipoprotein B-100; Innerarity *et al.*, 1990; Millar *et al.*, 2005). Presumably these adaptations would take place in order to meet the energy demands needed for smoltification and for reorganizing tissues in preparation to life in seawater, which could be related to the higher abundance in smolts of serine-rich coiled-coil domain-containing protein 2, stathmin and fibronectin, all related to cell and microtubule organization (Pankov and Yamada, 2002; Rubin and Atweh, 2004; Wang *et al.*, 2013). Curiously, serine-rich coiled-coil domain-containing protein 2 has been proposed as a reliable housekeeping gene in humans but this is unlikely to be the case in rainbow trout given the present results (Tilli *et al.*, 2016).

For the Smolt vs Post-smolt pools comparison, 11 DAPs were identified. They were related to countermeasures against hyper-osmotic stress, shown once again by the high abundance of serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C, and by ATP-sensitive inward rectifier potassium channel 12, a channel with the tendency to let potassium into cells (D'Avanzo *et al.*, 2010), being likely related to the seawater NKA pumps of rainbow trout (NKAα1b and NKCC1a). Finally, IGFBP2a was absent in the Post-smolt pool. The absence of this growth-repressing protein indicates that growth during the post-smolt phase (also called on-growing phase) would be enhanced with respect to the smolt phase (Duan *et al.*, 1999; Zhou *et al.*, 2008), which is in accordance with the repression of nonessential processes during smoltification.

Overall, the present study provides an in-depth characterisation of the rainbow trout blood plasma proteome, with 1,822 reliably identified and quantified proteins, analysed across three different developmental stages of the fish. Performing either fractionation alone or in combination with an enrichment step effectively maximized proteome coverage, supporting previous findings in human proteomics (Selvaraju and El Rassi, 2011). The effects of ProteoMiner^{TM} were explored in-depth, showing that this can increase the number of detected proteins due to the capability to decrease the masking effect on high-abundant proteins. In general terms, ankyrin-2 was invariably the most abundant protein in rainbow trout plasma, while other proteins such as DNA primase large subunit, actin, serum albumin, apolipoproteins, haemoglobin subunits, hemopexin-like proteins and complement C3 were generally among the most abundant proteins. DAPs between Parr and Smolt pools suggest preadaptations of smolts to seawater life, including prevention measures against hyper-osmotic stress and retinal changes, as well as downregulation in respect to the Parr pool of nonessential pathways. DAPs between Smolt and Post-smolt pools were related to coping with hyper-osmotic stress, retinal changes, and increased growth and copper excretion in Post-smolt pool. As

potential biomarkers, apolipoprotein B-100 would be the clearest candidate, being a highly abundant protein (second most abundant in Smolt pool) and significantly higher in Smolt pool than in Parr or Post-smolt pools. Therefore, it might be a robust smolt marker, increasing during the smoltification process and decreasing after the smolt window. However, being and important lipoprotein component, it is implicated in the transport of lipids in response to a wide variety of signals (Segrest *et al.*, 2001; Kreuter *et al.*, 2007; Klingenberg *et al.*, 2010) and its abundance might vary in response to processes not related to smoltification. Therefore, its suitability as smolt marker, as for the rest of potential biomarkers, needs further testing. For post-smolts, the lack of IGFBP2a might also be an interesting biomarker for growth potential. Other biomarker candidates would preferably be DAPs found in WP, due to the simplicity of detection respect to EP. However, each of these candidate biomarkers needs to be validated by seawater survival tests.

Chapter 5: A Peptidomic Approach to Biomarker Discovery for Smoltification using MALDI-TOF MS on Blood Plasma

5.1. Introduction

Currently, the most widely used smoltification marker, aside from the assessment of fish size and coloration (Hoar, 1988; Kendall *et al.*, 2015), is the measurement of NKA activity in fish gill (McCormick, 1993). A second, less often used method is the measurement of the transcription of NKA related genes in gill tissue, mainly *nkaα1a*, *nkaα1b*, and *nkcc1a* (Nilsen *et al.*, 2007). However, both of these tests rely on time-consuming methodologies with high running costs (respectively, kinetic assay measured by spectrophotometry and RT-PCR). Moreover, these tests are performed on gill tissue obtained using very invasive, and normally lethal, gill dissections. Finally, the accuracy of these tests in Atlantic salmon, which is likely to be similar in rainbow trout, has been reported to be very low: 60% for NKA activity and 57% for *nkaα1a* and *nkaα1b* transcription (McGowan, 2018).

Often, new diagnostic approaches are developed for biomedical clinical use, only reaching animal research several years afterwards. This might be the case for MALDI-TOF MS, which is currently one of the preferred platforms for identifying novel human disease biomarkers (Karpova *et al.*, 2010; Ng *et al.*, 2014; Hajduk *et al.*, 2016), but has not yet been used for biomarker discovery in a fish species. While all MS platforms have high-throughput capabilities, MALDI-TOF excels in its relative simplicity of use, inexpensive running costs, and the short amount of time it takes to produce results (Volmer *et al.*, 2007), often being able to run a sample without previous processing (Hsieh *et al.*, 2008; Preianò *et al.*, 2012; Montoya-Rodríguez *et al.*, 2015).

Biomarker studies have been performed on a variety of biofluids, such as urine (Albalat *et al.*, 2013), seminal plasma (Davalieva *et al.*, 2012), and blood plasma (Hortin, 2006). Of these, blood plasma would be the most easily accessible on a fish, which moreover contains the most complex and informative proteome of an organism (Anderson and Anderson, 2002; Jacobs *et al.*, 2005; Pernemalm and Lehtiö, 2014). However, although blood is information rich which is preferable for biomarker discovery because it increases the pool of potential biomarkers, studying proteins in blood is challenging and time consuming due to the need for depletion of high-abundant proteins and overnight protease digestion. Both of these steps can be avoided if only the peptide fraction (<20 kDa) is analysed, since high-abundant proteins are bigger in size and a protease digestion step is not

needed, thus significantly decreasing the time dedicated to sample preparation and analysis. Although the peptidome is a relatively small part of the proteome, several studies have shown that it is highly informative (Albalat *et al.*, 2013; Mansor *et al.*, 2013; Stalmach *et al.*, 2015), and that the naturally occurring peptides can be very accurate biomarkers (Carrette *et al.*, 2003; Petricoin *et al.*, 2006; Roscioni *et al.*, 2013).

Therefore, the aim of this study is to develop a method for the discovery of smoltification biomarkers in the plasma peptidome of rainbow trout.

5.2. Materials and methods

5.2.1. Fish and rearing conditions

As described in Chapter 2.

Parr and smolt plasma samples (n = 8) were used in the present experiment (Table 5.1). These samples were part of the plasma pools used in Chapter 4.

Table 5.1. Measurements in fish used for blood plasma analysis (values ± s.e.)

Pool	Sampling	Length (cm)	Weight (g)	Fulton index	NKA activity
Parr	3rd March	18.4 ± 0.71[b]	84.1 ± 8.73[b]	1.3 ± 0.02[b]	1.14 ± 0.12[b]
Smolt	11th May	22.5 ± 0.40[b]	158.2 ± 9.60[a]	1.4 ± 0.03[a]	11.0 ± 1.00[a]

Fulton index is measured in g cm^{-3}. NKA activity is measured in µmol ADP mg $protein^{-1}$ $hour^{-1}$. Different letters indicate significant differences.

5.2.2. Sampling

As described in Chapter 2 and 4.

5.2.3. Gill NKA activity

As described in Chapter 2.

5.2.4. Blood plasma cut-off filtering

Individual plasma samples were processed following Albalat et. al (2013)(Figure 5.1). Briefly, 150 µl of plasma and 150 µl of urea buffer (2 M urea (Sigma-Aldrich), 100 mM NaCl (Sigma-Aldrich), 0.0125 % NH4OH (Merck), and 0.01 % SDS) were added to a 20 kDa Centrisart® I centrifugal ultrafiltration unit (Stedim-Sartorius) and centrifuged at 3,400xg for 1 hour (Eppendorf Centrifuge 5418R). Then, 200 µl of filtrate was added to a, previously equilibrated with 25 ml of 25 % ammonia solution (Merck Millipore), Illustra NAP-5 column (GE Healthcare Life Sciences). Once the filtrate completely entered the column, 300 µl of 25% ammonia solution was added to the column and the flow through was discarded. Then, 700 µl of 25% ammonia solution was added to the column and the eluted sample was collected. Finally, eluted samples were freeze-dried (Christ Alpha 1-4 LSC).

5.2.5. MALDI-TOF MS analysis

Following results by Albalat *et al.* (2013), several dilutions of each analysed sample were tested (*i.e.* 1:1, 1:2, 1:4 and 1:8) to increase peptidome coverage. The eluted samples were resuspended in 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich) and spotted onto an MTP AnchorChip target plate (Bruker Daltonics) in quadruplicates. MALDI TOF MS analysis was performed using an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics). The matrix solution was 0.7 mg/ml α-Cyano-4-hydroxycinnamic acid (LaserBio Labs) in 90% ACN, 0.1% TFA, and 1 mM NH_4H_2PO4 (Sigma-Aldrich). One microliter of prepared plasma samples was spotted onto the target plate, dried, and then 0.8 µL of matrix solution was added. External mass calibration was performed every four spots using 0.6 µL PeptideCalibration Standard II (Bruker Daltonics).

Measurements were carried out in reflectron positive ion mode covering a mass range from 800 to 4000 kDa. Data acquisition parameters were as follows: ion source 1: 25 kilovolts (kV), ion source 2: 21.35 kV, lens: 10.50 kV, reflector 1: 26.30 kV, reflector 2: 13.80 kV, and laser power: 33%. For the calibrants, data was acquired from 1,000 satisfactory laser shots in 200 shot steps allowing 200 shots per raster spot, whereas for samples it was 3,000, 300 and 300, respectively. Samples (both original eluates and diluted samples) were analysed in tetraplicates.

5.2.6. MALDI-TOF MS Data processing

Each spectrum was visually inspected for quality control. Spectra with only noise, extremely low intensity, or presence of plastic contamination, were removed. MALDI-TOF MS output ASCII files were processed using R software (v. 3.4.2) and the R package 'MALDIquant', following the package's recommended settings (Gibb and Strimmer, 2012). Briefly, spectra were smoothed with the 21 point

Savitzky-Golay-Filter, baseline-corrected using TopHat method, and intensity was normalized by total ion current. Spectra were aligned at a signal-to-noise ratio of 4 and mass tolerance of 0.002 Da using the "lowess" method. Technical replicates (4 per each sample) were combined by averaging their intensity, and non-monoisotopic peaks were removed at a signal-to-noise ratio of 1.5 using the "MAD" method. Peak alignment into discrete bins (binning) was performed using the "strict" option at a tolerance of 0.005 Da and peaks that were found in less than 37.5% of the technical replicates were removed. Finally, a matrix of peak intensities to mass-to-charge (m/z) values was generated.

5.2.7. LC-MS/MS analysis of inclusion list

In order to identify peptides of interest (significantly different peptides in intensity between parr and smolt samples), LC-MS/MS analysis of three randomly selected samples (undiluted) was performed. In this case, the instrument used small detection ranges near the m/z of interest, rather than a completely untargeted approach. In all other aspects, LC-MS/MS analysis, data processing and protein annotation were as described in Chapter 4. Knowing the m/z of peptides detected by MALDI-TOF MS and by LC-MS/MS, peptide identifications were accepted if the difference in m/z of a peptide of interest by both methods was below 200 ppm.

5.2.8. Support Vector Machine (SVM) analysis

A supervised machine learning algorithm, SVM, was used to test whether MALDI-TOF MS data could be used to classify treated plasma samples as either parr or smolt. The R package 'e1071' was used (Dimitriadou *et al.*, 2009). SVM models are algorithms that find the D-1 hyperplane that linearly separates two or more classes of samples in a D dimensional space. They use the samples at the margins of each group, known as support vectors, to define the margins of the separating hyperplane. A C-classification algorithm of radial kernel was used. Gamma value and constraint violation cost were determined by the model itself by tuning. The 'training' process used intensities to m/z values of 80% of the samples (randomly selected), labelled as either parr or smolt samples, while the remaining 20% of the samples were used to test whether the model could correctly classify them, as visually explained in Figure 5.1. Starting at the random sample selection step, this analysis was run 100 times on the 8 biological replicates per developmental stage (n = 8) to measure the performance of the model.

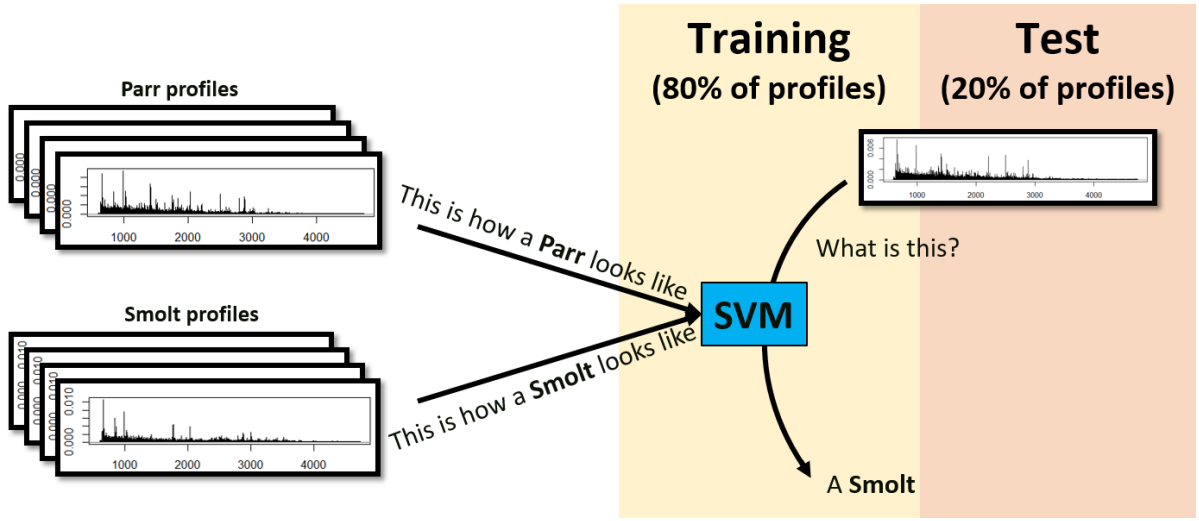


Figure 5.1. SVM pipeline.

5.2.9. Further data analysis and representation

Data representation was carried out in R. PCA and heatmaps were plotted as described in Chapter 4.

Raw spectra were visualized in FlexAnalysis 3.4 (Bruker Daltonics).

Venn diagram was plotted using the online tool Venny 2.0.2 (http://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html).

Pair-wise t-tests for each m/z value followed by 5% FDR post hoc test were performed in order to identify differentially abundant peptides. T-test was also performed to identify significant differences between the two developmental conditions in respect to their morphometric and NKA activity measures (Table 5.1).

5.3. Results

5.3.1. MALDI-TOF MS data

MALDI-TOF MS profiles were obtained for 8 parr and 8 smolt <20kDa plasma samples analysed at dilutions 1:1, 1:2, 1:4, and 1:8 (Figure 5.2). For undiluted samples (1:1 dilution), monoisotopic peak filtering, and peak binning, resulted in 2,219, and 327 remaining peaks, respectively. After removal of peaks that appeared in less than 37.5% of the samples, 159 peaks were detected and accepted as reliably identified and quantified naturally occurring peptides in the <20kDa fraction of rainbow trout

plasma (Figure 5.3 and 5.4). Similarly, processing of 1:2, 1:4 and 1:8 diluted samples resulted in the quantification of 147, 114, and 89 peptides, respectively. When comparing the results for different dilutions, only 50 peptides were detected in all four, with big variation in the detected peptide populations among dilutions.

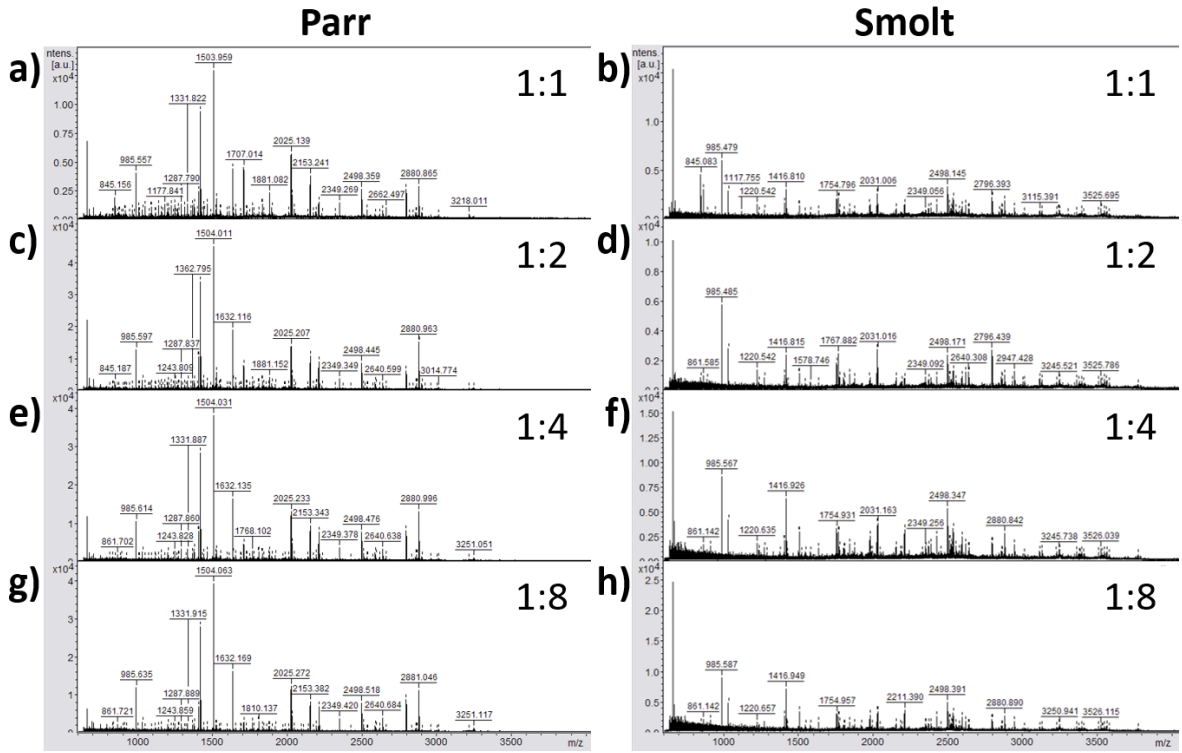


Figure 5.2. Representative MALDI-TOF profiles of a parr (a,c,e,g) and a smolt (b,d,f,h) <20kDa plasma peptides analysed at dilutions 1:1 (undiluted)(a,b), 1:2 (c,d), 1:4 (e,f), and 1:8 (g,h).

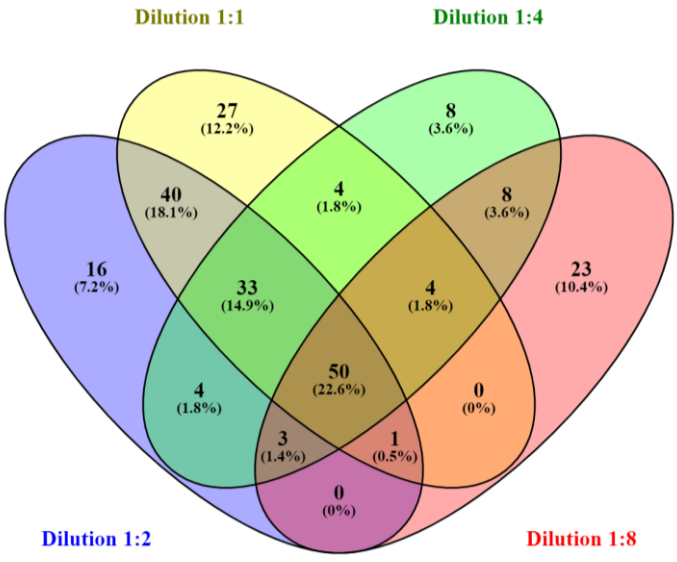


Figure 5.3. Venn diagram of peptides detected in samples analysed at different dilutions.

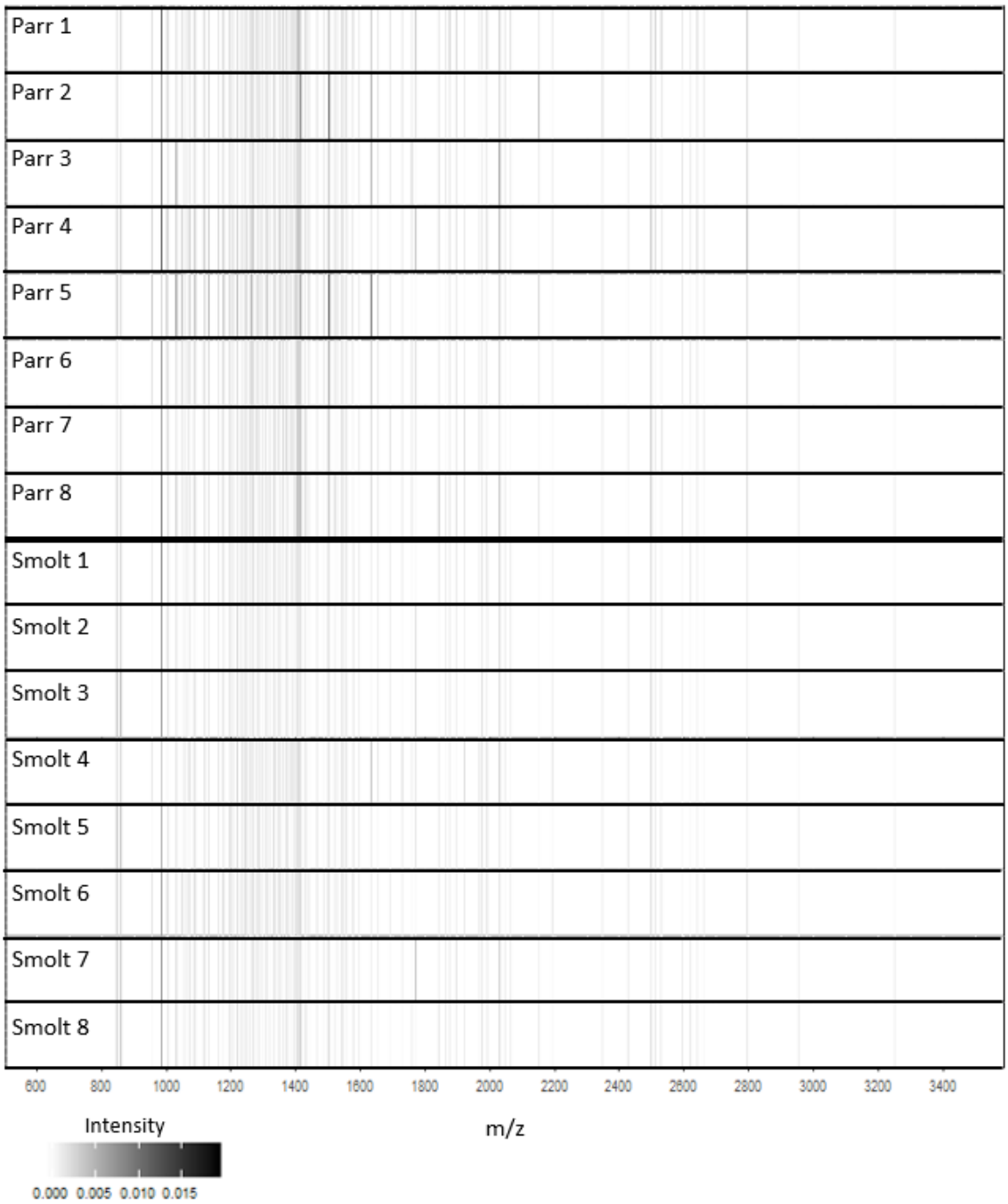


Figure 5.4. Detected naturally occurring peptides in parr and smolt rainbow trout.

5.3.2. PCA

PCA was used to visualize the relationship between replicates and the dissimilarity among developmental conditions. For undiluted samples, PC1 explained 28.2% of the variation and PC2 14.8% (Figure 5.5a). Two clusters corresponding to parr and smolt samples were clearly separated, mainly due to PC1. Contrarily, PCA of none of the diluted samples showed grouping according to parr and smolt (Figure 5.5b-d).

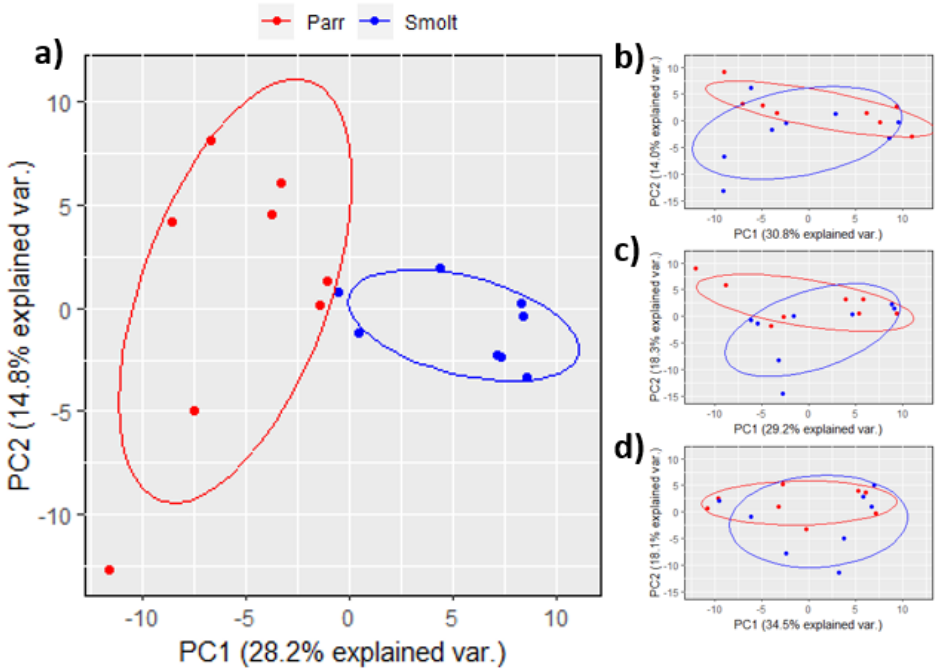


Figure 5.5. PCA of naturally occurring peptides in parr and smolt rainbow trout plasma samples. Samples were tested undiluted (a), and at dilutions 1:2 (b), 1:4 (c), and 1:8 (d). Points indicate individual replicates. Ellipses show 68% normal probability for each group.

Regarding the PCA of undiluted sample, the factor loadings of the main variables (*i.e.* peptides) explaining the variance of PC1 and PC2 are shown in Table 5.2.

Table 5.2. Top 10 peptides (m/z) explaining variance of PC1 and PC2 for PCA of undiluted samples. 'Loadings' indicate the correlation coefficient between peptides and PCs.

PC1		PC2	
m/z	**Loadings**	**m/z**	**Loadings**
2523.32	0.134	1271.09	0.174
3115.71	0.132	1256.73	0.172
1656.93	0.131	2498.38	0.160
3526.04	0.130	985.612	0.151
650.069	0.125	1173.68	0.150
2844.57	0.121	1842.01	0.141
2354.79	0.119	1007.6	0.141
2538.29	0.116	1405.83	0.131
1578.9	0.107	1233.96	0.128
2426.33	0.105	1321.06	0.126

5.3.3. Differentially abundant peptides

Statistical testing of undiluted samples revealed that 32 of the detected peptides presented significantly different intensities ($q < 0.05$) when comparing them in the two tested developmental conditions (Table 5.3). Ten of them were higher in smolts, while the other 22 were higher in parr. On the other hand, statistical analysis of data from 1:2, 1:4 and 1:8 samples reported no significantly different peptides.

Most of the major peptide contributors to PC1 (Table 5.2), with the exception of three, were among the list of differentially abundant peptides. Regarding PC2 contributors, only m/z 1405.83 was differentially abundant.

Table 5.3. Statistical parameters of differentially abundant peptides (m/z) in undiluted samples. Mean normalized peptide intensities are shown as x1000 times their actual values. 'Protein ID' indicates, for those that was possible, the accession number of the protein that the sequence of the detected peptide was matched to. 'Freq.': frequency.

MALDI-TOF MS					LC-MS/MS	
m/z	**Mean Parr**	**Mean Smolt**	**Freq. Parr**	**Freq. Smolt**	**Protein ID**	**Sequence**
861.20	2.17	3.72	0.75	1.00		
862.24	1.18	2.01	0.13	0.63		
1046.01	2.03	0.33	0.88	0.13		
1083.60	1.34	1.00	1.00	0.88		
1194.16	1.40	0.87	1.00	0.88		
1259.78	1.68	0.65	1.00	0.13		
1305.56	1.14	0.93	1.00	1.00		
1363.05	2.00	0.71	1.00	0.50		
1401.96	1.37	1.01	1.00	1.00		
1405.83	5.05	2.17	1.00	1.00		
1416.94	5.06	2.06	1.00	1.00		
1427.91	1.42	0.83	1.00	0.88	ENOB	GNPTVEVDLYTAK
1504.35	5.79	1.21	1.00	1.00		
1519.87	1.80	0.91	0.88	0.75		
1527.03	1.79	0.76	1.00	0.75		
1530.00	1.25	0.76	0.88	0.38		
1542.90	1.26	0.97	1.00	1.00		
1558.42	1.19	0.68	1.00	0.75	H1	AEVAPAPAAAAPAKAPK
1578.90	0.77	1.07	0.13	1.00		
1749.03	1.05	0.68	0.75	0.13		
1760.52	1.26	0.28	0.88	0.25		
1897.09	1.64	1.02	1.00	0.88		
1976.79	0.99	1.61	0.88	1.00		
2354.79	0.10	0.58	-	0.75		
2426.33	0.80	1.15	0.50	1.00		
2523.32	0.50	0.74	-	0.88	CO3	EALGLMQGKTYMIMGKSEDLHR
2538.29	0.86	1.28	0.50	1.00		
2640.50	1.39	0.68	1.00	0.88		
2662.50	0.94	0.61	0.75	0.13		
2796.65	2.66	1.01	1.00	1.00	NBEL2	EVFKERIGYSQLFDVLKSQGQPTK
3115.71	0.25	0.53	-	0.75		
3526.04	0.28	0.53	-	0.75		

5.3.4. LC-MS/MS analysis

LC-MS/MS analysis of the plasma samples detected 153 peptides. These were composed of between 6 and 74 aminoacids (although if over 50 aminoacids long they are technically considered proteins) and they were mapped onto 27 different proteins (Table 5.4). However, only 4 of these were of a similar m/z (200 ppm) as the peptides of interest (Table 5.3). Therefore, the using LC-MS/MS we were able to identify 12.5% of the significantly different peptides detected by MALDI-TOF MS (100*4/32).

Table 5.4. Peptides identified by LC-MS/MS. 'Peptide count' indicates the number of different peptides (*i.e.* different sequence) that were mapped onto a particular protein.

Accession	**Protein name**	**Peptide count**
ACTB	Actin, cytoplasmic 1	1
ACTB2	Actin, cytoplasmic 2	4
APA12	Apolipoprotein A-I-2	4
ATPB	ATP synthase subunit beta, mitochondrial	4
CO3	Complement C3	20
ENOA	Alpha-enolase	5
ENOB	Beta-enolase	6
FA150	Protein FAM150-like	2
H1	Histone H1	11
H2AX	Histone H2AX	7
H2B	Histone H2B	8
H32	Histone H3.2	3
H4	Histone H4	3
ION3	Intermediate filament protein ON3	4
ION3	Tubulin beta chain	5
M10B2	Putative helicase mov-10-B.2	7
N4BP1	NEDD4-binding protein 1	4
NBEL2	Neurobeachin-like protein 2	7
NLRC5	Protein NLRC5	6
PLSL	Plastin-2	9
PTPRF	Receptor-type tyrosine-protein phosphatase F	9
TBA	Tubulin alpha chain	7
TP8L3	Tumor necrosis factor alpha-induced protein 8-like protein 3	1
UBP12	Ubiquitin carboxyl-terminal hydrolase 12A	1
VIMB	Vimentin beta	2
VIME	Vimentin	10
ZN704	Zinc finger protein 704	3

5.3.5. SVM analysis

Accuracy of the SVM (*i.e.* proportion of correct choices) was 1 on 69% of the cases, 0.75 on 25%, 0.5 on 4%, 0.25 on 1% and 0 on 1% (Figure 5.6), and therefore, overall accuracy was of 0.90 ((69 + 25*0.75 + 4*0.5 + 0.25 + 0)/100). Understanding parr as '0' and smolt as '1', in terms of overall sensitivity (true smolts classified as smolts) and specificity (true parr classified as parr), the results were respectively 84.31% and 95.14%.

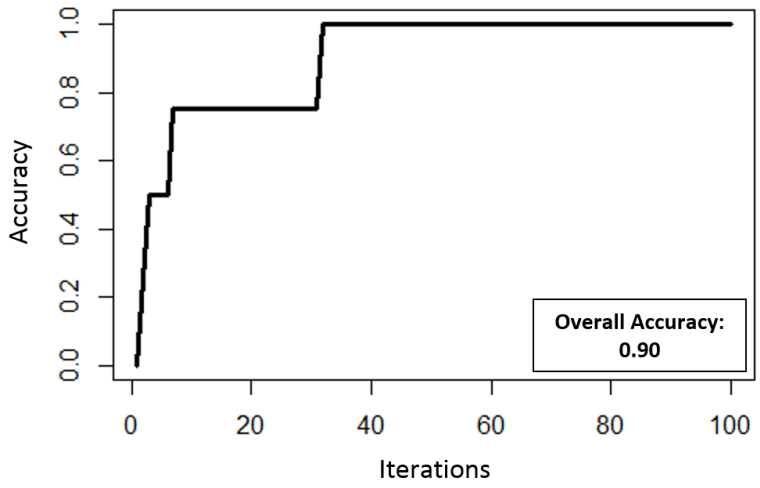


Figure 5.6. Receiver operating characteristic curve showing the diagnostic ability of the SVM model to correctly classify parr and smolt rainbow trout plasma peptidome samples.

5.4. Discussion

Current smolt marker tests are time-consuming, expensive, inaccurate, and rely on invasive or lethal dissections of gill (McCormick, 1993; McGowan, 2018). Therefore, there is an industry need for improved smolt markers. The present study represents a first approach towards the development of a new smolt marker test based on blood plasma, which can be sampled nonlethally (Anderson and Anderson, 2002; Jacobs *et al.*, 2005; Pernemalm and Lehtiö, 2014). Moreover, while the initial capital investment cost to buy a MALDI-TOF MS is high, the running costs are cheap and it allows for high throughput analysis of samples, making it one of the preferred methods for biomarker discovery in the biomedical field (Karpova *et al.*, 2010; Ng *et al.*, 2014; Hajduk *et al.*, 2016). The efficacy of the method is further increased when data is processed by machine learning algorithms such as SVMs (Timm *et al.*, 2008), which not only take into consideration the presence and abundance of the molecules of interest but also the differential ratios between them.

The analysis of <20kDa plasma by MALDI-TOF MS of diluted samples did not add peptides of interest (significantly different intensities among treatments) to those found in undiluted samples, unlike what was seen in a previous study (Albalat *et al.*, 2013). However, similar to the results of that study, the analysis of the samples at different dilutions resulted in very different peptide profiles. If the objective of the study had been to characterise the plasma peptidome, thus aiming at maximal peptide coverage, the dilution approach would have been key, adding 62 peptides to the total. Aimed at biomarker development, the most interesting results were found in undiluted samples, where 159 peptides were detected, which is similar to the number of peptides found in human plasma peptidome studies (Taneja *et al.*, 2011; Conraux *et al.*, 2013). PCA showed a clear separation between the two developmental stages, indicating the possibility of finding peptide smoltification biomarkers in the plasma peptidome. Indeed, 32 peptides were present in significantly different intensities in parr and smolt samples. However, individual molecules can be weak biomarkers when compared with those resulting from a multivariate analysis (Schwamborn *et al.*, 2007; Timm *et al.*, 2008; Alexandrov *et al.*, 2009). Therefore, SVM was implemented. Despite using a very limited amount of samples (n = 8), with other studies normally using n > 50 (Honda *et al.*, 2005; Pyatnitskiy *et al.*, 2011; Lawton *et al.*, 2014), the model achieved a high overall accuracy of 90%, with a higher specificity (95.14%) than sensitivity (84.31%), thus indicating that it was more accurate at identifying parr than smolts. Results from this study show good potential for the application of this approach as a seawater-readiness test, as it achieved much higher accuracy than those reported for NKA activity (60%) or *nkaα1a* and *nkaα1b* transcription (57%) (McGowan, 2018).

Very few of the differentially abundant peptides were identified by LC-MS/MS, despite having run the LC-MS/MS with an inclusion list (targeted approach). However, the proportion of identified peptides was similar to other studies (Mansor *et al.*, 2013). In fact, assumptions about the biological role of the peptide, if any, based on the protein of origin are likely to be wrong (J Kastin and Pan, 2010). This is due to the nature of peptides, which are rarely synthesized as such, often being fragments of degraded proteins (Rock *et al.*, 2002). LC-MS/MS identified 153 peptides, which were peptides from 27 parent proteins. Many of them were peptides derived from highly abundant proteins in plasma (*i.e.* actin, apolipoprotein, complements, histones, tubulin, etc.) (Li *et al.*, 2016) and, with the exception of protein NLRC5 and neurobeachin-like protein 2, all of them were present or had closely related variants found in the dataset of Chapter 4. Moreover, these results were very similar to those of Greening *et al.* (2010) in human blood plasma, who followed a similar workflow (20 kDa filtering and LC-MS/MS), and identified 44 proteins, with apolipoproteins and complements among them, from 266 peptides.

In conclusion, the described method has the potential to significantly improve current seawater-readiness assessment tools for rainbow trout, and likely also for other salmonids, using samples that can be obtained non-lethally. However, further work should focus on addressing a series of limitations. Firstly, while the classification showed promising results, its capabilities could still be improved if the number of samples in the 'training' dataset is increased. Secondly, while a validation of the model was performed on the 'test' dataset, further validation is needed using rainbow trout samples from different locations and sampled at different times. Furthermore, classification of the samples used in the present study into either parr or smolt based was done based on their NKA activity. However, in upcoming studies this should be done based on seawater performance after transfer. Admittedly, the method was relatively expensive and time-consuming due to cut-off filter and washing column steps. However, this could be easily avoided, after further investment in equipment, by using size-exclusion columns in an automatized LC-MALDI setup (Zucht *et al.*, 2005; Hölttä *et al.*, 2012).

Chapter 6. Characterization of the growth-stunted phenotype

6.1. Introduction

Fast growth is one of the most desirable attributes in farmed animals from an economical point of view, while stunted growth has a negative impact on production efficiency, profitability, sustainability, and often on animal welfare (Devlin *et al.*, 2000; Sartelet *et al.*, 2012). In this sense, while the production of seawater-transferred rainbow trout has increased steadily in recent years (Food and Agriculture Organization of the United Nations, 2019), a persistent problem has affected this sector: shortly after seawater transfer, a fraction of the animals die (around 10%) and another portion experience reduced growth (between 10-60%). Stunted animals (GS) are characterised not only by reduced growth but also by a marked decrease in condition factor and often show external lesions such as fin damage. GS seawater-transferred rainbow trout represent a problem that seems to be more pronounced during the summer months, with some companies choosing to stop their production during this season, thus abandoning one third of their potential production.

While there is still no literature tackling the specific issue of GS seawater-transferred rainbow trout, previous studies have identified a freshwater rainbow trout phenotype, as also seen in other salmonid species, that also occurs in aquaculture or laboratory conditions and presents stunted growth and reduced condition factor (Sloman *et al.*, 2000a; Sloman *et al.*, 2000b; Vindas *et al.*, 2016). Studies show that while reduced food intake is an important driver for the development of this freshwater phenotype, it is not enough to completely explain the reduced growth exhibited by the phenotype (Abbott and Dill, 1989; DiBattista *et al.*, 2006). A number of studies have linked GS to subordinate behaviour (Gilmour *et al.*, 2005), with characteristics of the phenotype including a higher standard metabolic rate (Sloman *et al.*, 2000a). Subordinate fish have also been shown to exhibit higher post-stress plasma cortisol levels, lower stressor avoidance (Sloman *et al.*, 2001; Damsgård *et al.*, 2019) and increased brain serotonin levels (Vindas *et al.*, 2016). From a metabolic perspective, as observed in rainbow trout and in a variety of other salmonid species, subordinate fish are characterised by increased hepatic protein catabolism (Mommsen *et al.*, 1999; DiBattista *et al.*, 2006) and lipid metabolism, with subordinate fish relying more on free fatty acids than on triglycerdies for energy obtention (Kostyniuk *et al.*, 2018). Moreover, differences in carbohydrate metabolism include a lower hepatic glycogen content, higher mobilization of stored glycogen, higher gluconeogenic potential (Ejike and Schreck, 1980; Sloman *et al.*, 2001; Gilmour *et al.*, 2012), and higher plasma glucose levels (Peters *et al.*, 1988; Elofsson *et al.*, 2000) in subordinate fish. Finally, in Atlantic salmon delayed

smoltification has been reported in the subordinate phenotype, also known as pace-of-life syndrome in this species (Damsgård *et al.*, 2019).

However, while some or all of the above-mentioned factors are likely to be relevant to the GS phenotype in rainbow trout, GS occurs after an artificial and forceful seawater transfer of the fish, which adds another layer of complication to the already complex issue. Furthermore, rainbow trout have a unique life-history, with their wild population being naturally divided into freshwater residents and sea-run animals, which is in turn related to smoltification rates (Christie *et al.*, 2011; Kendall *et al.*, 2015). Efforts to produce a strain with a single phenotype, either selecting the sea-run (Sharpe *et al.*, 2007; Christie *et al.*, 2011; Sloat and Reeves, 2014) or the freshwater-resident (Thrower and Joyce, 2005; Hayes *et al.*, 2012), have proved unsuccessful so far. From here arises the hypothesis that the fish that performed well in seawater could be natural migrants to seawater, while the ones that die or become GS could be natural freshwater-residents but were forcefully transferred to seawater.

In cases like this, with many possible explanations for a physiological alteration but still very little known, a first characterisation of the GS seawater-transferred rainbow trout could provide a holistic view of the underlying mechanisms that characterise this condition (Beale *et al.*, 2016; Raposo de Magalhães *et al.*, 2018; Karczewski and Snyder, 2018). This is where -omics technologies exceed, measuring the abundance of large numbers of posteriorly annotated biomolecules (*i.e.* transcripts, proteins, metabolites, lipids, etc.) of a sample in an untargeted manner. With this information it is possible to pinpoint the involved pathways before moving onto a targeted approach to study them in detail. In similar cases for humans and other animal species, -omic approaches have been successfully used (Liumbruno *et al.*, 2010; Jové *et al.*, 2014; Skorve *et al.*, 2015). As a target tissue, the liver, due to its central role in energy storage and mobilization, is the sample type that is more likely to reflect differences in physiology and metabolism (Burra, 2013). Both proteomic and lipidomic studies have been used to assess the effects on liver of differential feeding (Martin *et al.*, 2001; Jové *et al.*, 2014; Skorve *et al.*, 2015), stress (Wu *et al.*, 2016), and disease conditions (Martel *et al.*, 2012; Lee *et al.*, 2017), among others

The aim of the study is to characterise the GS phenotype by identifying differences among GS and FG seawater-transferred rainbow trout that can be used to explain the occurrence of GS. In order to accomplish this aim, the transcription, abundance or activity of proteins and hormones such as gill NKA, IGF-I, IGFBP1b, GHr1, CTSL, and cortisol was evaluated using targeted approaches. Moreover, the liver proteome and lipidome were compared in GS and FG groups to pinpoint molecules and processes that might be involved in GS occurrence.

6.2. Materials and methods

6.2.1. Ethics

Experimental work was ethically reviewed, approved and registered by the NARA and by the AWERB (088, University of Stirling, UK).

6.2.2. Fish and rearing conditions

Post-smolt rainbow trout with a weight of 247.9 ± 2.21 g (mean ± s.e.) at seawater transfer (N = 306) were used in this experiment. Fish were fed *ad libitum* using a standard commercial dry diet (Skretting AS) from automatic feeders according to temperature and fish size. Fish were kept indoors in tanks equipped with LED lights in a rainbow trout facility from Lerøy Vest AS (Bjørsvik, Hordaland, Norway). The fish were kept at continuous light, natural temperature, water flow at 0.4 L/kg/min and O_2 was above 80% saturation in the outlet.

The present experiment was carried out on a subset of samples from 60 (n= 30) tagged (Carlin) fish generated in Chapter 2. Out of the 306 fish that were transferred to seawater, the 30 fish with the highest Fulton index and SGR-W were used to represent FG, while the 30 fish with the lowest values for these variables were used for GS (Table 6.1).

Table 6.1. Measurements in fish used for each condition (n= 30) (values ± s.e.).

Samples	Length (cm)	Weight (g)	Fulton index	SGR-W
Fast-growing	30.8 ± 0.29^{a}	447.2 ± 12.30^{a}	1.5 ± 0.01^{a}	0.9 ± 0.03^{a}
Growth-stunted	29.3 ± 0.39^{b}	308.6 ± 11.95^{b}	1.2 ± 0.01^{b}	0.4 ± 0.05^{b}

Length, weight and Fulton index correspond to end-point sampling measurements. Fulton index is measured in g cm^{-3}. Specific growth rate is measured in % day^{-1}. Different superscript letters indicate significant differences.

6.2.3. Sampling

Non-lethal sampling for morphometrics and blood took place on the 5th of July, in freshwater. Fish were transferred to seawater on the same day and lethal sampling took place 9 weeks after on the 14th of September 2016.

For lethal sampling, fish were quickly dip-netted out of the tanks and euthanized by a lethal overdose of isoeugenol (AQUI-S). For each fish, weight and length were recorded. Blood was extracted using heparinised syringes and centrifuged at 3,500 xg for 10 min to obtain plasma, which was frozen at -80°C. The first gill arch of the fish was dissected out and preserved at -80°C in SEI buffer (Sucrose 250mM, Na_2EDTA 10mM, Imidazole 50mM (all Sigma-Aldrich)). Liver samples were either preserved

frozen at -80°C (for proteomic and lipidomic analysis) or preserved in RNAlater (for transcription analysis) according to manufacturer guidelines (overnight at 4°C and frozen at -80°C).

6.2.4. Gill NKA activity

NKA activity was measured in gill tissue collected from 17 randomly selected fish from each group (FG and GS) at the end-point sampling in seawater (n= 17).

NKA activity was measured as described in Chapter 2.

6.2.5. TR-FIA for plasma IGF-I

Circulating IGF-I levels were measured in plasma collected from 8 randomly selected fish from each group (n= 8). Measurements took place on the same fish before seawater transfer and at the end-point sampling in seawater.

TR-FIA protocol for plasma IGF-I was as described in Chapter 2.

6.2.6. Plasma cortisol

Plasma cortisol levels were measured in plasma collected from 15 randomly selected fish from each condition (n= 15) in freshwater. These 15 plus another 15 per group were measured in seawater (n= 30).

Plasma cortisol was measured using a custom ELISA in a 96-well plate. All wells except the 'non-specifics' received 100 µl cortisol antibody (East Coast Biologics); 1:3,000 in 50 mM $NaHCO_3$, 50 mM NaH_2CO_3, pH 9.6) and were incubated overnight at 4°C. The following day, the plates were washed three times with 200 µl/well wash buffer + Tween (100 mM Tris, 0.9% NaCl, 0.1% Tween20). Subsequently, non-specific sites were blocked by the addition of 200 µl blocking buffer (100 mM Tris, 0.9% NaCl, 0.1% Tween20, 2% Normal Calf Serum) to each well. Plates were covered and incubated for 1 h at RT on a plate shaker (300 rpm). Wells were emptied by decanting, after which 10 µl of standard (4–2,048 pg cortisol/10 µl assay buffer containing 100 mM Tris, 0.9% NaCl, 0.1% 8-anilino-1-naphthalene-sulfonic acid, 0.1% Tween20) in triplicate, or 10 µl of undiluted plasma in duplicate was added to designated wells. Non-specifics and B_0 received 10 µl assay buffer (both in triplicate). After the addition of standards and samples, 90 µl cortisol-HRP conjugate (1:3,000; East Coast Biologics) solution was added to all wells. Plates were incubated overnight at 4°C or 4 h at RT. The plates were then washed once with wash buffer + Tween, and twice with wash buffer without Tween. 100 µl 3,3',5,5'-Tetramethylbenzidine substrate at room temperature (Sigma-Aldrich) was added to each well. After 30 to 60 min (depending on the time required to develop a blue color) incubation in the dark on a plate shaker (300 rpm), 100 µl of stop solution (1M sulfuric acid) was added to all wells. Absorbance was measured within half an hour at 450 nm.

6.2.7. RT-PCR

RT-PCR analysis was performed on liver tissue collected from 8 randomly selected fish from each group at the end-point sampling in seawater (n= 8).

FG and GS individuals were analysed for liver of *igf-I*, *igfbp1b*, *ghr1* and *ctsl* mRNA abundance as previously described in Chapter 2 and using the primers presented in Table 6.2.

Table 6.2. Primers used for RT-PCR analysis and accession numbers of the gene sequences (GenBank).

Gene name	Primer sequence (5'>3')	Accession number
igf-I	TGCGGAGAGAGAGGCTTTTA AGCACTCGTCCACAATACCA	M81904
igfbp1b	AGTTCACCAACTTCTACCTACC GACGACTCACACTGCTTGGC	AF403539
ghr1	CGTCCTCATCCTTCCAGTTTTA GTTCTGTGAGGTTCTGGAAAAC	AF403539
ctsl	CAACTACCTGCAGGCACCTA ACATGATCCCTGGTCCTTGAC	AF358668
efα1	CCCCTCCAGGATGTCTACAAA CACACGGCCCACGGGTACT	AF498320

6.2.8. Liver proteome

6.2.8.1. Samples

TMT is a multiplex relative quantitation method by MS. Each of up to 10 trypsin-digested protein samples is labelled with a unique mass-tag, combined, and analysed in the same MS run. Upon MS fragmentation, mass-tags are released and used to measure relative protein abundance levels for each sample. In the present study, livers of 5 FG individuals and 5 GS individuals were selected to compare the liver proteome of the FG and GS phenotypes (Table 6.3).

Table 6.3. Measurements in fish used for proteomics and lipidomics.

Sample	Length (cm)	Weight (g)	Fulton index	SGR-W
FG.1	32.10	502.58	1.52	0.79
FG.2	32.30	505.79	1.50	0.94
FG.3	31.20	466.8	1.54	0.89
FG.4	30.80	475.89	1.63	1.22
FG.5	31.90	520.62	1.60	1.11
GS.1	27.70	257.14	1.21	-0.13
GS.2	26.00	204.16	1.16	0.06
GS.3	24.60	180.7	1.21	0.30
GS.4	26.80	223.95	1.16	-0.22
GS.5	28.70	284.94	1.21	0.52
FG	31.7 ± 0.28^{a}	494.3 ± 9.97^{a}	1.6 ± 0.03^{a}	1.0 ± 0.08^{a}
GS	26.8 ± 0.70^{b}	230.2 ± 18.56^{b}	1.2 ± 0.01^{b}	0.1 ± 0.14^{b}

Length, weight and Fulton index correspond to end-point sampling measurements. Fulton index is measured in g cm^{-3}. SGR-W is measured in % day^{-1}.

6.2.8.2. Liver lysis

Homogenization of 50 mg of liver (wet weight) was done in 1 ml of 0.1 M Tris-HCl pH 7.6 supplemented with 1% protease inhibitor cocktail (Roche) using a pestle motor mixer. SDS and DTT were added to the homogenates to a final concentration of 4% and 0.1M respectively. Samples were then incubated at 95°C for 5 min and cleared by centrifugation at 16,000xg for 10 min at RT.

6.2.8.3. TMT labelling

After measuring protein concentration by BCA, 100 µg of protein were processed for protein digestion. The following steps, protein reduction, alkylation, precipitation, trypsin digestion and TMT labelling of peptides were performed following manufacturer instructions (TMT10plex™ Isobaric Label Reagent Set, ThermoFisher Scientific), followed by a clean-up of the multiplexed sample using Hypersep SpinTip (ThermoFisher Scientific), according to manufacturer instructions. Finally, samples were dried using a vacuum drier (Savant DNA SpeedVac 110, Thermo Scientific).

6.2.8.4. LC MS/MS analysis of TMT

The multiplexed sample was analysed in a LTQ-Orbitrap XL LC–MSn mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray source and coupled to an Ultra High Pressure Liquid Chromatographer system (Waters nanoAcquity). Initially, 5 µL of sample resuspended in ultrapure water was loaded, desalted and concentrated in a BEH C18 trapping columns (Waters) with the instrument operated in positive ion mode. The peptides were then separated on a BEH C18 nanocolumn (1.7 µm, 75 µm × 250 mm, Waters) at a flow rate of 300 nL/min using an ACN/water

gradient; 1% ACN for 1 min, followed by 0−62.5% ACN over 21 min, 62.5− 85% ACN for 1.5 min, 85% ACN for 2 min and 1% ACN for 15 min.

MS spectra were collected using data-dependent acquisition in the m/z range 400−2,000 using a precursor ion resolution of 30,000, following which individual precursor ions (top 5) were automatically fragmented using collision induced dissociation with a relative collision energy of 35%. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 s and exclusion duration of 180 s. TMT tags on lysine residues and N termini (+229.1629 Da) an carbamidomethylation of cysteine (+57.021 Da) residues were set as fixed modifications, and oxidation of the methionine (+15.9949 Da) as variable modification.

6.2.8.5. LC MS/MS data analysis and sequence annotation for TMT

MS data was analysed using Proteome Discoverer (ThermoFisher Scientific). Peak integration allowed for a window tolerance of 20 ppm using the 'most confident centroid' method. Only high-confidence peptides were used for quantification ($q < 0.01$). Peptide relative quantification among samples was based on TMT label abundance. Protein identifications were based on at least one unique peptide. Only unique peptides were used for protein quantification. Data across samples was normalized based on protein median. Protein abundance was further normalized by dividing it in each sample by the total (sum of all 10 samples) of the abundance for that protein. A multiple t-test followed by FDR 5% was used to compare the abundance of each detected protein in both conditions.

Peptide sequences were annotated by database search against the rainbow trout SwissProt database, which was downloaded from MASCOT [downloaded in August 2018] and loaded into Proteome Discoverer. The initial search parameters allowed for a single trypsin missed cleavage, carbamidomethyl modification of cysteine residues, oxidation of methionine, acetylation of N-terminal peptides, a precursor mass tolerance of 10 ppm, a fragment mass tolerance of ±0.5 Da, and a FDR of 0.01. Those proteins identified as 'uncharacterised' in the rainbow trout genome were further searched by sequence homology against the Atlantic salmon, zebrafish, and human SwissProt databases, in this order of preference. Only homologies of E-value lower than 0.01 were accepted as valid.

Data was transformed by natural logarithm to satisfy the normal distribution and homogeneity of variance assumptions, tested with the Shapiro and Bartlett tests, respectively. Next, multiple t-test analysis and 5% FDR correction were used.

6.2.8.6. GO analysis

GO analysis was performed as explained in Chapter 2.

6.2.9. Liver lipidome

6.2.9.1. Individual samples

The same liver samples from FG and GS (n =5) described in the liver proteome section were used (Table 6.2).

6.2.9.2. Lipid extraction

Lipid extraction was carried out following the Folch method (Folch *et al.*, 1957; Christie, W. W. and Han, 2010). Briefly, 25 mg of liver (wet weight) sample were homogenized in 10 ml of chloroform/methanol (2:1), incubated on ice for 1 h, added 2.5 ml of 0.88% KCl, vortexed, incubated on ice for 5 min and centrifuged at 400xg for 5 min. Afterwards, the top layer was removed by aspiration and the lower layer was filtered through paper filters (No.1, Whatman). Solvent was evaporated under a stream of oxygen-free nitrogen and desiccated *in vacuo* overnight. Samples were stored under argon at -20^{o}C.

6.2.9.3. LC MS/MS analysis of lipids

Lipids were analysed by LC-MS using a Thermo Orbitrap Exactive MS (Thermo Scientific), equipped with a heated electrospray ionization probe and coupled to a Thermo Accela1250U HPLC system. All samples were analysed in both positive and negative ionization modes over the m/z range 200–2,000. Samples were injected into a Thermo Hypersil Gold C18 column (2.1 mm x 100 mm, 1.9 mm). Mobile phase A consisted of water containing 10 mM ammonium formate and 0.1% (v/v) formic acid. Mobile phase B consisted of 9:1 isopropanol/ACN containing 10 mM ammonium formate and 0.1% (v/v) formic acid. All solvents were LC-MS grade (Fisher Scientific). The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B was increased to 100% over 10 min and held for 7 min before re-equilibration with the starting conditions for 4 min.

6.2.9.4. LC MS/MS data analysis and lipid identification

The raw LC-MS profiles were processed and the protein abundance quantified with Progenesis CoMet v2.0 software (Non-linear Dynamics). Data analysis was done using the in-built ANOVA and FDR 5% tests. This was performed for data acquired in both positive and negative ionization modes.

After PCA, correlation of each lipid ion with PC1 (pCorr) was calculated. Lipid ions of pCorr > 0.90 (higher in GS) or pCorr < -0.90 (higher in GS) were searched against Lipid Maps (www.lipidmaps.org) for identification. Of these, lipids of q-value < 0.05 and fold change > 2 between FG and GS were used for further analysis.

6.2.9.5. General data analysis and representation

Data representation was carried out using R statistical software and R package ggplot2 (Wickham, 2009).

PCA and data representation were carried out using R and 'ggbiplot' package (Vu, 2011). Ellipses show 68% of Normal probability for each group.

DAPs were plotted in heatmap form showing individual sample abundance scaled by protein (*i.e.* by row) for liver proteins.

Unpaired t-test was used to test for differences in morphometric data, liver protein and lipid percentage, NKA activity, IGF-I abundance, cortisol abundance, and gene transcription among FG and GS groups. Data was transformed by either natural logarithm or square root to satisfy the normal distribution and homogeneity of variance assumptions, tested with the Shapiro and Bartlett tests, respectively. Similarly, paired t-test was used to test for differences between freshwater and seawater IGF-I abundance.

6.3. Results

6.3.1. NKA activity

NKA activity values after 9 weeks in seawater were not related to GS development as they showed no significant differences among FG and GS groups (p: 0.60, df: 32, t: 0.53). NKA activity values were 2.6 ± 0.30 µmol ADP mg protein^{-1} hour^{-1} on average (Figure 6.1).

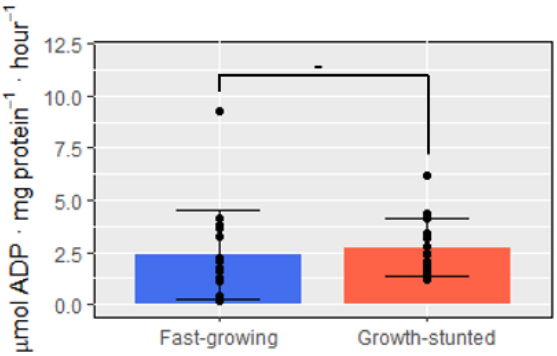


Figure 6.1. Gill NKA activity in FG and GS fish after 9 weeks in seawater. Error bars indicate s.d.

6.3.2. Plasma cortisol

Plasma cortisol levels showed no significant differences between FG and GS in either freshwater (p: 0.47, df: 28, t: 0.73) or after 9 weeks in seawater (p: 0.44, df: 58, t: 0.78) (Figure 6.2). However, cortisol levels were significantly higher in freshwater (average of 76.0 ± 10.67 ng ml^{-1}) than in seawater (average of 20.8 ± 4.19 ng ml^{-1}) ($p < 0.001$, df: 88, t: 5.76).

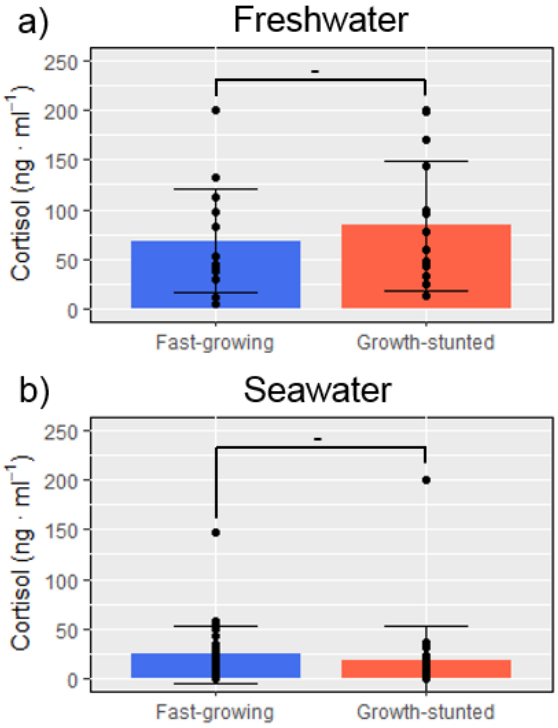


Figure 6.2. Circulating plasma cortisol levels in FG and GS fish prior (a) and 9 weeks after (b) seawater transfer. Error bars indicate s.d.

6.3.3. Plasma IGF-I

Circulating plasma IGF-I levels showed no significant differences between FG and GS in freshwater (p: 018, df: 14, t: 1.46) but did after 9 weeks in seawater ($p < 0.01$, df: 13, t: 3.09) (Figure 6.3). This significant difference was due to a significant increase for FG ($p < 0.01$, df: 7, t: 3.76), while plasma IGF-I levels did not vary significantly from freshwater to seawater for GS (p: 0.63, df: 7, t: 0.50).

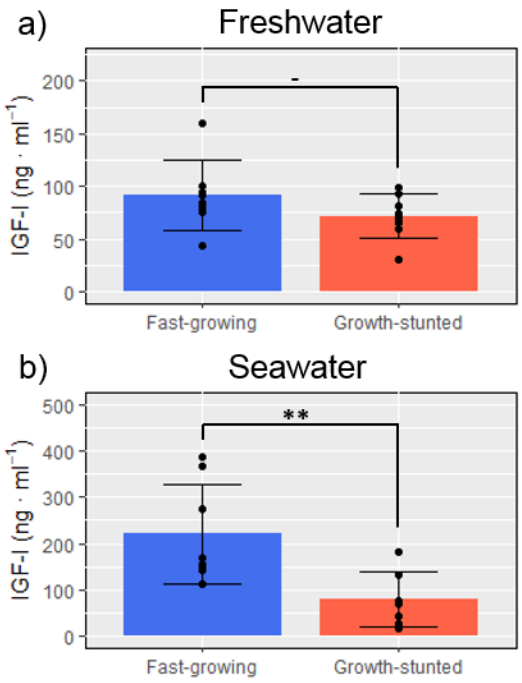


Figure 6.3. Circulating plasma IGF-I abundance in FG and GS fish prior (a) and 9 weeks after (b) seawater transfer. Error bars indicate s.d.

6.3.4. Liver transcription of *igf-I, igfbp1b, ghr1* and *ctsl*

Concordantly with plasma IGF-I abundance, liver *igf-I* transcription was higher in FG than in GS ($p < 0.01$, df: 14, t: 3.99). On the other hand, while not significant for *igfbp1b*, there was weak evidence (p-value < 0.1) indicating that its transcription might be higher in GS (p: 0.07, df: 14, t: 1.94). For *ghr1* (p: 0.10, df: 14, t: 1.73) and *ctsl* transcription (p: 0.39, df: 14, t: 0.90), no differences were found (Figure 6.4).

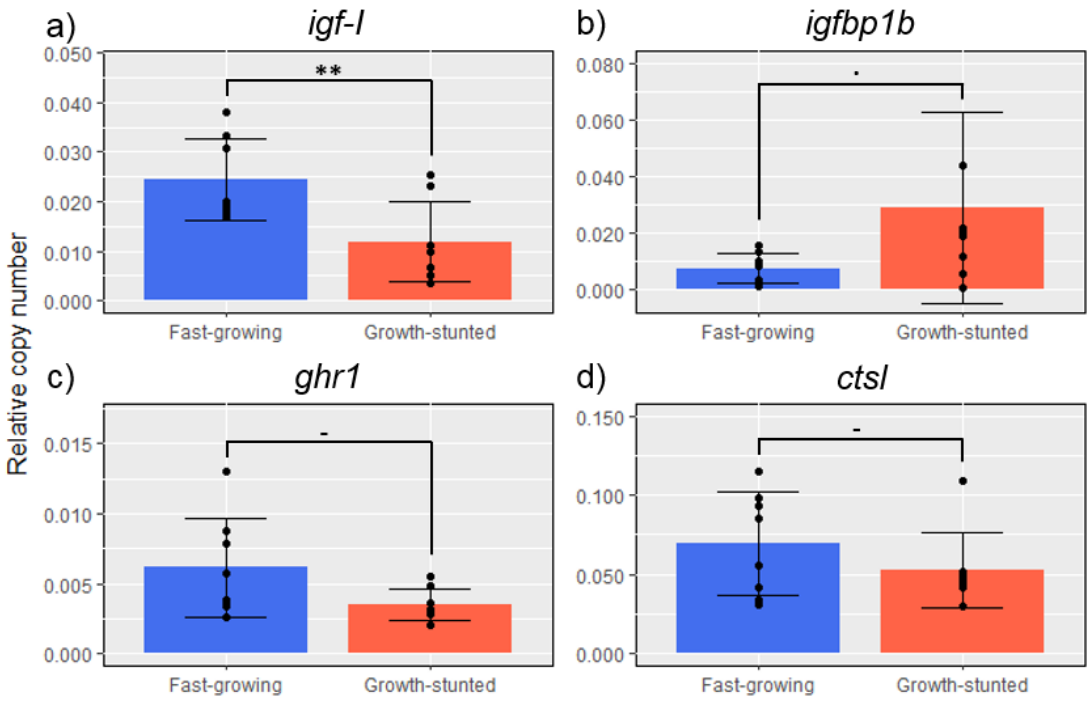


Figure 6.4. Liver transcription of growth-related genes *igf-I* (a), *igfbp1b* (b), *ghr1* (c), and *ctsl* (d) in FG and GS fish after 9 weeks in seawater. Error bars indicate s.d.

6.3.5. Liver proteome

6.3.5.1. Protein abundance

In terms of protein concentration, the liver composition of GS was shown to contain a significantly higher protein percentage than FG (FG: 30.7 ± 0.72, GS: 26.1 ± 0.58) ($p < 0.01$, df: 8, t: 4.94). Nevertheless, equal amounts of liver protein per sample were used in the proteomic analysis.

6.3.5.2. Detected proteins

After MS analysis of TMT labelled liver samples, a total of 308 different peptides that successfully incorporated a TMT label were detected. These peptides were mapped onto 99 quantified proteins (Appendix 6.1).

GO analysis revealed cellular process and regulation as the main biological process GO, cytoplasm, nucleus, extracellular components and ribosome for cellular component GO, and binding and catalytic as the main molecular function GO (Figure 6.5).

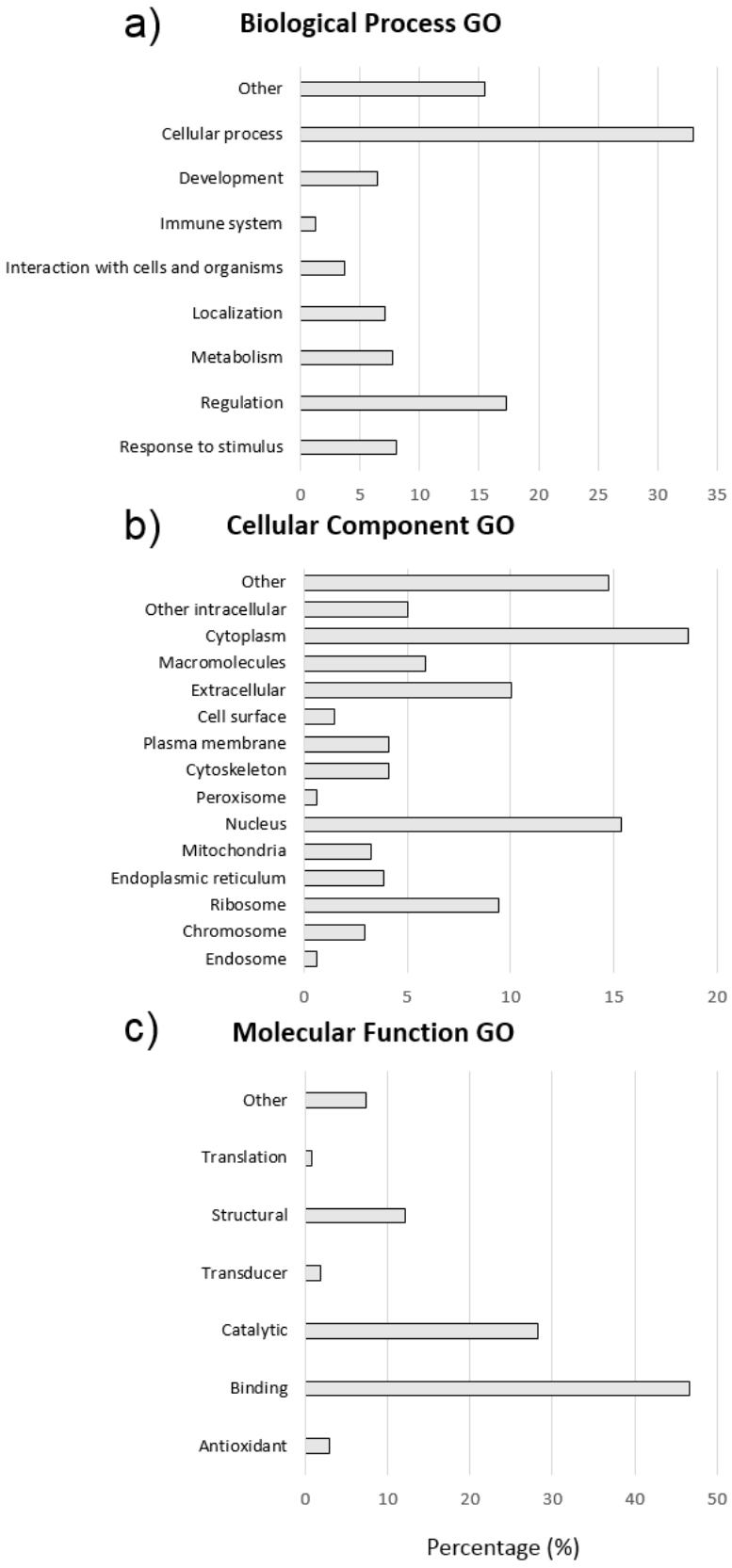


Figure 6.5. GO terms associated to proteins quantified using TMT.

PCA was used to visualize the relationship between replicates, and the dissimilarity among FG and GS. PC1 explained 38.9% of the variation between FG and GS while PC2 explained 14.4%, for a combined 53.3%. A distinction between the two tested conditions was possible, being driven by PC1 (Figure 6.6).

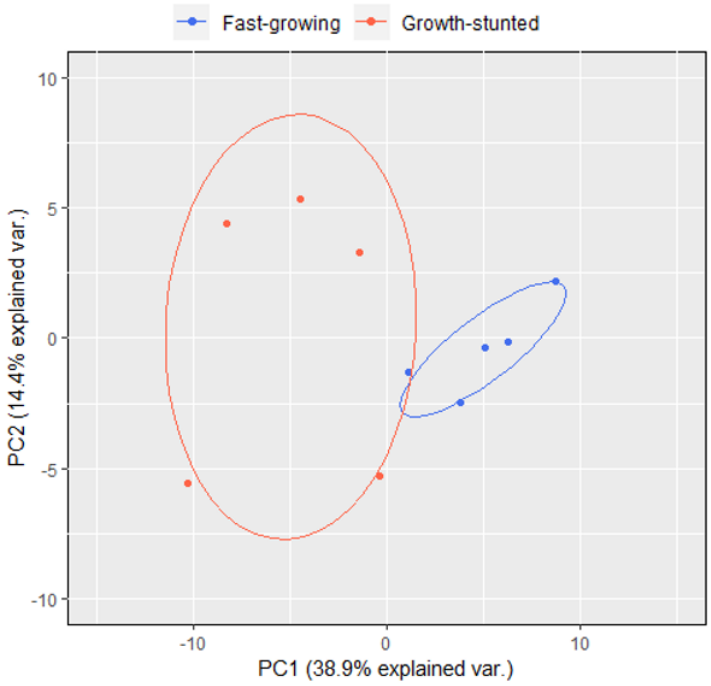
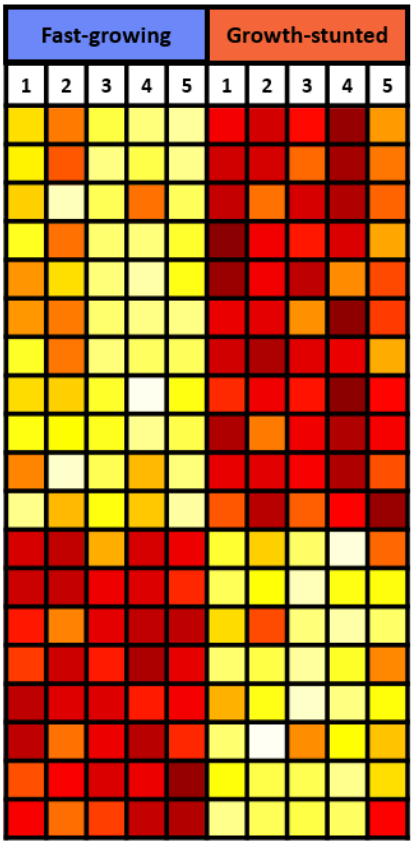


Figure 6.6. PCA of quantified proteins. Points indicate individual replicates. Ellipses show 68% Normal probability for each group.

6.3.5.3. Differential proteins

In total, 19 DAPs (q-value < 0.05) were detected when comparing the liver proteome of GS and FG (Figure 6.7). These proteins presented roles related to translation (*e.g.* 40S ribosomal protein S13, glycine N-methyltransferase), redox homeostasis (*e.g.* Protein disulfide-isomerase (PDI), catalase), oxygen transport (*e.g.* Alpha-globin IV), stress response (*e.g.* Heat shock protein (HSP) 90-alpha 1), and transport and metabolism of carbohydrates (*e.g.* Malate dehydrogenase, GDH/6PGL endoplasmic bifunctional protein) and lipids (*e.g.* Non-specific lipid-transfer protein).



Accession	Protein name
O95479	GDH/6PGL endoplasmic bifunctional protein
Q6UD79	Calreticulin
P01023	Alpha-2-macroglobulin
A0A060XS59	Protein disulfide-isomerase (I)
A0A060W539	Protein disulfide-isomerase (II)
A0A060WQP1	Protein disulfide-isomerase (III)
Q90474	Heat shock protein HSP 90-alpha 1
A0A060W2P7	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 1
C1BG76	40S ribosomal protein S13
A0A1S3LI61	Translocating chain-associated membrane protein
P51124	Granzyme M
A0A060VXL3	Annexin
A0A060YE44	Poly [ADP-ribose] polymerase
A0A060VZR8	Malate dehydrogenase
A0A060W7L7	Catalase (I)
A0A060X782	Catalase (II)
A0A060XET2	Glycine N-methyltransferase
P22307	Non-specific lipid-transfer protein
Q98974	Alpha-globin IV

Figure 6.7. Heatmap of protein abundance for 19 DAPs between FG and GS liver samples. Accession and protein name are retrieved from Uniprot (uniprot.org).

6.3.6. Liver lipidome

6.3.6.1. Lipid abundance

In terms of total lipid, the liver composition of FG and GS (FG: 15.4% ± 2.87, GS: 13.8% ± 0.97) were not significantly different (p: 060, df: 8, t: 0.54).

6.3.6.2. Detected lipids

After MS analysis of lipid extracted from liver samples, 6,456 lipid ions were detected in positive ion mode and 2,756 in negative ion mode.

After an initial PCA analysis, in positive mode PC1 explained 38.3% of the variation, while PC2 explained 13.4%. For negative mode data, PC1 and PC2 explained 34.4% and 18.9%, respectively. A clear distinction between the two tested conditions was possible in both cases (Figure 6.8).

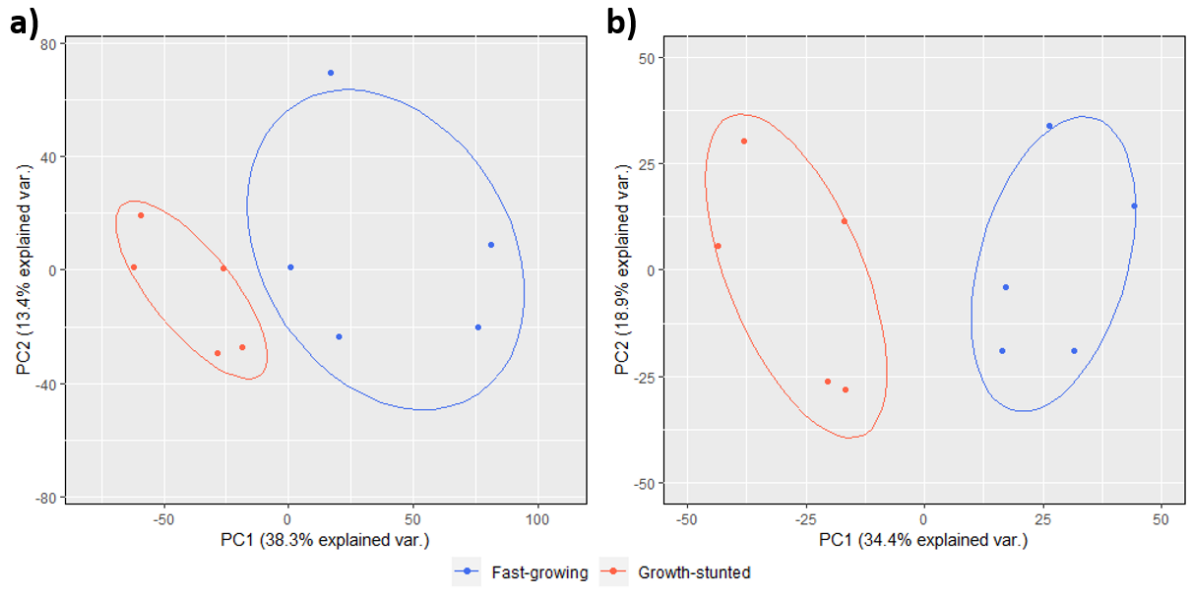


Figure 6.8. PCA of quantified lipid ions detected in positive (a) and negative ionization modes (b). Points indicate individual replicates. Ellipses show 68% Normal probability for each group.

6.3.6.3. Differential lipids

For positive ionization mode, 56 lipid ions presented pCorr > 0.90 (higher in GS) or pCorr < -0.90 (higher in GS), q-value < 0.05, and fold change > 2. Similarly for negative ionization mode, 59 lipid ions were differential in abundance (Figure 6.9). This lipid data in shown in Appendix 6.2.

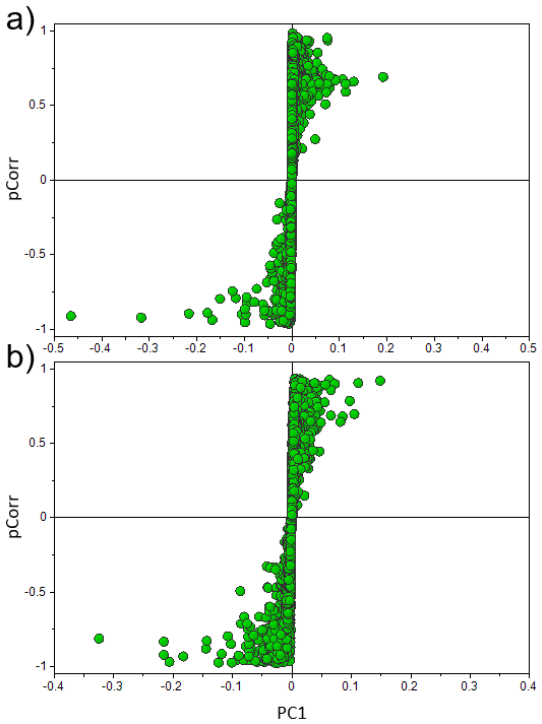


Figure 6.9. S plots of covariance of PC1 (x axis) vs correlation (y axis) for both positive (a) and negative (b) ionization modes.

Differential lipids corresponded to twelve major classes of lipid: cholesterol esters (CE), ceramides, cardiolipins (CL), diglycerides (DG), TG, phosphatidylcholines (PC), phosphatidylethanolamines (PE), lysoPC, lysoPE, phosphatidylinositol (PI), phosphatidylserines (PS), and sphingomyelin (SM).

Main differences were found in the phospholipid fraction (sphingolipids and glycerophospholipids). For instance, in FG liver, most of the glycerophospholipids that were significantly higher corresponded to the lipid classes PC and PS (48 out of 55). For GS, glycerophospholipids that were found to be significantly higher corresponded to the lipid classes CL, PC and PE (33 out of 43) (Table 6.4). Notable changes in sphingolipids were also found with FG showing 5 ceramides being significantly higher in FG compared to GS.

Table 6.4. Main lipid classes represented by differential lipids between FG and GS. Number of different lipid species detected in significantly higher abundance in each condition.

	Fast-growing	Growth-stunted
Glycerolipids		
Triglycerides	1	0
Diglycerides	1	3
Total	2	3
Glycerophospholipids		
Phosphatidylethanolamines (PE)	4	13
LysoPE	0	1
Phosphatidylcholines (PC)	18	10
LysoPC	0	2
Phosphatidylinositol (PI)	3	0
Phosphatidylserines (PS)	30	6
Cardiolipins (CL)	0	11
Total	55	43
Sphingolipids		
Sphingomyelin (SM)	1	2
Ceramides	5	0
Total	6	2
Sterol lipids		
Cholesterol esters (CE)	1	0
Total	1	0

6.4. Discussion

Seawater-transferred rainbow trout farming is a developing industry, having experienced a strong increase in production during the last three decades (Food and Agriculture Organization of the United Nations, 2019). However, the high incidence of GS fish has hindered its growth. Currently, the cause

of the development of this unwanted phenotype is still unknown. Understanding the underlying mechanisms that drive it could potentially result in optimized rearing strategies, feeding protocols and to the identification of molecular markers for the early detection of GS fish, which would greatly improve the sustainability, profitability and welfare of seawater-transferred rainbow trout farming. In the present study, an in-depth characterization of the GS was performed. Targeted approaches were employed to measure NKA activity, plasma IGF-I abundance, plasma cortisol levels and liver *igf-I*, *igfbp1b*, *ghr*, and *ctsl* transcription, while untargeted –omics approaches were used to study the liver proteome and lipidome of GS fish compared to FG fish.

Since growth is the attribute that most clearly distinguishes GS and FG fish, it would be expected that growth regulators are different between the two phenotypes. In this sense, IGF-I is a peptide hormone that promotes growth of fish (Wood *et al.*, 2005; Picha *et al.*, 2008; Beckman, 2011), along with GH and its receptor GHr, among others. On the other hand, IGFBP1b, which modifies the affinity of IGF-I for its receptor and represses growth (Shimizu *et al.*, 2006; Kawaguchi *et al.*, 2013; Kaneko *et al.*, 2019), and CTSL, an endopeptidase, are both induced under catabolic conditions (Salem *et al.*, 2006; Salem *et al.*, 2007). As expected, in this study, differences among some of these growth-regulatory molecules were found between FG and GS rainbow trout. Weak evidence that high *ifgbp1b* transcription might play a role in the development of GS was found, however, differences were not significant and further testing would be needed. However, the involvement of the growth-enhancing peptide hormone IGF-I was demonstrated, being significantly lower for GS at both plasma abundance and liver transcription levels. These results show the importance of the somatotropic axis in the regulation of growth and in the development of GS, consistently with nutritional restriction scenarios (Wilkinson *et al.*, 2006). While IGF-I abundance increased in FG during their time in seawater, levels in GS did not change. This confirms that GS development only occurs after seawater transfer.

The liver is a key organ for the accumulation and mobilization of energy reserves (Burra, 2013), with hepatosomatic index being significantly reduced in rainbow trout during starvation (Albalat *et al.*, 2006). Moreover, the liver is a key regulatory organ. In the present study, total protein concentration in GS livers was higher than in FG. Liver protein synthesis can vary in response to several processes, one of them being nutritional status (McMillan and Houlihan, 1992). The effects of a short-term starvation period would be a decrease in hepatic protein synthesis. However, in response to long-term starvation, protein production in liver can increase in rainbow trout, likely due to energy reserve depletion affecting other tissues (McMillan and Houlihan, 1992). A labelled proteomic approach (TMT) was used for the relative quantification of liver proteins present in both FG and GS rainbow trout. TMT can analyse up to 10 samples in one run, thus improving analytical power by reducing the technical bias caused by comparing samples analysed in different runs. However, it had not been used in

rainbow trout. To verify that detected proteins by this method were comparable to other methods, GO analysis was performed. GO analysis of identified proteins revealed that the majority of the proteins identified had regulatory functions, acted on a wide variety of cellular components (especially in cytoplasm, nucleus, extracellular domain and ribosome) and their main molecular activities were binding and catalytic. Indeed, the liver proteome detected using MS and TMT was similar to the liver proteome of other teleosts and vertebrates (Kiss *et al.*, 2011; Wang *et al.*, 2015; Qiu *et al.*, 2016).

As seen from the 19 identified DAPs, a variety of processes were significantly affected as a result of GS development. As a general trend, the liver proteome indicated differences in metabolism of energy reserves, mainly GDH/6PGL endoplasmic bifunctional protein, which oxidizes glucose-6-phosphate and therefore is implicated in glucose metabolism, being higher in GS (Clarke and Mason, 2003; White *et al.*, 2007), and malate dehydrogenase, which is related to carbohydrate metabolism, being higher in FG (Minarik *et al.*, 2002). These proteins reflect previously documented changes in hepatic glucose which were linked to hyperglycaemia (Sloman *et al.*, 2001; Gilmour *et al.*, 2012) and carbohydrate (Ejike and Schreck, 1980; Sloman *et al.*, 2001; Gilmour *et al.*, 2012) metabolism of GS freshwater rainbow trout and other salmonids. Changes in carbohydrate metabolism and hyperglycaemia can occur due to both starvation and other sources of stress (Moon, 2001; McCowen *et al.*, 2001).

HSP90 was higher in GS. This molecular chaperone promotes the maturation, structural maintenance and regulation of specific target proteins involved for instance in cell cycle control and signal transduction. Moreover, it also plays a role in the regulation of transcription factors, certain epigenetic modifiers, such as DNA methyl transferases, and participates in the release of histones from the promoter region of certain genes, thus promoting their gene transcription (Khurana and Bhattacharyya, 2015; Verma *et al.*, 2016; Pearl, 2016). In turn, HSP90 expression is activated in response to stress (Jarosz and Lindquist, 2010; Kaplan and Li, 2012). In this sense, Currie *et al.* (2009) showed that HSP90 transcription in liver increases due to fasting in rainbow trout. However, none of the DAPs shown in the present study were consistent with the list of DAPs presented in a rainbow trout starvation trial (Martin *et al.*, 2001), including Cathepsin D. Moreover, the low mortality registered during the seawater phase (less than 1%) suggests that, while GS may have been eating less, likely due to the influence of dominant fish (Abbott and Dill, 1989; DiBattista *et al.*, 2006), these fish were not starved. While DAPs were also not consistent with what is expected to change in the liver proteome of calorie restricted zebrafish, the main finding of Jury *et al.* (2008) was a downregulation of oxygen-binding activity, which is consistent with lower alpha-globin IV, an oxygen transport protein, in GS. However, this could also indicate higher exercise levels in FG, which is consistent with the lower activity in the GS fish described by Vindas *et al.* (2016) in Atlantic salmon. Finally, liver lipidome results are not consistent with starvation scenario for GS. While initially, given

the poor growth observed in GS and their low condition factor, the biggest differences between FG and GS livers were expected to be mostly related to the depletion of energy reserve species (*e.g.* TG, DG) in GS (Groener *et al.*, 1979; Kjær *et al.*, 2009; Jensen-Urstad and Semenkovich, 2012) results from this study did not support this hypothesis. Moreover, no differences in total liver lipid were found between FG and GS, with levels around 13-15%, in agreement with fed rainbow trout in other studies (Jezierska *et al.*, 1982; Albalat *et al.*, 2006; Furné *et al.*, 2012).

On the other hand, HSP90 can also be upregulated due to other stressors. Currie *et al.* (2009) indicated that an upregulation of the transcription of HSP90 in liver occurs during the first days of social interaction in freshwater rainbow trout. After 6 days, hepatic HSP90 transcription remained elevated in subordinate fish but not in dominant. Indeed, rainbow trout are aggressive towards conspecifics and GS fish often display fin damage, which is a sign of bullying by dominant fish (Moutou *et al.*, 1998; North *et al.*, 2006; Hoyle *et al.*, 2007). In this sense, the expression patterns of DAPs like alpha-2-macroglobulin (related to blood coagulation; Cvirn *et al.*, 2002; Ignjatovic *et al.*, 2007), granzyme M (a regulatory protease related to injury, inflammation and repair (Mahrus *et al.*, 2004; Ewen *et al.*, 2012; Hiebert and Granville, 2012), and annexin (an inflammation inhibitor protein related to injury and repair (Babbin *et al.*, 2008; Martin, G. R. *et al.*, 2008), might be related to injury and healing. However, unlike previously reported, cortisol levels were not higher in GS (Sloman *et al.*, 2001; Gilmour *et al.*, 2005; Jeffrey *et al.*, 2014). Regardless, previous studies only tested the short-term effects of this social stressor. In the situation of the present study, fish interacted for months. If cohabiting with dominant fish was indeed a stressor for GS, this stress might have become chronic, in which case no differences in plasma cortisol would be found, as cortisol levels decrease under chronic stress (Barcellos *et al.*, 2006; Fast *et al.*, 2008).

The liver lipidome showed important differences between the two phenotypes, mainly found in the phospholipid fraction (sphingolipids and glycerophospholipids). Phospholipids are key structural constituents of cellular membranes and of lipoproteins involved in the transport of dietary lipid from the intestine and liver to the rest of the body (Tocher, 2003; Thiam *et al.*, 2013). Changes in phospholipid composition can occur due to diet (Tocher *et al.*, 2008; Carmona-Antoñanzas *et al.*, 2015; Almaida-Pagán *et al.*, 2015), age (Almaida-Pagán *et al.*, 2014; Almaida-Pagán *et al.*, 2015), temperature (Hazel, 1990), or disease (Pettegrew *et al.*, 2001; Kuliszkiewicz-Janus *et al.*, 2005), among others. These phospholipid composition differences were mainly related to bigger numbers of differentially higher PE, lyso species (lysoPE and lysoPC) and CL in GS and bigger numbers of differentially higher PI, PS, PC and ceramides in FG. Moreover, higher rates of phospholipid transport between membranes in FG was indicated by the DAP non-specific lipid-transfer protein (Seedorf *et al.*, 1994; Stanley *et al.*, 2006). Sphingolipids contain a backbone of sphingoid bases and a set of

aliphatic amino alcohols that includes sphingosine. SM is a crucial sphingolipid component of cell membranes for their structural organization. In turn, cleavage of SM results in the liberation of ceramide, which regulates the intracellular activities of an array of kinases, phosphatases and transcription factors (Hannun and Luberto, 2000; Cutler and Mattson, 2001). Among other signals, SM cleavage occurs in response to growth factors, hormones and oxidative stress (Rao *et al.*, 2007). On the other hand, glycerophospholipids are a large family of glycerol-based lipids that are mainly involved in the formation of cellular membranes (Triebl, 2016). Biologically, the bigger number of differentially higher PC in FG is consistent with higher growth (Kanazawa, 1993; Kasper and Brown, 2003; Azarm *et al.*, 2013), while more PI can increase survival and reduce the occurrence of deformities (Geurden *et al.*, 1998; Carmona-Antoñanzas *et al.*, 2015). However, dietary administration of PE or PS has been shown to have little effect on fish performance (Kanazawa, 1985; Kanazawa, 1993; Geurden *et al.*, 1998). Furthermore, administration of lysoPC and lysoPE, several of which were significantly higher in GS, can be processed more easily but form less stable membranes (due to their simpler structure missing one of the two fatty acids found in PC or PE) and their effects on fish performance have been shown to be slightly negative (Geurden *et al.*, 1998; Tocher *et al.*, 2008; Triebl, 2016).

Being components of membranes, glycerophospholipids are affected by oxidative stress. This is especially the case for CL, due to their almost exclusive location in mitochondrial membranes where the electron-transport chain occurs, and where there is intense reactive oxygen species production (Hoch, 1992). CL are involved in the biogenesis, dynamics and organization of mitochondrial membranes, controlling their permeability and contributing to the assembly of mitochondrial protein complexes involved in respiration and energy production (Paradies *et al.*, 2011; Horvath and Daum, 2013). These lipids can be used as biomarkers for mitochondrial dysfunction (Paradies *et al.*, 2002; Paradies *et al.*, 2011). Therefore, CL are implicated in energetic balance (Paradies *et al.*, 2011; Horvath and Daum, 2013) and ceramides regulate a wide variety of molecular processes (Hannun and Luberto, 2000; Cutler and Mattson, 2001). While they are very different in composition and nature, both of these lipid classes are prone to peroxidation, which can lead to dysfunctional mitochondria for CL (Hoch, 1992) and to the induction of apoptosis for ceramides (Andrieu-Abadie *et al.*, 2001; Cutler and Mattson, 2001). In this sense, detected DAPS catalase (I and II) and protein disulphide-isomerase (I, II, and III) are both involved in cell redox homeostasis. Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen and its activity is used a biomarker of oxidative stress. Therefore, it is crucial enzyme in protecting the cell from oxidative damage by reactive oxygen species and it has been proposed as a marker and potential tool for the treatment of liver diseases like hepatitis and hepatocarcinoma (Salvi *et al.*, 2007). Moreover, this enzyme may also control bioenergetic

metabolism by regulating the activity of the Krebs cycle, respiratory chain, and ATP synthesis (Salvi *et al.*, 2007). On the other hand, PDI acts as a converging hub for hydrogen peroxide generation pathways, including oxidases and peroxidases (Laurindo *et al.*, 2012). Moreover, it is tightly connected to oxidoreductases, mitochondria, and NADPH oxidases, the three main mechanisms of oxidant generation (Laurindo *et al.*, 2012; Ali Khan and Mutus, 2014; Zeeshan *et al.*, 2016). Therefore, although PDI deficiency results in health conditions (Gromova *et al.*, 1999; Yi *et al.*, 2009), it represents a mechanism of positive oxidative stress regulation. Therefore, these two seemingly opposite DAPs, in combination with the dramatic differences in CL and ceramide lipid composition, seem to indicate that GS might be under higher levels of oxidative stress. In turn, this could be associated to dysfunctional hepatic cellular membranes and mitochondrial membranes and might explain their physiological problems. Indeed, hepatic oxidative stress induced by diet (Han *et al.*, 2011; Azaza *et al.*, 2015) or chemical exposure (Zhu *et al.*, 2008; Sun *et al.*, 2014) has been linked to decreased growth and feed efficiency in fish. Therefore, since dietary phospholipids are incorporated into organs, including the liver (Jordal *et al.*, 2007), it is possible that supplementation of lacking phospholipids into the diet of GS might mitigate their stunted growth (Kanazawa, 1993; Kasper and Brown, 2003; Azarm *et al.*, 2013). However, while this mitigation strategy might reduce the incidence of GS, it would not solve the initial cause of the occurrence of the phenotype.

Overall, results point at low IGF-I abundance and transcription as an important cause or consequence of the reduced growth experienced by GS. Moreover, HSP90 indicates that GS are under higher stress. Due to the inconsistency of the DAPs in the present study with those found in a starvation trial of rainbow trout (Martin *et al.*, 2001) and the lack of differences in overall lipid and in DG and TG in liver, starvation seems unlikely to be the cause of this stress. On the other hand, the high incidence of fin damage in GS fish seems to support social stress as an underlying cause, consistently with what leads to GS development in freshwater rainbow trout (Gilmour *et al.*, 2005; Currie *et al.*, 2009). Moreover, other GS freshwater rainbow trout characteristics were also identified, which were related to higher hepatic glucose (Gilmour *et al.*, 2012) and carbohydrate metabolism in subordinate animals (Gilmour *et al.*, 2012), and to changes in lipid metabolism suggesting that dominant fish rely more on TG for maintenance while subordinate fish rely on free fatty acids (Kostyniuk *et al.*, 2018). Regarding this last point, differences between groups in hepatic lipid composition affected phospholipids, indicating differences in membrane structure and dietary lipid transport (Tocher, 2003; Thiam *et al.*, 2013). Finally, both the liver proteome, though DAPs catalase and PDI, and the liver lipidome, through differential abundance of CL and ceramides, indicated that GS might suffer more hepatic oxidative stress than FG, which has been linked to growth performance and feed efficiency previously (Zhu *et al.*, 2008; Han *et al.*, 2011; Sun *et al.*, 2014; Azaza *et al.*, 2015). However, there is still potential in the

generated lipidome dataset. Further analysis would allow for the understanding of the biological relevance of the detected lipids, improving our comprehension of the lipid regulation in GS. Moreover, due to the lack of behavioural tests, the relationship between GS and the naturally freshwater-resident phenotype could not be tested. Finally, if social subordination is indeed a cause of GS development, fish should be regularly graded by size, thus impairing the establishment of hierarchies, reducing the stress of GS fish and increasing their welfare conditions (Ellis *et al.*, 2002; Martins *et al.*, 2006; Sneddon, 2006). Regarding their nutritional needs, supplementation of lacking phospholipids into their diet might mitigate their stunted growth (Kanazawa, 1993; Kasper and Brown, 2003; Azarm *et al.*, 2013).

Chapter 7: General discussion

Seawater-transferred rainbow trout aquaculture has become an attractive source of revenue in leading countries for Atlantic salmon production, like Norway, Chile or Scotland, as shown by the rapid increase in their production of this phenotype of rainbow trout in recent years (Food and Agriculture Organization of the United Nations, 2019). Moreover, being the salmonid that inhabits the broadest latitudinal range of environments due to artificial introductions around the world, interest in the aquaculture of this species has great potential to expand to more countries (Behnke, 2010). Industrial interest in this species is due to the need to diversify available aquaculture products, its resistance to infectious pancreatic necrosis (Okamoto *et al.*, 1993; Ozaki *et al.*, 2001), and their preference for brackish water compared to Atlantic salmon (Altinok and Grizzle, 2001). Due to this brackish water preference during the on-growing stage, seawater-transferred rainbow trout producers can make use of otherwise unexploited areas (*e.g.* fjords) which are less prone to sea lice infestations. However, most seawater-transferred rainbow trout aquaculture practices, including protocols to induce smoltification, have been imported from Atlantic salmon without evidence data supporting that these are indeed suitable for the species. As a result, the progress of this sector has been hindered by issues related to the transfer of these fish to seawater: especially during the summer months, large numbers of fish die (around 10%) or become GS (10-60%), resulting in significant economic losses. Current issues encountered by seawater-transferred rainbow trout farmers are likely related to a lack of understanding of the extrinsic (rearing conditions) and intrinsic (*e.g.* threshold size for smoltification, genetically determined phenotypic plasticity) factors governing rainbow trout smoltification and seawater adaptation, as well as a lack of reliable biomarkers to assess seawater-readiness in the species. Moreover, more information on the mechanisms driving GS fish development is needed in order to reduce their occurrence.

7.1. Smoltification in rainbow trout

In this study, smoltification in rainbow trout was consistent with previous literature on other salmonids. Firstly, fish experienced classical morphological changes, progressively becoming silvery in coloration and decreasing in Fulton index during the smolt window (Hoar, 1988). The onset of smoltification took place between March and April and the smolt window was reached at around mid-March or April and arguably lasted for 2 months until May, mid-May or even June, as determined by NKA activity and transcription of related genes. This is a shorter duration than the 3-4 month smolt window that 2+ steelhead trout experience (Wagner, 1974a; Dickhoff *et al.*, 1978; Negus, 2003). In

terms of osmoregulation in gill tissue, generally NKA activity during the parr stage was below 3 µmol ADP mg protein^{-1} hour^{-1}, while during the smolt window NKA activity was above 5 ADP mg protein^{-1} hour^{-1} on average. In this sense, there was big variability among experiments, with the measurements during the smolt window in 2016 (chapter 2) being above 7.5 µmol ADP mg protein^{-1} hour^{-1} on average. Nonetheless, fish from both experiments were likely to be osmocompetent in seawater during that period, as previous studies have reported smolt window values of between 3 and 4 µmol ADP mg protein^{-1} hour^{-1} (Madsen and Naamansen, 1989; Ewing *et al.*, 1994). In contrast, NKA activity values during the smolt for Atlantic salmon tend to be higher than 7 ADP mg protein^{-1} hour^{-1} (Berrill *et al.*, 2006; Handeland *et al.*, 2013). As expected, during the smolt window the transcription of the freshwater pump *nkaα1a* decreased to minimum levels, while *nkaα1b* transcription increased in a similar fashion to NKA activity (Nilsen *et al.*, 2007). Transcription of *nkcc1a* was also stable at high levels during the smolt window, however, in this case the transcription of this gene did not increase, as it was already high during the parr stage.

Liver transcription of genes related to growth and smoltification were also mostly in accordance to previous literature, with *igf-I* and *ghr1* transcription increasing during the smolt window (Mori *et al.*, 2001; Kiilerich *et al.*, 2007; Shimomura *et al.*, 2012). However, in this study *ghr1* transcription only increased sharply and transiently at the end of the smolt window. Transcription of the growth inhibitor and modifier of the activity of IGF-I, *igfbp1b*, did not show seasonal variation related to smoltification unlike what has been shown for other IGFBPs (Breves *et al.*, 2017). On the other hand, *ctsl* transcription followed a similar trend as *igf-I* or as the seawater pumps, indicating high rates of tissue reorganization during this period, which is consistent with major changes experienced by the liver (Sheridan *et al.*, 1985; Sheridan, Woo *et al.*, 1985; Sheridan, 1986).

Also for the DAPs identified in plasma between Parr and Smolt pools, most of the proteins that were identified were related to known processes that occur during smoltification or seawater adaptation. These DAPs were related to processes like the development of seawater tolerance (serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C; Watanabe *et al.*, 2018), changes in the retina (S-arrestin; Renninger *et al.*, 2011; Murthy *et al.*, 2014; Sullivan *et al.*, 2017), and increased metabolism of lipids (apolipoprotein B-100; Innerarity *et al.*, 1990; Millar *et al.*, 2005). Moreover, some changes were related to a lower resource investment towards some nonessential processes during smoltification, such as inhibition of sexual maturation (kinesin-like protein KIF20B; Wang, Xueying *et al.*, 2018), repression of some immune pathways (ras-GEF domain-containing family member 1B-A; Tam *et al.*, 2004; Epting *et al.*, 2007), and lower muscle performance (AMP deaminase; Fischer *et al.*, 2007) at the smolt stage.

On the other hand, due the overall lack of changes during smoltification of *pu.1, il1β, il4/13,* and *gata3* transcription in head kidney, haematopoiesis did not seem to be influenced by the smolt status of the fish. However, only a small set of genes were tested and others that are also related to haematopoiesis but were not tested might indeed change.

7.2. Desmoltification in rainbow trout

The desmoltification of rainbow trout started between May and June and was characterised by a marked decrease in seawater tolerance (decrease in NKA activity, and *nkaα1b* and *nkcc1a* transcription) and a recovery of freshwater tolerance (increase in *nkaα1a* transcription), as described in Atlantic salmon (McCormick *et al.*, 1997). Remarkably, *nkcc1a* transcription levels were lower in desmolted fish than in parr, suggesting that the osmotic stress of a seawater transfer would be more detrimental for fish after the smolt window than before. This was indeed shown by lower mortalities upon seawater transfer of parr steelhead trout in December than desmolts in June (Wagner, 1974b).

Transcription of *igf-I* and *ghr1* also decreased (Ágústsson *et al.*, 2001), which is likely related to a loss of hypo-osmoregulatory ability and, unlike previously described (Duston *et al.*, 1991; Høgåsen, 1998), of growth potential, as the SGR of desmolted fish was markedly reduced. Moreover, as utilization of energy reserves in liver decreases and their levels return to normal parr levels (Lundqvist and Eriksson, 1985; Li and Yamada, 1992), so did tissue reorganization, and this was reflected by a decrease in *ctsl.*

Finally, though this was affected by a seasonal increase in temperature, haematopoiesis, and especially *il4/13* transcription, decreased during desmoltification. Therefore, the immune capacity of desmolted fish, and specifically the production of B lymphocytes and activation of macrophages, would be compromised (Martinez *et al.*, 2009; Takizawa *et al.*, 2011; Sequeida *et al.*, 2017).

7.3. Seawater adaptation in rainbow trout

Seawater adaptation in rainbow trout was characterised by intermediate growth rate (more than parr yet less than smolt). The smolt status of the fish prior seawater transfer seemed to greatly affect variables like SGR or circulating IGF-I in seawater, being lower in fish that had gone through desmoltification. Transcription of tested growth-related genes did not vary after seawater transfer, as seen from *igf-I*, *ghr1, igfbp1b*, and *ctsl*, but this too might be dependent on the smolt status of the fish when transferred to seawater. However, circulating levels of IGFBP1b increased after seawater

transfer, seemingly contrary to chum salmon (Kaneko *et al.*, 2019), indicating negative endocrine control over growth during at least the first months at sea.

Interestingly, NKA activity levels during smoltification were higher (above 5 µmol ADP mg protein^{-1} hour^{-1} on average) than after more than 2 months in seawater (approximately 2.6 µmol ADP mg protein^{-1} hour^{-1} on average). This indicates that the hypo-osmoregulatory capacity achieved by the fish during smoltification was higher than what was is actually needed in seawater. However, it is relevant to point out that in this case fish were transferred to fjord water of 32‰ salinity. At full seawater (35‰ salinity), NKA activity might have been higher. Moreover, this decrease in NKA activity after seawater transfer is not consistent with previous literature in steelhead trout and other salmonids (Madsen and Naamansen, 1989; Uchida *et al.*, 1996; McCormick *et al.*, 2009). However, these studies only tested the short- to medium-term effects of seawater transfer (up to two weeks in seawater).

Regarding their plasma proteome, 11 DAPs were identified when comparing Smolt and Post-smolt pools. Proteins that were higher in Post-smolt were related to countermeasures against hyper--osmotic stress (serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C, ATP-sensitive inward rectifier potassium channel 12; D'Avanzo *et al.*, 2010). On the other hand, IGFBP2a, a growth-inhibiting protein (Duan *et al.*, 1999; Zhou *et al.*, 2008), was not expressed in Post-smolt. This would indicate that, regarding the effect of this protein, growth would be enhanced with respect to the smolt phase. Interestingly, neither the higher hypo-osmoregulatory capacity nor the higher growth capacity are backed by the NKA activity or growth, IGF-I abundance, or IGFBP1b abundance in post-smolts. These seemingly inconsistent measurements are likely indicative of a change in importance of the proteins regulating these processes in seawater.

7.4. Smolt markers

Although NKA activity has been shown to have relatively poor accuracy as a seawater-readiness biomarker (McGowan, 2018), no seawater survival tests were performed. Therefore, in this study the suitability of other biomarkers to assess seawater readiness had to be compared to NKA activity. Although given the limitations of NKA activity this approach might not be ideal, NKA activity is still the preferred seawater tolerance marker by both industry and scientific studies (Handeland *et al.*, 2013; Elsner and Shrimpton, 2018; McGowan, 2018). While NKA activity is able to provide a gross estimation of seawater tolerance, the transcription of related genes indicates the amount of energy allocated in seawater and freshwater tolerance, thus providing an estimation of the likeliness for fish to naturally

migrate (Healy *et al.*, 2018). Moreover, the transcription of NKA-related genes is more sensitive to transient changes in osmoregulation. However, the biological relevance of such changes is still unclear. Finally, NKA activity seems to be mainly driven by *nkaα1b*, while it does not reflect some of the changes in *nkcc1a* transcription (Nilsen *et al.*, 2007; Houde *et al.*, 2018). Therefore, NKA activity can be complemented by analysing *nkaα1a*, *nkaα1b* and *nkcc1a* transcription.

As suggested by Beckman (1999), genes and proteins from the somatotropic axis have potential as robust smolt biomarkers. Although the increase of the transcription of *igf-I* in liver took place approximately two weeks before the start of the smolt window, it followed a similar trend to that of *nkaα1b* or NKA activity. Therefore, this biomarker could be used to predict an NKA activity increase two weeks in advance. Moreover, *ghr1* peaks in transcription seemed to coincide with high seawater tolerance and low freshwater tolerance periods. Although it needs further testing, due to its clear and transitory increase, this could be a good biomarker to pinpoint the best seawater transfer window within the smolt window. However, further experimentation should corroborate if this information from gene transcription can also be obtained from the circulating IGF-I. Moreover, in order to measure transcription of genes in liver, animals need to be sacrificed, while blood plasma can be sampled non-lethally.

Plasma was shown to be an easily-accessible, highly informative biofluid (Anderson and Anderson, 2002; Jacobs *et al.*, 2005; Pernemalm and Lehtiö, 2014) that reflects changes that occur during smoltification through the study of its proteome. In this sense, a total of 965 proteins were identified and quantified in plasma across the parr, smolt and post-smolt life stages of rainbow trout. Ankyrin-2 was invariably the most abundant protein in all three life stages, while other proteins such as DNA primase large subunit, actin, serum albumin, apolipoproteins, haemoglobin subunits, hemopexin-like proteins and complement C3 were generally among the most abundant proteins. Importantly, 327 were only detected in EP thanks to the effects of a low-abundant protein enrichment strategy, thus greatly increasing proteome coverage. Moreover, a list of 17 proteins in plasma that significantly change during the smoltification process was obtained. Of these DAPs, the 7 that were detectable in WP are the most interesting as biomarkers, as they are present in higher abundance and therefore can be a measured more easily. In this sense, apolipoprotein B-100 might be the candidate with the most potential, as it was the second most abundant protein in the Smolt pool. However, being and important lipoprotein component, it is implicated in the transport of lipids in response to a wide variety of signals (Segrest *et al.*, 2001; Kreuter *et al.*, 2007; Klingenberg *et al.*, 2010) and its abundance might vary in response to processes not related to smoltification. Therefore, its suitability as smolt marker, as for the rest of potential biomarkers, needs further testing. Moreover, each of these candidate biomarkers needs to be validated by seawater survival tests.

Similarly, the plasma peptidome contains vast amounts of information that can be accessed with the right tools (Albalat *et al.*, 2013; Mansor *et al.*, 2013; Stalmach *et al.*, 2015). In the present study, 159 different peptides were detected in undiluted blood plasma from rainbow trout parr and smolts. The implementation of this information into a machine learning classification model, despite the small number of biological replicates (n = 8), was shown to be capable of classifying fish according to their smolt status with very high accuracy (90%). Therefore, MALDI-TOF MS analysis coupled with SVM data analysis has great potential as a biomarker test for smoltification. However, first the number of samples used for 'training' the model should be increased, and the samples used for 'testing' should come from different locations and sampled at different times. Furthermore, comparing fish that have successfully gone through the smoltification process and fish that did not from the same cohort would likely produce more accurate results, reducing the noise caused by comparing fish of different size and age. Moreover, as for the plasma proteome DAPS, the model needs to be further validated by seawater survival tests.

7.5. Spring smolt production

The two main environmental factors driving smoltification in salmonids are photoperiod and temperature (McCormick and Moriyama, 2000; McCormick *et al.*, 2002). To study their effects on the development of smoltification related traits (*i.e.* NKA activity, transcription of NKA-related genes) in rainbow trout, a trial testing several photoperiod regimes (*i.e.* APP, DPP, LL, SNP), and a second trial testing several photoperiod and temperature combinations (NT+LL, NT+SNP, HT+LL, HT+SNP), were performed on subsequent years (2016 and 2017 , respectively), at the same location, on 1+ fish of the same genetic background (AquaGen), and similar temporal spans.

In terms of photoperiod, the effects of its manipulation on rainbow trout proved relatively small when compared with the effects of photoperiod on other salmonids (Handeland and Stefansson, 2001). In this study only minor differences were observed due to treatment, or lack thereof, on NKA activity, transcription of osmoregulatory genes *nkaα1a*, *nkaα1b* and *nkcc1a*, growth, and transcription of growth-related genes *igf-I*, *igfbp1b*, *ghr*, and *ctsl*. In this sense, summer signals (APP, LL and SNP) produced very similar results, with APP inducing a longer smolt window and causing fish to have higher values of plasma IGF-I, which however was not backed up by differences in growth. Nonetheless, these differences were not sufficient to disregard the photoperiod that is currently being used in production systems, LL, as a suitable photoperiod choice. However, the effects of a winter signal (DPP) seemed to be detrimental when compared to summer signals in terms of growth, length of the smolt window, NKA activity, transcription of *igf-I*, and abundance of plasma IGF-I and growth.

For the next experiment, two summer signals, the one currently used in production setups, LL, and the one that has the most gradual increase in light hours, SNP, were combined with either NT or HT treatments to test the modulation effects that temperature has on photoperiod in terms of osmoregulation, growth and immune capacity (measured through transcription of *pu.1, il1β, il4/13a,* and *gata3*). Indeed, an intense modulation of the photoperiodic control of seasonal changes by temperature was shown, which was even able to negate the effects of photoperiod in cases like the transcription of *nkaα1a*, *pu.1* and *il4/13.* This modulation was proportional to temperature, decreasing as NT and HT drew progressively closer. On the other hand, *nkaα1b* was mainly driven by photoperiod. However, temperature did not affect the onset of smoltification, as also suggested for Atlantic salmon (McCormick *et al.*, 2002).

HT produced no clear advantages in terms of osmoregulation. However, it was able to significantly reduce *nkaα1a* which could make fish more likely to migrate to seawater (Sogard *et al.*, 2012). As per photoperiod, data suggests LL would be more suitable than SNP for both NT and HT scenarios in terms of the development of osmocompetence in seawater, with an optimal seawater transfer window in April, as seen from peaks in both *nkaα1b* and *nkcc1a* transcription during that month. Although a larger length and heavier weight were achieved and maintained during the whole freshwater phase by fish kept at HT, soon after TC this size advantage was progressively lost due to the compensatory growth that fish kept at NT experienced. In terms of transcription of haematopoiesis genes, HT resulted in lower transcription levels, especially for *pu.1* and *il4/13*, thus decreasing the proliferation of lymphoid cells and macrophages and potentially compromising the immune capacity of the fish (Pleguezuelos *et al.*, 2000; Iwasaki, H. and Akashi, 2007; Sequeida *et al.*, 2017). Since especially the adaptive immune response would be affected (*pu.1* and *il4/13*), the value of prophylactic measures would be reduced at HT while, at the same time, providing more suitable conditions for the proliferation of pathogens (Austin and Austin, 2012).

Overall, LL proved to be a suitable photoperiod regime for the induction of smoltification and having been shown to result in higher growth if maintained during long periods of time (Taylor *et al.*, 2005). Therefore, there are no grounds on which to disadvise current aquaculture practices in terms of photoperiod chosen. However, while there was variability among years, likely due to differences in natural temperature, the smolt window of fish kept at LL was reached before April in both cases, followed by a period of relatively high hypo-osmoregulatory competence until mid-May. Afterwards, the desmoltification process started. Therefore, the optimal seawater transfer window would be between April and May. Currently, after having been kept at LL, rainbow trout are transferred to seawater after reaching a desired minimum size, which normally occurs between April and June.

Results indicate that, by then, fish may have already desmolted, which is likely to have implications for GS occurrence.

7.6. Summer post-smolt production

Post-smolts were kept at LL to maximize growth during this phase. Growth rate seemed to be highly dependent on a timely seawater transfer. This was illustrated by the lower SGR-L of fish transferred after desmoltification in 2016 (below 0.2% day^{-1} on average), as opposed to a more timely seawater transfer in 2017 (above 0.25% day^{-1} on average). This difference in growth might have been related to IGF-I levels, which were higher in 2017 in both freshwater and seawater. Likely, high IGF-I levels prior seawater transfer are crucial towards growth (below 100 mg ml^{-1} in 2016 and above 175 mg ml^{-1} in 2017, on average), and they decrease during desmoltification, as seen for *igf-I* transcription. This also affected survival and occurrence of GS fish. While in 2016 the mortality rate was negligible (below 1%), 14% of fish were GS. On the other hand, in 2017 there were no GS fish. However, 6.74% of fish died. This indicates that occurrence of GS is reduced by a timely seawater transfer but those that become GS are more likely to die. This might be related to bigger differences between GS and FG and stronger competence (Gilmour *et al.*, 2005).

In terms of treatments, photoperiod in freshwater did not have strong effects on subsequent seawater performance, as suggested previously (Wagner, 1974a). However, lack of significant differences in growth between photoperiod treatments might be due to the seawater period not being long enough. In terms of temperature, while fish kept at HT were slightly bigger, these differences were likely to disappear had the seawater phase been prolonged. Overall, also in seawater, no grounds on which to disadvise current aquaculture practices were found, as LL seemed suitable for both NT and HT in both freshwater and seawater.

7.7. Growth-stunted phenotype characterization

As mentioned above, GS fish development appeared to be related to the smolt status of the fish on seawater transfer, with desmoltification increasing their occurrence. In this sense, their reduced growth was related to lower circulating IGF-I abundance and transcription in liver, which have been linked to fish growth in several species (Taylor *et al.*, 2005; Wood *et al.*, 2005; Beckman, 2011). On top of being less abundant, the action of this hormone is likely further inhibited by a higher abundance of circulating IGFBP1b (Kaneko *et al.*, 2019), which was significantly and inversely correlated with growth.

While the causes of GS development remain unconfirmed, a combination of proteomic and lipidomic approaches successfully provided a characterization of the phenotype and identified factors that are associated to GS fish. First of all, characteristics of socially subordinate GS freshwater rainbow trout were identified, which were related to higher carbohydrate (Gilmour *et al.*, 2012) and hepatic glucose metabolism (Gilmour *et al.*, 2012), and to changes in lipid metabolism suggesting that dominant fish rely more on TG for maintenance while subordinate fish rely on free fatty acids (Kostyniuk *et al.*, 2018). Rainbow trout are aggressive towards their conspecifics and subordination of GS would be consistent with their higher incidence of fin damage (Moutou *et al.*, 1998; North *et al.*, 2006; Hoyle *et al.*, 2007) and their elevated HSP90 levels (Currie *et al.*, 2009). The causes of this subordination might be related to GS fish being weaker due to a suboptimal seawater adaptation but this hypothesis needs further testing.

A general lack of differences between GS and FG on liver total lipid content and on energy reserve lipid species abundance (*e.g.* DG, TG), coupled with the low mortality during the seawater stage (less than 1%) and the dissimilarity of the liver proteome with that of starved rainbow trout (Martin *et al.*, 2001), suggested that starvation was not an important factor for GS development. Instead, results suggested processes related to the correct functionality of the liver. In this sense, hepatic lipid composition differences were mainly on phospholipids, indicating differences in membrane structure and dietary lipid transport (Tocher, 2003; Thiam *et al.*, 2013). More PC in FG is consistent with their higher growth (Kanazawa, 1993; Kasper and Brown, 2003; Azarm *et al.*, 2013), while differences in CL (higher in GS) could indicate higher mitochondrial activity in the livers of GS (Paradies *et al.*, 2011; Horvath and Daum, 2013). Moreover, hepatocyte cellular membranes of GS fish might be compromised by oxidative stress. This was suggested by higher PDI and lower catalase abundance in GS. While PDI is involved in hydrogen peroxide generation (Laurindo *et al.*, 2012), catalase is a crucial protein for protection against oxidative stress (Salvi *et al.*, 2007). Furthermore, differences in CL and ceramide composition are consistent with this hypothesis (Hoch, 1992; Andrieu-Abadie *et al.*, 2001; Cutler and Mattson, 2001).

Therefore, two possible lines of action can be adopted to try to reduce GS incidence. To impair the establishment of hierarchies and social dominance, fish should be regularly graded by size, thus reducing the stress of GS fish and increasing their welfare conditions (Ellis *et al.*, 2002; Martins *et al.*, 2006; Sneddon, 2006). Secondly, supplementation of lacking phospholipids into their diet might mitigate their stunted growth (Kanazawa, 1993; Kasper and Brown, 2003; Azarm *et al.*, 2013).

7.8. Future perspectives

Several future lines of research are suggested by the present work. First and foremost, potential biomarkers need validation. In order to do this, blood samples should be taken from tagged fish during their parr and smolt stages. The performance of these fish in seawater would then be used to validate potential biomarkers in blood (*e.g.* apolipoprotein B-100, MALDI-TOF SVM model, circulating IGF-I). Moreover, experimentation involving seawater transfer at different times, when different treatments present significant differences for the transcription of *nkaα1a*, *nkaα1b* and *nkcc1a*, followed by short and long term reassessment of the performance of the fish in seawater are needed to validate the relevance of each of this these NKA related genes as seawater-readiness markers. Moreover, this would also allow us to test the effects of salinity on rainbow trout smoltification. Secondly, a very clear effect of temperature on some genes that regulate haematopoiesis (especially *pu.1* and *il4/13*) was shown. Follow-up studies should focus on the demonstration of these effects using actual cell counts, performed by flow cytometry, coupled with immune challenge of fish held at different temperatures. This would have direct implications for aquaculture, likely for more fish species than just rainbow trout, and especially in warm climates. Regarding the plasma proteome, while a list of proteins related to the smoltification and seawater adaptation processes was provided in Chapter 4 and their possible biological implication was discussed, further work targeting each of these proteins specifically should be carried out to fully understand their role in such processes. Regarding GS fish, liver histology and oxidative stress assays should be carried out to confirm results in this thesis. Finally, formulation and elaboration of feeds that incorporate phospholipids needed by GS fish is required. Once this is accomplished, the next step will be to confirm whether grading fish by size and feeding them this new diet is an effective strategy to reduce GS occurrence.

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