The biological basis of smoltification in Atlantic salmon

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\textbf{ABSTRACT.} Chile is the second-largest producer of Atlantic salmon in the world, and the Chilean salmon production accounts for 27\% of the world’s production. One important step of the productive cycle in freshwater is the smoltification process that prepares the fish for the marine life stage. This review describes the biological basis of smoltification in Atlantic salmon, with particular attention on branchial osmoregulatory adaptations. We also discuss some of the infectious diseases and problems in smoltification (two of the main causes of losses in Chilean aquaculture) that could be related from a physiological point of view.

\textit{Key words:} Chilean salmon culture, smoltification, Atlantic salmon.

\section*{GENERAL BACKGROUND}

Aquaculture provides the world’s growing population with both food and livelihoods (FAO 2020). Currently, aquaculture is the fastest-growing sector in the animal production industry worldwide, with an annual increase of 7.5\% since 1970 (FAO 2020). According to the Food and Agriculture Organization (FAO) of the United Nations, aquaculture’s contribution to total fish production has risen steadily in all continents, and Chile is one of the top-ten aquaculture producers. These ten countries contributed to 88\% of the entire world’s production by mass. Moreover, China is the major producer country by far (FAO 2020). In 2019, the value of seafood exported by China was more than USD 20 billion while Chile exported about USD 5 billion of seafood in the same year\textsuperscript{1}.

Chilean aquaculture production relies heavily on salmonids farming (salmon and trout), accounting for 84\% of total Chilean aquaculture production and practically 100\% of all farmed fish. According to Subpesca-Chile, in 2019 Atlantic salmon (\textit{Salmo salar}) was the main species farmed in Chile, accounting for 58.7\% of total production, followed by Pacific salmon (\textit{Oncorhynchus kisutch}) with 36.2\%, and Rainbow trout (\textit{Oncorhynchus mykiss}) with 5.1\% (Subpesca Chile 2020).

Chile is the second-largest producer of Atlantic salmon in the world, with an annual average volume close to 800,000 tons between 2014\textsuperscript{2} and 2019\textsuperscript{3}. Chilean production of salmon species amounts for 27\% of the world’s production, while Norway continues to lead with 52\% of the share (Iversen \textit{et al} 2020). However, after the massive ISA virus infection in 2007, the Chilean government and the salmon industry put significant efforts into basic and applied research on many of the key aspects of salmon production to maintain Chile in a leading position worldwide (Olson and Criddle 2008, Martini Costa 2019, Iversen \textit{et al} 2020).

\section*{SUMMARY OF THE SALMON AQUACULTURE PROCESS}

In the last 40 years, the global salmon farming industry has switched from a small-scale operation to a mass-production scheme (Bjørndal and Aarland 1999, McLeod \textit{et al} 2006, Olson and Criddle 2008). The high level of industrialisation of salmon aquaculture is significant due to the incremental success of strategies to adapt the salmon wildlife cycle to a large-scale farming setting.

Wild salmonids begin their life in freshwater. Adult salmons spawn in freshwater, their eggs hatch into alevins, and then they begin their development into fry and parr. At this stage, environmental cues initiate the smoltification process, preparing the fish for downstream migration and entrance into seawater, where they will grow up as a marine, predatory species (Björnsson and Bradley 2007, Björnsson \textit{et al} 2011). This anadromous strategy confers reproductive and developmental advantages to salmon because it enables them to utilise a relatively safe environment provided by freshwater for reproduction, whereas juvenile migration towards the ocean allows...

\textsuperscript{1} \url{https://thefishsite.com/articles/rabo}

\textsuperscript{2} \url{https://www.salmonchile.cl/en/production/}

\textsuperscript{3} \url{https://www.salmonexpert.cl/article/industria-salmonicultora-se-acerca-al-milln-de-toneladas-de-produccin-anual/}
them to feed on a rich supply of fish and other marine organisms (Stefansson et al 2008). Thus, smoltification represents the key turning point in the anadromous life cycle of Atlantic salmon (McCormick 2009, Björnsson et al 2011).

The salmon production process was developed considering the biological background of this typically anadromous life cycle. The salmon production process can be divided into three steps (see figure 1): a) production of broodstock, roe, and fry in freshwater; b) production of smolts (smoltification) in freshwater; and c) growing farmed fish in seawater (Asheim et al 2011, Asche and Bjorndal 2011).

The production cycle of farmed salmon takes about three years on average. During the first year of production, the eggs are fertilised and the fish develop and grow to approximately 100 grams in a controlled freshwater environment (figure 1a and 1b) (Bergheim et al 2009). Subsequently, fish are transferred to seawater cages, where they continue growing until approximately 4-5 kg for 14-24 months (figure 1c). After reaching harvesting size, fish are transported to primary processing plants where they are slaughtered and gutted (Mowi 2020).

**SMOLTIFICATION IN THE SALMON PRODUCTION CYCLE**

In the productive salmon chain, the industrial smoltification phase (figure 1b) correlates directly with production efficiency because smolt quality impacts the indicator “yield per smolt” (harvest weight per smolt released). “Yield per smolt” is calculated as the fraction between harvest weight for each smolt transferred to seawater. This parameter is influenced by premature mortality rates, disease, temperature and growth attributes. The Faroe Islands reached a higher yield per smolt in the world: 4.87 kg in 2013. In Norway, the average yield per smolt was estimated at 3.71 kg, and in Chile, it was 3.58 kg in the same year.

The average yield per smolt for the Chilean salmon industry in 2013 was estimated at only 3.58 kg (Mowi 2020), and during 2015 it reached a minimum value of only 3.0 kg. After improving the production strategy, productivity reached a record level of over 4.4-4.5 kg per smolt during 2018, according to Aquabech4.

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4 https://www.fishfarmingexpert.com/article/chile-production-has-reached-record-levels/
In the yearling production system, smoltification occurs following the seasonal yearling signals (natural photoperiod and temperature) rather than being dictated by the regular artificial programming of salmon. To start the smoltification, the salmon must reach a size-related threshold to respond to the ambiental signals. During the yearling smoltification, temperatures are not expected to be a problem since ambiental water supply is used following the species’ natural smoltification period.

In the under-yearling production system, smoltification must be induced artificially through photomanipulation as the most common strategy, and the process takes place at a time of the year that is different to that dictated by regular biological, but artificially induced, programming. In this system, smoltification is induced by an artificial “winter signal” (alternating light and dark cycles of 12 hours), typically during late summer, when ambient temperatures are at their highest. In a typical photomanipulation program, the recommendation is to avoid a temperature drop during the transition from the first to the second period (Staurnes et al. 2001).

BIOLOGICAL BASIS OF PHYSIOLOGICAL CHANGES THAT OCCUR DURING SMOLTIFICATION

In nature, smoltification, also called parr-smolt transformation, is a complex adaptation process driven by the endocrine system that consists of several independent but coordinated developmental changes in the biochemistry, physiology, morphology, and behaviour of juvenile salmon (McCormick 2013). These changes possess a high energetic cost for the fish and correlate with decreased defenses related to the immune system (Pontigo et al. 2016); however, this process prepares the fish for downstream migration and transition to the marine life stage (Björnsson and Bradley 2007, Stefansson et al. 2008). Important components of the parr-smolt transformation are i) environmental cues, primarily photoperiod and temperature (Björnsson and Bradley 2007); ii) endocrine control of smoltification (Björnsson et al. 2011), and iii) physiological changes in osmoregulation that allow the smolt to thrive in high-salt environments (Clarke et al. 1996, McCormick 2013).

ENVIRONMENTAL REGULATION OF SMOLTIFICATION

Photoperiod and seasonal temperature fluctuations are two important environmental cues that work together to transform Atlantic salmon parrs into smolts (Clarke et al. 1996). In the Northern hemisphere, smoltification in wild salmon is complete by spring, when a rising temperature of 8-10 °C initiates wild smolt migration to seawater (Jonsson and Ruud-Hansen 1985, Clarke et al. 1996). The mechanism by which photoperiodic information is translated into a neuroendocrine response in teleosts has not been fully elucidated. Different preparatory photoperiods show differential gill NKA activity and expression patterns in ionocytes (van Rijn et al. 2020). Melatonin secretion by the pineal gland of salmonids can be directly stimulated by photoperiod (Falcón et al. 2007). Also, elevated temperature increases melatonin secretion, and the salmon pineal gland could be working as a photoperiod and temperature sensor (Nisembaum et al. 2020).

HORMONAL REGULATION OF SMOLTIFICATION

Parr-smolt transformation engages several endocrine signaling systems (see figure 2) (Björnsson et al. 2011). After salmons have reached a size- or growth-related threshold, the light–brain–pituitary axis is stimulated by photoperiod and seasonal temperature, resulting in simultaneous increments of growth hormone (GH), cortisol, and thyroid hormones (McCormick 2001). Besides its growth related functions, GH modulates intermediary metabolism and osmoregulatory mechanisms in fish by stimulating somatomedin activity, such as insulin-like growth factors IGF-1 and IGF-2 (McCormick et al. 1991, Madsen et al. 1995, McCormick 1996). GH and cortisol interact to control hyperosmoregulatory mechanisms in gills, gut, and kidneys, promoting increased salinity tolerance and changes in growth (weight to length ratio) and intermediary metabolism. In gills, cortisol and the GH/IGF-1 axis promote differentiation of salt-secreting ionocytes (see next section for details), a process that requires upregulation of three major osmoregulatory membrane transporters: the sodium-potassium ATPase (NKA), the sodium-potassium-2 chloride cotransporter 1 (NKCC1), and the cystic fibrosis transmembrane conductance regulator (CFTR) (Hwang et al. 2011).

Lastly, thyroid hormones regulate olfactory imprinting, metabolism, morphological changes such as silvering, and possibly behaviour (McCormick 2013), whereas prolactin in smoltification is thought to be a general inhibitor of most aspects of smolt development (Sakamoto and McCormick 2006). An increase in thyroid hormones (plasma T4) is detected in hatchery smolt after release and in wild smolt during migration (Iwata et al. 2003, McCormick et al. 2003). Also, plasma T4 increases after smolts are exposed to water with different chemical compositions (Hoffnagle and Fivizzani 1990) or during entry into estuarine environments (McCormick et al. 2013).

OSMOREGULATORY CHANGES IN GILLS DURING SMOLTIFICATION

As mentioned above, salmonids begin their life cycle in freshwater, where they are hyperosmotic to the external medium. Osmotic pressure favours water entry into the body and the loss of salt by diffusion across the gill. To compensate for this passive flow of water and ions to maintain homeostasis, the fish eliminates excess water as diluted urine and obtains salts from food in the intestine.
and water by active uptake through the gill’s epithelium. As they move into seawater, the osmotic gradient is reversed because the internal fluids of salmonids are approximately one-third the osmolarity of seawater, and they become hypooosmotic relative to the external medium. Accordingly, salmons lose water and gain salts by passive diffusion. As compensatory mechanisms, they drink seawater, reduce their urine production, and actively secrete salts across the gill’s epithelium through specialised cells called ionocytes, mitochondria-rich (MR) cells, or chloride cells (Clarke et al 1996).

One of the key events in osmoregulatory changes during the parr-smolt transformation involves fine-tuning of the ion-transporting machinery in the gill epithelia (Tipsmark et al 2008). Ion transport is primarily carried out by MR cells (Madsen et al 2015) and requires expression changes in NKA (D’Cotta et al 2000), NKCC1, and other critical proteins at the cell surface, such as CFTR and claudins (Hirose et al 2003, Hiroi et al 2005). In most euryhaline teleosts, upregulation of gill NKA (Kamiya and Utida 1969, Morgan et al 1997, Seidelin et al 2000) and NKCC1 (Pelis et al 2001, Tipsmark et al 2002, Wu et al 2003) are associated with seawater acclimation.

NaCl secretion by teleost gills, necessary in seawater life, is accomplished via secondary active transport of Cl\(^-\) and passive transport of Na\(^+\) (figure 3). The driving force for active transport is provided by NKA, which maintains intracellular Na\(^+\) at low levels and intracellular K\(^+\) at high levels, compared to the extracellular medium (Marshall and Grosell 2006). This NaCl secretion mechanism needs an additional condition to work in seawater: a thermodynamic requirement to recycle K\(^+\) out via conductive pathways (potassium channels, figure 3). The molecule responsible for this K\(^+\) transport is still unknown in salmonids, but several K\(^+\) channels may be involved in the function of MR cells in other teleosts (Marshall and Grosell 2006). One plausible candidate is the inward-rectifying K\(^+\) channel (eKir), highly expressed in gills of the seawater-acclimated Japanese eel (Suzuki et al 1999). Another candidate is a large-conductance, calcium-activated K\(^+\) channel (called BK for “Big K\(^+\)”), whose expression was recently detected in gills from the teleost fish Porichthys notatus (Rohmann
et al 2009). Also, an inward-rectifying K\(^+\) channel (Kir1.1 or ROMK for Renal Outer Medullary K\(^+\) channel) and a BK channel were detected in MR cells from Mozambique tilapia (Furukawa et al 2012) and, more recently, in the gills from rainbow trout and Atlantic salmon by our research team (Loncoman et al 2015).

The capacity to measure the activity or the expression of different molecular components of this salt secretion machinery has provided useful tools for examining smolt development, and it will be discussed with greater detail in the next section.

MARKERS OF SMOLTIFICATION

Historically, research on smoltification has focused on understanding smolt development in the aquaculture industry and, in particular, the need to control the timing and quality of smolt for the transfer of juveniles from freshwater into ocean net pens (McCormick 2013). Knowing exactly when the smolts are ready to be relocated to seawater is crucial for the productivity of salmon farmers. Increased tolerance to salinity is often measured by either increased survival or by lower plasma electrolytes and osmolality after direct transfer from freshwater to high salinity (a “seawater challenge” as an example of a salinity tolerance test) (Clarke et al 1996, McCormick 2013). The main disadvantage of salinity tolerance tests is that they fail to distinguish between seawater tolerance and transient adaptation: since some fish may show increased survival but ultimately fail to adapt to long-term seawater exposure (Iremonger 2008).

Currently, the use of molecular markers of smoltification (see table 1) are used to accurately determine the transfer time to seawater since many physiological
changes produced during the parr-smolt transformation are detrimental to continued life in freshwater, and they tend to revert relatively fast if the fish are not able to enter seawater environments before smoltification is complete (the "smolt window") (Stefansson et al. 2008). In most instances, the salmon industry uses a single enzymatic marker for smoltification: the enzymatic activity of gill NKA, which activity increases during the transition from parr to smolt (McCormick et al. 2013, Zaugg 1982, McCormick et al. 2009, Nilsen et al. 2007). This protein activity is often considered an indicator of smolt development (McCormick 1993, Clarke et al. 1996). The Chilean salmon industry uses NKA enzymatic activity in gills as a main marker to determine the timing of smolt transfer to seawater.

Many scientific publications describe that some of the salt secretion machinery’s critical molecules expressed in the gills of salmonids correlate with seawater adaptation. Atlantic salmon expresses two major NKA isoforms in distinct gill ionocytes. NKAα1a is the most abundant isoform in freshwater, whereas NKAα1b predominates in seawater (McCormick et al. 2009). Gill mRNA levels of NKAα1b increase during Atlantic salmon smolting, whereas NKAα1a mRNA decreases (Nilsen et al. 2007). Also, co-transporters (NKCC1), aquaporins, and ion channels (CFTR, ROMK, and BK) are expressed in gills covary with salinity adaptation (Tipsmark et al. 2010, Furukawa et al. 2012; McCormick 2013, Loncoman et al. 2018). Tipsmark et al. (2008) showed that Claudin10e mRNA levels, a family of membrane proteins that form tight junctions and thus determine transepithelial resistance and ion permeability, also increase during smolt development and after exposure to seawater. All these molecules are considered potential molecular markers of smoltification, but there is no available evidence to determine its true predictive value in decreasing fish mortality in seawater. Some of these alternative markers are being implemented in Chile for research purposes (Loncoman et al. 2015, Loncoman et al. 2018, Vargas-Lagos et al. 2018), except for the NKA subunits that some laboratories in Norway and Chile measure as a service (see table 1); but the salmon industry has not adopted these markers for routine tests, probably due to the lack of quantitative data about any advantage over NKA measurements alone.

The search for new possible markers for smoltification is ongoing using all available techniques, even high throughput analyses such as transcriptomics (Houde et al. 2020, Versen et al. 2020, West et al. 2020) and small RNA sequencing (Shwe et al. 2020).

### Table 1. Common laboratory methods to test smoltification.

| Name              | Technique              | Assay                                                                 | References                      |
|-------------------|------------------------|----------------------------------------------------------------------|---------------------------------|
| Gill NKA activity | Spectrophotometry      | Coupled assay to quantify ADP generated by ATPase activity by detecting the disappearance of NADH at 360nm | (McCormick 1993)               |
| Gill NKA activity | Spectrophotometry      | Detection of Pi generated by ATPase activity (Zaugg 1982) at 312nm or 578nm² | (Flik et al. 1983)             |
| NKA subunit mRNA expression | Real-Time PCR | PCR primers for specific detection of mRNA from NKA subunits a1a and a1b | (McCormick et al. 2009)        |
| NKA protein expression | Immunohistochemistry  | Antibodies against the NKA protein                                   | (McCormick et al. 2013)        |

SMOLT-WINDOW OR SMOLTIFICATION WINDOW

In industrial salmon production, survival and growth performance in seawater are the two major manifestations of smoltification. However, if smolts are prevented from reaching seawater exposure, several of the preparatory changes associated with marine life are reverted, a process known as desmolification in Atlantic salmon or Parr-reversion in Pacific salmonids (figure 4) (Hoar 1988, Duston et al. 1991, Stefansson et al. 1998). This natural reverting process closes the “smolt-window” during which smolts can enter and quickly adapt to seawater. Conceptually, the smolt-window (or smoltification window) is considered the period when physiological conditions to be transferred into seawater are at their optimal peak (Sharron 2015). The smolt-window is operationally associated only with the increase in gill NKA enzyme activity (figure 4).

The smolt window duration is in the range of 300-400 degree-days (d°C) (Stefansson et al. 1998, McCormick et al. 1999, Stefansson et al. 2008). In the salmon farming industry, the decision to transfer smolt into seawater is taken using a threshold value of NKA activity, without any consideration of its dynamic properties, i.e. whether NKA activity is on the rise (smoltification phase), reaching its peak, or in decline (desmoltification phase). Zydlewski and Zydlewski (2012) show that gill NKA activity measurements are predictive of performance during the first few days of acclimation but, after transfer, fish grew at the same rate, with no differences in fish size and growth rate, among groups with initially different gill NKA activities. They concluded that gill NKA expression in freshwater at the peak of smolting...
CURRENT CHALLENGES IN CHILEAN SALMON PRODUCTION: ARE SMOLTIFICATION AND INFECTIOUS DISEASES RELATED?

Aquaculture farming in Chile has experienced a high-speed growth, mainly due to favourable geographical and environmental conditions, but this fast growth, together with confinement systems and the presence of native life, increases the risk of infectious disease outbreaks. The 2019 annual reports from SERNAPESCA (SERNAPESCA 2019) describes the principal causes of mortality in the Chilean salmon aquaculture. This report pointed out that two of the leading causes are “unadapted” fish (fish with problems in osmoregulation by deficient smoltification, 11.2%) and infectious diseases (23.9%).

Historically, smoltification problems are the other leading cause of economic losses in Chilean salmon aquaculture because the amount of “delayers and unadapted” fish at the seawater stage is still significant, with an 11.2% in 2019, being the first one the infectious diseases (23.9%) as we previously mentioned (SERNAPESCA 2019). These groups consist of fish seeded in seawater during a suboptimal developmental phase of smoltification that prevents adaptation to their new environment. The “unadapted” are fish that do not adapt to the marine environment early on and die after entering the sea, mainly due to osmoregulatory problems. The “delayers” are fish that adapted relatively well after entering the sea but subsequently present impaired growth and productive performance5.

In recent years, some Chilean smoltification facilities have adopted the Norwegian production standards from their company owners, and they claim that the problems regarding smoltification have been solved. It is particularly surprising that despite the research focused on smoltification, very little has been studied regarding the impact of this transformation on the salmon’s immune system. Several earlier studies in smolts and post-smolts of Atlantic salmon produced evidence for a possibly weakened immunity. Decreased plasma lysozyme, IgM levels, and leucocyte levels were observed (Muona and Soivio 1992, Melingen et al 1995). In the last years, some groups have started to show some data of association between the increase in infectious diseases that occurs after transfer to seawater with an alteration in the immune response during smoltification. Post-smolts show a weak response against viral (Moore et al. 2017; Nuñez-Ortiz et al. 2018; Jensen et al. 2019) and also, their skin barrier to infection (Karlsen et al. 2018) or gut immune functions (Wang et al. 2020) are weak during the first post-smolt period.

Recent transcriptomics analysis have demonstrated repressed expression of genes associated with the immune system during smoltification and after seawater transfer (Johansson et al 2016, Krasnov et al 2016); opening the possibility of a biological relationship between two of the most important causes of death in Chilean aquaculture (infectious diseases and problems in smoltification) and a warning about the need for more scientific research regarding this relationship between two different and disconnected fields of study.

FUTURE PERSPECTIVES

Smoltification in salmonids continues to be a field of study in constant progress: new transporters are being discovered that change their expression during this process (Fleming et al 2019, Koltenyuk et al 2020, McKay et al 2020), and the parr-smolt transformation process is characterised in new strains of salmon in culture (van Rijn et al 2020), or wild salmon (Bernard et al 2019). Even different aspects of the process are evaluated (Nemova et al 2020), and the biological character of the process continues in development (Striberny et al 2021).

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5 https://www.salmonexpert.cl/article/pathovet-da-un-paso-en-la-prediccin-de-peces-desadaptados-y-rezagados/
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