Unlocking the archive: using the biochemical and isotopic composition of fish scales to understand the marine phase of Atlantic salmon

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Declaration

I hereby declare that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work and has not been taken from the work of others, save to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

The Atlantic salmon is a species of great importance culturally, economically and ecologically. Recent declines suffered by many populations have been linked to marine mortality, therefore a better understanding of the marine phase is needed to inform management decisions, slow population declines, and protect this iconic species. The overall aim of this research was to develop, validate and apply methods that unlock the information contained in Atlantic salmon scales to enhance our knowledge of the marine phase. Fish scales incorporate biochemical and isotopic signatures as they grow, acting as a chronological record of the fish's life history. Large, multi-decadal archives of Atlantic salmon scales are held by many organisations, containing vast amounts of data to be explored.

Stable isotopes of scales can be used to examine the diet, origin, and trophic level of prey of a fish, but inorganic carbonates on the scale surface can confound results. The carbon isotopic ratio ($\delta^{13}C$) of acid-treated and untreated scales from 208 Atlantic salmon was analysed. Acid-treatment had a negligible effect on $\delta^{13}C$ and therefore does not need to be performed prior to stable isotope analysis (SIA) of Atlantic salmon scales, saving scale material, time and money. A recent isotope-based geolocation tool suggests that the marine feeding location of salmon can be determined by correlating a time series of scale $\delta^{13}C$ with sea surface temperature (SST). To validate the method, SIA of archived scales from 100 fish (10 years of a 50-year period) caught at their feeding grounds west of Greenland was completed. The highest area of correlation between scale $\delta^{13}C$ and SST, the Labrador Sea, accurately represented the foraging location of the fish. This validation allows the results of the geolocation tool to be interpreted with increased confidence.

Cortisol, the most commonly measured stress hormone in fish, was recently extracted from fish scales as a measure of chronic stress. In this thesis, the method was adapted for use on Atlantic salmon scales and used to extract cortisol from the scales of 156 experimentally reared post-smolts that were exposed to 3 temperatures (6, 10.5 and 15°C) and varying starvation stressors. Cortisol increased significantly in fish kept at 15°C. Fluctuations occurred in fish at 6°C and in starved fish at 10.5°C, but a larger sample size is needed to determine the significance of these results. This research determined that scale cortisol is a suitable biomarker for temperature stress in Atlantic salmon and, due to optimisation to require lower weights of scale material, may open this method up to a wider range of species

and life stages. Using the method, the stability of cortisol was confirmed in archived scales, then cortisol was extracted from 120 archived scale samples (6 years over a 29-year period). No interannual trends were detected, and individual variability appeared to drive the differences in cortisol. Combining cortisol data with other analyses could help understand the factors affecting scale cortisol in Atlantic salmon.

This research illustrated the value of scales for examining the marine phase of Atlantic salmon, which may be key to preventing further declines. The methods developed and validated in this thesis can be used to determine marine feeding location and to examine the response of salmon to stressors experienced during their life cycle.

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Chapter 1. General Introduction

1.1 The importance of Atlantic salmon

The Atlantic salmon (*Salmo salar*, L.) has a long, storied relationship with humans; its cultural importance is represented in 10,000 year old paintings and carvings in Scandinavian rocks, remains of bones in Spanish caves dating back 40,000 years, and its presence on the Irish currency from 1928 to 2002 (Stafford-Langan, 2006; Hindar *et al.*, 2011). Worldwide, the salmon has become an integral part of the culture, diet, identity, economy and spiritual practices of the indigenous and local communities it supports (Myrvold *et al.*, 2019). Salmon stocks in the River Teno (Deatnu in the Sámi language) at the border of Norway and Finland, have sustained the Sámi people since they first arrived in the area at the end of the ice age (Pedersen, 2012; Holmberg, 2018) and salmon fishing is a community event that marks the beginning of a new season (Ween and Colombi, 2013). Salmon is an essential component of their diet and serves as a festive food; at times salmon were even used to pay taxes (Ween and Colombi, 2013; Holmberg, 2018). Atlantic salmon have similar importance for the Mi'kmaq people of Nova Scotia, who relied on smoked salmon as a staple to get them through the winter, but now often reserve the fish for special occasions due to dwindling population sizes (Denny and Fanning, 2016). As increasing regulations are placed on the fishery, the Mi'kmaq people worry they will lose the traditional knowledge and cultural value associated with salmon (Myrvold *et al.*, 2019).

The cultural importance of the Atlantic salmon is further evidenced by its presence in folklore and mythology. A hero of Irish lore, Fionn Mac Cumhaill, gained all the wisdom of the world by eating *an Bradán Feasa*, the Salmon of Knowledge (Cross and Slover, 1936). Other heroes and leaders are described as doing the "heroes salmon-leap" and adorning themselves in gold salmon brooches or salmon skin (Cross and Slover, 1936). In Welsh mythology, the hero Culhwch calls upon the wisest and most ancient of all the animals, the Salmon of Llyn Llyw, to help rescue an imprisoned child (Guest, 1902). The Atlantic salmon has given its likeness to coats of arms and artwork, and has inspired place names around the North Atlantic (Utredninger, 1999; Myrvold *et al.*, 2019).

Exploited for food and recreational purposes, Atlantic salmon are also of great economic importance across their range (Hindar *et al.*, 2011). However, current exploitation rates of wild salmon are declining as stock numbers dwindle and conservation measures are put in

place (ICES, 2021a). The 2020 nominal catch (round, fresh weight of fish caught and retained) of 915 tonnes was higher than 2019 but lower than previous five- and ten-year means (ICES, 2021a). Peak nominal catches were recorded in the late 1960s and '70s but the general pattern since then has been one of decline (Figure 1.1; (ICES, 2021a)).

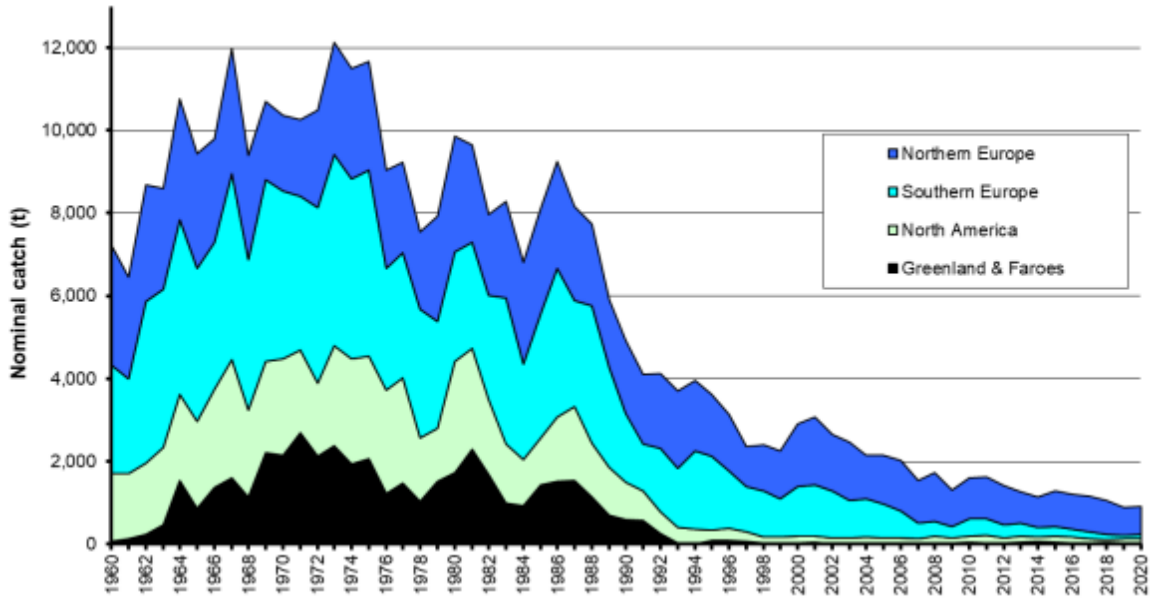

Figure 1. 1 Plot of nominal catch (round, fresh weight caught and retained) of *Salmo salar* in tonnes from 1960 to 2010 (2010 figures are estimates). "Northern Europe" refers to Norway, Russia, Finland, Iceland, Sweden and Denmark; "Southern Europe" refers to Ireland, UK (Scotland, England and Wales, and Northern Ireland), France and Spain; "North America" refers to Canada, USA, St Pierre et Miquelon (France); and "Greenland and Faroes" refers to Greenland and the Faroe Islands. Source: ICES (2021a)

Contrary to the trend in fisheries, salmon farming has been rapidly increasing since the 1980s and total worldwide production of farmed Atlantic salmon was estimated to be 2,638,000 tonnes in 2020 (Figure 1.2), higher than the previous five-year mean (ICES, 2021a). Salmon and trout accounted for 18% of the total value of internationally traded fish products in 2019 (FAO, 2021). In Ireland, salmon aquaculture production was worth €127 million to the economy in 2020, an increase of 13% from the previous year (BIM, 2021). Atlantic salmon are also harvested through ranching programmes, which involves the release of hatchery produced salmon into the wild at the smolt stage, with the intention of harvesting the full returning adult population (ICES, 1994). Ranched salmon are also released as part of

experimental research, for stock assessment purposes, and for angling (Marine Institute, 2020; ICES, 2021a).

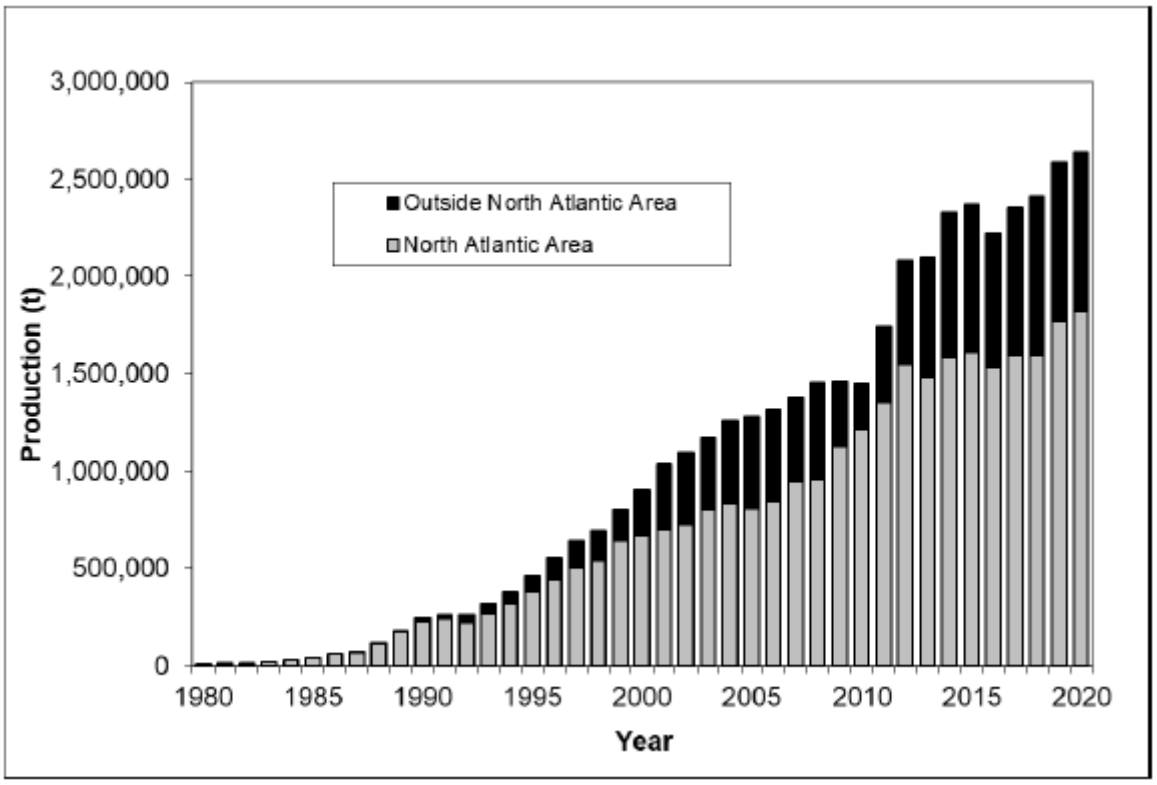

Figure 1. 2 Worldwide production of farmed *Salmo salar* from 1980 to 2020 (2020 is an estimated production figure) in tonnes round fresh weight. Source: ICES (2021a)

While wild salmon harvests are declining, recreational fishing is becoming increasingly economically important, and catch and release measures are increasingly common in rod fisheries (ICES, 2021a). A study in the rural west of Ireland village of Waterville, County Kerry, showed that tourist anglers spent an average of €103-182 per day in the local area, varying between Irish and international visitors (Inland Fisheries Ireland *et al.*, 2016). Anglers provide an economic boost to the locality when visiting to fish for Atlantic salmon and seatrout (*Salmo trutta*, L.), spending far more on average per day than a non-angling tourist (€68-89; (Fáilte Ireland, 2016)), and usually visiting Waterville outside of key tourist periods thereby extending the season for local providers (Inland Fisheries Ireland *et al.*, 2016). In 2012, up to 406,000 individuals participated in recreational angling in Ireland for multiple species including Atlantic salmon (Tourism Development International, 2013). Results from the survey estimated the economic impact of angling was €755 million, taking

indirect and induced impacts into account (Tourism Development International, 2013). Across the North Atlantic, anglers spent an estimated €300 – 500 million related to rod fishing for wild Atlantic salmon in 2017, including fishing permits, guides, lodging and other expenses (Myrvold *et al.*, 2019).

Job creation is another economic benefit of Atlantic salmon; recreational angling for Atlantic salmon and other species is estimated to support approximately 10,000 jobs in Ireland (based on 36 jobs per €1 million in expenditure; (Tourism Development International, 2013). The Irish seafood sector provided direct and indirect employment to 16,430 people in 2020 (BIM, 2021). In eastern Canada in 2010, it was estimated that Atlantic salmon-related income amounted to $128 million and 3,873 full time jobs (Gardner Pinfold, 2011). This study did not quantify those who were seasonally employed therefore the total number of people relying on salmon for income is expected to be double the number employed full time (Gardner Pinfold, 2011). In Scotland, the third-largest producer of farmed Atlantic salmon, 1,780 people were directly employed in smolt and fish production in 2016, with further jobs supported up- and down-stream (including feed services and processing, respectively) (Kenyon and Davies, 2018).

Finally, the Atlantic salmon and other anadromous salmonids are ecologically important; the presence of salmon in an ecosystem is an indicator of good environmental quality as they require clean, well-oxygenated water to survive (Crisp, 2008; Jonsson and Jonsson, 2011a). Atlantic salmon can strongly influence ecosystem structure and functioning and are recognised to have a keystone role (Willson and Halupka, 1995). The keystone importance of Atlantic salmon was demonstrated in the Rhine, where the decline of salmon began a cascade effect on the ecosystem, leading to declines or extinction of other fish species (Lenders, 2017). Baltic Sea salmon are credited with ecosystem services such as nutrient cycling, reducing sedimentation by agitating gravel beds while spawning, food web maintenance as a prey species at different life stages, and as a top predator indicator of toxins in the food chain (Kulmala *et al.*, 2012). From egg to adult, salmon are a source of prey and are crucial for the survival or reproduction of some species (Willson and Halupka, 1995). Pacific salmon (*Oncorhynchus spp.*) demonstrate the important role of salmonids in transporting nutrients between marine and freshwater systems (Naiman *et al.*, 2002). The fish accumulate nutrients in the marine environment and return to the freshwater ecosystem on their spawning migration, depositing the nutrients into the system if they die after spawning, thereby

subsidizing the local area (Naiman *et al.*, 2002). Carcasses such as these sustain productivity, support detritivorous species such as caddisfly larvae, and may even support the next generation of salmon juveniles (Richey *et al.*, 1975; Zhang *et al.*, 2003). Thus, changes in the abundance of salmon species could affect not only marine and freshwater ecosystems, but terrestrial systems also (Noakes, 2014).

1.2 Recent declines of Atlantic salmon

1.2.1 Global trends in declines

In recent decades, Atlantic salmon populations have suffered declines across their distribution (Parrish *et al.*, 1998). Reported catches provide the longest historical record of salmon abundance (Chaput, 2012) with the decline in catch sizes mirroring the species decline. However, there is variability in the global pattern across salmon distribution and not all stocks are declining. In the Northeast Atlantic, apart from the River Tenu/Tana, stocks in Finland, Norway, Sweden and Scotland were at full reproductive capacity in 2020, and there were increases in salmon returns in areas of Canada (Labrador, Newfoundland and Québec) in the Northwest Atlantic (ICES, 2021a). In Ireland, out of the 144 rivers for which scientific advice on salmon is provided, 85 are classified as High Risk, 32 as Moderate Risk, 16 as Low Risk, and only 11 are reported as Not at Risk (NASCO, 2021). According to the classification system, rivers classified as High Risk have reached <50% of conservation limits (NASCO, 2016). This emphasises the need to monitor and conserve Atlantic salmon on a population basis.

The complex, anadromous life cycle of the Atlantic salmon makes them vulnerable to many threats. The early phases of Atlantic salmon (egg, alevin, fry, and parr) take place in freshwater and last between one and eight years depending on latitude, genetics and environmental conditions (Crisp, 2000; Thorstad *et al.*, 2010). During this phase, salmon juveniles are territorial and benthic dwelling with social hierarchies (Thorpe *et al.*, 1992; Thorstad *et al.*, 2010). To prepare for their sea migration, salmon undergo a complex physiological and morphological transition into smolts which allows them to transition from life adapted in freshwater to the saline conditions of the marine environment and move downstream to start their migration in spring (Jonsson and Jonsson, 2011b). During the post-smolt year, the first year at sea, growth increases considerably, post-smolts must adapt to novel predators and food sources, and mortality is believed to be very high (Dingle, 1980;

Eriksson, 1988; Thorstad *et al.*, 2010). Atlantic salmon usually spend one to four years at sea (Jonsson and Jonsson, 2011b) and have been recorded migrating distances of almost 3,000 km to marine feeding grounds (Rikardsen *et al.*, 2021). Salmon return to their natal rivers as one-sea-winter (1SW or grilse, one winter spent at sea) or multi-sea-winter (MSW, more than one winter at sea) fish, ready to make their upstream migration to spawn (Jonsson and Jonsson, 2011c). Many Atlantic salmon survive spawning, going back to sea to feed and returning in future years as repeat spawners (Thorstad *et al.*, 2010).

However, life-history changes are accompanying declines in abundance; in many populations, MSW returns are declining at a higher rate than 1SW fish (Chaput, 2012). Individuals returning to French rivers decreasing in size and entering natal rivers later, particularly 1SW fish, between 1987 and 2013, (Bal *et al.*, 2017). Grilse in Scotland were also found to be returning later, especially in years where the fish were in poor condition (Todd *et al.*, 2008). In juveniles, timing of downstream migration was found to be associated with temperature and river flow, with initiation of the seaward migration occurring 2.5 days earlier per decade (Otero *et al.*, 2014). These shifts are believed to be related to global climate changes (Todd *et al.*, 2008; Otero *et al.*, 2014), which are also expected to affect timing of the hatching of Atlantic salmon eggs (Rooke *et al.*, 2019).

1.2.2 Underlying causes of declines

The marine phase is a critical period for survival of Atlantic salmon and declines in the marine phase appear to drive overall declines in abundance (Friedland, 1998; Drinkwater *et al.*, 2003). Many factors are at play in the marine phase and likely interact with each other, making understanding effects on Atlantic salmon complex. During the post-smolt life stage, salmon are susceptible to predation, competition for food, parasites and diseases (Hansen *et al.*, 2003). Growth of post-smolts from the Burrishoole River in the west of Ireland was found to be positively correlated with marine survival, confirming the importance of oceanic conditions that facilitate growth in the early marine phase (Friedland *et al.*, 2005; Peyronnet *et al.*, 2007). Marine mortality of post-smolts is thought to be heavily influenced by temperature; survival of Norwegian post-smolts was recorded to be positively correlated with a temperature range of 8-10°C on sea entry (Friedland *et al.*, 1998). However, synchronous declines across 1SW and MSW salmon that feed at different oceanic foraging grounds and are exposed to distinct ocean warming suggest that increasing SST cannot be the sole cause

of decline in Atlantic salmon, other factors must also be at play (Soto *et al.*, 2018). Cyclical climatic factors such as the North Atlantic Oscillation (NAO) and Atlantic Multi-decadal Oscillation (AMO) may also influence marine survival (Peyronnet *et al.*, 2008; Friedland *et al.*, 2009). These oscillations affect SST, the frequency of storms, movement of currents, and wind speed and direction (Hurrell, 1995; Hurrell *et al.*, 2003; Friedland *et al.*, 2009). Correlations between both the NAO and AMO indexes and Atlantic salmon survival have been recorded at different life stages (Peyronnet *et al.*, 2008; Friedland *et al.*, 2009). Many factors, including interactions between those factors, can be responsible for marine mortality of post-smolt and adult Atlantic salmon and there are currently still many unknowns.

Threats encountered by Atlantic salmon during their complex life cycle include overfishing, interactions with farmed salmon, migration barriers and climate change (Friedland *et al.*, 2009; Graham and Harrod, 2009; McGinnity *et al.*, 2009; Birnie-Gauvin *et al.*, 2017; Dadswell *et al.*, 2021). When NASCO (North Atlantic Salmon Conservation Organization) was established, an oversight meant that areas outside of the Exclusive Economic Zone (EEZ) were not being surveilled, leaving large areas over the range of Atlantic salmon open to illegal, unreported and unregulated (IUU) fishing (Dadswell *et al.*, 2021). Estimates suggest that legal and illegal unreported catch totaled 276 tonnes in 2020 (ICES, 2021a). While lower than the unreported catch of 2,788 tonnes in 1987 (ICES, 2021a), this is still a concern due to declining salmon numbers. Wild salmon are also at risk of becoming infected with pathogens or salmon lice (*Lepeophtheirus salmonis*, Krøyer) from farmed Atlantic salmon that spread easily through net pens (Finstad and Bjørn, 2011; Johansen *et al.*, 2011). Captive-bred escapees can cause considerably lower recruitment in the wild population, and negatively affect the ability of the wild salmon to adapt to warmer water temperatures in winter resulting from climate change (McGinnity *et al.*, 2009). In the freshwater environment physical barriers, including hydroelectric dams, weirs and bridges, can reduce the migration ability of salmon (Nieland *et al.*, 2015; Lawrence *et al.*, 2016; Birnie-Gauvin *et al.*, 2017). Factors in the freshwater stage that affect smolt growth and salinity tolerance, including deteriorating water quality, invasive species and higher run-off from the terrestrial environment, can have consequences for survival in the marine realm (Russell, I. *et al.*, 2012; Thorstad *et al.*, 2021).

Climate change is expected to greatly affect Atlantic salmon in many ways due to their complex life history (Graham and Harrod, 2009). Migratory species are particularly

vulnerable to climate change as they utilise environmental variables as cues in their life cycle (Friedland *et al.*, 2003a). Significant relationships have been recognised between long-term trends in phytoplankton, zooplankton and salmon abundances, SST in the Northeast Atlantic, and Northern Hemisphere temperature (NHT) (Beaugrand and Reid, 2003). Declines in zooplankton and salmon catches between 1960 and 2009 were accompanied by increases in SST, NHT, phytoplankton abundance, and AMO index (Beaugrand and Reid, 2012). This suggests that Atlantic salmon numbers will continue to decline as SST continues to rise, causing a northerly expansion of salmon stocks to find more suitable temperatures (Beaugrand and Reid, 2012). Post-smolt survival was likewise negatively correlated with SST in the southernmost habitats but positively correlated in the northernmost, suggesting the effect of increasing SST on post-smolt survival may vary with latitude (Olmos *et al.*, 2020). A temperature increase of 1°C in the Norwegian Sea in 2005 coincided with a decrease of zooplankton abundance by almost 50%, a sudden reduction of Atlantic salmon growth, and a lower number of 1SW returns (Vollset *et al.*, 2022). Declines of 2SW returns were recorded in North American populations, coinciding with reduced productivity, but some regional groups showed differing trends (Mills *et al.*, 2013). Broad scale studies such as these demonstrate the detrimental effects climate change can have on Atlantic salmon populations, and that effects experienced by populations can differ by location and life-history stage. As climate change progresses, adults completing their upstream migration are likely to face increased temperatures and decreased river flow, an important factor governing upstream migration rates (Todd *et al.*, 2011). Higher water temperatures and reduced river flow are the biggest threats to salmon currently near the southern limits of their distribution as suitable habitat is expected to decrease (Graham and Harrod, 2009; Jonsson and Jonsson, 2011d).

1.3 Management and restoration of Atlantic salmon

While Atlantic salmon numbers are declining at an alarming rate, management measures have been implemented with the aim of conserving populations. NASCO was established in 1984 to conserve, restore, enhance and manage wild Atlantic salmon (ICES, 2021a). River fisheries are managed by each individual state, but distant water fisheries that are comprised of fish from many different regions such as at West Greenland, are managed by the six signatory parties of NASCO (ICES, 2021a). Responsibilities are discharged via three Commissions: the North-east Atlantic Commission, the West Greenland Commission, and the North American Commission (Figure 1.3) (ICES, 2021a). In 1999, NASCO agreed on a

Precautionary Approach to protect the salmon resource and its environment (NASCO, 1998). The requirements of the approach include: setting conservation limits (CLs) and management targets for each river; developing stock rebuilding programmes for stocks below the CL; and formulating pre-agreed management objectives (NASCO, 1998). As part of NASCO, fisheries management measures are implemented, including the banning of drift nets in West Greenland in 2020 (ICES, 2021a). Mandatory catch and release measures have been employed in many areas and were implemented in Wales in 2020 (ICES, 2021a). In some regions, ranching programmes are used to supplement rod and line fisheries (ICES, 2021a).

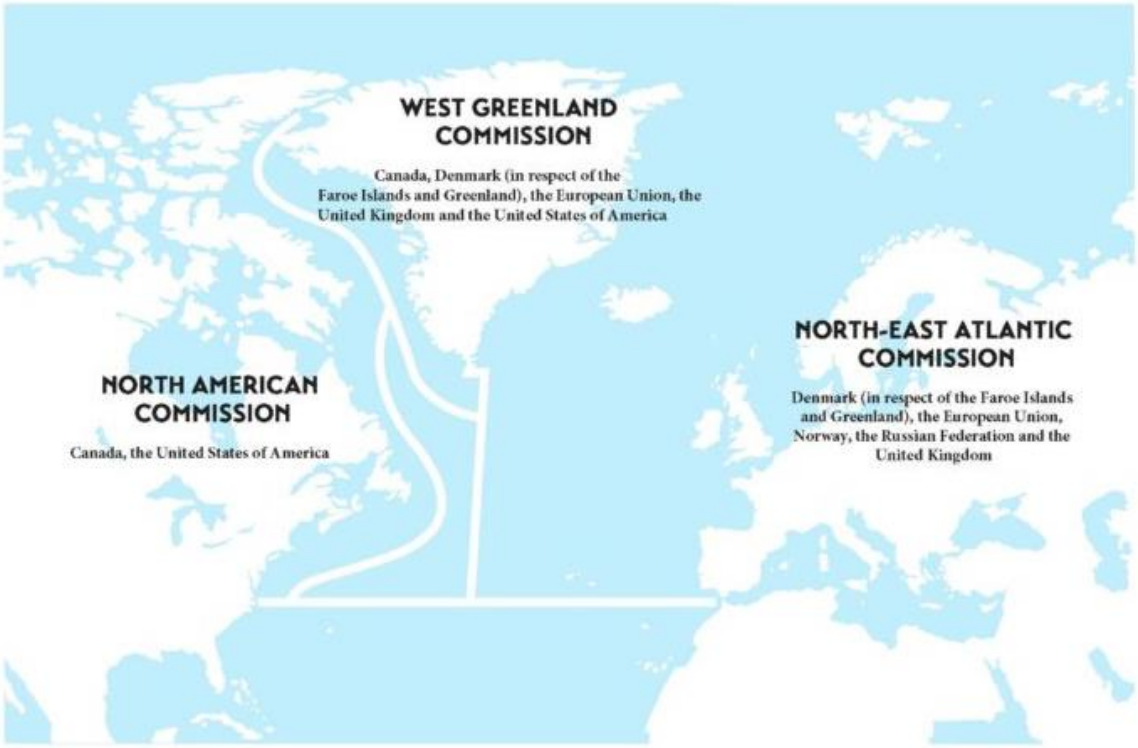

Figure 1. 3 Map showing the membership of the regional Commissions, as defined by the Convention for the Conservation of Salmon in the North Atlantic Ocean. The section of ocean between the commission boundaries is not included in any of the three commissions. Source: NASCO (2001), ICES (2021a)

Scientific advice is commissioned by NASCO from the International Council for the Exploration of the Sea (ICES) and presented in an annual report of the Working Group on North Atlantic Salmon (WGNAS) (Crozier *et al.*, 2004). ICES is an intergovernmental organisation with 20 member countries that undertakes research to give advice on the sustainable use of seas and oceans (ICES, 2021b). Working groups such as the WGNAS are

expert groups overseen by ICES that conduct studies and analyses to provide the basis of ICES scientific advice on a particular topic (ICES, 2021b). Each year, NASCO poses a series of questions to the WGNAS which are answered in the annual report to provide a comprehensive account of the status of salmon stocks in the three commission areas, including catch statistics, abundance estimates, return rates and estimates of unreported catches (ICES, 2021a). These data are then used to implement management strategies for Atlantic salmon populations.

Many restoration projects are underway with the aim of restoring fish stocks (Lennox *et al.*, 2021). Restoration strategies can be quite varied and include the addition of gravel to rivers to provide supplementary substrate for spawning (Barlaup *et al.*, 2008); removal of weirs and other migration barriers (Fjeldstad *et al.*, 2012); and supplementing the population with hatchery-reared juveniles (Kennedy *et al.*, 2012). Restoration methods have varying rates of success or failure and require thorough evaluation of the population in question prior to implementation (Lennox *et al.*, 2021). After reviewing a range of stressors affecting Atlantic salmon and restoration measures used, Lennox *et al.* (2021) recommended that the highest priority should be protecting and conserving existing Atlantic salmon populations and only executing restoration techniques when absolutely necessary.

1.4 Monitoring salmon at sea

For salmon, the high rates of marine mortality also make it imperative to understand their distribution at sea (Hobson *et al.*, 2010; MacKenzie *et al.*, 2011). However, monitoring Atlantic salmon at sea is made difficult by the vastness of their habitat and the extent of their migration (Graham *et al.*, 2010). Direct observation of such a widely dispersed, pelagic species is not possible so other methods need to be used (Graham *et al.*, 2010). Traditionally, tagging surveys have been based on mark recapture methods, enhanced by a NASCO Tag Return Incentive Scheme to encourage reporting of tag recoveries (NASCO, 1989). Internal and external tags recovered from salmon tagged between the 1960s and 2000s were analysed in conjunction with the Northwest Atlantic Salmon Tagging Database (ICES, 2007) to determine the distribution and growth of salmon from different origins caught at West Greenland (Reddin *et al.*, 2012). Various tracking methods have been used to monitor salmon at sea including data storage tags (DSTs; (Reddin *et al.*, 2004)) which store data until recovered, pop-up satellite archival tags (PSATs; (Rikardsen *et al.*, 2021)) which transmit

data via satellite, and acoustic telemetry methods (Barry *et al.*, 2020) which use acoustic transmitters and receivers to track multiple fish simultaneously (DeCelles and Zemeckis, 2014). The Marine Institute in Ireland runs a salmon tagging programme that involves injecting coded-wire microtags into the nasal cartilage of ranched smolts prior to release, and tags are recovered when the fish makes its return migration (Wilkins *et al.*, 2001). Methods involving genetics, including microsatellite (King *et al.*, 2001; Bradbury *et al.*, 2014) and SNP (single-nucleotide polymorphisms) baselines (Jeffery *et al.*, 2018), have been used to characterise the origin of fish caught in oceanic fisheries.

Both national and international programmes have been introduced to further our knowledge of the marine phase of the Atlantic salmon. SALSEA (Salmon at Sea) is an international programme that was established by NASCO to gain a comprehensive overview of factors contributing to marine mortality of salmon (NASCO, 2004). SALSEA-Merge, one of the surveys as part of the programme, was a partnership of nine European nations who completed marine surveys in the Northeast Atlantic in 2008 and 2009 (NASCO, 2012). Data collected included scales, tissues for genetic analysis, and oceanographic data, to provide a detailed overview of migration and distribution patterns of post-smolts in the Northeast Atlantic (NASCO, 2012).

The international sampling programme for the West Greenland fishery has samplers stationed at three communities (Sisimiut, Maniitsoq, and Qaqortoq) and samples are taken separately at Nuuk (ICES, 2020a). Samples include tissue, adipose fin clips, tag recovery, and biological characteristics (ICES, 2020a). From this programme, a time series of data from 1969 has been compiled, including length, weight, growth, and continent of origin of fish caught at West Greenland (ICES, 2019a; ICES, 2020a).

Launched in 2019, the SeaMonitor Project coordinated by the Loughs Agency in Northern Ireland will deliver a large telemetric acoustic array and spatial models aiming to support conservation measures in five species, including Atlantic salmon (Loughs Agency, 2022). Thus far, telemetry data from smolts tagged as part of the project have revealed a northerly migration pattern from smolts entering the Irish Sea in Northwest England (Green *et al.*, 2022). Collaborative programmes such as these are vital for monitoring migration patterns, marine distribution, and feeding locations of Atlantic salmon, expanding our knowledge of the marine phase.

1.5 Scales as recorders of life history

Fish scales are a useful tissue for a variety of analyses. Scales grow incrementally and are comprised of two layers: the hard external layer composed of calcium phosphate similar to the mineral apatite, over a basal plate composed mostly of collagen fibers (Fouda, 1979; Hutchinson and Trueman, 2006). Concentric ridges, or circuli, are laid down on the scale surface as it grows providing a record of the life history of the fish, including growth, spawning and seasonal slowing of the metabolism, making scales a suitable structure for age and growth analysis (Panfili *et al.*, 2002). As the fish grows and new collagen layers are formed, they incorporate chemical signatures such as stable isotopes, and glucocorticoid hormones including cortisol (Hutchinson and Trueman, 2006; Aerts *et al.*, 2015; Laberge *et al.*, 2020). These signatures act as a chronological record of biological, chemical and ecological events over the lifetime of the fish (Perga and Gerdeaux, 2003). This chronological record is particularly useful when studying migratory fish because the transition between freshwater and marine habitats produces a distinct growth check in the scale (Panfili *et al.*, 2002).

Scales can be sampled relatively quickly, simply and non-lethally. They are collected as part of routine sampling and can be stored at room temperature for an extensive amount of time (Tray *et al.*, 2020). As a result, many organisations have vast archives of scales spanning decades; the archive held by the Marine Institute in Ireland contains scales dating back almost a century (Tray *et al.*, 2020). Due to scale structure, archives such as these provide access to a large time series of life-history data, making them valuable resources. An extensive archive is also held by Fisheries and Oceans Canada (DFO) which contains Atlantic salmon scales taken as part of routine sampling at the West Greenland fishery. This archive contains over 20,000 scale samples dating back to the 1960s (ICES, 2020b).

Traditionally, scales have been used for retrospective analyses of age and growth, as scale circuli are deposited at a rate that is proportional to body growth (Heidarsson *et al.*, 2006). The use of scales for age and growth analysis allows for a time series of data to be analysed and compared to other factors such as marine survival (Peyronnet *et al.*, 2007), thereby gaining important insight into the structure of a population. Scale characteristics such as river age and the number of circuli in the first sea zone, can even be used to determine the continent of origin of Atlantic salmon (Lear and Sandeman, 1980). More recent advances in

scale methodology provide the opportunity to examine temporal changes in a variety of aspects of the life history of Atlantic salmon through methods such as stable isotope analysis (Trueman and Moore, 2007) and investigation of chronic stress responses (Aerts *et al.*, 2015).

1.6 Stable isotopes as a tool to reconstruct Atlantic salmon life history

Stable isotope analysis (SIA) is a robust tool that has a wide range of applications in ecological studies. Isotopes are atoms of the same element with different numbers of neutrons; stable isotopes are not reactive and do not decay (Fry, 2006). Due to their stability, stable isotopes provide a natural way to track elements in the environment (Fry, 2006). Stable isotopes are measured as the ratio of the heavy to light isotope and expressed using delta notation (δ) (McKinney *et al.*, 1950; Dawson and Siegwolf, 2007). Stable isotope signatures in the tissues of an animal reflect its diet and environment; as they eat, animal tissues become more enriched in isotopes such as carbon and nitrogen in a predictive manner (DeNiro and Epstein, 1978; Peterson and Fry, 1987; Hobson, 1999). As a result, isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are commonly measured to examine diet and trophic level in food web studies (Post, 2002). SIA can be used to answer a wide range of research questions, including distinguishing between farmed and wild salmon (Dempson and Power, 2004), reconstructing life history strategies (Hanson *et al.*, 2013), recreating animal movement and location (MacKenzie *et al.*, 2011), and distinguishing between the trophic niches of native and invasive species (Pacioglu *et al.*, 2019). Carbon and nitrogen stable isotope analysis is usually carried out using an elemental analyser (EA) coupled with a continuous flow-isotope ratio mass spectrometer (CF-IRMS) (Fry, 2006).

Dorsal muscle is often the tissue of choice in SIA of fish (Pinnegar and Polunin, 1999), but other tissues can also be analysed, including mucus (Church *et al.*, 2009), fins (Graham *et al.*, 2013; Hayden *et al.*, 2015) and scales (Hutchinson and Trueman, 2006; Trueman and Moore, 2007; Grey *et al.*, 2009). Sample preparation methodologies need to be adapted to account for characteristics of the tissue being analysed, such as the presence of lipids or inorganic carbonates (DeNiro and Epstein, 1978; Perga and Gerdeaux, 2003), or post-analysis models can be used (Kiljunen *et al.*, 2006). Scales are valuable tissues for reconstructing life history and migration patterns using SIA; they can be sampled non-lethally and provide a permanent record of isotopic changes, including diet and environment, over the lifetime of the fish (Trueman and Moore, 2007). The unique structure of the scale allows for analyses over an

extended time-period, and SIA of Atlantic salmon scales has, for example, been used to determine migratory connectivity of salmon in the Baltic Sea (Torniainen *et al.*, 2013), to relate $\delta^{13}C$ and $\delta^{15}N$ time series to environmental variables (Trueman *et al.*, 2012), and to identify anthropogenic nutrient input into riverine systems (Roussel *et al.*, 2014). However, the irreplaceable nature of archived scales increases the importance of methodological validation prior to completing destructive analysis.

1.7 Cortisol analysis

Cortisol is a steroid hormone released as a response to stress and is the main corticosteroid measured in fishes (Sadoul and Geffroy, 2019). Stress, the adaptive response of an organism to a stressor, invokes the same basic response pattern from organisms (Selye, 1950; Russell, E. *et al.*, 2012). Cortisol release is an attempt by the organism to manage stress and it can act as a biomarker to measure the severity of stressors (Russell, E. *et al.*, 2012; Baker and Vynne, 2014). When stressors such as air exposure, starvation, and extreme temperatures are experienced by a fish, cortisol activates energy stores and regulates the immune response, but sustained exposure to stressors can have damaging effects (Russell, E. *et al.*, 2012).

Cortisol is measured regularly in farmed Atlantic salmon for welfare purposes to ensure their environment is not causing stress (Barton, 2002). In fish, the most common method of measuring cortisol is in blood plasma (Cook, 2012). However, blood cortisol only provides a measure of acute stress and it can be influenced by the sampling process (Bertotto *et al.*, 2010); blood samples need to be taken within 3 minutes of capture (Birnie-Gauvin *et al.*, 2019) to avoid compromising the cortisol levels. Alternative, less-invasive matrices for cortisol measurement have been examined, including gill filaments (Gesto *et al.*, 2015), fin tissue, mucus (Bertotto *et al.*, 2010), and even water samples (Madaro *et al.*, 2018). Within the past decade, fish scales have been assessed as a matrix for the measurement of chronic stress (Aerts *et al.*, 2015) and scale cortisol has since been proven to be a reliable biomarker for temperature stress (Hanke *et al.*, 2019; Laberge *et al.*, 2020; Goikoetxea *et al.*, 2021). Aerts *et al.* (2015) used methanol as the extraction solvent and performed chromatographic analysis using HPLC-MS/MS (high performance liquid chromatography tandem mass spectrometry) to detect and quantify scale cortisol. Combined with the easy, non-invasive process of collecting scale samples, this method could provide a suitable means to examine

chronic stress in wild populations of Atlantic salmon, and to retrospectively analyse stress in populations over time by quantifying cortisol in archived scales.

1.8 Objectives and thesis outline

Despite being a well-studied species of ecological, economic, and cultural importance, there are many aspects of the complex life cycle of the Atlantic salmon that are still not fully understood. These knowledge gaps need to be addressed to inform conservation and management of the species. The overall aim of this thesis was to develop, validate and apply methods that utilise the wealth of data contained in scales to enhance our knowledge of Atlantic salmon. This validation is particularly important, given that the analyses are destructive and archived material is irreplaceable once analysed. The methods examined in this thesis can unlock information contained in vast archives of Atlantic salmon scales. Potential applications include determination of marine feeding locations, examination of temporal trends in foraging distribution, non-invasive measurement of stress responses, and investigation of temporal trends in and drivers of physiological stress during the marine migration.

This thesis consists of six chapters including this general introduction, four peer reviewed research papers that are published, under review or in preparation, and a discussion. An outline of each chapter, including aims and publication details, is described below.

Chapter 2:

This study addressed a methodological disagreement in the literature regarding the treatment of Atlantic salmon scales with hydrochloric acid to remove inorganic carbonates prior to stable isotope analysis. The results inform the development of an efficient protocol for preparing Atlantic salmon scales for stable isotope analysis. The objectives were:

- to examine scales from a large sample size of Atlantic salmon from different regions and life histories
- to determine if Atlantic salmon scales require acid-treatment prior to stable isotope analysis

This manuscript is published as:

O'Toole, C., Weigum, E., Graham, CT., White, P., Samways, K., Hayden, B., and Brophy, D. (2020). Acid treatment of Atlantic salmon (*Salmo salar*) scales prior to analysis has negligible effects on $\delta^{13}C$ and $\delta^{15}N$ isotope ratios. Journal of Fish Biology; 97:1285-1290. https://doi.org/10.1111/jfb.14501

Chapter 3:

The isoscape approach uses stable isotopes to pinpoint the feeding location of organisms; to apply it, baseline data may need to be collected from all prey items known to be consumed by the species, in parallel with the collection of tissues from the organism of interest. This prohibits the application of the isoscape approach to stable isotope analysis of archived scales, thereby eliminating a valuable data resource. An alternate approach developed by MacKenzie *et al.* (2011) does not require the collection of baseline data. Instead, it uses maps of the strength and significance of the correlation between time series of SST and carbon stable isotope ratios ($\delta^{13}C$) in scales to identify the most likely feeding areas. This method allows retrospective analysis of archived scales and has already been used in other studies to support inferences about where different populations and life stages are feeding. However, the assumptions of the method needed to be thoroughly validated to ensure these interpretations are robust

The objective of this study was to use stable isotope analysis of scales from Atlantic salmon of known feeding location (West of Greenland) over a 50-year time span (10 sampling years) to determine if the location predicted by the geolocation tool matches the known feeding location. Growth measurements and scale $\delta^{15}N$ were also analysed to examine variability in $\delta^{13}C$ and investigate assumptions of this geolocation approach.

This manuscript is currently in preparation for the target journal, Scientific Reports, as:

O'Toole, C., Brophy, D., Stroh, A., Weigum, E., Hayden, B., White, P., Trueman, CN., Robertson, MJ., and Graham, CT. (in prep.). Carbon isotope geolocation tool successfully identifies the foraging location of Atlantic salmon captured at sea.

Chapter 4:

Blood sampling is the most common method of extracting cortisol, a stress hormone, from fish. However, the method is invasive, can be affected by the sampling process, and only reflects acute stress, not chronic stress. Recent research has successfully extracted cortisol from fish scales, providing a non-invasive measure of chronic stress (Aerts *et al.*, 2015). In this chapter, this method was adapted for use with small amounts of Atlantic salmon scales and used to examine variability in cortisol from scales of experimentally reared post-smolts exposed to three temperature treatments and four feeding treatments. The objectives of this study were:

- to test if cortisol could be reliably detected in the scales of Atlantic salmon post-smolts
- to investigate relationships between temperature and feeding conditions with cortisol in the scales of Atlantic salmon post-smolts
- to evaluate scale cortisol as a potential biomarker for monitoring physiological stress responses to environmental change in wild Atlantic salmon

This manuscript is currently in preparation for the target journal, Science of the Total Environment, as:

O'Toole, C., White, P., Thomas, K., Ó Maoiléidigh, N., Fjelldal, PG., Hansen, T., Graham, CT., Brophy, D. (in prep.). Scale cortisol concentration in Atlantic salmon post-smolts is influenced by temperature.

Chapter 5:

Cortisol in the marine portion of scales of returning adult salmon from a multi-decadal scale collection were analysed to examine temporal trends in physiological stress during the marine phase. Scale cortisol was compared between years with contrasting meteorological conditions (positive and negative phases of the North Atlantic Oscillation). A simulated degradation study was also completed to determine the stability of cortisol in scales over time. The objectives were to:

- Determine if cortisol in Atlantic salmon scales remains stable during storage using simulated degradation

- Use cortisol measurements from the marine portion of scales from returning adult Atlantic salmon to provide an index of physiological state during the marine phase.

- Examine temporal trends in this index of physiological state over a 29-year period (6 selected years from 1989 to 2018)

- Examine relationships between physiological state and environmental (the North Atlantic Oscillation (NAO), SST and zooplankton abundance) and biological (fish size and growth) variables

This manuscript is currently in preparation for the target publication, the Journal of Experimental Marine Biology and Ecology, as:

O'Toole, C., White, P., Ó Maoiléidigh, N., Conroy, C., Stroh, A., Graham, CT., Brophy, D. (in prep.). Cortisol in scales of adult Atlantic salmon shows substantial individual variability with no annual signal.

Chapter 6:

The general discussion of this thesis addresses the overall findings of the four research chapters and examines these results in the context of exiting knowledge on Atlantic salmon, stable isotope analysis, and cortisol analysis. The impact of the results of this thesis on the use of historical scale archives to increase our understanding of the marine phase of Atlantic salmon is discussed, and recommendations for further research are made.

Chapter 2. Acid treatment of Atlantic salmon (*Salmo salar*) scales prior to analysis has negligible effects on $\delta^{13}C$ and $\delta^{15}N$ isotope ratios

Published as: O'Toole, C., Weigum, E., Graham, C.T., White, P., Samways, K., Hayden, B., Brophy, D. (2020). Acid treatment of Atlantic salmon (*Salmo salar*) scales prior to analysis has negligible effects on $\delta^{13}C$ and $\delta^{15}N$ isotope ratios. Journal of Fish Biology, 97:1285-1290. https://doi.org/10.1111/jfb.14501

2.1 Abstract

There is debate in the literature as to whether scales of fishes require acidification to remove inorganic carbonates prior to stable isotope analysis. Acid-treated and untreated scales from 208 Atlantic salmon from nine locations on both sides of the Atlantic were analysed for $\delta^{13}C$ and $\delta^{15}N$. Linear mixed effects models determined the effect of acid treatment to be statistically significant. However, the mean difference was small ($\delta^{13}C$: 0.1±0.2‰; $\delta^{15}N$: -0.1±0.2‰) and not of biological relevance. This study concludes that Atlantic salmon scales do not need to be acidified prior to stable isotope analysis.

2.2 Main body

Stable isotope analysis (SIA) is a powerful tool in ecology that can be used to determine the prey items of primary importance to consumers (Wieczorek *et al.*, 2018); identify the marine feeding location of individual organisms (MacKenzie *et al.*, 2011); establish migratory connectivity between populations (Torniainen *et al.*, 2013); and investigate the trophic position of consumers (Vander Zanden *et al.*, 1997). Dorsal muscle is typically the tissue of choice for SIA of fish (Pinnegar and Polunin, 1999) but alternative tissues have also been analysed, including fins (Graham *et al.*, 2013; 2014), mucus (Church *et al.*, 2009) and scales (Perga and Gerdeaux, 2003; Hutchinson and Trueman, 2006; Sinnatamby *et al.*, 2007; MacKenzie *et al.*, 2011; Torniainen *et al.*, 2013). Scales are an ideal tissue to analyse as they can be sampled non-lethally, relatively simply and quickly. In addition, data describing age and growth rates can also be obtained from the scales prior to SIA (Einum *et al.*, 2002; Hutchinson and Trueman, 2006). Many laboratories around the world hold vast archives of scales from various fish species, from which considerable amounts of invaluable data can be generated. Stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) are incorporated into the collagen layers of scales as they grow and can be used for analysis of diet, environmental

conditions, and trophic structure, making SIA of fish scales potentially very informative (Hutchinson and Trueman, 2006). Scale archives may include samples that span up to 100 years, thus providing exciting opportunities to gain unique insights into the lives of not only individual fish but also populations over large timespans. Analyses of archived scales can determine if migration pathways or feeding histories have changed over the span of the archive (MacKenzie *et al.*, 2011). Combining these analyses with environmental data could illuminate long-term patterns, including climate change effects.

In previous studies that use SIA of fish scales, various methods have been used to prepare the scales for analysis and to deal with the potentially confounding inorganic component of fish scales. In a study on whitefish (*Coregonus lavaretus*, L.) in Lake Geneva, Perga and Gerdeaux (2003) determined that exposing scales to HCl (hydrochloric acid) for 2 minutes was necessary during preparation for SIA to remove such inorganic carbonates from the scale which can be enriched in ^{13}C (Perga and Gerdeaux, 2003). However, acidification is not a desired step in preparing scales for SIA as it greatly increases the preparation time and has been shown to alter nitrogen isotopic composition, causing enrichment in ^{15}N (Bunn *et al.*, 1995; Pinnegar and Polunin, 1999). This would necessitate the analysis of twice as much material which is very time consuming, costly, and highly unfavorable when dealing with archived scales which may be limited in number. Schlacher and Connolly (2014) recommend that acidification should not be carried out as a general rule and the effects should be determined prior to analysis as acidification can affect both $\delta^{13}C$ and $\delta^{15}N$. Sinnatamby *et al.* (2007) completed a study on Atlantic salmon (*Salmo salar*, L.), yellow perch (*Perca flavescens*, Mitchill) and walleye (*Sander vitreus*, Mitchill) and did not find significant differences between acidified and non-acidified scales. As this contradicts Perga and Gerdeaux (2003), they suggested that the need for acidification of fish scales could vary between species, a possibility that was also proposed by Ventura and Jeppesen (2010), who suggest that varying mineral content in scales of different species could be responsible for disparities between previous studies. Additionally, dissolved inorganic carbon (DIC) in freshwater is usually depleted in inorganic ^{13}C values relative to seawater due to CO_2 input from decomposing terrestrial matter (Boutton, 1991). Therefore, uptake of inorganic carbon in fish scales could be affected by varied availability of DIC between marine and freshwater ecosystems. According to Trueman and Moore (2007), the apatite component of Atlantic salmon scales is less than 30% of the mass of the scale, indicating that acidification is not

necessary. However, while the regression models carried out by Sinnatamby *et al.* (2007) showed strong relationships between treated and untreated scale isotope ratios for the two freshwater species, yellow perch (R^2 = 0.997 for $\delta^{13}C$; 0.989 for $\delta^{15}N$) and walleye (R^2 = 0.989 for $\delta^{13}C$; 0.980 for $\delta^{15}N$), the relationship for Atlantic salmon was much weaker (R^2 = 0.455 for $\delta^{13}C$; 0.553 for $\delta^{15}N$). The $\delta^{13}C$ of acidified and non-acidified scales were not significantly different from each other, but the p value (0.052) of Atlantic salmon was somewhat inconclusive (Sinnatamby *et al.*, 2007).

The current study furthers the research of Sinnatamby *et al.* (2007) on Atlantic salmon scales. We focus mainly on marine feeding salmon as they are under-represented in other similar studies that focus on freshwater habitats (Perga and Gerdeaux, 2003; Ventura and Jeppesen, 2010). The aim is to examine a much larger sample size from many regions across the range of the fish, including Canada, Ireland and the UK, to determine if acidification to remove inorganic carbonates is necessary prior to SIA of Atlantic salmon scales. Our study includes scales from varying life histories including ranched, farmed and wild fish. This research is particularly important for Atlantic salmon as an increasing number of studies are carrying out SIA of both modern and archived salmon scales to better understand marine migrations and long-term trends.

Scale samples were obtained from Atlantic salmon in nine locations in Canada, Ireland, Northern Ireland and Wales in 2018. Our samples included adult Atlantic salmon from freshwater tanks, marine aquaculture pens, ranched adults returning to Ireland, and wild adult salmon returning to rivers in Eastern Canada and Europe. Scales were obtained from adult captive broodstock from the Tobique River, hereafter Tobique (n = 30), in northwestern New Brunswick, Canada during hatchery spawning. Samples from the Tobique population represent growth exclusively in a freshwater hatchery environment. The marine aquaculture fish were reared in the Bay of Fundy, New Brunswick, Canada in two separate aquaculture facilities: fish hereafter known as Aqua 1 (n = 30) were sampled during routine health screening while in sea cages; wild origin smolts, grown to maturity in modified net pens at the world's first Marine Conservation Farm (Aqua 2, n = 25), were sampled during the autumn tagging period and subsequently released back to their natal rivers. Adult salmon returning to Canadian rivers were sampled at the Big Salmon River (BSR, n = 24), Upper Salmon River (USR, n = 7) and Gaspereau River (Gaspereau, n = 8) in the Bay of Fundy, New Brunswick. Adult salmon returning to European rivers were sampled at fish traps in the

Bush River, Northern Ireland (hereafter Bush, n = 26), the River Dee, Wales (hereafter Dee, n = 48), and the Burrishoole River on the west coast of Ireland (hereafter Burrishoole, n = 10). Scales collected at the Burrishoole River were from ranched fish, reared until the smolt stage in a hatchery at the Marine Institute's Newport Research Facility in County Mayo, then released into Lough Furnace to begin their sea migration and sampled on their return as grilse.

Prior to analysis, all scales were soaked in distilled water for a minimum of 2 minutes and then scraped gently with a scalpel on both sides to remove any mucus. Suitable scales from each fish were chosen for imaging. Burrishoole scales were imaged using an Olympus BX51 compound microscope and ImagePro Plus software Version 6.3.1.542. All other scales were imaged using a Leica MZ16 A microscope with Auto-Montage Pro software. The scales were allowed to air dry following imaging. Scale material corresponding to the last summer at sea was excised under a dissecting microscope or magnifier to obtain a temporally distinct sample (MacKenzie *et al.*, 2011). Between 1 and 1.2mg of the scale cuttings were weighed into tin capsules (Elemental Microanalysis pressed tin capsules, 5 x 3.5mm) and folded for analysis. The remainder of the cut scales were submerged in 1M HCl for 2 minutes, then rinsed with distilled water and placed in an oven at 60°C for approximately 24 hours. Acidified scale sections were then weighed into tin capsules as above. All analyses were carried out at the Stable Isotopes in Nature Laboratory (SINLAB) at the University of New Brunswick, Fredericton, NB, Canada. A combination of CE NC2500 and Costech 4010 elemental analysers connected to either a Delta-Plus/Conflo II or a Delta^{Plus} XP/Conflo III continuous-flow isotope ratio mass spectrometer (CF-IRMS) were used for analysis of carbon and nitrogen isotopes. Stable isotope measurements are reported in the standard delta (δ) notation in parts per thousand (‰) relative to the international standards: Vienna Pee Dee Belemnite (VPDB) for carbon (Craig, 1957) and atmospheric air (AIR) for nitrogen (Mariotti, 1983). Isotope values were normalised using in-house secondary standards (USGS61, BLS (Bovine Liver Standard) and MLS (Muskellunge Muscle Standard)) which were all calibrated against International Atomic Energy Agency (IAEA) standards. To assess analytical accuracy, the following check standards were analysed: Nicotinamide, N_2, and CH_7. Repeated analysis of internal standards shows that the analytical precision was better than ± 0.2‰ for $\delta^{13}C$ and ± 0.3‰ for $\delta^{15}N$. Approximately 7% of samples were run in replicate to monitor instrument drift over time. Following acidification, some samples achieved low

weights of 0.6mg and below. These samples were run separately and the CF-IRMS was amplified for low-weight samples to achieve accurate results. All statistical analyses were carried out using R Version 3.5.2 in RStudio Version 1.2.5019.

Across locations, mean $\delta^{13}C$ values ranged from -16.7‰ to -14.9‰ in untreated scales and from -16.7‰ to -14.8‰ in acid-treated scales. Mean values for $\delta^{15}N$ ranged from 8.1‰ to 15.0‰ in untreated scales and 8.00‰ to 15.1‰ in treated scales. The difference between means at each location was small (from 0.0 ± 0.1 standard deviation to 0.3 ± 0.2 standard deviation; Table 2.1). Linear mixed effect models were used to examine the effect of acid treatment on $\delta^{13}C$ and $\delta^{15}N$ values in the scale. Three models were tested for each of $\delta^{13}C$ and $\delta^{15}N$ (models and AIC values displayed in Supplementary Table 2.1). For both $\delta^{13}C$ and $\delta^{15}N$, the best fitting model (based on AIC values: for $\delta^{13}C$, the best fitting model had an AIC value of 115.5, while the other two models had AIC values of 529.4 and 540.5; for $\delta^{15}N$, the best fitting model had an AIC value of 414.3, while the other two models had AIC values of 869.5 and 886.2) included treatment as a fixed effect with location and fish ID as random effects. The total explained variance indicated a good model fit (conditional $R^2 = 0.96$ for $\delta^{13}C$, 0.99 for $\delta^{15}N$), with a very small proportion of that variance attributed to the acid treatment (marginal $R^2 = 0.002$ for $\delta^{13}C$, 0.0005 for $\delta^{15}N$). The fixed effect model estimate indicated that $\delta^{13}C$ values of untreated scales were 0.07‰ (± 0.02‰ standard error) higher than $\delta^{13}C$ values of treated scales. $\delta^{15}N$ values of untreated scales were 0.08‰ (± 0.02‰ standard error) lower than $\delta^{15}N$ values of treated scales. Treatment had a significant effect on $\delta^{13}C$ ($p < 0.001$) and $\delta^{15}N$ ($p < 0.001$) values but the differences between acid-treated and untreated scales were negligible considering that analytical precision was estimated at ± 0.2‰ for $\delta^{13}C$ and ± 0.3‰ for $\delta^{15}N$. A linear mixed effect model was used to model the relationships between $\delta^{13}C$ in acid treated and untreated scales, with location included as a random effect (models and AIC values can be viewed in Supplementary Table 2.2). The marginal R^2 (0.899) and conditional R^2 (0.934) show that the majority of the variability in $\delta^{13}C$ of acid-treated scales is due to variation in $\delta^{13}C$ before treatment, with less than 4% of variability accounted for by location. A similar linear mixed model was run for nitrogen (models and AIC values can be viewed in Supplementary Table 2.2), where marginal R^2 (0.983) and conditional R^2 (0.989) show that less than 0.01% of variability in $\delta^{15}N$ of acid-treated scales was due to location.

Table 2. 1 The mean (± SD) of $\delta^{13}C$ and $\delta^{15}N$ isotope signatures and the difference between untreated and acid-treated Atlantic salmon scales for each location and the combined data.

				Carbon			**Nitrogen**		
				Mean $\delta^{13}C$ ± SD (‰)			Mean $\delta^{15}N$ ± SD (‰)		
Location	n	Region	Life History	Untreated	Acidified	Difference	Untreated	Acidified	Difference
Aqua 1	30	Canada	Aquaculture	-15.2 ± 0.2	-15.5 ± 0.3	0.2 ± 0.2	8.1 ± 0.2	8.0 ± 0.2	0.0 ± 0.1
Aqua 2	25	Canada	Aquaculture	-14.9 ± 0.2	-15.0 ± 0.2	0.2 ± 0.2	15.0 ± 0.3	15.1 ± 0.3	-0.1 ± 0.2
BSR	24	Canada	Wild	-14.9 ± 0.2	-14.8 ± 0.3	-0.0 ± 0.2	10.3 ± 0.4	10.3 ± 0.5	0.0 ± 0.2
Burrishoole	10	Ireland	Ranched	-16.4 ± 0.3	-16.6 ± 0.3	0.2 ± 0.2	10.6 ± 0.8	11.0 ± 0.8	-0.3 ± 0.2
Bush	26	N. Ireland	Wild	-16.7 ± 0.5	-16.7 ± 0.3	0.1 ± 0.3	11.6 ± 0.7	11.7 ± 0.7	-0.1 ± 0.2
Dee	48	Wales	Wild	-16.7 ± 0.3	-16.7 ± 0.3	0.0 ± 0.2	11.3 ± 0.6	11.5 ± 0.6	-0.3 ± 0.2
Gaspereau	8	Canada	Wild	-16.5 ± 0.3	-16.3 ± 0.6	-0.2 ± 0.4	11.6 ± 0.6	11.5 ± 0.6	0.2 ± 0.2
Tobique	30	Canada	Freshwater	-15.0 ± 0.2	-15.1 ± 0.2	0.1 ± 0.2	10.0 ± 0.2	10.0 ± 0.2	0.0 ± 0.2
USR	7	Canada	Wild	-15.2 ± 0.4	-15.1 ± 0.5	-0.0 ± 0.2	11.1 ± 1.9	11.2 ± 2.1	-0.2 ± 0.3
Combined	208			-15.7 ± 0.9	-15.8 ± 0.9	0.1 ± 0.2	11.0 ± 1.9	11.1 ± 2.0	-0.1 ± 0.2

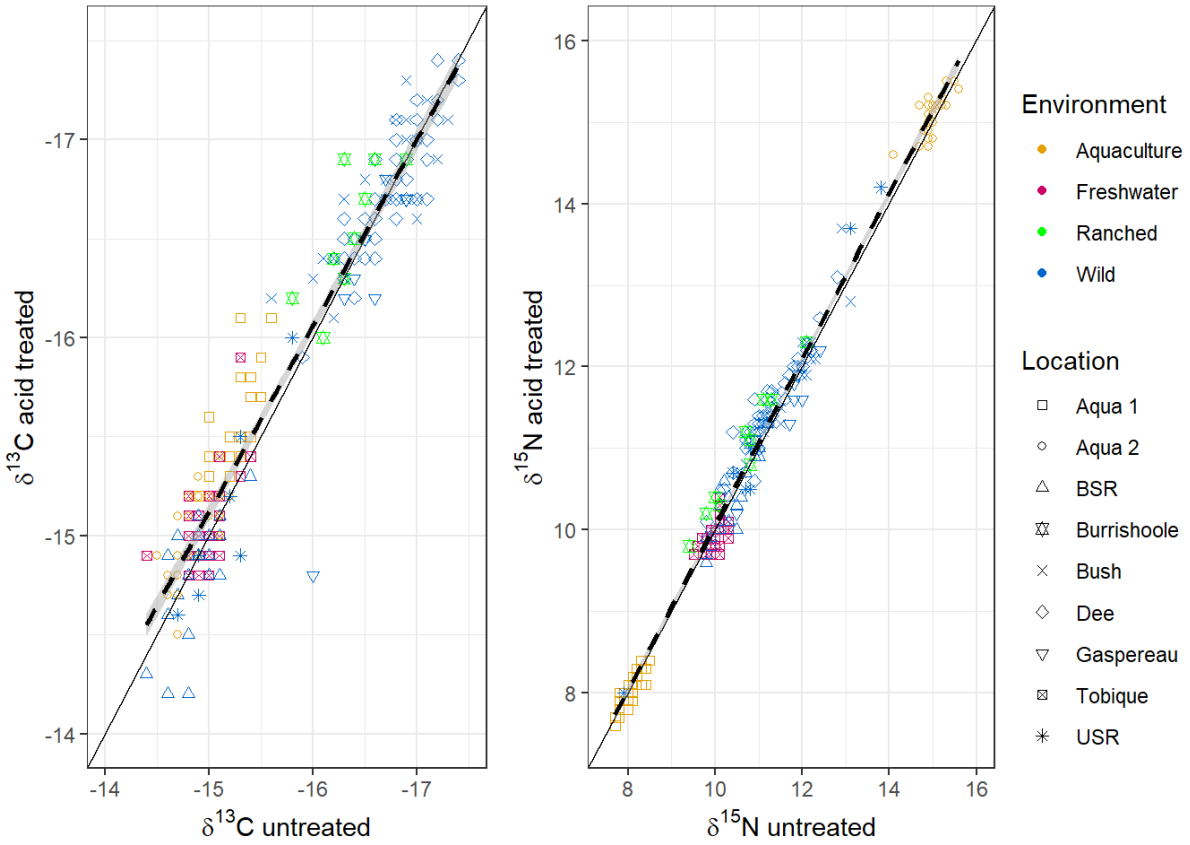

Figure 2. 1 Relationships between acid-treated and untreated $\delta^{13}C$ and $\delta^{15}N$ of Atlantic salmon scales. Continuous line shows 1:1 line, and dashed line shows the line of best fit for the data, included for illustrative purposes. Colours represent the life history environment of the fish, where Freshwater represents hatchery rearing and Ranched represents fish that were hatchery reared until the smolt stage when they were released to migrate to sea.

The model-estimated differences between acid-treated and untreated scales are too small to be biologically relevant. Kennedy *et al.* (2005) examined over 200 salmon fry stocked in 11 tributaries of the Connecticut River in eastern United States. Using stable isotopes of carbon and nitrogen, they were able to distinguish 7 out of 11 sites between 40 and 104 days after being stocked in the river with the difference in $\delta^{13}C$ between sites ranging from 0.25 to 6.1‰. As those fish were released from the same hatchery just four months prior to recapture, this confirms that a difference of 0.07‰ ± 0.02‰ for $\delta^{13}C$ in adult fish with a wide-ranging migration is not likely to be biologically relevant. The mean difference of -0.08‰ ± 0.02‰ for $\delta^{15}N$ is also not likely to be biologically relevant. $\delta^{15}N$ is most commonly used to estimate trophic position, and trophic fractionation of $\delta^{15}N$ is widely accepted to be ~3‰ on average (Post, 2002; McCutchan Jr *et al.*, 2003) and can vary from 1.3‰ to 5.3‰ and depending on

factors including diet, physiological stress and tissue type analysed (Minagawa and Wada, 1984; McCutchan Jr *et al.*, 2003; McMahon *et al.*, 2015). These values are considerably higher than the mean differences in $\delta^{15}N$ reflected in our analyses. Figure 2.1 outlines the relationship between acid-treated and untreated scale data for both $\delta^{13}C$ and $\delta^{15}N$. The slope of the relationship was significantly different from 1 for $\delta^{13}C$ ($p < 0.05$) but not for $\delta^{15}N$ ($p = 0.05$). The line of best fit deviates slightly from the 1:1 line for $\delta^{13}C$ where the freshwater samples are clustered. This suggests that further research is needed on the effect of acidification on scales of Atlantic salmon residing in freshwater environments. However, the difference between acid-treated and untreated scales were very small for the freshwater samples (0.1 ± 0.2‰ for $\delta^{13}C$). Boxplots of the difference in isotopic composition between untreated and acidified Atlantic salmon scales are displayed in Figure 2.2 and show differences are relatively low and constant across locations.

The data from this study show that treatment has a statistically significant effect on Atlantic salmon scales, contrary to the results of Sinnatamby *et al.* (2007), but the difference is very small and not likely to be of biological relevance. Therefore, this study finds that acidification has a negligible effect on Atlantic salmon scales and is not necessary prior to carbon stable isotope analysis, confirming the findings of Sinnatamby *et al.* (2007). The mean difference in this study is much smaller than that recorded by Perga and Gerdeaux (2003), where acidifying whitefish scales increased the mean $\delta^{15}N$ by 1.3 ± 0.3‰. It is possible that scales are affected differently by acidification depending on the species or the habitat occupied by the fish, i.e. freshwater versus marine. The majority of fish in this study had spent at least one year in the marine environment, but the Tobique fish were reared exclusively in freshwater. Tobique data showed very small differences between acid-treated and untreated scales (0.1 ± 0.2‰ for $\delta^{13}C$; 0.0 ± 0.2‰ for $\delta^{15}N$), much smaller than that reported by Perga and Gerdeaux (2003) who also examined fish that exclusively inhabited freshwater. This is consistent with the findings of Ventura and Jeppesen (2010) who suggest that the effect of acid treatment on scales may vary between species. This study has answered an important contradiction in the literature by investigating acid-treated and untreated scales from 208 Atlantic salmon. These fish were from nine different locations on both sides of the Atlantic and were from ranched, farmed, and wild life histories. Using a large sample size and a variety of locations, this study agrees with Sinnatamby *et al.* (2007) and concludes that acidification prior to stable isotope analysis is not necessary for Atlantic salmon.

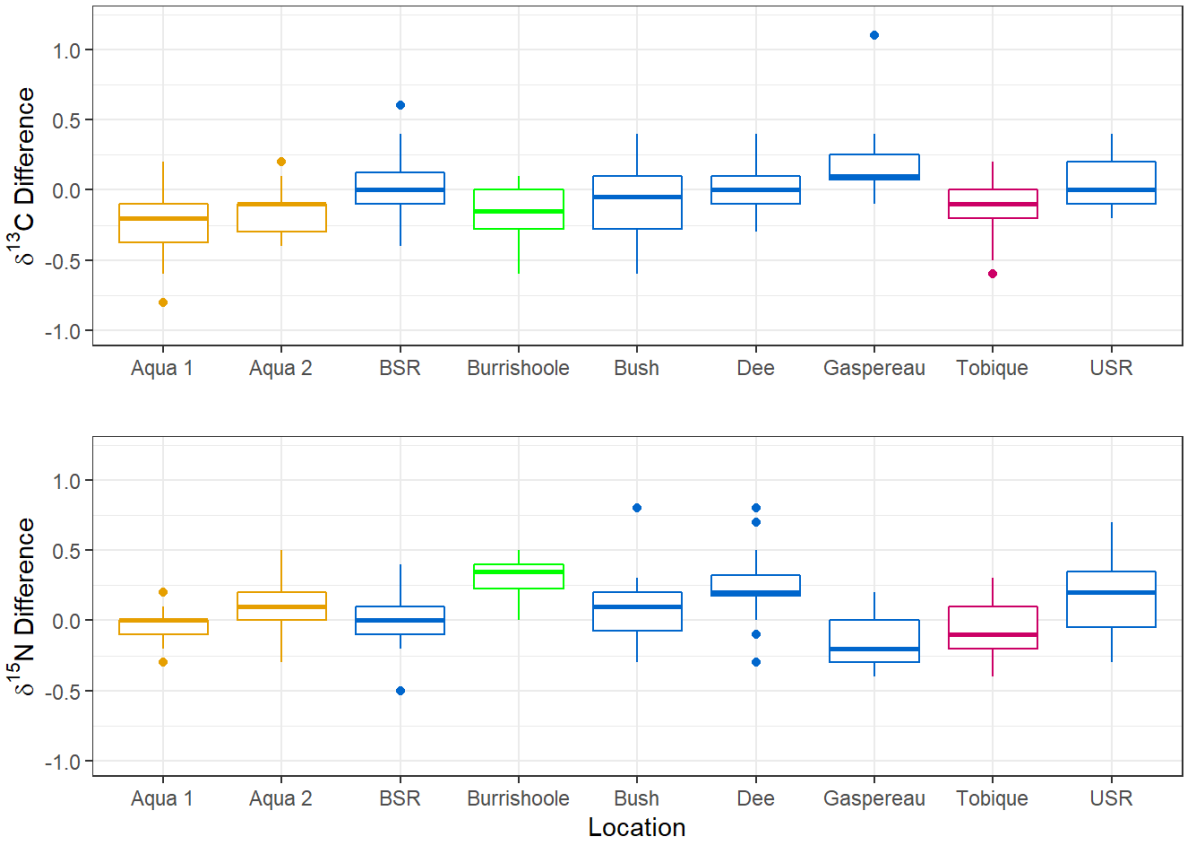

Figure 2. 2 Boxplots of the difference in $\delta^{13}C$ and $\delta^{15}N$ stable isotope ratios between untreated and acidified Atlantic salmon scales, organised by sampling location. The bold line in each box represents the median for each location. Colours depict life history environment as in Figure 2.1, where orange represents aquaculture, red represents freshwater, green represents ranched, and blue represents wild.

2.3 Acknowledgements

The authors would like to thank the Marine Institute for funding this project, and Ger Rogan, Elvira de Eyto, Signe Martin, Elizabeth Tray and Louise Vaughan for support in sampling the Mayo fish. The authors also thank Anne McGeachy, Andrea Prentice and Samantha Pettey for assistance running the analyses at SINLAB. We are also grateful to Dennis Ensing and Ian Russel who provided samples from Northern Ireland and Wales, respectively.

2.4 Supplementary material

Supplementary Table 2. 1 AIC of the model used is in bold text.

Model	**AIC value**
Carbon	
lmer(d13C ~ Treatment + (1|ID.CODE)	529.4103
lmer(d13C ~ (1|ID.CODE)	540.4616
lmer(d13C ~ Treatment + (1|ID.CODE) + (1|Location)	**115.5433**
Nitrogen	
lmer(d13N ~ Treatment + (1|ID.CODE)	869.4745
lmer(d13N ~ (1|ID.CODE)	886.1731
lmer(d13N ~ Treatment + (1|ID.CODE) + (1|Location)	**414.3275**

Supplementary Table 2. 2 AIC of the model used is in bold text.

Model	**AIC value**
Carbon	
lmer(d13C_AT ~ d13C + (1|Location)	**-31.53434**
lm(d13C_AT ~ 1)	526.32258
lmer(d13C_AT ~ (1|Location)	157.80334
lm(d13C_AT ~ d13C)	-16.60996
Nitrogen	
lmer(d13N_AT ~ d13N + (1|Location)	**-46.59116**
lm(d13N_AT ~ 1)	879.31711
lmer(d13N_AT ~ (1|Location)	428.87829
lm(d13N_AT ~ d13N)	-14.53787

Chapter 3. Carbon isotope geolocation tool successfully identifies the foraging location of Atlantic salmon captured at sea

3.1 Abstract

Stable isotope analysis is used to reconstruct foraging location of species that are difficult to track directly due to their wide dispersal in the marine realm. Carbon stable isotope ratios ($\delta^{13}C$) in fish tissues correlate with primary productivity, which in turn co-varies with ocean temperature. Therefore, time-series of $\delta^{13}C$ measurements from fish scales should correlate with the temperature of the water at the fish's foraging location over the same time period. On this basis, carbon stable isotopes in scales are used as a geolocation tool to infer the most likely feeding location of Atlantic salmon after they return to their natal rivers. This study aimed to ground-truth a $\delta^{13}C$-based geolocation tool (MacKenzie *et al.*, 2011) by using the method to infer feeding location of Atlantic salmon captured at feeding grounds off the coast of Greenland over a 50-year time span (10 sampling years). The scale $\delta^{13}C$ time-series was strongly correlated with sea surface temperature measurements in the Labrador Sea, showing that the geolocation tool could correctly identify the feeding location of the sampled fish. This validation increases confidence in reconstructions of Atlantic salmon migrations, supporting retrospective analysis of salmon scale archives to discern broad migration pathways and examine temporal changes in foraging patterns.

3.2 Introduction

Migratory species are morphologically, physiologically, and behaviorally adapted to the various conditions they must endure to safely complete their life history (McCormick *et al.*, 1998; Hobson, 1999). The Atlantic salmon (*Salmo salar*, L.) is an anadromous fish of ecological, cultural and commercial importance that undertakes extensive migrations between freshwater and marine habitats, covering distances of up to almost 3,000km (Rikardsen *et al.*, 2021). Movements between ecosystems make salmon particularly vulnerable to threats including illegal fishing (Dadswell *et al.*, 2021), barriers such as dams (Nieland *et al.*, 2015; Lawrence *et al.*, 2016), interactions with farmed salmon (McGinnity *et al.*, 2009; Vollset *et al.*, 2016; Forseth *et al.*, 2017) and climate change, particularly as they migrate between habitats which are subject to different climatic pressures (Graham and Harrod, 2009). Atlantic salmon and other diadromous fish can be particularly difficult to track in the marine zone due to their wide dispersal in a vast habitat (Graham *et al.*, 2010). Consequently, many

aspects of their life history remain a mystery. Atlantic salmon populations have suffered declines in recent decades, largely due to reduced marine survival (Friedland *et al.*, 1993; Friedland *et al.*, 2000; ICES, 2021a). It is therefore increasingly necessary to identify the location of salmon feeding to expand our understanding of the marine phase of the life cycle and to support conservation and management efforts.

Successful feeding is a key factor in survival of salmon at sea, therefore spatial and temporal factors that affect prey availability could influence marine survival (Rikardsen and Dempson, 2011). Salmon populations from many different countries are known to mix at certain feeding grounds (Parrish and Horsted, 1980; Reddin and Friedland, 1999), including West Greenland and the Faroe Islands (Hansen and Jacobsen, 2003; Jacobsen *et al.*, 2012; Reddin *et al.*, 2012; Gilbey *et al.*, 2017; Ó Maoiléidigh *et al.*, 2018). Marine climate conditions have been linked with growth and survival of Atlantic salmon of both North American and European origin (Friedland *et al.*, 2000; Friedland *et al.*, 2003b; Friedland *et al.*, 2005; Peyronnet *et al.*, 2007). However, trends in marine survival are not consistent among populations and can vary temporally and spatially, even between populations from the same region (Pardo *et al.*, 2021). Changes in survival of Atlantic salmon across its distribution may not reflect changes in individual populations (Pardo *et al.*, 2021), so it is important to understand distribution at marine feeding grounds at a population level to appropriately inform conservation and management strategies (MacKenzie *et al.*, 2011).

Various direct methods of observation have been used to collect data on Atlantic salmon during their marine phase, including pop-up satellite archival tags (PSATs) (Rikardsen *et al.*, 2021), data storage tags (DSTs) (Reddin *et al.*, 2004; Reddin *et al.*, 2006), and acoustic telemetry (Barry *et al.*, 2020). However, there are limits to these methods: tags can be very costly and may be too large for smaller fish, such as post-smolt salmon entering the marine environment (Thorstad *et al.*, 2010; Lacroix, 2013); and acoustic transmitters are limited by the number and location of receivers, while background noise can cause interference (Thorstad *et al.*, 2010; Leander *et al.*, 2019). Indirect methods that use natural markers, such as stable isotope analysis, provide an alternative method to infer migratory pathways and determine feeding locations of salmon at sea (Graham *et al.*, 2010; MacKenzie *et al.*, 2011).

Stable isotope signatures in animal tissues reflect those of the animal's environment and diet. Over time, tissues become more enriched in carbon and nitrogen isotopes than the plants and

animals they are eating (DeNiro and Epstein, 1978; Peterson and Fry, 1987; Hobson, 1999). Ultimately, all carbon and nitrogen isotope ratios (expressed using delta notation: $\delta^{13}C$ and $\delta^{15}N$, respectively) in a food web are derived from primary production and are reflective of the local environment (Trueman and Moore, 2007; Graham *et al.*, 2010). As an animal moves through isotopically distinct habitats, the isotopic composition of its tissues acts as a "tag" that can subsequently be used to track its movements by comparing with isotopic composition of local prey or primary producers (Graham *et al.*, 2010). As isotope ratios vary spatially across the marine environment, isoscapes (isotopic "maps") have been used to link the isotope ratios in an animal to the location where it incorporated isotopes into its tissues from the local food web (Graham *et al.*, 2010). Isoscapes are gaining traction as a geolocation method for fish species; for example, a dual isotope isoscape using $\delta^{18}O$ (oxygen isotopic ratio) and $\delta^{13}C$ shows great potential for examining fish migration in the Baltic Sea (Torniainen *et al.*, 2017), and $\delta^{13}C$ isoscapes were used to determine movement patterns of three species of tuna (Logan *et al.*, 2020). The isoscape approach requires a geochemical map of isotopic values for the habitats used by the animal; knowledge of fractionation factors that can offset the isotopic composition of the animal from baseline isotopic values; and an understanding of tissue turnover rates for the particular animal tissue being used (Hobson *et al.*, 2010; McMahon *et al.*, 2013). Extensive sampling is required to establish baseline spatial isotopic patterns, particularly if the study animal is a highly mobile marine predator (Graham *et al.*, 2010). MacKenzie *et al.* (2011) have suggested a novel approach for inferring the feeding location of Atlantic salmon from scale stable isotope signatures that removes the need for collecting baseline isotope data. This approach relies on the assertion that the fractionation of carbon isotopes during photosynthesis by phytoplankton co-varies with temperature, leading to higher phytoplankton $\delta^{13}C$ values in warmer waters (Laws *et al.*, 1995; Hofmann *et al.*, 2000; Barnes and Jennings, 2009; Graham *et al.*, 2010; MacKenzie *et al.*, 2011; Soto *et al.*, 2018). Sea surface temperature (SST) time series from putative feeding locations are paired with time series of carbon isotope ratios in scales of returning adult salmon; the area for which the correlation is the strongest is inferred to be the most likely location where the fish were feeding (MacKenzie *et al.*, 2011).

Fish scales are an ideal tissue for stable isotope analysis; they incorporate stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in the collagen layer as they grow, are relatively non-invasive to sample, are sampled routinely, and are easy to store (Trueman and Moore, 2007). Laboratories in many countries possess large multi-decadal collections of Atlantic salmon

scales dating back almost 100 years in some cases (Trueman and Moore, 2007; Tray *et al.*, 2020). The MacKenzie *et al.* (2011) approach has been applied to scale collections to examine the relationship between the decline of a Canadian Atlantic salmon population, ocean temperature and foraging location (Soto *et al.*, 2018), and to infer the location of marine feeding grounds used by Atlantic salmon returning to a Spanish river and UK river (Almodóvar *et al.*, 2020). The use of long-term SST datasets in place of empirical baseline data enables these retrospective analyses of archived samples. The geolocation tool appears to be effective for determining marine feeding locations of Atlantic salmon. However, it is important to validate the approach and to evaluate the reliability of the results by applying it to salmon captured at a known marine foraging location at sea.

A further advantage of using scales as the tissue for this analysis is that age and growth measurements can be retrospectively analysed. Concentric rings (circuli) on the scale surface provide a permanent record of the life history of a fish, including seasonal slowing of the metabolism (Panfili *et al.*, 2002) and the point of first ocean entry (Hogan and Friedland, 2010). Age and growth data traits are necessary for gaining a comprehensive understanding of a population and its growth dynamics (Panfili *et al.*, 2002), and in this case can be used to verify some of the assumptions of the approach being tested. The MacKenzie *et al.* (2011) approach assumes that $\delta^{13}C$ varies with SST. However, Atlantic salmon growth also increases with increasing temperature, to a certain point (Forseth *et al.*, 2001). With enough food to sustain an increased metabolic rate, Atlantic salmon post-smolts were shown to have the highest growth rate at 14°C (Handeland *et al.*, 2008). This is contradictory to other studies (Todd *et al.*, 2008), therefore we will examine the effect of SST and growth on $\delta^{13}C$. By examining the relationship between SST, $\delta^{13}C$ and growth measurements from Atlantic salmon scales in this study, we have the opportunity to determine if this assumption of the geolocation approach is valid.

This study aimed to determine the accuracy of the MacKenzie *et al.* (2011) method of geolocation by applying it to Atlantic salmon captured at feeding grounds off the coast of Greenland over a 50-year time span (10 sampling years). Stable isotope analysis of scales from this extended collection produced a time series of $\delta^{13}C$ that was paired with SST data for the North Atlantic to produce probability maps of potential feeding areas. Growth measurements and scale $\delta^{15}N$ were also analysed to examine variability in $\delta^{13}C$ and investigate assumptions of the geolocation approach.

3.3 Materials and Methods

3.3.1 Sample site and selection

Scale samples were obtained from Atlantic salmon caught in the West Greenland fishery (Figure 3.1), which is a mixed-stock fishery with contributions from many populations of North American and European origin (Bradbury *et al.*, 2016). The fishery generally opens in August and closes towards the end of October or when the quota has been reached (ICES, 2015). During the time frame of this study, salmon could only be targeted with hooks, fixed gillnets and drift nets, and the minimum mesh size has been 140mm since 1985 (ICES, 2019b). The salmon caught at the West Greenland fishery are primarily non-maturing one-sea-winter (1SW) individuals that were otherwise destined to become large multi-sea-winter (MSW) fish (Bradbury *et al.*, 2016).

All fish used for this study were caught between August 11th and September 20th in their respective years from 1968 to 2018. Years were chosen to include both positive and negative phases of the North Atlantic Oscillation (NAO) to ensure a good spread of temperature conditions. Scales were collected from 10 fish per year (n = 100; Table 1). Sampling was carried out in three locations in Greenland: Qaqortoq (southern Greenland, NAFO (Northwest Atlantic Fisheries Organization) division 1F), Nuuk (SW Greenland, NAFO division 1D) and Maniitsoq (SW Greenland, NAFO division 1C) (Figure 3.1). Fishing in these areas generally took place within 40 nautical miles of the shoreline (ICES, 2021a). Scales were gently scraped from the left flank of the fish at the standard sampling location, 3-5 rows above the lateral line and diagonally posterior to the dorsal fin (Shearer, 1992), and stored in paper envelopes until they were prepared for analysis. Scales collected in 2018 (n = 10) were specifically collected for this study, all other scales (n = 90) were obtained from Fisheries and Oceans Canada (DFO) from their extensive archive of scales caught in the West Greenland fishery.

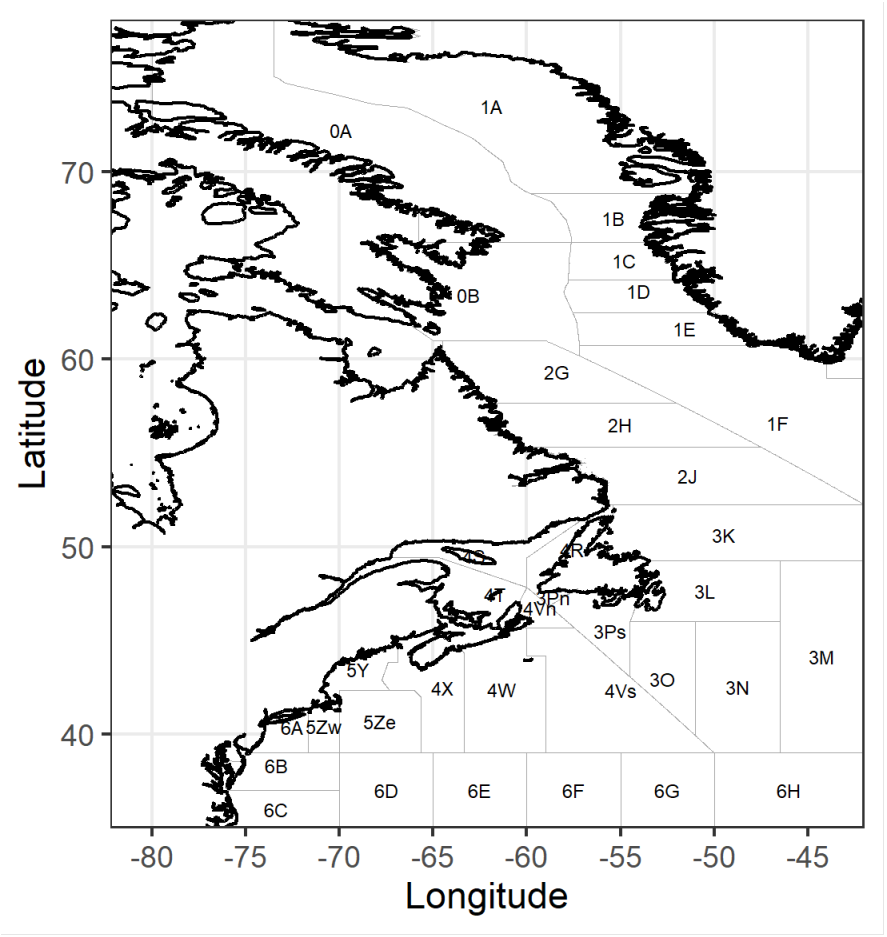

Figure 3. 1 Map showing the NAFO (Northwest Atlantic Fisheries Organization) divisions in the Northwest Atlantic. The land mass to the left of the map is north-eastern Canada and the United States, and the land mass in the top right of the map is southern Greenland. *Salmo salar* analysed in this study were caught in divisions 1C, 1D and 1F.

The fish used in this study were of North American, European, and unknown origin. Between 1968 and 2018, continent of origin and/or region of origin were determined by examination of scale characteristics and later by genetic analysis (Table 3.1). Up until 1981, samples were assigned continent of origin (COO) through a discriminant function analysis of five scale characters (Lear and Sandeman, 1980). The technique and database were later updated and used to classify samples until 1997 (Reddin, 1986; Reddin *et al.*, 1988; Reddin and Friedland, 1999). 1998 was a transition period, with COO determined using scale characteristics (Reddin, 1986) in addition to a combined genotypic/phenotypic approach (Reddin *et al.*, 1990; ICES, 1999). Samples from 2000 and 2010 were genotyped at 12 microsatellite DNA loci to determine COO and province of origin (King *et al.*, 2001). A microsatellite baseline with 15 loci (Bradbury *et al.*, 2014) and a Single Nucleotide Polymorphism (SNP) baseline

(Jeffery *et al.*, 2018) were used to classify fish from 2017 to continent and region of origin (ICES, 2018). The SNP method developed by Jeffery *et al.* (2018) was also used for COO and ROO (region of origin) assignation in 2018 (ICES, 2019b).

Table 3. 1 Sampling year, catch data, and origin details for the 100 *Salmo salar* individuals sampled for this study. NAFO div. refers to the NAFO (Northwest Atlantic Fisheries Organization) division (Figure 3.1) where the fish were caught, and the origin Eur. refers to Europe, N. Am. Refers to North America, and UnK. indicates the origin is unknown. Origin det. refers to the origin determination, method/technique refers to the method used to determine continent of origin (COO) and region of origin (ROO). Province of origin was determined for one year.

Year	**Landing location**	**NAFO div.**	**Catch dates**	**Origin**			**Origin det.**	**Method/ Technique**
				Eur.	**N. Am.**	**UnK.**		
1968	Nuuk	1D	20 Sept.	8	2		COO	Scale characteristics (Lear and Sandeman, 1980)
1976	Nuuk	1D	15 Sept.	9	1		COO	Scale characteristics (Anon, 1979)
1978	Nuuk	1D	05 Sept.	4	6		COO	Scale characteristics (Anon, 1979)
1984	Maniitsoq	1C	26 Aug.	5	4	1	COO	Scale characteristics (Anon, 1984)
1989	Nuuk	1D	01 Sept.	5	5		COO	Scale characteristics (Anon, 1990)
1998	Nuuk	1D	21 Aug.	4	5	1	COO	Scale characteristics (Reddin, 1986) & genotypic/phenotypic approach (Reddin *et al.*, 1990)
2000	Nuuk	1D	16 Aug.	1	9		COO & province of origin	Genetics; microsatellite analysis (King *et al.*, 2001)
2010	Nuuk	1D	24 - 27 Aug.	1	8	1	COO	Genetics; microsatellite analysis (King *et al.*, 2001)
2017	Maniitsoq	1C	13 - 18 Aug.		10		COO & ROO	Genetics; microsatellite (Bradbury *et al.*, 2014) and SNP (Jeffery *et al.*, 2018) baselines
2018	Qaqortoq	1F	11 - 17 Aug.	0	9	1	COO & ROO	SNP range-wide baseline (Jeffery *et al.*, 2018)
Totals				37	59	4		

3.3.2 Sample preparation

Prior to analysis, all scales were soaked in ultrapure water (18.2 mΩ) for a minimum of two minutes and then scraped gently with a scalpel on both sides to remove any mucus and traces of the storage envelope. Scales were not acid-treated to remove inorganic carbonates prior to analysis as acidification has a negligible effect on $\delta^{13}C$ in Atlantic salmon scales (Sinnatamby *et al.*, 2007; O'Toole *et al.*, 2020 (Chapter 2)). Suitable scales from each fish were selected for imaging for growth analysis and for preservation in an archive database (Tray *et al.*, 2020). Scales from 2018 were imaged using an Olympus BX51 compound microscope and ImagePro Plus software Version 6.3.1.542. Archived Greenland scales were imaged using a Leica MZ16 microscope with Auto-Montage Pro software. Following the protocol of MacKenzie *et al.* (2011), a temporally distinct sample was obtained using a scalpel under a dissecting microscope. The portion of the scale corresponding to the last summer at sea was excised to isotopically reflect the carbon from foraging during the most recent feeding period.

3.3.3 Stable isotope analysis

Between 1 and 1.2mg of excised scale material was weighed into tin capsules (Elemental Microanalysis pressed tin capsules, 5 x 3.5mm) and all analyses were carried out at the Stable Isotopes in Nature Laboratory (SINLAB) at the University of New Brunswick, Fredericton, NB, Canada. A combination of CE NC2500 and Costech 4010 elemental analysers connected to a DeltaPlus XP/Conflo III continuous-flow isotope ratio mass spectrometer (CF-IRMS) was used for analysis of carbon isotopes. Stable isotope measurements are reported in the standard delta (δ) notation in parts per thousand (‰) relative to the international standards, Vienna Pee Dee Belemnite (VPDB) (Craig, 1957) for carbon and atmospheric air (AIR) for nitrogen (Mariotti, 1983). The following in-house secondary standards, all of which were calibrated against International Atomic Energy Agency (IAEA) standards, were used to normalise isotope values: USGS61, BLS (Bovine Liver Standard) and MLS (Muskellunge Muscle Standard). To assess analytical accuracy, nicotinamide, CH_7 and N_2 were analysed as check standards. Repeated analysis of internal standards showed that the analytical precision was better than ± 0.1‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$. Over 2% of samples were run in replicate to monitor instrument drift over time.

3.3.4 Growth analysis

Growth analysis was carried out using scale images. Using ImagePro PLUS software Version 7.0, a straight line transect was taken from the scale focus to the scale edge and measurements were taken along this transect. All measurements were in mm. Freshwater radius (FW radius) was measured from the focus to the point of first entry to seawater (Figure 3.2). Marine radius was measured from first sea entry to the scale edge, and post-smolt growth (PSG) from sea entry to the end of the first winter at sea. In this study, the portion relating to growth after the last winter at sea (last marine) was also measured as it corresponds to the segment of scale that was excised for analysis. Total radius was measured along the full transect, from focus to scale edge.

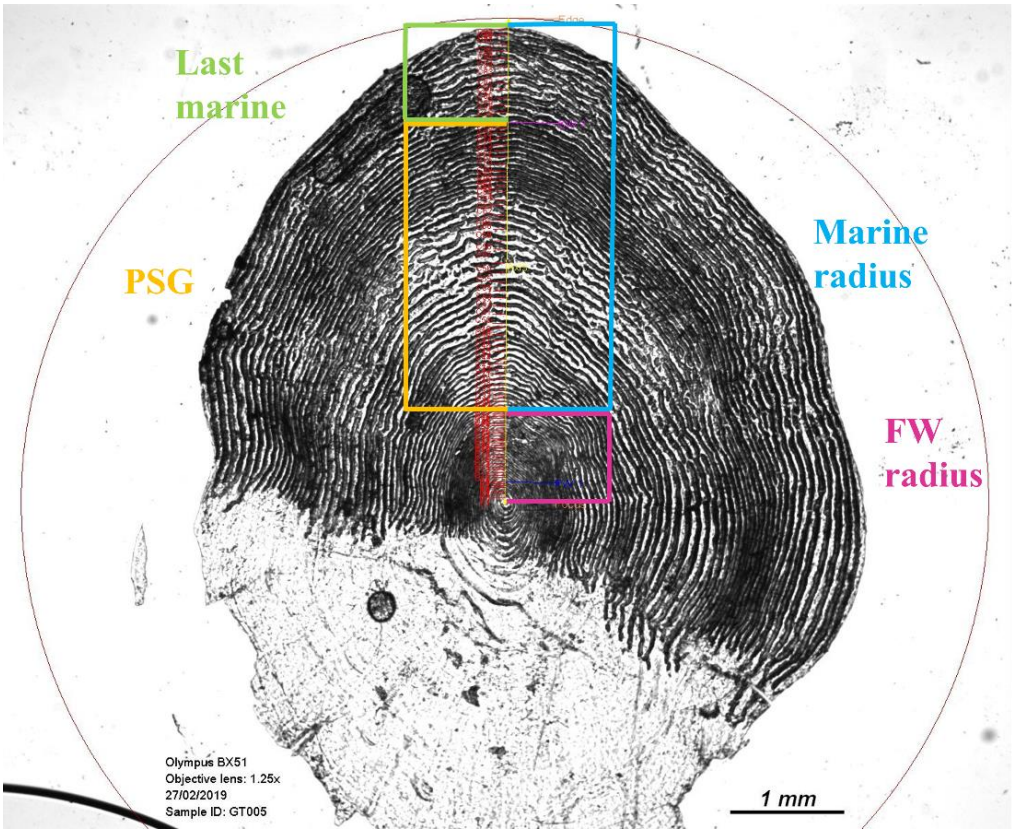

Figure 3. 2 Image of a one-sea-winter Atlantic salmon scale with a transect for growth measurements spanning from the focus to the scale edge. Four growth segments that were analysed in this study are marked on the scale: freshwater radius (FW radius, pink), post-smolt growth (PSG, orange), last marine (green) and marine radius (blue). Total radius spans the full length of the transect, from focus to scale edge.

3.3.5 Data analysis

3.3.5.1 Suess Effect

Since the industrial revolution, there has been a decrease in ^{13}C in the atmosphere due to the depletion of ^{13}C in fossil fuel derived CO_2 (Keeling, 1979). To account for these anthropogenic changes in $\delta^{13}C$, sample $\delta^{13}C$ values were corrected for the Suess Effect using the equation of Hilton *et al.* (2006):

$$\text{Suess correction factor} = a * \exp^{(b*0.027)}$$

where a is the annual rate of $\delta^{13}C$ decrease for the water body and b is the sample year minus 1850 (the onset of the industrial revolution). The annual rate of $\delta^{13}C$ decrease for the North Atlantic Ocean used in this study was -0.018‰ (Quay *et al.*, 2007; Mansouri *et al.*, 2021).

3.3.5.2 Application of the geolocation tool

A LOESS smoother with a span of 0.5 and polynomial order of 2 was applied to the $\delta^{13}C$ data from the Greenland Atlantic salmon scales to remove the influence of high frequency fluctuations in the time series, as described by MacKenzie *et al.* (2011). SST data were extracted from the HadIIST1 dataset (Rayner *et al.*, 2003), a monthly dataset from 1870 to present on a one-degree latitude-longitude grid. Following previous studies, 8-month summer (March to October) mean SST data were used to reflect the growth period that was excised from the scale (MacKenzie *et al.*, 2011; Soto *et al.*, 2018; Almodóvar *et al.*, 2020). Sample $\delta^{13}C$ and SST data were matched by year and the temporal covariance between scale $\delta^{13}C$, and SST were analysed in each one-degree grid square (MacKenzie *et al.*, 2011). Pearson's product-moment correlation coefficient and p value of the relationship between SST and scale LOESS-smoothed $\delta^{13}C$ were calculated using the Chelton method (Chelton, 1984; Pyper and Peterman, 1998). Degrees of freedom were adjusted for temporal autocorrelation. Maps with a one-degree grid square resolution were created to visually represent the range of p values and Pearson's r across the North Atlantic using a colour gradient. According to the MacKenzie *et al*. (2011) approach, the area with the strongest correlation between SST and temporal variations in scale $\delta^{13}C$ are the most likely feeding areas of the fish during their last summer at sea. Maps spanned from 40-80°N latitude and 105°W to 45°E longitude. To further test the approach, mapping was also carried out using the raw data values that had not been corrected for Suess Effect. This was to compare Suess corrected and uncorrected maps to determine whether application of a correction had an effect on the success of the

geolocation tool. Analyses were carried out using R Version 4.1.2 in R Studio Version 2022.02.0+443. Data visualisation was carried out using the ggplot2 package (Wickham, 2016). Maps were created using the packages maps (Becker *et al.*, 2021), rgdal (Bivand *et al.*, 2021) and colorRamps (Keitt, 2012) with the 10m coastline vector from Natural Earth. SST extraction utilised R packages raster (Hijmans *et al.*, 2021), ncdf4 (Pierce, 2021), chron (James *et al.*, 2020), lattice (Sarkar *et al.*, 2021) and RColorBrewer (Neuwirth, 2014).

3.3.5.3 Analysis of origin, growth and $\delta^{15}N$

$\delta^{13}C$ varies with SST and the correlation between these two factors forms the basis of the method by MacKenzie *et al*. (2011). To examine the relationship between SST and $\delta^{13}C$ using linear models, all SST values (8-month summer mean) within the area of highest correlation determined by the geolocation tool were extracted from the HadIIST1 dataset. The average temperature for this area was calculated for each year of the study. Linear mixed effects models were used to examine whether variability in $\delta^{13}C$ could be explained by other variables in addition to SST. All models included $\delta^{13}C$ as the response variable, *SST* as a fixed effect and *Year* as a random effect. The other fixed effects were *Origin, Length, FW radius, PSG, Marine radius, Last marine, Total radius* and $\delta^{15}N$. A series of model combinations were analysed, including pairwise interactions with *SST.* The best fitting model was selected based on Akaike Information Criteria (AIC) values and loglikelihood tests. Prior to running linear mixed effects models, four fish with unknown population origin were removed from the dataset. Statistical analyses were conducted using R Version 4.1.2 in R Studio Version 2022.02.0+443. Linear mixed effects models were carried out using the lme4 (Bates *et al.*, 2015) and lmerTest (Kuznetsova *et al.*, 2020) packages. Marginal and conditional R^2 values were calculated using the MuMIn package (Bartoń, 2020).

3.4 Results

3.4.1 Determining feeding location

Atlantic salmon scale $\delta^{13}C$ varied over the 10 sample years, and scales collected in 1984 were depleted in $\delta^{13}C$ compared to the other years (Table 3.2). More recent years (the 2000s) were enriched in $\delta^{13}C$ compared to earlier in the time series (Supplementary Figure 3.1). As expected, $\delta^{13}C$ varied with SST, though four outliers in 2018 were considerably depleted in $\delta^{13}C$ compared to the other fish from that year (Figure 3.3). When $\delta^{13}C$ was plotted against

$\delta^{15}N$, there were no longer any outliers (Figure 3.4). $\delta^{15}N$ ranged from 8.34 to 12.28‰ across the 10 sample years and also had lowest values in 1984 (Table 3.2, Figure 3.4).

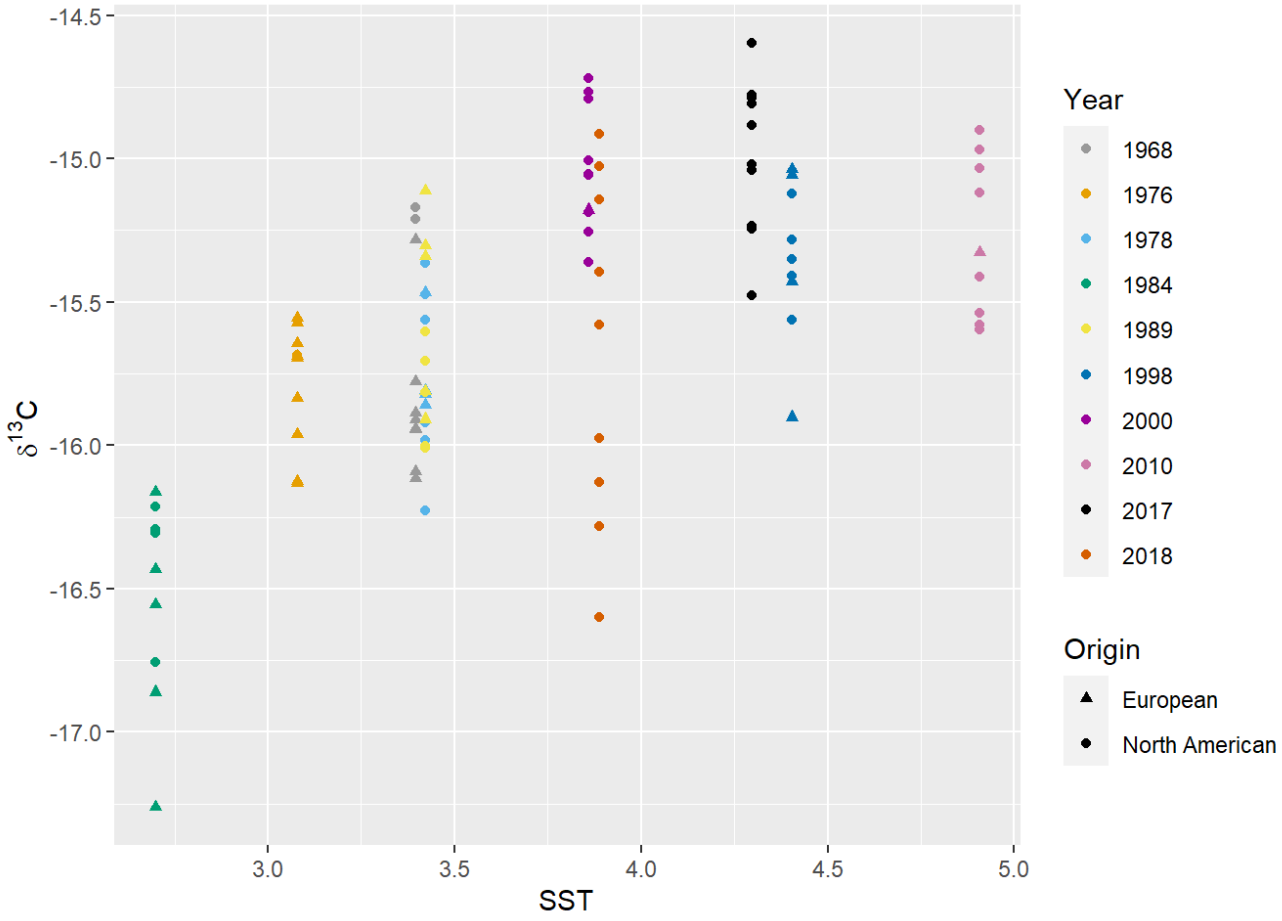

Figure 3. 3 Plot of sea surface temperature (SST) and scale $\delta^{13}C$ of 1SW *Salmo salar* of North American and European origin caught in the West Greenland fishery. Year is represented by colour and fish Origin is represented by shape.

Table 3. 2 Mean, minimum and maximum $\delta^{13}C$ and $\delta^{15}N$ (‰) from scales of 100 *Salmo salar* caught in the West Greenland fishery. Both Suess corrected and raw values are presented for $\delta^{13}C$. Length (cm) refers to the range of fork lengths of fish sampled in each year.

Year	**Suess corrected $\delta^{13}C$**			**Raw $\delta^{13}C$**			**$\delta^{15}N$**			**Length (cm)**
	Mean	**Min.**	**Max.**	**Mean**	**Min.**	**Max.**	**Mean**	**Min.**	**Max.**	**Range**
1968	-15.73	-16.11	-15.17	-16.17	-16.55	-15.60	9.21	8.56	9.84	65.1 – 69.0
1976	-15.79	-16.13	-15.55	-16.33	-16.67	-16.09	9.82	9.32	10.79	69.0 – 70.0
1978	-15.75	-16.23	-15.36	-16.32	-16.80	-15.93	9.59	8.85	10.26	61.0 – 67.0
1984	-16.45	-17.26	-15.62	-17.12	-17.93	-16.30	9.16	8.34	10.37	63.0 – 66.0
1989	-15.66	-16.01	-15.11	-16.43	-16.78	-15.88	9.63	8.90	10.47	65.0 – 68.0
1998	-15.32	-15.90	-15.04	-16.30	-16.88	-16.02	9.96	8.77	11.24	61.0 – 68.0
2000	-15.04	-15.36	-14.72	-16.07	-16.40	-15.75	9.94	9.47	11.20	60.0 – 68.1
2010	-15.28	-15.60	-14.90	-16.63	-16.95	-16.25	9.64	8.98	10.38	61.8 – 67.5
2017	-14.99	-15.48	-14.60	-16.62	-17.11	-16.23	11.18	10.43	12.26	61.5 – 68.5
2018	-15.66	-16.60	-14.92	-17.34	-18.28	-16.60	10.97	9.41	12.28	56.5 – 67.3

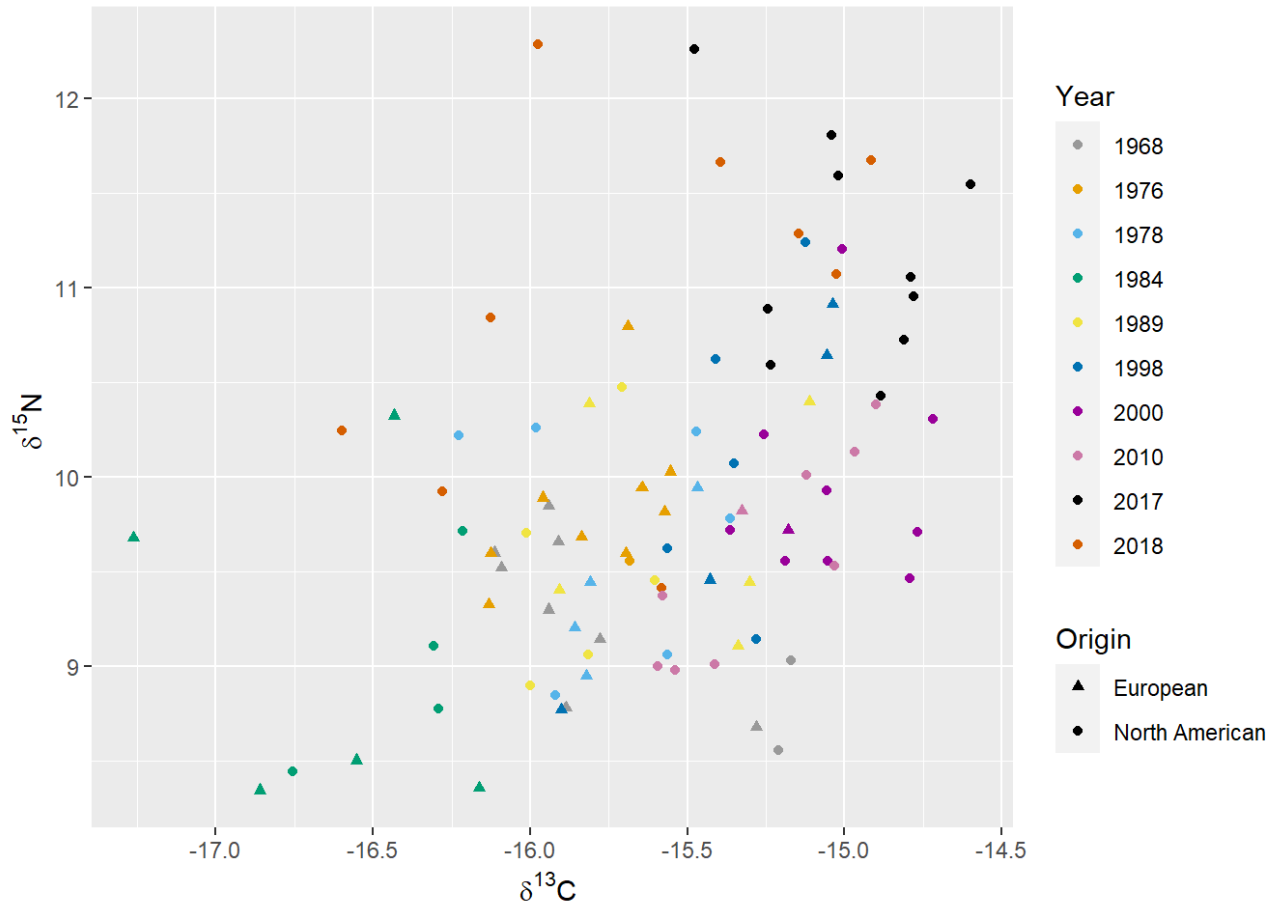

Figure 3. 4 $\delta^{13}C$ and $\delta^{15}N$ from scales of *Salmo salar* caught in the West Greenland fishery between 1968 and 2018 (10 sample years). Fish Origin is distinguished by shapes, and Year by colour.

Correlations of LOESS smoothed salmon scale $\delta^{13}C$ with SST varied spatially, and p values varied from 1.0 to 0.02 across the range of the map (Figure 3.5). The correlation coefficient (Pearson's r) ranged from -0.96 to 0.92 (Figure 3.5). Based on the strength and significance of the relationship between LOESS smoothed $\delta^{13}C$ and SST, the most likely feeding grounds of the Atlantic salmon in this study is in the Labrador Sea, off the coast of Newfoundland and Labrador from just north of Cape Freels in the south to Killiniq Island in Nunavut, at the northernmost tip of Labrador, and moving eastwards towards southern Greenland. This is the largest continuous area of high correlation determined by p value and correlation coefficient that overlaps between the two maps ($p \leq 0.025$, $r > 0.8$) and is the area where fish were most likely feeding at sea.

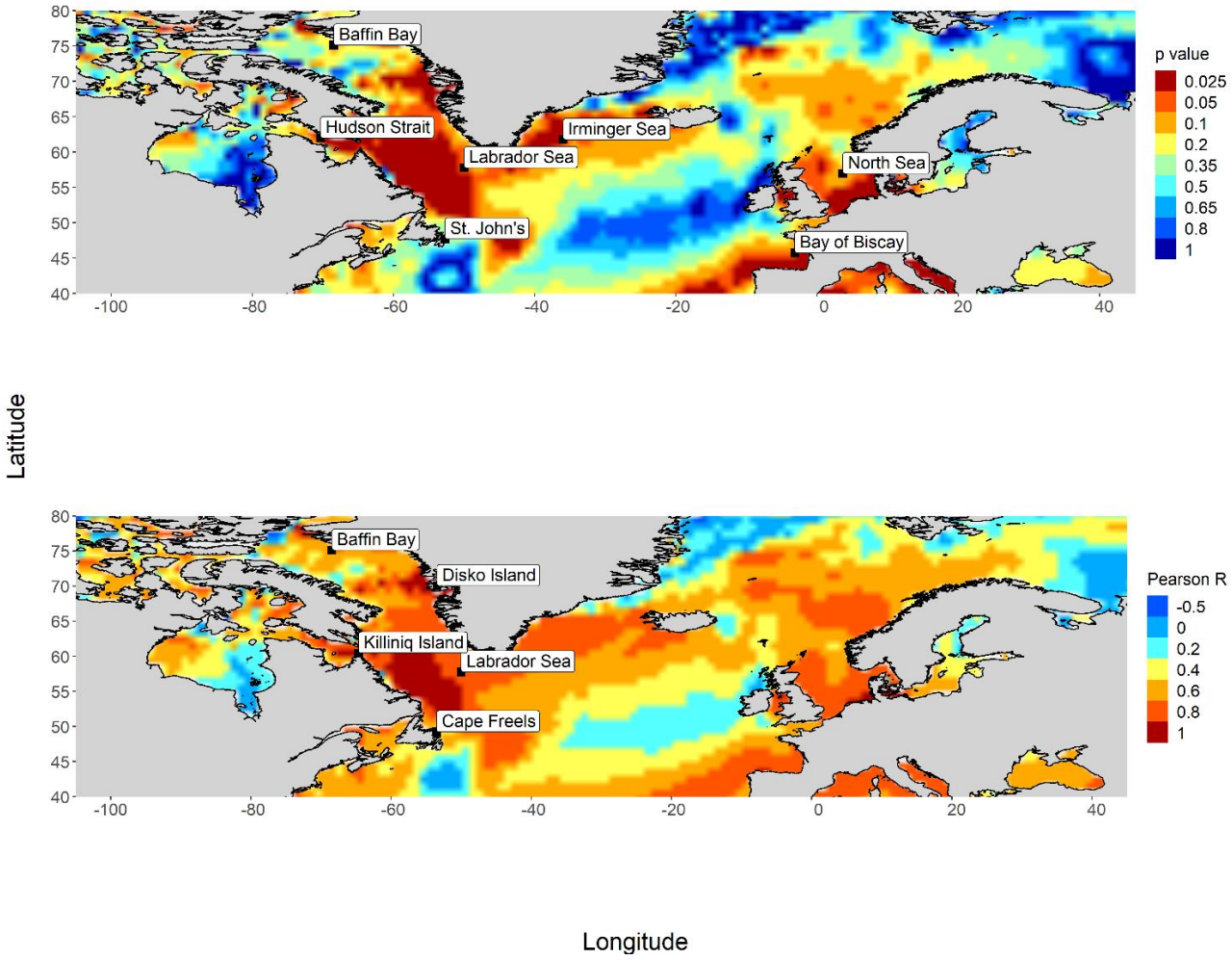

Figure 3. 5 Map showing the p values and correlation coefficient (Pearson's r) resulting from Pearson's product-moment correlations between archived *Salmo salar* scale $\delta^{13}C$ values and sea surface temperatures (SST). Correlations are based on a LOESS fit to temporal data (span = 0.5, polynomial order 2). The colour gradient indicates the strength of the correlation (p value, top map; Pearson's r, bottom map). The areas with the highest correlation (shown in red on the maps) are the most likely feeding areas of 1SW *Salmo salar* from ten sample years (1968, 1976, 1978, 1984, 1989, 1998, 2000, 2010, 2017, 2018).

Individually, the p value map (Figure 3.5) indicated multiple areas of high correlation ($p \leq 0.025$), including: a sizeable portion of the Labrador Sea, stretching from the Hudson Strait to St. John's in Newfoundland and Labrador; an area in Baffin Bay; the Irminger Sea; the southern North Sea; the Bay of Biscay and eastern Atlantic bordering Portugal; and some locations in the Mediterranean Sea. The Mediterranean Sea is not within the distribution

range of Atlantic salmon and is included as it falls within the latitude and longitude confines of the map.

The map based on Pearson's r (Figure 3.5) indicated the highest areas of association between scale $\delta^{13}C$ and SST (0.8 – 1.0) were: the largest area of correlation in the Labrador Sea, along the coast of Newfoundland and Labrador and spreading towards south-western Greenland; a small coastal area of Baffin Bay, near Disko Island in western Greenland; and a small coastal area of the North Sea. Both correlation methods need to be used in tandem to successfully determine the most likely feeding area of Atlantic salmon.

Maps created using raw data values that were not Suess corrected show correlations in very different areas than maps using Suess corrected data. The p value map (Supplementary Figure 3.2) suggests fish were feeding in the Eastern Atlantic or Hudson Bay in North America. The map based on Pearson's r indicated the most likely feeding area was in the Irminger Sea and central North Atlantic. The areas of highest correlation were not cohesive between the two maps and did not correspond to the feeding area of the fish in this study.

3.4.2 Analysis of origin, growth and $\delta^{15}N$

When linear mixed effects models were used to examine whether $\delta^{13}C$ was being affected by variables in addition to *SST*, the best fitting model included *SST*, *Total radius*, *Length* and $\delta^{15}N$ as fixed effects:

$$\delta^{13}C \sim SST + Total\ radius + Length + \delta^{15}N + (1|Year)$$

All fixed effects had a significantly positive effect on $\delta^{13}C$ (*SST*, p = 0.003; *Total radius*, p = 0.009; *Length*, p = 0.012; and $\delta^{15}N$, p = 0.008). This indicates that the larger the fish was and the higher its $\delta^{15}N$, the more enriched the $\delta^{13}C$ in the scale. The fixed effects in this model explained 53.3% of the variance in $\delta^{13}C$ according to marginal R^2 values, of which 41.2% was explained by *SST* alone. *Year* explained an additional 17.0% of variance (conditional R^2 = 0.703).

3.5 Discussion

This study successfully validated the carbon isotope-based geolocation approach developed by MacKenzie *et al*. (2011). The method effectively determined the most likely feeding location of Atlantic salmon caught in the West Greenland fishery between 1968 and 2018

was in the Labrador Sea. This region is a known feeding area for salmon of North American and European origin (Reddin *et al.*, 2012). Other known marine feeding grounds for Atlantic salmon include the Norwegian Sea (Haugland *et al.*, 2006; Ó Maoiléidigh *et al.*, 2018) and the Faroe Islands (Hansen and Jacobsen, 2003; Jacobsen *et al.*, 2012; Gilbey *et al.*, 2017). Some areas of the Norwegian Sea showed significant correlation between SST and scale $\delta^{13}C$ ($p = 0.025 – 0.05$, $r = 0.6 – 0.8$), but the area around the Faroe Islands did not show a significant correlation ($p = 0.35 – 0.5$, $r = 0.2 – 0.4$). Using the highest correlation as shown by the p value and Pearson's correlation coefficient in tandem, this approach was able to distinguish the feeding area in the Labrador Sea from those in the eastern Atlantic.

The southern Labrador Sea is likely to be an important overwintering ground for Atlantic salmon post-smolts, based on high catches of post-smolts in the area in autumn and 1SW fish in spring (Reddin and Short, 1991; Reddin and Friedland, 1993). In late summer and autumn, non-maturing adult salmon of Southern European and North American origin have been found in the Labrador Sea (Reddin and Friedland, 1993). At that stage, the fish are more likely to overwinter at sea than return to their natal river to spawn. While it can vary by location, Atlantic salmon generally spawn in the autumn and winter months (Power, 1981; Webb and McLay, 1996), and return from marine feeding grounds to begin their upstream migration several months prior to spawning (Fleming, 1996). The fish sampled for this study were all 1SW, having spent one winter at sea before being caught in West Greenland in August or September of their second summer. Based on timing, it is likely these fish were destined to be MSW fish and were not going to migrate to their natal river and spawn that year. This is consistent with the fish that are usually caught in the West Greenland fishery (Bradbury *et al.*, 2016).

Using the MacKenzie *et al.* (2011) approach, the Labrador Sea was determined as the most likely feeding area for MSW Atlantic salmon returning to the St. John River in New Brunswick, Canada (Soto *et al.*, 2018). Interestingly, both 1SW and MSW fish analysed by Soto *et al.* (2018) exhibited depleted $\delta^{13}C$ values in the early 1980s, similar to the obvious dip in $\delta^{13}C$ in fish from 1984 in this study. 1984 had the lowest SST in this study and had the lowest SST in all locations examined by Soto *et al.* (2018) apart from the Norwegian Sea, where 1995 was lower. As depicted by the assumptions of the MacKenzie *et al.* (2011) approach, increasing SST leads to higher $\delta^{13}C$ in primary producers (Laws *et al.*, 1995; Hofmann *et al.*, 2000; Barnes and Jennings, 2009; Graham *et al.*, 2010). Therefore, low SST

in the Labrador Sea in 1984 (2.7°C) is likely responsible for $\delta^{13}C$ depletion in both studies. Soto *et al.* (2018) reported weaker correlations than our study ($R^2 = 0.56$, p value < 0.001 for MSW, $R^2 = 0.28$, p value < 0.001 for 1SW; our study: $p \leq 0.025$, $r = 0.92$ in areas of highest correlation) for a wide range of areas across the North Atlantic. When specific regions were examined, correlations continued to get weaker and it was posited that the cohort in each year consisted of individuals returning from different feeding grounds (Soto *et al.*, 2018).

Atlantic salmon from the Rivers Sella (Spain) and Dee (UK) also showed the highest correlation with SST values in the western North Atlantic, particularly in the Labrador and Irminger Seas (Almodóvar *et al.*, 2020). Correlations with Phytoplankton Colour Index (PCI) suggested that the Irminger Sea was the most likely feeding area, but correlations between scale $\delta^{13}C$ values and SST or PCI were still quite low, with highest R^2 values of 0.42 (Almodóvar *et al.*, 2020) compared to the highest Pearson's r value of 0.92 in our study. The range of $\delta^{15}N$ in our study (8.34 - 12.28‰) is within the range of MSW fish returning to the rivers Dee (7.9 - 13.0‰) and Sella (9.3 - 13.6‰) (Almodóvar *et al.*, 2020). Similar to Soto *et al*. (2018), the lower correlation strength determined by Almodóvar *et al.* (2020) could suggest feeding in multiple feeding grounds over the time series.

The most likely feeding grounds for Atlantic salmon in this study, according to the MacKenzie *et al.* (2011) approach, correspond to results of other studies in the northwest Atlantic. Post-spawned kelts were tagged with PSATs in the Northwest Miramichi River in New Brunswick, Canada between late April and mid-May (Strøm *et al.*, 2017). Of the 8 tagged fish that left the Gulf of St. Lawrence, all utilised areas of the Labrador Sea and the diving behaviour of the fish indicated foraging (Strøm *et al.*, 2017). SALSEA (Salmon at Sea, a large-scale collaborative research project focusing on the post-smolt stage of the Atlantic salmon life cycle (NASCO, 2012)) surveys in August 2008 and September 2009 caught 107 salmon between the two years (22% adult, 78% post-smolt), the majority of which were caught in the Labrador Sea (Sheehan *et al.*, 2012). Acoustic tagging of Canadian post-smolts also demonstrated their use of the Labrador Sea (Chaput *et al.*, 2019). Maiden North American salmon tagged at West Greenland migrated to the Labrador Sea during autumn and winter, where they overlapped with fish tagged in Irish rivers (Rikardsen *et al.*, 2021), demonstrating the use of the region by Atlantic salmon from both North American and Southern European populations.

A considerable advantage of the MacKenzie *et al.* (2011) approach compared to isoscape methods is that it does not require the collection of extensive baseline data, allowing for substantially less sampling effort. An assumption of isoscapes is that the regional isoscape remains constant for the period of study (MacKenzie *et al.*, 2011). By using SST values from each sampling year, the approach developed by MacKenzie *et al.* (2011) takes temporal variation into account. Physical tagging methods are often constrained to studies on kelts which are large enough to withstand a tag (Lacroix, 2013; Strøm *et al.*, 2017; Rikardsen *et al.*, 2021); this method is more cost-effective than tagging and allows for analysis of salmon on their first sea migration, which is a critical life stage for survival (Friedland *et al.*, 2009; Thorstad *et al.*, 2012). Through retrospective analysis of archived scales, the approach by MacKenzie *et al.* (2011) facilitates the analysis of large time series of data, providing valuable insights into the foraging behaviour of individual populations over time. The approach could be further tested using fish caught at feeding grounds in the Norwegian Sea and around the Faroe Islands to determine if the feeding locations are reliably assigned for fish from the Eastern Atlantic also.

However, a disadvantage of the approach is the requirement of a large time series of data for analysis. In this study, scales from 100 fish over 10 years were analysed. Previous research involving this approach analysed scales from 523 salmon (MacKenzie *et al.*, 2011), 325 1SW and 307 MSW salmon analysed separately (Soto *et al.*, 2018), and 316 MSW salmon pooled from two rivers (Almodóvar *et al.*, 2020). Additionally, the method assumes that Atlantic salmon migrate to the same feeding grounds over the duration of the time series (MacKenzie *et al.*, 2011). It has been suggested that the location of feeding grounds, especially those along the polar front, may be altered in response to climate change (Rikardsen *et al.*, 2021). This assumption of the approach could pose a problem if feeding locations have changed drastically between years, particularly for salmon foraging in frontal areas (Rikardsen *et al.*, 2021). The solution to this could be found in a model similar to the physics-biogeochemistry model presented by Magozzi *et al*. (2017) but with annual rather than decadal resolution. A modelling method would need to be validated like the geolocation tool validated in this study.

The MacKenzie *et al*. (2011) approach cannot identify specific feeding grounds; the feeding location with the highest correlation in this study spans thousands of kilometres in area. However, it is useful for evaluating the relative likelihood of salmon feeding in broad areas such as known feeding grounds. In this study, when samples were taken from fish caught at

their feeding grounds, the correlation was very strong ($r > 0.8$, $p < 0.025$). In other studies, when fish were sampled on their return to their natal rivers, correlations were weaker (R^2 = 0.56, p value < 0.001 for MSW, R^2 = 0.28, p value < 0.001 for 1SW (Soto *et al.*, 2018); R^2 = 0.42 (Almodóvar *et al.*, 2020)), indicating that results could be more difficult to interpret when the fish in a time series were returning from multiple feeding grounds. This study highlighted the importance of Suess correcting scale $\delta^{13}C$ prior to utilising the geolocation tool. When raw data were used, the maps produced were remarkably different from each other and did not reflect the foraging location of the fish. It is therefore imperative to correct for Suess Effect prior to using this method.

Linear mixed effects models in this study validated the underlying assumptions of the approach developed by MacKenzie *et al.* (2011). Interannual variability in $\delta^{13}C$, a tenet of the approach, was confirmed by the best fitting model. Growth was also shown to have a positive effect on $\delta^{13}C$; freshwater and post-smolt growth did not significantly affect $\delta^{13}C$, but $\delta^{13}C$ increased with both increasing fork length and total scale radius. $\delta^{15}N$ had a positive relationship with $\delta^{13}C$ suggesting that some of the variation in $\delta^{13}C$ could have come from size or trophic level of prey (DeNiro and Epstein, 1978; Rau *et al.*, 1983). A plot of the relationship between $\delta^{13}C$ and SST (Figure 3.3) showed four outlying values in the samples from 2018. This suggests that some of the fish caught in 2018 may have been feeding under different environmental conditions or at a different trophic level than the rest of the samples. However, $\delta^{15}N$ for these fish suggest they were foraging on similar prey as the rest of the fish in this study, therefore the difference in $\delta^{13}C$ in these four fish is likely to be due to local environmental factors, or differences in inshore/offshore feeding (Dixon *et al.*, 2019). These small-scale variabilities did not skew the overall result of the MacKenzie *et al*. (2011) approach. The variability in $\delta^{13}C$ that was not explained by the models in this study could be due to differences in diet. The diet of North American and European Atlantic salmon caught at West Greenland has been changing in recent years as the proportion of capelin (*Mallotus villosus*, Müller) in gut contents decreased and the proportion of boreoatlantic armhook squid (*Gonatus fabricii*, Lichtenstein) and *Themisto* sp. (amphipods) increased (Renkawitz *et al.*, 2015). This could be due to the energy density of capelin which has decreased by approximately 33.7% since 1990 (Renkawitz *et al.*, 2015). Variability could also be linked to differing levels of fractionation, which has been shown to increase at higher temperatures (Barnes *et al.*, 2007).

This study is the first to validate the stable isotope geolocation tool developed by MacKenzie *et al.* (2011). The approach facilitates the analysis of many more individuals than would be possible using traditional methods such as tagging and mark/recapture methods. This method provides a tool to retrospectively analyse vast archives of scales to determine important feeding locations, providing valuable data for conservation efforts and expanding our knowledge of the mysterious, poorly understood marine phase of the Atlantic salmon. This study allows for the approach to be used with increased confidence and provides scale $\delta^{13}C$ values for 100 fish that can be used in future studies as a reference for the West Greenland and Labrador feeding grounds.

3.6 Acknowledgements

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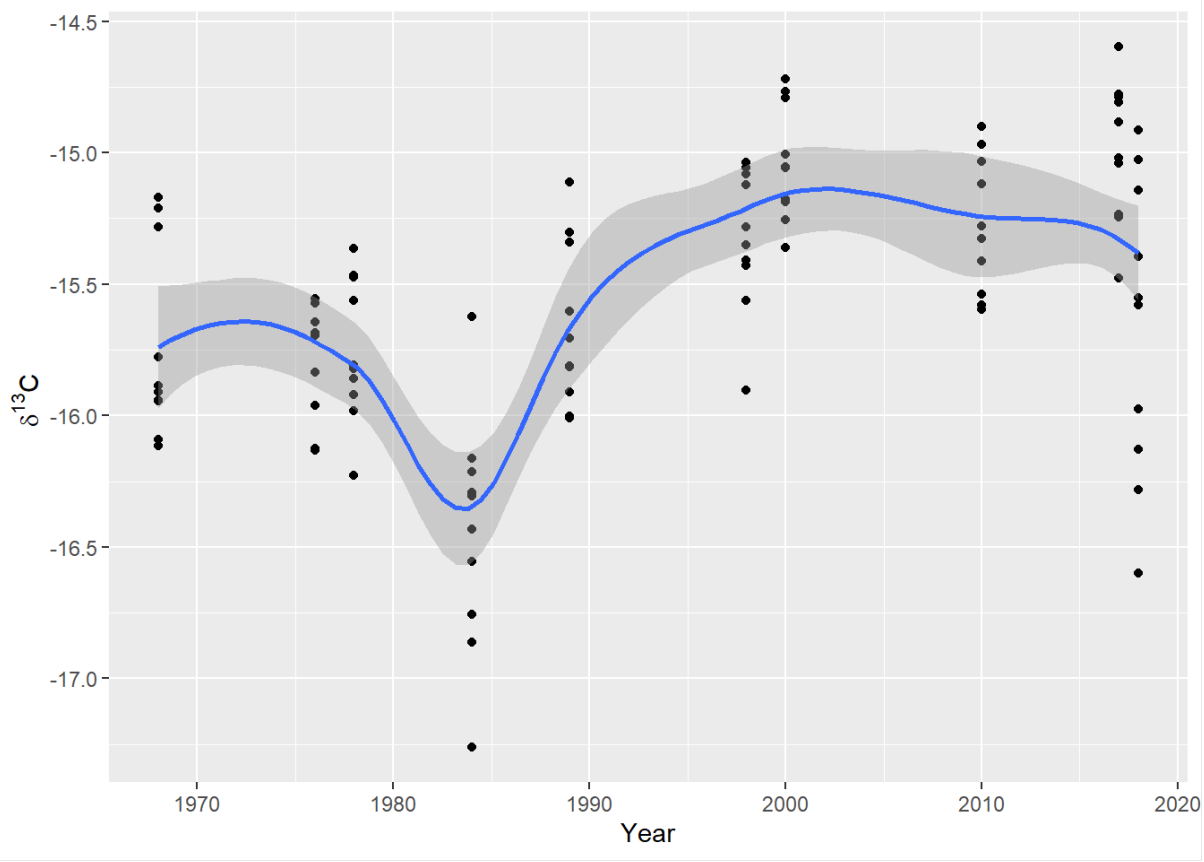
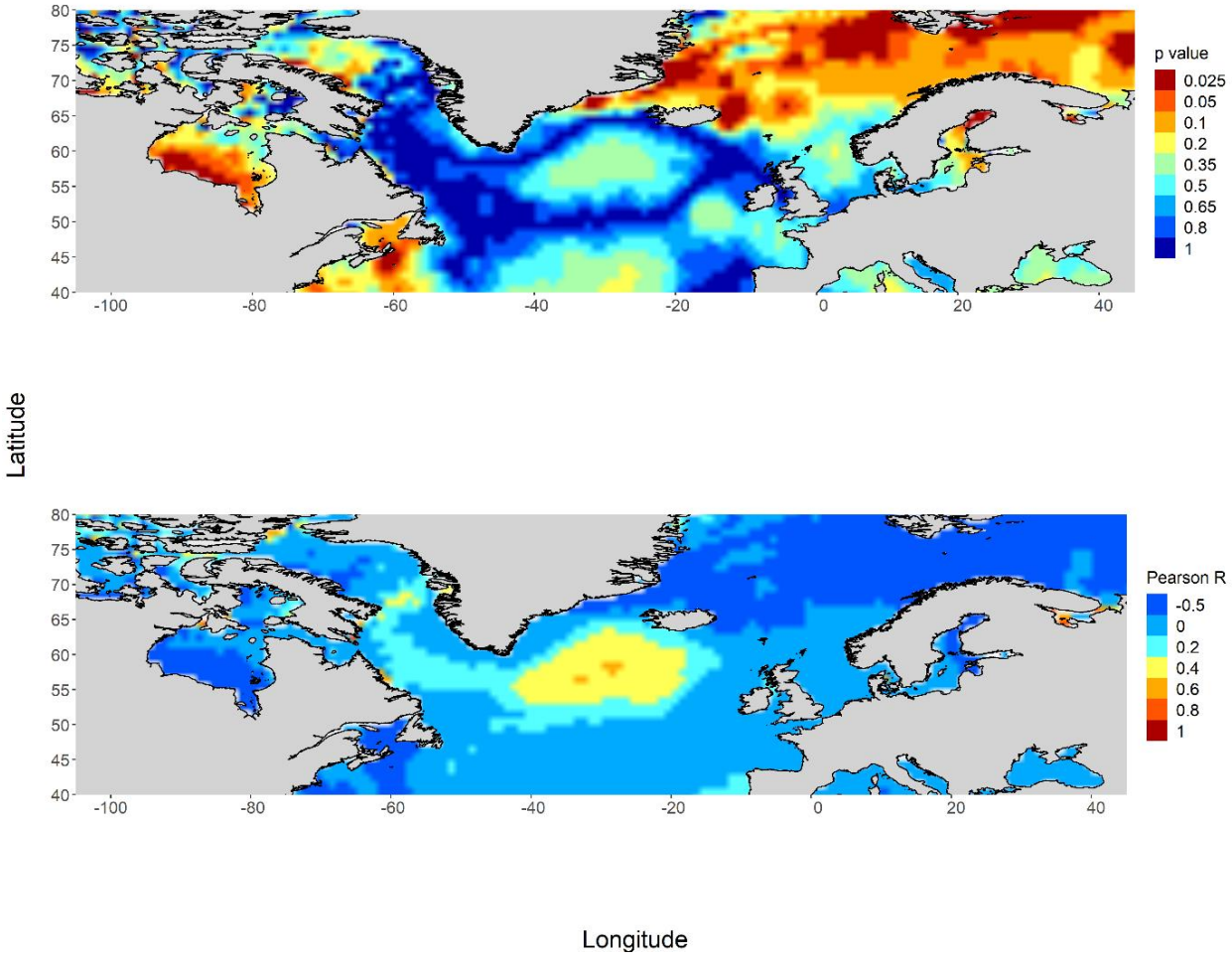
3.7 Supplementary material



Supplementary Figure 3. 1 Temporal trends in $\delta^{13}C$ of archived *Salmo salar* scales caught in the West Greenland fishery. A LOESS smoother was applied to the data (blue line) with a span of 0.5 and a polynomial order 2. The grey shading represents the confidence intervals of the smoothed fit.



Supplementary Figure 3. 2 Map showing the p values and correlation coefficient (Pearson's r) resulting from Pearson's product-moment correlations between archived *Salmo salar* scale $\delta^{13}C$ values and sea surface temperatures (SST). These maps were created using the same methods as Figure 3.5 but used raw data that were not corrected for Suess Effect. Correlations are based on a LOESS fit to temporal data (span = 0.5, polynomial order 2). The colour gradient indicates the strength of the correlation (p value, top map; Pearson's r, bottom map). The areas with the highest correlation indicate the most likely feeding areas of 1SW *Salmo salar* from ten sample years (1968, 1976, 1978, 1984, 1989, 1998, 2000, 2010, 2017, 2018).

Chapter 4. Scale cortisol concentration in Atlantic salmon post-smolts is influenced by temperature

4.1 Abstract

Anadromous fish are particularly vulnerable to anthropogenic and environmental stressors including pollution, rising temperatures, and changes in food availability. Knowledge of how fish respond to specific stressors can aid understanding of population declines and inform predictions of how populations will react to future environmental change. Cortisol in fish scales provides an ideal measure of chronic stress in fish and has been shown to fluctuate with temperature. This study is the first to examine the effect of temperature and feeding conditions on scale cortisol in Atlantic salmon (*Salmo salar*). Post-smolts were subjected to three different temperatures (6, 10.5 and 15°C) and four feeding/starvation treatments over a 12-week period. When fed constantly, scale cortisol was significantly higher in fish at 15°C than at 10.5°C. Cortisol concentration was also elevated at 6°C compared to 10.5°C. The cortisol increase at 15°C appeared to be a physiological response to the temperature increase and not a result of smoltification or maturation. Cortisol increases were also evident after starvation at 10.5 and 15°C but this result needs to be examined further. This study demonstrated that scale cortisol fluctuations can be detected in Atlantic salmon scales and may be a suitable biomarker for chronic stress caused by temperature conditions outside of the physiological optima. Scale cortisol analysis could be a valuable tool for monitoring stress responses to environmental change during the marine phase of wild Atlantic salmon populations, and retrospective analyses through scale archives.

4.2 Introduction

Wild fish are regularly exposed to environmental factors that cause stress, including varying food availability, risk of predation, infection, temperature changes, and pollutants (Schulte, 2014). Many of these stressors are influenced by climate change, which is expected to have a negative effect on numerous fish species (Free *et al.*, 2019), with impacts varying by species and life stage (Barbeaux and Hollowed, 2018). Chronic exposure to stressors can be detrimental to the long-term coping capacity of the fish, as prolonged physiological stress has consequences for growth, survival, and reproduction (Alfonso *et al.*, 2021). Physiological responses to climate change and other anthropogenic pressures have consequences for the management of commercial fisheries and the design of conservation measures to protect

culturally, ecologically and economically important species such as the Atlantic salmon (*Salmo salar*, L.).

Cortisol, a steroid hormone released as a response to stress, is an important biomarker that can be used to examine the effect of anthropogenic changes in the environment on fish (Sadoul and Geffroy, 2019) and can serve as a sensitive indicator to the severity of stressors (Baker and Vynne, 2014). Cortisol measurement is an essential tool for investigating coping mechanisms in fish species and is often used in an aquaculture setting (Barton, 2002). Cortisol is usually measured in blood plasma (Cook, 2012) however this approach is impractical for wild fish which can be widely dispersed and sensitive to disturbances. As well as being invasive, blood sampling can interfere with real-time cortisol levels and provides a measure of acute rather than chronic stress (Bertotto *et al.*, 2010; Cook, 2012; Aerts *et al.*, 2015; Sadoul and Geffroy, 2019). This restricts the use of cortisol as a marker of stress in wild populations (Pankhurst, 2011).

Fish scales incorporate glucocorticoids, including cortisol, as they grow and provide an ideal matrix for monitoring chronic stress due to their slow, persistent growth, and their ease of sampling (Aerts *et al.*, 2015). Recent investigations have shown that cortisol in fish scales reflects cortisol levels in blood plasma (Carbajal *et al.*, 2019), and provides a reliable measure of chronic stress (Aerts *et al.*, 2015; Laberge *et al.*, 2020). Due to their incremental growth, scales incorporate steroids such as cortisol over the lifetime of the fish and capture systemic cortisol exposure over a long time-period (Aerts *et al.*, 2015; Sadoul and Geffroy, 2019). Cortisol in the scale is not incorporated quickly enough to be affected by acute stress during sampling; it remains elevated longer than changes in plasma cortisol, and scale cortisol concentrations correlate to fluctuations in blood cortisol (Carbajal *et al.*, 2019; Laberge *et al.*, 2020). Scale cortisol has been used to examine chronic stress in milkfish (*Chanos chanos*, Forsskål (Hanke *et al.*, 2019)), rainbow trout (*Oncorhynchus mykiss*, Walbaum (Carbajal *et al.*, 2019)), goldfish (*Carassius auratus*, L. (Laberge *et al.*, 2020)) and European sea bass (*Dicentrarchus labrax*, L. (Goikoetxea *et al.*, 2021)) and provides an indicator of thermal stress (Hanke *et al.*, 2019; Laberge *et al.*, 2020; Goikoetxea *et al.*, 2021). Scale cortisol has been proposed as an ideal biomarker for examining stress responses of fish to environmental change (Goikoetxea *et al.*, 2021).

Anadromous fish such as Atlantic salmon are exposed to many environmental stressors during their dispersive life history and are particularly vulnerable to the effects of climate change (Graham and Harrod, 2009; Lassalle and Rochard, 2009). In recent decades, Atlantic salmon stocks in Europe have been declining. This decline has been attributed to many interacting factors including climatic conditions (Almodóvar *et al.*, 2018; Nicola *et al.*, 2018), food availability (Renkawitz *et al.*, 2015), interactions with farmed salmon (McGinnity *et al.*, 2009; Vollset *et al.*, 2016; Forseth *et al.*, 2017) and illegal fishing (Dadswell *et al.*, 2021). Future climate change could pose a further threat to the species; predictive modelling indicates a contraction of Atlantic salmon distribution by the end of the century (Lassalle and Rochard, 2009). The post-smolt period, when salmon first migrate into the marine environment, is considered a critical stage during which growth and survival has declined dramatically (Friedland *et al.*, 2009; Soto *et al.*, 2018). If exposure to elevated temperatures, reduced food availability or other stressors during the post-smolt period is reflected in scale cortisol levels, this biomarker could be used to examine physiological stress responses to environmental change during the marine migration. Scales are routinely sampled for many salmon populations across Europe and multi-decadal collections exist (Tray *et al.*, 2020). These archives provide material to investigate physiological stress responses to environmental change in salmon across broad temporal and spatial scales.

This study used an artificial rearing experiment to investigate if temperature and feeding conditions during the post-smolt stage of Atlantic salmon affect scale cortisol levels. The specific aims were:

(1) To test if cortisol could be reliably detected in the scales of Atlantic salmon post-smolts

(2) To investigate relationships between temperature and feeding conditions with cortisol in the scales of Atlantic salmon post-smolts

(3) To evaluate scale cortisol as a potential biomarker for monitoring physiological stress responses to environmental change in wild Atlantic salmon

It was expected that increased scale cortisol levels would be detected in fish kept at temperatures outside their optimum range and in fish that had food withheld during the experiment.

4.3 Materials and Methods

4.3.1 Rearing experiment

The fish used for this experiment were one-year old (1+, fork length ranging from 15 to 27.5cm) Atlantic salmon post-smolts from a Norwegian hatchery strain (AquaGen, Trondheim, Norway), reared for a study by Thomas *et al.* (2019). The fish had been reared in 6°C ambient freshwater at the Institute of Marine Research in Matredal, Norway, and were transferred to seawater at the beginning of the experiment. For the duration of the 12-week experiment, the fish were kept in 1 x 1 metre tanks with a salinity of 35‰ and a dissolved oxygen level of over 90%. The photoperiod used (24 hours of light and 0 hours of darkness (LD 24:0)) represented the conditions experienced in the Norwegian Sea in the month of May. Post-smolts were kept at three temperatures: 6, 10.5 and 15°C. Temperatures were gradually increased from the initial 6°C over 48 and 96 hours to reach 10.5 and 15°C respectively, to avoid the shock of a fast temperature increase. Four feeding treatments were used in the experiment: the control group were fed to excess for the duration (Constant), one group was starved for 7 days in week 8 (ST7), another group were starved for 14 consecutive days in weeks 7 and 8 (ST14), and the final group were starved for 28 days total in 7-day increments (weeks 4, 6, 8 and 10; ST28). A fractional factorial design was used; all feeding treatments were crossed with the 10.5°C temperature treatment, and two feeding treatments (Constant and ST14) were crossed with the 6 and 15°C temperature treatments (Figure 4.1). A total of 16 tanks with 60 fish per tank were included in the experiment with two replicate tanks held at each temperature × feeding combination (Figure 4.1). Mortality was assessed as 2.9% (Constant and ST14 feeding treatments at all three temperatures) and 1.9% (all four feeding treatments at 10.5°C) for the first 24 hours and was then negligible for the rest of the experiment (Thomas, 2018). Further detail of the experimental set-up can be found in Thomas *et al.* (2019). All experimental work on *Salmo salar* post-smolts was conducted ethically in accordance with the Norwegian Regulation on Animal Experimentation 1996.

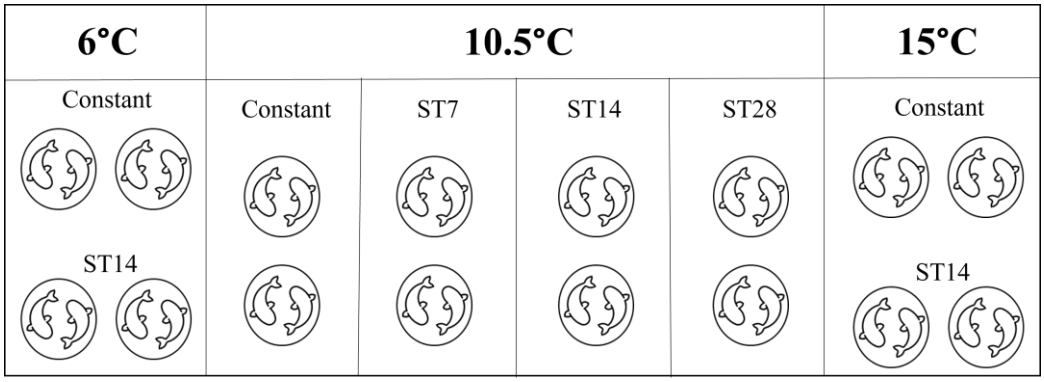


Figure 4. 1 Schematic of the rearing experiment for *Salmo salar* post-smolts. Each fish-pond icon represents a tank, with two replicate tanks for each temperature and feeding treatment combination (16 tanks total, 60 fish per tank; some 10.5°C tanks are duplicated between the two experiments). Three temperature treatments were used (6, 10.5, and 15°C), and four feeding treatments: Constant (control group that was fed constantly), ST7 (starved for 7 days during week 8), ST14 (starved for 14 consecutive days in weeks 7 and 8) and ST28 (starved for 28 days total; 7 days in each of weeks 4, 6, 8 and 10).

4.3.2 Post-smolt sampling

Three fish were randomly selected from each tank and killed at the beginning of each experimental week using a lethal dose of 2-Phenoxyethanol solution (0.6 ml l^{-1}). Length and weight measurements were recorded. Scale samples were removed from each fish from the standard scale sampling location on the left side posterior of the dorsal fin and above the lateral line (Shearer, 1992), then stored in paper envelopes. Scales were stored for approximately 6 years prior to analysis for this study.

To investigate the effect of temperature and feeding conditions on cortisol in scales, scale samples were selected from weeks 2, 9, 11 and 12 and prepared for cortisol analysis (n = 156). The experiment was conducted in two parts. In part 1, the effects of temperature were examined using samples from all three temperature treatments (6, 10.5 and 15°C). In part 2, the effect of feeding conditions on scale cortisol were examined using samples selected from the two feeding treatments (Constant and ST14) for the 6 and 15°C temperature treatments, and all four feeding treatments (Constant, ST7, ST14 and ST28) for the 10.5°C temperature treatment. The number of samples analysed from each temperature and feeding treatment is shown in Table 1. There was some overlap in the samples used in parts 1 and 2; the fish from the Constant and ST14 treatments at 10.5°C are included in both parts.

4.3.3 Scale preparation

Prior to analysis, all scales from each sample were cleaned by soaking for a minimum of 3 minutes in ultrapure water (18.2 mΩ) and then manipulated using clean forceps to gently remove mucous before cleaned scales were allowed to air dry. Each clean, dry scale was cut into small pieces in a glass petri dish using a scalpel. Cut scales were placed into pre-weighed centrifuge tubes and the weight (target weight 10mg) of each sample was recorded.

4.3.4 Scale cortisol analysis

A reference material was fabricated by cleaning and cutting the scales (8.63g) from a farmed adult salmon in accordance with the above method and then spiking with a solution containing 76ng/g of cortisol (Cerilliant). The spiked scales were kept in the freezer at -18°C prior to analysis as a reference material.

The extraction procedure was adapted from Aerts *et al.* (2015), with each extraction batch including 14 post-smolt scale samples, a procedural blank that did not contain any scales, and 10mg of the spiked reference scales in accordance with quality control procedures. 100mg of an internal standard containing 157.3-160.1 ng/g cortisol-d_4 (Cerilliant) was added to all samples to account for variability in the extraction and analysis processes, followed by 8ml of ROMIL Methanol 215 SpS (>99.9% assay). Samples were vortexed (VWR S42 and S040 Lab Dancer) for 30 seconds and then placed on a shaker (Stuart Scientific Flask Shaker SF1) at 800osc/min for one hour at ambient temperature. Samples were then placed in a Thermo Scientific Megafuge 8 benchtop centrifuge for 10 minutes at 3260g. The supernatant was carefully pipetted off into glass universal tubes and evaporated to almost dryness under nitrogen at 60°C in a TurboVap LV Concentration Workstation, until approximately 0.5ml remained in each sample. Samples were reconstituted in 5ml of H_2O/MeOH (80:20, v/v). GracePure SPE C_{18}-Max (500mg, 6ml) solid phase extraction (SPE) columns were conditioned on vacuum with 3ml MeOH followed by 3ml of ultrapure water. Samples were loaded using glass Pasteur pipettes, followed by 4.5ml of H_2O/MeOH (65:35, v/v). The samples were eluted with 3.5ml of H_2O/MeOH (20:80, v/v) and evaporated to almost dryness (approximately 0.5ml) at 60°C under nitrogen before being transferred to gas chromatography (GC) vials for analysis. Any samples that were below the quarter mark on the GC vial were topped up with some H_2O/MeOH (20:80, v/v). Equipment was cleaned and acid-washed between samples to prevent cross-contamination.

Samples were analysed on an Agilent 6890N Network GC (gas chromatography) system with an Agilent Tech 5975 Mass Selective Detector with helium as the carrier gas and a Trajan (SGE) capillary GC column HT8 (25m x 0.2mm, 0.25µm film thickness). The 189.1 *m/z* ion was used to identify cortisol, and the 306.1 *m/z* ion was used to identify cortisol-d_4. Following analysis, standards and samples were integrated and data were removed for further analysis. Reference scales and system suitability standards were used as quality control to check instrument consistency across runs, and procedural blank concentrations were used to calculate the limit of detection (LOD) of the GCMS (gas chromatography mass spectrometer). The samples (n = 5) with cortisol values below the LOD of 5.1ng/g were estimated using regression (Helsel, 1990; Higgins *et al.*, 2013).

4.3.5 Statistical analysis

Linear mixed effects models were used to investigate the effects of temperature (part 1 of the experiment) and feeding on scale cortisol (part 2). During post-hoc testing, a Holm adjustment (Holm, 1979) was used to minimise the chance of a Type I error. The adjustment accounted for the total number of pair-wise comparisons made across parts 1 and 2 of the analysis.

4.3.5.1 Experiment 1: effect of temperature on scale cortisol

To investigate the effects of temperature, the constantly fed fish were analysed for all temperature treatments for weeks 2, 9 and 12. *Temperature*, *Week* and *Sex* were included as fixed effects and *Tank* was included as a random effect to account for non-independence of measurements from the same tank. Cortisol data were transformed to achieve a normal distribution by raising to the power of 0.26, as determined by the Box Cox procedure. The response variable was $Cortisol\ (ng.g^{-1})^{0.26}$, and the full model included all terms and the interaction between the fixed effects:

$$Cortisol\ (ng.g^{-1})^{0.26} \sim Temperature*Week*Sex + (1|Tank)$$

The full model was compared to a series of less complex models and the best fitting model was selected based on Akaike Information Criteria (AIC) values and loglikelihood tests.

4.3.5.2 Experiment 2: effect of feeding on scale cortisol

The effect of feeding was analysed for each temperature separately. Week 2 was not included in this analysis as in that week fish were fed to excess across all treatments. Weeks 9 and 12 were analysed for fish in the 6 and 15°C temperature treatments. Fish at 10.5°C were analysed at weeks 9, 11 and 12. In each analysis, *Feeding*, *Week* and *Sex* were included as fixed effects and *Tank* was included as a random effect. At 6 and 15°C, *Feeding* was analysed at two levels: Constant and ST14. In the analysis of data from the 10.5°C treatment, *Feeding* was analysed at four levels: Constant, ST7, ST14 and ST28. The response variable was $Cortisol\ (ng.g^{-1})^{0.26}$. The full model at each temperature included the three fixed effects and their three-way interaction:

$$Cortisol\ (ng.g^{-1})^{0.26} \sim Feeding*Week*Sex + (1|Tank)$$

The best-fitting model was selected using the same procedure as described above. In all cases, if the variance contribution from the *Tank* random effect was found to be negligible, this term was omitted and the optimal models were refit using a general linear model. Post-hoc tests were used to compare each feeding treatment to the Constant treatment.

Changes in length and fish condition across the duration of the experiment were plotted using the full dataset from Thomas *et al.* (2019). Condition was calculated using residual values of the log of weight against the log of length (Fechhelm *et al.*, 1995; Blackwell *et al.*, 2000). A large positive mean residual value was considered to have a higher-than-average weight for its length. Pearson's product-moment correlations were used to test for correlations between the transformed cortisol and condition. A Holm adjustment (Holm, 1979) was used to minimize the chance of a Type I error.

All statistical analyses were carried out using R Version 4.1.2 in R Studio Version 2022.02.0+443. The NADA package (Lee, 2020) was used to estimate values for samples below LOD based on robust linear regression (Helsel, 1990). Linear mixed effects models were run using the lme4 package (Bates *et al.*, 2015), and the MASS package (Ripley *et al.*, 2022) was used for Box Cox transformation. The MuMIn package (Bartoń, 2020) was used to test all model combinations using the "dredge" function, and to determine marginal and conditional r^2 values. The emmeans package (Lenth, 2021) was used for post hoc testing. Data visualisation was carried out using the ggplot2 package (Wickham, 2016).

4.4 Results

4.4.1 Cortisol analysis

Cortisol was successfully extracted from the scales of 156 Atlantic salmon post-smolts and quantified using GCMS. Cortisol content of the samples ranged from 5.28ng/g to 215.93ng/g (measured cortisol, excluding estimated values for 5 samples below LOD) (Table 4.1).

4.4.1.1 Experiment 1: effect of temperature on scale cortisol

Linear mixed effects models analysed variation in scale cortisol between the three temperature treatments under constant feeding conditions. The variance explained by the *Tank* random effect was negligible so this effect was removed and the models were re-run using general linear models (GLMs). The best fitting model (Table 4.2) included *Temperature* as a fixed factor but not *Sex* or *Week*. The model explained 14.6% of the variance in cortisol concentrations, according to R^2 values. Post hoc testing showed that cortisol concentrations were significantly lower in fish in the 10.5°C temperature treatment than those at 6°C ($p = 0.016$) and 15°C ($p = 0.003$) prior to correction. Following Holm's adjustment of p values, cortisol concentrations in fish at 10.5°C were only significantly lower than those at 15°C ($p = 0.016$), not 6°C ($p = 0.080$).

Table 4. 1 Mean and range of scale cortisol content (ng/g), average fork length (cm), and sex of *Salmo salar* post-smolts at each temperature and feeding treatment combination at weeks 2, 9, 11 and 12 of the 12-week experiment. The four feeding treatments were as follows: Constant (control group that was fed constantly), ST7 (starved for 7 days during week 8), ST14 (starved for 14 consecutive days in weeks 7 and 8) and ST28 (starved for 28 days total; 7 days in each of weeks 4, 6, 8 and 10). Samples below the LOD (limit of detection) of the GCMS (n = 5) were estimated using regression. * denotes a group that includes an estimated value.

Week	**Feeding**	**Temp. (°C)**	**N**	**Cortisol (ng/g)**		**Length (cm)**		**Sex**	
				Mean	**Range**	**Mean**	**Range**	**M**	**F**
2	Constant	15	6	43.49	20.09 – 74.80	20.3	17.9 – 22.1	5	1
		10.5	6*	18.98	4.18 – 36.50	19.8	19.0 – 20.6	3	3
		6	6	42.81	10.91 – 79.16	20.2	19.5 – 21.3	3	3
	ST14	15	6	59.72	33.73 – 86.40	19.7	18.4 – 22.0	2	4
		10.5	6	18.13	6.03 – 45.75	19.8	18.8 – 20.5	1	5
		6	6	54.90	18.28 – 90.61	18.4	15.0 – 20.0	3	3
9	Constant	15	6	69.35	47.69 – 118.92	24.4	22.7 – 26.8	2	4
		10.5	6*	32.92	5.20 – 73.21	23.2	21.9 – 25.0	4	2
		6	6	69.36	9.05 – 126.47	21.3	20.3 – 22.5	4	2
	ST7	10.5	6	49.72	6.81 – 78.99	23.8	22.9 – 24.5	3	3
	ST14	15	6	27.65	7.77 – 71.72	23.3	21.3 – 25.0	3	3
		10.5	6	54.63	5.28 – 124.31	22.8	21.5 – 24.1	3	3
		6	6*	87.91	5.95 – 169.00	20.6	19.2 – 22.0	3	3
	ST28	10.5	6	49.29	27.97 – 91.51	23.1	20.5 – 25.7	2	4
11	Constant	10.5	6	69.16	21.95 – 168.42	24.5	22.6 – 27.0	4	2
	ST7	10.5	6*	35.72	6.58 – 63.95	23.9	23.0 – 25.3	3	3
	ST14	10.5	6	67.64	29.30 – 215.93	23.5	21.5 – 26.1	5	1
	ST28	10.5	6	54.31	31.32 – 74.68	22.6	20.8 – 25.0	3	3
12	Constant	15	6	48.23	16.26 – 85.50	26.2	25.2 – 27.5	3	3
		10.5	6	35.17	6.43 – 91.11	24.6	23.9 – 25.1	2	4
		6	6	37.78	6.00 – 50.11	21.4	19.9 – 22.4	4	2
	ST7	10.5	6	49.63	22.42 – 92.74	24.1	21.5 – 27.1	3	3
	ST14	15	6	57.37	20.99 – 191.16	24.9	22.6 – 27.1	2	4
		10.5	6	90.28	21.00 – 162.94	24.4	23.0 – 25.6	2	4
		6	6	59.27	5.98 – 140.86	21.0	19.2 – 22.4	2	4
	ST28	10.5	6*	48.76	7.14 – 65.36	23.8	22.6 – 26.0	3	3

Table 4. 2 Results of the best fitting general linear models based on AIC values and likelihood ratio test on the effect of temperature and feeding on scale cortisol from *Salmo salar* post-smolts, including summary data for coefficients of each model: the estimate (Est.), standard error (S.E.), t-value, and p value. The AIC value and adjusted R^2 of each model are also listed. Adj. p value refers to the p value following Holm adjustment. Cortisol was transformed to achieve a normal distribution.

Model	Coeff.	Est.	S.E.	t-value	p value	Adj. p value	AIC	R^2
Constant feeding treatment, all temperatures								
Cortisol $(ng.g^{-1})^{0.26} \sim$ Temperature	6 – 10.5°C	0.431	0.17	2.49	0.016	0.080	87.26	0.15
	6 – 15°C	-0.11	0.17	-0.66	0.514	0.514		
	10.5 – 15°C	-0.55	0.17	-3.15	0.003	0.016		
6°C, Constant and ST14 feeding treatments								
Cortisol $(ng.g^{-1})^{0.26} \sim 1$						0.200	53.00	
15°C, Constant and ST14 feeding treatments								
Cortisol $(ng.g^{-1})^{0.26} \sim 1$						0.200	40.36	
10.5°C, Constant, ST7, ST14 and ST28 feeding treatments								
(Cortisol $ng.g^{-1})^{0.26} \sim 1$						0.200	123.37	

4.4.1.2 Experiment 2: effect of varying periods of starvation on scale cortisol

Cortisol varied across treatments and appeared to increase after the starvation event in the 10.5°C treatment (Figure 4.2). Linear mixed effects models analysed variation in scale cortisol across the feeding treatments at each temperature. The variance explained by the *Tank* random effect was negligible so this effect was removed and the models were re-run using GLMs.

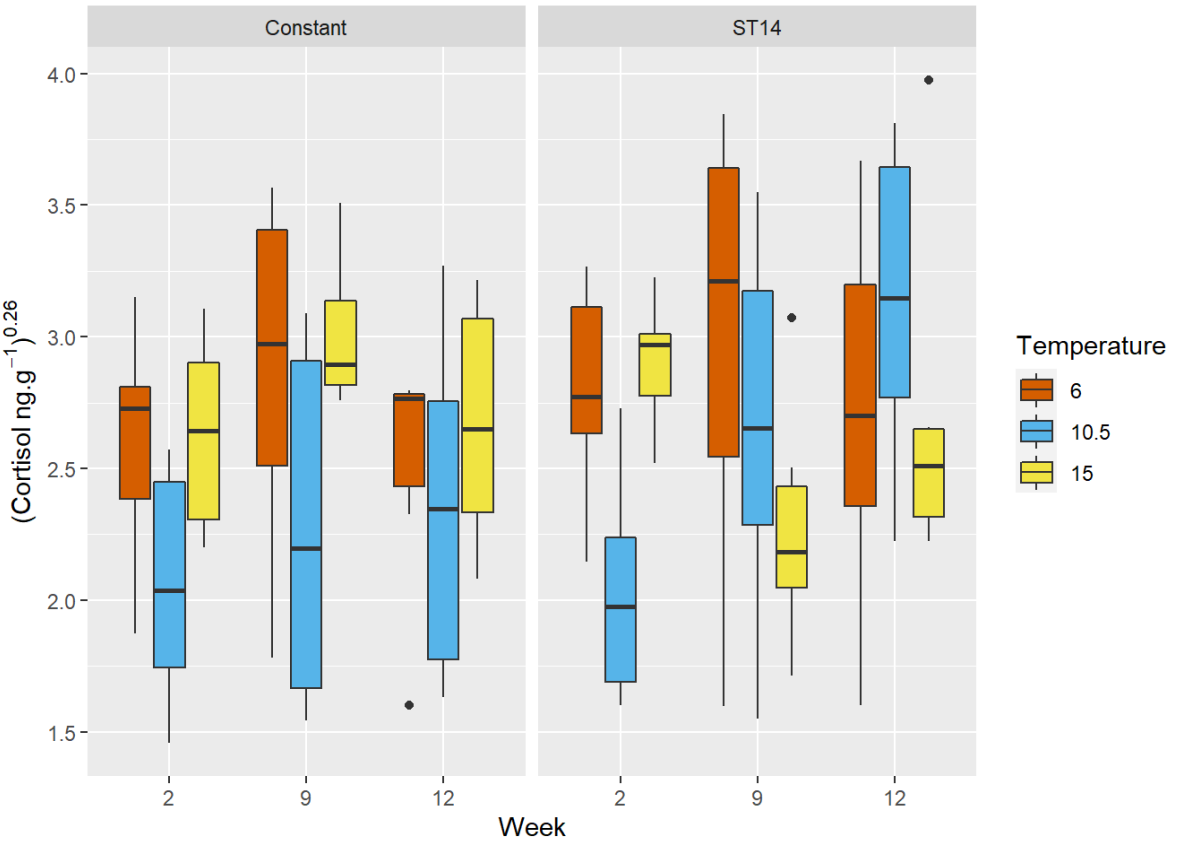


Figure 4. 2 Median and variation of Cortisol (ng.g^{-1})$^{0.26}$ in *Salmo salar* post-smolt scales in weeks 2, 9 and 12. Fish were kept at 6°C, 10.5°C and 15°C for a period of 12 weeks and were fed to excess throughout (Constant) or starved for 14 days at weeks 7 and 8 (ST14). Cortisol was transformed to achieve normal distribution. The bold line in each box represents the median of the data, the 25th and 75th percentiles are represented by the bottom and top limits of each box respectively. The bottom and top whiskers represent the smallest and largest values within 1.5 times the interquartile range from the 25th and 75th percentiles respectively, and the dots correspond to values >1.5 times and <3 times the interquartile range

For the analysis of data from the 6°C temperature treatment, the null model, which included no fixed or random effects, was the best fit (Table 4.2), indicating that there were no differences in scale cortisol concentrations between feeding treatments, weeks, or sexes at 6°C. At 15°C, there appeared to be a difference between feeding treatments in week 9 when plotted, particularly for males (Figure 4.3). However, when comparing scale cortisol between feeding treatments at 15°C, the null model, which did not include any fixed or random effects, was the best fit (Table 4.2). At 10.5°C, scale cortisol concentration increased after the

14-day starvation event, particularly in male fish (Figure 4.4). When all four feeding treatments from the 10.5°C temperature treatment were analysed, the best fitting model was the null model (Table 4.2).

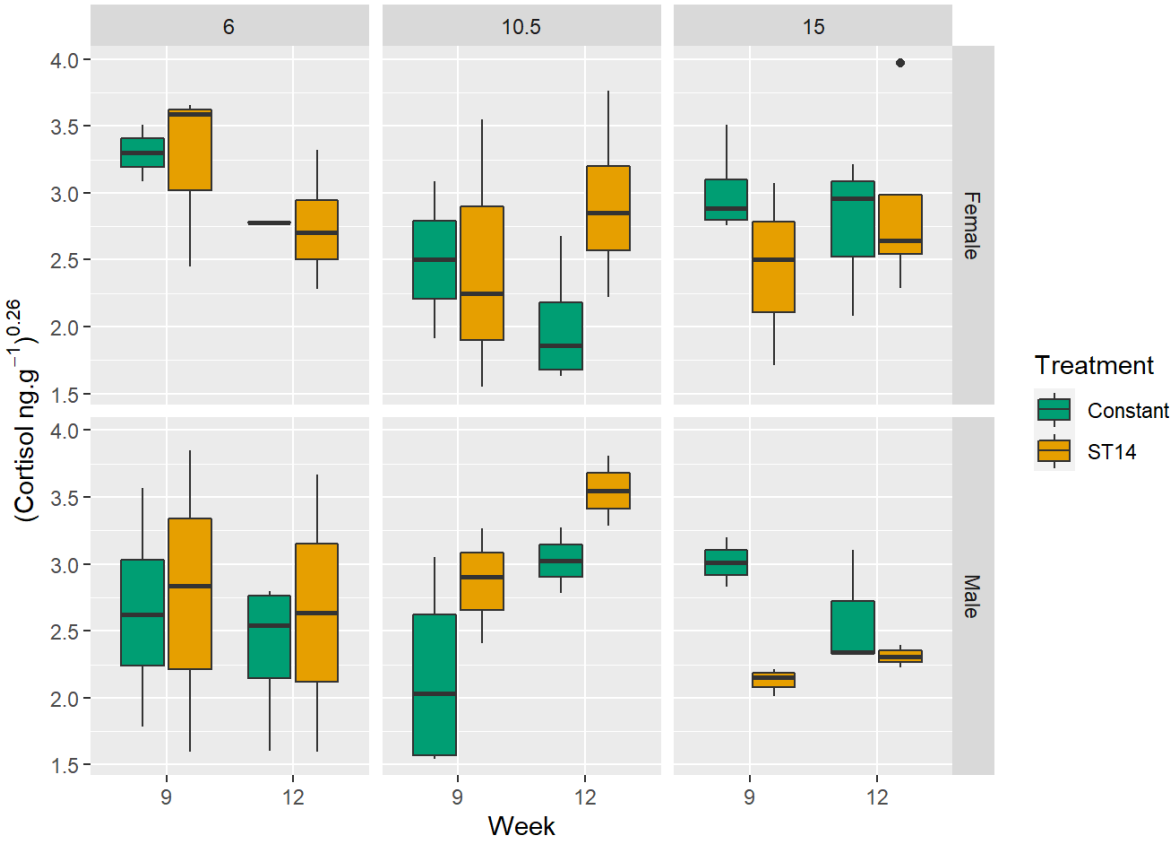


Figure 4. 3 Median and variation of Cortisol (ng.g^{-1})$^{0.26}$ in female and male *Salmo salar* post-smolt scales in weeks 9 and 12. Fish were kept at 6°C, 10.5°C and 15°C for a period of 12 weeks and were fed to excess for the duration (Constant) or starved for 14 days at weeks 7 and 8 (ST14). Cortisol was transformed to achieve normal distribution.

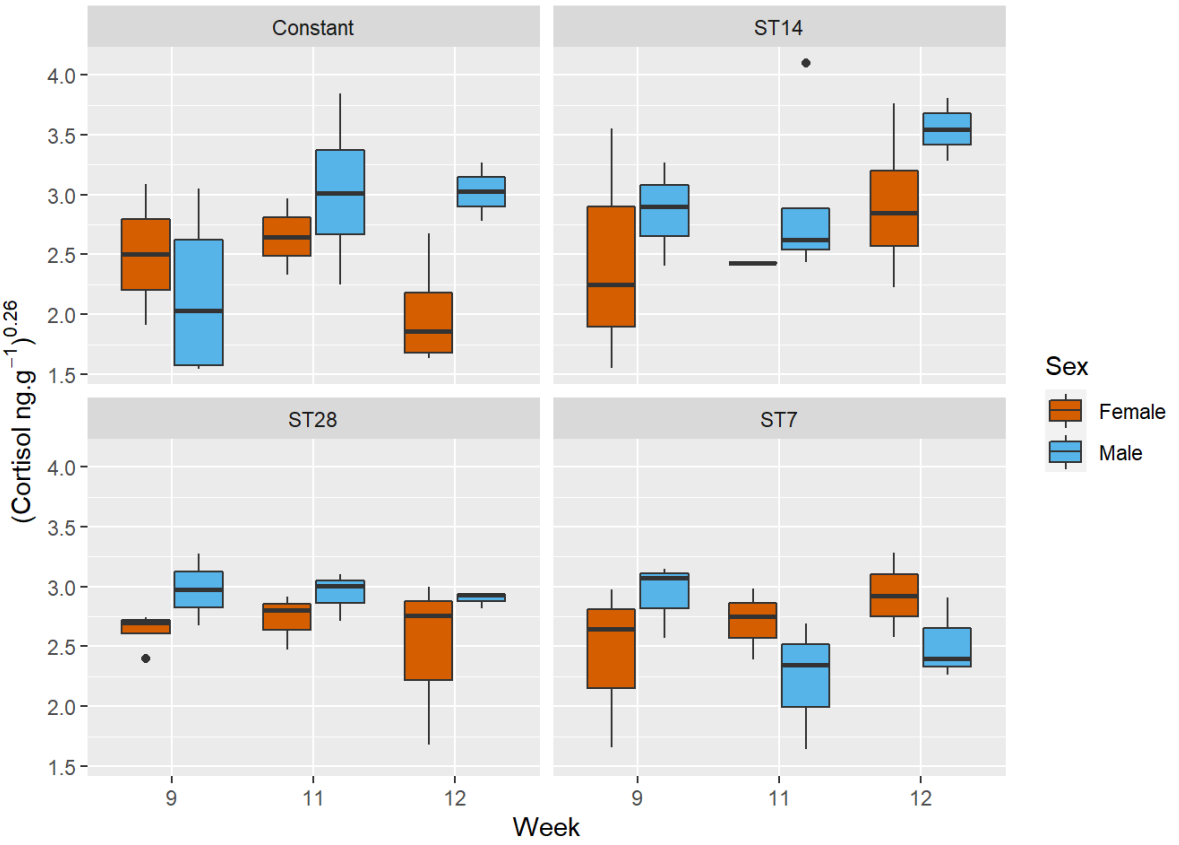


Figure 4. 4 Median and variation of Cortisol (ng.g^{-1})$^{0.26}$ in scales of female and male *Salmo salar* post-smolts over the 12-week study. Fish were kept at 10.5°C and four feeding treatments: Constant (the control group that was fed constantly), ST14 (starved for 14 days), ST28 (starved for 28 days in four 7-day periods) and ST7 (starved for 7 days). Cortisol was transformed to achieve normal distribution.

4.4.2 Comparison of growth rates between treatments

Thomas *et al* (2019) reported variation in growth rates between the temperature and feeding treatments. Changes in growth and condition during the duration of the experiment were plotted here to allow comparison with the cortisol measurements (Figure 4.5). From weeks 8-12, both scale and body growth increased with temperature. Fourteen days of starvation had no significant effect on scale growth or fork length at 6°C but caused reduced scale and body growth at both 10.5 and 15°C. Both scale growth and body growth were significantly lower in the ST28 treatment than the Constant treatment at 10.5°C (Thomas *et al.*, 2019). Starvation caused a reduction in condition which was evident at week 9 at all temperatures (Figure 4.5). By week 12, there was no difference in condition between the fish from the ST14 treatments compared to the Constant at all temperatures. At 10.5°C, fish from the ST28 treatment had

lower condition than the constantly fed fish, while fish from the ST7 treatment had a higher condition. According to Pearson's product moment correlations and following Bonferroni correction, there was no significant correlation between cortisol and fish condition for any treatment combination (p values ranged from 0.053 to 0.927).

Figure 4. 5 Mean (±standard error) fork length and condition of *Salmo salar* post-smolts during the 12-week study. Fish were kept at three temperature treatments (6, 10.5 and 15°C) and four feeding treatments: Constant (the control group that was fed constantly), ST14 (starved for 14 days), ST28 (starved for 28 days in four 7-day periods) and ST7 (starved for 7 days). Data from Thomas *et al.* (2019) used to create this figure.

4.5 Discussion

This research determined that cortisol can be detected and quantified in small quantities (down to 10mg) of scales from Atlantic salmon post-smolts. Scale cortisol was significantly higher at 15°C than at 10.5°C, indicating that Atlantic salmon post-smolts may be exposed to physiological stress at elevated temperatures; however, other factors contributing to cortisol

production should be considered. The increase in cortisol could be due to smoltification but, as a similar increase was noted at 6°C, this suggests the elevated cortisol was a stress response to temperature. There was some indication that fourteen days of starvation caused an increase in cortisol levels, but this effect was not significant after correction for multiple comparisons.

Some of the cortisol extracted from post-smolt scales could have resulted from factors not measured in this study or from naturally occurring shifts. The fish in this study completed the process of smoltification in freshwater and entered saltwater at the beginning of the 12-week experiment. Corticosteroids have been shown to increase during the smoltification process in coho salmon (Specker and Schreck, 1982; Barton *et al.*, 1985; Björnsson *et al.*, 2011) and may play an important role in maintaining sodium transport and increasing Na-K-ATPase (sodium-potassium-stimulated adenosine-triphosphatase) (Pickford *et al.*, 1970) which is involved in salinity tolerance (Epstein *et al.*, 1967). Plasma cortisol increases occurred simultaneously between February and May in Atlantic salmon pre-smolts exposed to three different photoperiods (Stefansson *et al.*, 1989), which correlated with previously recorded cortisol increases in spring associated with the stress of smoltification (Thorpe *et al.*, 1987). Cortisol plays an important role in regulating smolt development (McCormick, 2009) and may also be involved in regulating lipid metabolism in smolts (Sheridan, 1986). However, as fish in the various treatments in our study did not exhibit a uniform increase in cortisol over the duration of the experiment, factors other than smoltification must contribute to cortisol fluctuations. Combined with similar cortisol increases in both 15 and 6°C, this suggests that cortisol increased as a response to temperature rather than smoltification.

It is possible that sex played a role in scale cortisol increases in post-smolts in this study. Increased temperature combined with a LD 24:0 photoperiod have been shown to trigger maturation in male Atlantic salmon both during and directly following smoltification (Fjelldal *et al.*, 2011). Scale cortisol appears to be elevated in males compared to females at 10.5°C but not at 15°C, and *Sex* was not a significant factor in any of the models analysed for this experiment. Testing with a larger sample size could make elucidating differing responses between the sexes clearer. The high degree of variation within-treatment could have prevented the statistical identification of elevated cortisol in some treatments. Additionally, individual variability is likely to affect cortisol levels; fish with a high standard metabolic rate (SMR), often dominant fish, tend to utilise food reserves more quickly than fish with a low

SMR, making food deprivation a greater threat (O'Connor *et al.*, 2000). Social dynamics are also an important factor, as dominant and subordinate fish will react differently to stressors and have different baseline cortisol levels, and individual genetics could play a role (Doyon *et al.*, 2003; Jeffrey *et al.*, 2014). In this experiment, scale cortisol values showed a high degree of variability within each treatment and a large proportion of the variance was not explained by the experimental treatments. This suggests that individual variability played an important role, which may be linked to dominance hierarchies within each tank. While similar effects may be observed in wild fish exposed to periods of food shortage or temperature stress, the effects of competition and individual dominance traits would likely be different for wild fish compared to a laboratory reared population confined to tanks.

The effect of temperature on scale cortisol levels was evident in constantly fed fish. Temperatures above a species' natural optimum have been shown to elicit elevations in plasma cortisol (Chadwick *et al.*, 2015; Hanke *et al.*, 2019; Goikoetxea *et al.*, 2021). Standard and active metabolic rates of Atlantic salmon increase with increasing temperature (Hvas *et al.*, 2017), therefore we would expect higher cortisol levels due to the increased metabolic demand. In this study, scale cortisol levels were significantly elevated in constantly fed fish from the 15°C treatment compared to those in the 10.5°C treatment. The upper threshold for growth and development of Atlantic salmon post-smolts in seawater is around 18°C; however, food conversion efficiency and food intake have been shown to decline from 12.8°C for small fish (70-150g) and 14°C for larger fish (170-300g) (Handeland *et al.*, 2000; Handeland *et al.*, 2008). The fish analysed for scale cortisol in this study ranged from 32-250g. Cortisol increases were noted at similar temperatures in another study; Atlantic salmon post-smolts acclimated to four different temperatures (4, 8, 12 and 17°C) had significantly higher baseline cortisol levels (measured in water) with increasing temperature (Madaro *et al.*, 2018). The post-smolts at higher temperatures also reacted more quickly and intensely to additional stressors than those at the colder temperatures (Madaro *et al.*, 2018). While 15°C is within the temperature range experienced by Atlantic salmon during their life cycle, there are clear temperature limits for different life stages of fishes (Elliott and Elliott, 2010), and smolts from Norwegian rivers will usually enter seawater that is 8-10°C (Friedland *et al.*, 1998; Hvidsten *et al.*, 1998). These studies demonstrate that even small temperature increases above the optimum can result in cortisol increases related to physiological stress.

Post-smolts exhibit a preference for an 8-12°C temperature range (Friedland *et al.*, 2000; Holm *et al.*, 2000), and temperature has been positively correlated with post-smolt survival (Hansen and Quinn, 1998; Friedland *et al.*, 2000). The experimental fish in this study were reared at ambient conditions of 6°C during the freshwater phase, a temperature that wild Atlantic salmon juveniles experience in freshwater. However, 6°C is colder than temperatures that post-smolts would typically encounter during their migration to marine feeding grounds. 6°C is below the size-dependent temperature optima for food conversion efficiency and growth for Atlantic salmon post-smolts and would therefore cause food intake and digestion rates to decrease (Handeland *et al.*, 2008). Increased plasma cortisol in juvenile coho salmon (*Oncorhynchus kisutch*, Walbaum) resulted from acclimation to low temperatures (Allan, 1971). The post-smolts in this study also exhibited elevated cortisol concentration in the 6°C temperature treatment, though the relationship was no longer significant following Holm correction. This suggests that the low temperature may have been stressful for the fish, but further investigation with a larger sample size is needed confirm if this is the case. Growth of the experimental fish increased with temperature and did not reflect differences in cortisol. According to data from Thomas *et al.* (2019), the highest growth was achieved in the 15°C temperature treatment, which also had highest scale cortisol concentration. Fish in the 10.5°C temperature treatment had intermediate growth and the lowest scale cortisol concentration. The lowest growth was achieved by the fish kept at 6°C which, as mentioned, had higher cortisol concentrations than fish at 10.5°C, though not significantly so following correction for multiple comparisons.

Our results suggest that there are differences in scale cortisol between starved and fed fish, but the effect is complex with low statistical power. Condition and length measurements show that fish reacted differently to starvation at 10.5 and 15°C; at 10.5°C, skeletal growth recovered post-starvation, but not condition; the opposite happened at 15°C, where starved fish recovered their condition but not their length. Reports on the effect of starvation on cortisol in salmonids have been mixed. Depending on the study and the species, starvation can cause cortisol to increase, decrease, or not be affected at all (Bar, 2014). Plasma cortisol levels in overwintering female rainbow trout had a small but significant increase in fed fish and re-fed fish (starved for 63 days, then fed) compared to fish starved for 120 days (Pottinger *et al.*, 2003). A second study found no significant difference in plasma cortisol between rainbow trout starved for up to 65 days and those that were fed continuously over the same period (Milne *et al.*, 1979). In a third study, rainbow trout blood cortisol levels were not

significantly affected by starvation in 1+ fish, but 0+ fish had significantly elevated plasma cortisol levels for 2 weeks following food withdrawal (Sumpter *et al.*, 1991). Prolonged periods of starvation may lead to changes in scale cortisol, but the results of our study and others demonstrate that the relationships are complex and more difficult to detect than the effect of temperature.

The duration of the starvation period may not have been enough to trigger a marked stress response in the experimentally reared post-smolts. As part of their natural life cycle, fish experience periods of food shortage. Atlantic salmon juveniles that overwinter in freshwater reduce foraging effort during the winter to minimise predation risk and because feeding is less energetically profitable at low temperatures, instead relying on stored lipids for energy (Metcalfe and Thorpe, 1992; Bull *et al.*, 1996). This state of natural anorexia occurs due to appetite suppression, even when food is freely available (Metcalfe and Thorpe, 1992). Post-smolts starved for 4 weeks lowered their standard metabolic rate after 1 week of fasting and again after 3 weeks of fasting, but an increase in stress response (measured by mass-specific oxygen uptake rates) wasn't noted until 3 weeks of fasting (Hvas *et al.*, 2020). Adults cease feeding prior to entering freshwater on their return migration to spawn in their natal river (Kadri *et al.*, 1995), with lipid content decreasing from over 11% of body mass in summer to 2% or less of the wet mass following spawning (Jonsson *et al.*, 1997). These studies demonstrate that Atlantic salmon are adapted to periods of food deprivation, therefore they may not experience predictable stress from relatively short instances of starvation similar to the conditions in this experiment.

The method developed in this research enabled the successful detection of cortisol in the scales of Atlantic salmon post-smolts resulting from temperature stressors, along with other factors, over a period of 12 weeks. It also shows the potential for scale cortisol being measured to examine starvation as a stressor. Other authors have expressed a concern that scale cortisol analysis is not entirely non-invasive due to the amount of scale needed, particularly if sampled from a small fish (Sadoul and Geffroy, 2019). In previous studies 100mg (Aerts *et al.*, 2015) and 40-50mg (Carbajal *et al.*, 2019; Laberge *et al.*, 2020) of scale material was analysed. In our research, the method was optimised for use with 10mg of scale material, requiring less scales per sample which would therefore result in less intrusion on fish during non-lethal sampling. The lower sample weight makes the method more suitable for use on wild fish and for scale archives, which contain limited amounts of material. The

use of higher specification GCMS instrumentation could enable the further reduction of required scale material which could support the analysis of scale cortisol across a wider variety of samples and populations.

Analysis of cortisol in small volumes of scales from wild fish could be used to monitor stress responses during the marine phase in wild Atlantic salmon populations or to relate stress during the freshwater phase to subsequent survival. In Atlantic salmon and sea trout kelts, baseline blood cortisol levels are a good predictor of migration success (Birnie-Gauvin *et al.*, 2019); if cortisol in scales shows a similar relationship, it may provide a useful index of pre-migration condition that can be collected less invasively than blood samples. Future work could examine if brief and sudden exposure to temperatures close to the upper limit, as can occur during marine heatwaves, produces a detectable stress response in scale cortisol. In addition, scales may contain other markers of thermal responses such as heat shock proteins. For example, heat shock protein-70 in gill tissue showed a large magnitude threshold response when brook trout (*Salvelinus fontinalis*, Mitchill) were exposed to temperatures close to the thermal ecological limit for this species (Chadwick *et al.*, 2015). Heat shock proteins can be found in all cells of all organisms (Li and Srivastava, 2003) and in fish are detectable in fin tissue (Feldhaus *et al.*, 2010).

In conclusion, this study confirmed that cortisol can be reliably detected in Atlantic salmon post-smolt scales with sample weights as low as 10mg. Scale cortisol reflected rearing temperatures that were above or below the temperature preference of Atlantic salmon post-smolts, suggesting that it is a suitable biomarker for monitoring physiological stress responses in wild Atlantic salmon resulting from environmental changes. However, wild fish are exposed to a wide range of interacting stressors including sea lice (*Lepeophtheirus salmonis*, Krøyer) infestation (Poole *et al.*, 2000), maturation (Baker and Vynne, 2014), and climatic patterns (Friedland *et al.*, 2009), which could make direct stress responses to temperature difficult to establish. Further testing is required to determine if rearing post-smolts at 6°C elicits a significant increase in scale cortisol. The method also detected fluctuations in scale cortisol related to starvation periods which also needs to be further investigated to determine the relationship between scale cortisol and starvation. This research is a positive step towards a non-invasive measure of cortisol for Atlantic salmon. Additionally, this method could allow a retrospective analysis of chronic stress in Atlantic salmon over time, thanks to the availability of scales in vast, multi-decadal archives. This

analysis could provide the opportunity to examine how salmon have coped with environmental change factors that have already happened, giving insight into how they might react to the predicted changes that are yet to come.

4.6 Acknowledgements

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Chapter 5. Cortisol in scales of adult Atlantic salmon shows substantial individual variability with no annual signal

5.1 Abstract

Scale cortisol has been identified as a measure of chronic stress in fish and was revealed to reflect elevated temperatures experienced by Atlantic salmon. As levels of marine mortality continue to be high in many populations, links have been made to climate change, particularly increasing sea surface temperature (SST), and cyclical climatic forcings such as the North Atlantic Oscillation (NAO). Scales, which provide a record of the life history of a fish, are regularly used to examine the effect of conditions in the marine environment on growth. Scale cortisol concentrations may provide further information about the physiological state of salmon at sea and their response to stressors. In this study, the stability of cortisol over a simulated 96-week period was determined. Cortisol was then extracted for the first time from archived scales of 120 Atlantic salmon (6 years of a 29-year period). Climate factors and biological factors including sex and growth did not explain variability in scale cortisol. Individual variability, which could include maturation and smoltification, appeared to drive differences in cortisol more than interannual variability of shared factors. Future research could examine a smaller portion of scale for a more temporally distinct sample or combine analyses with other methods to further investigate the causes and effects of elevated cortisol.

5.2 Introduction

Stress refers to the non-specific, adaptive response of an organism to a disturbance, or stressor, that results from the attempt to acclimate to that stressor (Selye, 1950; Russell, E. *et al.*, 2012). All organisms have a basic reaction pattern to stress that is the same, regardless of the nature of the stressor, and is initiated and controlled by two hormonal systems (Selye, 1950; Schreck and Tort, 2016). Cortisol, a steroid hormone that is released as a stress response, is the main corticosteroid produced in fishes (Sadoul and Geffroy, 2019) and can be released in response to a variety of stressors such as extreme temperatures, low oxygen, starvation, confinement and handling (Barton, 2002). Cortisol mobilises energy stores, regulates the immune system response to stress (Russell, E. *et al.*, 2012) and can serve as a sensitive indicator to the severity of stressors (Baker and Vynne, 2014). It can be measured to investigate coping mechanisms in fish in response to both natural and anthropogenic cues. It

is often quantified in aquaculture to ensure the artificial rearing environment causes minimum stress during maximum production (Barton, 2002). Additionally, cortisol measurements can be used to examine the effect of anthropogenic changes in the environment on fish (Sadoul and Geffroy, 2019). Cortisol fluctuations can also occur naturally due to physiological changes, including smoltification (Specker and Schreck, 1982; Barton *et al.*, 1985), maturation, and reproduction in salmon (Baker and Vynne, 2014).

Cortisol is most commonly measured in blood plasma (Cook, 2012) and has been analysed extensively to address the effect of stressors in a variety of fish species. Investigations of cortisol levels in wild fish include the determination of baseline cortisol levels in sockeye salmon (*Oncorhynchus nerka*, Walbaum) during crucial life stages (Baker and Vynne, 2014) and the use of cortisol levels as a predictor of future migration success in salmonid kelts (Birnie-Gauvin *et al.*, 2019). However, blood sampling can itself be a form of stress to the organism being sampled (Bertotto *et al.*, 2010). The sampling process can confound the real-time cortisol levels, is invasive, and blood cortisol measures acute stress but does not give an indication of chronic stress (Bertotto *et al.*, 2010; Cook, 2012; Aerts *et al.*, 2015; Sadoul and Geffroy, 2019). Cortisol levels in the blood can vary depending on the time of day and on food intake, further complicating the interpretation of such data (Davenport *et al.*, 2006).

The detection of steroid hormones in human hair (Yang *et al.*, 1998) stimulated further research to identify alternative matrices for measurements of chronic stress. The scales of fish are an ideal matrix because they grow slowly, incorporate cortisol, and are easily sampled (Aerts *et al.*, 2015). Scale cortisol has recently been used to examine chronic stress in multiple species including the common carp (*Cyprinus carpio*, L. (Aerts *et al.*, 2015)), rainbow trout (*Oncorhynchus mykiss*, Walbaum (Carbajal *et al.*, 2019), and European sea bass (*Dicentrarchus labrax*, L. (Goikoetxea *et al.*, 2021). Under experimental conditions, cortisol levels in scales of Atlantic salmon post-smolts reflected the rearing temperature and were elevated at 15°C relative to 10.5°C (Chapter 4). This method could be applied to examining physiological responses in Atlantic salmon to rising sea temperatures or other stressors experienced in the wild.

The number of Atlantic salmon returning to their natal river to spawn following marine foraging migrations remains low despite reductions in fishery efforts, suggesting that other factors besides overfishing are affecting marine survival (ICES, 2021a). This increased

marine mortality has been linked to climate change (Friedland, 1998) through direct effects such as temperature changes and indirect effects such as food availability (Thorstad *et al.*, 2021). Climate impacts on Atlantic salmon have been linked to cyclical, broad scale climatic forcing, including fluctuations driven by changes in the North Atlantic Oscillation (NAO) (Friedland *et al.*, 2003a). A high, or positive, winter NAO index is associated with mild and stormy weather (Hurrell, 1995; Hurrell *et al.*, 2003) and has been shown to decrease sea-age at maturity in Atlantic salmon, while a positive NAO index in May increased growth rates (Jonsson and Jonsson, 2004). Climate change and an associated increase in the NAO index exposes Atlantic salmon at sea to higher temperatures, increased prevalence and intensity of storms, and shifting ocean circulation patterns (Hurrell, 1995; Hurrell *et al.*, 2003; Friedland *et al.*, 2009). Increasing temperature has been shown to result in an increased stress response in post-smolts (Madaro *et al.*, 2018), suggesting that these climatic changes may place salmon under increased physiological stress. Sea surface temperature (SST) also has strong links to long-term changes in phytoplankton and zooplankton abundance and productivity (Beaugrand and Reid, 2003; Beaugrand and Reid, 2012), suggesting that changing environmental factors may also cause food availability stress at higher trophic levels for marine foraging Atlantic salmon.

Salmon scales are routinely sampled across the species' distribution and can be stored easily at room temperature, with laboratories in many countries holding extensive collections of scales (Trueman and Moore, 2007; Tray *et al.*, 2020). These vast, multi-decadal archives contain a wealth of data dating back almost a century (Tray *et al.*, 2020). Many studies have utilised these extensive collections to examine the effects of long-term biological trends in Atlantic salmon, including the analysis of circuli patterns in archived scales to examine how environmental conditions at marine feeding grounds affect growth rates (Friedland *et al.*, 2005; Hogan and Friedland, 2010; Tillotson *et al.*, 2021). Scale cortisol concentrations may provide additional information about physiological state and give insight into responses to potential climate change mediated stressors such as shifting NAO indices, increasing SST, or fluctuating food availability.

This study examines the applicability of using cortisol from archived salmon scales as an indicator of physiological state during the marine phase. In addition to climate factors on a broad scale, biological factors on an individual scale will be examined to determine if cortisol concentration is influenced by sex or growth during certain life stages. As far as we are

aware, this is the first study to measure scale cortisol in archived Atlantic salmon scales. Therefore, prior to extracting cortisol from archived scales, we used scales from a single individual to carry out a stability study to determine if cortisol in scales remains stable over time.

The aims of this study were to:

1. Determine if cortisol in Atlantic salmon scales remains stable during storage using simulated degradation

2. Use cortisol measurements from the marine portion of scales from returning adult Atlantic salmon to provide an index of physiological state during the marine phase

3. Examine temporal trends in this index of physiological state over a 29-year period (6 selected years from 1989 to 2018)

4. Examine the relationships between physiological state and environmental (NAO, SST and zooplankton abundance) and biological (fish size and growth) variables

We hypothesised that in years with a positive NAO index, the increasing temperatures and changing food availability would be reflected in increasing scale cortisol levels.

5.3 Materials and Methods

5.3.1 Sample collection

5.3.1.1 Stability study

Scales were gently scraped from both flanks of an individual farmed Atlantic salmon, obtained fresh and whole from a fish shop, from behind the dorsal fin and above the lateral line. 16 samples were taken and each was placed into an individual acid-free paper envelope, the type that is routinely used to store archived scales (Tray *et al.*, 2020). Scale collections are usually held at room temperature. In general, according to Arrhenius' equation, the rate of a reaction approximately doubles for every temperature increase of 10°C (Ebrahim *et al.*, 2021). Therefore, with a standard room temperature of approximately 20°C, 12 weeks at 50°C represents approximately 96 weeks at room temperature. At the beginning of the experiment (week 0), two samples were placed on a shelf at room temperature to act as a room temperature control. Two samples were weighed and placed in crucibles into a chamber

furnace (Carbolite Gero ELF 11/14B) at 50°C. The remaining samples were placed in the freezer to limit potential degradation prior to entering the oven. Two samples remained in the freezer for the entire 12-week duration of the experiment as freezer controls. Every two weeks (weeks 2, 4, 6, 8 and 10), two samples were removed from the freezer and brought to room temperature. Each sample was weighed and placed in a crucible in its designated location in the furnace. At week 12, the experiment was finished. Time spent in the oven varied between samples from 2 to 12 weeks (Table 5.1). Freezer and room temperature controls did not spend any time in the oven. When the experiment was complete, scales were prepared for cortisol analysis as described below.

Table 5. 1 Experimental set-up of a stability study to examine the cortisol concentration in scales from the same Atlantic salmon (*Salmo salar*) over time. Controls were kept at room temperature and in the freezer for the duration of the 12-week experiment. Samples were moved from the freezer to the oven at 2-week intervals to simulate accelerated degradation.

Week	**n**	**Treatment**	**Time in oven (weeks)**	**Simulated time (weeks)**
0	2	Room temperature	0	N/A
	2	Freezer	0	N/A
	2	Oven	12	96
2	2	Oven	10	80
4	2	Oven	8	64
6	2	Oven	6	48
8	2	Oven	4	32
10	2	Oven	2	16
12	16	Samples removed from oven and freezer, all samples prepared for analysis		

5.3.1.2 Archived scales

Scale samples were taken from the scale archive kept by the Marine Institute's Newport Research Facility in County Mayo, Ireland. Scales were from ranched Atlantic salmon, released into the Burrishoole system as smolts, that were caught on their return to the Burrishoole catchment after one winter at sea. All scales were taken from the standard sampling location, behind the dorsal fin on the left flank, 2-3 rows above the lateral line (Shearer, 1992) and stored in standard scale envelopes in the archive. 20 fish were sampled from each of 6 years (n = 120). Years were chosen to reflect the positive (1989, 1999, 2018) and negative (1998, 2001, 2010) phases of the NAO.

5.3.2 Sample preparation

Using clean, dry equipment, archived scales were carefully removed from storage envelopes and placed in ultrapure water (18.2 mΩ) for a minimum of 5 minutes. On a dissection microscope, scales were gently scraped with a scalpel to remove mucous and residue from the envelope. Once clean, scales were allowed to air dry. One suitable scale from each sample was selected for age and growth analysis and preservation in the archive. The remainder of the scales in each sample were cut under the dissection microscope using a scalpel. The entire portion of scale relating to the marine phase was excised to obtain a temporally distinct section of scale for analysis. Excised scale segments were carefully cut into small pieces using a scalpel and placed in pre-weighed centrifuge tubes and the weight (target weight 10mg) of each sample was recorded.

Scales for the stability study were cleaned following the above procedure and allowed to air dry. A portion of the scale was not excised. The full scale was cut into small pieces and placed into centrifuge tubes for analysis. The weight (target weight 10mg) of each sample was recorded.

5.3.3 Cortisol analysis

Scales (8.63g) from a farmed adult salmon were cleaned and cut following the above method, then spiked with a solution containing 76ng/g cortisol (Cerilliant) to create a reference material. These scales were stored in the freezer at -18°C prior to analysis as a reference material.

Cortisol extraction was completed following the method described in Chapter 4, modified from Aerts *et al.* (2015). Each extraction batch of 16 included 14 scale samples, a procedural blank that did not contain any scales, and 10mg of spiked reference scales in accordance with quality control procedures. 100mg of an internal standard containing cortisol-d_4 was added to each sample, procedural blank and reference, followed by 8ml ROMIL Methanol 215 SpS (>99.9% assay). The concentration of the internal standard was 157.3ng/g cortisol-d_4 for all stability study samples and 6 archive batches and 155.4ng/g cortisol-d_4 for the remaining 3 archive batches. Each sample was vortexed (VWR S42 and S040 Lab Dancer) and then placed on a shaker (Stuart Scientific Flask Shaker SF1) at 800osc/min for one hour at ambient temperature. Samples were spun in a benchtop centrifuge (Thermo Scientific Megafuge 8) for 10 mins at 3260g. The supernatant was carefully removed using glass Pasteur pipettes and placed in glass universal tubes. These samples were evaporated to almost dryness (approximately 0.5ml) under nitrogen at 60°C in a TurboVap LV Concentration Workstation. Samples were reconstituted in 5ml of a H_2O/MeOH (80:20, v/v) solution. C_{18} solid phase extraction (SPE) columns (GracePure SPE C_{18}-Max 500mg, 6ml, and SEClute SPE C_{18}-Max 500mg/6ml) were conditioned on vacuum with 3ml MeOH followed by 3ml of ultrapure water. Samples were loaded into the SPE columns using glass Pasteur pipettes, followed by 4.5ml H_2O/MeOH (65:35, v/v). Samples were eluted with 3.5ml H_2O/MeOH (80:20, v/v) and evaporated to almost dryness (approximately 0.5ml) under nitrogen at 60°C. Samples were transferred to gas chromatography (GC) vials for analysis. Any samples below the quarter mark on the GC vial were topped up with some H_2O/MeOH (80:20, v/v). Equipment was cleaned and acid-washed between samples to avoid cross-contamination.

All samples were analysed on an Agilent 6890N Network GC system with an Agilent Tech 5975 Mass Selective Detector with helium as the carrier gas and a Trajan (SGE) capillary GC column HT8 (25m x 0.2mm, 0.25µm film thickness). Following analysis, standards and samples were integrated and data were saved for further analysis. Reference scales and system suitability standards were used as quality control to check instrument consistency across runs, and procedural blank concentrations were used to calculate the limit of detection (LOD) of the GCMS (gas chromatography mass spectrometer). No samples in the stability study were below the LOD of 4.9ng/g. Thirteen archived samples had recorded cortisol values that were below the LOD of 3.8ng/g. For these samples, the measured values were substituted with fill-in values, calculated using robust linear regression methods (Helsel, 1990; Higgins *et al.*, 2013) and these were carried forward to subsequent analysis.

5.3.4 Growth analysis

Scales were viewed under a compound microscope interfaced with a digital camera and PC. Images were captured and analysed using ImagePro PLUS Version 6.3 image analysis software. Growth analysis was completed using ImagePro PLUS Version 7.0. Measurements were taken along a straight line transect from the focus to the scale edge. Radii were measured in mm; freshwater radius (FW radius) was measured from the focus to the point of first sea entry, post-smolt growth (PSG) from first sea entry to the end of the first winter at sea, and marine radius was measured from first sea entry to the scale edge (Figure 5.1).

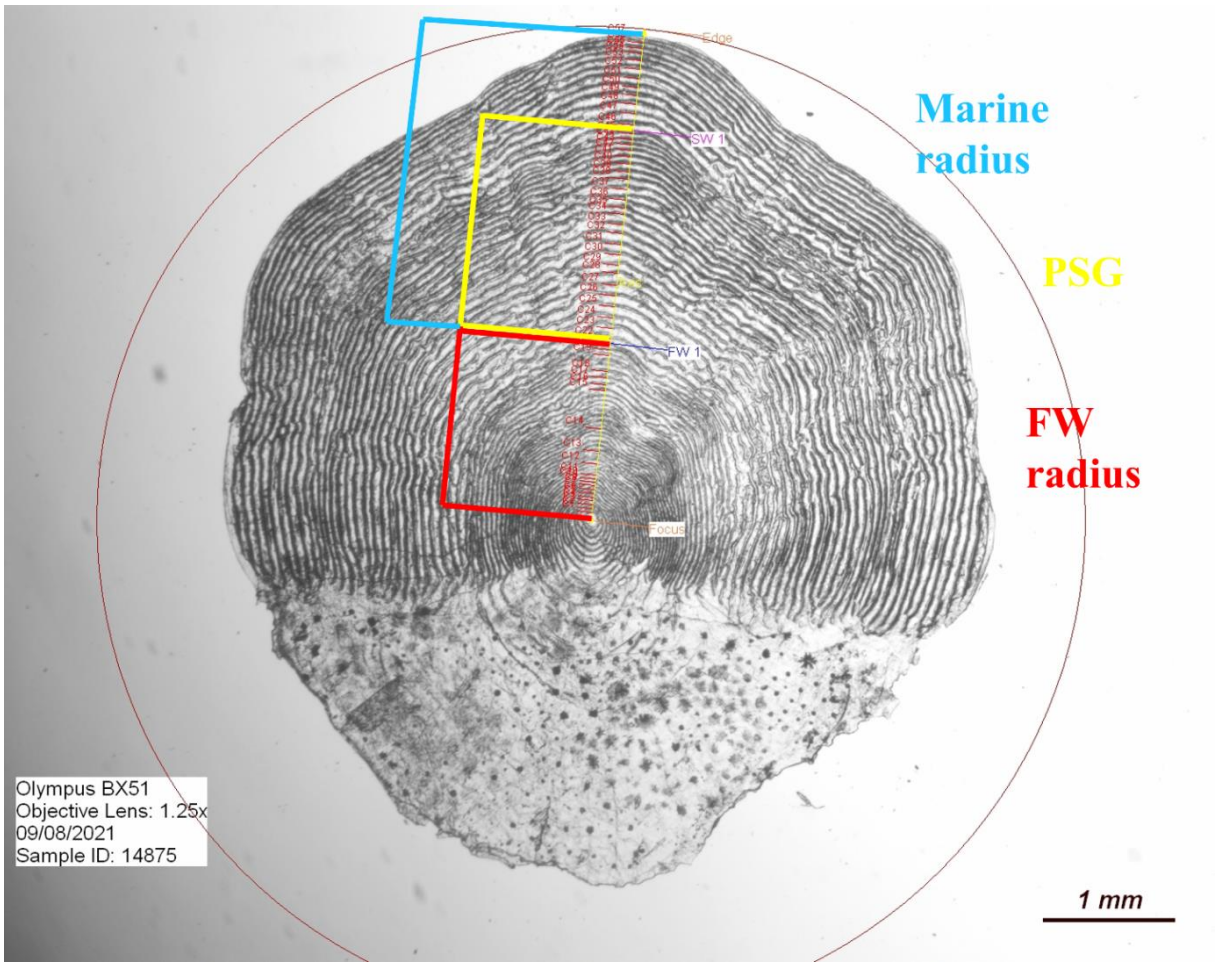

Figure 5. 1 Image of an archived scale from a one-sea-winter Atlantic salmon with a transect for growth measurements spanning from the focus to the scale edge. Three growth segments that were measured and analysed in this study are marked on the scale: freshwater radius (FW radius, red), post-smolt growth (PSG, yellow) and marine radius (blue).

5.3.5 Statistical analysis

5.3.5.1 Stability study

General linear models were used to investigate if scale cortisol concentrations varied significantly between the different treatment groups (room temperature, freezer and oven). *Treatment*, *Weeks in oven* and *Sample weight* were included as fixed effects in the models. The response variable was the cortisol concentration ($ng.g^{-1}$). The full model included all fixed effects:

Cortisol ~ Treatment + Weeks in oven + Sample weight

The full model was compared to a series of less complex models and the best fitting model was selected based on Akaike Information Criteria (AIC) values and loglikelihood tests.

5.3.5.2 Environmental data

Monthly mean SST data from the Norwegian Sea were generated from the ERSSTv5 dataset (Huang *et al.*, 2017). Winter mean SST (December of the previous year, January and February) and spring/summer mean SST (March to August) were calculated for each sample year in the cortisol dataset. Data from four regions (A1, B1, B2 and B4; areas around Ireland and the NE Atlantic) of the CPR (Continuous Plankton Recorder; https://doi.org/10.17031/1736) survey (Richardson *et al.*, 2006) were used to examine relationships between food availability and cortisol. Summer mean (June – August) was calculated for abundance data of four zooplankton groups (large copepods, small copepods, euphausiids and hyperiids), as well as the summer mean of all four groups together, for each year of the study apart from 2018 for which we did not have zooplankton abundance data. NAO Index data was obtained from the website of the Climate Analysis Section of the National Center for Atmospheric Research (NCAR, 2003). The mean value of the NAO index was calculated from March of the year prior to capture until the month before capture for each year; this period corresponded to the period during which the analysed portion of the scale was formed. Each year was then assigned an NAO category (positive or negative) based on the index value calculated.

5.3.5.3 Archived scales

Cortisol data were transformed to achieve a normal distribution by raising to the power of 0.46, as determined by the Box Cox procedure. Plots did not show any relationship between

the transformed cortisol concentration and SST or zooplankton abundance, therefore these factors were not included in any models. Linear mixed effects models were used to analyse inter-annual variability in scale cortisol and relationships with individual fish size and scale growth measurements. A series of models of varying complexity were analysed, including two-way interactions. In each analysis, the response variable was *Cortisol* $(ng.g^{-1})^{0.46}$, and *Year* was included as a random effect in all models. The explanatory variables tested for all sample years were: *NAO, Sex, Length, Marine radius, FW radius* and *PSG*. When samples from 1989, which lacked weight data, were omitted from the study, *Fish weight* was included in the analyses. The best fitting model was determined using AIC values and loglikelihood tests.

Separate linear models were used to examine the effect of *Year* on each of the size and growth measurements (*Length, FW radius, Marine radius, PSG* and *Weight*). *Length, PSG* and *Weight* data were log transformed to normalise the distribution. In each model, the size or growth measurement was the response variable and *Year* was the explanatory variable. The best fitting models were determined using AIC values and loglikelihood tests. All statistical analyses were carried out in R version 4.1.2 in R Studio version 2022.02.0+443. The ggplot2 package (Wickham, 2016) was used for data visualization and the NADA package (Lee, 2020) was used to estimate concentrations of samples below the LOD (Helsel, 1990).

5.4 Results

5.4.1 Stability study

When all samples and controls were analysed using GLMs, the best fitting model was the null model which included no fixed effects. This indicates storage conditions and simulated degradation had no significant effect on scale cortisol concentration (Figure 5.2).

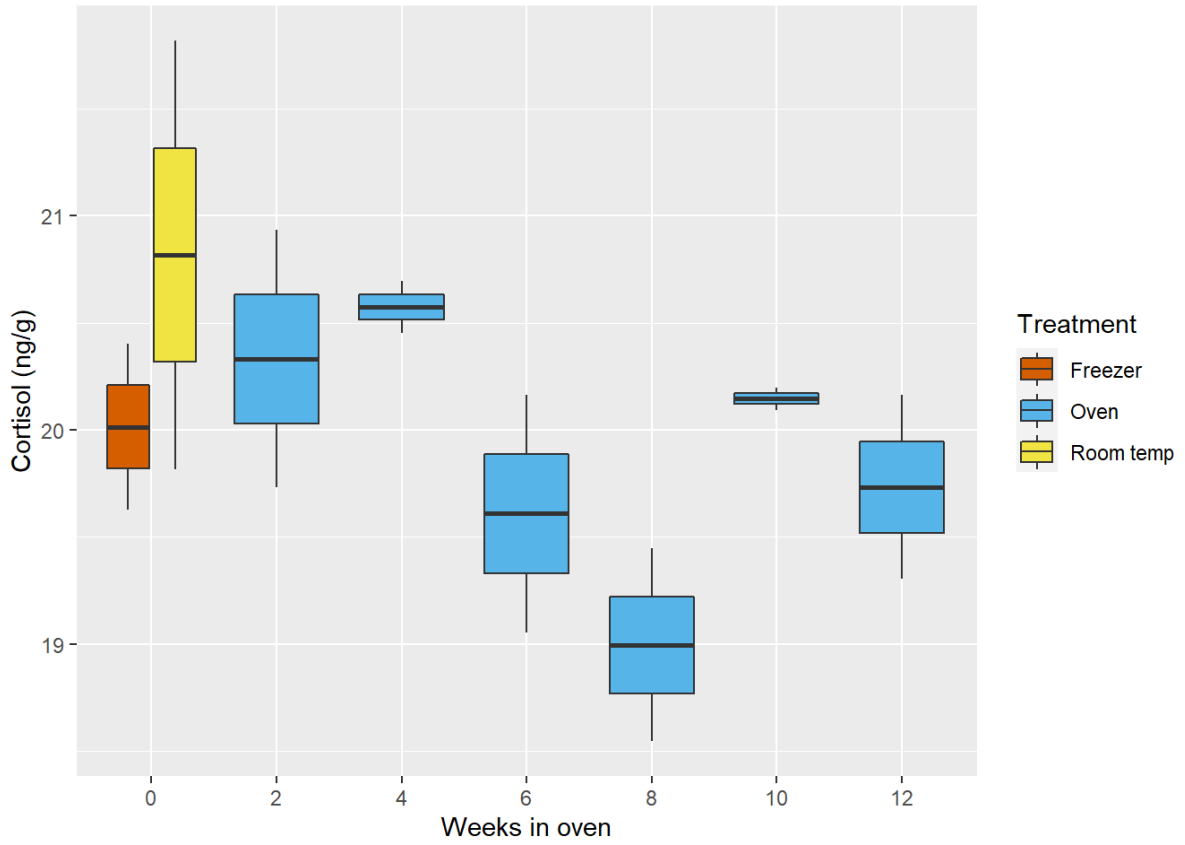

Figure 5. 2 Mean and variation of Cortisol (ng/g) in *Salmo salar* scales over a 12-week stability study. Data are coloured by treatment: samples that were kept at room temperature and in the freezer for the full 12 weeks as controls, and samples kept in the oven at 50°C for between 2 and 12 weeks to simulate degradation.

5.4.2 Archived scales

Cortisol was successfully extracted from the archived scales of 120 fish collected over 6 years. Cortisol concentrations ranged from 4.05 to 135.37 ng/g (measured cortisol, excluding estimated values for 13 samples that were below LOD). Overall, there was very little fluctuation in cortisol between years or phases of the NAO (Figure 5.3). The best fitting model for all linear mixed effects models analysing variation in cortisol from environmental and biological factors was the null model, which did not include any fixed effects. These results indicate that none of the tested factors had a significant effect on scale cortisol concentration in the Atlantic salmon in this study at a broad scale. Interannual variability was present in size and growth variables; *Year* had a significant effect on *Length* ($p = 0.0003$; Figure 5.4), *Marine radius* ($p = 0.014$; Figure 5.5), *FW radius* ($p = 7.83 \times 10^{-07}$; Figure 5.5)

and *Weight* ($p = 1.46x10^{-05}$; Figure 5.4). The best fitting model for *PSG* was the null model, indicating that *Year* did not have a significant effect on *PSG* in this study.

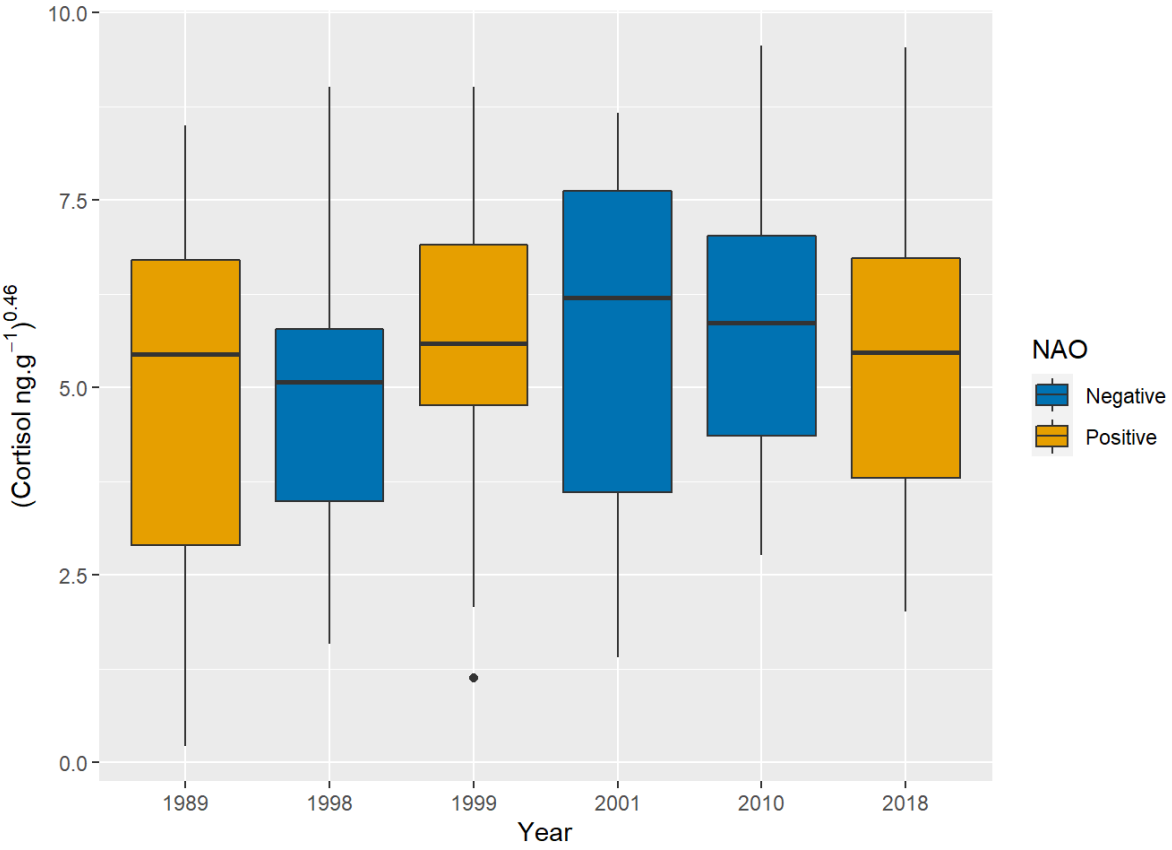

Figure 5. 3 Mean and variation of Cortisol $(ng.g^{-1})^{0.46}$ in archived scales of *Salmo salar* in 6 years over a 29-year period. Data are coloured by the phase of the NAO (North Atlantic Oscillation) that was dominant during the marine phase of the fish.

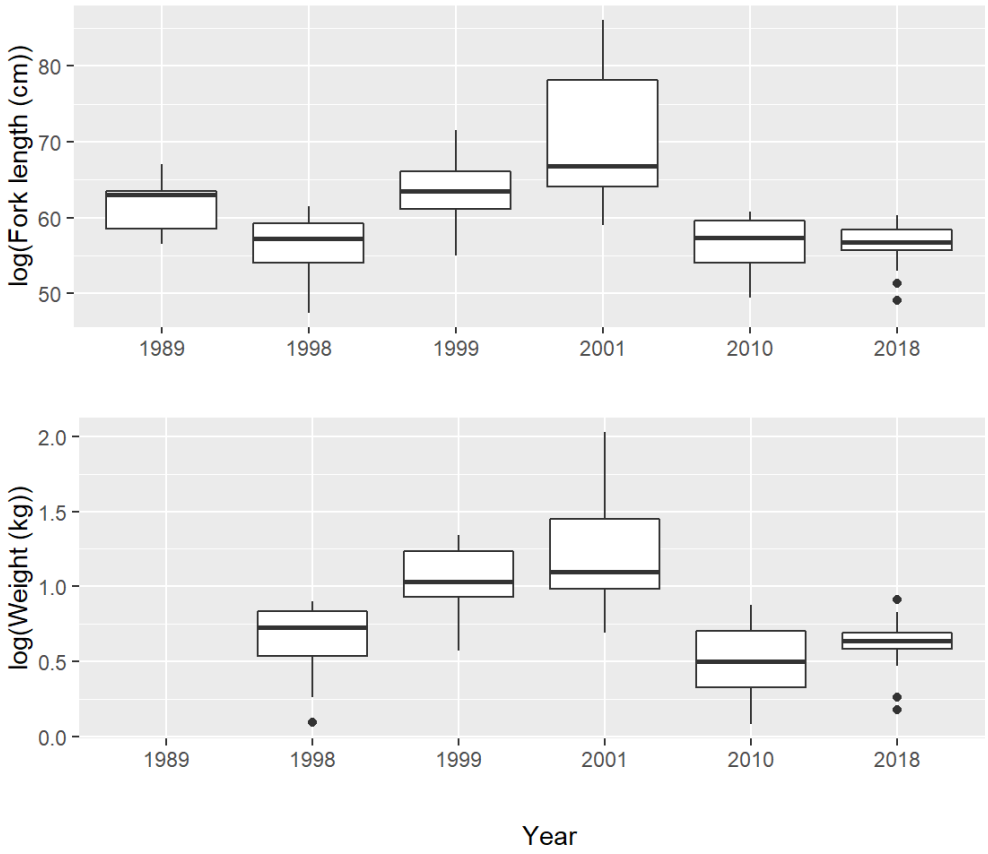

Figure 5. 4 Mean and variation in the log of fork length (cm) and log of weight (kg) of 120 *Salmo salar* from 10 sample years between 1989 and 2018. Weight data was not present for fish sampled in 1989.

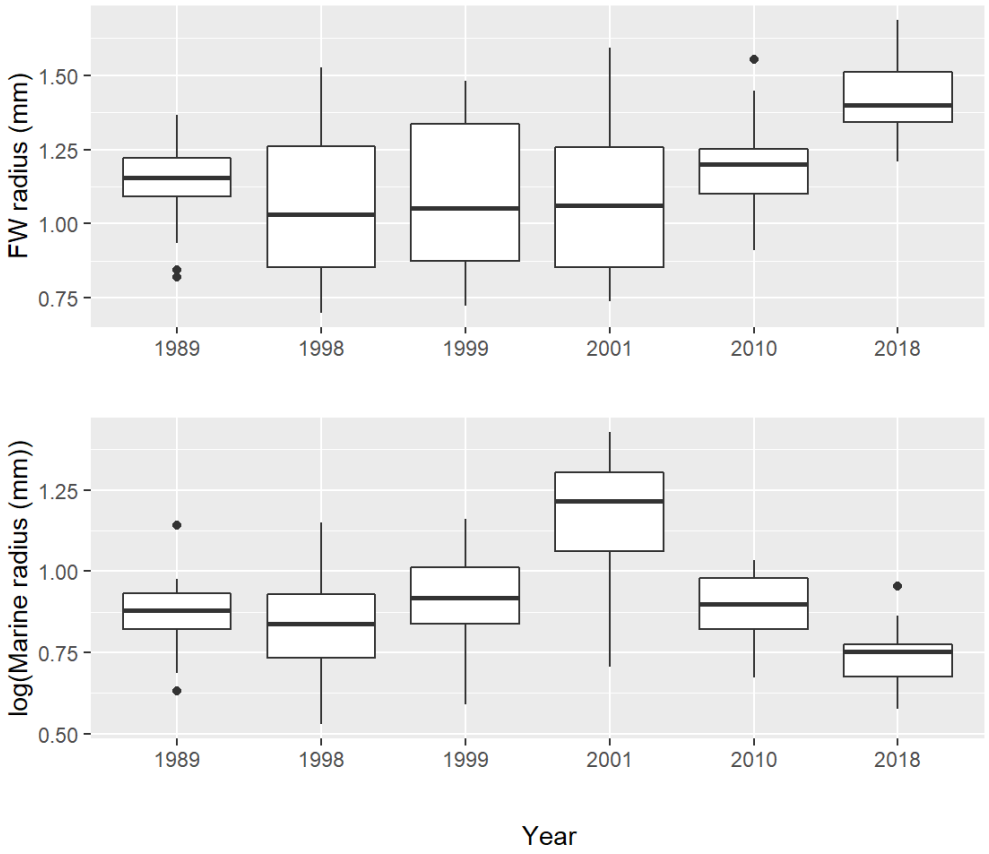

Figure 5. 5 Mean and variation in freshwater radius (FW radius) and log of marine radius of archived scales from 120 *Salmo salar*. Samples were taken from 10 years between 1989 and 2018.

5.5 Discussion

This study was the first to measure cortisol in archived Atlantic salmon scales and determined that scale cortisol did not degrade significantly over a simulated period of almost 2 years (96 weeks). This suggests that cortisol in scales that have been archived is likely to remain stable from the date of sampling, whether stored on a shelf at room temperature as is standard, or in the freezer. This is an important result, providing the opportunity to utilise the wealth of data contained in scale archives around the North Atlantic for studies on chronic stress in Atlantic salmon. This research also opens the potential for analysis of other species and alternative archived structures such as opercula (Grey *et al.*, 2009; Keaveney and Reimer, 2012). When scale cortisol concentration was analysed in archived Atlantic salmon scales from 6 years over a 29-year period, annual variability was recorded for growth and size but there was no annual signal in cortisol. Many studies have reported similar temporal changes in growth during the marine phase (Friedland *et al.*, 2000; McCarthy *et al.*, 2008). Our results indicate

that there is no clear annual signal linking cortisol and the NAO. The range and variability of cortisol concentrations detected across the 120 individual fish analysed indicates that individual variation in physiological state is driving cortisol fluctuations, rather than factors affecting all of the fish. It must also be noted that the current study was completed on returning fish, therefore did not take into account fish that did not survive the return journey to their natal river.

The scale cortisol concentrations recorded in this study were similar to the range of values recorded in experimentally reared post-smolts (5.28 - 215.93ng/g) held under varying temperature (6 °C, 10.5 °C and 15 °C) and feeding conditions (constant and interrupted food supply) (Chapter 4). This suggests that the fish in this study displayed similar levels of variability in physiological state to the experimental fish, although they likely experienced differing stressors over their life histories. The individual variability in scale cortisol may reflect a combination of local scale environmental processes, biological, behavioural and genetic effects at an individual level. Examining cortisol effects at an individual level can be difficult and patterns may differ between species based on varying life history traits (Bessa *et al.*, 2021). Social hierarchy can influence individual cortisol levels as dominant fish generally exhibit lower basal cortisol levels than subordinates (Bessa *et al.*, 2021). However, this varies by group size; in small groups, differing cortisol levels between dominant and subordinate fish are more prominent; in larger groups, dominant fish can even exhibit higher cortisol levels than subordinates, possibly because in larger groups there are more challenges of dominants by subordinates (Bessa *et al.*, 2021). Similarly, subordinate rainbow trout exhibited chronically higher basal cortisol levels than dominants, yet cortisol increases due to an acute netting stressor were significantly smaller in subordinates than dominants (Jeffrey *et al.*, 2014). The high levels of individual variability in cortisol in this study could reflect local-scale social hierarchies. Starvation has had varying effects on blood cortisol, causing increases in some studies and life stages and no effect in others (Milne *et al.*, 1979; Sumpter *et al.*, 1991). Maturing members of a population, such as the fish in this study, cease feeding on their return migration prior to entering freshwater (Kadri *et al.*, 1995). As not all fish in the population stop feeding concurrently (Kadri *et al.*, 1997), any resulting shifts in cortisol are likely to differ between individuals. Differing levels of infestation from the salmon lice (*Lepeophtheirus salmonis*, Krøyer), which is known to elevate plasma cortisol levels (Bjorn and Finstad, 1997; Poole *et al.*, 2000), could also account for individual differences in scale cortisol.

Cortisol has an important function in Atlantic salmon maturation and reproduction; the fish in this study were 1SW fish sampled on their return migration and would have been preparing to spawn and may have had increased cortisol concentrations. Plasma cortisol studies detected an increase in cortisol in maturing salmonids, with the effect more pronounced in females than in males (Donaldson and Fagerlund, 1970; Pickering and Christie, 1981; Kubokawa *et al.*, 2001; Baker and Vynne, 2014). The cortisol concentrations reported in the current study were integrated across the full marine phase, and a relationship between sex and cortisol was not detected. Cortisol also plays a role in smoltification which would likely be reflected in the marine portion of the scale. Plasma cortisol increases have been recorded in salmonids going through smoltification (Donaldson and Fagerlund, 1970; Baker and Vynne, 2014), and the hormone is believed to be involved in salinity tolerance (Epstein *et al.*, 1967). Due to the role of cortisol in osmoregulation, the hormone is also likely to fluctuate in the salmon in this study as they readjust to freshwater in preparation for their upstream migration. However, the scale cortisol measurements in this study probably reflect general stress responsiveness across the marine phase rather than more short-term cortisol fluctuations resulting from maturation, reproduction, or osmoregulation.

When multiple stressors are experienced by a fish at the same time, they can interact, making it difficult to predict or understand the effect (Petitjean *et al.*, 2019). Fish experience many different stressors during their marine migration, making it difficult to distinguish between factors that are causing cortisol fluctuations. In future analyses, excising a smaller portion of scale could allow for more detailed analysis of factors affecting cortisol over a shorter period. This study examined the full marine period of the scale so that a lower number of archived scales were required for the analysis. This limited the specificity of our investigation as Atlantic salmon can cover distances of up to 3,000km during their marine migration (Rikardsen *et al.*, 2021). This method for extracting cortisol from archived scales may give more informative results if completed in conjunction with tracking methods such as the archival tags used by Strøm *et al.* (2018) or isotopic methods to determine marine foraging location (MacKenzie *et al.*, 2011). Knowing the location of the fish during the timeframe associated with the portion of scale being analysed would enable the analysis of more factors.

Future investigation into the causes and consequences of the individual variability in cortisol could help to establish the application of scale cortisol measurements as an indicator of physiological state during the marine phase of Atlantic salmon. Genetic analyses could help

understand components of the individual stress response; both aggression levels and stress responsiveness show heritable variation in Atlantic salmon (Holm and Fernö, 1986; Fevolden *et al.*, 1991; Garcia de Leaniz *et al.*, 2007). Similarly, individual variability in cortisol could have a genetic basis, as suggested by the breeding of fish for their response to confinement stress (LeBlanc *et al.*, 2011). Further research could examine if high cortisol during the marine phase has consequences for lifetime reproductive fitness by combining scale cortisol analysis with genetic parentage analysis that includes observation of reproductive behaviour and success, similar to studies by Neff *et al.* (2015) and Milot *et al.* (2013). A sustained elevation of cortisol can have physiological costs in fish, including reduced longevity and reproductive success (McConnachie *et al.*, 2012). Increased cortisol may have an immunosuppressive effect and can make fish more susceptible to common bacterial and fungal pathogens (Pickering and Pottinger, 1989; Espelid *et al.*, 1996). Chronic exposure to higher cortisol levels may reduce the ability of the fish to cope with additional stressors (Barton *et al.*, 1987). These potential long-term effects of cortisol elevation illustrate the importance of understanding the causes and effects of cortisol increases.

This study was the first to demonstrate that cortisol can be extracted from archived scales of Atlantic salmon, and a reference of scale cortisol concentrations for salmon dating back as far as 1989 has been started. It was also determined that cortisol does not degrade significantly over a simulated two-year period. Further analysis is needed to determine the causes and consequences of the cortisol fluctuations seen in this study. Examination of scale cortisol at an individual level or in tandem with other analyses, such as genetics or location data, could have the potential to reveal temporal patterns in stress responses of Atlantic salmon to environmental changes over time.

5.6 Acknowledgements

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Chapter 6. General discussion

Much of the current research on Atlantic salmon (*Salmo salar*) is directed towards understanding factors in the marine phase that contribute to mortality. This thesis explored the use of Atlantic salmon scales as a resource to investigate this critical period of the life cycle by developing, validating, and applying methods that can be used to address knowledge gaps and enhance our understanding of the marine phase. This has resulted in the contribution of scale preparation methodology specifically tested for Atlantic salmon prior to analyses of $\delta^{13}C$ and $\delta^{15}N$ (Chapter 2), and validation of a SIA (stable isotope analysis) geolocation tool that can be used with increased confidence to track the marine feeding location of Atlantic salmon populations over time (Chapter 3). A method to extract cortisol from Atlantic salmon scales has been developed to provide a measure of chronic stress and has been used successfully to show that cortisol is influenced by rearing temperature during the post-smolt stage (Chapter 4). The stability of cortisol in scales stored for extended periods has been confirmed and the method has been applied to archived scales to examine individual and interannual variability (Chapter 5). In this section of the discussion, an overview of the advances made by each chapter is presented. This is followed by a synthesis of the ways information from scales can advance understanding of the marine phase of Atlantic salmon and some suggestions for future research.

The results of this thesis have an important place in the application of stable isotopes in current and future research. Through analysis of scales from 208 fish with a variety of life histories including wild, ranched and farmed individuals and from both marine and freshwater habitats, it was confirmed that frequently applied acidification of scales to remove inorganic carbon components of scales prior to analysis has a negligible effect on the $\delta^{13}C$ of Atlantic salmon scales. This concurred with the findings of Sinnatamby *et al.* (2007), expanding on their results with a larger sample size, wider geographical range of samples, and more conclusive statistical analyses. While the results differed from those of Perga and Gerdeaux (2003) in their analysis of whitefish, this is consistent with their suggestion that the effect of acidification may be species-dependant due to inter-specific differences in scale mineral content (Ventura and Jeppesen, 2010). This suggests that it may be necessary to examine the effect of the acidification step on $\delta^{13}C$ prior to applying stable isotope analysis of carbon to scales from a new species. A clear conclusion that acid treatment of Atlantic salmon scales is not required prior to SIA means samples do not need to be analysed in

duplicate to measure both $\delta^{13}C$ and $\delta^{15}N$; this saves both time and money. In addition, it means that fewer scales are needed for SIA. This is crucial because it enhances the feasibility of applying SIA to scale archives which hold limited numbers of valuable scales for each individual. The results from Chapter 2 were of immediate benefit when preparing archived scales for SIA in Chapter 3 of this thesis, as half the number of scales was requested from the Greenland archive than would have been necessary if acidification was required.

In Chapter 3, scale $\delta^{13}C$ and $\delta^{15}N$ were analysed from 100 fish caught at their foraging grounds in the West Greenland fishery, representing 10 years out of a 50-year period. This analysis successfully validated the isotope-based geolocation tool proposed by MacKenzie *et al.* (2011); based on correlations of the stable isotope time series with SST (sea surface temperature), the marine feeding grounds of the fish were correctly identified. This tool is of great importance as it enables the inclusion of archived scales that could not otherwise be analysed in geolocation studies owing to a lack of year-of-sampling specific baseline data. There is a prevalent and urgent need for a more comprehensive understanding about the marine phase of Atlantic salmon as low return rates to natal rivers persist. In this context, the MacKenzie *et al.* (2011) method has been applied across several populations to determine the feeding location of returning adult Atlantic salmon (Soto *et al.*, 2018; Almodóvar *et al.*, 2020). The validation of the method allows the results of these and future studies to be robustly interpreted with increased confidence and an improved understanding of its strengths and weaknesses. Additionally, data generated during this validation provide baseline measurements of $\delta^{13}C$ and $\delta^{15}N$ for Atlantic salmon caught in the West Greenland fishery.

By using SST data from across the North Atlantic Ocean from each year of the analysis, the MacKenzie *et al.* (2011) geolocation tool uses both spatial and temporal analyses to determine feeding location. A great advantage of this method is that it could facilitate analysis of fish that are too small for tracking by physical tagging methods. Many studies suggest that survival in the first year at sea is critical to marine survival (Friedland *et al.*, 1993; Friedland *et al.*, 2009; Pardo *et al.*, 2021). The geolocation tool by MacKenzie *et al.* (2011) can be used on 1SW fish, but limitations imposed by scale architecture may prevent the analysis of the post-smolt growth period due to overplating effects (Hutchinson and Trueman, 2006). Dixon *et al.* (2015) examined three growth periods from the scales of 1SW fish: the first summer, first winter, and second summer, plus the portion of scale related to the full marine period from scales from the same fish. They confirmed that overplating had a

significant effect on stable isotopes of the earlier growth years. However, while the full marine growth zone was found to be a reasonable weighted average of assimilated diet at sea, it reflected $\delta^{13}C$ and $\delta^{15}N$ values closer to the first summer at sea than the second. The authors suggested the marine zone of the scale mostly reflects the diet and environmental conditions of the post-smolt phase. While the whole marine zone is still biased due to overplating, it provides the best proxy for stable isotopes incorporated in the first summer (Dixon *et al.*, 2015), and may be useful for examining the post-smolt period using the MacKenzie *et al.* (2011) geolocation tool. The effect of overplating could be examined in more detail using scales sampled from the same fish at two points in its life cycle. For example, samples could be taken on river exit or during tagging, and then again during tag recovery, when caught at marine feeding grounds, or during upstream migration, to examine the difference in isotopic composition of corresponding portions of scale at two different time periods.

An assumption of the method is that Atlantic salmon return to the same feeding grounds over the duration of the time series being studied (MacKenzie *et al.*, 2011). Therefore, the method may not accurately determine the feeding location of fish that actively select optimum feeding locations at polar fronts, the location of which can vary interannually with temperature (Rikardsen *et al.*, 2021). An oceanic front is a boundary between distinct water masses with large temperature and salinity gradients (Bakun, 1996; Raj *et al.*, 2019). Fronts are highly productive regions where Atlantic salmon foraging behaviour has been recorded (Hedger *et al.*, 2017; Raj *et al.*, 2019; Rikardsen *et al.*, 2021). Data from Atlantic salmon kelts tagged with PSATs revealed that individuals migrated rapidly towards polar frontal areas, where parts of the North Atlantic Current were adjacent to cold polar waters (Rikardsen *et al.*, 2021). If the salmon follow the front as it moves with global warming, this interannual variability is likely to affect the accuracy of the MacKenzie *et al.* (2011) method as the fish will not be returning to the same location for every year of the time series. Magozzi *et al.* (2017) utilised a coupled ocean physics-biogeochemistry model, NEMO-MEDUSA (Madec and the NEMO Team, 2008; Yool *et al.*, 2013), to predict the spatio-temporal $\delta^{13}C$ of phytoplankton at one degree and monthly resolution over a 10-year period. The model uses relevant properties including SST, concentration of dissolved CO_2 and phytoplankton growth rates, to predict phytoplankton $\delta^{13}C$ across ocean scales (Magozzi *et al.*, 2017). Predicted phytoplankton $\delta^{13}C$ values across the North Atlantic can then be used as an isoscape for geolocation studies on Atlantic salmon. This method has decadal resolution,

but a similar model with annual resolution could be less susceptible to error if salmon feeding at oceanic fronts were to move with increasing temperatures as each year of samples could be analysed separately, and the full time series would not need to be analysed together as in the MacKenzie *et al.* (2011) method. A model-predicted method would require similar validation as was completed for the MacKenzie *et al.* (2011) geolocation tool in this thesis.

To further understand factors affecting Atlantic salmon during their life cycle, cortisol, a stress hormone, was examined in scales. Many studies have measured plasma cortisol in salmonids for a variety of purposes, including to determine the role of cortisol during fasting in rainbow trout (Pottinger *et al.*, 2003), as an indicator of migration timing in Atlantic salmon kelts (Pottinger *et al.*, 2003; Birnie-Gauvin *et al.*, 2019), and to determine the thermal ecological limit of brook trout (*Salvelinus fontinalis*) (Chadwick *et al.*, 2015). In this thesis, cortisol was measured in the scales of Atlantic salmon for the first time. Using an extraction protocol modified from Aerts *et al.* (2015), who developed the method for use on common carp scales, cortisol was extracted from the scales of experimentally reared Atlantic salmon post-smolts as a measure of chronic stress (Chapter 4). These fish had been exposed to a series of temperature and starvation stressors over a 12-week period. Cortisol was successfully extracted from the scales of 156 post-smolts and quantified using GCMS. The method was adapted from UPLC-MS/MS (ultra-performance liquid chromatography tandem mass spectrometry) used by Aerts *et al.* (2015) to suit the instrumentation available that had the precision required. The method was also optimised to analyse low weights of scale; 10mg compared to the 100mg analysed by Aerts *et al.* (2015) due to the size difference between scales of Atlantic salmon and common carp. This opens the method up to a greater range of species and life stages, and to archived samples. There was a wide range of scale cortisol concentrations observed between individuals, from 5.28 to 215.93ng/g.

The results of this study revealed that scale cortisol can be used as an indicator of temperature stress in Atlantic salmon. Increases in cortisol concentration in both temperature treatments that were outside the physiological optimum for Atlantic salmon post-smolts suggested the response was caused by the temperature treatments, not a result of normal physiological events such as smoltification or maturation. To further the use of this method, scale cortisol could be analysed in scales from fish which have experienced extreme events, such as a heatwave or drought. Many areas, including the National Index Burrishoole catchment in the west of Ireland, have extensive records of environmental data in addition to scale collections,

which could be used to investigate whether a short, sharp increase in temperature or the stress of a drought is reflected in an increase in scale cortisol. Further research is needed to determine if scale cortisol is a suitable biomarker to identify a stress response to starvation. Combining scale cortisol analysis with data on fish condition and gut content analysis could provide an alternative way to examine the effect of starvation on scale cortisol. Future research could also examine the analysis of other biochemical markers in scales such as heat shock proteins. Heat shock proteins have been detected in fin and gill tissues (Feldhaus *et al.*, 2010; Chadwick *et al.*, 2015) and are found in all cells (Li and Srivastava, 2003), therefore should be measurable in scales. Heat shock proteins may provide an alternative means of examining the effect of increasing temperatures on Atlantic salmon rather than scale cortisol which can fluctuate as a result of a wide variety of stressors.

Chapter 5 demonstrated that scale cortisol concentrations are not altered in storage during a simulated 96-week period. This is a notable result as it allows for the analysis of cortisol in archived scales with increased certainty that cortisol in the scale has not degraded over time. Scale cortisol was then successfully extracted from the archived scales of 120 fish from 6 years over a 29-year period (1989 – 2018). These scale samples came from the archive kept at the Newport Research Facility of the Marine Institute in Furnace, Co. Mayo. Similar to the post-smolt analysis, there was a wide range of cortisol measured, from 4.05 to 135.37ng/g. There were no significant interannual patterns in cortisol discernible from models or plots. A range of environmental and biological factors were tested but none were significant according to linear mixed effects models, though annual variability was recorded for size and growth factors. Individual variability seemed to be driving the cortisol fluctuations and appeared to have a large influence on scale cortisol concentration. Further research is needed to investigate the cause and effect of increased cortisol levels during the marine phase of Atlantic salmon. Combining scale cortisol analysis with tagging studies could be beneficial as scale cortisol concentrations could be linked with the environmental history of the fish, including SST experienced while at marine feeding grounds.

The results of Chapter 5 suggest that many stressors interact during the marine phase which, combined with the high level of individual variability recorded, means that determining the effect of a particular stressor on scale cortisol is currently challenging. Some individuals appear to exhibit a more pronounced stress response than others, and cortisol input from physiological changes such as smoltification and maturation further complicates

interpretation of such data. Even under controlled laboratory conditions, there was considerable individual variability in the post-smolt study (Chapter 4). It may be beneficial to examine whether the amount of cortisol accumulated by a fish in the marine phase has an effect on reproduction or survival later in life. Scale cortisol concentrations could be combined with survival data to determine whether there is a connection between cortisol concentration and survival rates between years at a population level, similar to how plasma cortisol was shown to be a predictor of migration success in kelts (Birnie-Gauvin *et al.*, 2019). As plasma cortisol has been shown to negatively affect reproduction (Campbell *et al.*, 1992; Cook *et al.*, 2011), the effect of scale cortisol concentration on reproductive success could be examined by linking scale cortisol with a genetic parentage study. By studying a population for multiple generations, it would be possible to measure the reproductive success of an individual and compare it to the scale cortisol accumulated during the marine phase. This could help determine whether the amount of cortisol accumulated by a fish affects its reproductive output.

Further research is needed to untangle the complexity of scale cortisol. If the individual variability in cortisol can be deduced, archived scales may provide a medium with which to examine how fish responded to stressors in the past, and how to predict the effect future stressors will have. The analysis of cortisol in archived scales to infer the effect of a specific factor acting on the fish in the marine phase may not be beneficial until further testing is done due to the interacting factors and the destructive nature of the analysis.

6.1 Synthesis and future directions

This thesis examined the use of scales as recorders of life history that can be used to investigate what Atlantic salmon experience in the marine phase, and how they respond. The use of scales as a research tool has changed greatly over time, from basic understanding of scale structure and classification (Goodrich, 1907; Fouda, 1979), to growth analyses (Doyle *et al.*, 1987; Treble *et al.*, 2008; Marco-Rius *et al.*, 2013; Thomas *et al.*, 2019) and determining country of origin based on surface characteristics (Lear and Sandeman, 1980). In more recent years scales have been used to reconstruct diet histories (Estrada *et al.*, 2005; Roberts *et al.*, 2022) and migration patterns (Torniainen *et al.*, 2013; Ruokonen *et al.*, 2019; Guiry *et al.*, 2020). The research in this thesis has further advanced the data inferred information that can be gleaned from contemporarily sampled and archived scales and will help enhance our understanding of the life cycle of the Atlantic salmon; marine foraging

location can be determined retrospectively from archived scales with increased confidence, and cortisol can be extracted from Atlantic salmon scales as an indicator of physiological state during the marine phase.

Methodologies are constantly evolving; there is much we still do not know about the potential of scales. Recent research shows further possibilities for analysing archived scales; Genotyping-in-Thousands by sequencing (GT-seq) was used on archived scale and fin samples from a population of kokanee (sockeye) salmon (*Oncorhynchus nerka*) that had suffered years of severe population decline, followed by a recovery (Setzke *et al.*, 2021). This analysis was able to determine that genetic diversity of the population was not lost, despite the drastic reduction in numbers over a 12-year period. This tool was successfully demonstrated on degraded archived scale samples and provides a new method with which to reconstruct genetic diversity, population size and structure from historical samples (Setzke *et al.*, 2021).

The methods described in this thesis emphasise the importance of properly maintaining archived scale collections for future analysis. Scales are a valuable, versatile tissue but, without proper care, sources of irreplaceable data could be lost. An open-source database model and archiving system has been implemented at the Marine Institute's Newport Research Station to manage the multi-decadal archive (Tray *et al.*, 2020). Termed the Irish Fish Biochronology Archive (IFBA), one of the aims of this database is to act as a roadmap for the development of infrastructures to keep samples safe and accessible, and to facilitate open access to data (Tray *et al.*, 2020). Cooperation between agencies, both nationally and internationally is important to ensure best practises are implemented to protect vast archives such as IFBA. A newly formed ICES expert group now provides a forum for such international collaboration. The Workshop on Scale, Otolith Biochronology Archives (WKBIOARC) met in 2020 with representatives from 12 jurisdictions/countries to collaborate on finding solutions for establishing, managing and maintaining biochronology archives (ICES, 2020a). Cooperation such as this is necessary to get the most out of scales through sharing expertise and connecting archives from different regions for more powerful broad-scale analyses (ICES, 2020a). Through collaboration, accessibility of archived samples and both current and future methodologies such as those described in this thesis, the information contained within scales can be used to enhance our understanding of the mysterious marine phase of the Atlantic salmon.

Appendices

Appendix 1: Details of collaborations, training and dissemination activities

During the course of my PhD research, I was involved in several collaborative, training and dissemination activities. These activities helped me to develop as a researcher, including gaining new skills and establishing important collaborations with accomplished researchers in the field. Through dissemination activities, I was able to discuss my research on both scientific and public stages, nationally and internationally. My PhD research benefited greatly from these opportunities, and from successful funding applications.

Publications

O'Toole, C., Weigum, E., Graham, C.T., White, P., Samways, K., Hayden, B. and Brophy, D. (2020). Acid treatment of Atlantic salmon (Salmo salar) scales prior to analysis has negligible effects on δ13C and δ15N isotope ratios. *J Fish Biol*, 97 (4)**,** 2020/10/01, 1285-1290.

Poster Presentations

Ecology and Evolution Ireland Conference 2019, Galway, Ireland

Oral Presentations

NoWPaS Conference 2018, Oulanka, Finland

NoWPaS Conference 2019, Rowardennan, Scotland

Ecology and Evolution Ireland Conference 2019, Galway, Ireland (flash presentation)

Seminar at the University of New Brunswick, 2019

Public seminar for Sea Synergy Marine Awareness and Activity Centre, 2020, online

Guest seminar to Prof Colin Adams' research group, 2020, online

Marine Institute's Burrishoole Research Day, 2021, online

Unlocking the Archive Workshop, Marine Institute, Co. Mayo, Ireland, 2022

ICES/PICES Early Career Scientist Conference (upcoming, 2022), Newfoundland, Canada

Courses

Survivors' Guide to Stable Isotope Analysis II, Ortigia, Sicily (7-day course, 2018)

ICES Training Course on Scientific Writing and Publishing for Marine Scientists, online (4-day course, 2021)

Linear Models and Bayesian Statistics Course, 2019, Ecology and Evolution Ireland

Laboratory Safety, 2018, Galway, Ireland

Gas Safety Training, 2018, Galway, Ireland

Collaborations

Dr Brian Hayden, Director of the Stable Isotopes in Nature Laboratory (SINLAB) in the University of New Brunswick, NB, Canada. I began a collaboration with Dr Hayden with a 10-day research visit to his lab at UNB to analyse samples for Chapter 2. We continued our collaboration when I was awarded a Dobbin Atlantic Scholarship, allowing me the opportunity to spend a month at SINLAB to complete analyses for Chapter 3.

Dr Clive Trueman, University of Southampton, England. Dr Trueman gave advice on the geolocation tool validation in Chapter 3. He also provided data and advice for future validation of a geolocation modelling tool.

Dr Anna Sturrock, University of Essex, England. Dr Sturrock is interested in utilising my cortisol method to examine cortisol in fish scales and to determine if cortisol can be detected in fish eye lenses.

Dr John Strøm, The Arctic University of Norway and the Institute of Marine Research, Norway. Dr Strøm provided scales from Norwegian Atlantic salmon with associated tagging data for use in the future validation of a geolocation modelling tool.

Research Grants

Dobbin Atlantic Scholarship, Ireland Canada University Foundation (€4,400)

Marine Institute Networking and Travel Grants

- €660
- €750

Travel and Accommodation Grant, ICES/PICES Conference (upcoming, $720 CAD)

Miscellaneous

Organising committee for NoWPaS 2019 Conference

Attended ICES expert group workshop, the Workshop on Scale, Otolith Biochronology Archives (WKBIOARC), 2020, Galway, Ireland

Supervision of student interns:

- Co-supervision of three IMBRSea MSc student interns (2 months, 2019)
- Full supervision of one College of Science undergraduate placement student (6 months, 2021)

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