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BRIEF COMMUNICATION

Examining fish scale biomineral from Atlantic salmon populations

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Abstract

Fish scale microchemistry can be used to make life-history inferences, although ecological studies examining scale composition are relatively rare. Salmon scales have an external layer of calcium phosphate hydroxyl apatite (HAP). The structure, hardness, and calcium content of this layer have been shown to vary within and between species. This variation may lead to misinterpretation of trace element profiles. This study uses backscatter scanning electron microscopy with electron dispersive spectrometry to compare scales from salmon populations and to present a more detailed analysis of scale HAP than was previously available. Our findings extend the range of salmon populations for which HAP Ca is available and confirm previous findings that the HAP Ca is relatively invariable within this species.

KEYWORDS
fish scale, microchemistry, salmon, SEM

Biomineralized tissue in fish, such as otoliths, scales, and bones, grows incrementally and incorporates trace elements from the surrounding environment into its structure (Panfili et al., 2002; Trofimova et al., 2020). Microchemical composition (e.g., trace elements) of fish biominerals can vary between groups of fish due to influences of the environment (Ryan et al., 2019), diet (Bilton & Robins, 1971), and genetics (Clarke et al., 2011). Variation in fish biomineral trace element signals can be used to distinguish life-history patterns (Thorrold et al., 1998; Vu et al., 2022). Because they are sampled non-lethally, fish scales are a particularly useful biomineral sample type in studies of vulnerable species such as Atlantic salmon (*Salmo salar* L.). From a conservation perspective, analyses involving non-invasive sampling methods, such as trace element studies using fish scales, are valuable for improved understanding of salmonid life history (Tray et al., 2022).

Trace element microchemistry of salmonid scales has previously been used to differentiate wild and farmed fish (Adey et al., 2009), track farm origin (Flem et al., 2017, 2018), and distinguish between marine and freshwater habitat use (Ryan et al., 2019). Fish scale architecture has been the subject of research in material sciences (Arola et al., 2018; Ikoma et al., 2003; Torres et al., 2008) although studies specifically examining salmonid scale structure to inform ecological microchemical analyses are rare. For example, biomineralization processes underlying the uptake and post-depositional behavior of trace elements in salmonid scales are not well understood (Ryan

et al., 2016; Tray et al., 2022), even though these processes are of critical importance for correct interpretation of chemical signals in scales.

Salmon scales have an external layer composed of calcium phosphate hydroxyl apatite (HAP). Previous work from Flem et al. (2005) has provided a mean calcium value of 37.4± 0.4% for salmon scales from the pre-smolt stage from four Norwegian salmon populations. This value was calculated using a scanning electron microscope equipped with electron dispersive spectrometry (SEM-EDS), and this study included scales from two fish farm stocks, one cultivated stock from a river and one wild local stock of salmon. However, there are limited data to support the view that calcium concentrations are near invariant within salmon HAP layers.

Some studies have indicated differences in major elemental components of fish scale HAP. Arola et al. (2018) reported differences in scale Ca/P values between species, whereas Kihara et al. (2007) found that scale calcium content can be affected by diet within juvenile red sea bream. Specifically, results from the Arola et al. (2018) study show that differences in Ca/P ratios are due to differences in apatite structure; in some species scales were harder and more calcified than in others. This prompts more investigation into the variation in the calcium content of the apatite layer in scales. This also warrants further investigation into the comparability of scale HAP between different ontogenetic and geographic/genetic groups of salmonids.

Ca is used as an internal standard in laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) studies for scale chemistry (Flem et al., 2017; Ryan et al., 2019). If Ca were found to vary significantly between samples in a trace element study, this might introduce artifacts when comparing LA-ICP-MS analyses of elements normalized to Ca. The normalized elemental concentrations would be consistent within a sample, but not between samples, meaning that apparent differences in HAP chemistry might be exaggerated, or masked, due to differences in HAP Ca concentration. If HAP calcium varies between fish from different groups, it could introduce error into the calculation of trace element ratios and would potentially mask accurate signals and inferences of life history.

This study uses backscatter electron microscope equipped with electron dispersive spectrometry to characterize structure and compare major elemental ratio values between scales taken from different salmonid populations to better inform interpretations of trace elements in salmonid scales. Here, high-resolution SEM images of salmon scales were presented, and elemental concentrations from various populations were compared. Scales were sourced from wild Canadian salmon (post-smolt, marine phase) obtained through the North Atlantic Salmon Conservation Organization's Greenland sampling programme (Sheehan et al., 2021) and Irish salmon captured at an ecological monitoring facility (Marine Institute) in Ireland (Tray et al., 2020). Scale composition was compared between three wild fish of Canadian origin, sampled on a marine feeding ground off the coast of Greenland, two wild fish captured as grilse in a river in Ireland (i.e., salmon that return to spawn after one winter at sea) and three fish reared in a hatchery in Ireland, released to the wild as smolts and captured as grilse returning to spawn. Fish life stages were confirmed using scale reading techniques (Shearer, 1992). The results improve the understanding of salmonid scale structure, which is essential to ensure their accurate usage in microchemical studies.

Scale cleaning preparation methods were adapted from Courtemanche et al. (2006). Briefly, scales were rinsed in Millipore Milli-Q water, and debris was brushed off with a small blunt acid–washed paint brush. Samples were cleaned using 5% hydrogen peroxide and rinsed thrice in Milli-Q water, and air-dried between two glass slides overnight (Adey et al., 2009). Molds were prepared using SeriForm cylindrical mounting cups (25 mm internal diameter) for cross-sectional analysis. Dried scales were placed within the mold and mounted using EpoThin cold curing Epoxy resin. The molds were placed in an oven at 30°C overnight to harden. After curing, the molds were cut cross-sectionally (down the longest axis of the scale), with an IsoMet low-speed precision saw, fitted with a 12.7 mm diamond wafering blade. After cutting, the sections were ground on a stainless-steel plate for 80 s to remove saw marks. The sections were polished on a LaboPol-21 automatic polisher, fitted with diamond grit polishing cloths (6 μm for 10 min, followed by 1 μm for 10 min). The sections were soaked in 5% hydrogen peroxide for 15 min to remove leftover organic material, air-dried, and carbon-coated (16 nm thick).

The material characterization using backscatter scanning electron microscopy equipped with electron dispersive spectrometry (BSEM-EDS) analysis was carried out in the iCRAG Lab at Trinity College Dublin in Ireland. Samples were placed in a Tescan TIGER MIRA3 VPFE-SEM (variable pressure field emission), equipped with BSE and two Oxford Instruments X-Max 150 mm^2 detectors. The instrument was calibrated using three standards: (1) Apatite-Wilberforce, Ontario, Canada, (2) Tugtupite-Greenland, and (3) Durango Fluroapatite, Mexico. Aztec software was used for quantitative point analysis (Astimex 53 calibration) and elemental mapping.

Teleost scales have two distinct compositional layers (Fouda, 1979). The lower layer, called the basal plate, is comprised of collagen. Salmon scale cross sections from this study revealed two zones in all samples: the external HAP layer and the basal plate (collagen). The collagen is partly mineralized, and stacked in lamellar sheets demonstrated clearly in Figure 1a. As the scale grows, the new/lower lamellar sheets push the existent/older collagen upwards, a phenomenon known as underplating (Hutchinson & Trueman, 2006). The calcified upper external layer is a matrix of collagen and HAP (Gil-Duran et al., 2016), which is the material measured in salmon scale microchemical trace element studies. As the fish grows, the surface area of the scale increases, and the external layer periodically forms a circular ridge, called a circulus, at the edge of the scale (Fisher & Pearcy, 2005; Tzadik et al., 2017) creating growth bands, which can be used to age the fish (Panfili et al., 2002). Images of salmonid scale circuli in cross section are shown in Figure 1a.

An elemental map of HAP calcium was created from a cross section of a scale from an Irish wild salmon (Figure 1c). Slight variation in colouration (e.g., calcium elemental composition) can be seen on the compositional map. Fish scale HAP and collagen were recognizable in the cross sections of all scales used in this study. The circuli ridges on the BSEM images were clearly identifiable. To our knowledge, this is the first study to present BSEM images of cross sections of salmonid scale circuli. Some images (most notable in the middle figure of Figure 1a) appear to have the "underplating" phenomena

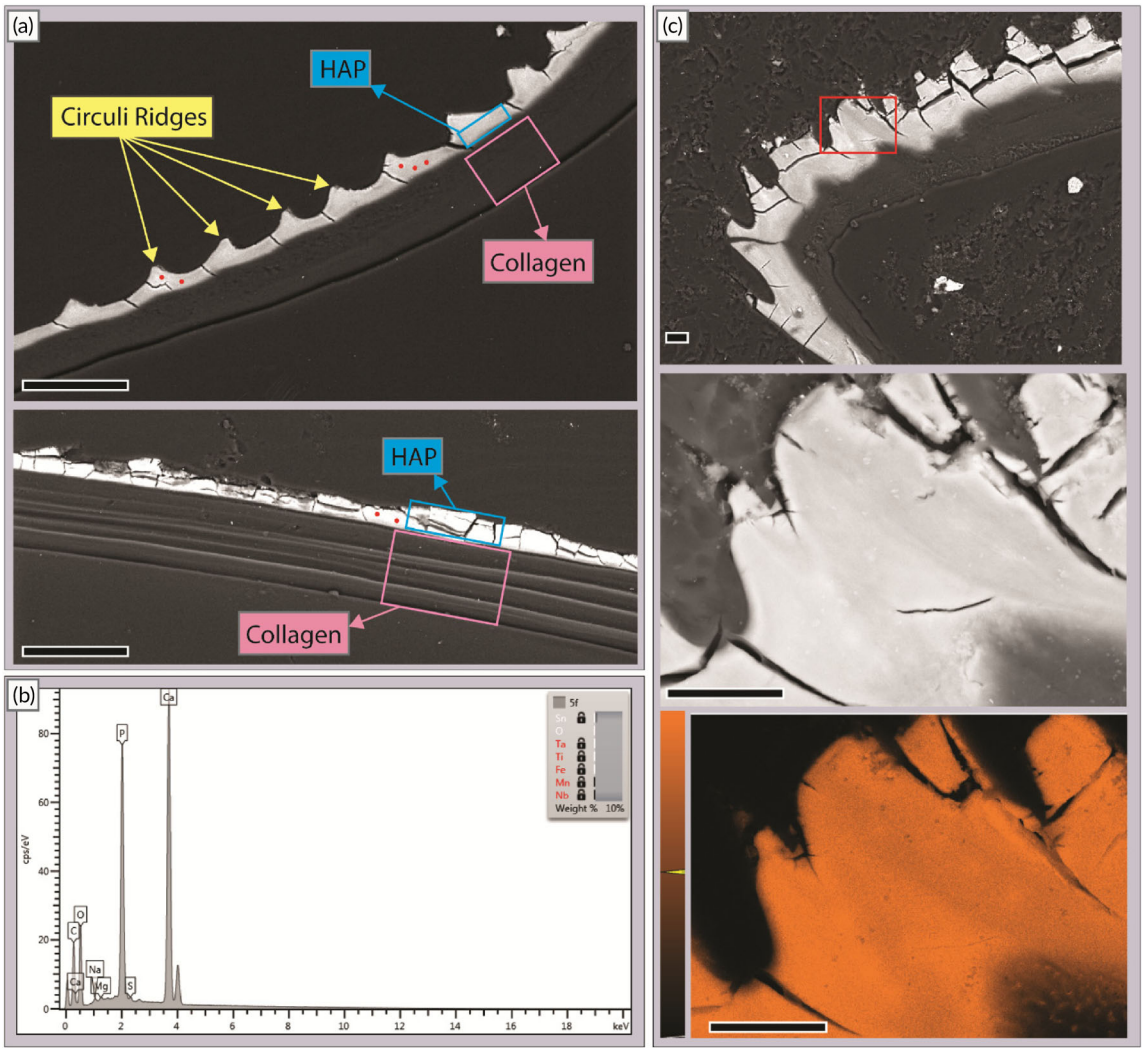

FIGURE 1 (a) Images of salmon scale cross sections; red points indicate locations for 10 electron dispersion spectrometry (EDS) measurements; settings for images were HV: KV; Mag: 679x; WD: 15 mm; scan speed: 6; circuli ridges, hydroxyl apatite, and collagen regions have been annotated in upper and lower images, respectively. Scale bar is 25 μm. (b) An example of a spectrum peak (Scale 9219) resulting from an EDS measurement. (c) Elemental maps, upper images with red box indicate the location of map on scale hydroxyl apatite (HAP), the middle image shows the backscatter scanning electron microscopy (BSEM) image of the map region, and the lower image shows calcium concentration map (scale id: X12-27); darker colouration indicates low concentration (elemental concentration scale bar, left side of bottom image). Scale bar is 25 μm.

within the collagen layer, as described by Hutchinson and Trueman (2006). Furthermore, the HAP layer was variable in thickness. This apparent change in HAP thickness could be an artifact of scale orientation with respect to the plane of cutting when preparing the sample mounts.

The positions of EDS point measurements taken to compare elemental differences between groups are indicated in Figure 1a. Figure 1b provides an example of a scale HAP spectrum profile. Ten EDS spectrum measurements were taken for each of the eight scales, giving a total of 80 measurements for the study. The Tescan TIGER MIRA3 VPFE-SEM provides EDS measurements in atomic weight %. When using EDS, and where full elemental quantification is anticipated, the total % recovered of a material typically equals 100%. Biomineral EDS data are often presented "normalized" to account for innate water content. The data have undergone a treatment to account for "missing" material (raw weight % of element ÷ total % of material recovered = normalized weight % of element). It was anticipated that salmon scale EDS totals would be <100%, and slightly variable due to dissolved OH^- groups within scale HAP. Thus, subsequent EDS elemental ratio data can provide insight into salmon scale structure, as ratios do not depend on % material recovered. To investigate inherent differences in salmon scale HAP composition, elemental ratios for major components of HAP (Ca/P and Ca/O) were used.

Data were analysed using R, version 1.1.383 (R Core Team, 2015) with packages lmerTest (Kuznetsova et al., 2017), pwr (Champely, 2020), effsize (Torchiano, 2020), and pbkrtest (Halekoh & Højsgaard, 2014). The mean and range for CaP, CaO, and %Ca are presented in Table 1 for hatchery Irish grilse (HIG), wild Canadian post-smolts (WCP), and wild Irish grilse (WIG). The HAP calcium values are comparable to previously reported values (Flem et al., 2005). Linear mixed effects (LME) models were used to analyse variability in major HAP ratios (Ca/P; Ca/O). FishID was included as a

TABLE 1 The mean and range for the groups of fish used in this study.

Fish group	HIG ($n = 30$)	WCP (n = 30)	WIG ($n = 20$)
CaO			
Mean (SD)	0.896 (0.0112)	0.887 (0.0180)	0.885 (0.0141)
Min, max	0.861, 0.911	0.855, 0.924	0.862, 0.904
CaP			
Mean (SD)	1.89 (0.0365)	1.86 (0.0579)	1.87 (0.0335)
Min, max	1.78, 1.94	1.74, 1.98	1.81, 1.92
% Ca			
Mean (SD)	37.0 (0.38) %	36.7 (0.61) %	36.6 (0.44%)
Min, max	35.8%, 37.4%	35.5%, 37.8%	35.8%, 37.2%

Note: The groups are hatchery Irish grilse (HIG), wild Canadian post-smolts (WCP), and wild Irish grilse (WIG).

random effect, to account for correlation between repeated measurements within each scale, and FishType (hatchery and wild) and FishRegion (Ireland/Canada) were included as fixed effects. To avoid inflation of type I error rates due to the small sample size, which can occur when using maximum likelihood estimation (McNeish, 2017), the significance of the fixed effects was further assessed by employing estimation with a Kenward-Roger correction (Kenward & Roger, 1997; Oberfeld & Franke, 2013). The mixed effects model results found that the fixed effects, FishRegion and FishType, and covariates did not significantly improve the fit when compared to the null model in both Ca/P and Ca/O. The result indicates that fish origin (Canada/Ireland) and fish type (hatchery/wild) do not impact the major elemental composition of the HAP layer of their scales.

A retrospective effect size determination and power analysis (two-group independent sample *t*-tests) was conducted for the fish groups used in the mixed effects models to investigate if similarity of groups was due to the low sample size (i.e., type II error) or truly reflected a lack of variation in scale composition ratios. Effect size was measured using the Hedges g estimate, which is appropriate when dealing with sample sizes less than 20 (Grissom & Kim, 2005). The parameters set to detect type II error were: desired power level = 0.8; alpha level = 0.05; effect size = Hedges g value. The Hedges g estimate confirmed that for the comparisons of elemental ratios between scales from Canadian and Irish fish, the effect sizes were negligible for both Ca/P and Ca/O (0.08 and −0.11, respectively). For the hatchery and wild scales the effect sizes for both Ca/P and Ca/O were −0.91 and −0.64, respectively. The power analysis suggests that with a larger sample size (20 and 39) this study may have detected a difference in CaO and CaP, respectively, between hatchery and wild fish.

Overall, the results indicate that the composition of scale HAP does not vary significantly between fish of different geographic origins, captured at different points during their life history. Although no difference was detected in composition between scales from wild and hatchery fish, further analysis using a larger sample size would be needed to confirm this result. To our knowledge this study represents the most comprehensive examination of Ca variations in salmon scales to date. BSEM-EDS was used to present high-resolution images and an elemental map of scale HAP and internal microstructure. This study presents a more detailed analysis of scale HAP composition than was previously available by analysing scales from different types of fish and fish from different regions. Our findings affirm the use of microchemical analyses of trace elements and isotope ratios in salmon scales from different geographical regions for ecological life-history research.

AUTHOR CONTRIBUTIONS

Elizabeth Tray: Conceptualization, methodology, formal analysis, investigation, writing - original draft, writing - review & editing, visualization. **Deirdre Brophy**: Conceptualization, methodology, formal analysis, investigation, writing - review & editing, visualization, supervision, funding acquisition, project administration, validation, resources. **Elvira de Eyto**: Methodology, formal analysis, writing - review & editing, visualization. **Niall Ó'Maoileidigh**: Resources, funding acquisition, project administration, supervision. **Tim Sheehan**: Resources, writing - review & editing. **Ian Bradbury**: Resources. **Quentin G. Crowley**: Conceptualization, methodology, resources, formal analysis, investigation, writing - review & editing, visualization, funding acquisition.

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