

**Gill leptin mRNA expression analysis  
during smoltification of Arctic charr  
(*Salvelinus alpinus*)**

By

Shou Wang

FSK-3910 Master thesis in International Fisheries Management (30 credits)



Institute of Aquatic Biology (IAB)  
Norwegian College of Fishery Science  
University of Tromsø  
Norway  
May 2009

## ACKNOWLEDGMENTS

I would like to deeply thank the following people who, during the several months of my thesis research, provided me with helpful assistance. Without their suggestions and encouragement, my thesis would likely not have matured within the limited time. Firstly, I would especially like to thank my supervisor, Even H. Jørgensen for his willingness to accept me as his student and extremely patient guidance during the study period. I have developed tremendous respect of his knowledge and responsibility and I am grateful to work with him on this project. He introduced me into the world of fish physiology research and showed me with the attitude of being a good scientist. I would like to thank Jo J. Aarseth and Eirik Frøiland for their valuable guidance and advice during laboratory work. Thanks are also due to Bjørn Erik Bye for assisting me during laboratory work. I am also grateful to A. Christine Brown for helping me with grammar correction on my thesis. The encouragement and suggestions from my colleague Zuobing Zhang are gratefully acknowledged. I am fortunate to have worked with such a nice and friendly group of people and am looking forward to the challenges we face in further study of this subject.

## SUMMARY

In teleost fish, gill plays an important role of controlling ion transport mechanisms for the maintenance of ion- and osmoregulatory homeostasis in both fresh water and seawater. Leptin mRNA expression has been found in several fish species, and was also found in gills of Arctic charr (*Salvelinus alpinus*) in this study. However, no study has been reported on the function of leptin in the gills of fish. The question arise f Leptin may have some function related to ion- and osmoregulation in fish gills. In this study, Arctic charr were sampled during their smolting period in freshwater. Seawater tolerance, as well as gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity of the fish increased from winter to spring in these fish, showing that they smoltified. There was no change in gill leptin mRNA expression during and after smolting in the fish acclimated to freshwater and hence, the results gave no evidence for a paracrine role of leptin in the regulation of the adaptational changes in gill during smolting. It can not be excluded, however, that leptin may be involved in the changes taking place in the gill in association with seawater exposure, and future studies must include sampling of gill tissue, and analyses of leptin mRNA expression after seawater transference.

Key words: Leptin, Arctic charr, smoltification,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, gill

## CONTENTS

	Page No.
1. Introduction	1-4
2. Material and Methods	5-13
2.1 Experiment design	5-6
2.2 Plasma osmolality and gill Na <sup>+</sup> , K <sup>+</sup> -ATPase activity analyses	6
2.3 Gill mRNA level analyses for leptin expression	6-13
2.3.1 Total RNA extraction	6-7
2.3.2 DNase I treatment to remove genomic DNA	7-8
2.3.3 Reverse transcription	8-9
2.3.4 Qualitative analyses of leptin expression	9-11
2.3.5 Quantitative analyses of leptin expression	11-13
2.4 Statistical analysis	13
3. Results	14-17
3.1 Indices of smoltification	14-16
3.2 Quantitative PCR (q-PCR)	16-17
4. Discussion	18-23
4.1 Smolting	18-20
4.2 Leptin	20-22
4.3 Importance to fisheries management	22-23
5. Conclusion	23
6. Reference	24-28
Appendix	29-30

## 1. INTRODUCTION

A substantial proportion of teleost fishes are either hyperosmotic (brackish and freshwater species) or hyposmotic (marine species) to the aquatic environment in which they live. Salmonid fish (belonging to the family *Salmonidae*) are diadromous species which can migrate between freshwater and marine environments at different stages of the life history, depending on their genetic constitution (e.g. species) and environmental factors. They spawn, and grow up in freshwater, after which they undertake one or several feeding migrations to the sea, and this life strategy is termed anadromy (Rounsefell, 1958). Before seaward migration, most anadromous salmonids undergo a series of physiological, biochemical and behavioural changes that are pre-adaptive for marine residency. The transformation, from freshwater adapted parr to a seawater adapted smolt, is termed parr-smolt transformation, smoltification or smolting (Hoar, 1988; Beouf, 1993). Smoltification is a dynamic phenomenon, corresponding to information deeply inscribed in the genome, expressing itself at a specific time in the life of the fish under both internal control (nervous and endocrinological), and external synchronization with ecological factors (mainly photoperiod and temperature: Hoar, 1976, 1988; Boeuf, 1993).

The Arctic charr (*Salvelinus alpinus*) is the world's northernmost distributed freshwater fish species. They have a circumpolar distribution and the anadromous life history strategy in this species is restricted to oligotrophic northern lakes with access to the sea (Johnson, 1980). The anadromous Arctic charr have a seasonal migration rhythm which involves a short (1-2 months) feeding residency in coastal seawater every summer and residency in freshwater for the rest of the year (Johnson, 1980; Klemetsen *et al.*, 2003).

The gill is the main external organ for regulating ion balance in teleost fishes. Many studies have been devoted to gill physiology during smoltification of salmonids. Several reviews present the role of the gill in osmoregulation, this organ becoming of

the highest importance in seawater (Payan *et al.*, 1984; McCormick *et al.*, 1989; Avella and Bornancin, 1990). The gill epithelium is the site of active sodium and chloride uptake in freshwater fishes, and active excretion of these ions in marine fishes (Evans *et al.*, 1999).  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (known as the sodium pump) operates at the basolateral membrane of gill chloride cells to transport sodium and chloride into the fish (in fresh water) or out of the fish (in seawater). ATP is energizing the pump, which drives the formation of sodium and potassium gradients (Karnaky *et al.*, 1976). In the anadromous Arctic charr, studies have demonstrated that wild, downstream migrating fish display increased hypoosmoregulatory ability (Halvorsen *et al.*, 1993; Nilssen *et al.*, 1997; Gulseth *et al.*, 2001) and higher gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity than freshwater-resident Arctic charr (Damsgård, 1991; Arnesen *et al.*, 1995). It has also been shown that, during seawater acclimation of anadromous Arctic charr, increased hypoosmoregulatory ability is mainly attributed to increased gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity (eight-fold), rather than increased gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase protein levels (three-fold) (Bystriansky and Ballantyne, 2006). Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity may be regulated by multiple mechanisms and not determined only by enzyme number. It was proposed by Towle *et al.* (1977) that  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity is also modulated post-translationally, potentially through modifications of the properties of the membrane that surrounds the enzyme. The activity of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase has been shown to be sensitive to the lipid composition of its surrounding membrane environment (Ottolenghi, 1975; Stekhoven and Bonting, 1981) and correlated to several physical membrane properties such as phospholipid composition (Vemuri and Philipson, 1989) and fatty acyl chain length (Marcus *et al.*, 1986).  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity has also been reported to correlate with membrane cholesterol content in many tissues, including rainbow trout kidney and intestine (Crockett and Hazel, 1997). Modulation of membrane lipids may be responsible for the rapid rise (after 30 min) of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in the gill of killifish, *Fundulus heteroclitus* exposed to high salinity (Towle, 1981). Pre-adaptive increase in hepatocyte fatty acyl desaturation/elongation activities were found in freshwater during smoltification in Atlantic salmon (Tocher *et al.*, 2000). But apparent lack of correlation was found

between gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and gill basolateral membrane lipid composition in Arctic charr acclimated to freshwater (Bystriansky and Ballantyne, 2006). Environmental salinity may also alter gill lipid composition, and hence lead to a modulation of gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity (Bystriansky and Ballantyne, 2006). However, the influence of changing membrane composition of fish gills on ion regulatory mechanisms has not been adequately studied, and very little, if any, knowledge exists on the (endocrine) mechanisms regulating membrane composition.

Leptin is a member of the class-1 helical cytokine family produced primarily by adipose tissue in mammals (Zhang *et al.*, 1994). Its major role in regulating food intake, energy homeostasis, and reproduction in mammals has now been comprehensively studied and confirmed (Friedman, 2002). The leptin cDNA coding in fish was first identified and reported in the pufferfish, *Takifugu rubripes* (Kurokawa *et al.*, 2005). Later, the gene encoding for leptin has been cloned and reported for two salmonid species: the rainbow trout, *Oncorhynchus mykiss* (Murashita *et al.*, 2008) and Arctic charr (Frøiland *et al.*, 2009). Recent studies have shown that the liver, rather than adipose tissue, is the major leptin-expressing tissue in the common carp, *Cyprinus carpio* (Huising *et al.*, 2006), rainbow trout (Murashita *et al.*, 2008) and Arctic charr (Frøiland *et al.*, 2009). However, leptin gene expression has also been found in many other tissues including the gill in Japanese medaka, *Oryzias latipes*, (Kurokawa and Murashita, 2009). Amino acid sequences of human leptin and pufferfish leptin share only 13.2% identity, but the gene arrangement around the leptin gene and the three-dimensional (3D) structural protein model are well conserved between human and pufferfish leptin (Kurokawa and Murashita, 2009). In a recent study it was shown that recombinant trout leptin exerted a strong suppression of food intake in rainbow trout (Murashita *et al.*, 2008), showing that the role of leptin in regulating appetite and energy homeostasis is highly conserved in vertebrates. In addition to the role of leptin in regulating appetite and energy homeostasis, it has been shown that leptin stimulates cell proliferation and differentiation in a wide range of tissues in mammals (Gat-Yablonski and Philip, 2008) and participates in the

regulation of fat metabolism (Friedman, 2002). The presence of the leptin gene in fish gills means that leptin has a function in regulatory processes in the gill. Taking into consideration the substantial changes occurring in the gill during smoltification (i.e. membrane composition and chloride cell differentiation and proliferation), it is tempting to imagine a role of leptin in these processes.

On this background the present study was undertaken to investigate 1) if the leptin gene is present in the gill of Arctic charr, and 2) if there is any change in gill leptin mRNA expression during the smoltification process which correlates with parameters such as time, length, condition factor, plasma osmolality, and gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity. To do so, a smoltification experiment was set up with anadromous Arctic charr, which were examined for smolting indices (gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and hypoosmoregulatory ability). Along with the sampling of gill filaments for  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity analyses, filaments were also taken for leptin mRNA analyses. The experiment also included a third objective, namely to train myself in classical molecular techniques involved in quantifying specific gene expression.



## 2. MATERIALS AND METHODS

### 2.1 Experiment design

The experiment was performed with 2-year-old hatchery-reared offspring of anadromous Arctic charr originally caught in Lake Vårflusjøen, Svalbard (79°N) in 1990. Before and during the experiment, the fish had been held under ambient water temperature and natural light conditions (transparent roof, 69 °N). Fish were fed commercial dry feed (Skretting, Stavanger, Norway) in excess. In January 2008, a total of 209 fish were sorted out from the stock tank and transferred to a 3000 litre, cylindrical tank with running freshwater, in which they were held throughout the experiment period. Water supply was continuously adjusted to maintain oxygen saturation above 90%.

At seven sampling dates (February 20<sup>th</sup>, April 18<sup>th</sup>, May 9<sup>th</sup>, June 6<sup>th</sup> and 19<sup>th</sup>, July 3<sup>rd</sup> and 17<sup>th</sup>), 10 fish were taken out from the cylindrical tank by a dip-net and killed with an overdose (120 ppm) of benzocaine. Small pieces of gill filaments were then sampled from the second gill arch at the left side of the fish. The gill filaments were kept in ice cold SEI-buffer (0.3 M sucrose, 0.02 M Na<sub>2</sub>-EDTA, and 0.1 M imidazole) and frozen at -80 °C for analyses of gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity. Thereafter the whole gill tissue at the right side of the fish was excised, wrapped in aluminum foil, frozen in liquid nitrogen, and stored at -80 °C for later analyses of gill leptin mRNA expression.

Immediately following the freshwater sampling, a new sub-sample of 10 fish were taken out by a dip-net from the 3000 litre cylindrical tank and transferred to a 300 litre tank filled with running, full-strength (34 ‰) seawater at 6 °C. They were held in the tank, without being fed, for 48 hours, after which they were removed by a dip-net and killed in an overdose (120 ppm) of benzocaine. Blood was sampled from the caudal vein with lithium heparinized (30 USP units) vacutainers. The blood samples were held on ice until centrifugation (6000 xg, 10 min). Plasma were separated and stored

at -20 °C until analyses for osmolality. Fork length ( $\pm 0.1$  cm) and body weight ( $\pm 0.5$  g) of all fish sampled were recorded at the time of sampling.

## 2.2 Plasma osmolality and gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity analyses

Plasma samples were thawed on ice, vortexed for thirty seconds and analyzed for osmolality with a Fiske One-Ten Osmometer (Fiske Associates, MA, USA). Gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity was analyzed by a standard microassay procedure (McCormick, 1993). Both these analyses were performed by my colleague Bjørn Erik Bye.

## 2.3 Gill mRNA level analyses for leptin expression

Both qualitative and quantitative Polymerase Chain Reaction (PCR) analyses of leptin mRNA expression levels were performed in the experiment.

### 2.3.1 Total RNA extraction

Total RNA for analysing leptin mRNA level was extracted using TRIzol Reagent® (Invitrogen, USA) from a total number of 70 samples (10 fish for each sampling, 7 sampling dates). For each sample, the whole gill tissue wrapped in aluminum foil was taken out from -80 °C freezer and thawed on ice. Approximately 50 mg of gill filament was cut out using sterile blades on a 15 cm Petri dish and added to 1 ml trizol. Blades and petri dishes were changed between samples. To minimize RNA degradation, only 4 samples were processed at a time and all procedures were performed as quickly as possible. The gill filaments in trizol were sonicated on ice for a few pulses until no large pieces of tissue were observed. The sonicator was washed for 10 minutes in 1M NaOH, 2 minutes in 100% ethanol, 1 minute in 1mM NaOH, 1 minute in Milli-Q water and 1 minute in a second Milli-Q water solution sequentially between samples. For each sample, after sonication, the mixture was transferred to an autoclaved eppendorf tube. 200  $\mu$ l chloroform (CHCl<sub>3</sub>) was then added to the eppendorf tube. After vortexing for 30 seconds, samples were centrifuged for 15 minutes at 13,000 rpm at 4 °C. The transparent upper aqueous phase (~300 $\mu$ l) containing total RNA was transferred to a new eppendorf tube. 500  $\mu$ l of isopropanol

was added to the RNA phase. After vortexing, the mixture was centrifuged for 20 minutes at 13,000 rpm at 4 °C. The isopropanol phase was removed carefully, leaving the RNA pellet at the bottom of the tube. The RNA pellet was washed in 1000 µl of 75 % ethanol. After vortexing, the mixture was centrifuged for 10 minutes at 13,000 rpm at 4 °C. The ethanol phase was then removed. The pellet was dried in air at room temperature for 5 minutes. 30 µl Ultra Pure Water was added to the tube and incubated for 5 minutes at 65 °C to dissolve the RNA pellet. More water and longer incubation was needed if the pellet was not completely dissolved. Total RNA was quantified using a Nanodrop 1100 spectrophotometer. RNA integrity was checked by 1% agarose gel electrophoresis as shown in Figure 1. Total RNA extracted from all samples had high purity ( $A_{260}:A_{280} > 1.8$ ) and integrity (5S, 18S and 28S bands can be clearly observed instead of smear).

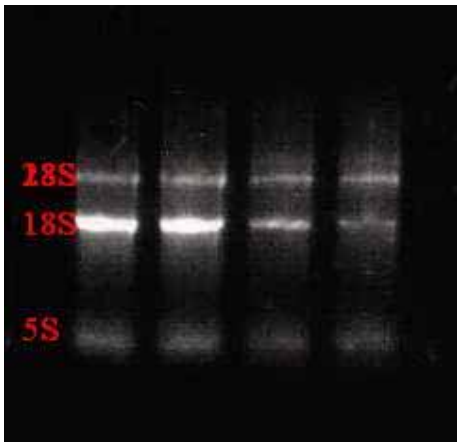


Figure 1. 1% agarose gel for total RNA quality check

Three rRNA bands (5S, 18S and 28S) can be seen (Figure 1), showing there was not much degradation during the extraction procedure and mRNA quality was good for later reverse transcription.

### 2.3.2 DNase I treatment to remove genomic DNA

All 70 samples were treated with DNase I (Invitrogen, USA) using the following steps. 20 U DNase I and 5 µg of total RNA were added to an eppendorf tube. Water

was added to make up to the total reaction volume to 10 $\mu$ l. After incubation for one hour at 37 °C and then 10 minutes at 65 °C, 30 out of 70 samples were randomly chosen to perform two PCR tests using the DNase I treated total RNA as templates to check if all genomic DNA were removed. Two primer pairs were designed to bind to any DNA templates in the samples. If genomic DNA were not removed completely by DNase I, there will be some PCR product amplified. The primer pair used in PCR1 was 5'-ACTACGACCGCCTGTACAGCCTGAGGAACA-3' (forward) and 5'-CATCACTAGGTTTCAGTTTGGTCCTTTGCT-3' (reverse). In PCR2, following primer pair was used: 5'-CAGTGCCCAACTTCTTTGTTGGCTCCCTGC-3' (forward) and 5'-TAACCCGATGTTTCACTTGAATCACCAGCA-3' (reverse). All primer pairs were purchased from Operon (Eurofins MWG Operon, USA). The PCR program for both PCR tests was 95° C 1min, [94° C 30s, 60°C 1min, 68°C 30s]X3, [94°C 20s, 55°C 1min, 68° C 30s]X3, [94° C 20s, 50° C 1min, 68° C 30s]X3, [94° C 20s, 42°C 1min, 68°C 30s]X3, [94°C 20s, 37°C 1min, 68°C 30s]X30, 68°C 7min. PCR protocols were shown in Table I in Appendix.

No bands were observed in 1% agarose gel electrophoresis performed after DNase I treatment, which indicated that genomic DNA was removed. Only RNA templates were in the DNase I treated samples for reverse transcription of cDNA.

### 2.3.3 Reverse transcription

Reverse transcription is a process to synthesize cDNA from mRNA. A short double-stranded sequence is needed at the 3' end of the mRNA which acts as a start point for the reverse transcriptase (an RNA-dependent DNA polymerase). This is provided by the poly (A) tail found at the 3' end of most eukaryotic mRNA to which a short complementary synthetic oligo-nucleotide (oligo dT primer) is hybridized. 1 $\mu$ g of DNase I treated total RNA was mixed with 1  $\mu$ l oligo dT primer (100  $\mu$ M) (Thermo scientific, ABgene, UK) to synthesis the first strand cDNA for each sample. Water was added to make up total volume to 11  $\mu$ l. After incubation for 5 minutes at 65 °C, the mixture was incubated on ice for 4 minutes. 1  $\mu$ l dNTP (10 mM)(Promega,

Madison, USA), 4  $\mu$ l 5X buffer, 2  $\mu$ l DTT (20mM), 0.5  $\mu$ l RTase (Takara, Japan), 0.5  $\mu$ l RNAsin (Promega, Madison, USA) and 1  $\mu$ l H<sub>2</sub>O were added to each reaction tube. The reaction tube was incubated for 1 hour and 30 minutes at 42 °C and then for 15 minutes at 70 °C. cDNA for all 70 samples were synthesized.

#### 2.3.4 Qualitative analyses of leptin expression

Assessment of cDNA quality was performed by amplifying partial  $\beta$ -actin gene using PCR.  $\beta$ -actin is often used as a housekeeping gene to check the quality of cDNA for specific gene expression. The sequence of the primer pair to amplify the  $\beta$ -actin fragment (ca. 100bp) was 5'-AGAGCTACGAGCTGCCTGAC-3' (forward) and 5'-GCAAGACTCCATACCGAGGA-3' (reverse). The primer pair was purchased from Operon (Eurofins MWG Operon, USA). The PCR program to amplify the  $\beta$ -actin fragment was 95 °C 1min, [95 °C 15sec, 60 °C 15sec, 72 °C 30sec]X30, 72 °C 5min. PCR protocol can be found in Table II in Appendix.

1% agarose gel electrophoresis was performed with each sample to check the PCR products.  $\beta$ -actin fragments were amplified using cDNA from all 70 samples indicating the high quality of cDNA ready for leptin gene expression. Samples with more than one band or which had bands at wrong positions were noted down and reverse transcription was performed again until all samples showed the  $\beta$ -actin band.

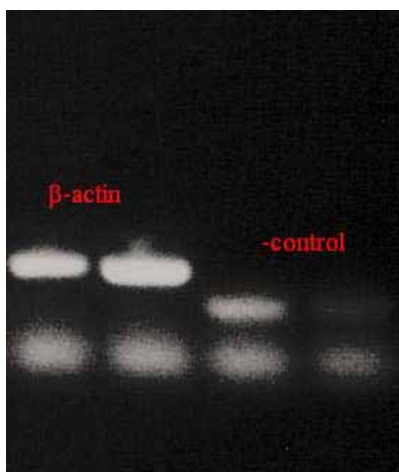


Figure 2.  $\beta$ -actin PCR products in 1% agarose gel

The two left lanes (Figure 2) were loaded with cDNA samples as templates, a strong band around 100 bp ( $\beta$ -actin) can be observed.  $\Phi$ 174 ladder was used to identify the size of  $\beta$ -actin band (not shown here). The two right lanes (negative control) were loaded with water instead of sample as templates. Lower bands in all four lanes could be the primer bands with the sizes around 20bp. One of the negative controls showing two bands might be due to non-specific self-binding of primers (primer dimer).  $\beta$ -actin fragments amplified by PCR in all cDNA samples showed that cDNA quality was good. Since we didn't know if the leptin gene was expressed in the gill sample of Arctic charr or not, cDNA quality check by amplifying  $\beta$ -actin fragment was important to avoid an incorrect conclusion caused by experimental error in previous steps.

#### Leptin gene amplification by PCR

PCR protocols for leptin gene amplification are shown in Table III in Appendix. The leptin primer pair (same as one used in q-PCR later) is listed in Table 1. Touch down PCR program for leptin was 95 °C 1min, [95 °C 15sec, \*68 °C 30sec, 68°C 30sec] X10(\* represents annealing temperature decreased 1 °C per PCR cycle from 68 °C to 58 °C in 10 cycles), [95 °C 15sec, 65 °C 30sec, 68°C 30sec]X30, 68 °C 10min.

The leptin cDNA (474bp) had been cloned and sequenced in our lab. The two leptin primers were used here to amplify a partial leptin gene length of 146bp. The sequence of the partial gene is (primer sequences are underlined):  
 5'-TGTCGCGCTGCCCAGGTCGCCAGCAGAAACAGACAGGAGAGGGTGGGT  
 TGGAGGAGGCTCTGAAGGACAGTGTCAGGAAGTTTGGTCTGAGTGTGTGT  
 CCAGTGGCACTAAACAGACTCAAGGGCTACCTCGATCGGCTACTGCTG-3'

All cDNA of the 70 samples from all sampling dates were randomly picked to perform the leptin gene amplification PCR. 1% agarose gel electrophoresis was performed with each sample.  $\Phi$ 174 ladder was loaded to indicate the size of PCR

products. Negative controls were added to each PCR test using ultra pure water instead of cDNA as templates.

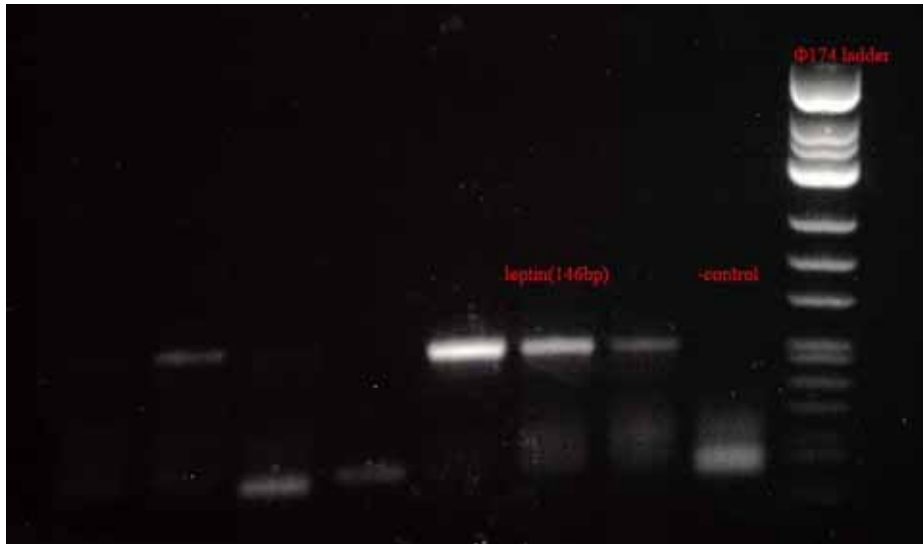


Figure 3. 1% agarose gel electrophoresis to detect leptin PCR product.

One sample from each sampling date was grouped together for one PCR amplification (10 groups). PCR products of 7 samples plus 1 negative control (water as template) are shown in Figure 3. Bands around 146bp were leptin. Strong leptin bands represented strong leptin mRNA expression in the gills for specific samples.

### 2.3.5 Quantitative analyses of leptin expression

Gill leptin mRNA levels were measured by quantitative-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA). 18S, instead of  $\beta$ -actin, was used as an internal control (housekeeping gene) and the average of leptin mRNA level from 10 samples in the first sampling date (Feb 20<sup>th</sup>) as calibrator. The leptin mRNA level measured is the relative quantification of leptin gene expression. Calculations were performed based on the instruction manual provided by Applied Biosystems, USA.

### Standard curve

One sample showing high leptin expression in qualitative analyses was chosen for leptin gene amplification using touch down PCR (the same touch down PCR program as in the qualitative analyses). PCR products were serial diluted 10 times (10X each time) to generate standard curves for leptin and 18S. 7 diluted PCR products were selected and used to generate one standard curve for each q-PCR plate. Primers used in PCR are shown in Table 1.

Table 1. Leptin and 18S primers and sequences used in touch down PCR (standard curve) and quantitative PCR (q-PCR).

Primer	Sequence	Probes
Leptin forward	5'- TGTCGCGCTGCCAGGTCGCCAGCA-3'	SYBR Green
Leptin reverse	5'- CAGCAGTAGCCGATCGAGGTAGCCC-3'	SYBR Green
18S forward	5'- TGTGCCGCTAGAGGTGAAATT-3'	SYBR Green
18S reverse	5'- CGAACCTCCGACTTTCGTTCT-3'	SYBR Green

\*All primers listed were purchased from Operon (Eurofins MWG Operon, USA).

### Quantitative PCR setting

cDNA for each sample was diluted 10X for leptin and 10,000X for 18S and used as the templates for q-PCR in duplicates. 70 samples were divided into two plates for leptin and 18S (4 plates in total). 5 samples at the same sampling dates were placed in one q-PCR plate to minimize the error caused by actual q-PCR efficiencies among plates. In each plate, a standard curve was generated. The primers and sequences are shown in Table 7. Q-PCR program used was: 50 °C 2min, 95 °C 10min, [95 °C 30sec, 60 °C 30sec, 72 °C 30sec]X40. The detector read the fluorescence in every cycle after 30sec at 60 °C. A dissolve program, as follows, was added to observe the melting temperature: 95 °C 15sec, 60 °C 20sec, 95 °C 15sec. This is to reveal if the right



product was amplified. Q-PCR protocols are shown in table IV in Appendix.

## 2.4 Statistical analysis

All data were presented as mean  $\pm$  standard error of means (s.e.m). Condition factor was calculated using the formula  $[K = W \cdot L^{-3} \cdot 1000]$ , where W was fish mass (g) and L was fork length (cm). Statistical analysis was performed using Statistica 6.1 (Statsoft, Inc. Tulsa, OK, USA). A parametric test (ANOVA, general linear model (GLM)) was used to reveal possible effects of time on gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in the fish that were sampled in freshwater and on plasma osmolality in the fish that were sacrificed after the 48 h seawater test. In the latter test, fork length was included as a continuous predictor of plasma osmolality. ANOVA was also used to reveal possible effects of time on gill leptin mRNA expression measured by q-PCR in all fish sampled. A post-hoc, Tukey test was used to reveal where differences occurred. Results were considered to be significant when the probability level was lower than or equal to 0.05.

### 3. RESULTS

#### 3.1 Indices of smoltification

Body mass, fork length and condition factors of Arctic charr sampled in freshwater at the various sampling dates are presented in Table 9. Plasma osmolalities after the seawater tests and gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity data of the fish sampled in freshwater are plotted in Figure 4.

Table 9. Mean (s.e.m.) body mass (g), fork length (cm) and condition factor (CF) of the Arctic charr sampled at the various dates in freshwater.

Sampling dates	n	Body mass (g)	Fork length (cm)	CF
February 20 <sup>th</sup>	10	192 (11.6)	26.8 (0.5)	0.99 (0.03)
April 18 <sup>th</sup>	10	166 (23.1)	25.5 (0.7)	0.95 (0.06)
May 9 <sup>th</sup>	10	169 (16.3)	25.3 (0.6)	1.02 (0.05)
June 6 <sup>th</sup>	10	186 (21.5)	25.7 (0.90)	1.07 (0.06)
June 19 <sup>th</sup>	10	238 (33.9)	27.1 (0.94)	1.12 (0.08)
July 3 <sup>rd</sup>	10	230 (37.8)	26.1 (1.29)	1.17 (0.06)
July 17 <sup>th</sup>	10	294 (24.0)	29.1 (0.76)	1.17 (0.03)

The ANOVA test showed that there was a significant effect of time ( $F = 2.44$ ;  $p < 0.05$ ) on condition factor. However, the Tukey post-hoc test could not reveal any pairwise, significant differences between dates in condition factor.

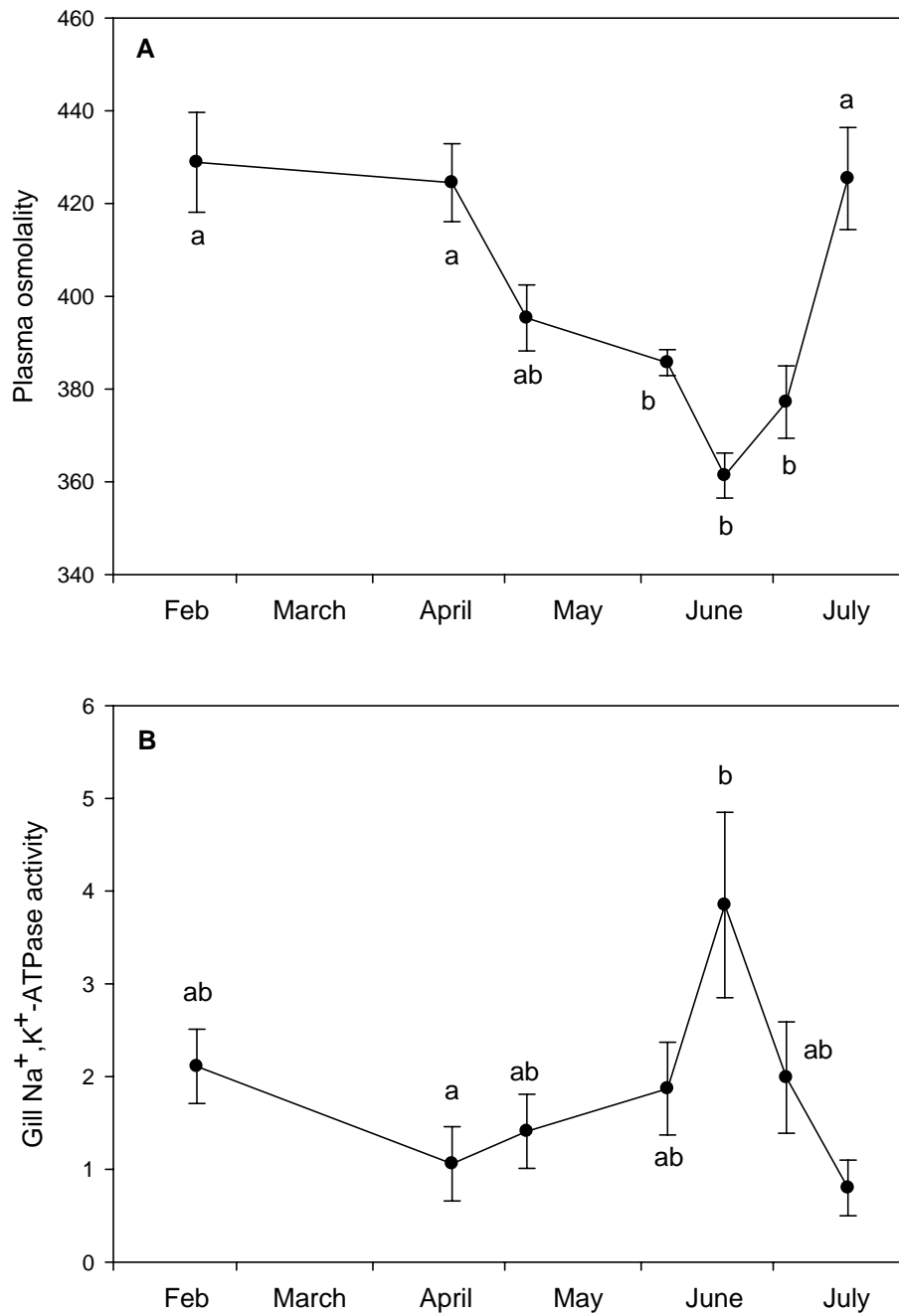


Figure 4. (A) Seasonal change in plasma osmolality (mOsmol) of Arctic charr after 48 h seawater tests and (B) gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities ( $\mu\text{mol Pi mg prot.}^{-1} \text{h}^{-1}$ ) of Arctic charr sampled in freshwater. Values represent means  $\pm$  s.e.m. and different letters denote dates that are significantly different.

Plasma osmolalities after the seawater (SW) tests were significantly effected by time ( $F = 8.59$ ;  $p < 0.001$ ) but not by fork length ( $F = 0.28$ ;  $p > 0.05$ ). Plasma osmolalities were high after the seawater tests in winter, but decreased gradually from April until a nadir in June, after which there was an abrupt increase again until July when the level was similar to those observed during winter. Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was significantly affected by time ( $F = 2.95$ ;  $p < 0.05$ ) but not fork length ( $F=0.13$ ;  $p > 0.05$ ). A post-hoc Tukey test on plasma osmolalities showed that there were significant differences between sampling dates: between April 18<sup>th</sup> and 3 sampling dates (June 6<sup>th</sup>, June 19<sup>th</sup>, July 3<sup>rd</sup>), also between the 3 sampling dates and July 17<sup>th</sup>. Activity was low in April after which there was a 3-fold increase until mid-June and an abrupt decrease until mid-July when the activity was similar to that in April.

### 3.2 Quantitative PCR (q-PCR)

Table 10. Standard curve data from each plate, 18S representing the house keeping gene and leptin the leptin gene.

	Slope	Intercept	R <sup>2</sup>	Threshold
18S plate 1	-3.250127	38.825527	0.996142	0.02928645
18S plate 2	-3.529757	41.250614	0.993853	0.1420303
leptin plate 1	-3.255512	37.134991	0.998285	0.1191765
leptin plate 2	-3.181794	36.726902	0.994460	0.1339628

Thresholds were set manually at an early exponential phase for all samples. A linear standard curve was fitted for each plate with R<sup>2</sup> around 0.995. We conclude that q-PCR efficiency for all samples in each plate should be almost the same from the standard curves. Relative leptin mRNA levels could then be measured by calculation from the q-PCR data. These data are shown in Figure 5.

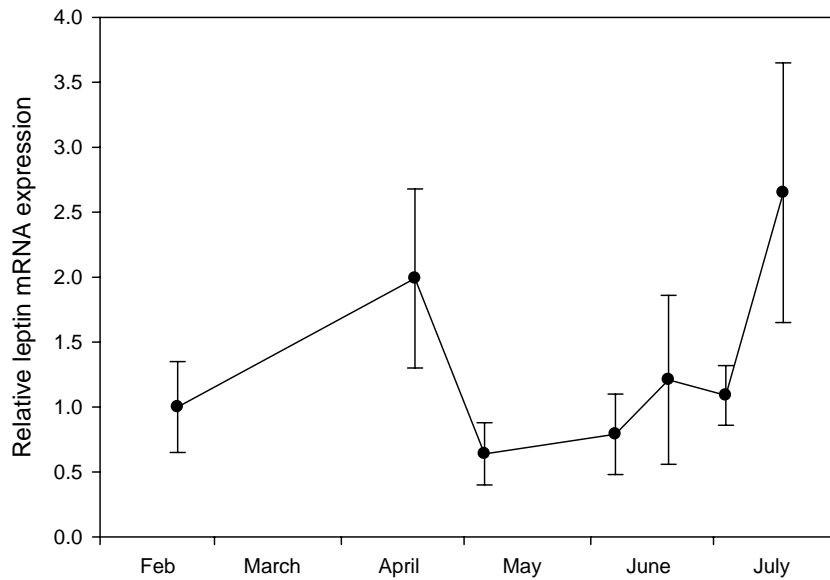


Figure 5. Relative gill leptin mRNA expression in Arctic charr sampled in freshwater at the various sampling dates.

Gill leptin mRNA expression were neither significantly effected by time ( $F = 1.07$ ;  $p > 0.05$ ), fish length ( $F = 0.32$ ;  $p > 0.05$ ) nor condition factor ( $F = 0.41$ ;  $p > 0.05$ ). Gill leptin mRNA level increased slightly in early April, but the individual variation was large at that point and the increase was not statistically significant. After returning to a similar level as in February, gill leptin mRNA level gradually increased from May to late July. Again there were large individual variations in leptin mRNA expression accompanying the increase in July.

## 4. DISCUSSION

Leptin mRNA expression was found in gills of Arctic charr in this study. To the question: “what is the role of leptin if the gene is expressed in the gills of Arctic charr?”, my first guess would be that it might have some function related to ion- and osmoregulation of fish because of the major function of the gill in ion- and osmoregulation. In this study, Arctic charr were sampled during the smoltification period in freshwater. Seawater tests were performed to examine the development of hypoosmoregulation, and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities were measured at various dates to reveal the smolting process in these fish. The activity of gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, an enzyme directly involved in ionic extrusion in seawater, can be used as a valuable indicator of seawater adaptability in migrating salmonids (Folmar and Dickhoff, 1980; Hoar, 1988). Quantitative PCR was performed in order to examine possible changes in gill leptin mRNA during the smoltification. Concurrent changes in gill leptin expression and smoltification indices (e.g. gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity) would provide indication of a role of leptin in the gill associated with the smoltification process. However, the data obtained in the experiment gave no indication of such an association.

### 4.1 Smolting

Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities increased significantly in the smoltifying Arctic charr. A peak in enzyme activity was reached in mid June, after which the activity decreased to the pre-smolting level by mid July. This observed increase in gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity is thought to be an important adaptation which enables smolts to successfully acclimate to the marine environment (Hoar, 1988). Since fish subjected to a seawater challenge test in early and mid June (when gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities reached a peak) had a significantly lower plasma osmolality than in winter and early spring after the seawater test, it confirms that gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity represents a predictive and reliable index of smolt development in anadromous Arctic charr, as in many other salmonids (McCormick *et al.*, 1987). The low plasma

osmolality after the seawater test in mid-June (~360 mOsm) indicate that the fish in the present study developed full seawater tolerance since these plasma osmolality levels are close to those seen in fully smoltified Atlantic salmon after 24 h seawater tests (Strand *et al.*, 2007).

Smolting is a progressive sequence of events and does not refer only to the last changes occurring at the end of the stay in freshwater. It is a gradual process starting several weeks before readiness to enter the marine environment (Boeuf, 1993). Many processes involved in pre-adaptation (e.g. those associated with metabolic changes), are linked to the need to grow rapidly in seawater (Dickhoff *et al.*, 1997). A stunting phenomenon is commonly observed in mariculture; fish do not grow, or cease growing, in seawater, probably due to incomplete smolt development (Boeuf, 1993). Smolting is also influenced by the growth in the juvenile stage. In Atlantic salmon, it has been shown that juvenile groups split into two modes in the distribution of length or weight (Boeuf *et al.*, 1985). The larger individuals reach the size threshold for smolting before the small fish, and consequently smoltify and leave the river one year before their smaller siblings. In Atlantic salmon, the growth in length is very high near the end of smolting, and the condition factor ( $K=W \cdot L^{-3} \cdot 1000$ , W, weight in g, and L, fork length in cm) decreases at the same time, from a range of 1.25-1.40 in the Atlantic salmon pre-smolt to 0.90-1.00 in the smolt (Hoar, 1988). In the Arctic charr, on the other hand, the condition factor increased during the smoltification period from 0.95 in April to 1.12 near the end of smolting in mid-June. Condition factor decreased in smolting Atlantic salmon due to increased length growth and utilization of energy (fat) reserves (Boeuf, 1993). Concentrations of liver and muscle cholesterol decrease, and triacylglycerol is used extensively during smolting (Sheridan *et al.*, 1983). Lipid depletion results primarily from reduced triacylglycerols, the major lipid storage compounds (Sheridan and Kao, 1998). Glycogen depletion results from increased glycogen phosphorylase a activity and decreased glycogen synthetase activity, while lipid depletion results from increased triacylglycerol lipase activity and decreased fatty acid synthesis (Sheridan *et al.*, 1985). These metabolic changes in Atlantic

salmon are important for fuelling the smoltification process (Sheridan, 1989) and the resulting change to a more slim body shape is considered to be adaptive for the change in life style from a bottom dwelling parr to a pelagic, actively swimming smolt (Hoar, 1988). The lack of any decrease in condition factor in Arctic charr during smoltification, as shown here indicate lower utilization of metabolic reserves such as lipid in smolting Arctic charr than in smolting Atlantic salmon. This indicates that the smoltification process is less costly in the anadromous Arctic charr than in the Atlantic salmon. For example, there is normally a several fold higher increase in gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in salmon (Nilson *et al.*, 2003) than in charr (Jørgensen *et al.*, 2007; this study). Notably, as evident in Figure 4, variation of gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity of Arctic charr sampled in June was much larger than variation of plasma osmolality of the fish. It may suggest that a high hypoosmoregulatory ability and salinity tolerance is not only caused by high gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity in Arctic charr. It may also imply that the Arctic charr have established a pelagic life long before they start to smoltify.

#### 4.2 Leptin

Leptin is an important hormone for regulating feed intake and balance in mammals (Friedman, 2002). Pufferfish leptin shares only 13.2% amino acid identity with human leptin, but the gene arrangement around the leptin gene and the three-dimensional (3D) structural protein model are well conserved between human and pufferfish leptin (Kurowaka and Murashita, 2009). Within mammalian classes, amino acid sequences of leptin are highly conserved (>80%). On the other hand, leptin sequences showed higher diversity (18-48% identical) within fish in a phylogenetic analysis (Kurowaka *et al.*, 2005). Mammalian leptin is primarily produced by adipose tissue (Zhang *et al.*, 1994). Leptin has impact on two central peptide signaling systems: it inhibits the orexigenic NPY/AGRP system; stimulates the anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript system (Arora, 2006; Meister, 2007). It is also involved in regulation of linear growth and cell differentiation and proliferation in



mammals (Gat-Yablonski and Philip, 2008). Recent studies have shown that the liver, rather than adipose tissue, is the major leptin expressing tissue in pufferfish (Kurokawa *et al.*, 2005), common carp (Huisin *et al.*, 2006), rainbow trout (Murashita *et al.*, 2008) and Arctic charr (Frøiland *et al.*, 2009). The amino acid sequence of fish leptin is very different from that of mammalian leptin. Therefore, previous functional studies of human and rat leptin used in fish have provided contradictory results on the role of this hormone in fish (Silverstein and Plisetskaya, 2000). However, the results of recombinant trout leptin injection in rainbow trout showed a strong anorexigenic effect, with a decrease in hypothalamic expression of NPY mRNA and increase in POMC mRNA (Murashita *et al.*, 2008). Effects of leptin on other processes in fish, for example skeletal growth and lipid metabolism, has so far not been reported.

The expression of leptin in fish gills (Kurokawa and Murashita, 2009) has probably nothing to do with the appetite regulating action of leptin seen in mammals and fish. In the present study I wanted to see if there are changes in gill leptin expression during the smolting process, to provide evidence for, or against, a paracrine role of leptin in the changes taking place in the fish gill during the smolting process. Such a role could, for example, be associated with the changes in gill lipid composition taking place during smolting. Lipid composition in the gills of Masu salmon (*Oncorhynchus masou*) changes during parr-smolt transformation, shown mainly by decreasing levels of triglycerides (Takeuchi *et al.*, 1990). Reduction of the triglyceride proportion in gill and body lipids were also observed in steelhead (*Salmo gairdneri*) (Sheridan *et al.*, 1983). Further, it could be possible that leptin is involved in the stimulation of differentiation and proliferation of the chloride cells that takes place during smolting in anadromous salmonids (McCormick, 2001). The results provided no evidence for a role of leptin in the gill during smoltification, since there were no changes in leptin expression.

On the other hand it has been shown that seawater exposure, *per se*, stimulates

development of gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and changes in lipid composition (Bystriansky and Ballantyne, 2006). It can therefore not be excluded that leptin is involved in processes taking place after seawater exposure and further studies should include sampling of gill tissue during the first period after transfer to seawater. Fish seem also to commonly possess two types of leptin (A and B type) genes, derived from whole-genome duplication early in the teleost lineage (Kurokawa and Murashita, 2009). The Arctic charr leptin gene cloned in a recent study was a typical salmonid leptin gene (A type), but it is likely that the Arctic charr also have the B type leptin gene (Frøiland *et al.*, 2009). Functional differences between these two leptin types have not been reported yet, but future studies on the role of leptin (endocrine and paracrine) should also include the B type leptin. Further, a regulation at the receptor level can not be excluded. The fish leptin receptor gene was first identified in marine medaka, *Oryzias melastigma* (Wong *et al.*, 2007) and in Japanese medaka, *Oryzias latipes* (Kurokawa and Murashita, 2009). Quantitative and qualitative analysis of leptin receptor mRNA expression needs to be performed to examine the function of leptin in the gills of Arctic charr and other anadromous salmonids.

#### 4.3 Importance to fisheries management

The study of parr-smolt transformation has become very important in various aspects of the management of natural populations of anadromous salmonids and in the rapidly growing mariculture industry. Since salmonids transform into a seawater state in the freshwater phase, before entering the sea, the physiological changes are therefore not triggered by the external salinity but by a ‘specific genetic program’ (Boeuf, 1993). External factors such as temperature and photoperiod synchronize the timing of the parr-smolt transformation (Hoar, 1976, 1988; Folmer and Dickhoff, 1980; Wedemeyer *et al.*, 1980). In the wild, migratory behaviour seems to be controlled by physiological status, but very little is known about the mechanisms triggering migration (seawater preference) (Ojima and Iwata, 2007). Moreover, smolts of many salmonid species remain a few days in the brackish water to adapt to full salinity seawater progressively (Zaugg *et al.*, 1985). In salmon reared in captivity, the

fish are normally transferred directly to seawater. These fish may have problems adapting to the seawater environment if they possess an incomplete smolt status. Some big fish may survive, but not grow (the stunting phenomenon). The true smolt stage (so called smolt-window), which allows full adaptation to seawater followed by good growth, lasts for a very short time (Hoar, 1988). Changes in general body shape and coloration are not sufficient by themselves to determine true smolt status. Seawater tests and gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity analysis are necessary for reliable information about smolt status. In both intensive marine culture, where on-growing salmon are kept in net pens or ponds in the sea, and extensive culture, where the fish is released in rivers or coastal areas and recaptured at later stages, a good smolt quality is the key to maximize production. We must therefore have a deep understanding of both the physiological and behavioural aspects of smolting and there is still a paucity of knowledge, particularly regarding the behavioral part of the smolting process.

## **5. CONCLUSION**

Leptin mRNA was found to be expressed in the gill of Arctic charr. We measured plasma osmolality and gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity to reveal the smolt status of the fish. Quantitative PCR were performed to see if gill leptin expression correlates with smolt indices. Seawater tolerance, as well as gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity, increases during the smoltification process. There was no change in gill leptin mRNA expression during and after smolting in the fish acclimated to freshwater. Results gave no evidence for a paracrine role of leptin in adaptational changes in gill during smolting. Further study of the possible role leptin in the fish exposure to seawater is needed.

## 6. REFERENCE

- Arnesen, A.M., Lysfjord, G., Damsgård, B., 1995. Smolt characteristics of small first-time migrant, and resident Arctic charr, *Salvelinus alpinus* (L.), from a river system in northern Norway. *Aquac. Res*, 26, 809-818.
- Arora, S., 2006. Role of neuropeptides in appetite regulation and obesity-A review. *Neuropeptides*, 40, 375-401.
- Avella, M. and Bornancin, M., 1990. Ion fluxes in the gills of freshwater and seawater salmonid fish, in *Comparative Physiology*, Vol. 6 (eds R.K.H. Kinne, E.Kinne-Saffran and K.W.Beyenbach), Basel, Karger, pp. 1-13.
- Boeuf, G., Le Roux, A., Gaignon, J.L. and Harache, Y., 1985. Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity and smelting in Atlantic salmon *Salmo salar* L. in France. *Aquaculture*, 45(1-4), 73-81.
- Boeuf, G., 1993. Chapter 4: Salmonid smolting: a pre-adaptation to the oceanic environment, in *Fish Ecophysiology*, (eds J.Cliff Rankin and Frank B.Jensen), Chapman & Hall, London, pp. 106-135.
- Bystriansky, J.S. and Ballantyne, J.S., 2006. Gill  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity correlates with basolateral membrane lipid composition in seawater- but not freshwater-acclimated Arctic char, *Salvelinus alpinus*. *Am J Physiol Regul Integr Comp Physiol*, 292(2), 1043-1051.
- Crockett, E.L. and Hazel, J.R., 1997. Cholesterol affects physical properties and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in basolateral membranes of renal and intestinal epithelia from thermally acclimated rainbow trout. *J Comp Physiol*, 167B, 344-351.
- Damsgård, B., 1991. Smolting characters in anadromous and resident Arctic charr, *Salvelinus alpinus* (L.) *J. Fish Biol*, 39, 765-774.
- Dickhoff, W.W., Beckman, B.R., Larsen, D.A., Duan, C. and Moriyama, S., 1997. The role of growth in endocrine regulation of salmon smoltification. *Fish Physiol Biochem*, 17, 231-236.
- Evans, D.H., Piermarini, P.M. and Potts, W.T.W., 1999. Ionic transport in the fish gill epithelium. *J Exp Zool*, 283, 641-652.
- Folmar, L.C. and Dickhoff, W.W., 1980. The parr-smolt transformation (smoltification) and seawater adaptation in salmonids. A review of selected literature. *Aquaculture*, 21(1), 1-37.

- Friedman, J.M., 2002. The function of leptin in nutrition, weight, and physiology. *Nutr. Rev.*, 60, S1-S14; discussion S68-S84, 85-87.
- Frøiland, E., Murashita, K., Jørgensen, E.H. and Kurokawa, T., 2009. Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. *General and Comparative Endocrinology*, in press.
- Gat-Yablonski, G. and Philip, M., 2008. Leptin and regulation of linear growth. *Current Opinion Clin. Nutr. Met. Care*, 11, 303-308.
- Gulseth, O.A., Nilssen, K.J., Iversen, M., Finstad, B., 2001. Seawater tolerance in first-time migrants of anadromous Arctic charr, *Salvelinus alpinus*. *Polar Biol*, 24, 270-275.
- Halvorsen, M., Arnesen, A.M., Nilssen, K.M., Jobling, M., 1993. Osmoregulatory ability of anadromous Arctic charr, *Salvelinus alpinus* (L.), migrating toward the sea. *Aquac. Fish. Manage*, 24, 199-211.
- Hoar, W.S., 1976. Smolt transformation: evolution, behaviour and physiology. *J. Fish. Res. Bd Can.*, 33, 1233-1252.
- Hoar, W.S., 1988. The physiology of smolting salmonids, in *Fish Physiology*, Vol. 11B (eds W.S.Hoar and D.J. Randall), Academic Press, New York, pp. 275-343.
- Huising, M.O., Geven, E.J., Kruiswijk, C.P., Nabuurs, S.B., Stolte, E.H., Spanings, F.A., Verburg-van Kemenade, B.M., Flik, G., 2006. Increased leptin expression in common carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. *Endocrinol*, 147, 5786-5797.
- Johnson, L., 1980. The Arctic charr *Salvelinus alpinus*, in *Charrs: Salmonid fishes of the Genus Salvelinus* (eds E.K.Balon and Dr.W.Junk), The Hague, pp.15-98.
- Jørgensen E.H., Aas-Hanson, Ø., Moriyama, S., Iwata, M. and Strand, J.E.T., 2007. The parr-smolt transformation of Arctic charr is comparable to that of Atlantic salmon. *Aquaculture*, 273, 227-234.
- Klemetsen, A., Amundsen, P.A., Dempson, J.B., Jonsson, B., Connell, M.F., Mortensen, E., 2003. Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish*, 12, 1-59.
- Karnaky, K.J., Kinter, L.B., Kinter, W.B. and Stirling, C.E., 1976. Teleost chloride cell. II. Autoradiographic localization of gill Na<sup>+</sup>, K<sup>+</sup>-ATPase in killifish *Fundulus*

- heteroclitus* adapted to low and high salinity environments. *J Cell Biol*, 70, 157-177.
- Kurokawa, T., Uji, S. and Suzuki, T., 2005. Identification of cDNA coding for a homologue to mammalian leptin from pufferfish *Takifugu rebripes*. *Peptides*, 26, 745-750.
- Kurokawa, T. and Murashita, K., 2009. Genomic characterization of multiple leptin genes and a leptin receptor gene in the Japanese medaka, *Oryzias latipes*. *Gen. Com. Endocrinol*, 161, 229-237.
- Marcus, M.M., Apell, H-J, Roudna, M., Schwendener, R.A., Weder, H-G, and Lauger, P., 1986. Na<sup>+</sup>, K<sup>+</sup>-ATPase in artificial lipid vesicles: influence of lipid structure on pumping rate. *Biochim Biophys Acta*, 854, 270-278.
- McCormick, S.D., 1993. Methods for nonlethal gill biopsy and measurement of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity, *Can. J. Fish. Aquat. Sci.*, 50, pp. 656–658.
- McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. *Amer. Zool*, 41, 781-794.
- McCormick, S.D., Saunders, R.L., Henderson, E.B. and Harmon, P.R., 1987. Photoperiod control of parr-smolt transformation in Atlantic salmon (*Salmo salar*): changes in salinity tolerance, gill Na<sup>+</sup>, K<sup>+</sup>-ATPase activity and plasma thyroid hormones. *Can. J. Fish. Aquat. Sci.*, 44, 1462-1468.
- McCormick, S.D., Moyes, C.D. and Ballantyne, J.S., 1989. Influence of salinity on the energetics of gill and kidney of Atlantic salmon *Salmo salar*. *Fish Physiol. Biochem.*, 6 243-254.
- Meister, B., 2007. Neurotransmitters in key neurons of the hypothalamus that regulate feeding behaviour and body weight. *Physiol. Behav.*, 92, 263-271.
- Murashita, K., Uji, S., Yamamoto, T., Rønnestad. and Kurokawa, T., 2008. Production of recombinant leptin and its effects on food intake in rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol(B)*, 150, 377-384.
- Nilson, T.O., Ebbesson, L.O.E. and Stefansson, S.O., 2003. Smolting in anadromous and landlocked strains of Atlantic salmon. *Aquaculture*, 222(1-4), 71-82.
- Nilssen, K.J., Gulseth, O.A, 1998. Summer seawater tolerance of small-sized Arctic charr, *Salvelinus alpinus*, on Svalbard. *Polar Biol*, 20, 95-98.
- Ojima, D and Iwata, M., 2007. The relationship between thyroxine surge and onset of

- downstream migration in chum salmon (*Oncorhynchus keta*) fry. *Aquaculture*, 273(2-3), 185-193.
- Ottolenghi, P., 1975. The reversible delipidation of a solubilized sodium-plus-potassium ion-dependent adenosine triphosphatase from the salt gland of the spiny dogfish. *Biochem J*, 151, 61-66.
- Payan, P., Girard, J.P. and Mayer-Gostan, N., 1984. Branchial ion movements in teleost: the roles of respiratory and chloride cells, in *Fish Physiology*, Vol. 10B (eds W.S. Hoar and D.J. Randall), Academic Press, New York, pp. 39-63.
- Rounsefell, A.G., 1958. Anadromy in North American salmonidae, *Fishery Bulletin* 131, Vol. 58, Washington, pp. 171-183.
- Sheridan, M.A., 1989. Alterations in lipid metabolism accompanying smoltification and seawater adaptation of salmonid fish. *Aquaculture*, 82, 191-203.
- Sheridan, M.A. and Kao, Y.-H., 1998. Regulation of metamorphosis-associated changes in the lipid metabolism of selected vertebrates. *Am. Zool.* 38, pp. 350-368.
- Sheridan, M.A., Allen, W.V. and Kerstetter, T.H., 1983. Seasonal variation in the lipid composition of the steelhead trout *Salmo gairdneri* Richardson, associated with the parr-smolt transformation. *J. Fish. Biol.*, 23(2), 125-134.
- Sheridan, M.A., Allen, W.V. and Kerstetter, T.H., 1985. Changes in the fatty acid composition of steelhead trout *Salmo gairdneri* Richardson, associated with the parr-smolt transformation. *Comp. Biochem. Physiol.*, 80B, 671-676.
- Silverstein, J.T. and Plisetskaya, E.M., 2000. The effects of NPY and insulin on food intake regulation in fish. *Am. Zool.*, 40, 269-308.
- Stand, J.E.T., Johnsen, H.K., Arnesen, A.M., 2007. Parr-smolt transformation in hatchery reared offspring of one domesticated and two wild populations of Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 273, 250-256.
- Stekhoven, F.S. and Bonting, S.L., 1981. Transport adenosine triphosphatases: properties and functions. *Physiol Rev*, 61, 1-76.
- Takeuchi, T., Sampekalo, J., Nomura, T. and Watanabe, T., 1990. lipid contents and classes in gill of masu salmon *Oncorhynchus masou* during the parr-smolt transformation. *Nippon Suisan Gakkaishi*, 56, 1527-1528.
- Tocher, D.R., Bell, J.G., Dick, J.R., Henderson, R.J., McGhee, F., Michell, D. and

- Morris, P.C., 2000. Polyunsaturated fatty acid metabolism in Atlantic salmon, *Salmo salar* undergoing parr-smolt transformation and the effects of dietary linseed and rapeseed oils. *Fish Physiology and Biochemistry*, 23, 59-73.
- Towle, D.W., 1981. Role of Na<sup>+</sup>, K<sup>+</sup>-ATPase in ion regulation by marine and estuarine animals. *Mar. Biol. Lett*, 2, 107-122.
- Towle, D.W., Gilman, M.E. and Hempel, J.D., 1977. Rapid modulation of gill Na<sup>+</sup>, K<sup>+</sup>-ATPase dependent ATPase activity during acclimation of the killifish, *Fundulus heteroclitus* to salinity change. *J. Exp. Zool.*, 202, 179-185.
- Vemuri, R. and Philipson, K.D., 1989. Influence of sterols and phospholipids on sarcolemmal and sarcoplasmic reticular cation transporters. *J Biol Chem*, 264, 8680-8685.
- Wedemeyer, A.H., Saunders, R.L. and Clarke, W.L., 1980. Environmental factors affecting smoltification and early marine survival of anadromous salmonids. *Mar. Fish. Rev.*, 42. 1-14.
- Wong, M., Yu, R., Ng, P., Law, S., Tsang, A. and Kong, R., 2007. Characterization of a hypoxia-response leptin receptor (omLepR(L)) cDNA from the marine medaka (*Oryzias melastigma*). *Mar. Pollut. Bull*, 54, 797-803.
- Zaugg, W.S., Prentice, E.F. and Waknitz, F.N., 1985. Importance of river migration to the development of seawater tolerance in Columbia River anadromous salmonids. *Aquaculture*, 51, 33-47.
- Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature*, 372, 425-432.



## APPENDIX

Table I. PCR1 and PCR2 protocols to detect existence of genomic DNA.

PCR1 (per reaction)		PCR2 (per reaction)	
10X buffer	2.5 $\mu$ l	10X buffer	2.5 $\mu$ l
dNTP	0.5 $\mu$ l	dNTP	0.5 $\mu$ l
Primer forward (10pm)	1.0 $\mu$ l	Primer forward (10pm)	1.0 $\mu$ l
Primer reverse (10pm)	1.0 $\mu$ l	Primer reverse (10pm)	1.0 $\mu$ l
Finn polymerase	0.3 $\mu$ l	Finn polymerase	0.3 $\mu$ l
H <sub>2</sub> O	19.2 $\mu$ l	H <sub>2</sub> O	17.7 $\mu$ l
DNase I treated sample	0.5 $\mu$ l	PCR1 product	2.0 $\mu$ l
Total volume	25 $\mu$ l	Total volume	25 $\mu$ l

Table II: PCR protocol for cDNA quality check ( $\beta$ -actin fragment amplification)

cDNA quality check PCR (per reaction)	
10X buffer	2.5 $\mu$ l
dNTP	0.5 $\mu$ l
$\beta$ -actin forward (10pm)	1.0 $\mu$ l
$\beta$ -actin reverse (10pm)	1.0 $\mu$ l
Finn polymerase	0.3 $\mu$ l
H <sub>2</sub> O	19.2 $\mu$ l
Sample	0.5 $\mu$ l
Total volume	25 $\mu$ l

Table III. PCR protocols for leptin gene amplification

Leptin gene expression PCR (per reaction)	
10X buffer	5.0 $\mu$ l
dNTP	0.5 $\mu$ l
Leptin forward (10 $\mu$ m)	1.0 $\mu$ l
Leptin reverse (10 $\mu$ m)	1.0 $\mu$ l
Finn polymerase	0.5 $\mu$ l
H <sub>2</sub> O	38.5 $\mu$ l
MgCl <sub>2</sub>	1.5 $\mu$ l
cDNA	2.0 $\mu$ l
Total volume	50 $\mu$ l

Table IV. Q-PCR protocols

Leptin q-PCR (per reaction)		18S q-PCR (per reaction)	
Leptin forward (5 $\mu$ m)	1.5 $\mu$ l	18S forward (5 $\mu$ m)	1.5 $\mu$ l
Leptin reverse (5 $\mu$ m)	1.5 $\mu$ l	18S reverse (5 $\mu$ m)	1.5 $\mu$ l
SYBR Green	12.5 $\mu$ l	SYBR Green	12.5 $\mu$ l
cDNA+H <sub>2</sub> O (10X)	9.5 $\mu$ l	cDNA+H <sub>2</sub> O (10X)	9.5 $\mu$ l
Total reaction volume	25 $\mu$ l	Total reaction volume	25 $\mu$ l