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Novel Scale Chemistry of *Salmo Salar* L. – New Linkage of Otolith and Scale Chemistry to M74 Reproductive Disorder

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Abstract

Baltic salmon suffer from maternally transmitted M74 reproductive disorder, which causes yolk-sac fry mortality due to thiamine deficiency. This study investigates trace elemental analyses of Baltic salmon applied on otoliths and scales and their possible link to identifying M74. Using laser-ablated inductively coupled plasma mass spectrometry, 170 female salmon from two Swedish rivers were chemically analysed. Egg thiamine concentrations were measured using chromatography. This study shows that novel scale chemistry aligns with traditional otolith chemistry regarding salmons' environmental and physiological patterns. Comparison between two chemical age-readers, using Mg:Ca, found 73% and 70% agreement for otoliths and scales, respectively. Comparing both structures revealed 51 – 58% agreement precision between both readers. Different degrees of vaterite formation were found in 48% of included otoliths but were not significantly correlated to M74. The chemical analyses mirrored trace elemental patterns reflecting salinity (Sr:Ca) and fresh- and saltwater transition (Ba:Ca) in both structures, revealing that scale chemistry can be used for migration studies. The Sr:Ba ratio in scales from severely thiamine deficient salmon (thiamine concentration < 0.4 nmol/g) showed significantly lower mean levels than healthier fish (> 8 nmol/g) throughout the whole life cycle, indicating that severely deficient salmon use coastal habitats more than healthier fish. The Sr:Ba ratio in scales correlated negatively to trace elements known for tracking pollution (Pb, U, Zn, and Cu). Additionally, a significant increase of B over life in the otoliths of severely deficient fish was discovered. Scale Sr:Ba shows novel promising results as a non-lethal sampling alternative for tracking M74 in Baltic salmon, with potential as a biomarker already early in life.

Keywords: Thiamine, M74, Salmon, Otolith chemistry, Scale chemistry, laser-ablation coupled inductively plasma mass spectrometry

Contents

Popular Science Summary

In the Baltic Sea, salmon are valued for the ecological, economic, and cultural benefits. They are anadromous fish, meaning their lifecycle starts at the river until they are strong enough to migrate out to sea, where they spend most of their life. They return to the river when they are ready for spawning. Thus, salmon have to adapt to different salinity levels (using osmoregulation), currents, temperatures, and more. In addition to these natural challenges, salmon have encountered other obstacles, such as anthropogenic structures, climate change, and diseases which contributed to the decline of salmon populations over the years. One disease commonly affecting salmon is the M74 reproductive disorder caused by thiamine deficiency, a vitamin B1, required for metabolic processes. When M74 persists, hatched eggs develop into yolk-sac fry (small, young fish that carry a food supply attached to them, in the form of a sack of egg yolk), but a high risk of mortality follows as the thiamine contents are too low for proper development. The underlying causes for the thiamine deficiency are yet largely unknown. In addition, biological markers that indicate M74 are missing, and the current thiamine analyses are complicated and time consuming. Furthermore, most studies are performed on hatchery fish only, thereby underestimating the incidence of M74.

To study major life-history events in fish, otoliths (ear stones) have widely been used. These stones are located near the brain, making studying the specimen of interest lethal. Thus, an alternative, non-lethal sampling method is desired. It was therefore investigated if either the chemical profile of the otolith, the scale, or both, could indicate a possible link to M74.
A total of 174 salmon, as part of the annual sampling procedure performed by the Swedish University of agriculture science, were sampled for both their otoliths and scales. The thiamine concentration was measured in 109 of them. Thiamine testing was performed by stripping the eggs of spawning females and measuring the concentration within these. More so, otoliths and scales underwent elemental imaging to determine the trace elemental composition of both structures.

A new approach to studying known trace elemental proxies on fish scales has been developed and showed promising results. Firstly, reading the chemical profiles of Barium (Ba) and Strontium (Sr) over the polished otolith cross-sections was previously known to indicate migration patterns. Secondly, by reading Magnesium (Mg), Phosphorous (P), and Zinc (Zn) over the same cross-section, the physical parameters, and age of the individual fish can be determined. First results show that chemical analysing of scales represents similar patterns as otoliths regarding both environmental (Sr:Ca, Ba:Ca) and physical (Mg:Ca, Zn:Ca, and P:Ca) parameters. Especially Mg has shown to aid in determination of the first year, while Zn and P were useful for additional years. Furthermore, it was found that severely thiamine deficient fish had significantly lower Sr:Ba values than healthier fish, in both scales and otoliths, suggesting that these fish lived in habitats closer to the coast. Therefore, Sr:Ba in scales is proposed as a new promising non-lethal biomarker for tracking M74.

Introduction

Keystone species are organisms that have an unproportionally huge effect on both their ecosystem and the survival of other species within this system (Paine, 1995). The Atlantic salmon (*Salmo salar* L.) is a major keystone species in the Baltic Sea with great ecological importance. Salmon are responsible for nutrient exchange between water bodies, serve as a food source for many organisms and control the food web (Hilderbrand et al., 2004). Unfortunately, they have suffered from a decline in great numbers over the past five decades (LaMere, Mäntyniemi & Haapasaari, 2020). While anthropogenic stressors such as overfishing are obvious contributors to the decline, other parameters such as limited access to spawning rivers (Romakkaniemi et al., 2003), predation and prey availability (Mäntyniemi et al., 2021), climate change (Eliason et al., 2011) and diseases are contributing factors.

Salmon lifecycle

Baltic salmon live an anadromous lifecycle, meaning they are born in rivers, migrate to and grow large at sea and return to their natal stream for spawning (Figure 1). Their lifecycle thus begins with fertilization of redds, a female nest of eggs that remain within the gravel during winter before the eggs develop into embryos and hatch during spring. The hatched salmon, called alevin, still have their yolk-sac attached to them for additional nutritional support. Once the sac is fully digested, the now called fry can feed to develop into smolts and then begin their migration towards the sea, where the time spent at sea varies between one to seven years, most commonly three or four years (Allan & Ritter, 1977). Once they are ready to spawn, the salmon return to their natal stream, stop feeding and prepare for spawning. While many salmon die after spawning and provide nutrients to the habitat (Webb et al., 2007), it is also possible for Baltic salmon to survive and repeat to spawn multiple times (Ducharme, 1969).

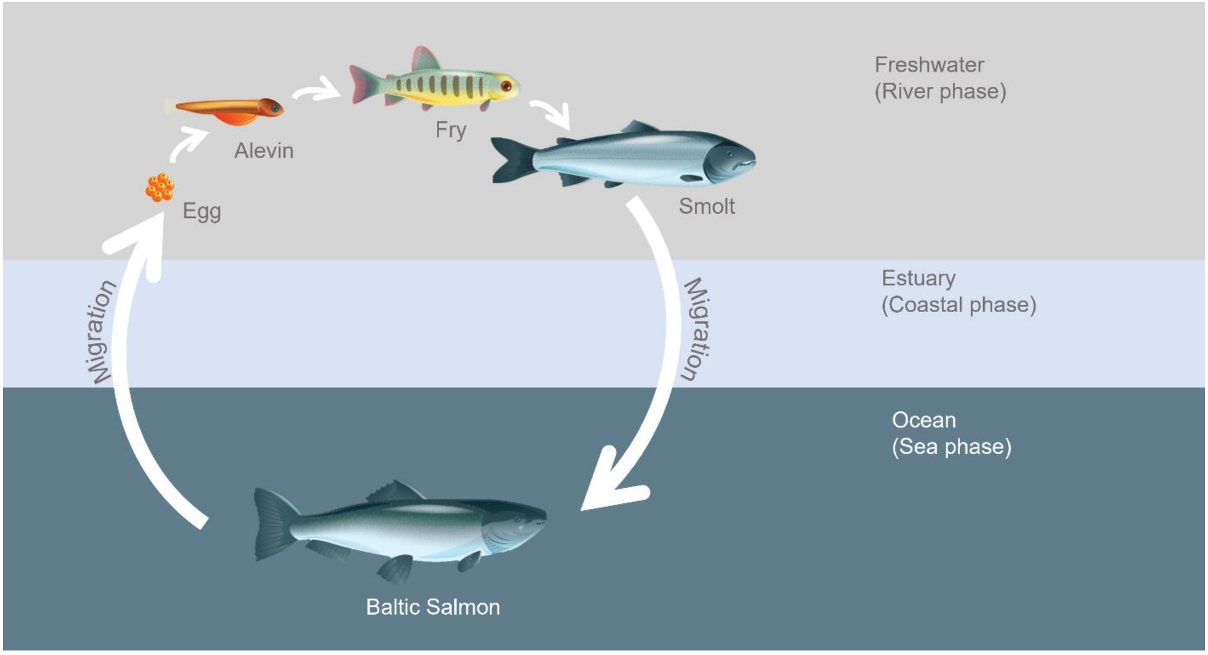

Figure 1. Simplified lifecycle of the Atlantic Salmon. Illustration from Macrovector/Shutterstock.com

Causes of decline

Salmonids, including the Baltic salmon, suffer from pollutants and contaminants that cause diseases and increase mortality. Additionally, overexploitation and anthropogenic modifications, such as hydropower plants and dams, have altered the natural ecosystem in rivers and formed obstacles for anadromous fish to return to their spawning habitat (Nilsson & Berggren, 2000). In Sweden alone, more than 10,000 dams are distributed over 80% of all existing rivers (Norstedt, 2008). While many fish ladders and hatchery stations have contributed to compensate for the loss of the population, genetic variation has yet greatly suffered (Hannson & Langefors, 2001).

Diseases are additionally contributing to the decrease of salmon in the oceans. Especially the M74 syndrome or M74 disorder (M = miljöbetingad, Swedish for environmentally induced, and 74 for being discovered in 1974), a reproductive disorder that increases the risk of yolk-sac fry mortality, causes concern. The occurrence of M74 shows to be linked to low thiamine contents within eggs and broodstock (Keinänen et al., 2012). Thiamine, a vitamin B1, is found in cells as thiamine monophosphate (TMP), Thiamine diphosphate (TDP), and non-phosphorylated thiamine. TMP serves as a reservoir for transforming thiamine and TDP while TDP is an important cofactor for various vital enzymes that regulate cell metabolism and convert energy into building blocks of the cell (Gustafsson et al., 2021). Thiamine deficiency thus disrupts the metabolism of carbohydrates, lipids, proteins, and major metabolic pathways (Engelhardt et al., 2020, Gustafsson et al., 2021).

Thiamine acquisition is mainly determined by the fish's diet as salmon predominantly feed on sprat and herring (Karlsson et al., 1999). Increasing fat content in sprat correlates to an increased need for thiamine for salmon (Hylander et al., 2020). The underlying causes of the deficiency in the Atlantic salmon remain largely unknown (Hylander et al., 2020). It is discussed that increased redox enzyme activities and biotransformation could contribute to M74 as disturbed metabolic processes (Vuori & Nikinmaa, 2000). Furthermore, it is unclear whether certain pollutants, especially polychlorinated biphenyls and 2,3', 4,4',5-pentachloro diphenyl ether could be correlated to M74, as shown in a study conducted by Vuorinen et al. (1997) or not, as contradicted by Asplund et al. (1999). Symptoms of living fish with the disease include reduced heart rate, abnormal swimming patterns, and impaired coordination (Vuori & Nikinmaa, 2000). In sacrificed fish, a pale and small spleen and necrotic cells can be observed. Although the causal factors are not yet fully discovered, treatment with thiamine has shown to be effective (Vuori & Nikinmaa, 2000).

Monitoring the occurrence of M74 is executed by stripping the eggs from spawning females and observing their colour and development. It was previously discovered that the red pigment and antioxidant astaxanthin causes various colour changes among eggs, where strong orange colour indicates healthy eggs and thiamine deficient eggs express a pale colour (Pickova et al., 1998). When the thiamine concentration is measured, levels below 0.4 nmol/g indicate acute thiamine deficiency and thus definite yolk-sac fry mortality, 3.9 nmol/g indicates the threshold for mortality in offspring, and 9.3 nmol/g measures the threshold for 20% of reduced growth (Balk et al., 2016).

Other diseases, such as red-skin disease (RSD), were also frequently reported over the last decade. RSD causes clinical signs in salmon that include but are not limited to wounds, hemorrhages, and pathogenic infections along the abdomen of the fish. Identified pathogens from the examination areas include bacteria, fungi, and viruses, but the cause of RSD cannot be linked to a specific specimen (Weichert et al., 2020).

Additionally, the ongoing climate change has its impact. Global warming causes physical changes to the water temperature as well as to the water flow, salinity, and acidity of the ecosystems. These alterations automatically affect the biological environment by actively promoting the growth of cyanobacterial species leading to eutrophication (Lürling et al., 2018). The Thünen Institute of Fisheries Ecology of north Germany reported that two microscopic algae, *Heterosigma akshiwo* and *Chattonella verruculosa*, caused an excessive algal bloom in 2001, from which over 400 salmon died (Johann Heinrich von Thünen Institute, 1999-2002).

Due to their anadromous lifecycle, salmonids pass through various habitats throughout their lifespan and must be able to adapt to physical, biological, and environmental variation, including changes in current, temperature, and salinity of the different water bodies (Schiewe, 2013).

Monitoring migration patterns

Several tracking methods, such as external marks with fin clips and satellite tags or internal marks, as well as recapture methods, have aided in retrieving snapshot insights into certain population dynamics in the past (Skalski, Buchanan & Griswold, 2009). However, these methods are limited by showing only temporal patterns and are often cost-intensive. Trace elemental chemistry has majorly improved this research field (Sippel et al., 2015).

Otolith and Scale Chemistry

To study major life-history events in fish, otolith chemistry has become an essential tool (Campana & Thorrold, 2001). Otoliths are biominerals, mainly consisting of aragonite (calcium carbonate) but also a small amount of organic matrix proteins, that are situated in the hearing and balancing system, located in the vestibular system near the brain (Campana, 1999, Thomas & Swearer, 2019). Each bony fish has three pairs of otoliths (sagitta, lapillus and asteriscus), all with variations in shape and size among species (Campana & Thorrold, 2001, Popper, Ramcharitat & Campana, 2005). Otolith growth is based on an accumulation of material layers around the core, forming growth zones in alternating opaque and translucent rings representing slow and rapid growth, respectively (Figure 2) (Campana & Thorrold, 2001).

Especially the strong correlation between otolith- and somatic growth has previously shown promising results as the otoliths continue to form growth zones throughout the fish's entire life which can be chemically analysed (Hüssy et al., 2020). Age determination is performed by counting the number of growth zones (Campana & Thorrold, 2001). The growth zones additionally contain various trace elements that are primarily absorbed through the gills or gut wall from the surrounding environment (Watanabe et al., 1997). The absorbed elements remain in the blood plasma and are passed through to the endolymph, following incorporation into the otolith (Kalish, 1989).

Microchemical analysing techniques of the otolith trace elemental composition provide detailed information about changes in the environmental conditions and fish physiology, which can be applied to study migration behaviour, provenance, population structure, hypoxia exposure, age, and metabolic activity (Hüssy et al., 2020). Although this is a promising and reliable tool, it is not ideal for studying vulnerable or endangered species as it remains invasive to the studied fish. Moreover, while otoliths primarily consist of aragonite, an alternative polymorph of calcium carbonate known as vaterite can sometimes form. This deformation has a nearly four times higher prevalence in farmed salmon than wild salmon, evidently partly caused by faster growth (Reimer et al., 2016). These vateritic structures impair the hearing and balancing system of the fish, are irreversible and can be distinguished by their rather transparent, crystalline structure (Figure 3) (Reimer et al., 2016).

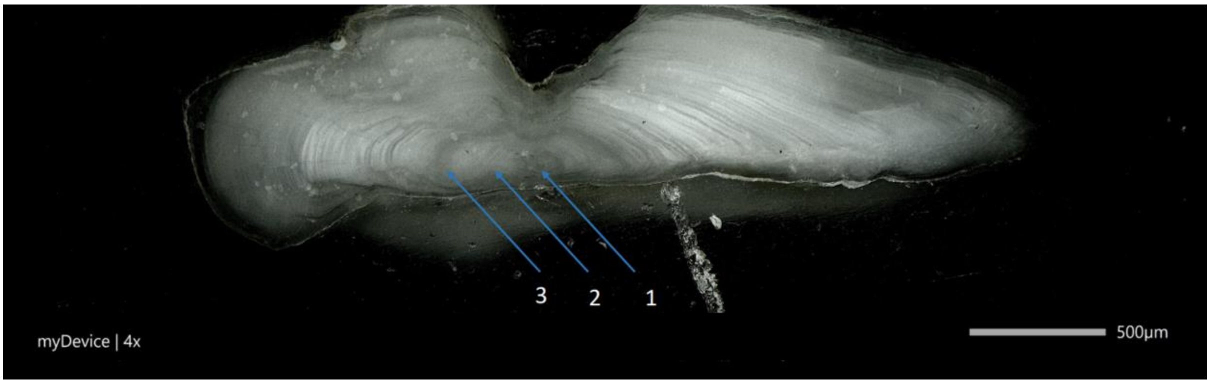

Figure 2. Transverse section of an otolith: (1) core, (2) opaque zone, resembling fast growth and (3) translucent zone, resembling slow growth (3).

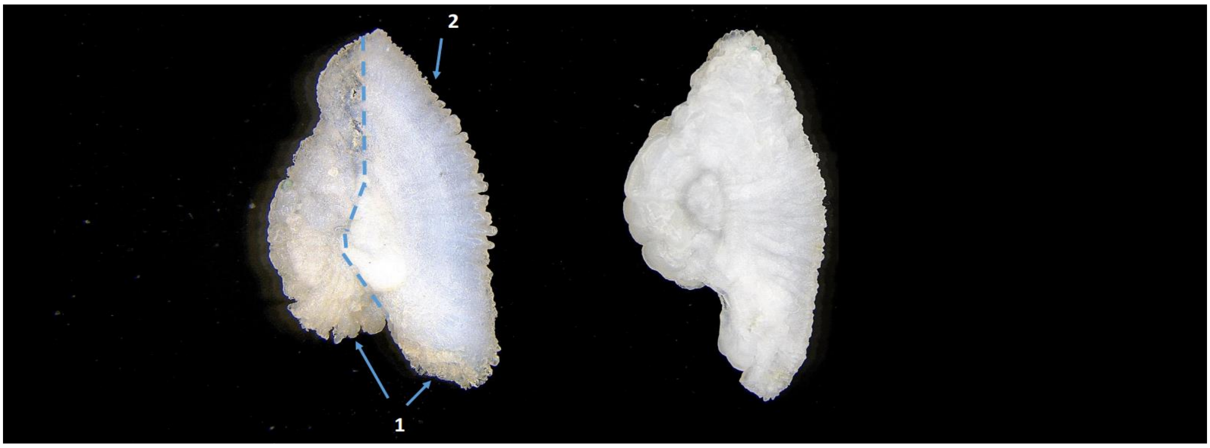

Figure 3. Left otolith (1) Indicating vaterite formation, clearly separated from (2) which shows the aragonite structure. Right otolith being fully aragonite.

In addition to otoliths, other calcified structures containing growth zones, such as opercula, vertebrae, rays spines, and scales, have been elementally analyzed. With the increasing demand for a nonlethal sampling alternative, especially scales have earned growing interest. Salmon have cycloid scales, comprised of an inner collagen layer and a surface layer of calcium-based salts (Hall, 2015). Concentric rings known as circuli are added to the scales with fish growth. The collagen is arranged in layers, growing from the center (focus) outwards, layering upon the previously built layer. Seasonal variation is shown due to slow growth in winter and the formation of narrow circuli, termed annuli (Ostrander & Elliott, 2000). Scales are considered one possible alternative as measuring the width of the annuli makes it possible to retrospective-calculate age and reconstruct growth history patterns (Panfili, Tomás & Morales-Nin, 2009).

Trace elements and Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)

LA-ICP-MS is a widespread method that helps to determine the compositional analyses of solid materials, including but not limited to otoliths and scales. Increasing sensitivity, precision, and accuracy allows for element- and isotope analyses of these materials. The LA-ICP-MS system has a pulsed laser operated at atmospheric pressure using matrix-matched calibration standards. This method reveals spatial trace elements which show the individual's migration patterns and age,nd can thus be applied to study certain life history events in migratory fish (Koch & Günther, 2010).

Aim

The aims of this study were to

(1) Find a non-lethal sampling alternative for identification of M74 reproductive disorder in order to replace the current, egg-stripping technique
(2) Develop monitoring techniques to extend the knowledge on environmental and physiological factors of salmon with linkage to M74
(3) Compare and validate trace elemental analyses of scales as non-lethal sampling alternative to otolith chemistry to reveal age and migration patterns

Materials and Methods

Ethical considerations

The salmon structures, otoliths, and scales were collected as part of the fish health monitoring program by the Swedish Veterinarian Institute (SVA) as well as the annual sampling procedure at the hatchery in Älvkarleby (Dalälven river) and Norrfors (Umeå river) from the Swedish University of Agricultural Sciences. The care and use of experimental animals complied with animal welfare laws and regulations set by the Swedish Government and the Swedish Board of Agriculture. The study followed guidelines and policies approved by the Swedish Board of Agriculture (permission to use animals for scientific purposes, license nos. 31–1069/12 and 5.2.18–443/17) and permissions from the Gothenburg Animal Ethics Committee for the specific surveys (license nos. 255–2012 and 126–2017). The samples used here were covered by a permit from the Swedish Board of Agriculture (license nos. 6.7.18–13.720/2019).

Sample collection

Over October and November 2021, a total of 174 female salmon were collected from two Swedish rivers, Dalälven (n=110) and Ume/Vindelälven (n=64) (Figure 4). All included fish were tagged with PIT tags, small radio transponders that allow for assigning of digital identification numbers. The fish were sacrificed and both otoliths and approximately 20 scales of every individual were collected and documented. The scales were sampled above the lateral line between the posterior edge of the dorsal fin and the anterior edge of the anal fin using small knives.

Figure 4. Map of the Baltic Sea, with the two sample locations, where X2 shows the Ume/Vindelälven near Umeå and X1 shows Dalälven near Älvkarleby. Illustration from Peter Hermes Furian/shutterstock.com.

Thiamine testing

Following the thiamine testing manual (Appendix I), around one tablespoon of unfertilized eggs from each female salmon was collected and sieved to remove excess liquid and improve the thiamine assays. The eggs were frozen and stored in zip-lock bags at -20°C. From each fish, the total body weight and length were documented. The eggs underwent aqueous extraction followed by reversed-phase ion-pair chromatography ESI+ (electrospray) MS-MS (Manne Larsson, 2020).

To investigate possible associations between M74 and the trace elemental concentration, each individual fish was assigned a defined group based on the degree of severity of M74, thereby incorporating literature research and known thresholds according to the thiamine concentration measured in nanomole (nmol) (Table 1).

Table 1. Degree of severity of thiamine deficiency, from Severe to None.

	Severe	**High**	**Moderate**	**Mild**	**None**
	0 – ≤ 0.4nmol/g	> 0.4 - ≤ 3nmol/g	> 3 - ≤ 5nmol/g	> 5-≤ 7nmol/g	> 7-9 nmol/g
n=	17	49	49	25	25

This grouping allows for comparison of severely deficient fish to other groups which enables identification of possible indications of impact of certain trace elements that decrease or increase thiamine deficiency, when comparing the severely deficient fish to the healthy individuals.

Sample preparation

Scales and otoliths were collected from all individuals. Both structures were cleaned with deionized water followed by ethanol. Pictures of the otoliths were taken on both sides, front and back, using a Jenoptik GRYPHAX ® camera and a stereo microscope (Leica M205 C). The same microscope was used to image the selected and mounted scale before cutting.

Preparations of scales

After selecting non-damaged, clear scales, it was ensured that only non-regenerated scales were included. Regenerated scales differ from the other scales as they lack compact circuli around the core of the scale. Next, the sides of the scales were cut to increase the capacity of the glass slides (Figure 5). Using double sided tape, the scales were mounted and labelled on the petrographic glass slides with the inner, smooth part facing the glass slide.

Preparations of otoliths

Through visual assessment, aragonite sagittal otoliths were selected and placed in deionized water for cleaning, then added to mounts. Epoxy was mixed with Struers Epoxy Fix and added to the mounted otoliths. Once solidified, the otoliths were ground into transverse cross-sections, starting from the rostrum down (Figure 6) using a MetaServ® 250 Grinder-Polisher (Buehler). Each section was hand-polished using grinding papers varying between P400 to P2500, until the inner core was exposed. For vaterite-rich otoliths, flat grinding was chosen. Regardless of the grinding method, the uppermost layer was polished and treated with liquid diamond (Kemet) type WX Xstr 1 micron to allow for clear reading. Each otolith was polished to a size between 3.5 and 4mm before mounting them to glass slides with double-sided tape.

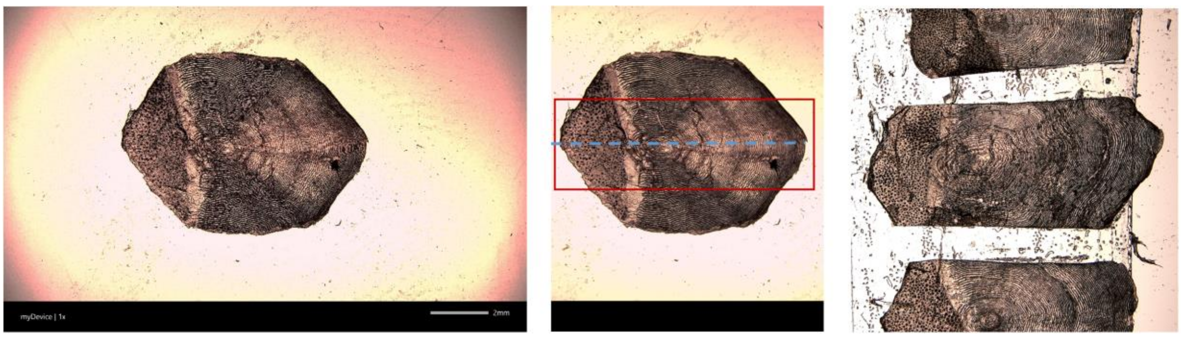

Figure 5. From left to right showing the full scale, red box on the middle picture providing an example of how the scales were cut to increase the space capacity on the glass slides and the right picture shows the final result. Blue dashed line indicates the section read by the laser.

Examination of the otoliths revealed vaterite formation, wherefore the otoliths were grouped according to the amount of perceptible vaterite (Table 2). This also led to the exclusion of four fish as both their otoliths were strongly affected by the vateritic structure.

Table 2. Grouping of the otoliths based on the amount of vaterite visible through the microscope.

Group	Amount of vaterite
1	0%
2	<10%
3	<50% but not less than 10%
4	>50% but not more than 90%
5	>90%

Both scales and otoliths were sent for LA-ICP-MS laser analysis at Lund University, Sweden.

Trace elements and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)

The LA-ICP-MS analyses of the salmon otoliths and scales were performed at the Department of Geology at Lund University in Lund, Sweden. The surface of the scales and cross-sections of the otoliths were ablated prior to analyses to avoid contamination. The laser beam ablated material along a line-transect from the dorsal edge through the core to the ventral edge, following the maximum growth axis on the cross-sections of the otoliths (Figure 6, Figure 7). For the scales, the laser beam ablated material from the focus to the edge (Figure 7). The material was consequently transferred to the mass spectrometer, where trace elemental concentration was detected as counts per second (cps). Two silicate glass reference materials, NIST 610 and 612 (National Institute of Standards and Technology, USA, Jochum *et al.*, 2011), were analyzed to assess instrument performance and correct for machine drift and mass bias. Matrix-matched reference materials were used as primary standards, with the calcium carbonate reference standard MACS-3 (Jochum *et al.* 2012) for the otoliths and apatite standard MAPS5, developed by USGS (United States Geological Survey) for the scales. The reference materials were analyzed before, between, and after samples. The isotope 43Ca was used as an internal standard. Detailed instrumental settings are described in Supporting Information, Appendix II. Data reduction of the analyses were carried out using Iolite software (v 3.63) (Paton et al., 2011).

Included elements were Lithium (^{7}Li), Boron (^{11}B), Sodium (^{23}Na), Magnesium (^{25}Mg & ^{26}Mg), Phosphorous (^{31}P), Potassium (^{39}K), Titanium (^{49}Ti), Chromium (^{52}Cr), Manganese (^{55}Mn), Copper (^{63}Cu), Zinc (^{66}Zn), Strontium (^{88}Sr), Iodine (^{127}I), Barium (^{137}Ba), Mercury (^{202}Hg), Lead (^{208}Pb), Uranium (^{238}U), Sulphur (^{33}S) for otoliths and scales with additionally Iron (^{57}Fe) and Cobalt (^{59}Co) for the scales. The known proxies are illustrated in table 3.

Trace elements reflecting different proxies were quantified as parts per million (ppm) and reported in a ratio to calcium ^{43}Ca. For otoliths, the calculated Ca in ppm was used, but for scales from spawning females, resorption of Ca risked altering the results and therefore a mean value of 37.4 ± 0.4% Ca previously reported in Atlantic salmon scales (Flem et al., 2005) was used.

Table 3. Known trace elemental proxies within otoliths and scales.

Trace elements	Known proxies
	Environment
Sr	Sr in otoliths indicates movement between freshwater and marine habitats (Halden & Friedrich, 2008) where low salinity regions correspond to low levels of Sr (Kraus & Secor, 2004, Hale & Swearer, 2008)
Ba	Ba, in otoliths can act as marker for transition between fresh- to saltwater (Hale & Swearer, 2008; Tabouret et al., 2010), reflecting the ambient water concentration (Bath et al., 2000; Elsdon and Gillanders, 2005)
Sr:Ba	Otolith Sr:Ba indicates the relationship between freshwater, costal and offshore habitats (Heimbrand et al., 2020)
Mn	Mn reflects hypoxia exposure in otoliths (Limburg et al., 2011, Altenritter, Cohuo & Walther, 2018) Sr, Ba and Mn in fish scales can indicate provenance (Wang et al., 2016) and migration (Campell et al., 2015)
	Physiology
Mg	Mg in otoliths indicates the metabolic status (Limburg et al. 2018).
P	For chemical age estimation, the Zn ratios and visual observation correlate, oscillatory distribution in otoliths (Halden & Friedrich, 2008) while more recently also the proxies for growth and metabolic activity, Mg and P, have shown to be suitable elements to identify age as they show high seasonal variation in concentration (Heimbrand et al., 2020)
Zn	Zn in Baltic salmon otoliths show seasonal variations (Limburg & Elfman, 2017), suitable for ageing, indicate somatic growth (Thomas et al., 2019), and can in female fish act as a spawning indicator (Sturrock et al. 2015)
Mn	Mn in otoliths can reflect rapid somatic growth to some extent, especially for juvenile fish (Heimbrand et al,. in prep)
	Pollution
Pb **Hg** **Cu** **Zn** **U**	Pb, Hg, Zn and Cu bioaccumulate in fish scales, indicating metal contamination (Shakir et al., 2020) U has shown to bioaccumulate in otoliths as an indication of contamination (Mounicou et al., 2019)

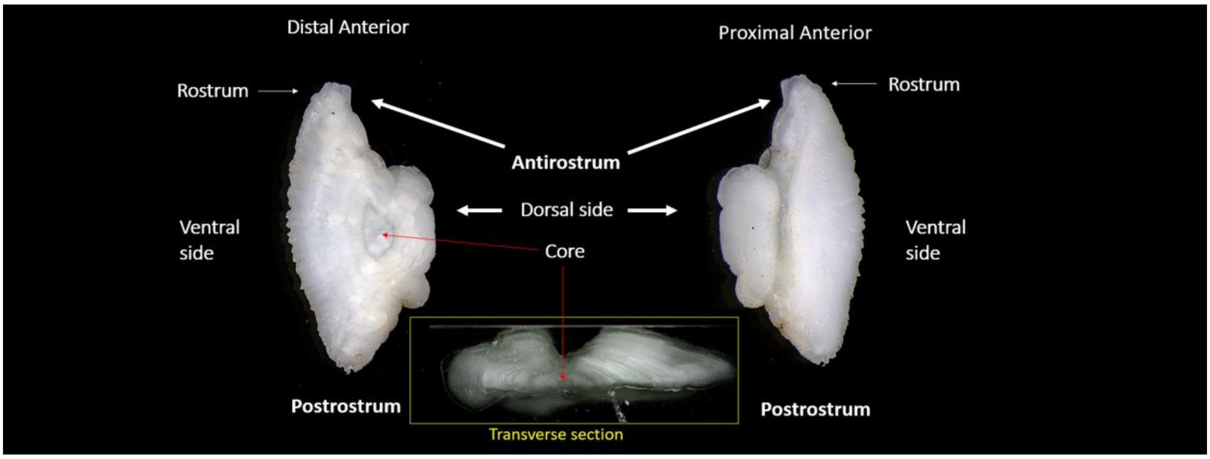

Figure 6. Anatomy of the otolith. The yellow box shows the transverse cross section when the otolith is polished down from the rostrum to the core.

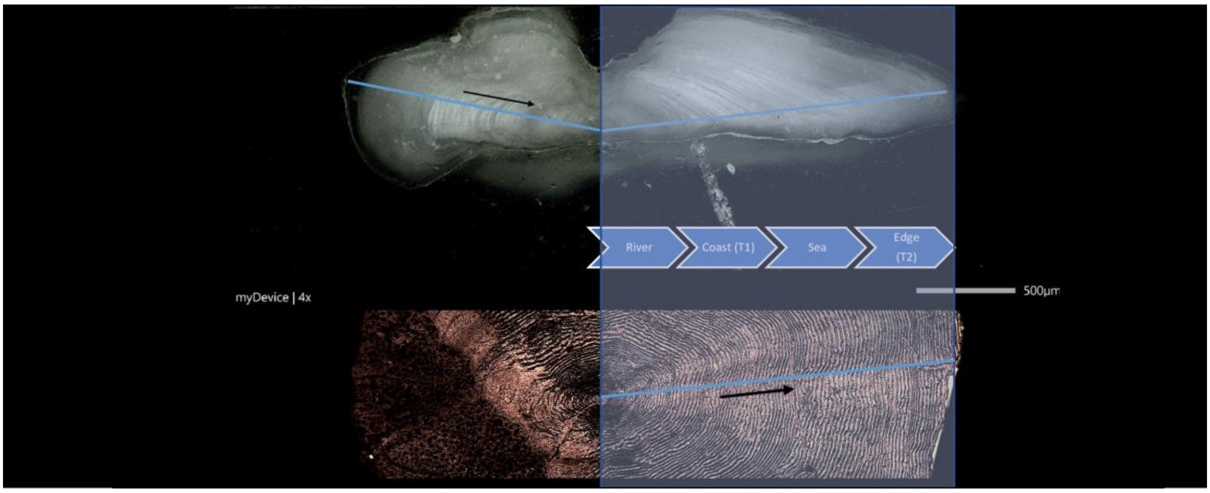

Figure 7. Otolith (top) and scale (bottom). Blue line on each structure shows the laser transect, black arrows indicate the direction. Blue box shows the analyzed part of each structure.

Chemical data reading

After the reduction of the laser analyses, an Excel template was created. The Mg:Ca, P:Ca and Zn:Ca ratios were used for age estimation for both structures, Sr:Ca and Ba:Ca/Mn:Ca ratios for migration pattern determination (Figure 8, Figure 9, Table 3).

The analyses included separating the data into several life-phases, with detailed insight into the core, measuring the distance from the core in micrometre (µm), and the core being defined between 0 and 400 µm and data entries for every 100 µm step. In a similar matter, the edge of the otolith was defined as the last 60 µm from the time point of death; thus, the outer rim of the otolith was measured in steps of 15 µm, 30 µm and 60 µm.

Following a validation method developed by Hüssy et al. (2020b), the distance from the core of the otolith to the first minima visible in the chemical profile of Mg:C, Zn:Ca and P:Ca ratios can be measured to define the first year of life. This is continued from the first minima to the second minima, defining the second year, and so forth (Figure 8).

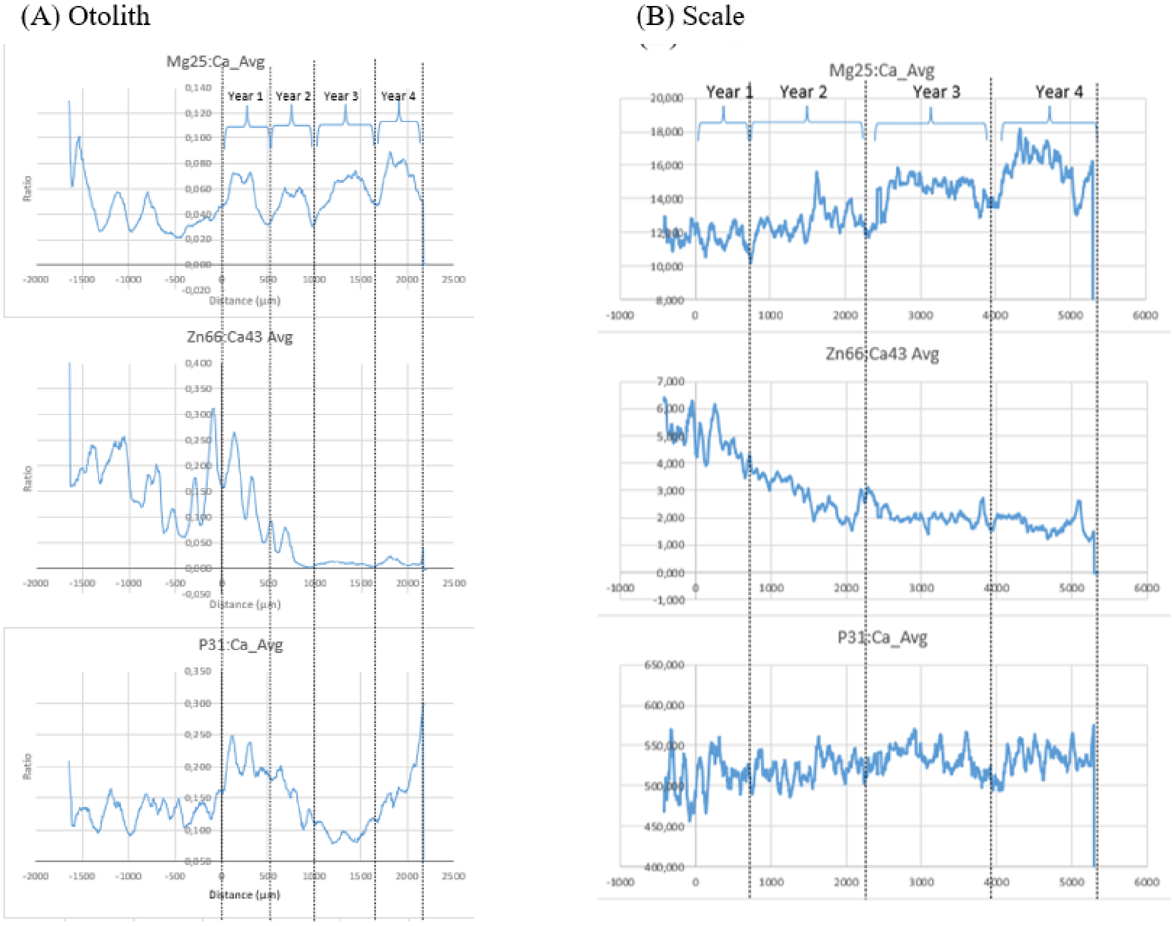

Figure 8. Chemical age determination using Mg:Ca, Zn:Ca and P:Ca ratios on the ventral side (right side of the y-axis) of the otoliths (A) and scales (B).

The major migration patterns were documented using Sr:Ca and Ba:Ca/Mn:Ca for both structures. This implied that low Sr:Ca corresponds to low salinity, thus the time spent at the river after birth until smoltification, and an increase in Sr:Ca indicates the migration towards the ocean as this ratio documents the respective increase in salinity of the environment. A steady higher concentration of Sr:Ca shows how long the fish remained at the ocean. Within the last µm of each structure, a drop in Sr:Ca was observed, representing that the fish enter an environment with lower salinity levels which suggests the migration back toward their natal river for spawning (Figure 9). Supporting the observations through Sr:Ca, Ba:Ca in otoliths and Mn:Ca in scales aid in visualizing coastal signals. When the fish migrates toward the sea, the Sr:Ca ratio rises and the Ba:Ca/Mn:Ca ratios peak, followed by a decline, indicating a change in salinity among the surrounding waters. This pattern, although inversed, is observed when the fish migrates back to the coast (Figure 9). As the transverse section for the otoliths was laser ablated from the dorsal edge through the core to the ventral edge, either the ventral or dorsal side can be read for chemical ageing due to symmetrical growth, which furthermore allows for determination of the core. However, only the longer ventral side of the otolith was read in this study (Figure 8, Figure 9).

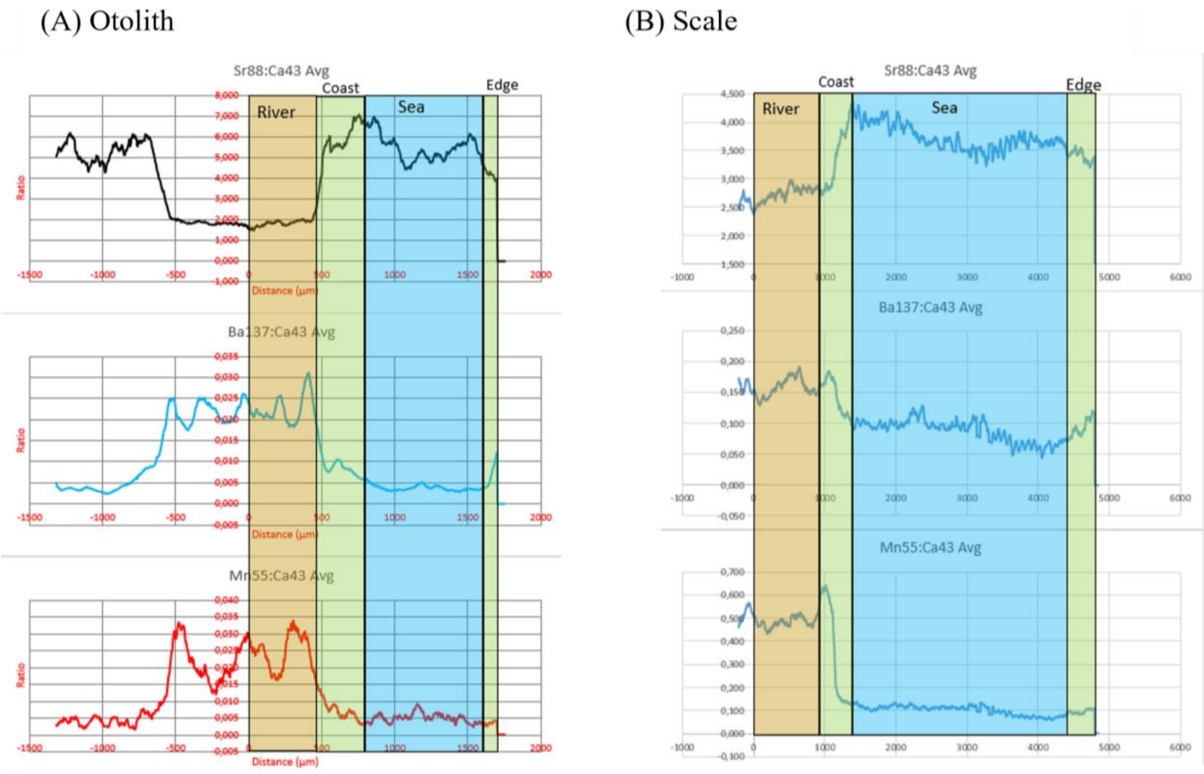

Figure 9. Determination of migration pattern using Sr:Ca, Ba:Ca and Mn:Ca on (A) otoliths (ventral side (right side of the y-axis) and (B) scales. Example with Fish number U32, Umeå.

Once the different life stages were established, all included elements were determined based on the major life history events. This provided detailed input in the concentration of each element during the River-, Coastal- (migration to sea), Sea- and edge (migration to river) phases. The elemental concentrations in all life-phases were reported as average, minima concentration and maxima concentration. More so, using the elements for metabolic activity and age determination, each included trace element was also reported in their ratio to calcium during every year of life.

Data analysis

The statistical analyses of this thesis were carried out in R version 4.1.2 (R Core Team, 2021) and Excel. Blind age-reading was performed and statistically tested for agreement analyses and age bias among two age readers and both structures. The degree of thiamine deficiency was accessed, and the two most contrasting severity groups were correlated to all available time points and the correlation coefficient (R^2) and p-value were calculated. For all performed tests, the significance level was set at 0.05.

To find possible links between certain trace elements to thiamine, TMP or TDP, the data was reduced and simplified, with focus on the average of each element during the ocean (sea-) phase and the average during spawning migration (edge-phase). Thereby, the two most contrasting severity groups based on the degree of thiamine deficiency, severe and none, were included. The Hmisc package was applied to compute significance levels for Pearson and Spearman correlations to determine the R^2 and p-values for all the possible pairs in the created data table, after which the data was filtered to retrieve only significance values with $p < 0.05$.

Results

Novel scale trace elemental chemistry

Laser-ablation coupled plasma mass spectrometry is an essential tool to reconstruct life-history events in fish. As several trace elements are already known to represent specified proxies among fish otoliths, this study compared the results from trace elemental analyses of Baltic salmon otoliths to their respective scales. Applying the same analysing technique as for otoliths, where the ratios Mg:Ca, Zn:Ca, and P:Ca, measured in parts per million (ppm), evidence seasonal variation and thus reflect both age and metabolic activity, was used to access whether scale chemistry has overlapping features as otolith chemistry and revealed similarities among patterns. To validate these findings, help of an expert traditional age-reader was used where a sporadic random selection of scales were aged based on their visual appearance and counting visible annual growth zones to ensure adequate chemical age reading.

The estimate percentage agreement and variation were assessed to confirm salmon otolith and scale chemistry validity. Table 4 presents the readings of two age readers, with over 70% agreement between both readers for each structure and over 50% agreement between the assigned ages of each structure. The age bias among both readers reveals variation of up to two years when comparing the structures, although the majority aligns (Figure 10). Otolith and scale ageing reached an acceptable agreement; however, otoliths display more distinct seasonal patterns and are easier to interpret due to long use of the method.

Table 4. Agreement of ageing between both readers and both structures. Data based on Appendix III.

Comparison	Percentage agreement	Coefficient of variation
Otoliths (n=60)	73,3 %	5,3
Scales (n=60)	70,0 %	5,4
Both structures (n=60): Reader 1	51,7 %	10,3
Both structures (n=60): Reader 2	58,3 %	8.1

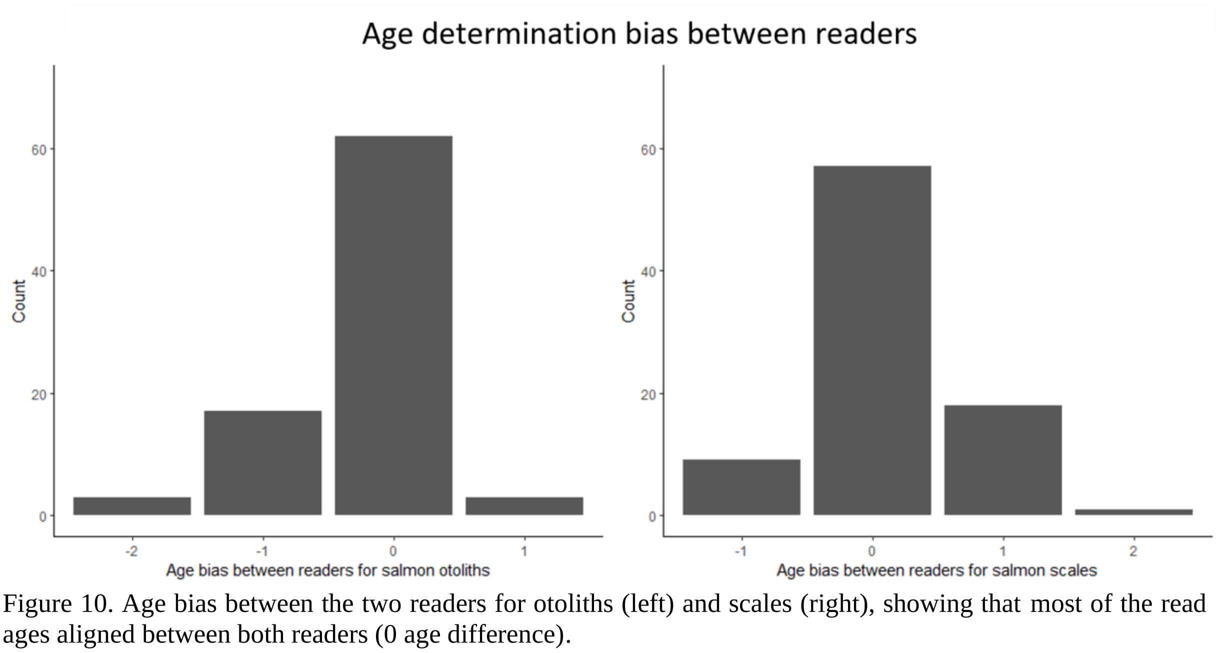

Figure 10. Age bias between the two readers for otoliths (left) and scales (right), showing that most of the read ages aligned between both readers (0 age difference).

Regarding the environmental parameters, variations among the Sr:Ca ratio and the Ba:Ca ratio were observed in the otoliths and corresponding seasonal variation patterns were discovered in the scales. While Sr:Ca follows a similar pattern in both structures, Mn:Ca was used as an additional coastal signal to Ba:Ca in scales (Figure 9).

Statistical analyses of potential proxies for M74

For identification of potential elements that could be applied as proxies for M74, the results from both scale and otolith chemistry were compared using the proxies for environmental patterns, where Sr represents the salinity levels of the surrounding water, and Ba suggests the freshwater/coastal signal. Additionally, the measured thiamine concentrations among the eggs of each individual fish allowed for assigning the fish into different groups, based on the severity of thiamine deficiency (Table 1). A comparison among the thiamine deficient fish (group: severe) and not-thiamine deficient fish (group: none) was performed.

Focusing on these migration patterns using Sr:Ca and Ba:Ca in correlation to thiamine concentration showed that fish from the Älvkarleby river with severe thiamine deficiency had lower Sr and higher Ba values, revealing that they returned to the coast/river earlier than healthier fish. This pattern is seen in both scales and otoliths, while more prevalent and significant in the otoliths (Strontium: p=0.027) (Appendix IV).

Additional tests were performed focusing on the Sr:Ba ratio, as the two environmental trace elements correlate to one another. This showed a clear coastal signal at the edge, but also at the river and sea phase, for the severe group in comparison to other groups. More so, especially the difference between the most severely thiamine deficient group and the least severe deficient group is significant in the scales (Figure 11) as the severely deficient fish have a Sr:Ba value significantly lower compared to healthier fish. These results clearly suggests that severely thiamine deficient fish use coastal habitats as their main living ground to a higher degree than healthier individuals who transition out to the ocean.

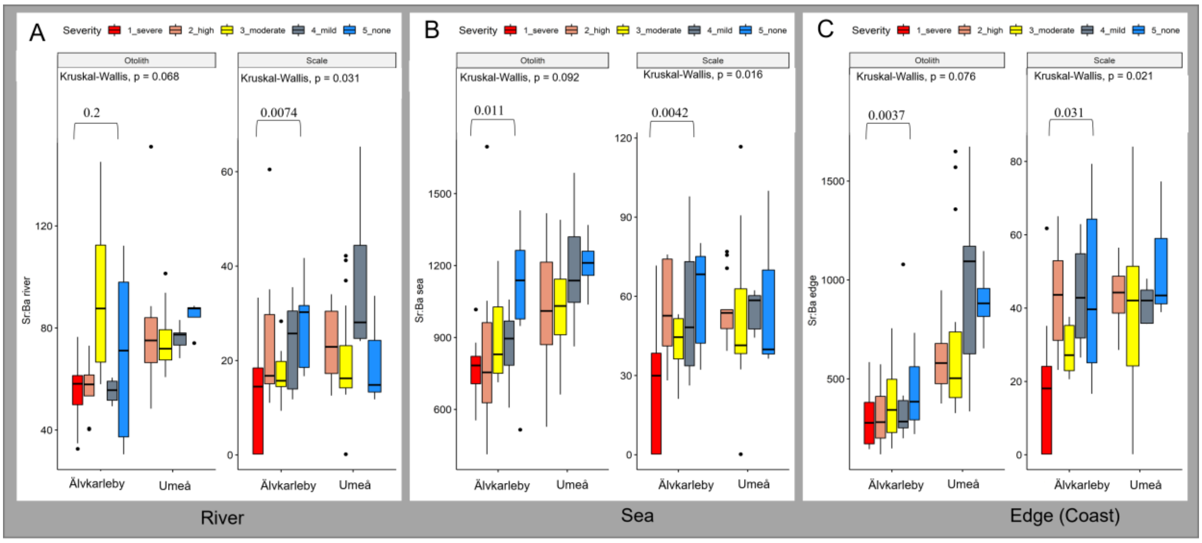

Figure 11. Ratios of Sr:Ba correlated to thiamine content in both otoliths and scales in both Rivers. A) showing Sr:Ba at river phase, B) showing Sr:Ba at the sea phase and C) showing Sr:Ba at the edge.

Furthermore, the severe group shows mean ratios of scale Sr:Ba of 26 ppm and 20 ppm in the sea and at the edge, respectively. All other groups have mean ratios significantly higher, especially the least-severe (none) group has values of 60 ppm and 45 ppm, respectively (Appendix V). Further comparisons within the most severe group indicated that the Sr:Ba ratio shows to be negatively correlated to the trace elements which are known for tracking pollution, such as Pb, U, Cu and Zn (Figure 12). Significant correlations have been found in scales throughout the entire life, but most outstanding at the edge (end of life) (Figure 12).

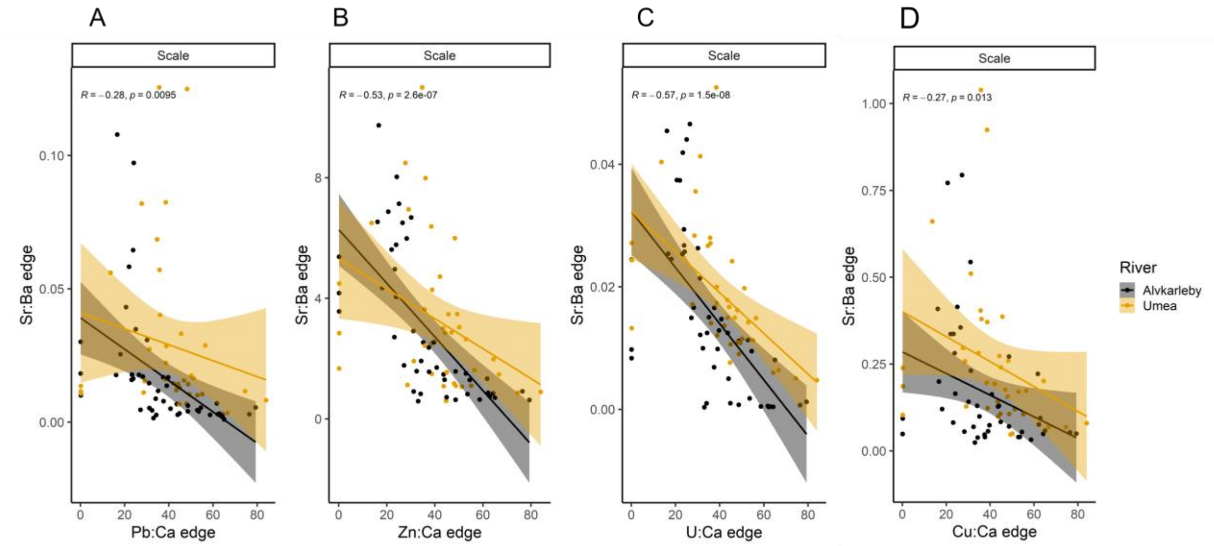

Figure 12. Correlation plots between Sr:Ba ratio (y-axis) to A. Pb:Ca, B: Zn:Ca, C. U:Ca and D. Cu:Ca at the edge.

Possible links between certain trace elements to Thiamine, TMP or TDP

As thiamine exists as free thiamine and furthermore is converted to TDP, a cofactor for several vital enzymes involved in metabolism, and TMP, a degradation product, all three of these forms were measured and correlated to the concentration of the trace element analyses in both structures, scales, and otoliths.

Among the otoliths of the severe group, there are strong positive correlations of Sr:Ca during the sea-phase to TDP, while Na:Ca during the migration phase negatively correlates to TDP. More so, U:Ca at sea and Cr:Ca and Ba:Ca during spawning migration have a strong negative correlation to thiamine (Figure 13 A). In contrast, the otoliths of the group with the lowest severity of deficiency (none) show a strong positive correlation of Mg:Ca during spawning migration to thiamine, indicating that their metabolic activity is higher compared to fish with lower thiamine concentrations. Several elements among the otoliths of this severity group correlate positively to TDP and TMP, while the Sr:Ba average at both included life-phases negatively correlated to TDP and TMP (Figure 13 B).

In the scales of the severe group, the elements displaying correlation to thiamine or TDP are I:Ca at sea and B:Ca during spawning migration which correlates positively and negatively, respectively (Figure 13 C). The possible proxy for I is currently unknown. The not-severe group displays only positive correlations to TDP and TMP, none to thiamine (Figure 13 D).

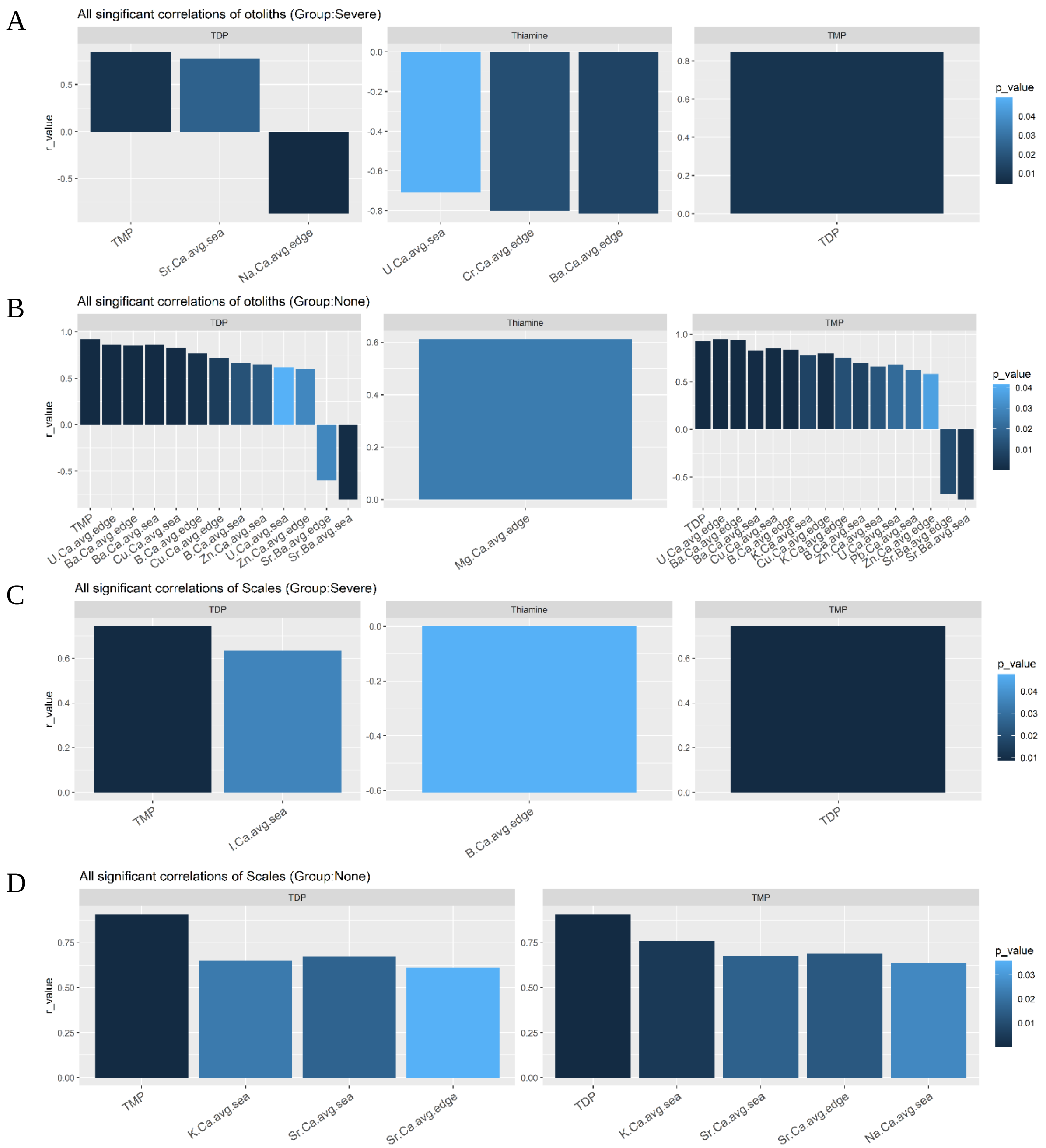

Figure 13. Significant correlations between trace elements to thiamine, thiamine diphosphate (TDP) and thiamine monophosphate (TMP), with (A) Otoliths of the severe group; (B) Otoliths of the not-severe (none) group; (C) Scales of the severe group; and (D) Scales of the not-severe (none) group. For D, there were no significant correlations to thiamine. The R-value is shown on the y-axis and the elemental ratios on the x-axis. The colour gradient indicates the degree of significance. Positive values show the strength of positive correlations and negative values show the strength of negative correlations.
Abbreviations: “avg.sea” = average of all values from time spent at sea. “avg.edge” = average during spawning migration.

One scale of one thiamine deficient fish from the Ume/Vindelälven (Umeå) was selected for whole-region mapping, thus, a selected region of the scale was fully laser ablated. Almost all trace elements reflect two major life-history events as two circuli (not shown). Especially interesting to observe is the increase in Pb over the lifespan and heavier concentrations of B and Cr toward the end of life (Figure 14). A paired T-test was performed to investigate whether these patterns reflect in all severe group fish. For otoliths, no change of Pb and Cr was detected over time (p=0.25 and p=0.58, respectively), while the increase of B (p=0.004) was. In scales, the concentration of Pb and B increased over time (Pb: p=0.013, B: p=0.004) but there was no change in Cr (p=0.13). The same elements were compared in the not severe group (none), with the otoliths showing a significant increase in Cr (p=0.036) and B (p=0.012) but not in Pb (p=0.4). The scales showed the same pattern, a significant increase of Cr (p=0.0001) and B(p=0.01) but not in Pb (p=0.06).

Additionally, Kruskal-Wallis one-way ANOVA was performed to find possible significant correlations between the severe and not-severe (none) group regarding these three elements, and no significant difference was found for either element. Thus, it was further tested whether the change in concentration of these elements over life was significantly different between either severity group. This showed a significant difference in B among the otoliths over time (p=0.0001), while it was not significant in the scale (p=0.84) nor in either structure for Cr or Pb. While there is no significant difference in B between the two severity groups at the end of life, it can be concluded that fish with severe thiamine deficiency generally might encounter bigger proportions of B throughout their life compared to healthier fish.

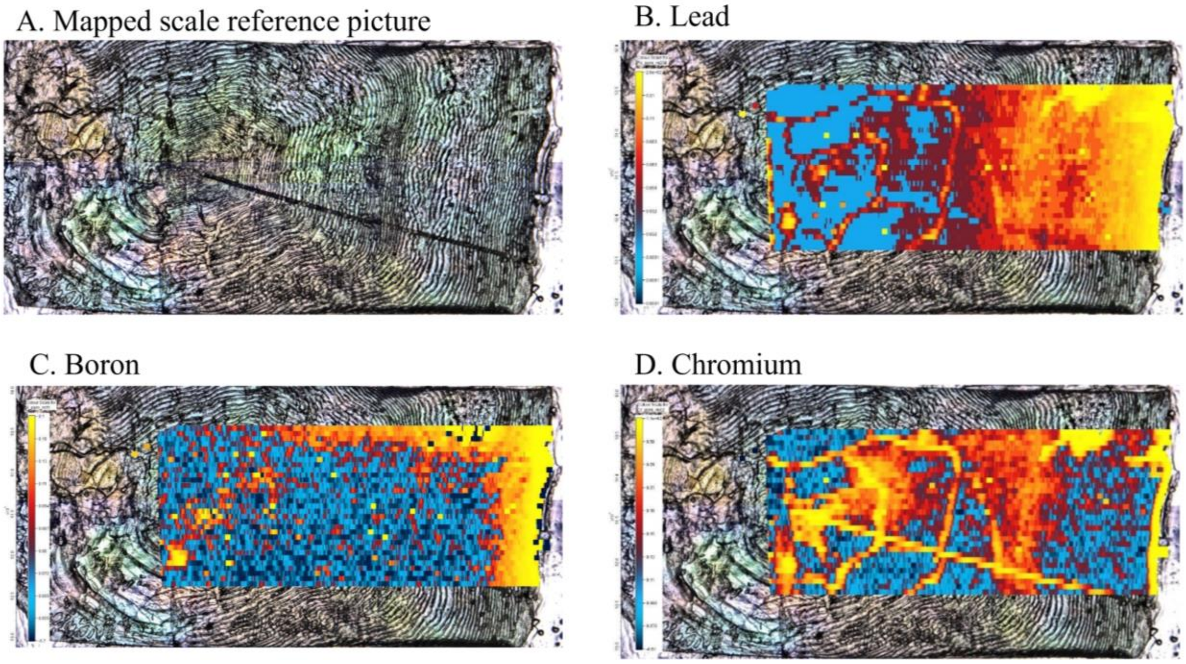

Figure 14. Mapping of a scale. A. Reference picture. Black line showing where the laser has been run previously across the entire life. B. Lead concentration. C. Boron concentration. D. Chromium concentration. Heatmap ranging from low concentration (blue) to high concentration (yellow).

Vaterite formation and M74 linkage

Otoliths consist of aragonite calcium carbonate structures, but a polymorph of vaterite formation sometimes forms. To access whether the vaterite formation of the otoliths could possibly indicate a link to M74 disorder, the otoliths were visually accessed and categorised into different groups based on the extent of vaterite present (Table 2). This showed that out of the 170 salmon included, 87 (~51%) fish had no vaterite formation on either otolith*, 53 (~32%) had at least one vaterite otolith, and 29 (~17%) fish had both otoliths affected by vaterite formation (Table 5, Figure 15 A). To investigate if there is a link between M74 disorder and vaterite formation on the otolith, the thiamine data for each fish was compared with the observed vaterite formation (Figure 15 B) without clear results.

Table 5. Number of salmon otoliths from the different regions that were affected by vaterite formation.

	Both Otoliths 0%	**At least* one otolith >10%**	**Both otoliths >10%**	**At least* one otolith >90 %**	**Both otoliths > 90%**
Umeå	39%	34%	17%	17%	4%
Älvkarleby	33%	34%	16%	17%	5%
Total	**37%**	**34%**	**16%**	**17%**	**5%**

* In some cases, only one otolith was retrieved.

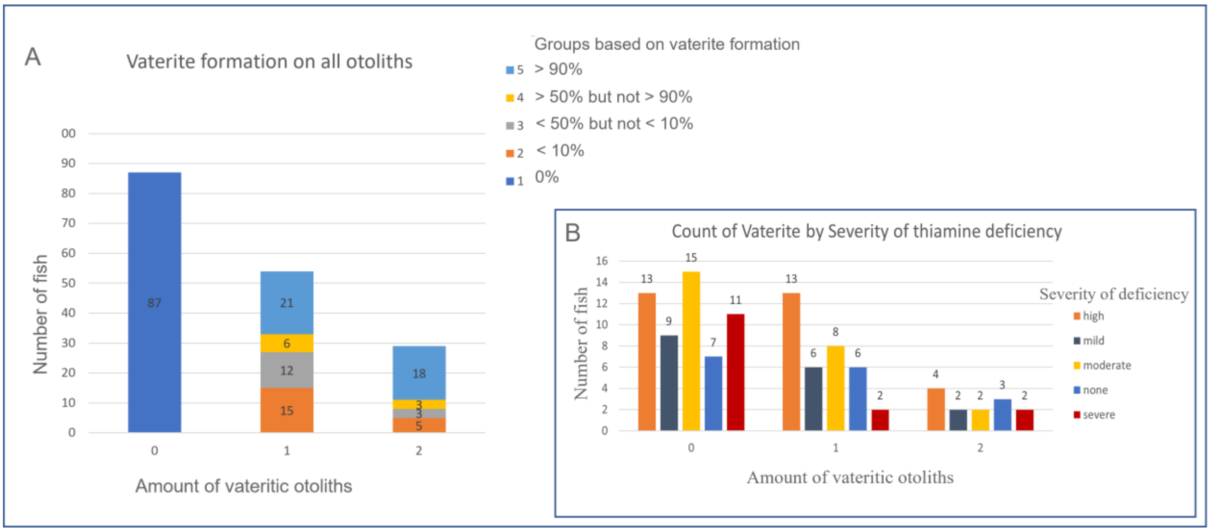

Figure 15. (A) Table chart showing that out of 170 fish otoliths, 87 had no otolith with vaterite formation, 54 had at least one otolith with vaterite and 29 had two vateritic otoliths. Of the 87 fish with no vaterite formation, 14 did not have a second otolith for comparison. Out of the 54 fish with at least one otolith (for 2 fish, only one otolith was found) affected, 21 were affected over 90%, 6 had vaterite formation on more than 50% but not more than 90%, 12 had less than 50 but not less than 10% of vaterite, and 15 were mildly affected with less than 10 %but not 0%. Out of the 29 fish with both affected otoliths, 18 had vaterite on over 90% of their surface, 3 had more than 50% of vaterite, 3 had less than 50%, and 5 less than 10% of vaterite. (B) Comparison among the thiamine severity groups based on how many otoliths were affected by vaterite formation.

The salmon included in this study were sampled in October and November 2021, however, they spent unequal time at their respective hatchery station prior to sampling. The catch-day data for the Älvkarleby river allowed to perform additional testing to investigate if the time spent at the hatchery possibly influences the severity of thiamine deficiency. Thus, a prediction error plot showed a weak positive correlation (R^2 = 0.05) between the amount of thiamine concentration observed in each fish with regard to the duration at the hatchery station, which shows that a longer time at the hatchery station slightly increases the thiamine concentration in the individual fish (Figure 16).

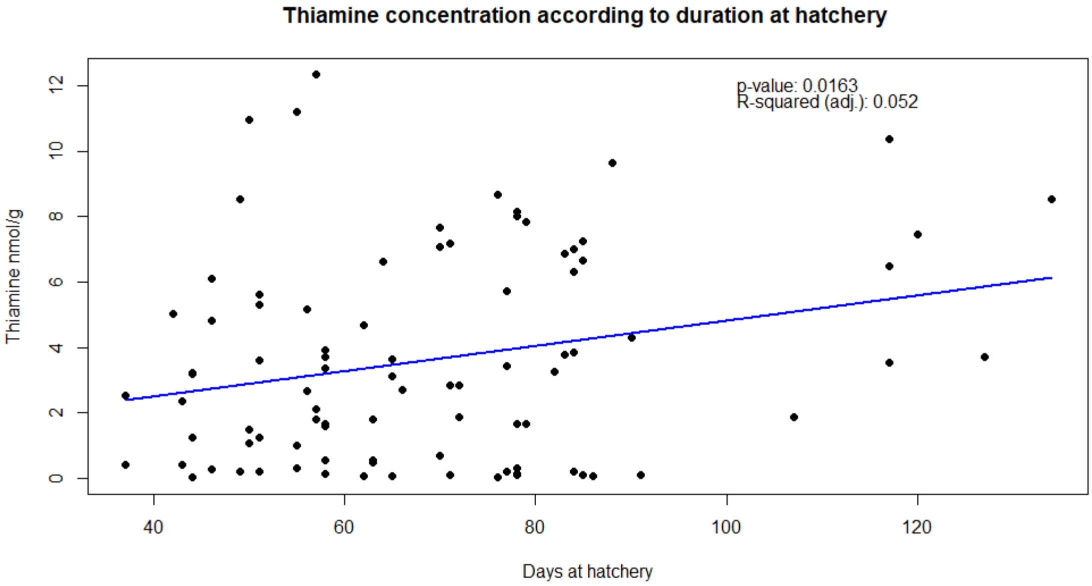

Figure 16. The days spent at the hatchery station displayed at different groups. Overall significant p-value with slight increase of thiamine with increase of duration at hatchery.

Discussion

Apart from one pilot study, this is the first study that comprises the application of trace elemental analyses on salmon scales and otoliths with linkage to thiamine deficiency. Otolith chemistry is regarded as the gold-standard for other fish species to study major life-history events through microchemical analysis techniques as the trace elemental composition provides detailed information on environmental and physical changes these fish undergo (Hüssy et al., 2020). With increasing demand to find a non-lethal sampling method with focus on studying vulnerable or endangered species, fish scales were already long under consideration and have in this study shown promising results for the first time.

Novel scale chemistry for determination of age

This study demonstrates that trace elemental chemistry can be applied on scales, and that scales express similar metabolic activity (Figure 8) and migration patterns (Figure 9) as otoliths. Additionally, this is the first known study attempting to use seasonal variations in trace elements incorporated into fish scales for interpreting age. Although previously untested, the chemical ageing of scales and otoliths mostly agreed among age readers (Figure 10). Minimal variation of one to two years of age among the two structures was found, but rarely occurred. This could be because (1) this is a novel and previously untested method; (2) that chemical analyses of scales display cracks in the structure and resorption during anadromous migration with eroded edges that could cause misinterpretation; and (3) variation of growth rates of the different structures influenced this. Generally, otoliths grow slower in colder environments (Campana & Thorrold, 2001), and as salmon return for spawning during autumn and stop feeding (Webb et al., 2007), the slower growth of their otoliths might not chemically display the age precisely at the edge, while the scale possibly could.

Even if the last possible explanation is to be assumed, it needs to be taken into account that scales stop growing with the end of the somatic growth of the fish, wherefore this method is limited to either young-lived fish or fish caught prior to reaching full length (Hüssy et al., 2020). Nevertheless, salmonids are optimal study subjects for scale chemical analyses as they continue to grow throughout their lifetime (Behnke & Tomelleri, 2002).

Environmental parameters for identification of M74

Focusing on the trace elements that display environmental variation and migration patterns, Sr:Ca and Ba:Ca, it was observed that thiamine deficient fish have a significantly lower amount of Sr:Ca in their otoliths compared to the healthy groups (Appendix IV, A). While a slight decrease of Sr:Ca in the severe group was also observed in the scales, the difference in the not-severe (none) group was insignificant. A mirrored pattern is displayed for Ba:Ca, as the severely deficient group reflects a significantly higher amount of Ba:Ca in their otoliths compared to the healthy groups (Appendix IV, B). These results lead to comparison of the Sr:Ba ratio among the different life-stages, suggesting that severely thiamine deficient female salmon spend more time at the coast/river during their whole life in comparison to healthier groups. The change in habitat might affect prey availability (Pickova et al. 2003) and pollutants. Further investigations have illustrated that the scales additionally reflect a negative relationship between the Sr:Ba ratio and pollution-indicating elements, which shows increased uptake of pollutants from the river (Figure 12). It was previously studied that Hg, Zn and Pb are found in higher concentrations within the Swedish freshwater environment when compared to the Baltic Sea (Gustavsson et al., 2010). The elevated levels in severe M74 fish and the connection to pollution-indicating elements might thus be explained by the higher outflow of metals from rivers, closer to civilization and industry.

The otoliths do not seem to reflect these measures (not shown), possibly because of the difficulty to incorporate these trace elements into the aragonite structure (Wassenburg et al., 2016). Although Zn could be a possible indicator for pollution, it was recently found that the Baltic salmon otoliths display annually recurring patterns of Zn with higher accumulation in summer and lower in winter (Limburg & Elfman, 2017).

Also interesting is the strong negative correlation between Ba:Ca average edge to thiamine among the severe group of the otoliths, which shows to be positively correlated to both TDP and TMP in the healthier (none) group (Figure 12). However, these correlations represent only a small part of the available dataset, and additional comparisons could be performed. The dataset comprises the average at the edge (representing the time point of spawning migration) and the average at the sea phase. It lacks the river phase (thus, the beginning of life) and the migration out toward the sea. While the maxima and minima at each life stage and the inner core and outer edge were also determined, these data were not included here but will be used for future studies when this project is extended.

Sample locations and important considerations

The sampled fish in this study originated from two sample locations on Sweden’s east coast, from the Ume/Vindelälven in the Gulf of Bothnia and Dalälven, approximately 500 km further south in the Baltic Sea (Figure 4). Based on the location variation, the water temperature difference affects the eggs' hatching and, thus, the release from the respective hatchery stations as the release is dependent on the growth status. The salmon hatchlings at Dalälven river usually remain within the hatchery for one year and are set free in early May. For Ume/Vindelälven, the release of one-year-old salmon occurs in the middle of June, while two-year-old salmon is released at the end of May. Both hatchery stations release the fish through a drainage system that allows for exiting below hydropower dams at both locations. More detailed information can be found in table 6.

This information aids trace elemental analyses when investigating ageing and migration patterns. For example, the first minima in Mg concentration indicates the end of year-one, which should occur before or during the increase of Sr, which indicates the movement of salmon towards the ocean. While also wild fish were sampled as they returned to the same natal river for spawning, a similar pattern can be assumed.

Table 6. Detailed information about the hatchery stations at the respective sampling locations.

	Dalälven (Älvkarleby, Uppsala County)	**Ume/Vindelälven (Norrfors, Umeå county)**
Release	Release of salmon after one or two years ➢ Both at beginning of May	Release of salmon after one or two years ➢ 1 y/o Salmon beginning/mid-June ➢ 2 y/o Salmon: End of May
Body size (gr) at time point of release	1 y/o salmon: 30g average 2 y/o salmon: 80g average	1 y/o salmon: 40-50g average 2 y/o salmon: 80-100g average
Location of release	Released through drainage system of hatchery flushed out into main river downstream (below hydropower station)	Released through drainage system of hatchery flushed out into river downstream (below hydropower dam)

Correlation between trace elements and measured thiamine concentration

Correlation matrices were created to find possible correlations between either of the thiamine forms and either of the elements. The two structures were differentiated to find overlapping correlations (Figure 13). None of the significant correlations among the severe group found in one structure was displayed in the other. However, a significant positive correlation between K:Ca during the sea phase and TMP was found in both structures of the not-severe (none) group. To investigate whether this is a possible marker for M74, additional studies should be performed where sample groups are large enough to separate wild and hatchery fish, as an increase was found to distinguish farmed from wild fish according to a study performed on sea bass (Arechavala-Lopez, Milošević-González & Sanchez-Jerez, 2016). Furthermore, the otoliths of the least severe group (none) showed strong positive correlations between thiamine and Mg:Ca during spawning migration (Figure 13 A) which might represent increased metabolic activity. This correlation was not seen in the corresponding severe group, possibly indicating disturbed metabolic processes among the severely deficient group. This was earlier found by Vuori & Nikinmaa (2000) who concluded that increasing redox enzyme activities and biotransformation are possible contributors to M74, showing distressed metabolic processes.

Although the 2-D mapping of a scale from a thiamine deficient fish showed increases in B, the observed increase over life was only significant in the otoliths. Regardless, the general increase of B over the course of life was significantly different in thiamine deficient fish compared to healthier fish, allowing for the assumption that indicates that they encountered bigger proportions of B throughout their life (Figure 13). B is generally found in sea water in the form of boric acid and contributed to the waterbody's alkalinity (Lee et al., 2019). More so, B is an essential trace element which plays essential roles in cross-linking of carbohydrates of plant and algal cells (Carrano et al., 2009), but the proxy for salmon otoliths and scales is yet unknown.

Vaterite formation and M74

Lastly, as stated in the results, vaterite formation was observed on at least one otolith in almost 32% of all studied fish, and 17% of the fish even had both otoliths affected. It could not be concluded whether there is a link between the vaterite formation and M74 disorder due to great variety of vaterite formation among the different thiamine groups (Figure 14, B) and uneven results. Moreover, the examination of vaterite formation was limited to visual assessment and further testing, thereby physically analysing the structure of the otoliths might allow for further conclusions but was not researched here. Additional studies for further information on this matter have not been found.

Optimization efforts and future perspectives

Since chemical ageing for scales is novel, seeing seasonal patterns among the chemical data of scales is challenging. Generally, the knowledge of traditional ageing was very helpful, allowing for clarification within the patterns observed in the chemical files and ensuring higher quality of the data analyses from the beginning. Another challenge was the amount of vaterite formation on the otoliths (Figure 10). Firstly, this led to the exclusion of some of the sample fish as none of their otoliths was in good condition, and secondly lowered the quality of the laser analyses due to deformed vaterite structure, making microchemical data analysing critical (Word et al., 2022).

Most sampled salmon showed visible lesions and haemorrhages along the abdomen of the fish that might be signs of RSD (Weichert et al., 2020). However, this study did not document the presence of visible clinical signs wherefore possible linkage of multiple underlying health causes that contribute to M74 were not analysed. However, the sampled fish was subject to multiple independent studies, and if this project were to be continued, a possible correlation between the external fungal infections and M74 should be performed.

Conclusions

The findings of the current study show that thiamine deficient fish spend a longer time in the riverine regions, thus, the pre-spawning period is extended, and their thiamine resources might be exhausted. With trust in the gold standard of trace elemental analyses, thus, the otolith chemistry, first application of scale chemistry were possible as a comparison study. As salmonids have to be able to adjust to different salinity levels when they pass through various habitats (Schiewe, 2013), which could be a possible indicator for reduced osmoregulation, wherefore the salmon might require extended time to adjust from saltwater to freshwater as thiamine plays essential roles in energy metabolism (Lonsdale, 2006). This theory was previously demonstrated in a study conducted by Manzetti et al. (2014), with findings that the free thiamine is connected to the electrolyte balance. Furthermore, salmon undergo a rather long fasting period before spawning (Karlsson and Karlström, 1994), during which previously established thiamine acquisitions are depleted, and energy is lost.

The great individual variation in thiamine concentration could originate from large-scale abiotic changes that occurred in the feeding area of the southern Baltic Sea (Majaneva et al., 2020). With severely deficient fish not migrating as far out as healthier fish, also the prey availability is affected. More so, presence of pollutants in the coastal regions has shown to further influence the severely deficient fish. The investigations of possible impact of the duration spent at the hatchery station has shown a weak positive relationship where a longer stay positively influences the thiamine concentration, which needs further examination (Figure 16).

Although the diet of salmon is linked to M74, the reason for the fluctuating levels of thiamine is yet not completely known. It is discussed that a change in the pre-existing food web has caused the increase of M74 (Pickova et al. 2003). These changes, possibly resulting from eutrophication and variation in salinity, cause alterations of the phytoplankton and antioxidants, which in turn also affect the zooplankton population and the base of the food web on which prey fish rely on (Pickova et al., 2003). Although the initial origin of thiamine availability lies in phytoplankton, the affected areas with recurrent M74 disorder did not show a decrease in phytoplankton and have been reported as sufficient for the salmon's diet (Pickova et al., 2013).

This study is the first of its kind to propose Sr:Ba in scales as a novel, promising non-lethal biomarker for M74. The mean levels of Sr:Ba in salmon with severe thiamine deficiency is significantly lower than in healthy salmon. These lower levels can be seen also earlier in life than during spawning, which suggests that non-lethal sampling of female salmon caught in the sea might be possible for monitoring thiamine deficiency. Further studies should be conducted on male salmon to evaluate if scale Sr:Ba could be an appropriate biomarker for both genders regarding thiamine deficiency. As scale Sr:Ba reflects the environment, evaluation of scale Sr:Ba in severe thiamine deficient salmon from other rivers is recommended.

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Appendices

Appendix I.

Insamlingsförfarande: Ägg som samlas in för tiaminanalys, ska kramas från honan över en sil för att på så vis filtrera bort överflödig vätska (Fig. 1) som annars skulle orsaka fel i tiaminanalyserna. Provet av ägg, ungefär 1 matsked, samlas in från obefruktade ägg och fryses in som ett platt paket i en ziplock påse (e.g. Mingrip, som SLU-Aqua tillhandahåller på begäran). Provpåsen märks upp med en identifieringskod för hon laxen. Proven ska frysas in så snart som möjligt (minst -20° C, gärna ännu kallare). Proverna ska skickas med frystransport till SLU-Aqua så snart som möjligt, inpackat i en kylbehållare med torr-is. Ta kontakt med Elin Dahlgren innan proven skickas för att säkra att de tas emot vid leverans på SLU. Följande biologiska mått och prov samlas in samtidigt med samma id kod som paketet med ägg:

- Total kroppsvikt och total kroppslängd
- ca 10 fjäll för åldersbestämning. Åldersbestämning av laxen sker på SLU-Aqua.

Fig.1 Kramning av ägg över en sil.

Fig. 2. Platta paket av ägg i zip lock påsar redo att frysas in.

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Translation:

Collection procedure: Eggs collected for thiamine analysis should be squeezed from the female over a sieve to filter out excess liquid (Fig. 1) that would otherwise cause errors in the thiamine assays. The sample of eggs, about 1 tablespoon, is collected from unfertilized eggs and frozen in a flat package in a ziplock bag (eg Mingrip, which SLU-Aqua provides on request). Sample bags are marked with an identification code for female salmon. Freeze the samples as soon as possible (at least -20 ° C, from morning). The samples must be sent with Brattransport to SLU-Aqua as soon as possible, packed in a cooling container with dry ice. Contact Elin Dahlgren before sending them for proof that they will be received upon delivery at SLU. The following biological measurements and samples are collected simultaneously with the same id code as packed with eggs.

Appendix II. Setup for the laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) at the Department of Geology at Lund University in Lund, Sweden.

Laser ablation system	**Lund University**
Make, Model & type	Photon machines, Analyte G2 excimer laser
Ablation cell & volume	HelEx 2-volume cell with eQC
Laser wavelength	193 nm
Pulse width	<4 ns
Fluence	2 J/cm^2 on otolith and MACS-3 and 3 J/cm^2 on NIST glass
Repetition rate	14-15 Hz (otoliths), 12 Hz (scales)
Scan speed	10 µm/s (otoliths), 15 µm/s (scales)
Spot size	35 x 75 µm (otoliths) 60 x 50 µm and 40 x 100 µm (scales)
Background collection	30 seconds prior to each analysis
Ablation setup	Line scan; 30 seconds on standards
Cell carrier gas flow	0.8 l/min He
ICP-MS Instrument	
Make, Model & type	Bruker Aurora Elite Quadrupole ICP-MS
RF power	Ca. 1300 W
Make-up gas flow	Ca. 0.95 l/min Ar
Detection system	Single collector discrete dynode electron multiplier or DDEM
Data reduction	
Software	Iolite v3.63
Primary standard	NIST SRM 610 and 612- preferred values from Jochum et al. 2011. OR MACS-3 for otoliths and MAPS for scales (Prefered values from GeoReM, 8/2019)
Secondary standard(s)	NIST SRM 612
Internal standard	^{43}Ca - Ca mean concentration in otolith set to 38 wt.% ^{43}Ca - Ca mean concentration in scales set to 37.4 wt.%

Appendix III. Results of ageing of both structures from two age readers as well as their difference.

R1 Oto.	R2 Oto.	R1 Sc.	R2 Sc.	Diff. Oto.	Diff. Sc.	R1 Oto.	R2 Oto.	R1 Sc.	R2 Sc.	Diff. Oto.	Diff. Sc.
4	4	4	4	0	0	**4**	5	3	3	-1	0
4	4	3	4	0	-1	**4**	4	4	3	0	1
3	4	4	4	-1	0	**3**	4	4	4	-1	0
3	3	4	5	0	-1	**4**	4	4	3	0	1
4	4	4	4	0	0	**4**	4	4	4	0	0
4	4	4	4	0	0	**4**	4	4	4	0	0
4	4	4	4	0	0	**4**	5	4	3	-1	1
4	4	5	4	0	1	**5**	5	4	4	0	0
5	4	4	4	1	0	**4**	3	5	4	1	1
4	4	4	4	0	0	**3**	3	5	3	0	2
4	4	4	3	0	1	**3**	5	5	4	-2	1
4	4	4	4	0	0	**4**	4	4	3	0	1
4	4	4	4	0	0	**3**	4	4	4	-1	0
3	3	3	3	0	0	**4**	4	4	4	0	0
3	4	4	4	-1	0	**4**	5	4	4	-1	0
4	5	3	3	-1	0	**3**	5	4	5	-2	-1
4	5	3	3	-1	0	**3**	3	4	4	0	0
5	5	4	4	0	0	**3**	4	4	4	-1	0
3	3	4	4	0	0	**3**	4	4	4	-1	0
4	4	4	5	0	-1	**3**	3	4	3	0	1
4	4	4	3	0	1	**4**	4	4	4	0	0
4	4	3	3	0	0	**4**	3	4	4	1	0
4	4	4	4	0	0	**4**	4	4	4	0	0
4	4	4	4	0	0	**4**	4	4	4	0	0
4	4	3	3	0	0	**4**	4	4	4	0	0
4	4	4	3	0	1	**3**	3	4	5	0	-1
5	5	4	3	0	1	**3**	3	4	4	0	0
4	5	4	3	-1	1	**3**	3	4	4	0	0
4	4	4	4	0	0	**3**	3	4	4	0	0
4	5	4	4	-1	0	**4**	4	4	4	0	0
4	4	4	4	0	0	**4**	4	4	5	0	-1
5	5	3	4	0	-1	**3**	4	5	5	-1	0
4	4	4	3	0	1	**3**	3	4	4	0	0
4	4	4	4	0	0	**3**	5	4	4	-2	0
4	4	4	4	0	0	**3**	3	4	4	0	0
4	4	4	3	0	1	**3**	4	3	3	-1	0
4	4	4	3	0	1	**3**	4	4	4	-1	0
3	4	4	4	-1	0			4	4	0	0
4	4	4	3	0	1			4	4	0	0
		4	4	0	0			4	4	0	0
		4	4	0	0						

	4	4	0	0	
	5	4	0	1	
	4	5	0	-1	
	4	5	0	-1	

R1 = Reader 1, R2 = Reader 2, Diff. = Difference, Oto. = Otoliths, Sc. = Scale.

Appendix IV

Although the overall p-value determined by Kruskal-Wallis one-way analyses of variance showed only significance among the Sr:Ca average edge values in the Scales (Figure IV, A), there was still a significant increase in Sr:Ca average between the most severe and least severe group (p=0.027). For the Ba:Ca average at the edge, none of the structures nor rivers showed to be significant overall, but a significant decrease between the most severe and least severe group of the otoliths for Älvkarleby (p=0.027) and between the second most severe (high) and least severe (none) for Umeå otoliths (p=0.0078) was observed. Additionally, all severe fish originated from the Älvkarleby river (Figure IV, B).

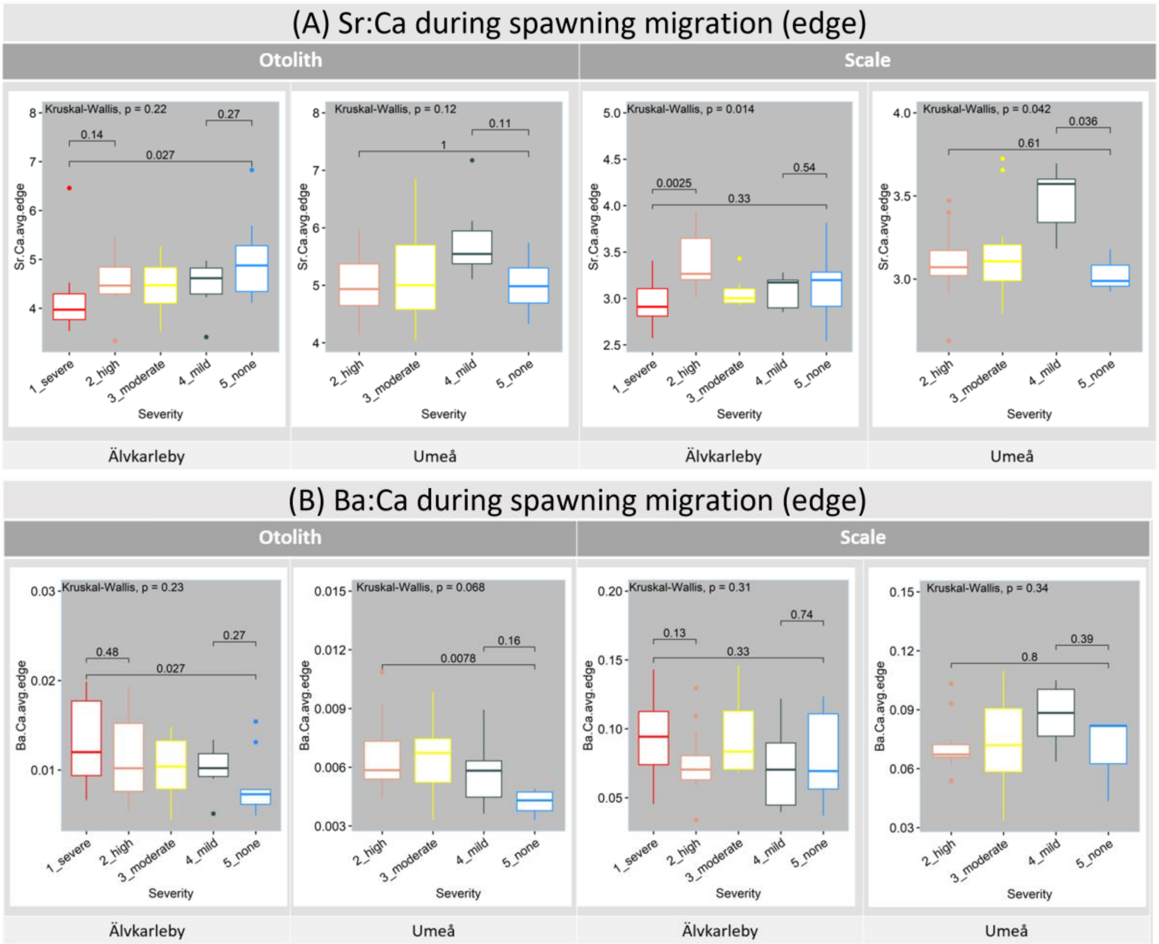

Figure IV. Ratios of A. Sr:Ca and B. Ba:Ca correlated to thiamine content in both otoliths and scales in both Rivers

Appendix V. Mean values of Sr:Ba for severity groups in river, sea and at the edge of salmon otoliths and scales from Älvkarleby and Umeå

Severity group	Structure	River	Sr:Ba river	Sr:Ba sea	Sr:Ba edge
Severe	Scale	Älvkarleby	12,66	26,06	19,88
High	Scale	Älvkarleby	23,07	53,90	42,90
Moderate	Scale	Älvkarleby	17,20	41,45	28,81
Mild	Scale	Älvkarleby	23,43	55,17	43,71
None	Scale	Älvkarleby	28,14	59,46	45,32
High	Scale	Umeå	23,62	55,24	42,68
Moderate	Scale	Umeå	20,39	50,21	38,41
Mild	Scale	Umeå	37,31	54,59	41,29
None	Scale	Umeå	20,10	58,76	52,28
Severe	Otolith	Älvkarleby	54,95	777,21	302,03
High	Otolith	Älvkarleby	56,43	841,27	320,57
Moderate	Otolith	Älvkarleby	93,11	901,37	388,02
Mild	Otolith	Älvkarleby	55,27	865,98	418,93
None	Otolith	Älvkarleby	72,56	1103,86	436,70
High	Otolith	Umeå	78,63	1014,99	592,16
Moderate	Otolith	Umeå	74,64	1027,98	708,38
Mild	Otolith	Umeå	75,88	1188,37	956,85
None	Otolith	Umeå	84,49	1207,39	890,66