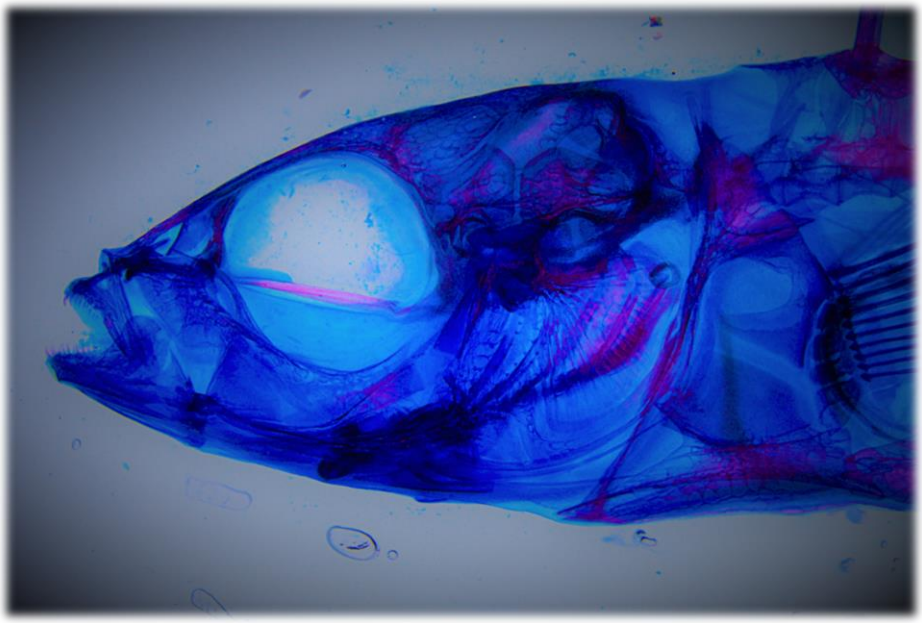
# The Ionomics of Three-Spined Stickleback on North Uist: Elemental Investigation of an Ecological Chemical Mosaic

## PhD thesis



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# Abstract

How does the abiotic chemistry of the environment affect biotic evolution? Organisms require approximately 25 elements to construct themselves, and the occurrence of these varies spatiotemporally in the environment, yet we know little about the elemental composition of animals beyond that of the commonest elements (C, N, P, K). Ionomics is the emerging study of the elemental sum of an organism's ion makeup (i.e. the ionome), which focuses on the potential importance, relevance, and accessibility of all important compositional elements. These have become increasingly accessible through Inductively Coupled Plasma Mass Spectroscopy. The chemical and pH variation across the island of North Uist in the Scottish Western Isles, combined with the three-spined stickleback populations that inhabit the various lochs provides a unique study system with which to better understand chemical consequences for evolution and inform on how rapid changes to water chemistry and pH could affect fish populations. Multi-element analysis of fish and environmental components was combined with a common garden experiment, reciprocal water rearing and a developmental approach of staining for bone ossification, to investigate population ionomic variation, genotype vs. phenotype, adaptation and the consequences of water chemistry. Results showed that fish ionomics were population specific and directly affected by both water chemistry and nutritional availability. Invertebrate populations of prey items did not exhibit the same trends in site specific ionomics, though taxa specific differences were found and fish stomach contents indicated potential selective feeding behaviours. Ionomics exhibits plasticity under common chemical conditions, but has a genetic component and fish are still readily identifiable to their population of origin on an elemental level. Water rearing chemistry directly impacted hatching rates and hatching mortality as well as fish ionomics, growth and development. These results demonstrate not only the application of ionomics and multi-element analysis to questions of ecology, evolution and development, but

also how such approaches can be integrated with more well established techniques and extremely suitable study systems to gain a better understanding of how biotic and abiotic components of environments interact to influence fish development, adaptation and evolution.

# Acknowledgements

I am extremely fortunate to have so many people that I wish to thank. Firstly, I would like to express my gratitude to my supervisors Professor Andrew MacColl and Dr Scott Young for everything I have learnt from them. Andrew's supervision has been essential in aiding me to temper my enthusiasm, focus my efforts and he has been supportive of me throughout my time at Nottingham, particularly during the strange and unpredictable circumstances we all found ourselves in during Covid-19. I will forever be grateful for his guidance and the potential he saw in me (even when he hid the cake from me during fieldwork. I may forgive, but I won't forget). I may always be the kid in the sweetshop when it comes to research and all the wonders of the natural world, but I feel I have at least learned to be more sensible with my pocket money. Much like Andrew, Scott's enthusiasm for ideas matches my own and he provided invaluable perspectives and technical support to this PhD. I have greatly enjoyed discussions with them both as this project has developed and I look forward to further exploring ideas with them in the future. It has been such exciting and rewarding research to work on, and I am lucky to have been chosen for it.

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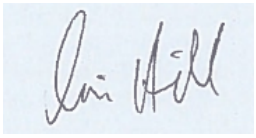
Thanks to my siblings James and Sarah for being amazing people, and to my friends and family for all their love and support throughout my life.

Finally, I have not the words to properly thank my parents Ian and Tina, who instilled and nurtured a passion for the natural world and science in me from as far back as I can remember. I would never have made it this far without their love and never ending support. Life has been good to me.

# Declaration

Unless otherwise acknowledged, the work presented in this thesis is original. No part has been submitted for another degree at The University of Nottingham or elsewhere. Any views expressed in the dissertation are those of the author.

Signed: Iain Hill 

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# Contents

# Chapter 1. General Introduction

## 1.1 Summary

For this thesis, I conducted a detailed study of the elemental composition (ionomics) of three-spined stickleback (*Gasterosteus aculeatus*) (hereafter stickleback). This study involved examination of eight freshwater sites on the island of North Uist, Outer Hebrides, and the stickleback populations that inhabit them. Samples of fish and multiple ecological components were analysed to assess sources of elemental variation. Selected populations were reared in common garden experiments to compare differences in elemental assimilation among populations and determine if there is a genetic component to the ionomics of examined populations. These same populations were then used for reciprocal water rearing experiments to assess the effects of sudden shifts in water chemistry of rearing environments.

## 1.2 Origins of Ionomics

In the full scope of evolutionary ecology, chemistry can sometimes be overlooked, especially on the elemental level, but is a fundamental component in the development of all life (Lane 2015). The composition of animals, including fish, in terms of genes, proteins and metabolites (Andersson and Georges, 2004; Cossins *et al.,* 2005; Crollius *et al.,* 2005) has been a topic of focus in recent history, with increasing progress in more recent years (Eenennaam *et al.,* 2014; Roesti *et al.,* 2015; Stockwell *et al.,* 2016; Salzburger 2018). However, a great deal less is known about the fundamental elemental components of animals and how elements are incorporated actively through behaviour and nutrition (Behmer *et al.,* 2001; Fortes *et al.,* 2016) and through functions such as homeostasis (Bury *et al.,* 2003; Wilson *et al.,* 2003; Dolomatov *et al.,* 2012). The elemental

composition of environments is especially relevant to freshwater fish as they are limited in their ability to change the nature of the chemical conditions they encounter relative to terrestrial species.

Ecological stoichiometry ('ES'), using the principles of chemical stoichiometry, was developed as a field to further understand interactions of the chemical component of ecology (Elser *et al.,* 1996; Elser and Urabe, 1999; Leroux, 2018). A now more established field (Hessen *et al.,* 2013), ES equation balances the raw currency of nutritionally relevant elements in the context of environmental energy and biomass as they transition through producers, consumers and nutrient recycling. Additional focus has been applied to specific applications, particularly in the field of evolutionary ecology, biogeochemical ecology and nutrient cycling (Hendrixson *et al.,* 2007; Leroux *et al.,* 2012; Collins *et al.,* 2017; Gonzalez *et al.,* 2018).

In contrast to ES, which focuses on a small number of 'crucial' elements, Ionomics is an emerging discipline that focuses on the potential importance, relevance and accessibility of the more comprehensive ~25 elements. These elements form the building blocks of life (Fig. 1.1), and are present in both abiotic and biotic arrangements (Silva and Williams, 2001; Kaspari and Powers, 2016). A more inclusive focus on multiple elements is particularly relevant when considering the multiple trace elements, micronutrients and metals that are vital to a range of biological processes (Bury *et al.,* 2003; Eide, 2006; Festa and Thiele, 2013; Kambe *et al.,* 2015; Khaliq *et al.,* 2018).

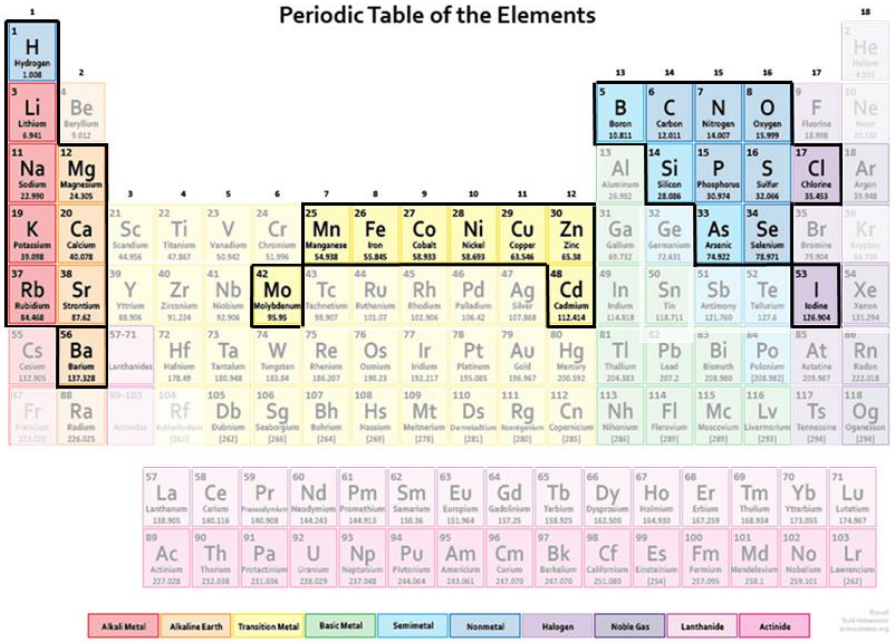



Figure 1.1 Elements necessary for and/or associated with organism composition and function, selected for analysis (highlighted).

The genome supports and perpetuates living systems through the integrated actions of the transcriptome, proteome, metabolome and ionome with these four biochemical processes making up the functional biochemical elements of genomics (Salt, 2004). Transcriptomes, proteomes and metabolomes represent the sum of expressed genes, proteins and metabolites of an organism, respectively. The ionome is the elemental sum of an organism and is involved in multiple fundamental biological processes including assimilation, anabolism and homeostasis (Miwa *et al*., 2009; Puig *et al.,* 2009). To date the majority of work on ionomics has been on plants (Salt, 2008; Baxter, 2009; Huang and Salt, 2009), and yeast (Eide *et al*., 2005; Yu *et al.,* 2012), with only a relatively small amount of work on complex animals (Yoshida *et al.,* 2014; Ma *et al.,* 2015). The latter have often been in the context of exploratory, general studies, which refine methodology, but often lack relevance in an environmental context. This is currently expanding in scope and an argument can be made to consider the ionome as a

combination of phenotypes rather than simply an elemental accumulation (Baxter, 2015).

## 1.3 Study system and stickleback

The island of North Uist lies in the Outer Hebrides off the west coast of Scotland. Three-spined stickleback populated the island since the last ice age, and have undergone an adaptive radiation, due in part to differences in chemical composition of the water bodies that formed as sea levels first dropped and then rose again (Magalhaes *et al*. 2016; Dean *et al*., 2019). North Uist exhibits calcium rich sand to the west, while the east is rich in peat which results in acid conditions. The combination of this unique mosaic of chemical variation (Waterston et al. 1979; MacColl *et al*., 2013; Haenel *et al*. 2019) coupled with the associated range of variation in both water bodies and stickleback makes for an ideal study species in an unrivalled study system to explore questions about ionomic variation, nutrient co-limitation and chemical consequences to biotic evolution.

Stickleback have long been recognised as a model organism to explore questions of evolution and ecology, due to their geographic range, adaptability and phenotypic plasticity. Post glacial systems of freshwater fish are particularly well suited to investigating adaptive divergence (Skulason *et al.,* 2019). Marine colonisation by stickleback of freshwater habitats has occurred many times independently and the genetic characteristics of this adaptation to chemically and ecologically varying conditions is well documented (Jones *et al*., 2012). Morphological changes in bony amour composition of stickleback, associated with adaptive responses to freshwater environments, are also well recognised (Magalhaes *et al*. 2016). However, variance in ionomics through the application of multi-element analysis is a relatively recent development in research approaches.

Rudman *et al*., (2019) conducted experiments to compare the ionomics of populations of high-plated marine and polymorphic plated freshwater stickleback, both wild-caught and lab reared, under conditions of saltwater

and freshwater. Multi-element analysis revealed low plated and high plated freshwater morphs of the same genetic population, had distinct ionomic differences when lab reared, mostly influenced by concentrations of Se, Zn, V and K. Comparisons of wild-caught marine and freshwater fish exhibited ionomic distinction associated with differences in Sr, As, Ba, Li and Mn. Lab reared marine and freshwater fish differed in their elemental composition most strongly in concentrations of B, Cd, Ba and Mn when reared under saltwater conditions and Na, K, Ba and Mn when reared in freshwater. These non-bone elemental changes demonstrate ionomic adjustments play a role in rapid adaptation of nutrient assimilation and ion regulation to differences in ecological chemistry, with particularly strong divergence between marine and freshwater stickleback ionomics recorded regardless of rearing water salinity. Such examples of ionomic differences and plasticity in response to environmental chemistry are useful in terms of investigating genotype-environment interactions of elemental composition and assimilation, but are yet to be examined in the context of a range of freshwater populations that inhabit varying chemical conditions.

## 1.4 Application

In order to gain a greater understanding of how abiotic and biotic processes interact on an ionomic level, a wider focus on environmental components is required with a more focused and refined application of sampling and analysis. Some work has been carried out in the context of comparing the elemental composition among species from different locations (Prater *et al.,* 2019) and within species from different conditions (Fallah *et al.,* 2011; Rudman *et al*., 2019). Other work has compared environments of generally similar geographical qualities (Asakura *et al*., 2014). Presently, elemental values of vertebrates and environments have yet to be combined in a single study to assess how the ionomic values of a vertebrate consumer relate to the chemical composition of their environment and particularly to examine how this varies between sites. To further the investigation of potential mechanisms, a greater degree of focus should also be given to the available

food sources in the environment (suspended particles, zooplankton and invertebrates), and the chemical nature of the environmental aspects themselves (water content and sediment composition). How consumers gain access to, retain and balance the nutrients they require from ecosystems with varying chemical compositions is particularly interesting as some trace elements can prove to be highly toxic when present in biological systems in quantities that exceed requirement, just as the lack of them can also prove fatal (Fraga, 2005). Unlike plants, which are generally fixed in their physical situation and therefore limited by their direct contact with the immediate environment, animals generally have more opportunity to seek out nutrition through a wider range of available sources.

## 1.5 This project

This research primarily related the elemental composition of the study species to its site of origin, seeking to firstly, address the hypothesis that **fish populations will exhibit site specific ionomics in response to the chemistry of their home environment**. To test the hypothesis that **population specific ionomics will have a heritable aspect**, common garden experiments were conducted (see Chapter 2). Secondly, to determine **how the chemistry of the environmental components relate to the ionomics of fish across sites**, extensive abiotic and biotic environmental sampling was conducted across multiple sites on North Uist. Analysis of these samples was also used to test the hypothesis that **invertebrate (prey item) populations will also exhibit site specific ionomics**, though less distinct than fish populations, which would potentially have a cumulative effect in elemental concentrations as they travelled up the trophic levels through consumption (see Chapter 3). Finally, I examined the interactions and consequences of water chemistry by rearing fish in reciprocal water conditions in field-based experiments to test the hypotheses that **rearing water chemistry will significantly affect fish ionomics, and development, particularly ossification** and that **effects will be more pronounced for alkaline populations under acidic water conditions**. This

stage of investigation helped to draw out not only any differences in homeostatic function between fish populations, but also informed how radical shifts in water chemistry can influence development, survivability and thus potentially evolution (Chapter 4).The multiple and integrative approach of investigation detailed above was carried out to gain greater insights into freshwater evolutionary ecology, adaptation and how chemical changes in the environment may manifest in regards to sustainability and evolution of the species they impact.

## 1.6 Elemental selection for analysis

Of the 30 or so elements accessible through Inductively Coupled Mass Spectrometry (ICPMS), 20 were selected to focus on in analysis at the beginning of this research in 2018 and are as follows: Na, Mg, P, S, K, Ca, Li, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, Ba (see Appendix; Table A.1). These elements were selected based on the available literature reviewed at the time for their involvement and association with, whole animal elemental composition. From an initial starting point (Leroux, 2018), Kaspari and Powers (2016) was particularly informative for an initial list of: Na, Mg, P, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, which was subsequentially cross referenced for known biological roles. Ma *et al*., (2015) informed the inclusion or further elements, with Li, As and Cd included due to their ready uptake in animals and while recognised as toxic in excess, some research suggests Li has a potential role as an essential nutrient in small doses (Shahzad et al., 2016; Jakobsson et al., 2017). Rb was added due to its association with K and potential role in stimulating metabolism (Nyholm and Tyler, 2000; Anke *et al*., 2005). Sr and Ba were included due to their association with Ca, an element of particular interest in regards to bone morphology of stickleback on North Uist (Giles, 1983).

# Chapter 2. Investigating ionomic variation of three-spined stickleback populations on North Uist

## 2.1 Abstract

How does the chemical composition of abiotic environments influence biotic evolution? Organisms require approximately 25 elements to construct themselves, and the occurrence of these varies spatiotemporally in the environment, yet we know little about the elemental composition of animals beyond that of the most common elements (C, N, P and K). Elemental variation at the individual or population level could have significant consequences for organismal adaptation and environmental interactions. The adaptive radiation of three-spined stickleback inhabiting lochs on the island of North Uist, Scotland provides an ideal study system to explore the effects of elemental variation at both the individual and population level. The multitude of water bodies on the island were formed, inhabited by three-spined stickleback and isolated as the last ice age receded, and therefore demonstrate large differences in chemical composition. In order to examine both environmental and organismal elemental variation, samples of water and three-spined sticklebacks were collected from different lochs. Elemental compositions of fish were then compared, so as to determine site-specific variations in ionomics. The whole fish ionomics relate directly to the pH of water at individual sites; this was further examined through 'common garden' rearing experiments that indicate a genetic- and population-specific component to elemental acquisition, assimilation, and composition of fish analysed using Inductively Coupled Plasma Mass Spectrometry (ICPMS).

## 2.2 Introduction

Organisms must adapt to environments that are constantly in flux in order to take advantage of ecological niches and in direct response to environmental pressures imposed by their habitat by both biotic and abiotic factors (Sultan, 2015). Research on evolutionary adaptations in the past has focused on morphological variation manifested as adaptive radiations in response to the availability of niches and environmental resources, e.g. Darwin's finches (Grant and Grant, 2002). However, the chemistry of environments and organism physiology also play a crucial role in survival (Lane, 2015) and recent developments have yielded insights into how species may adapt through more subtle mechanisms. While progress in this field is ongoing, we currently know far more about the genome than we do about the precise elemental composition of all but a handful of organisms. Gaining a mechanistic understanding of how animals gather, balance and maintain the elemental resources that are vital to survival, is crucial to attaining further insights of how species adapt to chemical variations in the environment.

Ecological stoichiometry has provided valuable insights into how elements transition through biological systems, but only focuses on a relatively small number of vital elements (C, N, P and K) (Hessen *et al*., 2013). Ionomics is the emerging study of the elemental sum of an organism's ionic composition (i.e. the ionome), which focuses on the potential importance, relevance and accessibility of ~25 elements. Developments in analytical techniques such as Inductively Coupled Plasma Mass Spectrometry (ICPMS) now enable greater exploration of the chemical interactions between organisms and their environments (Salt *et al*., 2008). These elements form the building blocks of life and are present in both the abiotic and biotic components of ecosystems (Kaspari and Powers, 2016). An inclusive focus on multiple elements is particularly relevant when considering trace elements and micronutrients that are vital to a range of biological processes (Bury *et al*., 2003). To date, the majority of studies on ionomics has been on plants (Salt et al., 2008; Salt, 2004; Huang and Salt, 2016) and yeast (Eide *et al*., 2005; Yu *et al*., 2012),

with little research on complex animals (Yoshida *et al*., 2014; Ma *et al*., 2015). Ionomic research such as this now suggests considering the ionome as the result of a combination of phenotypes rather than simply an arbitrary elemental accumulation (Baxter, 2015). How consumers in ecosystems with varying chemical compositions gain access to, retain and balance the nutrients they require is particularly interesting and relevant for the purposes of this study. For example, balance is imperative when a high concentration of some trace elements can result in toxicity, while too low a concentration could be fatal (Rajkowska, 2015).

Three-spined stickleback (Gasterosteus aculeatus), hereafter referred to as 'stickleback', make non-bone growth related changes to their ionome when transitioning from marine to freshwater environments (Rudman *et al*. 2019). Fish in freshwater have to further adapt to individual chemical environments in which they can be isolated. Since the last ice age, stickleback populations have colonised the island of North Uist in the Scottish Western Isles and adapted to environments with diverse aquatic chemistries (Magalhaes *et al*., 2016). They now occupy water that ranges from seawater to freshwater; the latter may vary from naturally eutrophic-alkaline to dystrophic-acidic. The combination of this unique mosaic of chemical variation (Waterston et al., 1979; Haenel *et al*., 2019), suggests that stickleback is an ideal study species in an unrivalled study system to explore questions about ionomic variation, nutrient co-limitation and chemical consequences for biotic evolution.

Much of the research on animal ionomics to date have been exploratory, general studies (Ma *et al*., 2015) which have sought to refine methodology. Some studies have compared the elemental composition of species from different locations (Prater *et al*., 2019), or within species from different conditions (Fallah *et al*., 2011), and considered the elemental content of environments (Asakura *et al*., 2014). Currently, these approaches are yet to be combined to understand how animal ionomics interacts with the multiple facets of the environments that surround and sustain them. This chapter

aims to address the following questions: 1) What is the extent of individual variation within populations? 2) What is the extent of variation among populations and to what extent is this variation maintained under common garden conditions?

## 2.3 Methods

### 2.3.1 Study Site and Species

The island of North Uist lies in the Western Isles off the west coast of Scotland. The surface geology of North Uist exhibits calcium rich sand (‘machair’) to the west, while the east comprises peat over ancient metamorphic rock, giving rise to acidic conditions (Waterston et al., 1979). These geological circumstances result in chemical variation across multiple lochs. This thesis focuses on ten such lochs (see Appendix; Table A.2), designated: OBSE, REIV, SAND, HOST, DUIN, GROG, TORM, CHRU, SCAD and BHAR (Fig. 2.1). These lochs were chosen to include sites that are isolated and disconnected from other water bodies as well as those that are linked directly to the sea. Using information from an initial environmental investigation, REIV, TORM, and BHAR were chosen for more detailed investigation under common garden conditions due to a number of relevant factors. These three sites are freshwater lochs, relatively isolated, and contain populations of fish that live in a range of chemical conditions and water pH values. Environmental surveys have indicated that these three sites have a range of nutritional availability, in terms of prey numbers and items. The three sites also represent varying productivity levels with REIV being eutrophic/mesotrophic, while TORM and BHAR are oligotrophic (Singkam, 2016).

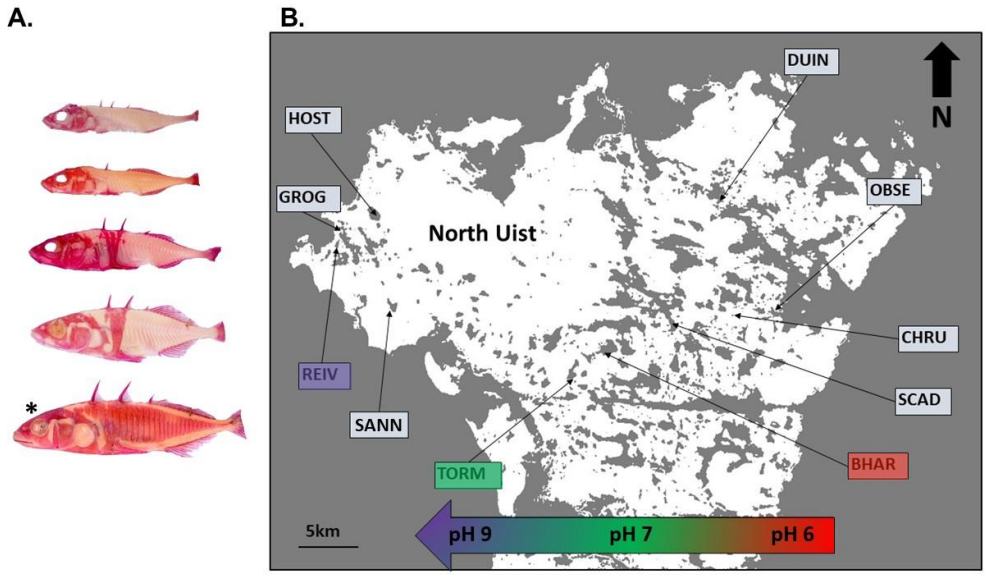



Figure 2.1. Examples of morphological (A) differences between marine sticklebacks (*) and resident stickleback (top four fish). Map of North Uist and the focal study sites (B). Coloured sites indicate those selected for common garden experiments with differing pH conditions. (Base map courtesy of L.L. Dean).

### 2.3.2 Wild Fish Sampling

Fish were captured using unbaited minnow traps (Gee traps, Dynamic Aqua, Vancouver, Canada), which were left for approximately 24 h. Traps were set along 50-150 m stretches of the shoreline in a depth of water approximately 0.4-3 m. Adult fish, varying in size, sex, and breeding condition were collected. Fish were humanely killed by anaesthetic overdose of MS222, after which they were rinsed with DI H2O, numbered using waterproof paper tags, weighed and photographed. Samples (fish) were then wrapped in cling film and frozen until further processing could take place. A portion of individuals in breeding condition were retained to produce in-vitro crosses.

### 2.3.3 Common Garden Experiment

In order to investigate the effects of diet, assimilation, and home environment on the ionome of the selected stickleback populations, multiple in-vitro crosses were made, from 11/05/19 to 13/05/19, from fish sampled from each loch. Males in breeding condition, indicated by nuptial colouration, and gravid females were identified and selected from each of the three lochs. For each cross a male and female from the same loch were selected at random. Fish were euthanised with a lethal dose of MS222, and eggs stripped from the female were fertilised with sperm from the testes extracted from the male. Eggs were initially kept separated by loch of origin and family in temporary storage tanks, which were filled with softened tap water that had been treated with 2-3 drops of methylene blue, and kept well aerated using air stones. Fertilised eggs were transported back to the aquarium in the University of Nottingham Life Science Building on 18/05/19 and hatched within the period 24/05/19 - 27/05/19, in separate tanks (Fig. 2.2).

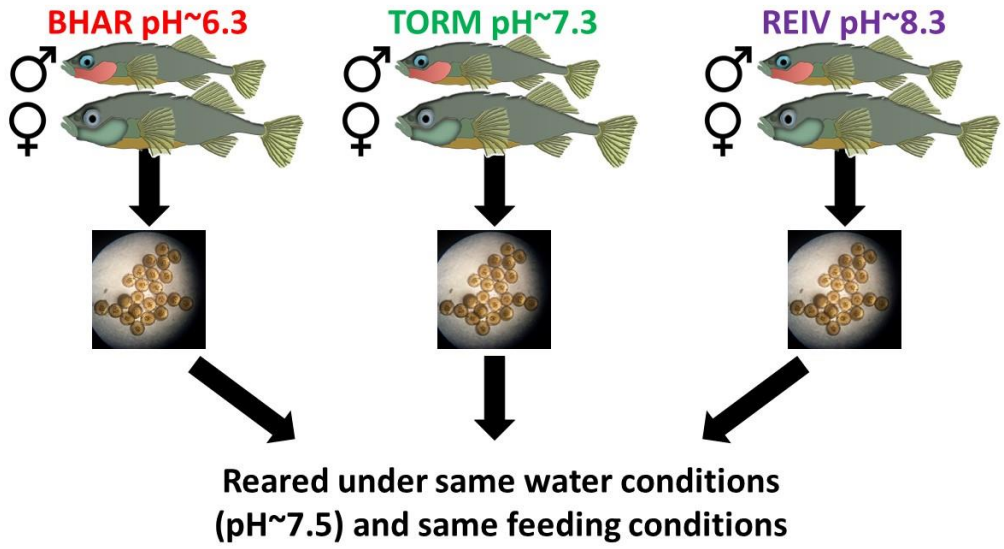



Figure 2.2. Design of common garden rearing experiment. Loch and approx. water conditions of origin of fish shown at the top. Conditions of rearing environment at the University of Nottingham shown at the bottom.

Aquarium tanks were filled with filtered water from the local mains supply in Nottingham and remained at a pH ~7.5 (+/-0.3). Water quality in tanks was carefully monitored and regular water changes carried out to ensure suitable rearing conditions (See Appendix; Fig. A.1). Families were kept in separate tanks and fed initially on Artemia sp. before transitioning to ZM complete feeds (ZM, www.zmsystems.co.uk) from ZM-100 (80-200 microns) through to ZM-200 fry food (0.2-0.3 mm), ZM-300 (0.3-0.5 mm) and ZM-400 (0.5-0.8 mm) as fish grew. The ZM feeds were carefully administered to be proportionate to fish numbers and mass to maintain an even availability of nutritional resources. The feed exhibits mildly hydrophobic qualities that results in dispersal across the water surface before dropping slowly in the water column, which limits the possibility of a dominant fish within the group monopolising the food source. ZM feeds were also confirmed to have consistent chemical composition, using Inductively Coupled Plasma Mass Spectrometry (ICPMS) analysis, with less variation than other available feeds, such as bloodworm (See Appendix; Fig. A.2).

Fish were reared at an ambient temperature of 13$^{o}$C. Two months post-hatching, fish were anaesthetised with MS222 every two weeks, to be weighed and measured until an appropriate mass for elemental analysis was reached. Fish were reared for approximately 139 days post-hatching at which point they had reached a sufficient mass for analysis. All fish were then euthanised with an overdose of MS222 and final weight and standard length measurements taken, before freezing. Representative samples, by mass, of 20 fish per population were selected, stomach contents were removed for analysis and the digestive system rinsed with DI H2O. These fish were then freeze dried, and their ionomic composition measured by ICPMS.

## 2.3.4 Water Samples

### 2.3.4.1 Field water samples

Loch water was collected using three 1 L sterile bottles which were filled at three points along the trapping range for each site approximately 1 m from the shoreline. From the top of each bottle, 20 ml of water was drawn into a syringe and expressed through a 25 mm * 0.22 µm filter (Chromotography.com, Runcorn, Cheshire, UK) into a universal container containing 1 ml 10% HNO3 in DI H2O. Samples were kept at room temperature until they could be analysed.

### 2.3.4.2 Lab water samples

In addition to regular water quality checks, water samples were taken from random tanks in the laboratory aquarium (A95) every two weeks for ICPMS analysis. From randomly selected tanks, 20 ml of water was drawn into a syringe and expressed through a 25 mm * 0.22 µm filter (Chromotography.com, Runcorn, Cheshire, UK) into a universal container containing 1 ml 10% HNO3 in DI H2O. Samples were kept at room temperature until they could be analysed.

## 2.3.5 Inductively Coupled Plasma Mass Spectrometry (ICPMS)

ICPMS enables determination of the concentration of multiple elements within a sample, making it an ideal analysis method to apply to ionomic studies. Solid samples must first be digested in acid, diluted and sometimes filtered to remove any residual particles, resulting in a colloid-free aqueous sample for analysis (Fig.2.3). Analysis involves the delivery of the sample to a nebulizer via a peristaltic pump, at which point it is aspirated into an argon plasma (~ 5000 $^{o}$ C) and ionized to singly-charged positive ions. A vacuum pump draws ions through nickel skimmer cones and a small pressure of helium prevents re-combination of elements to polyatomic species. The four charged bars of the quadrupole separates the ions by mass to charge ratio

with an oscillating electrical current and these are then sequentially received by the electron-capture detector. By comparison of the sample to reference standards and operational blanks, concentrations of individual elements can be determined.

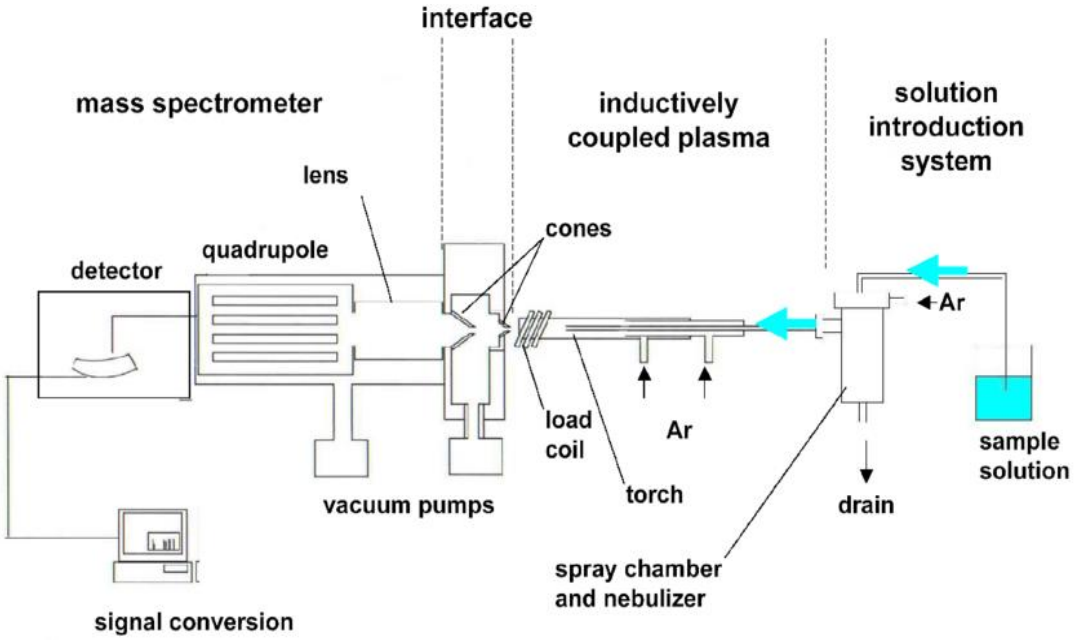



Figure 2.3. Schematic diagram of an ICPMS instrument (Gilstrap, 2009).

## 2.3.6 Fish processing

Stomach contents were dissected from partially defrosted fish and rinsed in DI H2O at the University of Nottingham. Stomach contents were transferred into individual 1.5 ml Eppendorf tubes for further analysis. Remaining viscera were rinsed with DI H2O and placed back with the fish. The fish were then placed into individual 5 ml Eppendorf tubes. All tubes were stored at -80$^{o}$ C for further processing. Following freeze drying, fish were crushed and put through an additional night of freeze drying. These samples were then microwave-digested with Primar PlusTM grade HNO3 (65%) and diluted with DI water for ICPMS analysis. Gravid fish were excluded from analysis at this stage in order to establish an ionomic baseline at a population level, as

previous research has identified changes in elemental concentrations associated with egg production and development (Hogstrand *et al*., 1996).

## 2.3.7 Fish armour values

Fish armour values were used from measurements collected in 2013 (Magalhaes *et al*., 2016) that covered various measures of multiple fish (30-40 fish per population) including: number of plates, length of dorsal spines 1 and 2, length of pelvis spine, length of pelvis, height of pelvis and length of biggest plate.

## 2.3.8 Data analysis

### 2.3.8.1 General Linear Mixed Effect Model (GLMM)

To model variation in fish size across populations in relation with the pH of home lochs, a general linear mixed model (Gamma family with an inverse link) was used to predict dry mass (g) with pH and sex of fish as fixed variables using the “lmer” function in lme4 as well as the packages lmerTest and MuMIn in R version 4.0.3 (2020-1010). Population (representing differences between home lochs) was used as a random effect. GLMMs were selected on the basis of AIC values to identify the best fit model.

### 2.3.8.2 Principal Component Analysis (PCA)

Principal Component Analysis was undertaken on the concentrations of 20 different elements from the whole fish ICPMS analysis using the “prcomp” function in base R (R Core Team 2022). The loadings were used to determine which variables contributed most to the variation of each PC, considering those with a value >0.30 to be contributing and >0.40 to be strongly contributing. A scree plot and loading table were used to determine an inflection point when the Eigenvalue dropped below the threshold of one. These relevant PCs were then extracted and run in a multivariate analysis of variance (MANOVA), using the “manova” function in R using population, pH, sex, and mass as explanatory variables.

A linear mixed model (LMM) was fitted (estimated using REML and nloptwrap optimizer) to predict PC1 of wild-caught fish ionomics (explaining the most variation) with pH and dry mass of fish as fixed effects and population as a random effect. Model selection was done on the basis of AIC values and explanatory variables were dropped from the model as necessary during model selection.

#### 2.3.8.3 Between Class Analyses (BCA)

As PCA does not provide a test of statistical significance and in order to clarify distinctions in ionomics at population level, a BCA (also known as a Between Site Analysis) was employed, which is less agnostic than PCA and allows the partitioning of sites (populations) from ecological data by class or site means into groupings as an aspect of the analysis. This was done using the “BCA” function in the R package ade4. From the initial PCA, components were selected before the standard deviation dropped below the threshold of one. These components were then included in a BCA and plotted using the adegraphics package. The “randtest” function in ade4 was then used to determine if there was a significant difference between sites. The criteria of the randtest is the ratio of the between-inertia (variance of the data) to the total inertia and a p-value was simulated from default 999 permutations.

#### 2.3.8.4 Linear Discriminant Analysis (LDA)

This analysis uses jack-knife re-sampling, by comparing one sample to the next and groups them by similarity to estimate group classifications using the “predict” function in the MASS package in R. This effectively groups them ‘blind’ and group designations are given at the end to see how well the groups separate by the variance in the data. LDA is more agnostic than BCA though the aim is the same, to highlight the differences between groups. While BCA maximises the between-class inertia (ability to split the variation into different groups in order to explain the variance), LDA maximises the between-class inertia relative to the total inertia.

#### 2.3.8.5 Correlation Heat Maps and Matrices

In order to better visualise the PCA results, elemental concentrations from all wild fish across populations were configured into a correlation matrix using the “reshape” function in R and then plotted into a correlation heat map displaying Pearson correlation coefficient values indicating the strength of correlations. Values (+/-) of 0.7 and higher were considered highly correlated while values (+/-) between 0.5 and 0.7 were considered to be moderately correlated; colour coding was used to indicate positive (red) or negative (blue) correlations.

Correlations were then visualised using the qgraph package in R to display elements as nodes with connecting line thickness indicating strength of correlations and colour of line corresponding to positive (red) and negative (blue) correlations. The “spring” argument was then applied that uses the “Fruchterman-Reingold” algorithm that invokes spring tension to arrange more correlated nodes closer together. This approach enables clusters of more correlated elements across populations of fish to be identified.

#### 2.3.8.6 Generalised Linear Models

Dry mass (g) of wild fish and their lab reared counterparts in the common garden experiment, were modelled using the “glm” function in R with population as an explanatory variable using a Gamma family and an inverse link, due to right skewed data. Models were fitted with AIC values.

#### 2.3.8.7 General Linear Models

PCA results from analysis of armour values were modelled using the “glm” function in R against the first two components of the ionomics PCA of wild fish using mean population values and the number of plates in armour ran to compare in separate models as these measures were found to be collinear to the first principal component of armour values. This was done with a Gaussian family and an identity link.

## 2.4 Results

The various water bodies of North Uist exhibit a range of pH conditions across the island and between sites (Table 2.1). As wide scale differences between sea water and fresh water have already been established, as well as ionomic adaptations in stickleback associated with this shift in water chemistry (Rudman *et al*., 2019), it was decided for analysis to focus specifically on differences between freshwater sites and the populations that inhabit them. This excluded sites OBSE and DUIN from further analysis.

Table 2.1. Range of water pH and salinity across all ten study sites on North Uist, 2019 and 2021.

| **Site** | **OBSE** | **REIV** | **SANN** | **HOST** | **DUIN** | **GROG** | **TORM** | **CHRU** | **SCAD** | **BHAR** |
|---|---|---|---|---|---|---|---|---|---|---|
| **pH 2019** | 9.5 | 9.0 | 8.9 | 8.5 | 8.4 | 8.3 | 7.0 | 6.6 | 6.5 | 6.3 |
| **pH 2021** | 9.62 | 8.48 | 8.77 | 8.43 | 8.79 | 8 | 7.12 | 6.83 | 6.16 | 6.09 |
| **pH average** | 9.56 | 8.74 | 8.84 | 8.47 | 8.6 | 8.15 | 7.06 | 6.72 | 6.33 | 6.2 |
| **Salinity ppt** | 22.2 | 0.18 | 0.15 | 0.12 | 13.86 | 0.12 | 0.06 | 0.05 | 0.05 | 0.05 |

Across the freshwater sites mean fish mass increased with pH (Fig. 2.4) indicating that mass (g) of fish was significantly predicted by both pH ($\chi2(1) = 7.29$, $p=0.007$) and sex ($\chi2(1)=4.66$, $p=0.03$).

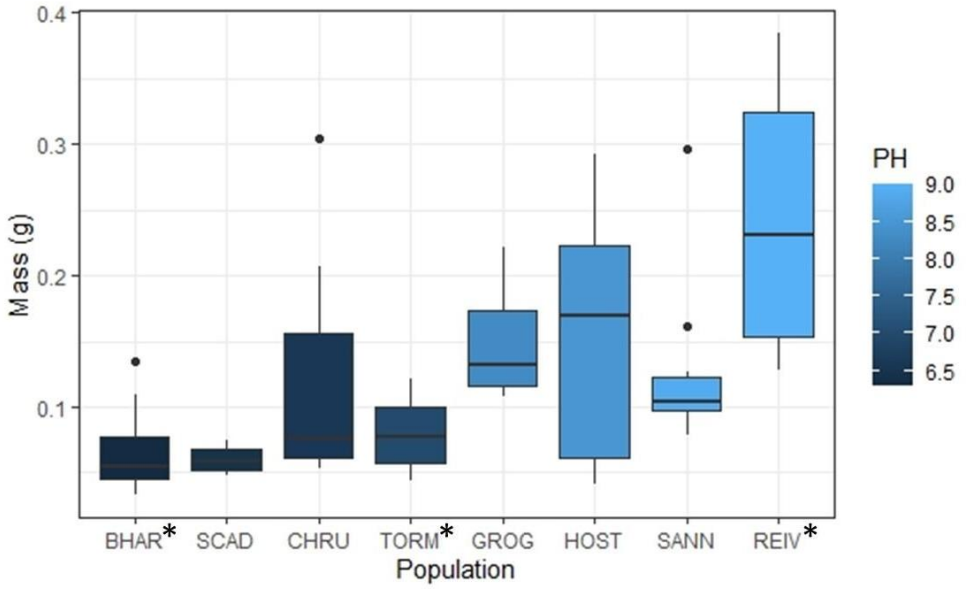



Figure 2.4. Variation in dry mass of sampled stickleback across a freshwater pH gradient on North Uist, May 2019. Populations are ordered from acidic to alkaline, left to right. Populations selected for common garden experiment are indicated by *.

## 2.4.1 Whole fish ICPMS analysis from freshwater sites

### 2.4.1.1 Principal Component Analysis

The elements with the highest loadings on PC1 were P and Ca with a smaller contribution from Rb (Table 2.2). The variance explained in the PC2 axis is a combination of elements including Na, Mg, K, Fe, Se and Cd. The first six PC's accounted for 73% of the total variation in the data before the standard deviation dropped below the threshold of one (Table 2.2). These first six PC variables were modelled in a MANOVA which showed ionomic variance of whole fish was significantly affected by pH (df=1, Residual df=70, Pillai=0.92, $p<0.001$), population (df=6, Residual df=70, Pillai=2.75, p=<0.001), sex (df=1, Residual df=70, Pillai=0.51, $p<0.001$), and mass (df=1, Residual df=70, Pillai=0.36, $p<0.001$) across the six PC's. (Figure 2.5). In a LMM with population as a random effect, PC1 was significantly related to

both pH of home loch conditions ($F(1,6.94)=21.01$, $p=0.003$) and mass of fish ($F(1,75.45)=14.58$, $p<0.001$).

Table 2.2. PCA loadings of elemental concentrations of whole fish with highlights showing the highest loadings driving the variation of the data. Darker blue indicating stronger loadings.

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 |
|---|---|---|---|---|---|---|
| **Na** | -0.157 | -0.306 | 0.150 | -0.144 | 0.245 | -0.317 |
| **Mg** | -0.178 | -0.304 | -0.307 | 0.048 | 0.107 | -0.085 |
| **P** | -0.424 | -0.081 | 0.011 | -0.027 | 0.133 | 0.214 |
| **S** | -0.243 | -0.272 | -0.257 | -0.186 | -0.075 | -0.048 |
| **K** | -0.161 | -0.310 | -0.186 | -0.200 | -0.080 | -0.421 |
| **Ca** | -0.409 | -0.014 | 0.013 | 0.008 | 0.162 | 0.289 |
| **Li** | -0.034 | 0.050 | -0.230 | 0.480 | -0.168 | -0.111 |
| **Mn** | 0.134 | -0.036 | -0.285 | -0.403 | -0.252 | -0.028 |
| **Fe** | 0.049 | -0.360 | 0.282 | 0.359 | 0.010 | -0.044 |
| **Co** | -0.067 | -0.194 | 0.183 | 0.327 | -0.427 | -0.038 |
| **Ni** | 0.001 | -0.012 | 0.001 | 0.078 | 0.393 | -0.286 |
| **Cu** | -0.239 | -0.050 | -0.173 | -0.042 | -0.232 | 0.472 |
| **Zn** | 0.093 | -0.060 | -0.394 | 0.316 | 0.239 | -0.035 |
| **As** | -0.223 | 0.229 | -0.203 | 0.354 | 0.069 | -0.160 |
| **Se** | 0.218 | -0.364 | -0.045 | 0.052 | -0.024 | 0.220 |
| **Rb** | -0.344 | 0.095 | -0.131 | 0.118 | -0.329 | -0.083 |
| **Sr** | 0.094 | -0.286 | -0.143 | 0.073 | 0.369 | 0.400 |
| **Mo** | -0.245 | -0.213 | 0.310 | -0.063 | -0.139 | -0.097 |
| **Cd** | 0.223 | -0.349 | 0.145 | 0.116 | -0.181 | 0.096 |
| **Ba** | 0.291 | -0.129 | -0.389 | -0.014 | -0.177 | -0.049 |
| | | | | | | |
| **Std. Dev** | 2.128 | 1.936 | 1.418 | 1.246 | 1.194 | 1.112 |
| **% of Variance** | 0.23 | 0.19 | 0.10 | 0.08 | 0.07 | 0.06 |
| **Cumulative %** | 0.23 | 0.41 | 0.51 | 0.59 | 0.66 | 0.73 |

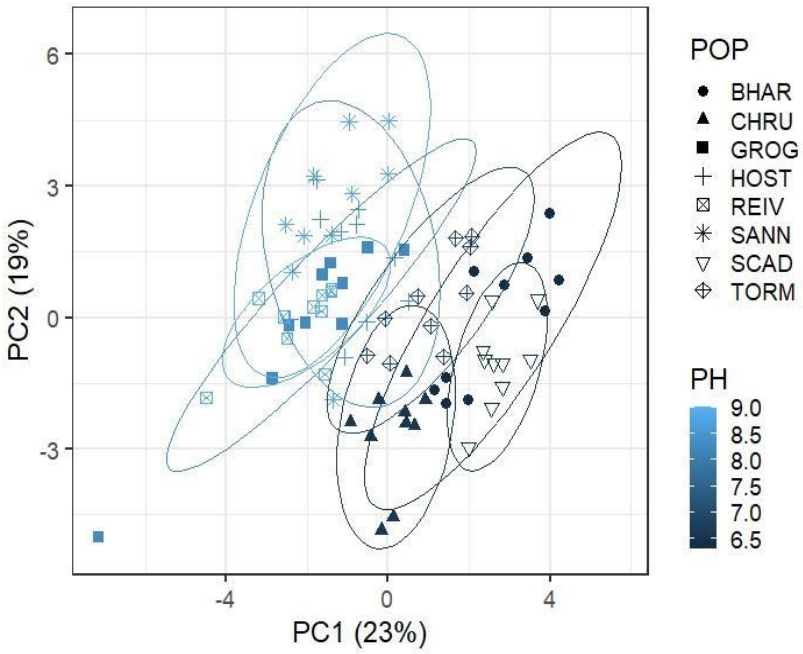



Figure 2.5. Visualisation of principal component analysis of the ionomic composition of wild caught fish (n=10 per population). Colours indicate pH of home site waters and population indicated by point shape and ellipses.

### 2.4.1.2 Between Class Analysis

BCA revealed ionomic distinction between fish populations with greater clarity than PCA explaining 47% of the total inertia (ability to split the variation into different groups in order to explain the variance) (See Fig. 2.6).

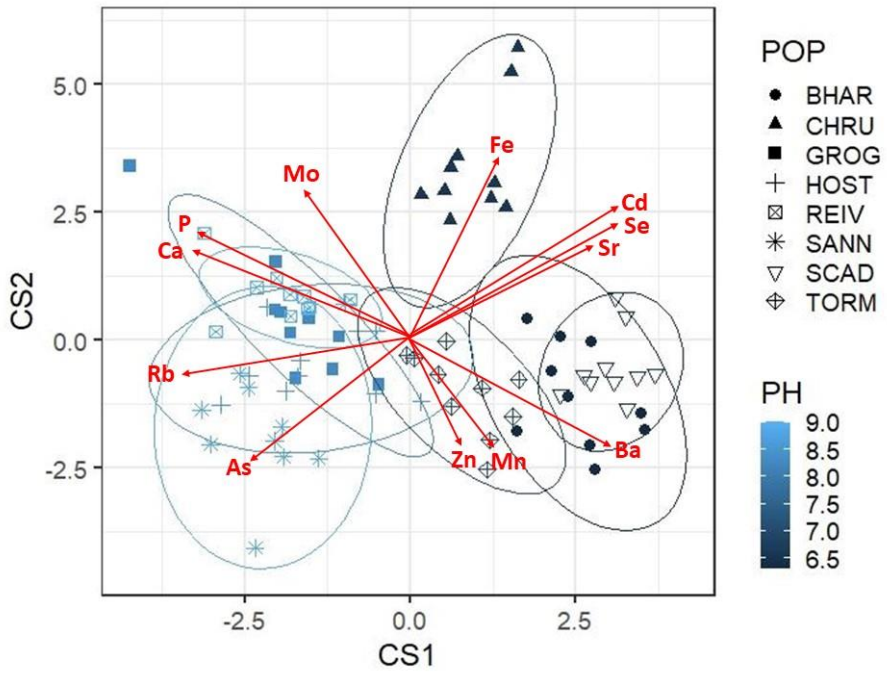



Figure 2.6. Visualisation of between class analysis of the ionomic composition of wild caught fish (n=10 per population). Colours indicate pH of home site waters and population indicated by shape and ellipses. Red arrows indicate main vectors, labelled by element.

The BCA was well fitted to the PCA with 47% of total inertia coming from differences between site comparisons. The variance between populations in terms of fish ionomics was significantly different (p=0.001, 999 permutations). Much like the PCA, the first axis related most strongly to concentrations of P, Ca and Rb in populations from alkali conditions and higher Ba concentrations in fish from more acid conditions (Fig. 2.6). Differences between BCA and PCA results included the highlighting of higher Fe concentrations in CHRU fish in particular.

### 2.4.1.3 Linear Discriminant Analysis

LDA predicted population groupings based on elemental composition of wild fish with 100% accuracy. This indicates fish were extremely well grouped into populations by elemental concentrations of whole fish and fish ionomics is population-specific at examined sites.

### 2.4.1.4 Correlation Matrix

Although there were positive correlations between pH and some elements, such as Ca in whole fish, there were also negative correlations between pH and other vital elements such as Mn (Figs. 2.7 and 2.8). In order to gain a clearer picture of direct correlations in elemental concentrations across populations (See Appendix; Figs. A.3-22), heat maps were used (Fig. 2.9) to visualise these relationships.

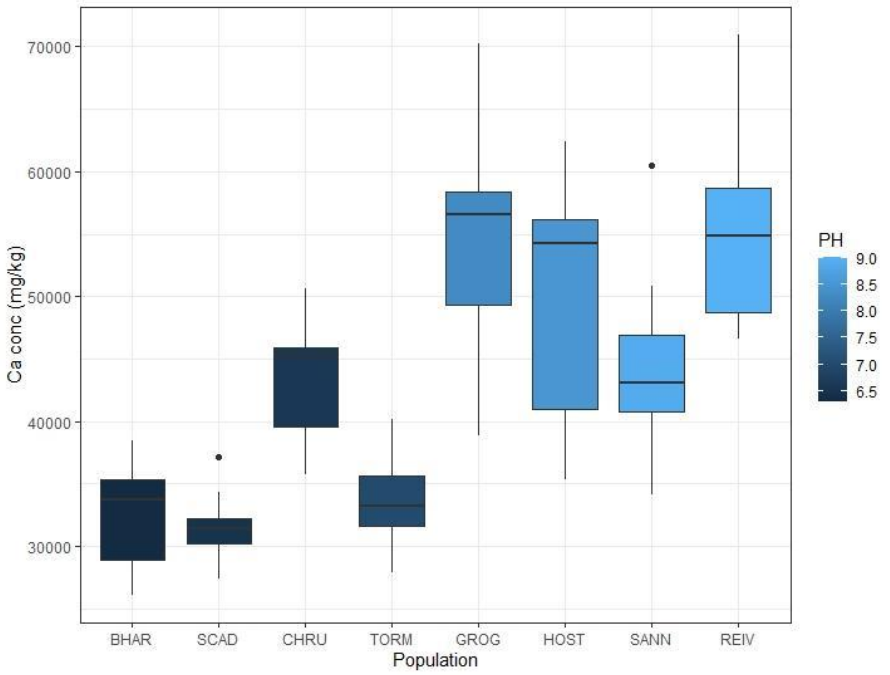



Figure 2.7. Variation in calcium concentrations of whole fish between populations from freshwater sites on North Uist, ordered by loch water pH.

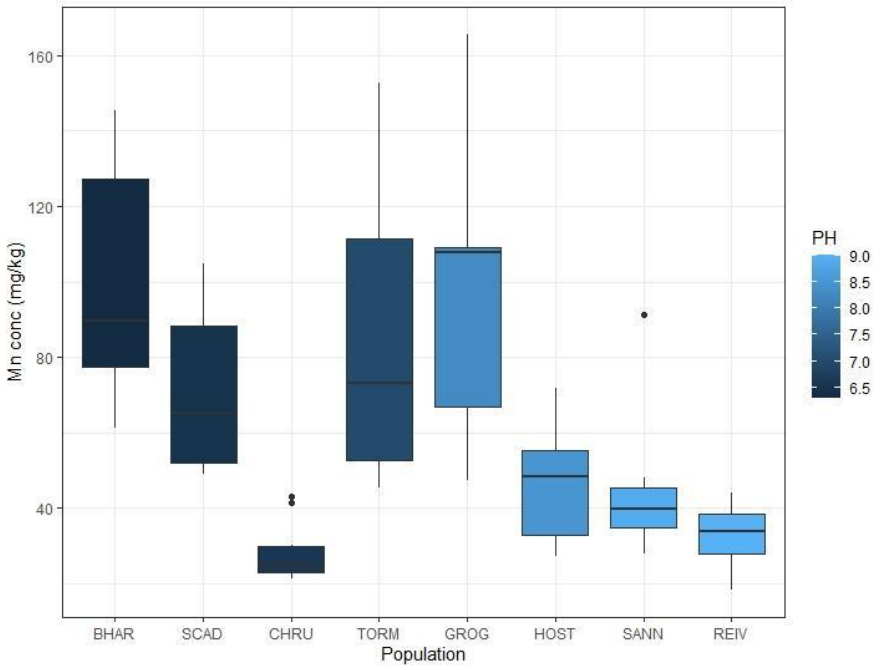



Figure 2.8. Variation in manganese concentrations of whole fish between populations from freshwater sites on North Uist, ordered by loch water pH.

Phosphorus and calcium were strongly positively correlated elements across all fish from all populations (r = 0.97, Fig. 2.9); Cd and Se were also highly correlated at 0.69. Moderately positive correlations include Fe and Cd, Fe and Se, S and Mg, As and Rb, Mg and K, Na and K, Se and Sr, S and K. Moderately negative correlations included Ba and Ca, Ba and P.

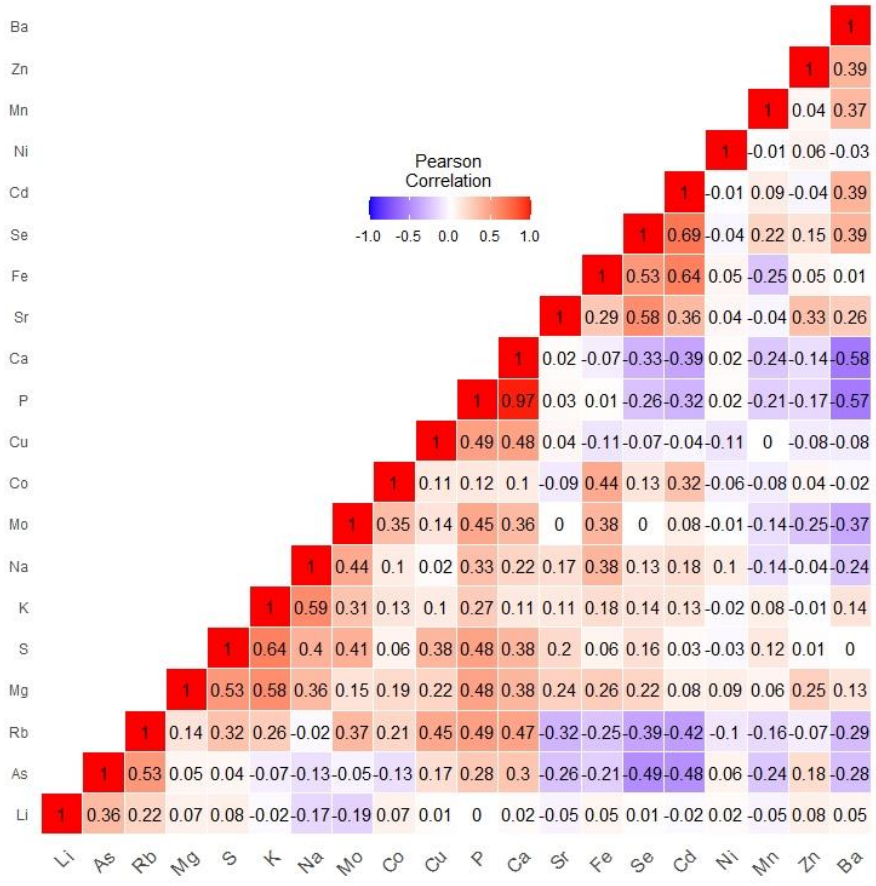



Figure 2.9. Correlations of elemental concentrations of whole fish from freshwater sites on North Uist.

### 2.4.1.5 Correlations with Qgraph

When visualised with the elements represented as separate nodes, the same negative correlations were seen in the relationship between Ba and both Ca and P as well as a clearer depiction of the positively correlated elements (Fig. 2.10A). When the nodes are 'sprung' to indicate strength of correlations by proximity, three particular clusters of positively correlated elements were revealed: 1) Fe, Cd, Se and Sr; 2) Ca, P, Rb and Cu; 3) Na, Mg, K and S (Fig. 2.10B).

Plotting the negative correlations of Ba with both Ca and Sr (Figs. 2.11 and 2.12) reveals that, although the relationship between Ba and Ca is negative across populations, within populations there are positive correlations, increasing as water conditions become more acidic, with a generally positive

relationship between Ba and Sr as water conditions become more alkaline. In both instances HOST is the exception, trending in the opposite direction from the other populations. This highlights the complexity of such chemical interactions in fish ionomics as well as the variability of elemental trends among, and within, different populations.

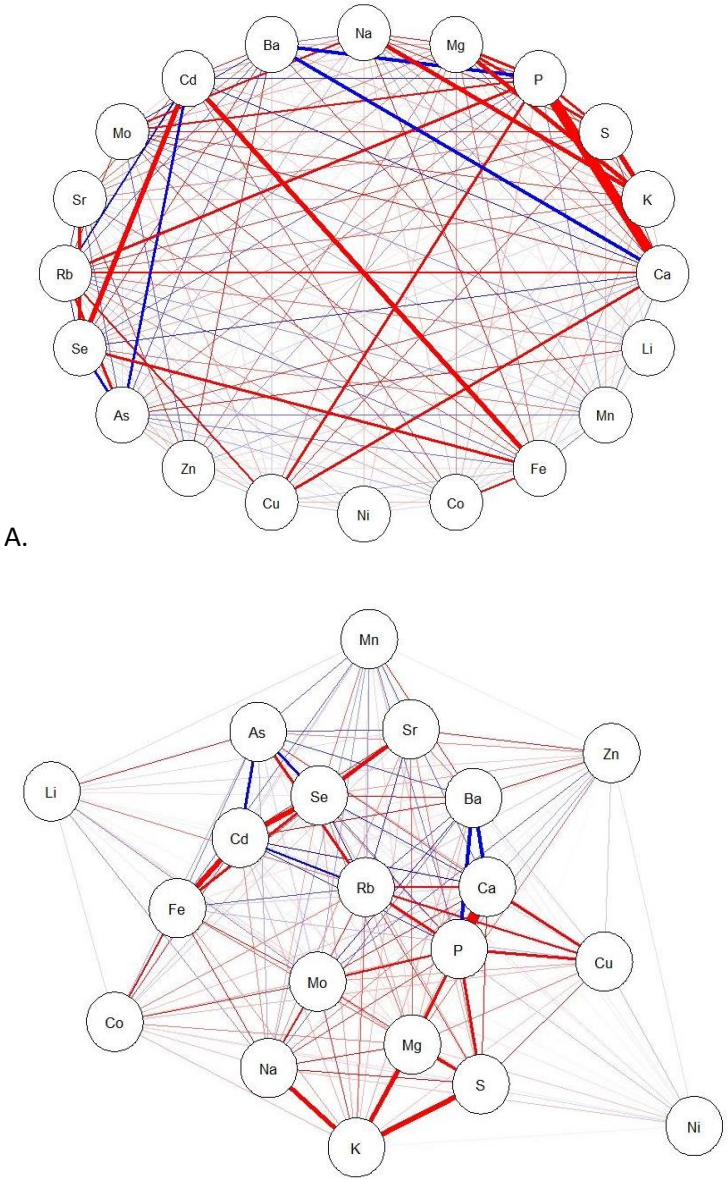



Figure 2.10. Correlations of elemental concentrations of whole fish from freshwater sites on North Uist. Blue lines are negative correlations, red lines are positive correlations with the width of the line (A) and proximity to one another (B) indicating the strength of elemental correlations.

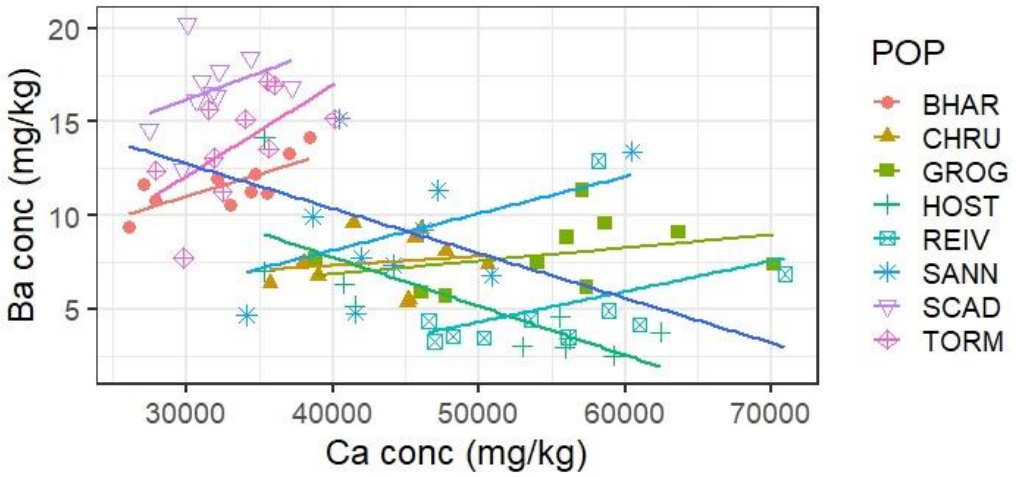



Figure 2.11. Trends of correlations for Ca and Ba concentrations in whole fish across populations (dark blue line) and within populations, indicated by colour and point shape.

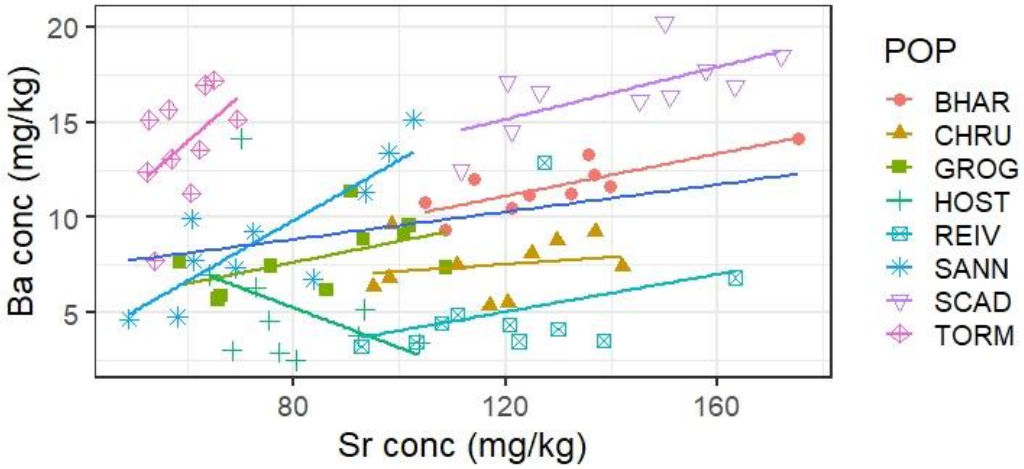



Figure 2.12. Trends of correlations for Sr and Ba concentrations in whole fish across populations (dark blue line) and within populations, indicated by colour and point shape.

## 2.4.2 Relating Armour Values to Ionomics PCA

The loadings for PC1 of the armour data PCA were relatively even across all armour values (Table 2.3) which also accounted for a large proportion of the total variance (86%), while PC2 only accounted for around 5% of total variance and was driven by length of dorsal spines 1 and 2. When modelled

against the ionomic PC1 (highest loadings were Ca, P and Rb) and PC2 (highest loadings for Na, Mg, K, Fe, Se and Cd), there was a significant relationship between PC1 armour and PC1 ionomics (Table 2.4), but the same was not true for PC2 armour. There was also a significant relationship between PC1 ionomics and number of plates, but PC2 ionomics exhibited no significance interactions, neither with the armour PCs nor the number of plates.

Table 2.3. PCA loadings of armour values of fish, with highlights showing the highest loadings driving the variation of the data. Darker blue indicating strongest loadings.

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 |
|---|---|---|---|---|---|---|
| **Dorsal Spine 1 Length** | 0.387 | 0.811 | -0.232 | 0.313 | -0.134 | 0.153 |
| **Dorsal Spine 2 Length** | 0.396 | -0.567 | -0.511 | 0.391 | -0.264 | 0.195 |
| **Pelvic Spine Length** | 0.420 | 0.018 | -0.092 | -0.617 | -0.479 | -0.452 |
| **Pelvis Length** | 0.396 | -0.121 | 0.820 | 0.300 | -0.249 | 0.052 |
| **Pelvis Height** | 0.424 | -0.053 | 0.048 | -0.497 | 0.394 | 0.643 |
| **Length of Biggest Plate** | 0.425 | -0.062 | -0.036 | 0.176 | 0.682 | -0.564 |
| | | | | | | |
| **Std. Dev** | 2.272 | 0.550 | 0.497 | 0.368 | 0.309 | 0.245 |
| **% of Variance** | 0.86 | 0.050 | 0.041 | 0.023 | 0.016 | 0.01 |
| **Cumulative %** | 0.86 | 0.910 | 0.952 | 0.974 | 0.99 | 1 |

Table 2.4. Results of GLMs fitted to the relationship of mean population values for ionomics PCs 1 and 2 to armour PCs 1 and 2, and number of plates (n.plate). Statistical significance indicated by *.

| | Estimate | Std. Error | D.F. | t-value | p-value |
|---|---|---|---|---|---|
| **PC1 ionomics ~ PC1 armour + PC2 armour** | | | | | |
| (Intercept) | 0.09 | 0.19 | - | 0.21 | 0.84 |
| PC1A | 0.86 | 0.09 | 1 | 9.6 | 0.0002 *** |
| PC2A | -0.26 | 0.73 | 1 | -0.35 | 0.72 |
| **PC1 ionomics ~ number of plates** | | | | | |
| (Intercept) | -2.29 | 0.5 | - | -4.6 | 0.003 ** |
| n.plate | 0.82 | 0.14 | 1 | 5.72 | 0.001 *** |
| **PC2 ionomics ~ PC1 armour + PC2 armour** | | | | | |
| (Intercept) | -0.007 | 0.58 | - | -0.01 | 0.99 |
| PC1A | -0.22 | 0.28 | 1 | -0.79 | 0.48 |
| PC2A | -0.3 | 2.3 | 1 | -0.13 | 0.9 |
| **PC2 ionomics ~ number of plates** | | | | | |
| (Intercept) | 0.21 | 0.95 | - | 0.23 | 0.83 |
| n.plate | -0.08 | 0.27 | 1 | 0.79 | 0.79 |

## 2.4.3 Whole fish ICPMS analysis from common garden study

### 2.4.3.1 Common Garden Conditions

Although elemental concentrations of the aquarium tank water were lower than some of the selected lochs for elements including Ba, Fe, Mn, Sr and Zn when compared to BHAR, other crucial elements were present at much higher concentrations than those found in home loch water, including Ca, K, Mg, Na and S. ICPMS analysis also demonstrated that while there was substantial variance in the elemental composition of different bloodworm batches (commonly used as a complete fish feed), the ZM feeds used for the common garden rearing were much more consistent in their composition and so more consistent in their nutritional content.

#### 2.4.3.2 Growth Rates

Wild caught fish from the three focal populations differed significantly in their adult mass (Fig. 2.4), increasing with pH ($\chi 2$ (2) = 60.26, p <0.001). The largest of these as adults were REIV fish from alkali conditions with TORM (neutral conditions) and BHAR (acid conditions) fish averaging much smaller masses, though TORM fish on average achieve a greater adult size than BHAR fish.

Initially, REIV fish exhibited the most growth in terms of mass (Fig. 2.13A), but their growth stalled and the BHAR fish caught up to reach a similar weight by the end of the six week period. TORM fish grew the least in terms of mass compared to the other two populations. The growth of REIV fish, in terms of standard length (SL), was overall similar to that of TORM fish (Fig. 2.13B); BHAR fish were the longest of all three populations throughout the course of the experiment.

Under common garden conditions (pH 7.5), lab-reared fish showed a marked difference between populations in average size (Fig. 2.13C) when compared to wild caught fish from the same populations (Fig. 2.4). Mean fish mass differed significantly by population ($\chi 2$ (2) = 7.00, p = 0.03). However, unlike the wild caught fish, the mass of lab-reared REIV fish was much less, on average, compared to fish from the other two populations; BHAR fish were the largest fish under common garden conditions but the smallest population by mass in the wild.

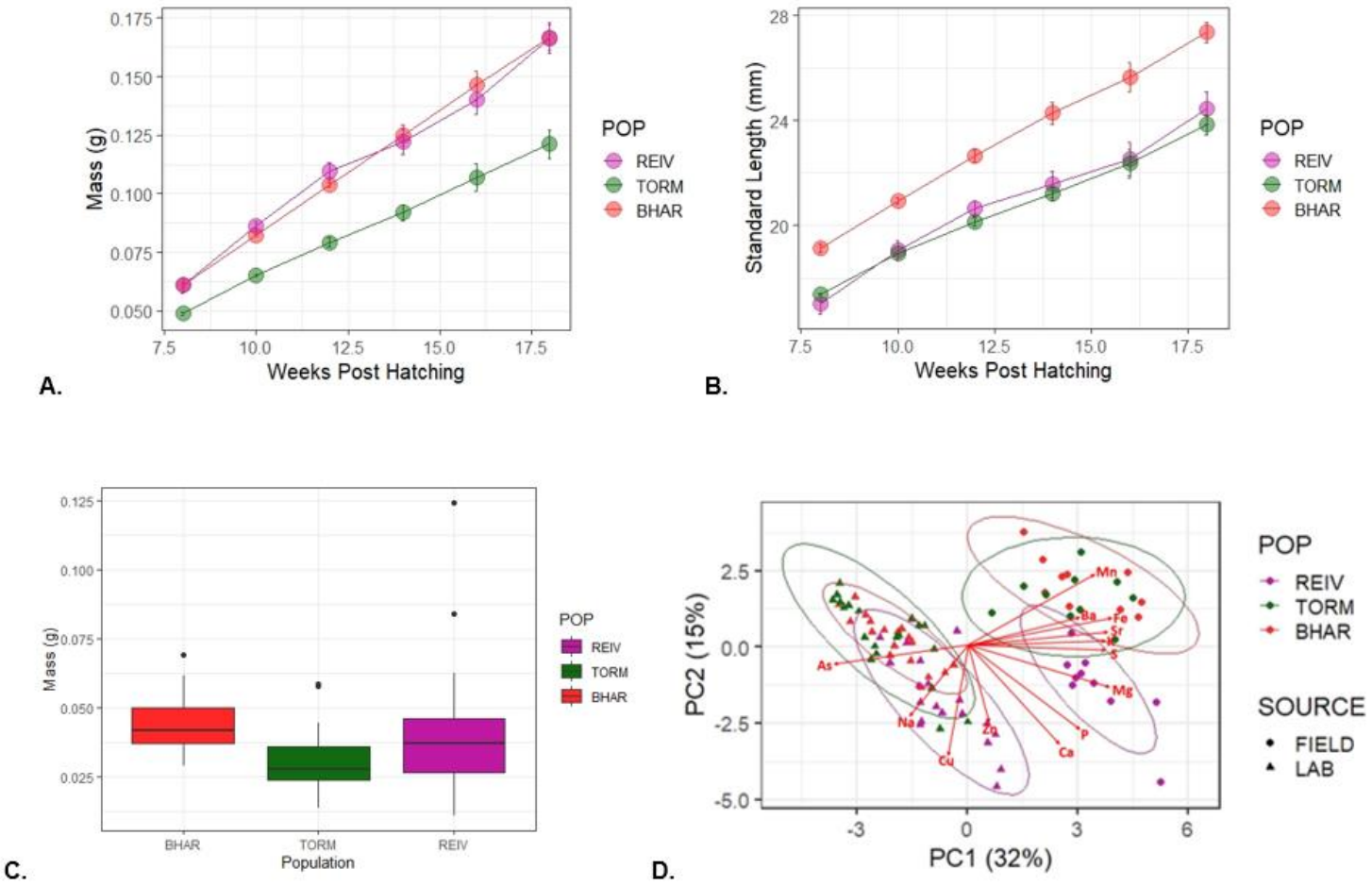



Figure 2.13. Average mass (g) (A) and length (mm) (B) of fish by population (POP, indicated by colour). Measured points represent weight and standard length measurements of all fish, which were two weeks apart. Dry mass of lab-raised fish from the three populations selected for common garden experiments, representing home loch pH conditions of around 6 (BHAR), 7 (TORM) and 8 (REIV) (C). Visualisation of combined principal component analysis of the ionomic composition of wild caught fish (field) compared to lab reared fish. Populations are indicated by ellipses and colour. Treatment (source) indicated by shape and red arrows indicate main loadings, labelled by element (D).

#### 2.4.3.3 Principal Component Analysis

When wild caught fish and lab reared fish were compared using PCA, they were identifiable by both population and treatment with 95% confidence ellipses (Fig. 2.13D). The first 5 PCs accounted for 73% of the variation in the data before the standard deviation dropped below the threshold of 1 and these components, when analysed in a MANOVA indicated that ionomic variance was significant affected by both population (df=2, Resdf=86, Pillai=0.845, $p<0.001$) and treatment (df=1, Resdf=86, Pillai=0.939, $p<0.001$). While differences in treatment were mainly driven by differences in Mg, S, K, Mn, Fe, As and Sr along the PC1 axis, difference between populations were driven by Na, P, Ca, Mn, Cu and Zn along PC2 axis with REIV fish more distinct in their elemental composition than BHAR and TORM.

#### 2.4.3.4 Linear Discriminant Analysis

Fish were grouped and predicted to their home loch populations with 94% accuracy. Two fish from TORM were incorrectly grouped into BHAR, while three fish from BHAR were incorrectly grouped into TORM. REIV fish were grouped with 100% accuracy. Despite being reared under the same water conditions and nutritional availability, fish were still readily grouped to home loch populations based on their elemental content alone, suggesting ionomic consistency with the wild fish analysed from their home lochs.

## 2.5 Discussion

The data presented in this chapter successfully demonstrates that the stickleback examined in this study exhibit elemental variation in their whole body composition (ionome) that is population specific and relates to the water pH of home lochs. It also indicates that while rearing fish under common conditions of water chemistry and nutritional availability affect ionomics of selected populations, they are still readily identifiable by population and are accurately grouped to their wild counterparts. Taken as a whole, these results indicate a genetic component to systems controlling elemental acquisition and retention resulting in population level differences in whole fish ionomic composition. Substantial variation in water chemistry among

freshwater lochs has resulted in morphological variation in the stickleback populations, including adaptions in body size and composition of bone armour, consistent with previous studies on North Uist (Waterston et al., 1979; Magalhaes *et al*., 2016; Singkam, 2016; Haenel *et al*., 2019).

ICPMS results combined with PCA effectively revealed the complexity of how different elemental concentrations interact across freshwater fish populations that have adapted to their unique chemical circumstances. Average fish mass was significantly different between sites and positively correlated with home loch pH. There was also a wider range in adult body size with higher pH and richer resource conditions, as previously shown by Singkam (2016), which links to fish living longer in nutrient rich more alkaline conditions. Though previously well explored elements, such as Ca, positively correlate in fish tissue concentrations with pH, other elements such as Mn are negatively correlated with pH in whole fish concentrations increasing as home water pH decreases.

Elements that drive variation across populations include Ca and P which are likely linked directly to bone composition of armour and morphological structure that also vary greatly between fish inhabiting lochs of differing pH and chemical conditions. Other elements appear to play a key role in ionomic variation (Rb, Na, Mg, K, Fe, Se and Cd) with the biological role of some currently better understood than others. In some instances, broad correlations in elemental concentrations across fish are more nuanced within populations and even population-specific in their interactions.

Large differences in the mass of wild caught fish are indicative of environmental differences between populations (Magalhaes *et al*., 2016) as are differences in ionome, with REIV fish being significantly greater in size and more distinct in their elemental content than the typically smaller fish from TORM and BHAR. When these selected wild populations were grown in common water chemistry and with access to the same nutritional content, differences in mass and SL were much reduced between populations. These results are consistent with previous studies (Marchinko and Schulter, 2007; Barrett *et al*., 2008) that found bone-reduced morphs of stickleback have an

early stage advantage under common conditions, where Ca and P are readily available, due to the lack of investment in bone growth. It should also be noted, that while the age and life history of wild caught fish is ambiguous, lab-reared fish were standardised in terms of fertilisation date, environmental conditions, and maturation state. Lab rearing reduced environmental effects, such as temperature fluctuation, parasitism and predation that directly impact development, fitness and mortality.

Elemental analysis revealed a shift in the ionome of common garden reared fish which is consistent with the findings of Rudman *et al*. (2019). In Rudman's study, when examining the effect of rearing conditions with a focus on differences between saltwater and freshwater, they also found stickleback retain ionomic distinction at the population level, while populations were also significantly affected in their elemental content through common garden rearing. For this thesis, consideration should be given to the fact that common garden fish were only reared for six months and so were not sexually mature in the interpretation of these results as this can also significantly alter elemental concentrations of fish tissues (Hogtrand *et al*., 1996). With caveats aside, the results presented are consistent with the findings of Rudman *et al*. (2019), where stickleback from varying home chemical conditions exhibit plastic shifts in ionome, while retaining population level distinctions, when reared under common conditions.

To address the main aims of this chapter, populations from freshwater lochs on North Uist were distinct at the ionomic level. Such ionomic differences would be expected when comparing populations from salt and freshwater, though not necessarily when comparing among freshwater bodies in such close geographical proximity. However, the ionome is plastic and responsive to the immediate chemical conditions and nutritional availability of the environment in which fish are reared. Fish from oligotrophic conditions (BHAR) also grew much more efficiently in term of standard length when compared to fish from eutrophic/mesotrophic conditions (REIV), when placed under circumstances of the common garden and the same nutritional availability. BHAR fish even surpassed the growth of REIV fish in terms of

final dry weight, which are significantly larger than BHAR fish under home loch conditions. The results suggest that while genotype and phenotype are critical, surrounding environmental chemistry and nutritional availability also play a key role in growth and determining elemental content.

## 2.6 Conclusions

In addition to already well recognised patterns of morphological divergence, the results in this chapter demonstrates ionomic, population specific variance in stickleback inhabiting chemically diverse conditions, as well as the complexity of elemental interactions between fish and the water that surrounds and sustains them. Multi-element analysis approaches provide exciting opportunities to further explore adaptive changes in fish, in response to ecologically and genetically influenced elemental assimilation and retention. The stickleback populations examined in this thesis have been separated under differing chemical conditions for thousands of years in their home lochs. Site-specific ionomics of fish populations are likely the result of a combination of abiotic, dietary, plastic and evolutionary differences and appear to relate most directly to the chemistry of the water they inhabit. Questions remain in regards to which aspects of elemental uptake from the environment are controlled by nutritionally active assimilation and which are the result other forms of elemental uptake such as homeostasis.

# Chapter 3. Relating the ionomics of three-spined stickleback populations to their chemical environment.

## 3.1 Abstract

How are animals composed from the various elements, in multiple forms, that surround and sustain them? Nutritional availability varies within habitats both spatially and temporally in various biotic and abiotic forms, and animals must adapt to optimise their acquisition of vital elements in order to survive. Three-spined stickleback inhabit water bodies of varying chemistry across the island of North Uist, Scotland and exhibit unique, population specific ionomic qualities. However, little is currently known about how such freshwater fish are formed on the ionomic level as well as which aspects of their chemical environment influence the variation of their elemental composition. Various environmental components were sampled from multiple freshwater lochs and analysed using Inductively Coupled Plasma Mass Spectrometry (ICPMS). These different environmental components were then examined for site specific qualities and cross analysed with the ionomics of stickleback populations that inhabit them to determine likely sources of elemental variance previously identified in the fish they surround. While differences in stickleback bone morphology relate directly to water chemistry, other elemental differences between populations were also closely linked to available nutritional resources, and while invertebrate prey populations did not exhibit site specific ionomics, stickleback stomach contents were site specific. Water chemistry plays a critical role in stickleback ionomic variation, but this is also true for nutritional elements of their environment with a potential indication of selective feeding behaviours. These insights provide a greater understanding of how water chemistry and nutritional availability interact to influence the ionomics of freshwater fish.

## 3.2 Introduction

Biotic and abiotic factors interact and affect one another in cycles of complexity and balance that form the environment. Ecological stoichiometry has aimed to address this complexity through the lens of chemical currency, tracking the flow of vital elemental resources through biological processes that in turn affect, and are effected by, the abiotic media that surround and sustain them (Sterner and Elser, 2002; Elser *et al*., 2007; Marleau *et al*., 2015). While early forms of investigation laid important foundations in understanding ecological balance and elemental interactions, ionomics provides insights into a wider range of vital nutrients and trace elements beyond the most currently well understood, C, N, P and K, that were addressed by ecological stoichiometry.

Nutritional elements are vital to an organism's survival and exist within their environment in a range of biotic and abiotic forms (Kaspari and Powers, 2016). The availability of such elements is often limited by a number of factors which include the chemical nature of the environment (Harpole *et al*., 2011), the adaptability of the organisms that are supported by it (Sultan, 2015; Gonzalez *et al*., 2018) and other aspects of the environment (Leroux, 2018), such as temperature, precipitation and time of year (Correa and Winemiller, 2014). Elements pass through the ecosystems and food chain by way of producers and consumers (Elser and Urabe, 1999; Atkinson *et al*., 2013), related biological processes such as excretion, and biogeochemical interactions (Battin *et al*., 2016; Gill and Finzi, 2016).

Populations with variable resources adapt to nutritional availability (Behmer *et al*., 2001; Correa and Winemiller, 2014). In some cases, this can lead to countergradient variation (Craig and Foote, 2001), by which organisms have developed greater efficiencies in resource acquisition and retention in order to compensate for environmental deficiencies. The ability of fish to self-select nutrition through feeding behaviours is increasingly better understood (Fortes *et al*., 2016). These selective feeding behaviours include examples of fish balancing their diets solely by association of visual cues to postingestive consequences (Fortes-Silva *et al*., 2012).

This section seeks to understand differences in observed stickleback ionomics on a population level, by investigating the potential contribution of site-specific nutritional resources. This will include examination of the elemental composition of different components of the surrounding environment including invertebrate prey sources and seston/zooplankton at individual sites. Stomach contents analyses then aid in clarifying the dietary intake of fish and potentially indicate any selective feeding behaviours in response to the elemental composition of invertebrate prey populations.

This chapter further investigates the hypothesis that in addition to the stickleback populations examined, broad ionomic differences will be observed in the invertebrate populations among sites as these nutritional sources would have a cumulative effect magnified to the stickleback at the higher trophic level. Additionally, it seeks to explore whether the relationship of water pH to elemental variation in fish is consistent in other aspects of the environment.

## 3.3 Methods

### 3.3.1 Environmental component sampling

In order to gain a clearer understanding of how various components of the environment contribute and interact with the ionomic composition of the fish populations they surround and support (Fig. 3.1), both biotic and abiotic samples were taken from each freshwater site on North Uist (see Chapter 2; Fig. 2.1).

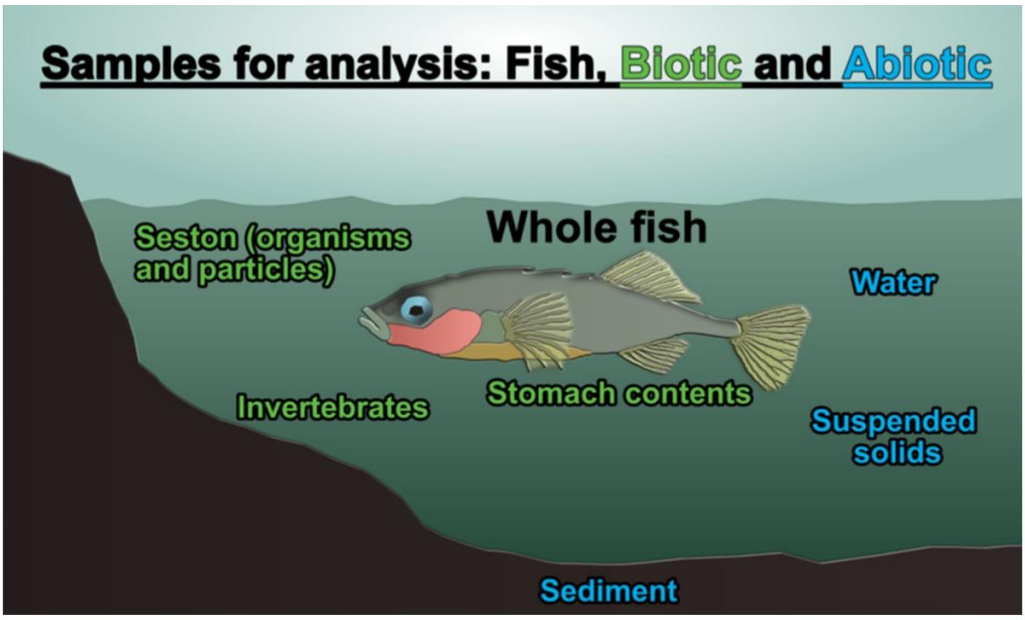



Figure 3.1. Diagram of biotic and abiotic environmental components sampled from each site.

#### 3.3.1.2 Water

Water was collected using three 1 L sterile bottles which were filled at three points across the trapping range of sticklebacks for each site, approximately 1 m from the shoreline. From the top of each bottle, 20 ml of water was drawn into a syringe and expressed through a 25 mm * 0.22 µm filter (Chromotography.com, Runcorn, Cheshire, UK) into a universal container with 1 ml 10% HNO3 in DI H20. Samples were kept at room temperature until they could be analysed using Inductively Coupled Plasma Mass Spectrometry (ICPMS).

#### 3.3.1.3 Suspended solids

Each of the three collected 1 L water samples were vacuum filtered to collect suspended solids. The filter paper (GE Healthcare, Life Sciences, Whatman, Buckinghamshire, UK) of each was wrapped separately in tin foil and frozen for further analysis.

#### 3.3.1.4 Sediment

Sediment was collected three times per site, approximately 2-3 m from the shoreline, using an Ekman grab sampler (Wildco Instruments, Wildlife Supply

Company, Buffalo, New York, USA) to bring up the top 2-3 cm of sediment, which was then stored in universal containers and frozen until further processing could take place.

#### 3.3.1.5 Seston

To investigate the potential role of nutritional availability in influencing the ionomic composition of fish at the various sites, seston and small invertebrates from each site were collected by dragging a plankton net (53 µm mesh) approximately 3 m from the shoreline for 20 m. The mouth of the plankton net had a diameter of 30 cm resulting in 1,410 L of water being sampled over the 20 m distance. The net was attached to a 1.5 m pole and held out to the side of the researcher, away from the shoreline, during sampling to avoid disturbance of the water and sediment affecting the sample. Seston was collected down the 110 cm conical net into a 300 ml catchment bottle. This 300 ml catchment bottle was then vacuum filtered and the filter paper from each site was wrapped separately in tin foil and frozen for further processing and analysis.

#### 3.3.1.6 Invertebrates

Multiple samples of invertebrate populations were taken from each site to investigate both nutritional availability and any potential site-specific variation in ionomics at a lower trophic level than stickleback. To sample invertebrates a ‘three-minute sampling’ as informed by Biggs *et al*. (1998), was conducted followed by a core sample. Initially, a brief survey of the shoreline identified potential mesohabitats. Two minutes were then spent collecting invertebrates in all identified mesohabitats by using a combination of kick sampling, and netting around plants and other obstructions. One minute was spent searching for any invertebrates that may be attached to the bottom of rocks or hidden by large pieces of material within the environment. Next a core of 7.7 cm diameter x approximately 20 cm of the sediment bottom was extracted and sieved through a 1.4 mm mesh and a 0.5 mm mesh to separate any benthic invertebrates present within the core. Samples of invertebrates were sorted to a family level where possible. Sorted

invertebrates were transferred into Eppendorf tubes by family and sample method and frozen for further processing and analysis.

#### 3.3.1.7 Stomach contents

Stomach contents were dissected from partially defrosted fish and rinsed in DI H2O at the University of Nottingham. Stomach contents were transferred into individual 1.5 ml Eppendorf tubes and viscera, rinsed with DI H2O, placed back with the fish. Fish were then placed into individual 5 ml Eppendorf tubes. All tubes were stored at -80$^{o}$ C for further processing.

### 3.3.2 Sample processing and analysis

All field samples (excluding water samples, which could be analysed without additional processing) were freeze-dried overnight to remove any moisture and establish an accurate dry weight. These samples were then acid-digested, diluted and analysed using ICPMS alongside known values and operational blanks for comparison and control.

Following freeze drying, invertebrates were crushed, and these samples were then microwave digested with concentrated HNO3 and diluted with DI water for ICPMS analysis. In order to gain sufficient mass for analysis, some invertebrate samples were pooled into groupings after identifying which were likely prey items for stickleback (identified as ‘POTENTIAL PREY’ in data analysis). This was based on size in relation to average adult stickleback gape and stomach contents.

Suspended solids, sediment and seston samples were acid digested in aqua regia using a combination of HNO3 and HCL on heating blocks prior to dilution and assay using ICPMS. Two samples from TORM site and all three samples from BHAR site were unviable and not included in analysis.

Stomach contents were acid-digested on a hot plate using concentrated HNO3 (65%; Primar PlusTM) employing an adjusted protocol that allowed for a reduced level of sample dilution with both HNO3 and DI water for more accurate results using the small sample quantities involved.

### 3.3.3 Data analysis

PCA was conducted on the ICPMS results of 20 different element concentrations from each environmental component that was sampled and analysed using the “prcomp” function in base R (R Core Team 2022). The loadings were then used to determine which variables contributed most to the variation of each PC, considering those with a value >0.30 to be contributing and >0.40 to be strongly contributing (though this threshold was lowered when overall scores were relatively small in some select cases). A scree plot of Eigenvalues and loading table were used to determine an inflection point when the Eigenvalue dropped below the threshold of one. These PCs were then extracted and run in a multivariate analysis of variance (MANOVA), using the “manova” function in MASS with site and water pH as explanatory variables.

To model any variation in elemental composition across sites in relation to pH of home loch, PCs (generally PC1) from the PCA of each environmental component were used as response variables in a linear mixed model (LMM), with site as a random effect and pH as a fixed variable (estimated using REML and nloptwrap) using the “lmer” function in lme4 as well as the packages lmerTest and MuMIn. Model selection was conducted using AIC values (Burnham and Anderson, 2002) to identify the best fitting model.

PCA does not provide a test of statistical significance, therefore, BCA (also known as a Between Site Analysis) was used in order to clarify distinctions in elemental composition at the site level, BCA is less agnostic than PCA and allows the partitioning of sites from ecological data by class or site means into groupings as an aspect of the analysis. This was done using the “BCA” function in the R package ade4. From the initial PCA, components were selected before the standard deviation drops below the threshold of one. These components were then included in a BCA and plotted using the adegraphics package. The “randtest” function in ade4 was then used to determine if there was a significant difference between sites. The criteria of the randtest is the ratio of the between inertia (variance of the data) to the total inertia and a p-value is simulated from a default 999 permutations.

To confirm sample groupings by site, an LDA analysis was conducted on each set of environmental ICPMS results using a jack-knife approach, by comparing one sample to the next and grouping them by similarity to the estimated group classifications using the “predict” function in the MASS package in R. The jack-knife effectively groups variables ‘blind’ and group designations are input at the end to determine how well the groups separate by the variance in the data. LDA is more agnostic than BCA, though the aim of highlighting the differences between groups is the same. While BCA maximises the between-class inertia (ability to split the variation into different groups in order to explain the variance), LDA maximises the between-class inertia relative to the total inertia.

For every PCA conducted, the mean site value of the first principal component was calculated for use as explanatory variables in a GLM (Gausssian family and identity link). Models were conducted using the “glm” function in R against the first two components of the ionomics PCA of wild fish using the mean site values to assess potential relationships between the stickleback ionomics and the abiotic and biotic environmental factors that surround them and likely form nutritional resources, as well as the stomach contents of the fish themselves. Suspended solids were excluded from this stage of analysis as BHAR was not represented in these samples due circumstances detailed above (see 3.3.2).

## 3.4 Results

### 3.4.1 Water analysis

Sodium, Mg, K, Ca, Rb and Sr had the highest loadings on PC1 (Table 3.1), while the elements that were most highly correlated on the PC2 axis were Li and Mn and, to a lesser extent, Zn and Cd. The first four components explained 81% of the variation in the data (Table 3.1). These PC variables were then extracted and analysed with a MANOVA which showed that the elemental variance of the water samples was significantly affected by both pH ($df=1$, Residual $df=40$, Pillai$=0.97$, $p<0.001$) and site ($df=6$, Residual $df=40$, Pillai$=2.09$, $p<0.001$).

Analysis of water samples shows distinct site-specific differences in chemical composition and elemental concentrations (Fig. 3.2) with the highest concentrations of most elements exhibited by the water of REIV (See Appendix; Figs. A.3-22). The main axis of elemental variation (PC1; 53.3% variation) is clearly related to the pH variation among the lochs ($F(1,6)=29.03$, $p=0.002$). Higher Fe concentrations were associated with more acidic conditions (Fig. 3.2).

Seventy percent of the total inertia (ability to split the variation into different groups in order to explain the variance) was explained by the BCA, indicating significant ($p=0.001$, 999 permutations) elemental differences between sites. Water samples were grouped through LDA with 100% accuracy, indicating highly site specific loch water chemistry.

Table 3.1. PCA loadings of elemental concentrations of water samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings.

| | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| **Na** | 0.300 | 0.033 | -0.045 | 0.007 |
| **Mg** | 0.298 | 0.020 | -0.012 | 0.029 |
| **P** | 0.180 | 0.242 | 0.258 | 0.305 |
| **S** | 0.282 | -0.022 | -0.103 | -0.131 |
| **K** | 0.292 | -0.079 | 0.182 | 0.027 |
| **Ca** | 0.298 | 0.049 | 0.014 | -0.070 |
| **Li** | 0.046 | 0.522 | -0.269 | 0.024 |
| **Mn** | -0.002 | 0.471 | 0.251 | -0.257 |
| **Fe** | -0.214 | 0.147 | 0.089 | 0.487 |
| **Co** | 0.069 | 0.246 | -0.186 | 0.557 |
| **Ni** | 0.103 | 0.171 | -0.573 | -0.080 |
| **Cu** | 0.178 | 0.172 | -0.334 | -0.119 |
| **Zn** | 0.072 | -0.293 | -0.113 | -0.188 |
| **As** | 0.253 | -0.159 | 0.089 | 0.264 |
| **Se** | 0.263 | -0.192 | 0.112 | 0.230 |
| **Rb** | 0.299 | -0.015 | 0.025 | 0.064 |
| **Sr** | 0.295 | -0.109 | 0.058 | 0.009 |
| **Mo** | 0.269 | -0.063 | -0.081 | 0.009 |
| **Cd** | -0.055 | -0.297 | -0.450 | 0.196 |
| **Ba** | 0.223 | 0.211 | 0.154 | -0.218 |
| | | | | |
| **Std. Dev** | 3.265 | 1.607 | 1.287 | 1.180 |
| **% of Variance** | 53.3 | 12.9 | 8.3 | 7 |
| **Cumulative %** | 53.3 | 66.2 | 74.5 | 81.5 |

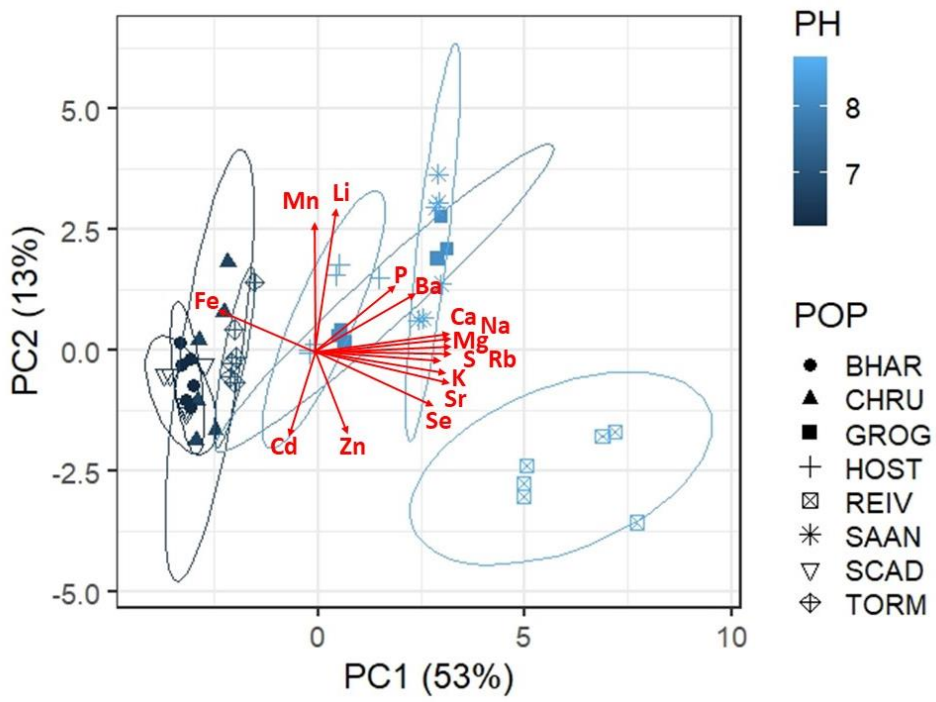



Figure 3.2. Visualisation of principal component analysis of the elemental composition of water samples (n=6 per site). Colours indicate pH and site indicated by point shape (POP) and ellipses. Red arrows indicate highest loadings on PCs labelled by element.

### 3.4.3 Suspended Solids analysis

The first four PCs accounted for 90% of the cumulative variance and, when modelled in a MANOVA, showed that, taken together, PCs 1-4 of elemental variance in suspended solids were significantly affected by both pH (df=1, Residual df=12, Pillai=0.96, $p<0.001$) and site (df=5, Residual df=12, Pillai=1.84, $p=0.02$).

The elements with the highest loading on PC1 were Fe, Se and Rb (Table 3.2) and this was not significantly related to pH of loch water ($p>0.05$). However, the highest loadings on PC2 were S, Ca, Sr and, to a lesser extent, Mn and Zn; this PC was more strongly related to pH ($F(1,5.08)=5.80$, $p=0.06$) when run in a LMM, although PC2 only accounted for 14% of the total variance. Potassium was excluded from analysis because concentrations fell below the detection limit.

Forty-seven percent of the total inertia was explained by BCA with a significant difference (p=0.008, 999 permutations) between sites, indicating site-specific differences in the composition of suspended solids. Samples were identified by LDA to site level with 100% accuracy, including TORM which only had one sample available to be included in analysis.

Table 3.2. PCA loadings of elemental concentrations of suspended solids samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings.

| | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| **Na** | -0.170 | -0.211 | 0.451 | 0.034 |
| **Mg** | -0.253 | -0.285 | -0.015 | 0.136 |
| **P** | -0.283 | 0.151 | 0.033 | 0.061 |
| **S** | -0.149 | 0.422 | -0.102 | 0.226 |
| **Ca** | -0.041 | 0.445 | 0.436 | -0.207 |
| **Li** | -0.289 | -0.120 | 0.055 | -0.011 |
| **Mn** | -0.169 | 0.308 | -0.324 | 0.180 |
| **Fe** | -0.293 | -0.094 | 0.028 | 0.051 |
| **Co** | -0.289 | -0.074 | 0.073 | 0.061 |
| **Ni** | -0.230 | -0.039 | -0.070 | -0.360 |
| **Cu** | -0.198 | 0.125 | -0.293 | -0.424 |
| **Zn** | -0.222 | 0.317 | -0.143 | 0.166 |
| **As** | -0.286 | -0.090 | -0.025 | -0.035 |
| **Se** | -0.290 | -0.059 | 0.034 | -0.030 |
| **Rb** | -0.290 | -0.045 | 0.006 | -0.005 |
| **Sr** | -0.018 | 0.397 | 0.517 | -0.079 |
| **Mo** | -0.248 | -0.218 | 0.238 | -0.055 |
| **Cd** | 0.007 | 0.031 | -0.129 | -0.701 |
| **Ba** | -0.262 | 0.123 | -0.165 | 0.065 |
| | | | | |
| **Std. Dev** | 3.348 | 1.657 | 1.357 | 1.165 |
| **% of Variance** | 59.0 | 14.4 | 9.7 | 7.2 |
| **Cumulative %** | 59.0 | 73.4 | 83.1 | 90.3 |

### 3.4.4 Sediment analysis

The most highly correlated elements on PC1 were S, Li, Fe and Cd, while the highest loadings on PC2 were Cu and, to a lesser extent, Rb (Table 3.3). The first five components explained 89% of the variation in the data and, when

these PC variables were extracted and analysed with a MANOVA, it was apparent that the elemental composition of sediment was significantly affected by both pH (df=1, Residual df=16, Pillai=0.97, $p<0.001$) and site (df=6, Residual df=16, Pillai=3.72, $p<0.001$) across these five PCs. However, when modelled in LMMs with site as a random effect, there was no significant ($p>0.05$) relationship of pH with either PC1 or PC2.

BCA revealed there was a significant (p=0.001, 999 permutations) difference between sites with 78% of the total inertia explained by differences between sites in the elemental composition of the sediment. Samples were identified with 100% accuracy to their loch of origin with LDA, indicating site-specific elemental concentrations in the sediment.

Table 3.3. PCA loadings of elemental concentrations of sediment samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings

| | PC1 | PC2 | PC3 | PC4 | PC5 |
|---|---|---|---|---|---|
| **Na** | -0.278 | 0.123 | 0.238 | 0.049 | -0.273 |
| **Mg** | -0.157 | 0.127 | -0.063 | -0.415 | -0.334 |
| **P** | -0.094 | 0.057 | 0.246 | 0.238 | 0.538 |
| **S** | -0.344 | 0.196 | 0.024 | 0.012 | 0.012 |
| **K** | 0.119 | 0.208 | 0.299 | -0.273 | -0.036 |
| **Ca** | -0.283 | 0.199 | 0.225 | 0.177 | -0.109 |
| **Li** | 0.304 | 0.263 | 0.115 | -0.057 | 0.080 |
| **Mn** | -0.038 | -0.130 | -0.279 | -0.463 | 0.047 |
| **Fe** | 0.291 | 0.289 | -0.096 | 0.051 | -0.151 |
| **Co** | 0.285 | 0.245 | -0.170 | 0.127 | -0.135 |
| **Ni** | 0.282 | 0.265 | -0.141 | -0.108 | 0.094 |
| **Cu** | 0.088 | 0.388 | 0.180 | -0.088 | 0.216 |
| **Zn** | -0.172 | 0.262 | -0.326 | -0.129 | -0.097 |
| **As** | 0.028 | 0.257 | -0.275 | 0.373 | -0.278 |
| **Se** | -0.283 | 0.168 | -0.208 | -0.125 | 0.076 |
| **Rb** | 0.149 | 0.298 | 0.329 | -0.204 | -0.002 |
| **Sr** | -0.268 | 0.158 | 0.254 | 0.140 | -0.209 |
| **Mo** | -0.044 | 0.201 | -0.347 | 0.311 | 0.277 |
| **Cd** | -0.293 | 0.241 | -0.179 | -0.014 | 0.122 |
| **Ba** | -0.191 | 0.137 | -0.077 | -0.279 | 0.424 |
| | | | | | |
| **Std. Dev** | 2.635 | 1.924 | 1.915 | 1.458 | 1.172 |
| **% of Variance** | 34.7 | 18.5 | 18.4 | 10.6 | 6.9 |
| **Cumulative %** | 34.7 | 53.2 | 71.6 | 82.2 | 89.1 |

### 3.4.4 Invertebrate analysis

Both family richness and total dry mass of collected invertebrates tended to increase with loch water pH (Table 3.4) and overall invertebrate numbers varied by site.

Table 3.4. Field survey invertebrate counts, identified where possible to family level.

| **Aquatic Invertebrates** | **REIV** | **SANN** | **HOST** | **GROG** | **TORM** | **CHRU** | **SCAD** | **BHAR** |
|---|---|---|---|---|---|---|---|---|
| Hemiptera-Corixidae (water boatmen) | 46 | 49 | 4 | 6 | 19 | 0 | 0 | 112 |
| Rhynchobdellida (leech) | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other leeches | 0 | 7 | 9 | 10 | 0 | 3 | 0 | 1 |
| Bloodworm | 7 | 0 | 21 | 0 | 0 | 0 | 1 | 0 |
| Other annelids | 6 | 0 | 23 | 0 | 2 | 0 | 0 | 0 |
| Other small worms/larvae | 0 | 0 | 2 | 11 | 0 | 7 | 6 | 0 |
| Zygoptera (damselfly larvae) | 5 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Trichoptera (caddisfly larvae) | 5 | 11 | 1 | 2 | 2 | 7 | 7 | 3 |
| Ephemeroptera (mayfly larvae) | 0 | 2 | 0 | 4 | 7 | 0 | 0 | 5 |
| Tabanidae (horsefly larvae) | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Other diptera | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gammarus | 25 | 22 | 11 | 101 | 7 | 2 | 5 | 0 |
| Other shrimp | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| Hydrachnidia (water mites) | 1 | 17 | 1 | 0 | 2 | 3 | 1 | 4 |
| Hydrophilidae (water scavenger beetle) | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Dytiscidae* (great diving beetle) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| Other coleoptera (2 species at CHRU) | 3 | 0 | 0 | 0 | 0 | 106 | 2 | 0 |
| *Radix balthica* (wandering snail) | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Small black snails | 0 | 672 | 113 | 266 | 0 | 0 | 0 | 0 |
| Large snails | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 1 |
| Other gastropods (small) | 10 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| Bivalves (cockles) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Family Richness** | **13** | **8** | **10** | **10** | **6** | **8** | **6** | **7** |
| **Total Dry Mass (g)** | **0.810** | **0.957** | **0.264** | **0.618** | **0.079** | **0.272** | **0.066** | **0.229** |
| **pH (2019)** | **9** | **8.9** | **8.5** | **8.3** | **7** | **6.6** | **6.5** | **6.3** |

Potassium had the highest loadings on PC1, followed by P and Mo, while Ca, Li, Mn and Ba were most highly correlated on PC2 (Table 3.5). The first four components explained 74% of the variation in the data. These PC variables were then extracted and analysed with a MANOVA which showed that ionomic variance of sampled invertebrates was not significantly affected by pH ($df=1$, Residual $df=26$, Pillai=0.25, $p>0.05$) or site ($df=6$, Residual $df=26$, Pillai=0.49, $p>0.05$).

Initial visualisation of elemental variance for invertebrates suggested that it was more evenly distributed between sample points than for water samples, but was similar to the variance for whole fish data. However, there was no clear difference in the ionomic composition of invertebrates between sites (Fig. 3.3) and a LMM revealed no significant relationship between invertebrate ionomics and pH ($p>0.05$).

Table 3.5. PCA loadings of elemental concentrations of invertebrate samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings.

| | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| **Na** | 0.150 | 0.179 | 0.299 | -0.020 |
| **Mg** | 0.289 | -0.100 | 0.248 | -0.028 |
| **P** | 0.294 | 0.240 | 0.245 | 0.035 |
| **S** | 0.219 | 0.276 | 0.267 | -0.114 |
| **K** | 0.393 | 0.029 | -0.017 | 0.212 |
| **Ca** | -0.241 | -0.311 | 0.070 | -0.237 |
| **Li** | 0.007 | -0.294 | -0.004 | -0.010 |
| **Mn** | 0.176 | -0.326 | -0.055 | -0.019 |
| **Fe** | 0.250 | -0.231 | -0.265 | 0.002 |
| **Co** | 0.206 | -0.048 | -0.325 | -0.128 |
| **Ni** | 0.270 | -0.285 | -0.173 | 0.017 |
| **Cu** | 0.059 | -0.107 | 0.417 | -0.211 |
| **Zn** | 0.196 | 0.241 | -0.303 | -0.247 |
| **As** | 0.134 | -0.288 | 0.045 | -0.393 |
| **Se** | 0.159 | 0.176 | -0.157 | -0.374 |
| **Rb** | 0.218 | 0.009 | 0.298 | -0.247 |
| **Sr** | -0.210 | -0.264 | 0.184 | -0.248 |
| **Mo** | 0.296 | -0.116 | -0.047 | 0.377 |
| **Cd** | 0.072 | 0.199 | -0.274 | -0.448 |
| **Ba** | 0.261 | -0.302 | 0.106 | 0.022 |
| | | | | |
| **Std. Dev** | 2.363 | 2.233 | 1.637 | 1.244 |
| **% of Variance** | 27.9 | 24.9 | 13.4 | 7.7 |
| **Cumulative %** | 27.9 | 52.9 | 66.2 | 74 |

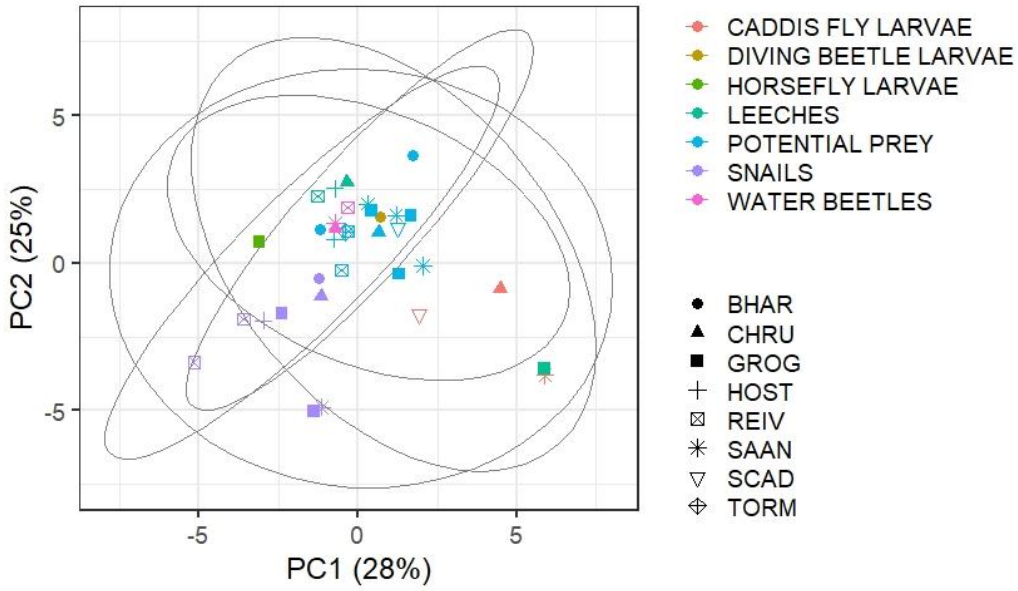



Figure 3.3. Principal component analysis of the ionomic composition of wild caught invertebrates. Points are coloured by family type. Site of origin is grouped by point shape and ellipses. Potential prey in the legend indicates invertebrates most likely to be consumed as a food source by stickleback.

Invertebrate taxa sample data were separated into groups by family (Fig. 3.3). Those indicated here simply as 'POTENTIAL PREY' were invertebrates which were specifically suspected of being consumed by stickleback (See 3.4.5). Snails exhibited higher relative concentrations of Ca and Sr, but otherwise there was little clear separation by taxa. However, when taxon was included in a LMM in addition to pH ($p>0.05$), there was a significant difference in PC1 among invertebrates at a family level ($F(6,23.06)=8.65$, $p<0.001$). This was confirmed when the ionomic variation of invertebrates was modelled in a MANOVA with taxa included, indicating that PCs 1-4 were significantly affected by both pH (df=1, Residual df=20, Pillai=0.59, $p=0.003$) and invertebrate type (df=6, Residual df=20, Pillai=2.28, $p<0.001$), but not significantly affected by site (df=6, Residual df=20, Pillai=0.94301, $p>0.05$).

BCA showed there was no significant difference between sites ($p<0.05$) with only 19% of the total inertia explained by variance in the ionomics of sampled invertebrates. However, Invertebrate samples were predicted with 88% accuracy to loch of origin with LDA.

### 3.4.5 Potential Prey Analysis

Potential prey items were tightly clustered in the centre on the PCA (Fig. 3.3) and so these were further separated out to test whether their ionomic variance was obscured by inclusion in the analysis with other invertebrate species types (Table 3.6). Though these represent a relatively low number of sample points, and too few too run a MANOVA or LMM, the most highly correlated elements on PC1 were Na, Mg, Ca, Li, Cu and Sr (Table 3.7), while the highest loadings on PC2 were K, Fe, Zn and Ca. In addition, LDA predicted potential prey samples to loch of origin with 100% accuracy.

Table 3.6. Invertebrates considered to be potential prey and dry mass of seston/zooplankton collected.

| **Aquatic Invertebrates** | **REIV** | **SANN** | **HOST** | **GROG** | **TORM** | **CHRU** | **SCAD** | **BHAR** |
|---|---|---|---|---|---|---|---|---|
| Hemiptera-Corixidae (water boatmen) | 46 | 49 | 4 | 6 | 19 | 0 | 0 | 112 |
| Bloodworm | 7 | 0 | 21 | 0 | 0 | 0 | 1 | 0 |
| Other annelids | 6 | 0 | 23 | 0 | 2 | 0 | 0 | 0 |
| Other small worms/larvae | 0 | 0 | 2 | 11 | 0 | 7 | 6 | 0 |
| Zygoptera (damselfly larvae) | 5 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ephemeroptera (mayfly larvae) | 0 | 2 | 0 | 4 | 7 | 0 | 0 | 5 |
| Other diptera | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gammarus | 25 | 22 | 11 | 101 | 7 | 2 | 5 | 0 |
| Other shrimp | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| Hydrachnidia (water mites) | 1 | 17 | 1 | 0 | 2 | 3 | 1 | 4 |
| **Potential Prey Total Dry Mass (g)** | **0.125** | **0.104** | **0.056** | **0.153** | **0.079** | **0.016** | **0.038** | **0.194** |
| **pH (2019)** | **9** | **8.9** | **8.5** | **8.3** | **7** | **6.6** | **6.5** | **6.3** |
| **Total Dry Mass Seston Collected (g)** | **0.031** | **0.008** | **0.024** | **0.003** | **0.080** | **0.354** | **0.154** | **0.022** |

Table 3.7. PCA loadings of elemental concentrations of potential prey samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings.

| | PC1 | PC2 | PC3 | PC4 | PC5 |
|---|---|---|---|---|---|
| **Na** | -0.325 | 0.090 | -0.015 | -0.230 | -0.049 |
| **Mg** | -0.296 | -0.002 | -0.034 | -0.378 | -0.042 |
| **P** | -0.226 | 0.264 | 0.224 | -0.193 | 0.073 |
| **S** | -0.269 | 0.288 | 0.133 | 0.020 | 0.190 |
| **K** | -0.086 | 0.377 | 0.297 | -0.052 | 0.176 |
| **Ca** | -0.305 | -0.210 | -0.132 | -0.032 | 0.057 |
| **Li** | -0.291 | 0.087 | 0.063 | 0.330 | -0.153 |
| **Mn** | -0.180 | 0.272 | 0.159 | 0.251 | -0.318 |
| **Fe** | -0.031 | 0.298 | -0.272 | -0.141 | -0.434 |
| **Co** | 0.122 | 0.124 | -0.245 | -0.177 | -0.561 |
| **Ni** | -0.020 | 0.162 | -0.423 | 0.140 | 0.037 |
| **Cu** | -0.304 | -0.192 | -0.030 | -0.124 | 0.109 |
| **Zn** | 0.184 | 0.322 | -0.123 | -0.284 | 0.152 |
| **As** | -0.259 | -0.087 | -0.319 | 0.157 | 0.176 |
| **Se** | -0.010 | 0.218 | -0.413 | 0.068 | 0.366 |
| **Rb** | -0.220 | 0.288 | 0.141 | 0.267 | -0.028 |
| **Sr** | -0.301 | -0.212 | -0.157 | -0.107 | 0.042 |
| **Mo** | 0.098 | -0.130 | 0.355 | -0.346 | -0.005 |
| **Cd** | 0.175 | 0.300 | -0.141 | -0.295 | 0.255 |
| **Ba** | -0.277 | -0.100 | -0.060 | -0.323 | -0.153 |
| | | | | | |
| **Std. Dev** | 2.716 | 2.001 | 1.847 | 1.460 | 1.113 |
| **% of Variance** | 36.9 | 20.0 | 17.1 | 10.7 | 6.2 |
| **Cumulative %** | 36.9 | 56.9 | 73.9 | 84.6 | 90.8 |

### 3.4.6 Seston analysis

Seston samples, which were mostly comprised of zooplankton, exhibited the highest dry mass at CHRU and the mass among lochs was roughly inverse to the dry mass of potential prey samples (Fig. 3.4).

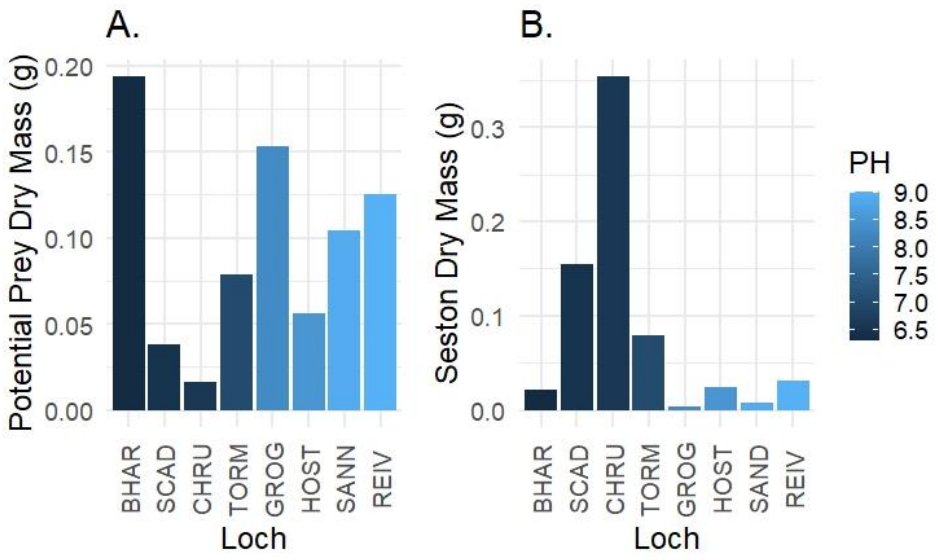



Figure 3.4. Dry mass of potential prey (A) and seston/zooplankton (B) sampled from each site, pH of loch water indicated by colour.

The variation in PC1 was driven by fairly even loadings of Na, Mg, P, S, As, Se, Mo and Cd while the highest loadings on PC2 was Mn and Li and Fe to a lesser extent (Table 3.8). The first three components accounted for 97% of the total variance in data, but there were too few data points to model in a LMM or MANOVA.

Table 3.8. PCA loadings of elemental concentrations of seston samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings.

| | PC1 | PC2 | PC3 |
|---|---|---|---|
| **Na** | -0.256 | 0.051 | 0.031 |
| **Mg** | -0.259 | 0.066 | 0.004 |
| **P** | -0.254 | 0.143 | -0.036 |
| **S** | -0.257 | 0.104 | -0.029 |
| **K** | -0.243 | 0.212 | -0.072 |
| **Ca** | 0.101 | -0.034 | -0.631 |
| **Li** | -0.234 | -0.265 | -0.060 |
| **Mn** | -0.105 | -0.515 | 0.207 |
| **Fe** | -0.227 | -0.268 | 0.127 |
| **Co** | -0.250 | 0.024 | -0.037 |
| **Ni** | -0.104 | -0.442 | -0.294 |
| **Cu** | -0.247 | 0.132 | -0.163 |
| **Zn** | -0.245 | 0.014 | -0.014 |
| **As** | -0.261 | -0.007 | -0.026 |
| **Se** | -0.255 | 0.126 | -0.031 |
| **Rb** | -0.247 | 0.159 | -0.072 |
| **Sr** | 0.086 | -0.085 | -0.637 |
| **Mo** | -0.256 | 0.121 | -0.032 |
| **Cd** | -0.254 | -0.003 | -0.044 |
| **Ba** | -0.154 | -0.482 | 0.036 |
| | | | |
| **Std. Dev** | 3.819 | 1.619 | 1.449 |
| **% of Variance** | 72.9 | 13.1 | 10.5 |
| **Cumulative %** | 72.9 | 86.1 | 96.6 |

### 3.4.7 Stomach Contents analysis

Phosphorus had the highest loadings on PC1, followed by Ca, Zn and Sr. In PC2 the highest loadings were Fe and Co, and to a lesser extent Mn and Ni (Table 3.9). Visualisation of PCs 1 and 2 (Fig. 3.5) revealed a central clustering of sample points without clear distinction by loch pH. A LMM of PC1, with site as a random effect, indicated that the elemental composition of stomach contents was not significantly ($p>0.05$) affected by pH of the loch of origin. However, a MANOVA modelled using the first seven PCs (accounting for 80% of the total variance) showed that stomach contents were

significantly related to both pH (df=1, Residual df=16, Pillai=0.88, p<0.001) and site (df=6, Residual df=16, Pillai=2.21, p<0.001).

BCA indicated there was a highly significant (p=0.001, 999 permutations) difference in stomach contents between sites driven by 39% of the total inertia; LDA identified stomach contents samples to loch of origin with 100% accuracy.

Table 3.9. PCA loadings of elemental concentrations of stomach contents samples with highlights showing the highest loadings driving the variation of the data. Darker blue indicates stronger loadings.

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 |
|---|---|---|---|---|---|---|---|
| **Na** | -0.123 | 0.271 | -0.321 | 0.183 | -0.217 | 0.116 | -0.182 |
| **Mg** | -0.274 | 0.110 | -0.160 | -0.196 | 0.160 | 0.073 | 0.460 |
| **P** | -0.405 | 0.097 | -0.083 | -0.078 | 0.056 | -0.127 | 0.072 |
| **S** | -0.136 | 0.203 | 0.144 | 0.234 | 0.029 | 0.587 | -0.135 |
| **K** | -0.213 | 0.228 | -0.405 | -0.005 | -0.037 | 0.025 | 0.053 |
| **Ca** | -0.301 | -0.022 | 0.064 | 0.177 | -0.358 | -0.445 | -0.010 |
| **Li** | 0.112 | 0.014 | 0.109 | 0.217 | 0.602 | -0.364 | 0.085 |
| **Mn** | -0.091 | -0.301 | 0.256 | 0.039 | -0.058 | 0.098 | 0.129 |
| **Fe** | -0.064 | -0.456 | -0.087 | -0.083 | -0.134 | -0.052 | 0.207 |
| **Co** | -0.134 | -0.449 | 0.095 | 0.007 | 0.097 | 0.078 | -0.061 |
| **Ni** | -0.047 | -0.319 | -0.343 | -0.175 | 0.005 | 0.042 | -0.328 |
| **Cu** | -0.277 | 0.005 | 0.175 | 0.101 | 0.352 | -0.110 | -0.424 |
| **Zn** | -0.306 | 0.060 | 0.140 | 0.136 | 0.294 | 0.261 | 0.141 |
| **As** | -0.214 | 0.032 | 0.203 | -0.475 | 0.039 | -0.062 | -0.076 |
| **Se** | -0.079 | -0.012 | -0.422 | 0.154 | 0.329 | -0.147 | 0.082 |
| **Rb** | -0.268 | 0.109 | 0.104 | -0.499 | 0.030 | 0.059 | 0.136 |
| **Sr** | -0.388 | 0.051 | 0.097 | 0.202 | -0.207 | -0.298 | -0.166 |
| **Mo** | -0.107 | -0.274 | -0.316 | -0.151 | 0.171 | 0.148 | -0.377 |
| **Cd** | -0.112 | -0.282 | -0.200 | 0.316 | -0.040 | 0.114 | 0.389 |
| **Ba** | -0.281 | -0.187 | 0.183 | 0.241 | -0.081 | 0.193 | -0.021 |
| | | | | | | | |
| **Std. Dev** | 2.067 | 1.860 | 1.774 | 1.304 | 1.135 | 1.081 | 1.019 |
| **% of Variance** | 21.4 | 17.3 | 15.7 | 8.5 | 6.4 | 5.8 | 5.2 |
| **Cumulative %** | 21.4 | 38.7 | 54.4 | 62.9 | 69.3 | 75.2 | 80.4 |

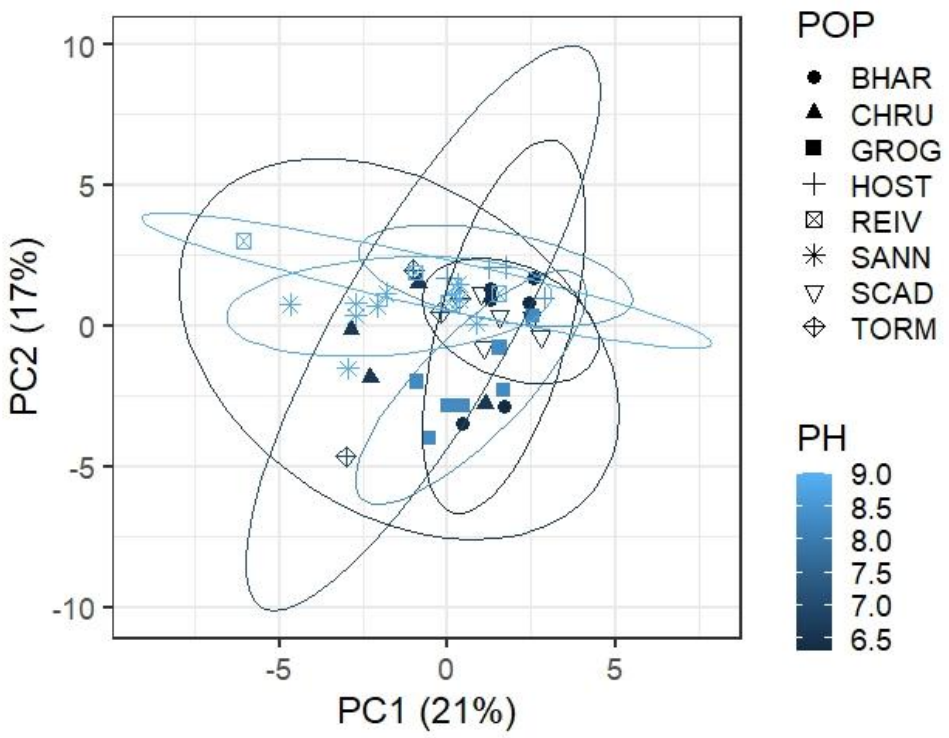



Figure 3.5. Visualisation of principal component analysis of the elemental composition of stomach contents samples from wild caught fish. Colours indicate pH and site is indicated by point shape (POP) and ellipses.

### 3.4.8 Across site analysis of fish ionomics and environmental variation

The highest loadings on PC1 of wild caught fish ionomics were Ca, P and Rb (Table 3.10) and while the mean population scores for this PC were not significantly related to most of the environmental components sampled (Table 3.11), there was a highly significant relationship with loch water chemistry. PC2 of the fish ionomics exhibited a significant correlation with PC1 of water, but was also significantly related to prey and seston PC1 scores (Fig. 3.6). There was also a potential correlation with stomach contents, although this was not quite significant.

Table 3.10. Summary of highest loading elements and percentage of variance covered on PCs from fish and environmental sampling PCAs.

| Principal Component | Highest Loading Elements | % of Variance |
|---|---|---|
| PC1 fish | Ca, P, Rb | 23 |
| PC2 fish | Na, Mg, K, Fe, Se, Cd | 19 |
| PC1 water | Na, Mg, K, Ca, Rb, Sr | 53 |
| PC1 sediment | S, Li, Fe, Cd | 35 |
| PC1 invertebrates | P, K, Mo | 28 |
| PC1 prey | Na, Mg, Ca, Li, Cu, Sr | 37 |
| PC1 seston | Na, Mg, P, S, As, Se, Mo, Cd | 73 |
| PC1 stomachs | P, Ca, Zn, Sr | 21 |

Table 3.11. The relationship between population mean fish ionomic scores (means of PC1 and PC2) and population mean PC values of different environmental components. Models were fitted with GLMs. Statistical significance indicated by *.

| | Estimate | Std. Error | D.F. | t-value | p-value |
|---|---|---|---|---|---|
| (Intercept) | 0.28 | 0.40 | - | 0.69 | - |
| PC1 water | 1.15 | 0.46 | 1 | 2.52 | <0.0001 *** |
| PC1 sediment | -0.83 | 0.39 | 1 | -2.13 | 0.32 |
| PC1 invertebrates | 0.44 | 0.84 | 1 | 0.52 | 0.14 |
| PC1 prey | -0.74 | 0.61 | 1 | -1.21 | 0.62 |
| PC1 seston | 0.09 | 0.15 | 1 | 0.59 | 0.95 |
| PC1 stomachs | -0.83 | 0.55 | 1 | -1.51 | 0.13 |
| (Intercept) | 0.24 | 0.23 | - | 1.04 | - |
| PC1 water | 0.54 | 0.26 | 1 | 2.10 | 0.001 ** |
| PC1 sediment | 0.24 | 0.22 | 1 | 1.08 | 0.48 |
| PC1 invertebrates | -1.17 | 0.48 | 1 | -2.45 | 0.75 |
| PC1 prey | -0.66 | 0.35 | 1 | -1.90 | 0.001 ** |
| PC1 seston | 0.45 | 0.08 | 1 | 5.28 | <0.0001 *** |
| PC1 stomachs | -0.54 | 0.31 | 1 | -1.72 | 0.085 |

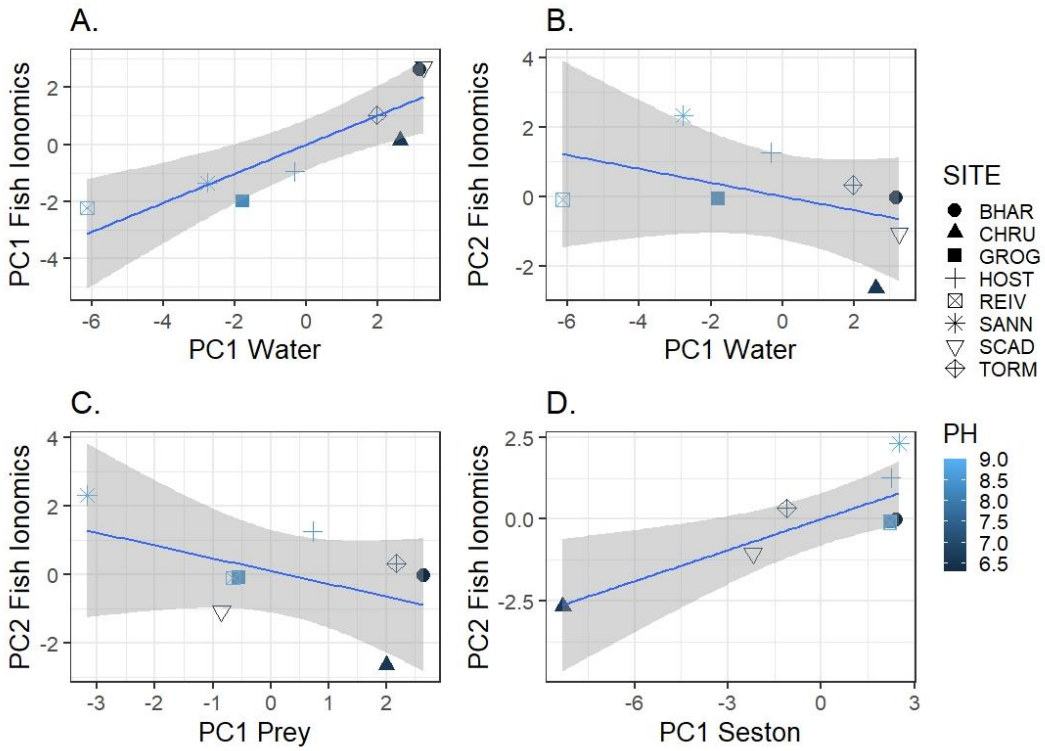



Figure 3.6. Relationships of mean scores for PC1 fish ionomics to PC1 water (A), and PC2 fish ionomics to PC1 water (B), PC1 prey (C) and PC1 seston (D). Site indicated by shape and pH of loch water indicated by colour.

## 3.5 Discussion

It is evident from the analysis and results discussed in this chapter that loch water chemistry plays a clearer role in the ionomics of resident stickleback populations than other surrounding environmental factors. PC1 of fish ionomics, which is mainly driven by the variance of elements associated with bone (see Chapter 2), was highly related to water chemistry and had no significant relationship with any other environmental component. However, PC2 of fish ionomics, which is mainly driven by variation of elements not associated with bone (and is consistent with the findings of Rudman *et al*. 2019), is related to water chemistry, but more strongly associated to nutritional sources of home loch environment. While differences in bone composition are morphologically distinguishable among populations of stickleback (Magalhaes *et al*., 2016), PC2 captures more subtle chemical

variation in stickleback population ionomics and demonstrates the valuable insights that can be gained from analytical approaches such as ICPMS. Additionally, these results can also provide greater insight into how the elemental composition of freshwater fish can be influenced by multiple environmental factors.

Though the initial hypothesis of broad site-specific ionomics across invertebrate populations was rejected in the course of analysis, potential prey species and seston (another nutritional resource) have a significant role in some aspects of the stickleback elemental acquisition and nutritional balancing, namely relating to PC2 (highest loadings of Na, Mg, K, Fe, Se and Cd). This was confirmed with stomach contents analysis to a certain extent as there appears to be a relationship with the second PC of stickleback ionomics across populations, but not quite to a significant level. One potential explanation for this would be selective feeding behaviours and/or contributing factors such as microbiome or nutritional resources including algae/plant life that were not covered in this environmental sampling and analysis and could account for high loadings of P and Zn in stomach contents.

As stated above, it was predicted that invertebrate populations would also exhibit site-specific ionomic distinctions between sites similar to those observed in the fish populations which would carry up the food chain through accumulation. However, the broad ionomic composition of the invertebrate populations that were sampled appeared to be less site-specific and more homogenous in their elemental content within and between sites, although significant differences were observed between species groups. One potential explanation for this could be higher mobility of invertebrates between the various sites on the island, resulting in less site-specific adaptation than fish, which tend to be more isolated and thus more affected by the specific water chemistry they experience over time. LDA was able to predict site of origin for invertebrate samples, but this could be in response to differences in species abundance between sites combined with elemental differences between species though subtler than those observed between stickleback populations.

A more in-depth study of the invertebrate populations could yield more specific answers in regard to these inconsistencies.

While aspects of suspended solids PC2 were affected by pH in a similar relationship to water and stickleback elemental composition, the same is not true for sediment. While sediment composition is significantly affected by site and pH of loch water when modelled across PCs, the same clear relationship shown for fish and water samples is not present in sediment PCs 1 and 2. Sediment composition was site specific, however, and clearly a different set of circumstances dictate how these elements are accumulated rather than the biotic components of the environment. Invertebrate samples PC1 also showed no significant relationship with pH if taxa was not included as a factor and the same was true for stomach contents PC1. There were too few data points to test pH relationships with potential prey or seston samples, but further sampling could help to test this in future studies.

## 3.6 Conclusions

This chapter sought to explore sources of elemental variation in the environment of ionomically distinct populations of stickleback. Through the examination of a range of environmental samples, it has demonstrated that while invertebrate populations broadly do not share the same relationship with pH differences across sites as observed in stickleback, they do display significant differences in their ionomics among taxa. In addition, while water chemistry was shown to be the primary driver in the site/population specific fish ionomics, souces of nutritional availability also likely plays a key role in how these fish have adapted over time to the specific environmental circumstances that surround and sustain them. These results help to explain how fish interact with the components of their environment on an elemental level. The consequences of water chemistry interactions and responses to potential shifts in this aspect of their environment will be addressed in the next chapter.

# Chapter 4. Examining the interactions and consequences of water chemistry through reciprocal water rearing.

## 4.1 Abstract

How do water rearing conditions and rapid shifts in water chemistry affect the ionomics and development of freshwater fish populations? Rearing environments affect development and phenotypic plasticity, influencing the evolution and divergence of species. Freshwater fish that have colonised de-glaciated habitats, offer opportunities to explore adaptive diversity through integrative approaches of ecological, evolutionary and developmental (eco-evo-devo) frameworks. Three-spined stickleback populations, inhabiting the varying chemical conditions across the island of North Uist, were reared in three systems set up on the island with loch water to mimic natural chemical conditions. Loch water and pure crosses of fish representing alkaline, neutral and acidic water conditions were used for reciprocal water rearing experiments in order to better understand the effects of water chemistry on more passive processes of elemental assimilation (homeostasis and gill function), while nutritional availability was standardised across treatments. Water conditions of the rearing environment impacted hatching rates and hatching success, as well as fish growth rates, ionomics and bone development. All fish appeared to benefit from alkaline water rearing, while fish from the alkaline home conditions struggled to hatch in water with a lower pH. Fish from acidic home conditions appeared to be more adaptable to sudden shifts of water chemistry. These results reflect broad adaptive phenotypic trends of fish inhabiting the island and give us a better understanding of how elemental variation in rearing environments influence ionomics, development, phenotypic plasticity and evolution.

## 4.2 Introduction

Environmental factors critically inform developmental processes that influence phenotypic plasticity but understanding these ecological and developmental (eco-devo) interactions is an ongoing process (Danchin *et al*., 2011; Sultan, 2015). Integrative ecological, evolutionary, and developmental (eco-evo-devo) frameworks seek to address the complexity of phenotypic variation through the application of novel theories and unique approaches to particularly well-suited systems. These include aspects such as freshwater fishes that inhabit recently de-glaciated environments (Skulason *et al*., 2019). Studies that take into account factors such as ontogenetic plasticity by translocating fish between differing environmental conditions (e.g. Pilakouta *et al*., 2023), have the potential to help us understand the selection shaping the evolution of extant diversity. Currently it is not known to what extent water rearing conditions and rapid shifts in water chemistry affect fish populations and influence their ionomic composition and development.

In addition to active assimilation through nutritional acquisition and gut absorption, aquatic animals can absorb elements from the surrounding environment in a number of other ways. Pathways include absorption through skin (Glover *et al*., 2013; Ferrie *et al*., 2014, Glover *et al*., 2016) and gill epithelium (Bury and Grosell, 2003; Blewett and Goss, 2017). These processes represent alternative modes of assimilation to active feeding behaviours and involve the uptake of elements that can be beneficial (Hogstrand *et al*., 1996; Bury *et al*., 2003) and in some cases, detrimental (Bury *et al*., 1998; Blewett and Leonard, 2017).

Rearing water conditions have been demonstrated to affect ionomics of stickleback in comparisons of freshwater to saltwater (Rudman *et al*., 2019), but such findings are yet to be tested over multiple chemically varied freshwater sites. Stickleback populations on the island of North Uist offer the opportunity to better explore the developmental effects and consequences of adaptation to chemically differing environments (Haenel *et al*. 2019) as well as gain insights into physiological impact of potential ionregulatory

disturbances in response to sudden changes in freshwater chemistry (Zimmer *et al*., 2021).

This chapter investigates the hypothesis that the water chemistry of differing rearing conditions would significantly affect ionomics and development, especially bone formation. These effects would be particularly evident in fish from alkaline home water when exposed to acidic rearing water, as these acidic conditions differ more greatly from current and ancestrally experienced environments (Haenel *et al*., 2019). The effects would be reduced in fish from acidic home water that have adapted to more extreme ecological conditions (Haenel *et al*., 2019) when exposed to more alkaline rearing water.

## 4.3 Methods

### 4.3.1 Experimental overview

In order to investigate the effect of water chemistry and homeostasis on fish ionomics, feeding treatments were kept the same across populations and experimental groups, while fish were reared in their own and each other's water conditions. Fish were reared over a 7-week field season on the island of North Uist, starting at the beginning of May 2021. Stickleback were used from pure crosses of three of the populations previously examined (Chapter 2; REIV, TORM and BHAR) whose home loch water exhibit differences in chemistry, elemental availability and pH. The three lochs are part of a pH gradient, chosen to represent an alkaline (REIV pH = ~8.3), neutral (TORM pH= ~7.3) and acidic (BHAR pH= ~6.3) conditions (Fig. 4.1). Inductively Coupled Plasma Mass Spectrometry (ICPMS) was used to analyse the elemental composition of fish and water samples, while bone and cartilage of sampled fish were stained to identify any developmental differences between fish reared under different loch water conditions.

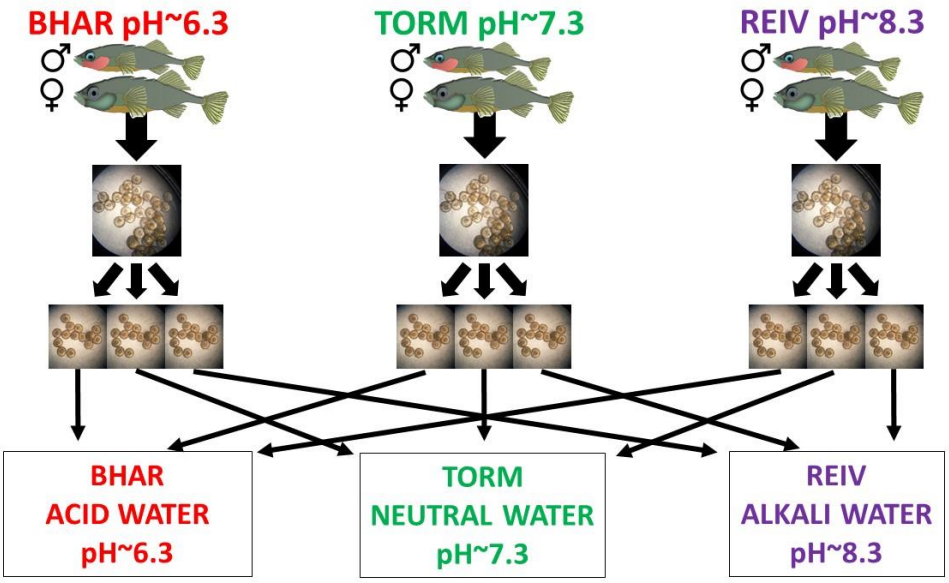


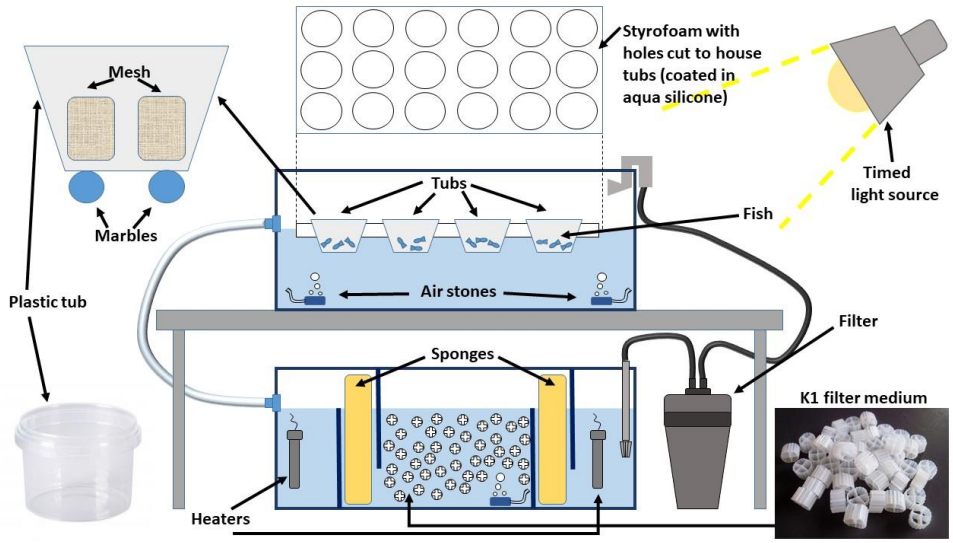

Figure 4.1. Design of reciprocal water rearing experiment. Loch and approx. water conditions of origin of fish shown at the top. Conditions and origin of rearing water shown at the bottom.

### 4.3.2 Field rearing systems

Three identical breeding systems (Fig. 4.2) were filled with filtered water from the three study sites and the natural microbiomes were allowed to populate the systems and K1 filter medium, which was included in systems to increase the surface area for microbiome to grow and maximise water filtration. Water was initially passed through a 100 µm filter sock into 25 L jerry cans and then vacuum pumped through lab grade filter paper (~20 µm), to allow the microbiome to pass through but exclude any parasites or organisms that could act as a potential food source. This was done as soon as possible upon arrival on North Uist to allow ample time for the microbiome to settle and begin stabilising the water chemistry of the systems. Weekly water changes from filtered loch water were also employed to ensure rearing systems mirrored the natural loch chemistry as closely possible. Lighting was timed to natural cycles of sunrise and sunset for the time of year using angled full spectrum aquarium lights in combination with a large skylight in the roof of the temporary laboratory. Rearing chambers consisted of 1L food

safe, chemically inert plastic containers, modified with mesh screens at the sides and bottom to allow water to flow through and suspended on a Styrofoam float that was sealed using a coating of silicone sealant (Everflex Aqua Mate).

Figure 4.2. Design for fish rearing systems used in reciprocal water rearing experiment.




### 4.3.3 Crosses

Multiple straight cross families were made from all three populations at which point eggs from each clutch were evenly divided into three treatment groups, one for each water condition, during the fertilisation process. Fertilisation was carried out in petri dishes containing embryo medium (1 ppt NaCl in dechlorinated H2O), prior to eggs being placed into separate petri dishes according to family and rearing water treatment combinations, and followed by 50% water changes with corresponding rearing system water. This was done once per day over the course of two days to acclimate eggs to rearing water conditions before placing them into rearing systems to hatch. Due to the lower number of eggs in TORM and BHAR clutches (BHAR females in particular, produce smaller clutches of ~30 larger eggs than the other two populations), six families were used from each of these populations while three families were used from REIV. Feeding was ad libitum initially with paramecium, then with artemia cultured using ZM HUFA enrichment (ZM,

www.zmsystems.co.uk) supplement in the artemia's rearing bottles. To reduce the possibility of changes to chemical conditions from natural loch water chemistry, methylene blue was not used to treat eggs.

### 4.3.4 Water samples

Water samples were initially taken from all three lochs (REIV, TORM and BHAR) and then from filtered loch water, and all three systems once per week to determine how closely the rearing tanks chemical conditions mirrored those of the natural environment. This was done by expressing 20 ml of sample water through a 25 mm * 0.22 µm filter (Chromotography.com, Runcorn, Cheshire, UK) into pre-loaded universal tubes containing 1ml of 10% NNO3 in DI H2O. These were examined using ICPMS analysis, requiring no further processing. Daily monitoring ensured pH remained constant in experimental tanks before and after water changes with filtered loch water.

### 4.3.5 Hatching difficulties and developmental delays

With a limited amount of time in the field season to grow fish into the size range necessary to generate enough mass for ICPMS analysis, the aim was to use heaters in the rearing system's sumps to raise the water temperature to the optimum growth range for stickleback (18-21$^{o}$C) once the eggs had hatched. However, an unexpected drop in ambient temperature (to around 9-10$^{o}$C) shortly after eggs were placed into the rearing systems threatened to put fish growth heavily behind schedule. In response, heaters were turned on (this was originally planned to take place once eggs had finished hatching) and the rearing system temperature was gradually raised to 19$^{o}$C over the course of two days.

Once hatching had begun (12 days post-fertilisation), it quickly became clear that while fry from all three populations were hatching with high success in the alkaline conditions, the REIV eggs were slower to hatch or unsuccessful under the two lower pH water conditions. This also lead to fungus growing on the deceased REIV fry which spread to eggs within the clutch and caused

additional eggs to become unviable. These difficulties also affected TORM fish, but with much lower frequency, though an entire family of TORM eggs in the neutral system was lost due to fungus. During this period, any infertile or fungus-afflicted eggs were removed from clutches to reduce further loss of viable eggs.

These complications led to very few REIV fish surviving under the lower pH conditions to the completion of hatching; further deaths of hatched fry, combined with a sudden rise in ambient temperature threatening to push the rearing systems beyond 21$^{o}$C, caused shutting down of the heaters across all three systems. From this point the rearing system's temperature was ambient dependent and not constant. In addition, 24 TORM fry from the alkaline water system and 12 REIV fry from the neutral water system escaped into the sump tank and were excluded from live counts.

Under the lower and fluctuating ambient temperature conditions, it became clear towards the end of the field season, that fry would not grow large enough to conduct ICPMS analysis and staining for bone development. In response it was decided that approximately 40 TORM fish should be transported from each system/water treatment back to Nottingham along with approximately 125 L of filtered water from each loch, to facilitate rearing fish to an adequate size in the aquariums at the Life Sciences Building. TORM fish were chosen due population viability as well as being from the neutral pH condition. Transport of the juvenile TORM fish back to Nottingham meant pooling families together into one transport container per water treatment for the journey due to space constraints.

### 4.3.6 Fish rearing in Nottingham

TORM fish were successfully transported back to the Life Sciences Building at the University of Nottingham where three 100 L capacity tanks had been prepared. One tank was set up for each pH water source (which were kept aerated along with system filter pumps during transport) and rearing system filter pumps were set up for each corresponding tank, so the pre-established biome could continue water filtration. Tanks were set up with approximately

70 L of water each, leaving approx. 50 L of loch water from each source. Remaining water was kept aerated for weekly water changes to tanks and weekly water samples were taken for analysis as described above from the rearing systems. Juvenile TORM fish were reared under these conditions at 13$^{o}$C for a further 41 days until they reached a developmental stage just prior to the point at which cartilage finishes converting to bone and reached a sufficient weight for ICPMS analysis.

### 4.3.7 Degree day calculations

Degree day calculations were applied as outlined by Chezik *et al*. (2013). A To value of 5$^{o}$C, was applied to the stickleback development period of 50 days post-fertilisation (dpf) at 20$^{o}$C at which point all major bone features are fully formed (Currey *et al*. 2017) to determine a figure of 750 degree days (DD) when bone conversion from cartilage would be complete. Applying the same DD calculations to fish for this experiment, TORM fish were grown for 85 dpf (44 days in the field rearing systems with fluctuating ambient temperatures, followed by 41 days in aquarium tanks at 13$^{o}$C) or 730 DD.

### 4.3.8 Fish sampling

For sampling, TORM fish were not fed for 24 hrs prior to being euthanised with an overdose of MS222, weighed and measured. A sub-sample of fish were selected (10 from each treatment group) evenly from across the range of mass for bone and cartilage staining to provide an accurate representation of the variation in development between water rearing treatment groups. This involved submerging fish in 4% buffered PFA for 4 hours, rinsing initially with DI H2O and replaced with 70% ETOH at which point they were stable and prepared for staining. All remaining fish were rinsed with DI H2O and immediately frozen at -80oC and stored for ICPMS analysis.

### 4.3.9 ICPMS Sample processing and analysis

Following freeze drying, sampled fish were acid-digested on a hot plate using concentrated HNO3 (65%; Primar PlusTM) employing an adjusted protocol

that allowed for a reduced level of sample dilution with both HNO3 and DI water for more accurate results using the small sample quantities involved.

### 4.3.10 Fish staining and bone measures

Staining fish for bone and cartilage involved creating a juvenile stickleback staining protocol by incorporating aspects of Walker and Kimmel (2007), Armstrong (2019), and Pilakouta *et al*. (2023). Bijous containers holding the individual fish were numbered 1-30, in order to blind treatment groups, and once staining of subsampled TORM fish was completed, eyes and viscera were removed prior to imaging with a digital microscope. ImageJ (Schneider *et al*. 2012) was used in order to determine the standard length (mm) of each fish and then the segmented line tool was used to measure the length (mm) of how much of each fish's spine consisted of vertebrae that had been fully converted to bone from cartilage, from the juncture of the operculum and spine to the end of the caudal peduncle (Fig. 4.3).

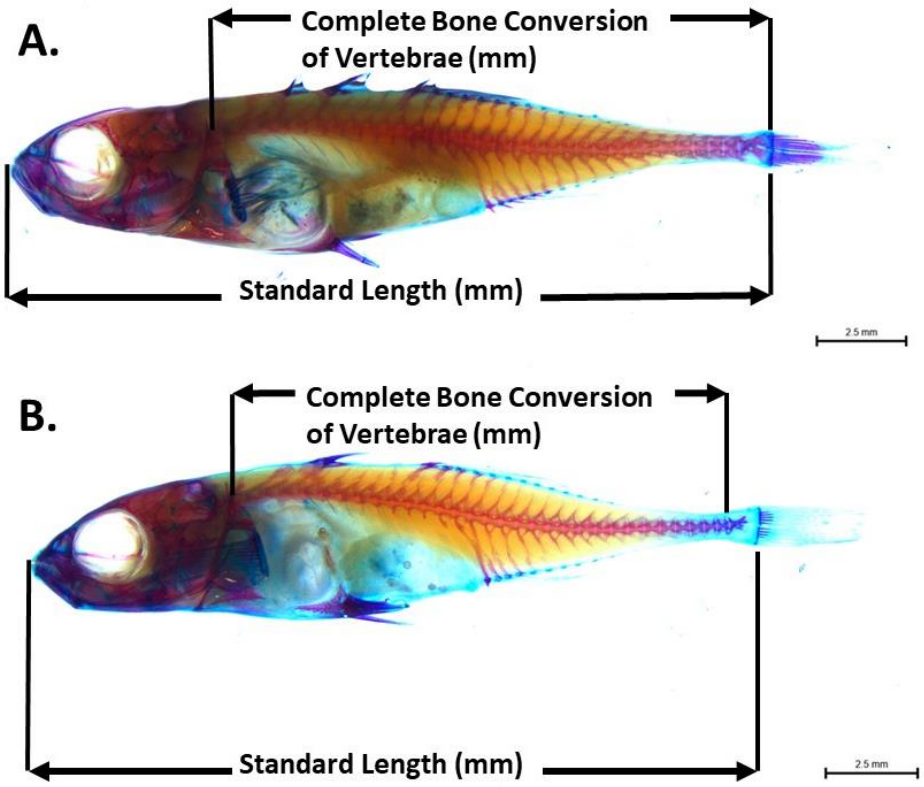



Figure 4.3. Measures used to calculate replacement of cartilage (blue) with bone (red) of stained TORM fish, demonstrating complete bone conversion (A) and partial bone conversion (B) of spine.

### 4.3.11 Data analysis

PCA was conducted on the ICPMS concentration data for 20 elements in both water samples and reared TORM fish using the “prcomp” function in base R (R Core Team 2022). The loadings were then used to determine which variables contributed most to the variation of each PC, considering those with a value >0.25 to be contributing and >0.40 to be strongly contributing. A scree plot of Eigenvalues and the loading table were used to determine an inflection point when the Eigenvalue dropped below the threshold of 1.0. These PCs were then extracted and run in a multivariate analysis of variance (MANOVA), using the “manova” function in MASS with site and water pH as explanatory variables for water samples and treatment (loch water) used as explanatory variable for reared TORM fish.

Egg hatching outcomes were recorded separately as a binomial response for eggs that hatched successfully, eggs that resulted in fry dying due to hatching difficulties, and eggs that died due to fungus. These results were fitted with a generalised linear mixed effect model (GLMM) using the “glmer” function in the R package lme4 with hatching outcome as the response variable, rearing water and population as fixed effects and egg clutch (division into each system), nested within family as a random effect (binomial family and a logit link).

Mass of reared TORM fish from ICPMS and staining were used as a response variables in GLMs, with rearing water as an explanatory variable. Models were conducted using the “glm” function in R, with a Gamma family and an inverse link. Differences in specific elemental concentrations of reared TORM fish required log transformation to reduce overdispersion. Once this was done GLMs were fitted with elemental concentrations as a response variable and rearing water as an explanatory variable, using a Gaussian family and an identity link.

In order to standardise for allometric effects of fish size on bone development in stained TORM fish, a linear model was initially conducted using the “lm” function in R with ossification of vertebrae (See 4.3.10) as the response variable and SL as the explanatory variable. The residuals of this model were then extracted (as this represents the variance in the data not accounted for by differences in body size) and modelled using the “glm” function in R, as the response variable with treatment of water rearing conditions as the explanatory variable (using a Gaussian family and identity link). Of the 10 fish sub sampled for staining from the TORM fish reared under alkaline water conditions, one fish was found to have dwarfism and was removed from analysis as an outlier.

## 4.4 Results

### 4.4.1 Rearing conditions: water from sites of target populations

The highest loadings on PC1 included Na, Mg, S, K, Ca, Co, As, Se, Rb, Sr and Mo (Table 4.1) and were indicative of higher concentrations of all these elements in REIV loch water (Fig. 4.4) while higher Fe concentrations were associated with the lower pH conditions of TORM and BHAR. The elements that were most highly correlated on the PC2 axis were P, Ni, Zn and Ba. The first four components explain 92% of the variation in the data and when these components were extracted and modelled with a MANOVA it showed that elemental variance of water was significantly affected by both pH (df=1, Residual df=31, Pillai=0.55, $p<0.001$) and loch of origin (df=2, Residual df=31, Pillai=1.74, $p<0.001$).

Table 4.1 PCA Loadings of elemental concentrations of rearing water from lochs, with highlights showing the highest loadings driving the variation of the data. Darker blue indicating the strongest loadings.

| | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| **Na** | 0.268 | -0.024 | 0.070 | -0.036 |
| **Mg** | 0.273 | -0.019 | -0.020 | -0.051 |
| **P** | 0.105 | 0.312 | -0.273 | 0.516 |
| **S** | 0.267 | 0.027 | -0.039 | -0.045 |
| **K** | 0.273 | 0.041 | -0.026 | -0.003 |
| **Ca** | 0.272 | 0.006 | -0.061 | -0.037 |
| **Li** | -0.147 | 0.193 | 0.497 | 0.274 |
| **Mn** | -0.130 | -0.106 | -0.526 | 0.329 |
| **Fe** | -0.250 | 0.012 | -0.237 | 0.071 |
| **Co** | 0.251 | 0.103 | -0.219 | 0.051 |
| **Ni** | 0.019 | 0.545 | 0.044 | 0.334 |
| **Cu** | 0.246 | 0.008 | 0.209 | 0.024 |
| **Zn** | -0.089 | 0.427 | -0.048 | -0.522 |
| **As** | 0.270 | -0.024 | -0.044 | -0.054 |
| **Se** | 0.271 | 0.028 | -0.043 | -0.032 |
| **Rb** | 0.272 | 0.061 | -0.030 | 0.037 |
| **Sr** | 0.272 | -0.009 | -0.064 | -0.050 |
| **Mo** | 0.268 | 0.054 | -0.074 | -0.034 |
| **Cd** | -0.143 | 0.183 | -0.466 | -0.349 |
| **Ba** | -0.048 | 0.565 | 0.087 | -0.144 |
| | | | | |
| **Std. Dev** | 3.643 | 1.540 | 1.321 | 1.035 |
| **% of Variance** | 66.37 | 11.85 | 8.72 | 5.34 |
| **Cumulative %** | 66.37 | 78.22 | 86.90 | 92.30 |

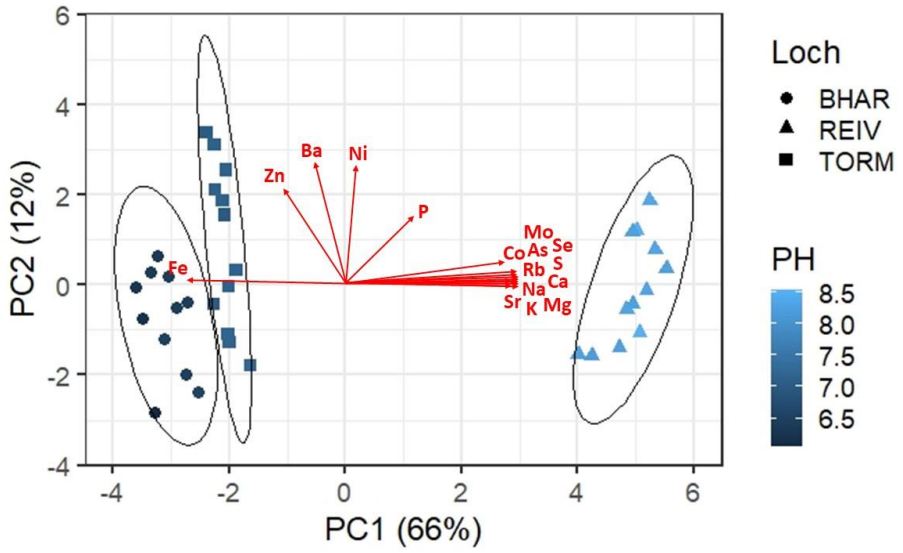



Figure 4.4. Visualisation of principal component analysis of chemical composition of weekly samples of rearing water. Source loch indicated by shape and ellipses, pH indicated by colour and red arrows indicate main loadings, labelled by element.

### 4.4.2 Hatching success and mortality

Hatching rates of the three populations reared under their own and each other's water conditions varied greatly (Fig. 4.5). REIV fish exhibited reduced hatching success as water pH decreased; fish from all three populations had the highest hatching success and fastest hatching rates in the most alkaline conditions. Hatching success was significantly affected by both rearing water ($\chi2(2) = 9.31$, $p=0.01$) and population ($\chi2(2) = 11.52$, $p=0.003$).

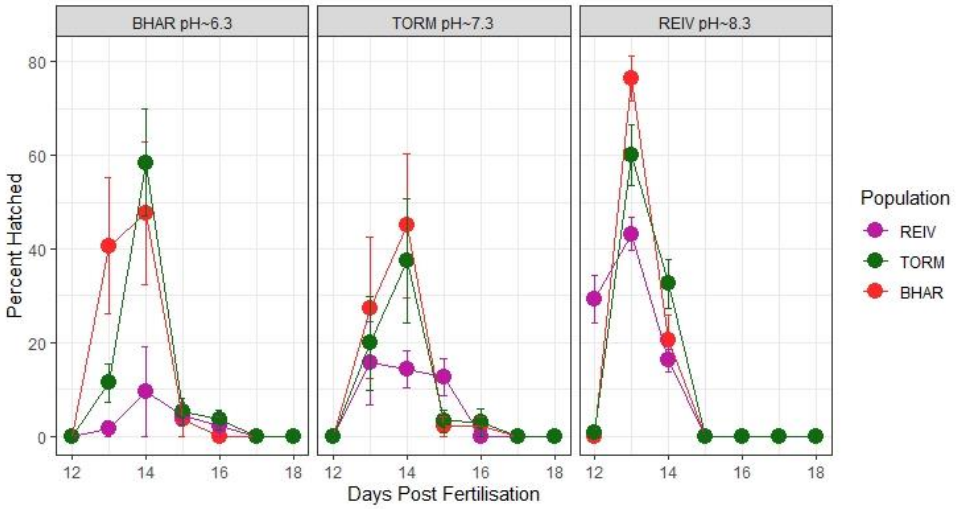



Figure 4.5. Hatching success in rearing systems as a percentage of starting number of eggs. Labelled by rearing water conditions and coloured by loch of origin (Population).

It became clear that REIV fish in particular were struggling to hatch in the two lower pH water conditions and dying as a result. In these cases, the egg chorions appeared to be more flexible than normal, making it difficult for fry to break free (Fig. 4.6). Once these hatching difficulties were identified and monitored, it was evident that this form of mortality affected REIV fry most acutely (Fig. 4.7) and appeared to be in direct response to decreased pH in the other two rearing systems. TORM and BHAR fish appeared to experience these hatching difficulties also, but with a much lower frequency than REIV fish. This form of hatching failure was significantly related to both rearing water ($\chi 2(2) = 21.95$, $p<0.001$) and population ($\chi 2(2) = 18.51$, $p<0.001$).

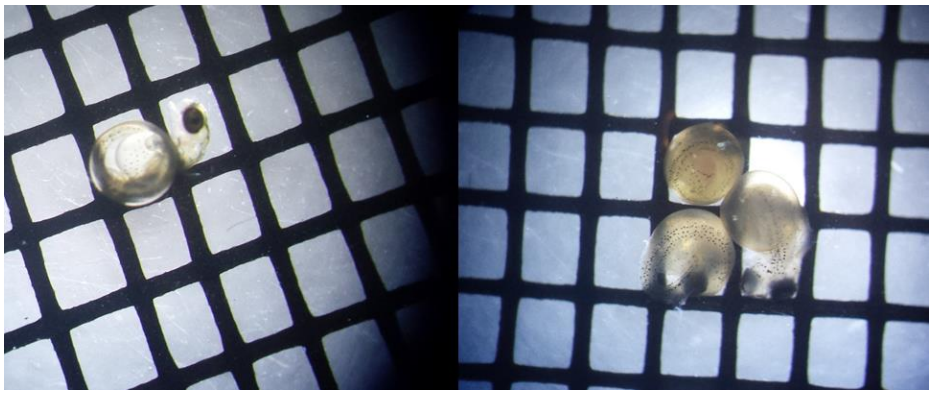


Figure 4.6. Images of fish that died while trying to hatch in rearing systems. Note that fry appear normally formed, but trapped in the chorion.

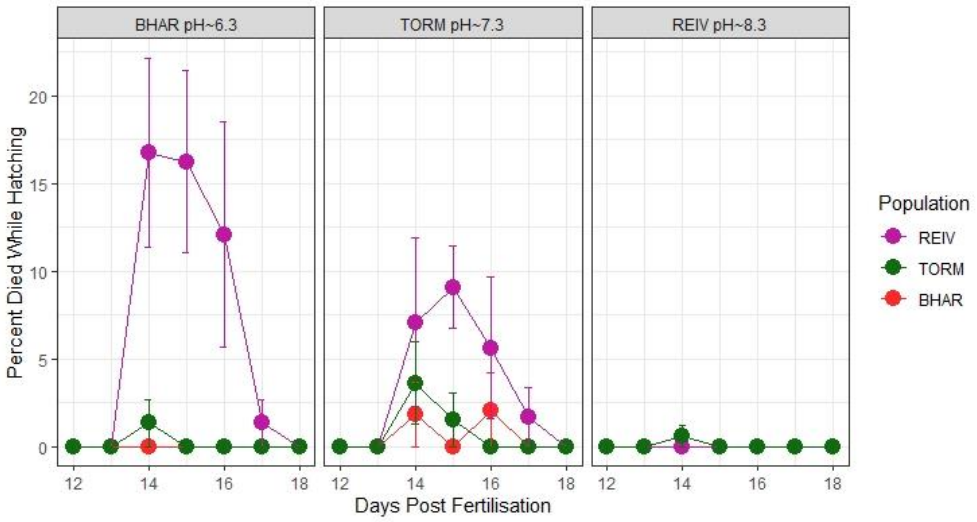



Figure 4.7. Counts of fish that died while attempting to hatch in rearing systems as a percentage of starting numbers of eggs. Labelled by rearing water conditions and coloured by loch of origin (Population).

Without methylene blue treatment, fungus did cause mortality among eggs across populations and systems, though the effects of this form of mortality was more limited under alkaline water conditions (Fig. 4.8). Fungus related mortalities were significantly related to rearing water ($\chi 2(2) = 6.03$, $p=0.049$), but not population ($p>0.05$).

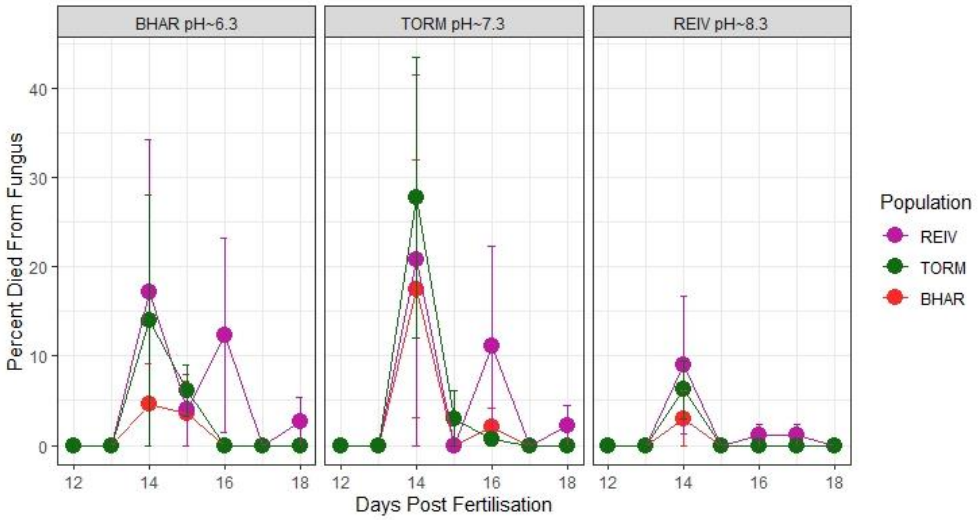



Figure 4.8. Counts of fish that died from fungus in rearing systems as a percentage of starting numbers of eggs. Labelled by rearing water conditions and coloured by loch of origin (Population).

### 4.4.3 Elemental composition of TORM fish

TORM was the only population for which sufficient fish could be reared to a size at which elemental analysis could be conducted on the fish. The highest loading elements on PC1 were Mg, Mn, Co, Sr and Ba (Table 4.2) which were most associated with TORM fish reared in BHAR loch water (Fig. 4.9). Elements that were most highly correlated on the PC2 axis were K, followed by Mg, P, S, Ca, Zn and Rb. The first five components explained 71% of the total variation in the data; subsequent extraction and analysis in a MANOVA indicated that fish ionomics were significantly affected by rearing water conditions ($df=2$, Residual $df=81$, Pillai=1.06, $p<0.001$).

Table 4.2. PCA Loadings of elemental concentrations of TORM fish reared under different loch water conditions, with highlights showing the highest loadings driving the variation of the data. Darker blue indicating the strongest loadings.

| | PC1 | PC2 | PC3 | PC4 | PC5 |
|---|---|---|---|---|---|
| **Na** | 0.145 | 0.127 | -0.283 | 0.231 | 0.406 |
| **Mg** | 0.253 | 0.287 | 0.263 | -0.055 | -0.151 |
| **P** | -0.208 | 0.372 | 0.055 | 0.156 | -0.142 |
| **S** | -0.019 | 0.337 | -0.109 | -0.006 | -0.172 |
| **K** | -0.126 | 0.395 | -0.102 | 0.018 | 0.053 |
| **Ca** | -0.127 | 0.371 | 0.181 | 0.076 | -0.235 |
| **Li** | 0.248 | 0.182 | 0.215 | -0.045 | 0.182 |
| **Mn** | 0.317 | -0.074 | 0.335 | 0.109 | -0.143 |
| **Fe** | 0.127 | -0.089 | 0.269 | -0.156 | 0.099 |
| **Co** | 0.379 | 0.076 | 0.246 | -0.085 | -0.016 |
| **Ni** | 0.152 | 0.129 | 0.103 | -0.085 | 0.655 |
| **Cu** | 0.238 | 0.106 | -0.210 | 0.288 | 0.208 |
| **Zn** | 0.112 | 0.282 | -0.309 | -0.263 | -0.027 |
| **As** | -0.149 | 0.146 | 0.296 | 0.208 | 0.195 |
| **Se** | 0.185 | 0.162 | -0.191 | 0.558 | -0.107 |
| **Rb** | -0.247 | 0.295 | 0.140 | -0.189 | 0.040 |
| **Sr** | 0.363 | 0.135 | 0.094 | -0.022 | -0.151 |
| **Mo** | 0.211 | -0.178 | 0.041 | 0.309 | -0.237 |
| **Cd** | 0.244 | -0.005 | -0.384 | -0.109 | -0.173 |
| **Ba** | 0.268 | 0.107 | -0.221 | -0.458 | -0.088 |
| | | | | | |
| **Std. Dev** | 2.265 | 2.108 | 1.499 | 1.185 | 1.027 |
| **% of Variance** | 25.65 | 22.22 | 11.23 | 7.02 | 5.27 |
| **Cumulative %** | 25.65 | 48.87 | 59.10 | 66.13 | 71.40 |

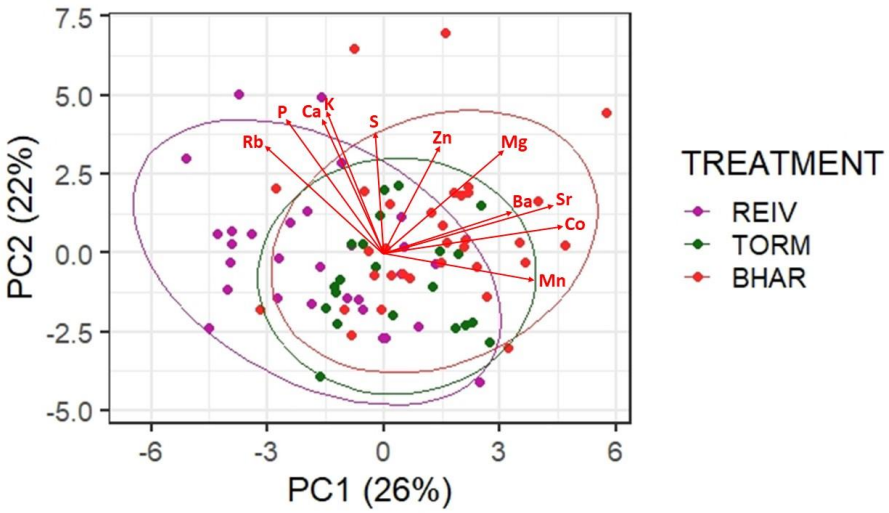



Figure 4.9. Visualisation of principal component analysis of the ionomic composition of TORM fish reared under different water conditions (TREATMENT). Red arrows indicate main loadings, labelled by element.

### 4.4.4 TORM fish elemental uptake

TORM fish mass was significantly affected by rearing water ($\chi^2$ (2)=15.76, p<0.001), with the largest fish being those reared in alkaline water and the smallest those reared in acidic water (Fig. 4.10).

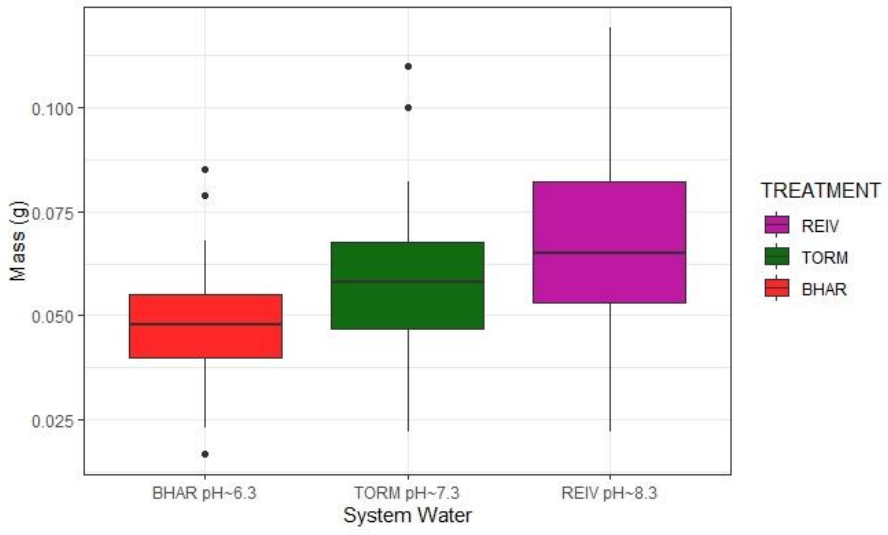



Figure 4.10. Wet mass of TORM fish reared under differing water conditions. Coloured by source of loch water (TREATMENT).

Against the trend of higher mass, there were significantly ($\chi^2$ (2)=48.18, p<0.001), higher concentrations of Zn in TORM fish as pH of rearing water decreased, though these differences followed the trend of available Zn in the three water rearing conditions (Fig. 4.11).

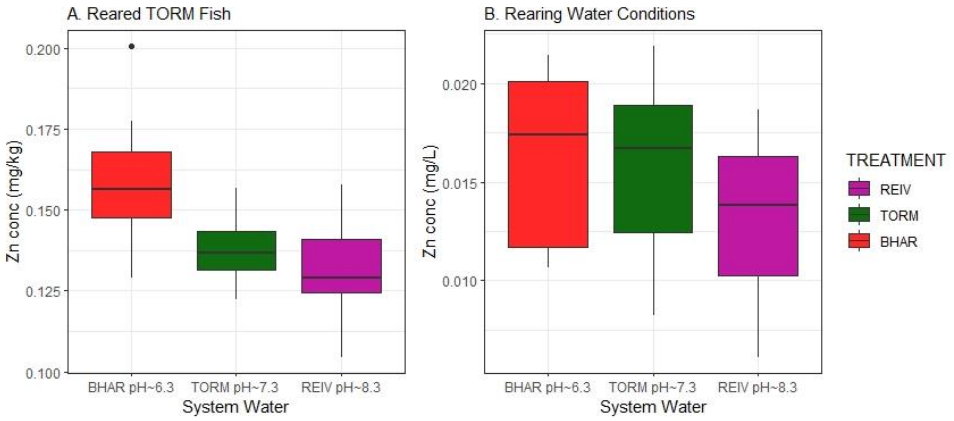



Figure 4.11. Zinc concentrations in dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

However, TORM fish reared in acidic water had significantly higher levels of Na in their tissues ($\chi^2$ (2)=10.19, p=0.006) than expected from the gradient of Na availability in their rearing water (Fig. 4.12). Concentrations of Mg in TORM fish followed a similar trend to Na, with significantly ($\chi^2$ (2)=6.21, p=0.045) higher concentrations of Mg present in fish against the availability of Mg in rearing water (Fig. 4.13).

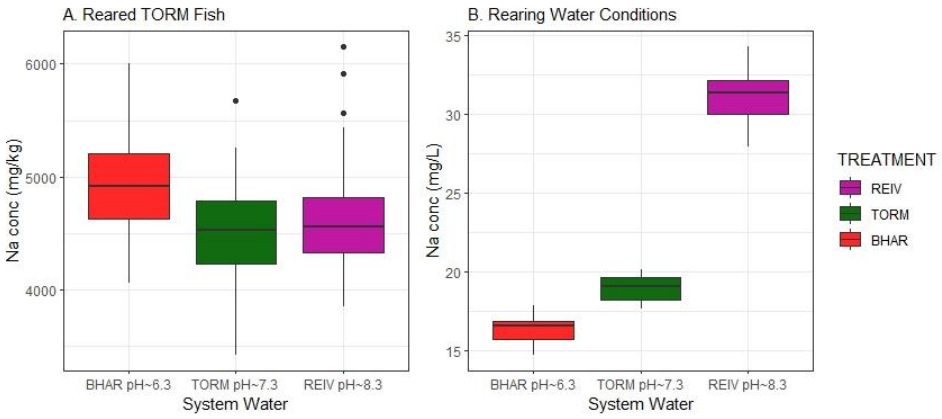



Figure 4.12. Sodium concentrations dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

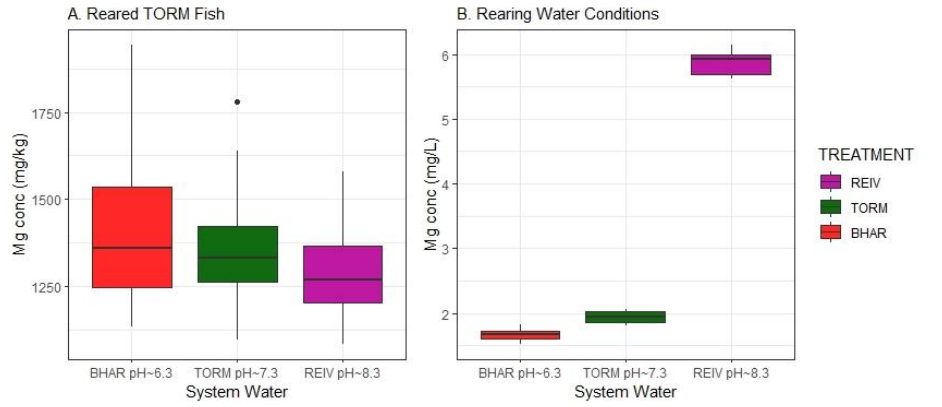



Figure 4.13. Magnesium concentrations dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

Concentrations of K in fish differed significantly by treatment ($\chi 2$ (2)=6.59, p=0.037), with the lowest levels present in TORM fish reared in neutral conditions, though this goes against the lower availability of K in acidic rearing water (Fig. 4.14).

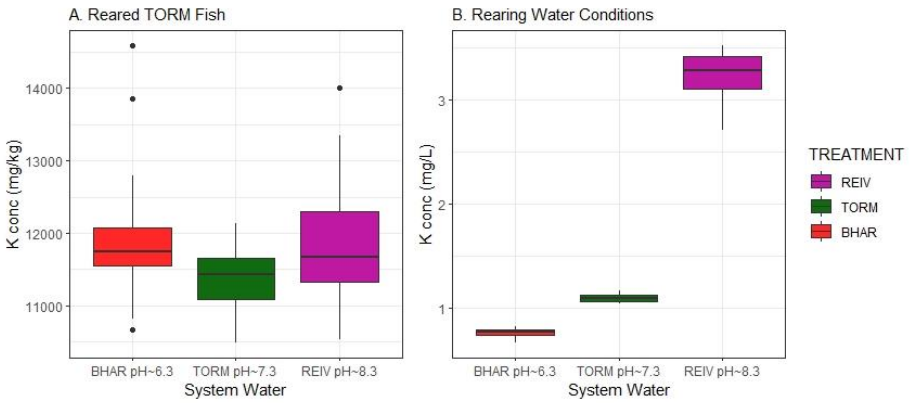



Figure 4.14. Potassium concentrations dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

## Bone associated elements

There was no significant ($p>0.05$) difference in Ca concentrations in fish between treatment groups, despite much higher availability of Ca in REIV water. In contrast, there were significant ($\chi2$ (2)=7.69, df=2, p=0.02) differences in P levels between treatment (Figs. 4.15 and 4.16).

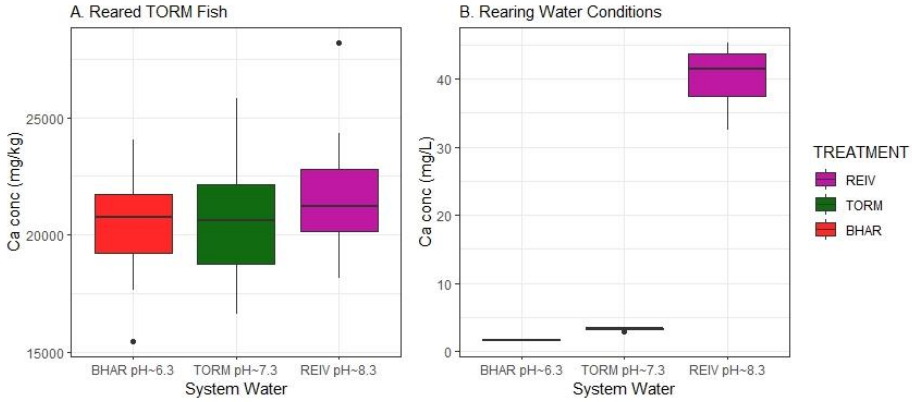



Figure 4.15. Calcium concentrations dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

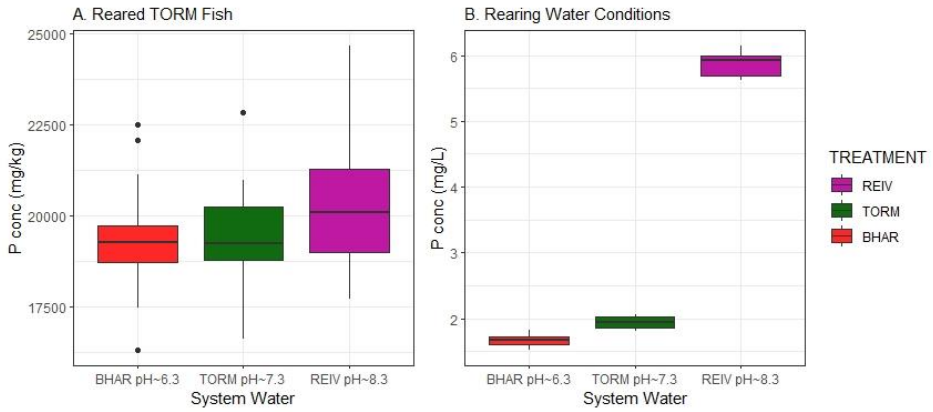



Figure 4.16. Phosphorus concentrations dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

In contrast to P and Ca levels in TORM fish, Sr was present at significantly ($\chi 2$ (2)=16.92, df=2, $p<0.001$) higher levels in fish reared under water conditions with the lowest Sr levels (Fig. 4.17) while fish reared in alkaline water exhibited the lowest Sr levels, despite this water containing by far the highest concentrations of Sr. However, this trend is similar to the pattern in freshwater populations across North Uist (Fig. 4.18), where Sr concentrations in fish increased as pH dropped below 7 against the gradient of Sr concentration in loch water.

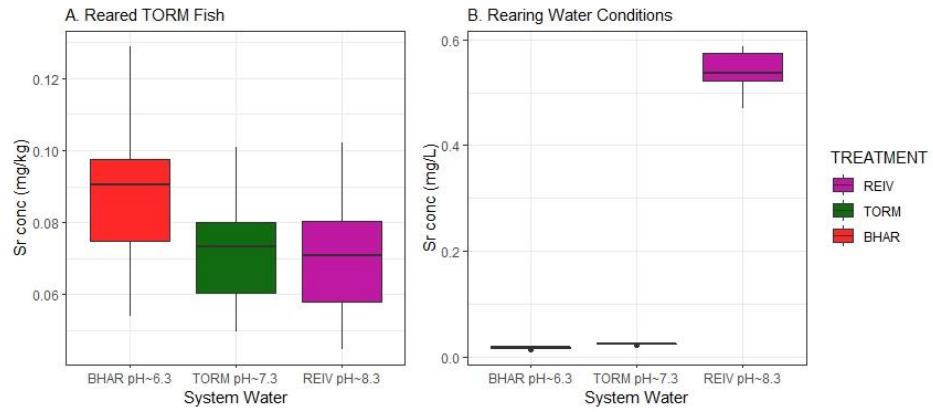



Figure 4.17. Strontium concentrations dry mass of whole TORM fish (A) and rearing water conditions (B), coloured by source of loch water (TREATMENT).

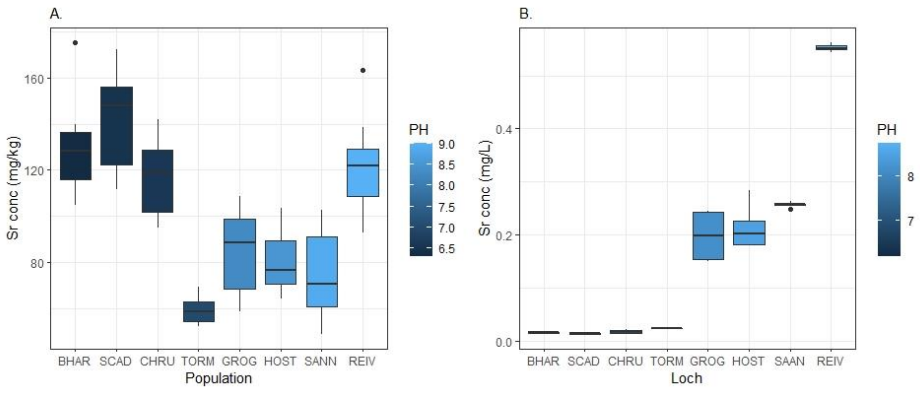



Figure 4.18. Variation in strontium concentrations of whole fish (A) between populations and home loch water (B) from freshwater sites on North Uist, ordered by loch water pH.

## 4.4.5 TORM fish bone development

The mass of TORM fish subsampled for staining differed significantly ($\chi^2(2)=15.47$, $p<0.001$) by rearing water conditions (Fig. 4.19) and was reflective of the fish analysed with ICPMS (Fig. 4.10), suggesting accurate representation of the fish from the three different treatment conditions.

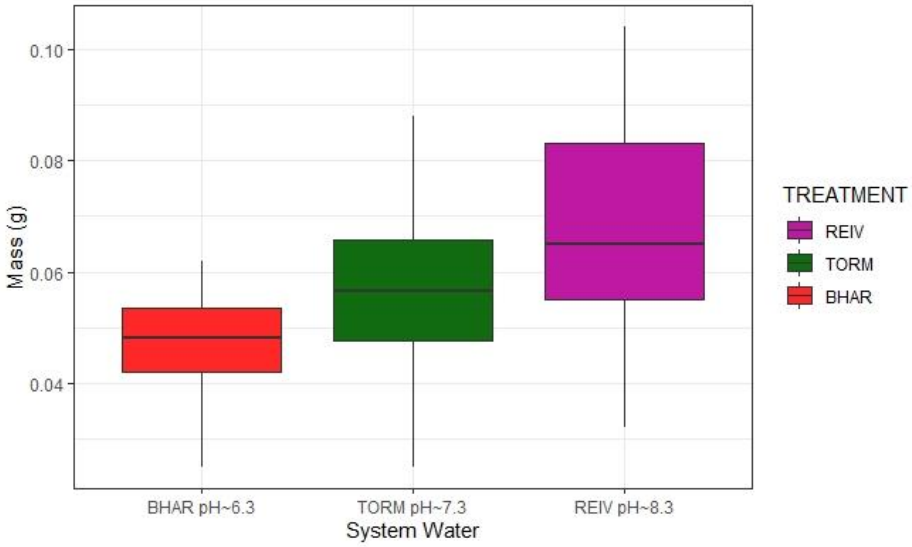



Figure 4.19. Wet mass of TORM fish reared under differing water conditions and sub sampled for staining for bone and cartilage; coloured by source of loch water (TREATMENT).

Once allometric differences of bone development in response to differences in body size had been accounted for, there was still a significant ($\chi 2$ (2)=7.74, p=0.02) difference in bone development between treatment groups (Fig.4.20). This was visually confirmed with TORM fish, size-matched where possible, to less than 0.3 mm, and indicated that ossification was taking place at a significantly slower rate in response to decrease in pH of water rearing conditions (Fig. 4.21).

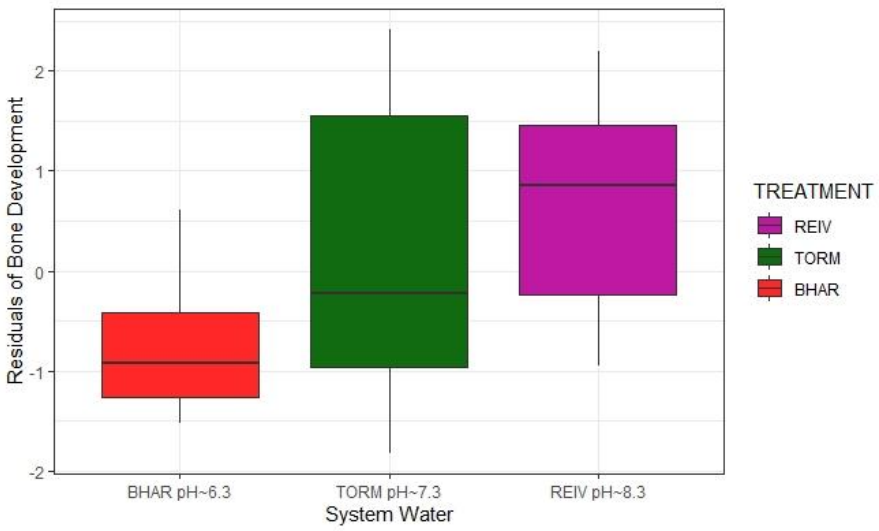



Figure 4.20. Residuals of TORM fish bone development once allometry had been accounted for; coloured by source of loch water (TREATMENT).

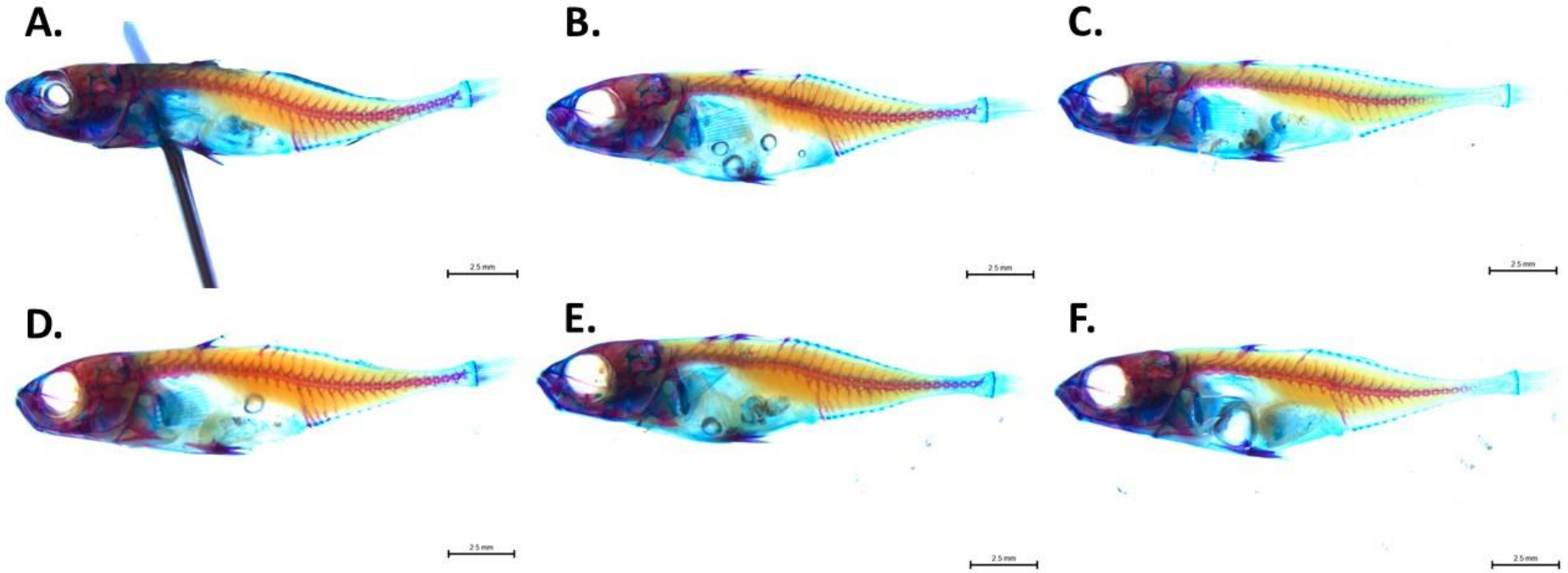



Figure 4.21. Comparison of bone development in stained TORM fish reared in: A. REIV WATER (S.L. =16.88mm), B. TORM WATER (S.L. =16.64mm), C. BHAR WATER (S.L. =16.91mm), D. REIV WATER (S.L. =17.5mm), E. TORM WATER (S.L. =17.22mm), F. BHAR WATER (S.L. =17.41mm).

## 4.5 Discussion

The water in which fish were reared affected their hatching rates and hatching success, ionomics (including specific elemental uptake and retention), growth and development. REIV fish from alkaline home loch conditions particularly struggled with unexpected hatching difficulties that increased as pH of the rearing water decreased. Fish from all three examined populations appeared to benefit from being reared in alkaline water conditions both in hatching success and increased hatching rates over a shorter period of time. These results support the hypothesis that fish that have adapted to more extreme acidic conditions (Haenel *et al*., 2019) are more adaptable across a range of pH, while fish from alkaline conditions are more susceptible to negative impacts from sudden changes in water chemistry. It is notable that while fungal mortalities did occur, this form of mortality did not significantly affect a specific population of the three examined and also appeared to be reduced under alkaline conditions. Hatching difficulties in stickleback populations however, have been linked to differences in egg size, lifestyle and duration of adaptation to fresh water conditions, when exposed to rapid shifts in water chemistry (Kassen *et al*., 1995).

TORM fish exhibited a significant plastic shift in ionomics in response to rearing water and much of the variation among treatment groups involved differences in concentrations of essential elements not associated with bone growth. These results are consistent with the findings of Rudman *et al*. (2019). However, Na, Mg, and K concentrations were highest in fish reared under water conditions where these elements were most scarce. This suggests some form of element-specific compensation mechanism that allows TORM fish to accumulate certain vital elements against the gradient of their availability in the surrounding environment. Bone associated elements (Ca and P) were consistently high in both alkaline water and the TORM fish reared under those alkaline conditions, which was consistent with increased bone development of fish within that treatment group. In contrast, Sr levels were significantly highest in TORM fish reared under acidic water conditions,

where Sr availability was the lowest out of the three water treatments. This result is consistent with Sr concentration trends of populations of acidic fish across the island of North Uist and could indicate incidental uptake of Sr as a result of increased efforts to attain Ca or the compensatory uptake of Sr to bolster bone density in the absence of Ca (Kolodziejska *et al*., 2021). Rudman *et al*., (2019) also recorded increased uptake of Sr in high-plated marine stickleback in comparison to low-plated freshwater stickleback when reared under freshwater conditions of low Sr concentrations. These higher levels of Sr uptake in marine fish exceeded those of wild caught marine fish and may be indicative of the same response of increased Sr assimilation efficiency in response to constrained environmental Ca availability.

The water rearing environment not only significantly affected the growth of TORM fish with an increase and decrease relative to home water conditions in more alkaline and acidic rearing conditions respectively, but also altered the developmental trajectory of the fish in terms of bone conversion from cartilage. These body size and developmental trends follow the broad differences in reduced body size and bone composition of fish populations across North Uist in relation to decreasing pH (Magalhaes *et al*., 2016) and could potentially be indicative of plastic responses in TORM fish to the ecological, developmental, and evolutionary pressures that have influenced the divergence of fish populations inhabiting the varied chemical conditions across the island.

Space limitations during transport back to Nottingham required TORM families to be pooled together and represented a single generation. Nevertheless, this still represents a successful, novel, multi-family approach to gaining greater insights of the chemical evolutionary adaptive divergence of a globally unique, post-glacial freshwater system. The genetic drivers of observed differences in ionomics, body size and development merit further investigation.

## 4.6 Conclusions

These results demonstrate the potential of integrating multi-elemental analysis with more well established investigative techniques in order to examine physiological and developmental consequences of the chemical environment. Further applications of this approach include conservation and re-location (Zimmer *et al*., 2021), aquaculture and investigation of fundamental evolutionary questions (Skulason *et al*., 2019).

# Chapter 5. General Discussion

## 5.1 Ionomics and approaches

When conceptualising the ionome as a fourth level of genomic analysis (in addition to the transcriptome, the proteome and the metabolome), I initially thought in terms of coloured building blocks (representing different elements) constructing an organism, surrounded by other blocks of different colour making up the environment around them. Organisms are surrounded by assortments of different coloured blocks in various forms at certain times, and the question then became, would organisms build themselves to specific proportions of different coloured blocks, by seeking them out from their surroundings or simply arbitrarily gather the blocks that were abundant and immediately available? Life is a condition at odds with entropy and the idea of selective processes targeting what coloured blocks an organism is made from became increasingly intuitive when considering not only the physical barriers organisms construct as their exterior to separate their composition from the relative chaos that surrounds them, but also the multiple forms of complex biological avenues they employ to ensure the coloured blocks they incorporate into their forms are ones that will aid in their composition, rather than hinder. DNA from a certain perspective is essentially extremely well organised groups of elements instructing other elements how to behave. This is of course a massive oversimplification, but one that I found useful at the beginnings of this project. Regardless of the density of the mediums that support and surround us, we are all constantly ‘swimming’ in chemical soup.

Famous examples such as Darwin’s Finches lay the groundwork for now well recognised morphological variation in organisms and highlights extensively studied evolutionary phenomena (Grant and Grant, 1989; Irschick *et al*., 1997; Magalhaes *et al*., 2016), but elemental adaptation has only been accessible and examined relatively recently. The Park Grass experiment, which started in Wales in the 1850’s and is still ongoing, provided indications of how organisms can adapt and diversify to make the most of the elemental circumstances in which they find themselves among a chemical mosaic

(Snaydon, 1970). Such ecological observations were followed with the exploration of the currency of vital elements through ecological stoichiometry (Sterner and Elser, 2002; Andersen *et al*., 2004) and expanded upon (Silva and Williams, 2001; Fraga, 2005; Kaspari and Powers, 2016), to a more inclusive view of element-organism interactions that formed the concepts of ionomics, which are explored in this thesis. Early ionomics work also focused on plants (Salt *et al*., 2008; Baxter, 2009; Huang and Salt, 2016), with a relatively small and recent focus on animal populations (Yu *et al*., 2012; Ma *et al*., 2015; Rudman *et al*., 2019). An interesting parallel to the sedentary lifestyle of plants and isolated populations of freshwater fish, is that both are hostage to their immediate chemical surroundings and must make the most of these conditions or fail to continue (Lane, 2015; Sultan, 2015). Fish are intimately connected to the chemistry of their immediate surrounding when compared to terrestrial animals as the water they inhabit, containing numerous dissolved elements, makes up their living space, larder and latrine.

When considering what elements to include in analysis at the beginnings of this project (see Chapter 1 and Appendix; Table A.1), I relied on several literature resources that were extremely informative (Ma *et al*., 2015; Kaspari and Powers, 2016; Leroux, 2018). The work of Silva and Williams (2001), a vital resource (especially at the time it was published), indicated that the biological function of boron in animals was inconclusive. More than 20 years since, there is great deal of research into the importance of boron such as its role in metabolism, immune function, cell membrane function and embryonic development (Khaliq *et al*., 2017; Abdelnour *et al*., 2018; Bialek *et al*., 2019). There are other examples of our understanding expanding to include the biological importance of elements that were previously less well understood (Kolodziejska *et al*., 2021; Mehri, 2020; Yan *et al*., 2022). The biological role of rubidium for instance, is currently ambiguous, but was included in analysis due to its chemical similarity to and association with, potassium and has a suspected role in stimulating metabolism (Nyholm and Tyler, 2000; Anke *et al*., 2005). While I think the list of elements examined for this project are appropriate and important to maintain consistency throughout the time it was conducted, were I to be asked now ‘which elements are relevant?’ I would

probably say ‘all of them’. All elements interact in their biotic and abiotic forms through a constantly shifting chemical world. I’ve come to the conclusion that much like ecology in general, everything matters depending on the questions asked, the approaches and time taken to investigate, and the technologies available with which to tease apart the contributing factors. None the less, the elemental list used for analysis throughout the course of this project is contextually relevant at the time of its selection, in terms of functional elements that make up animals as a whole.

While whole animal ionomics is extremely useful to examine broad elemental variation across and among populations, there are also a number of research applications to investigate differences in elemental concentrations across individual organs. Such approaches have been conducted in an exploratory fashion in mammals including examination of brain, heart, kidney and liver tissue (Ma *et al*., 2015), and for certain elements in specific organs of fish (Hogstrand and Haux, 1996; Cooper *et al*., 2006: Yoshida, *et al*., 2014) In an ideal world, I would have run all the fish’s organs separately and then could have the best of both worlds, being able to retroactively deduce the whole fish ionomics from the sum of its parts; but practicality, resources, time and the particularly small sample weights associated with running ICPMS on individual organs, currently did not allow for this approach. Such organ based approaches could provide further details of organ specific function for elemental accumulation, allocation and mitigation of detrimental levels of certain elements (Rajowska and Protasowicki, 2012; Webster *et al*., 2013), as well as indicating differences in elemental uptake efficiency if combined with lab based Stable Isotope Analysis and radioisotope experiments and with a particular focus on gill function (Sackville *et al*., 2022).

## 5.2 Field sampling and common garden experiment

Water chemistry plays a critical role in the population specific ionomics of stickleback examined for this research and correlates directly with differences in bone morphology, but also contributes to elemental differences in composition not associated with bone (see Chapters 2 and 3). Rudman *et al*., (2019) also reported non-bone related ionomic shifts in stickleback

associated with water chemistry transitions from saltwater to freshwater. However, in addition to water chemistry, the availability of nutritional resources of prey populations of invertebrates also highly contributes to those same elemental differences in fish that are not associated with bone. The lack of broad site specific ionomics across invertebrate populations was surprising but, in many cases, could be accounted for by mobility between sites meaning they simply do not have to adapt to specific chemical circumstances in the same way fish are required to. This would also make sense in that the zooplankton populations, making up the bulk of seston samples, would be less likely to exhibit mobility between sites and do correlate highly with fish ionomics (even more so than larger prey invertebrates analysed), though unfortunately, there were too few seston samples collected for a site specific analysis. Stomach contents correlations were near significant, indicating the potential for specific feeding behaviours, but that too would require more data to confirm and there is also the possibility of these results being influenced by stomach microbiome (Rennison *et al*., 2019). Nutritional contributors such as algae were not investigated in depth as part of this research, but may have some inclusion in seston samples, and could also be a part of these organism-environment interactions at the ionomic level. Of the three populations examined through the common garden rearing experiment, though from very different home water chemistry, all exhibited plastic shifts in ionomics when reared under the same chemical conditions and yet were still identifiable to the populations of their origins in their elemental composition. These results further reinforces the idea that population specific differences in stickleback ionomics are underpinned by genetic mechanisms of elemental selectivity and are likely adaptive responses to the water chemistry in which they have been isolated in addition to other potential environmental contributors.

Three-spined stickleback are remarkable animals in their plasticity, adaptation and global success at colonising numerous environments. They have long been recognised as an ideal study species for multiple ecological, evolutionary and developmental approaches. The adaptive radiation system present on North Uist is a particularly well suited example for the

investigation of ionomics and the approaches undertaken for this project, but there are a number of areas for which I simply did not have time (mostly due to Covid-19) to collect the necessary data or in some cases examine the data fully that I have collected. The island and the fish populations that inhabit it represent an ever-expanding list of questions and research opportunities, still to be explored. For example, gravid females were excluded from analysis in this project due to the need to standardise results and recognition that these kinds of body changes also alter biochemical properties of fish (Hogstrand *et al*., 1996). I had planned on analysing gravid female fish with ICPMS and running their egg clutches separately through analysis. This would not only provide clarity on direct elemental investment female fish devote to their egg clutches, which could be used to compare differences between populations and clutch size, but could also be compared to cases of females afflicted with the parasite *Schistocephalus solidus*. By analysing the parasite mass separately also, this could be used to determine the nutritional burden of such parasites and how they directly impact fish fecundity.

## 5.3 Reciprocal water rearing experiment

When hatching difficulties of REIV fish in lower pH conditions were identified (see Chapter 4), I conducted a small development series to gain a better understanding of what transpired. This involved going through the process of making an additional three families of pure crosses from each of the three examined populations and evenly separating clutches into the three water treatments upon fertilisation of eggs in the same process used at the beginning of the main experiment. In this instance though, eggs were kept in petri dishes with twice daily water changes from the rearing systems and daily photographs were taken of eggs and hatching fry until all hatching was complete. These eggs were kept at a relatively steady 16$^{o}$C (+/- 1$^{o}$C) and while mortality rates were much lower than they were in the clutches hatched into the rearing systems, REIV eggs (from alkaline home conditions) took 7 days to complete hatching in neutral water and 9 days under acidic conditions (though in both lower pH conditions, these extended periods mostly ended in hatching failure and mortality), while the eggs in all other

population and rearing water combinations took only 2-3 days to complete hatching. The photos from this series were also used to confirm that BHAR eggs (from acidic home conditions) were significantly larger than the eggs produced by the other two populations examined.

While stickleback that produce fewer larger eggs are associated with fish that have adapted to a freshwater benthic lifestyle for a longer period of time (Kassen *et al*., 1995), and while this could be indicative of a greater investment in smaller number of young due to a more stable environment (Pianka, 1970; Cassill, 2019), a by-product of these differences could result in a larger stronger fry with a larger surface area of egg from which to break out, that may help to mitigate such hatching difficulties observed during the course of Chapter 4, under lower pH freshwater conditions. Though the hatching mortalities recorded in the course of the reciprocal water rearing experiment may have been the result of an interaction with both lower pH and temperature (as heaters were in use for the rearing systems), a world where water is trending to become both warmer and more acidic (Caldeira and Wickett, 2003; Archer *et al*., 2004) could make these results ever the more relevant.

## 5.4 Reflections and future research

Through the differences in rearing water treatment in Chapter 4, plastic changes observed in TORM fish (from neutral home conditions) appear to reflect adaptive trends of stickleback populations across the island of North Uist in terms of reduced size and bone development, in response to decreasing pH levels. This could potentially be capturing a glimpse of environmental evolutionary pressures that have helped to shape the diversity of fish in the adaptive radiation across the island through observation of the development of a single generation. Cross breeds between isolated populations of stickleback are currently viable, especially under lab/aquarium conditions which tend to be pH neutral, but this does not speak to potential differences in wild: lifestyle, mating behaviour, sperm viability or parental care. Eggs were fertilised in embryo medium for the rearing experiment, prior to placement in water rearing treatments and fertilisation rates under different

water conditions among populations could also be further explored. Were for instance, a mating pair of REIV fish to find themselves in the acidic conditions in which BHAR fish live, it is difficult to say they would be likely to thrive given the reduction in hatching rate and hatching success recorded, during the research for this thesis. This is combined with greater bone mass associated with fish that inhabit more alkali conditions on the island and reduced bone development displayed by TORM fish under acidic conditions. If such trends of potential ecological reproductive isolation continued in response to lower pH water, with adaptations including: smaller fish, less bone and fewer, larger eggs to help mitigate hatching difficulties, then some populations on the island could represent the potential beginnings of a speciation event induced through chemical circumstance and immigrant inviability (MacColl and Chapman, 2010). A great deal more research would be necessary to confirm such a speculation though. Rearing into multiple generations and a combination of crosses and water rearing treatments could help to examine traits associated with adaptation to differences in water chemistry (MacColl, 2011), combined with a more comprehensive genetic approach to both the developmental/morphological differences among populations and associated with loch water chemistry (Ravinet *et al*., 2018; Bolnick *et al*., 2023), as well as any genetic mechanisms influencing the elementally specific uptake and retention recorded during the research for this project.

An additional speculation regarding TORM fish presented was the following: would reduced bone mass mean less attachment sites for muscle that could require more efficiently working muscle tissue as a result (the strongest muscles in the human body are the tongue and heart because they work without structure)? This could account for certain aspects of the results from chapter 4, that indicate the preferential uptake and retention of elements that were in shorter supply in their rearing treatment, as these include elements that, in addition to other vital roles, are essential for muscle function. Observationally, TORM fish are particularly quick and nimble when compared to fish from other populations, and comparative behavioural studies (startle response tests on young fish for example) combined with chemically varying

rearing treatments and pre and post ICPMS analysis could be used to clarify potential differences between populations in regards to muscle function and elemental uptake and utilisation. The development measure of spine ossification employed for this research was straightforward and effective for the goals of this project. However, a larger data set collection and application of morphometrics could also be used for a more in depth analysis of potential difference in water rearing treatment leading to other developmental effects, such as position and shape of bone structure and body proportions (Pilakouta *et al*., 2023).

A number of challenges were presented in trying to capture a point of development at which not only would fish be a suitable size for ICPMS analysis, but also when bone conversion from cartilage would be close to complete. While there has been older research on stickleback growth and development (Swarup, 1958; Allen and Wootton, 1982), more recent exploration of stickleback development is relatively scarce (Lefebure *et al*., 2017; Currey *et al*., 2017). These resources proved extremely useful in the timing of sample collection for Chapter 4, and Currey *et al*. (2017) address the intra population variance they recorded. However, the range of variation they reported, appears to be much more uniform than that exhibited by the TORM fish raised for this study. There was a surprising amount of variance both in terms of size and ossification among fish of the same population that were fertilised on the same day and sampled at the same time point (Fig. 5.1). The fish examined for this study also appeared to be generally slower in their development than the populations examined by Currey *et al*. (2017), and inconsistencies in results could be affected by differences in the stickleback populations examined, sample size and sampling methods. It is notable that while TORM fish were the only fish investigated at this stage of the experiment, both due to transport constraints and their suitability representing fish from neutral water conditions, they are also the only population on North Uist that do not have ‘armoured’ lateral plates and so investigation into developmental differences of other fish populations as well as size and developmental variation within sampling groups merits further investigation. I was successful in that the largest, most well developed fish

had indeed reached the bone conversion point I was aiming for, but in terms of development, it is often inferred that populations are somewhat homogenous. However, within population variation is always present and a vital component of diversity, phenotypic plasticity and speciation (Danchin *et al*., 2011). This form of within population/family/group developmental variance observed in the fish examined for this thesis could also be investigated further.

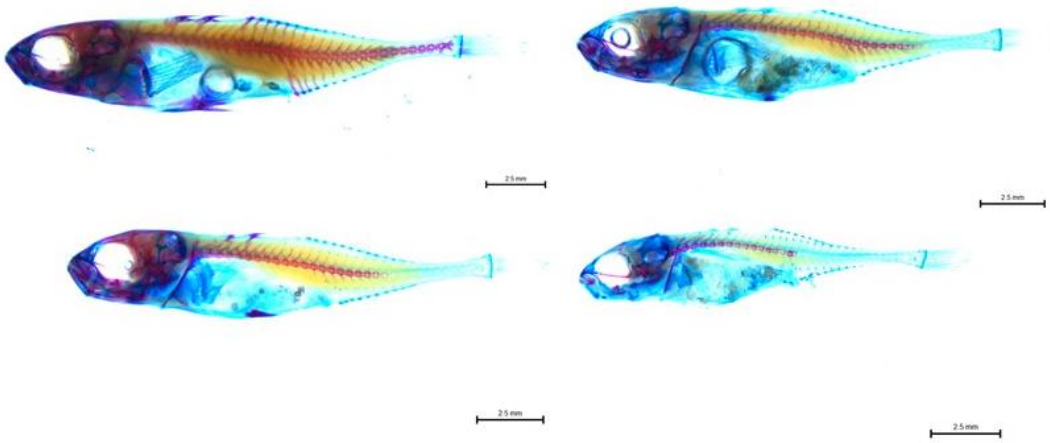


Figure 5.1 TORM fish reared under their own neutral water conditions, exhibiting difference in both size and bone development.

Commercially available microbiome products used to establish bio-filters in aquarium tanks would not have functioned in the pH range examined in the Chapter 4, thus naturally occurring microbiomes were brought in with filtered loch water in order to populate the rearing systems under the logical assumption, that much like the fish, they too had adapted to live under the different conditions of water chemistry. The microbiome is an increasingly well recognised, vital component of biological systems in ecology (Ni *et al*., 2014; Colston and Jackson, 2016; Rennison *et al*., 2019) and so further efforts could be taken into investigating the nature of the microbiome supporting the fish and ecosystems of the different lochs on North Uist to determine how they have adapted and if they are ancestrally related. In regards to another essential component of biome, invertebrate and zooplankton communities sampled, represent relatively small data sets and

additional collection and investigation could help to clarify why the population specific ionomics recorded in the stickleback were not present at this level. As with most ecological studies, the data collected for this project represent a temporal snapshot of the field season (relatively short periods between March and May) and sampling at additional times of year could help to gain insights into how conditions vary over the course of seasons. More data would always be nice, but sadly not always practical.

Among other future plans, I intend to apply the data I have collected to model a chemical speciation water analysis using the Windermere Humic Aqueous Model (WHAM) with Scott Young. This could also be used in combination with geo-biodiversity approaches (Patru-Stupariu *et al*., 2017), and a meta-analysis of research involving chemical concentration data of stickleback tissues to help build a model of how underlying geology, water chemistry and fish ionomics interact at the population level that could potentially be applied to better understand other aquatic systems that exhibit differences in environmental chemistry over a wider geographic area. Sodium links to fish growth could be explored (Chevalier, 2001), as well as the loch CHRU, which exhibits elevated iron levels across multiple environmental components of the site and fish (see Chapters 2 and 3). Iron is an essential micronutrient that can be harmful in particular to gill function if present at detrimental levels (Bury and Grosell, 2003; Bury *et al*., 2003). Investigation of this site could yield further insights into the long-term effects of living within an iron saturated environment, as well as opportunities to examine iron uptake in fish from this population via translocation into conditions of lower iron availability and vice versa for fish from iron deficient conditions.

## 5.5 Conclusions

Results from this project indicate that stickleback ionomics on North Uist is a heritable trait at the population level, but is also subject to plasticity and informed by multiple environmental components, most notably, water chemistry and nutritional availability, with potential differences in selective feeding behaviours. Fish adapted to more extreme acidic conditions appear to be more adaptable to sudden changes in water chemistry than fish from

more ancestrally familiar alkali conditions (Haenel *et al*., 2019) and the chemical nature of their rearing water directly impacts their hatching rates, hatching success, ionomics, growth and development (Table 5.1).

Table 5.1. Summary of hypotheses, questions and results included in this thesis.

| Chapter | Main hypotheses/questions | Results |
|---|---|---|
| 2 | a. Fish populations will exhibit site specific ionomics in response to the chemistry of their home environment.<br>b. Population specific ionomics will have a heritable aspect. | a. Accepted. Elemental composition of fish was site/population specific.<br>b. Accepted. Populations remained ionomically distinct under common garden conditions. |
| 3 | a. How does the chemistry of environmental components relate to the ionomics of fish across sites?<br>b. Invertebrates (prey items) will also exhibit site specific ionomics. | a. Water chemistry appears to be the primary driver of ionomic variation of fish populations, though nutritional availability and selective feeding behaviours are likely to also play a key role.<br>b. Rejected. Broadly, invertebrate ionomics did not exhibit the same site specific qualities as fish. |
| 4 | a. Rearing water chemistry will significantly affect fish ionomics, and development, particularly ossification.<br>b. Affects will be more pronounced for alkaline populations under acidic water conditions. | a. Accepted. Rearing water chemistry affected fish ionomics, growth and development.<br>b. Alkali fish showed significant difficulties in hatching, becoming more acute as rearing water pH decreased. Fish from lower pH home environments appeared to benefit from rearing in more alkali water. |

While my research has left me with more questions than answers (I am told by people wiser than myself that this is quite standard), my hope is that the work presented in this thesis may serve as foundations for others to build and expand upon. I often feel as though I have barely scratched the surface, but the work I have undertaken has demonstrated not only the application of ionomics and multi-element analysis to questions of ecology, evolution and development, but also how such approaches can be integrated with more well established techniques and extremely suitable study systems (Skulason

*et al*., 2019). Integrative approaches can help us better understand interactions between the biotic and abiotic components of environments on the levels of chemical niche, conservation and relocation (Zimmer *et al*., 2021), as well evolutionary trajectories and responses of adaptation to sudden changes in chemical circumstances. Ionomics is an emerging field with many exciting potential applications in research. Humans have become the dominant species on the planet through chemical manipulation of the environment and while the results of these manipulations are still in progress (Abraham *et al*., 2013; Jacquin *et al*., 2020; Malik *et al*., 2020; Spence and Tingley, 2020; Shahjahan *et al*., 2022), further understanding the effects of elemental variation on ecology, evolution and development can help us to mitigate the negative consequences of a rapidly shifting chemical world.

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# Appendix

Table A.1. Summary of elements focused on in analysis, abbreviation and machine detection limit. Limit of Detection determined by 3 times the standard error of 10 operational blanks from stomach contents digest run.

| Element | Abbreviation | Limit of Detection (mg/kg) |
|---|---|---|
| Arsenic | As | 4.39451E-06 |
| Barium | Ba | 0.000424141 |
| Cadmium | Cd | 1.90759E-05 |
| Calcium | Ca | 0.171832473 |
| Cobalt | Co | 7.44142E-06 |
| Copper | Cu | 0.000956565 |
| Iron | Fe | 0.00427755 |
| Lithium | Li | 3.75831E-05 |
| Magnesium | Mg | 0.014101009 |
| Manganese | Mn | 0.000174712 |
| Molybdenum | Mo | 2.1431E-05 |
| Nickel | Ni | 0.000250004 |
| Phosphorus | P | 0.059918918 |
| Potassium | K | 0.013776145 |
| Rubidium | Rb | 6.17302E-06 |
| Selenium | Se | 4.65073E-06 |
| Sodium | Na | 0.028670487 |
| Strontium | Sr | 0.000855344 |
| Sulphur | S | 0.497937871 |
| Zinc | Zn | 0.012513923 |

Table A.2. Summary information of lochs studied for this thesis.

| Loch Name | Code | pH (2019) | Salinity ppt | Average Depth (m) | Area (km2) | GPS Coordinates |
|---|---|---|---|---|---|---|
| Ob nan Stearnain | OBSE | 9.5 | 22.2 | 1.23 | NA | 57.602615, -7.170043 |
| Loch na Reivil | REIV | 9 | 0.18 | 0.74 | 0.061 | 57.610897, -7.5142355 |
| Loch Sanndaraigh | SANN | 8.9 | 0.15 | 0.77 | 0.155 | 57.58663, -7.461821 |
| Loch Hosta | HOST | 8.5 | 0.12 | 4.24 | 0.258 | 57.627483, -7.4899836 |
| Loch an Duin | DUIN | 8.4 | 13.86 | 2.07 | NA | 57.64204, -7.2113123 |
| Loch Grogary | GROG | 8.3 | 0.12 | 0.69 | 0.141 | 57.6151, -7.5105968 |
| Loch Tormasad | TORM | 7 | 0.06 | 1.89 | 0.211 | 57.562363, -7.316438 |
| Loch a' Chadha Ruaidh | CHRU | 6.6 | 0.05 | 0.82 | 0.021 | 57.59369, -7.197437 |
| Loch Sgadabhagh | SCAD | 6.5 | 0.05 | 3.05 | 5.516 | 57.585506, -7.239042 |
| Loch a' Bharpa | BHAR | 6.3 | 0.05 | 4.14 | 0.539 | 57.57059, -7.3011456 |

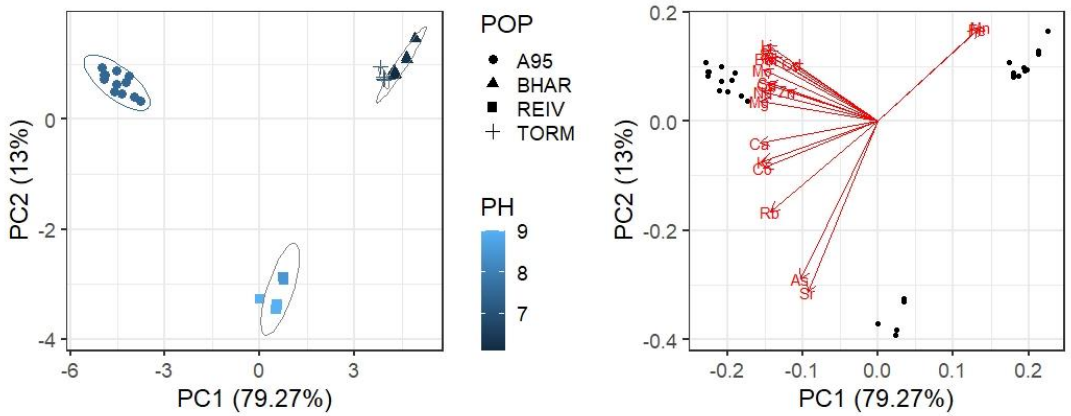


Figure A.1. Visualisation of principal component analysis of the chemical composition of common garden water conditions (A95) and home loch water conditions (left). Principal component analysis biplot of common garden and home loch water conditions. Red arrows indicate main loadings, labelled by element (right).

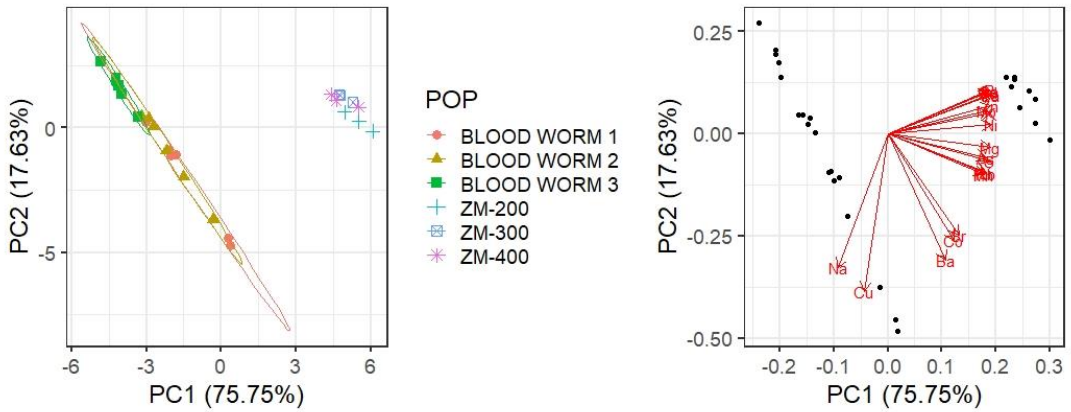


Figure A.2. Visualisation of principal component analysis of the chemical composition of common garden feeds used (ZM-200-400) and samples from three batches of blood worm feed (left). Principal component analysis biplot of common garden feed and blood worm batches. Red arrows indicate main loadings, labelled by element (right).

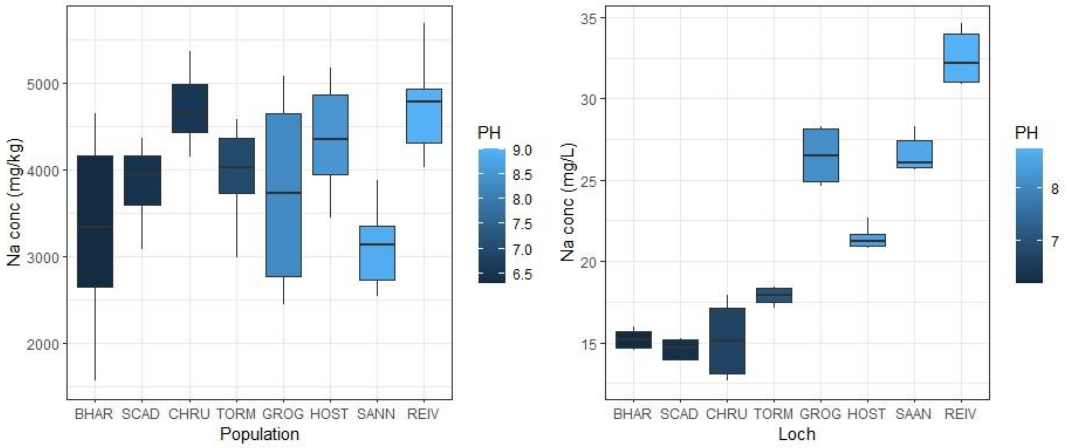


Figure A.3. Sodium levels of wild caught fish (left) and home loch water (right).

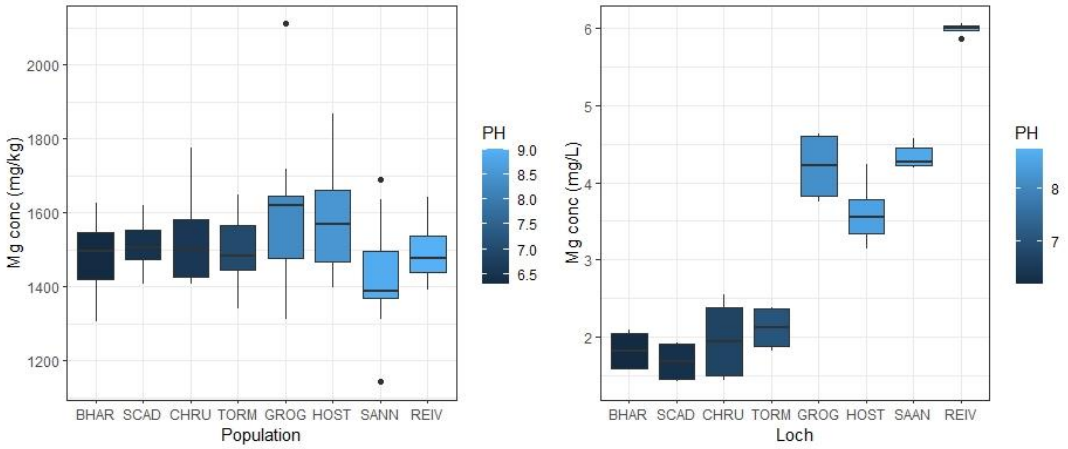


Figure A.4. Magnesium levels of wild caught fish (left) and home loch water (right).

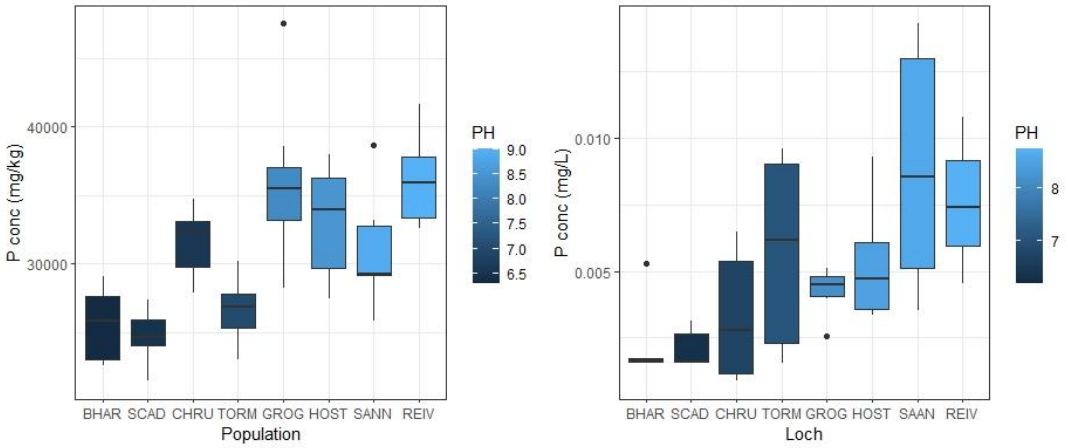


Figure A.5. Phosphorus levels of wild caught fish (left) and home loch water (right).

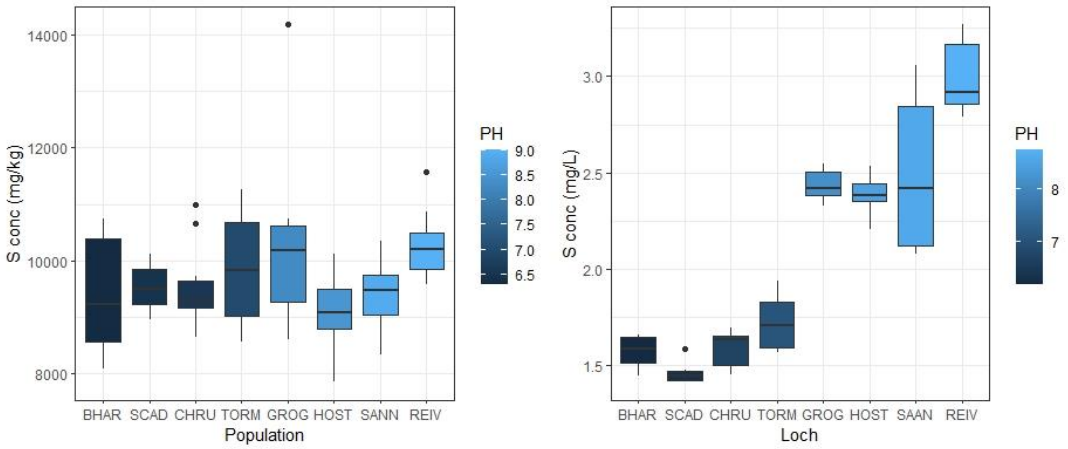


Figure A.6. Sulphur levels of wild caught fish (left) and home loch water (right).

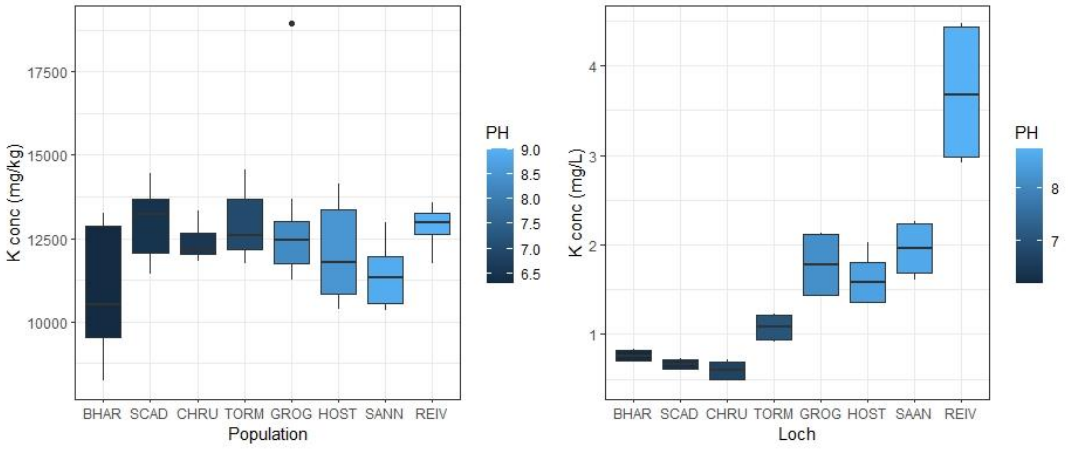


Figure A.7. Potassium levels of wild caught fish (left) and home loch water (right).

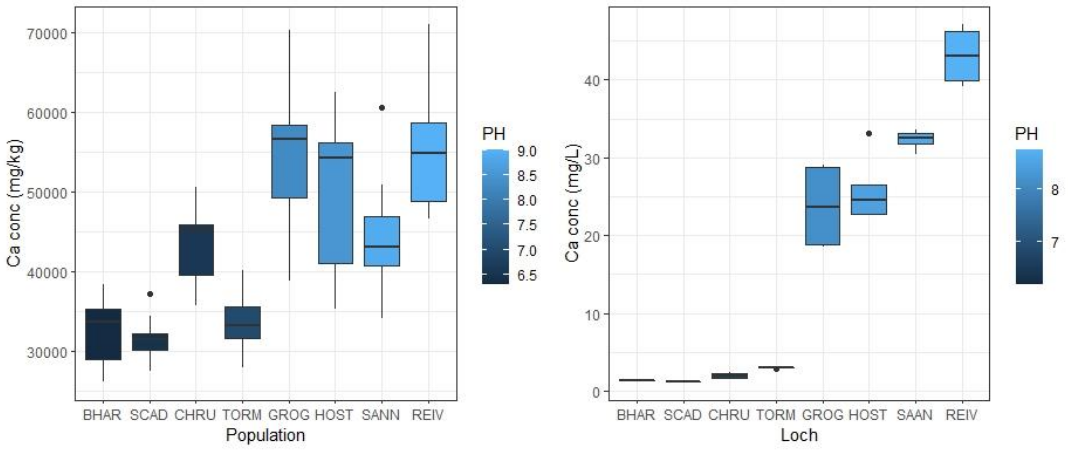


Figure A.8. Calcium levels of wild caught fish (left) and home loch water (right).

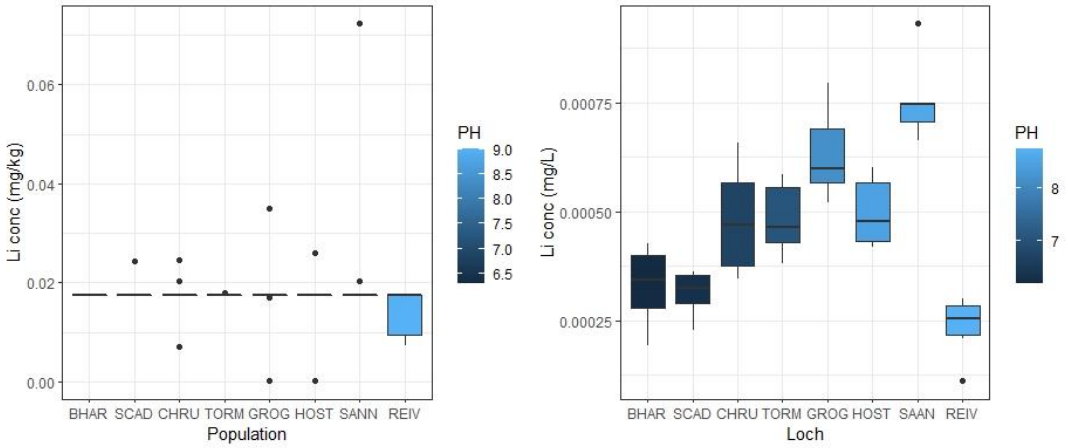


Figure A.9. Lithium levels of wild caught fish (left) and home loch water (right).

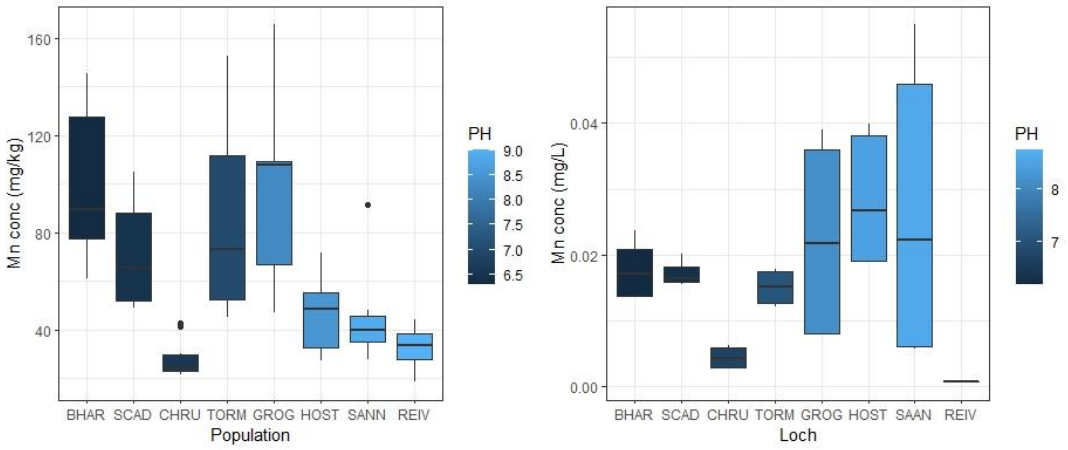


Figure A.10. Manganese levels of wild caught fish (left) and home loch water (right).

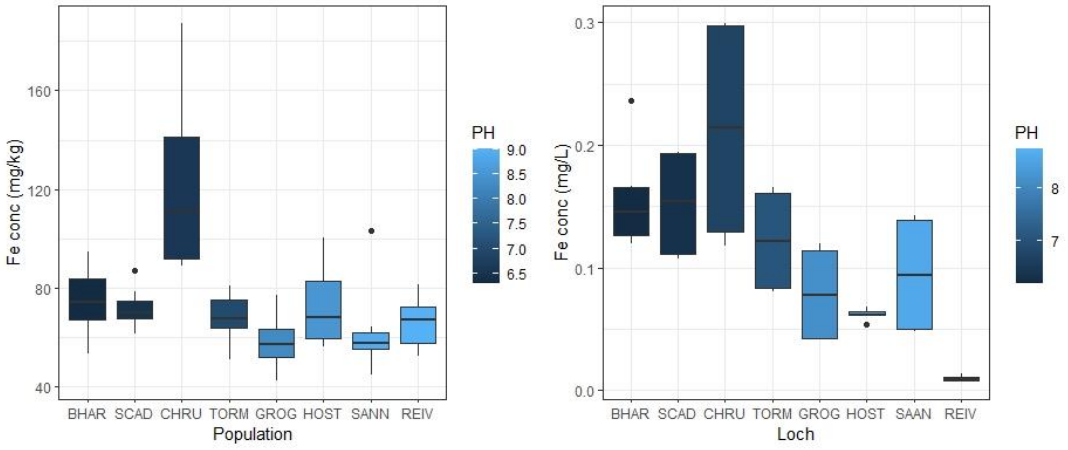


Figure A.11. Iron levels of wild caught fish (left) and home loch water (right).

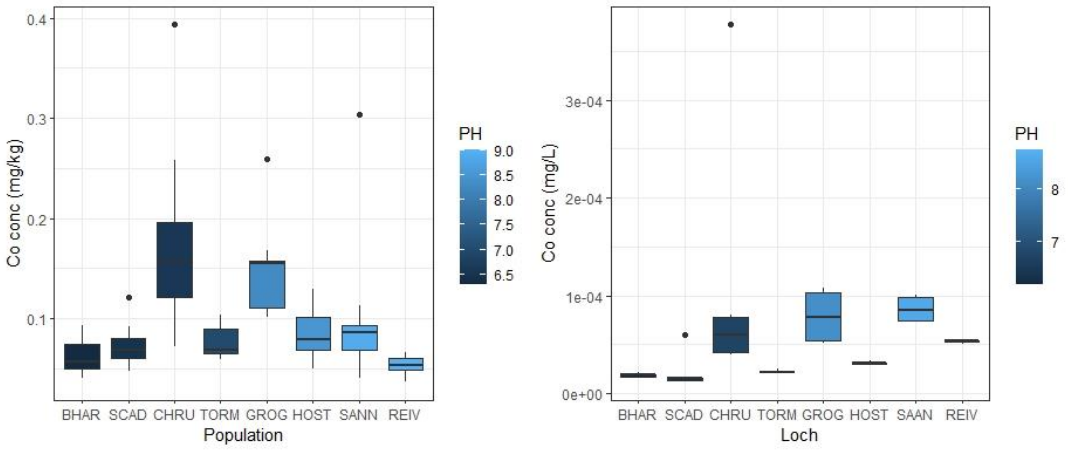


Figure A.12. Cobalt levels of wild caught fish (left) and home loch water (right).

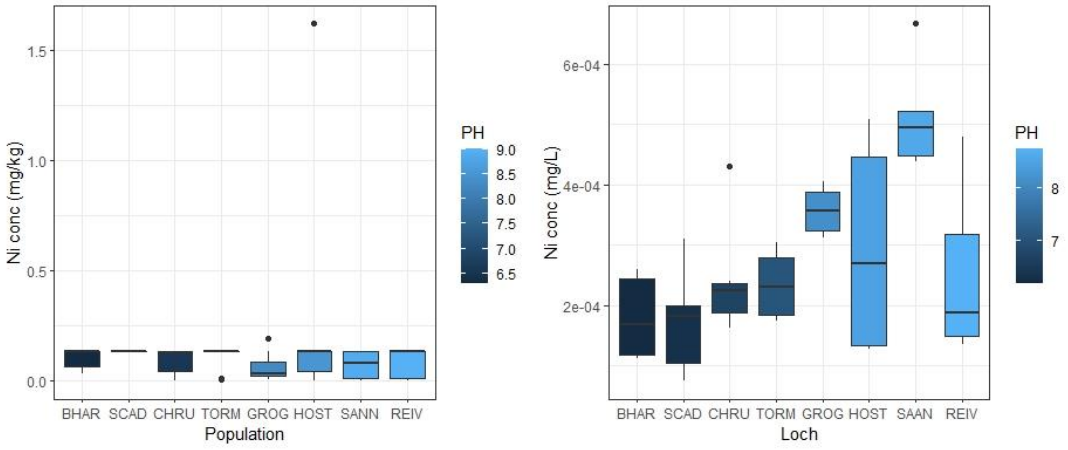


Figure A.13. Nickel levels of wild caught fish (left) and home loch water (right).

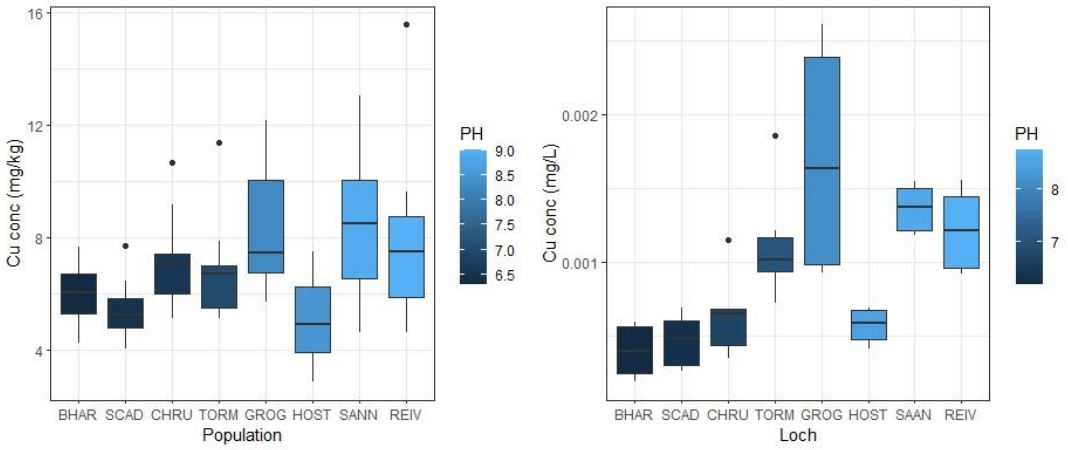


Figure A.14. Copper levels of wild caught fish (left) and home loch water (right).

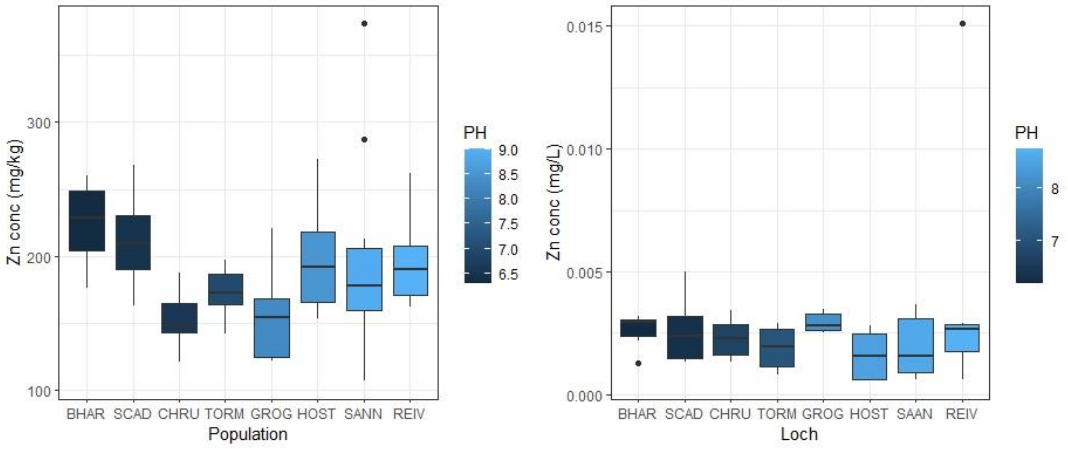


Figure A.15. Zinc levels of wild caught fish (left) and home loch water (right).

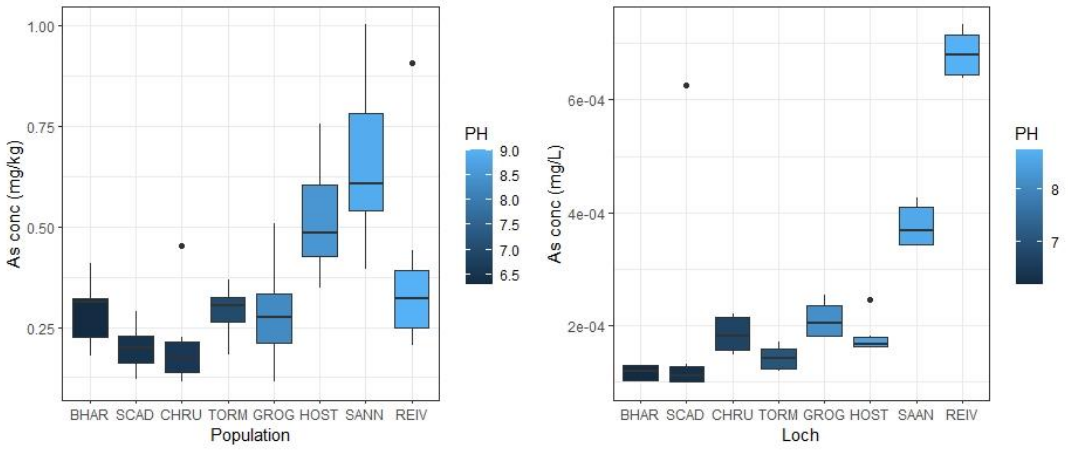


Figure A.16. Arsenic levels of wild caught fish (left) and home loch water (right).

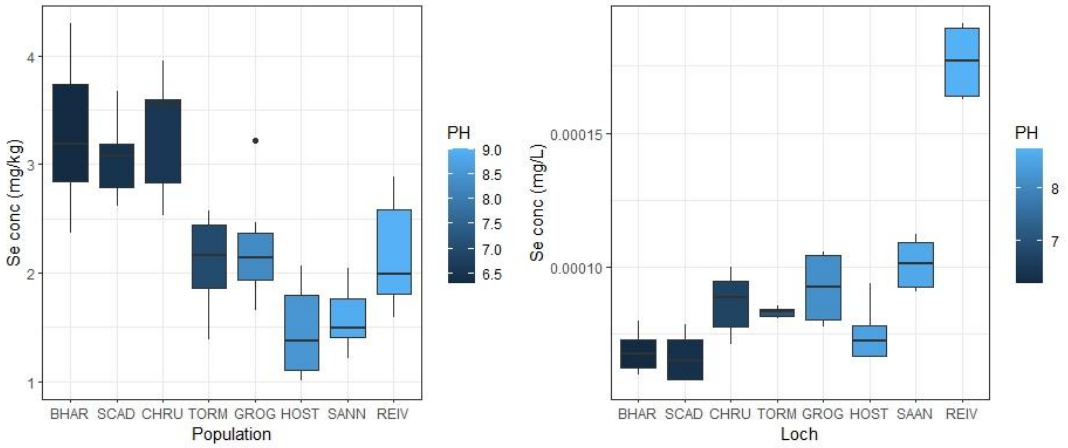


Figure A.17. Selenium levels of wild caught fish (left) and home loch water (right).

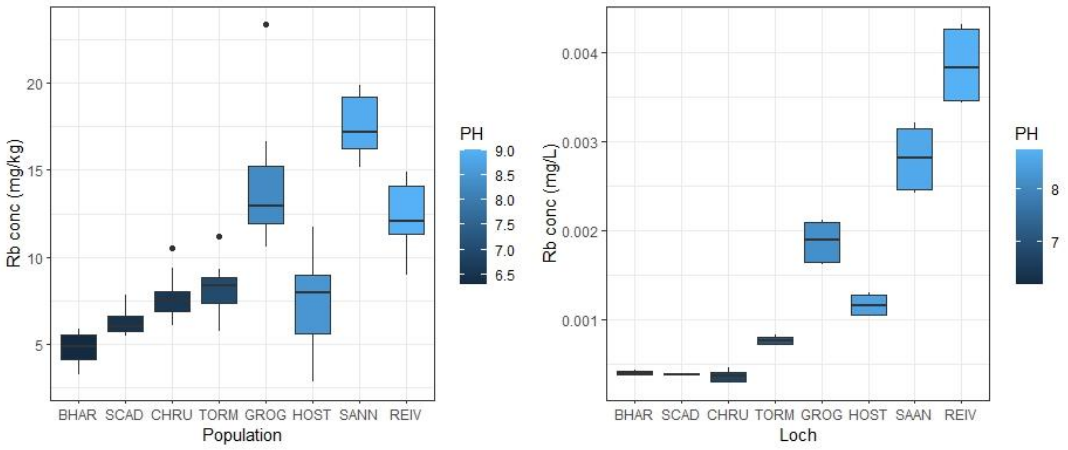


Figure A.18. Rubidium levels of wild caught fish (left) and home loch water (right).

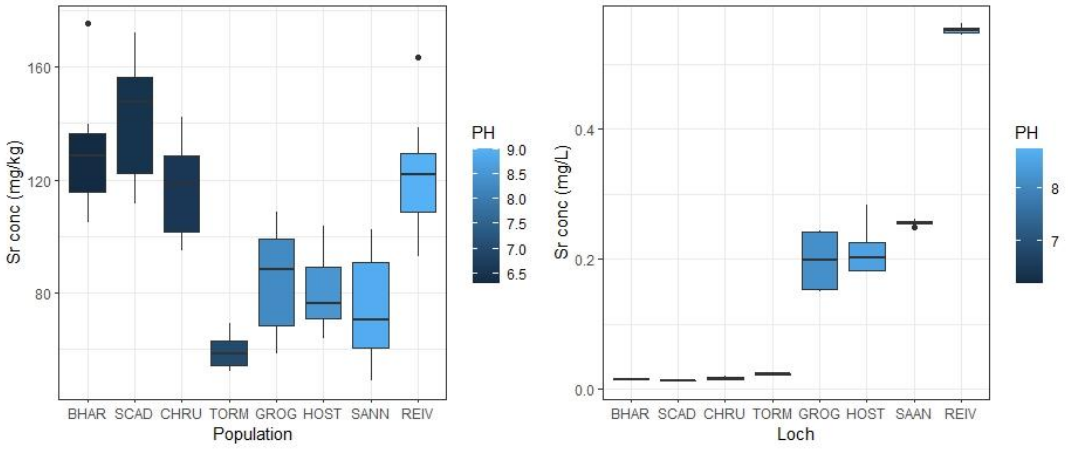


Figure A.19. Strontium levels of wild caught fish (left) and home loch water (right).

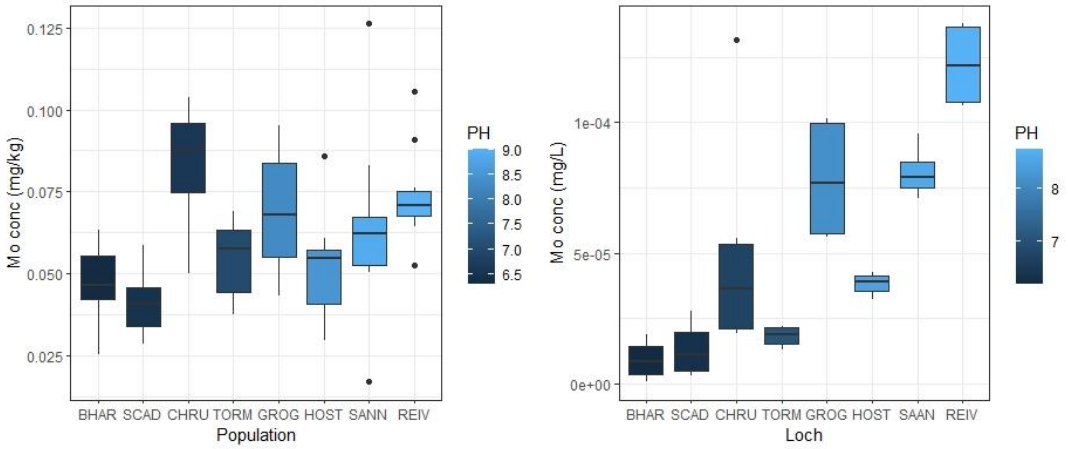


Figure A.20. Molybdenum levels of wild caught fish (left) and home loch water (right).

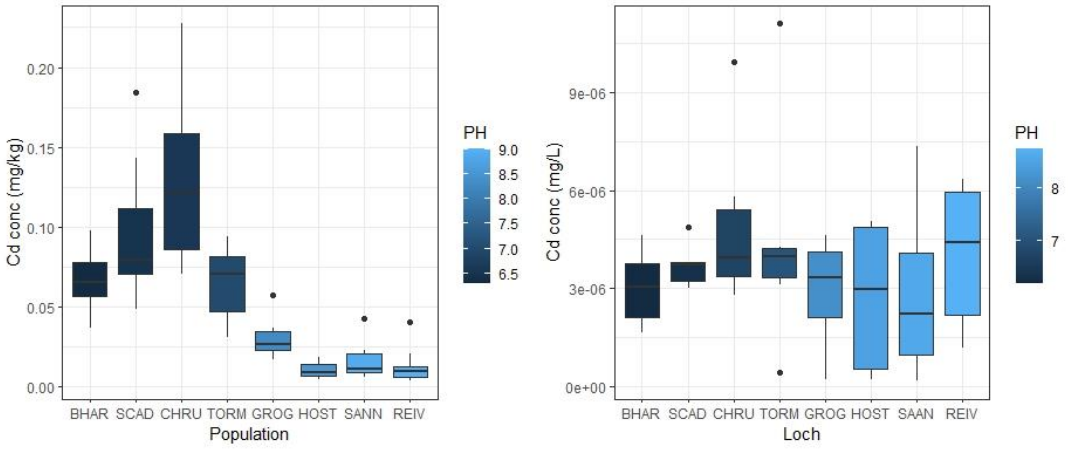


Figure A.21. Cadmium levels of wild caught fish (left) and home loch water (right).

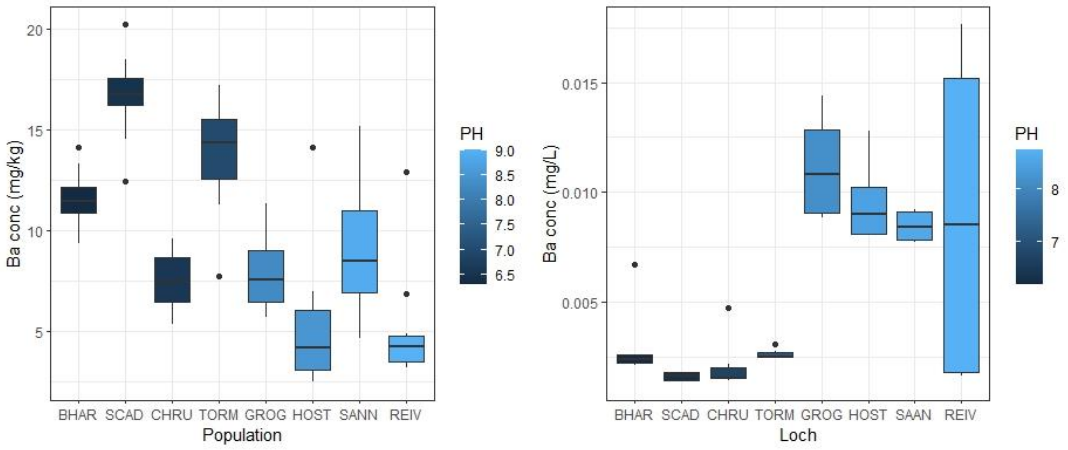


Figure A.22. Barium levels of wild caught fish (left) and home loch water (right).

Data used for this thesis (including dry digest weights and mg/kg (mg/L in the case of water samples) elemental concentrations) are available in:

**HILL, Iain 1434299-Data-Supplementary1**.