PHENOTYPIC VARIATION OF NATIVE FISH SPECIES ALONG AN IONIC GRADIENT

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ABSTRACT

Heterogeneous environments can promote phenotypic divergence among conspecific populations as a result of local adaptation or phenotypic plasticity. However, each process can be limited by various constraints, such as high gene flow, limited genetic variation, temporal fluctuations, or developmental constraints. We focus on the constraint that might be imposed when some populations are found in locations likely made stressful owing to low levels of an essential nutrient. We specifically use fish scales to investigate potential local adaptation to a limiting nutrient (calcium) in a continuous heterogeneous environment across multiple species. Scales are important to fish through their multiple functional consequences, including protection against predators, influence on swimming efficiency, and potential for calcium storage. Indeed, calcium is an essential element of scales that serves as the foundation for biomineralization and an important contributor to strength and flexibility. If scalar calcium simply reflects calcium availability, we would expect higher levels of scalar calcium in fish from calcium-rich water, as compared to fish from calcium-poor water. To test this "passive response" hypothesis, we collected three fish species (pumpkinseed, logperch, and yellow perch) at multiple sites across a dissolved calcium gradient in the Upper St. Lawrence River and analyzed their scalar calcium concentrations using atomic absorption and Inductively Coupled Plasma Optimal Spectrometry (ICP-OES). Contradicting this "passive response" hypothesis, we did not detect strong or consistent relationships between scalar calcium concentrations and water calcium concentrations. Instead, all three species maintained a relatively narrow level of calcium in their scales across the wide environmental gradient. We propose an "active homeostasis" hypothesis, wherein fish from calcium-poor water are better able to uptake, mobilize, and deposit calcium than are fish from calcium-rich water. We further suggest that the inability of some invasive fish to colonize calcium-poor water might reflect an evolutionary history that did not favor such homeostatic ability.

RÉSUMÉ

Les environnements hétérogènes peuvent favoriser la divergence phénotypique parmi des populations conspécifiques en raison de l'adaptation locale ou de la plasticité phénotypique. Cependant, chaque processus peut être limité par diverses contraintes, telles qu'un flux de gènes élevé, une variation génétique limitée, des fluctuations temporelles ou des contraintes de développement. Nous nous concentrons sur la contrainte qui pourrait être imposée lorsque certaines populations se trouvent dans des endroits susceptibles de devenir stressants en raison de leur faible teneur en nutriments essentiels. Nous utilisons spécifiquement des écailles de poisson pour étudier l'adaptation locale potentielle à un élément nutritif limitant (le calcium) dans un environnement hétérogène continu avec multiples espèces. Les écailles sont importantes pour les poissons du fait de leurs multiples conséquences fonctionnelles, notamment la protection contre les prédateurs, l'influence sur l'efficacité de la nage et le potentiel de stockage du calcium. En effet, le calcium est un élément essentiel des écailles qui sert de base à la biominéralisation et contribue de manière importante à la force et à la souplesse des écailles. Si le calcium dans les écailles reflète simplement la disponibilité de calcium dans l'eau, nous nous attendons à des niveaux plus élevés de calcium chez les poissons provenant d'eaux riches en calcium, par rapport aux poissons provenant d'eaux pauvres en calcium. Afin de valider cette hypothèse, nous avons recueilli trois espèces de poissons (crapet soleil, fouille roche zébré et perchaude) à plusieurs sites dans un gradient environnemental de calcium dissous dans le haut du fleuve Saint-Laurent et analysé les concentrations de calcium dans les écailles par absorption atomique et spectrométrie optimale à plasma induit par couplage inductif. Contrairement à l'hypothèse de réponse passive, nous n'avons pas détecté de relation forte ou cohérente entre les concentrations de calcium dans les écailles et les concentrations de calcium dans l'eau. Nous avons plutôt trouvé que les trois espèces ont maintenu un niveau relativement étroit de calcium dans leurs écailles au travers du large gradient environnemental. Nous proposons une hypothèse d'homéostasie active, selon laquelle les poissons provenant d'eaux pauvres en calcium sont mieux en mesure d'absorber, de mobiliser et de déposer du calcium dans leurs écailles que ceux provenant d'eaux riches en calcium. Nous suggérons que l'incapacité de certains poissons envahissants à coloniser des eaux pauvres en calcium est le résultat d'une histoire évolutive qui ne favorise pas une telle capacité homéostatique.

ACKNOWLEDGEMENTS

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PREFACE

THESIS FORMAT

This thesis has been written in agreement with the manuscript-style thesis guidelines set by Graduate and Postdoctoral Studies at McGill University. It is composed of a manuscript with a general introduction and general conclusion. References are formatted for submission to *Freshwater Biology*.

CONTRIBUTION OF AUTHORS

All research conducted for this thesis was performed under the supervision of Dr. Andrew Hendry from McGill University and Dr. Alison Derry from Université du Québec à Montréal. This work would not have been possible without their input and guidance. The project idea was a collaborative effort between myself, Alison Derry and Andrew Hendry. I implemented the data collection over the summers of 2017 and 2018 and analyzed the data myself. Data collection was made possible by the help of Louis Astorg, Sandrine Beaumont-Comeau and Freedom Sorbara. I prepared the thesis with input from Alison Derry and Andrew Hendry. This thesis will be formatted for future publication in a peer-reviewed journal. The current target is *Freshwater Biology*.

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GENERAL INTRODUCTION (LITERATURE REVIEW)

The goal of my thesis was to look at scalar calcium concentrations across an environmental gradient for three native fish species. My research was conducted in the Upper St. Lawrence River around Lac St. Louis and Lac des Deux Montagnes where the system is defined by an important spatially continuous environmental gradient. The following general introduction will set the stage for my manuscript by providing relevant information about multiple topics that are directly linked to the outcomes of my research. Such topics include heterogeneous environments and local adaptation, parallelism, ecophysiology and calcium in fish, countergradient variation and plasticity, and finally, the Upper St. Lawrence system. I conclude this general introduction with the objectives of my research.

HETEROGENEOUS ENVIRONMENTS AND LOCAL ADAPTATION

I am investigating the potential local adaptation of fish along a spatially continuous environmental gradient. This section offers general information about local adaptation in such heterogeneous environments.

Heterogeneous environments and challenging environmental conditions pose important challenges for natural populations who are continuously adapting to increase fitness in their local habitat (DeFaveri and Merilä, 2014). Local environmental differences are associated with divergent selective pressures which in turn promote resident advantages to the locally adapted populations, resulting in local adaptation (Williams, 1966; Travisano and Rainey, 2000; Kawecki and Ebert, 2004). Local adaptation is more commonly observed in distinct habitat types with divergent selective pressures such as lake vs. stream type stickleback (Berner *et al.*, 2008; Kaeuffer *et al.*, 2012) but is also overserved in continuous environmental gradient with high gene flow (Endler, 1973). In such continuous habitats, organisms locally adapt to certain "points" along the environmental gradient. An example of adaptation in such environmental gradients is the locally adapted common gobies (*Pomatoschistus microps)* to a salinity gradient in the Baltic Sea where nest resources quantity and quality, mussel size (used as nest substrate), and male body size decrease along the salinity gradient (Mück and Heubel, 2018). Alternatively, continuous heterogeneous environments with high gene flow can promote phenotypic plasticity (the capacity of an organism to change its phenotype in response to biotic or abiotic environmental factors) over local adaptation (Stomp *et al.*, 2008; Lande, 2009; Schoeppner and Relyea, 2009a; Molina-Montenegro *et al.*, 2012; Sassenhagen *et al.*, 2015).

Local adaptation is the result of an interaction between the genotype and the environment (Kawecki and Ebert, 2004). A common way to test such an interaction and to detect the genetic basis of adaptation is through reciprocal transplant experiments (Waser and Price, 1985; Niewiarowski and Roosenburg, 1993). However, these types of experiments are often practically limited which restricts their feasibility. Moreover, it can be difficult to detect the key environmental factors driving local adaptation in the field. An alternative option to reciprocal transplant experiments are common garden experiments in a laboratory setting. Common garden experiments allow isolation of the environmental factors driving selection, if the key factors are taken into account in the experiment. An important limitation of common garden experiments to consider is that certain genotypes may be better adapted to laboratory conditions giving them a significant advantage (Kawecki and Ebert, 2004; Winkler and Van Buskirk, 2012). In addition to these limitations, the observation of local adaptation can be confounded by other factors such as drift or migration that also lead to a genotype by environment interaction resembling local adaptation (DeFaveri and Merilä, 2014). These confounding factors can be ruled out by using population replication in the field. Indeed, different populations experiencing the same environmental pressures should respond in a similar manner (Langerhans & DeWitt, 2004) commonly referred to as parallel or convergent evolution. Studying the independent evolution of phenotypically similar traits in distinct lineages provides an ideal opportunity to identify the underlying mechanisms of potential adaptive evolution.

Local adaptation and/or phenotypic plasticity might be limited in improving local fitness when environmental gradients are in a key "limiting nutrient". Nutrients are used by organisms to survive, grow, and reproduce (National Research Council, 1993). A limiting nutrient is determined by the mass balance between essential elements such as C, P and N (Ensminger *et al.*, 1993; Wetzel, 2001). The nature of the limiting nutrient varies from one system to another. In general, nitrogen is often the key limiting nutrient in coastal and marine systems and phosphorus is the key limiting nutrient in temperate freshwater systems (Howarth and Marino, 2006). However, in some cases, other nutrients are limiting in aquatic systems such as iron or silicate (Del Amo *et al.*, 1997; Johnson *et al.*, 1999). For example, silicate has been recognized as a key limiting nutrient which determines the growth rate and biomass of diatoms and other phytoplankton (Kennington *et al.*, 1999; Wassmann *et al.*, 1999; Wu and Chou, 2003). Other examples of limiting nutrients include calcium limitation in crustaceans (Cairns and Yan, 2009). Low amounts of dissolved calcium ions have been shown to reduce survival of amphipod species and decrease growth rates in multiple zooplankton species such as daphniids, Bosmina, and cyclopoids (Zehmer *et al.*, 2002; Azan and Arnott, 2018).

ECOPHYSIOLOGY AND CALCIUM IN FISH

Fish scales are an understudied adaptive trait. Scale strength and flexibility is determined by the amount of calcium deposition. In our study system, the most important ion to vary along the environmental gradient is dissolved calcium (Ca^{2+}). Thus, to look at the potential adaptation of fish species along the environmental gradient, we measured calcium concentration in fish scales at different sites along the gradient. The section below discusses ecophysiology and calcium function and regulation in freshwater teleosts.

Ecomorphology is the study of the relationship between morphological traits (or physiological adaptation-for the purpose of my work) and the environment suggesting that body morphology (or physiology) evolves as an adaptive response to environmental challenges (Wainwright and Reilly, 1994). Historically, and as per its definition, ecological work has been done in the field whereas functional morphology work has mostly been done in a laboratory setting (Winemiller *et al.*, 1995). However, the study of ecomorphology has to be brought into nature to understand how organisms perform in their natural environment. Research at the interface of morphology and ecology in nature has been expanding but is still somewhat limited (Winemiller *et al.*, 1995; Langerhans and Reznick, 2010; Aguirre *et al.*, 2019). Fish offer an ideal opportunity to study the association between form and function in their natural environment (Aguirre, 2009). In fact, associations between form and function is known to be especially strong in fish, such that many species have strong signals of local adaptation in ecological tolerances (Tobler *et al.*, 2011; McDonnell and Chapman, 2015) and morphology (Webb, 1984; Ehlinger and Wilson, 1988; Berner *et al.*, 2008; Langerhans and Reznick, 2010). However, there exists only a very limited number of studies that look at fish scale physiology in the context of ecomorphology (Aguirre *et al.*, 2019).

Scales are a hard, flattened, skeletal element found in the skin of fish (Rotllant *et al.*, 2005). In teleost fish, scales enclose an important part of body calcium in the form of calcium carbonate ($CaCo_3$) and hydroxyapatite ($Ca_5(PO_4)_3(OH)$) (Loewen *et al.*, 2016). The functional roles of scales are diverse, yet underexplored, despite knowing that they serve multiple purposes in the survival of fish (Arendt *et al.*, 2001). Examples include predator protection by acting as a protective layer (Reimchen, 1988; Bereiter-Hahn and Zylberberg, 1993), swimming performance by reducing drag by breaking the boundary layer (Aleyev, 1977) and as a calcium and phosphorus pool by maintaining homeostasis

(Bereiter-Hahn and Zylberberg, 1993). Scales are also an important site of calcium metabolism and deposition (Crichton, 2008).

Calcium is also involved in many physiological functions such as muscle fiber contraction, intracellular messaging, and reproduction (Crichton, 2008). Most importantly calcium serves as the foundation for biomineralization (Crichton, 2008) where 99% of the whole-body fraction of calcium is stored in biomineralized structures such as otoliths, scales, and bones (Flik *et al*., 1986). Although fish can uptake calcium from their diet, this source of calcium is rather minimal and they mostly acquire it directly from the surrounding water (Simkiss and Wilbur, 1989). Fishes, compared to non-bony fishes, regulate calcium through an open system where their scales serve as a reservoir that stores calcium (Dacke, 1979). Under certain conditions such as starvation, sexual maturation and reproduction (Rotllant *et al*., 2005) calcium is reabsorbed from the scales and remobilized into its ionic form (Metz *et al*., 2014; Loewen *et al*., 2016).

PARALLELISM

We expect that different species that are at the same sites along the environmental gradient have more similar levels of scalar calcium, than individuals of the same species at different sites. Such an outcome would be a symptom of parallelism (or convergence). The following section discusses parallelism (or non-parallelism) in nature.

Different populations experiencing the same environmental pressures are expected to respond in a similar adaptive manner (Langerhans & DeWitt, 2004), whether through adaptive genetic divergence or phenotypic plasticity. When this phenomenon occurs, it is commonly referred to as parallelism or convergence, and – when genetically based – parallel or convergent evolution. Parallel evolution is the independent evolution of similar traits in unrelated species that inhabit similar environmental conditions (Conway Morris, 2003, 2008, 2010). A textbook example of this is the evolution of streamlined body shapes for fast swimming animals. In fact, although unrelated, dolphins, sharks, tunas and ichthyosaurs have all evolved similar streamlined body shapes. Other examples include the independent evolution of caffeine production such as in coffee, tea and cacao plants (Denoeud *et al*., 2014) or the independent evolution of a similar social structure in ants and termites (Losos, 2017).

Parallelism is most commonly studied by comparing multiple populations of a same species in both similar and different habitats (Oke *et al*., 2016). There are also multiple examples of parallelism between different species that share a similar environment (Langerhans and DeWitt, 2004). Few studies have evaluated parallelism across multiple species in a single "location" within the same continuous habitat (Rosenblum *et al*., 2017). Yet this approach is important because assessing parallelism across an environmental gradient, when the different "replicates" (population pairs or species) are in different locations, runs the risk of mistaking spatial variation in the nature of the environmental gradient for non-parallelism in response to a common environmental gradient (Oke *et al*., 2019). Exploring the adaptive responses of multiple species at single locations along environmental gradients within continuous habitats, can therefore allow replicated variation in factors that influence adaptive evolution and plasticity, as well as reduces analytical noise.

Langerhans and Dewitt (2004) argued that evolution is shaped by both unique histories as well as similar natural selective forces (Hendry and Kinnison, 2001; Langerhans and DeWitt, 2004) meaning when species are exposed to similar environmental gradients, their patterns of divergence will have both shared and unique elements (Langerhans and DeWitt, 2004). I explore this concept of parallelism across multiple populations of multiple species in a single continuous environment. Table 1. illustrates how my work will fill the current knowledge gap in the literature.

Table 1. General analytic framework modified from Langerhans and Dewitt (2004) to study shared and unique responses to a common environmental gradient within and among species where S is species, E is environmental gradient and P is population.

	Parallelism		
	Among species (Langerhans and DeWitt 2004)	Within species (Oke et al. 2016)	Among and within species (Sanderson et al.)
Population (P)		✓	✓
Species (S)	✓		✓
Environmental gradient (E)	✓	✓	✓
P × S			✓
P × E		✓	✓
S × E	✓		✓
P × E × S			✓

COUNTERGRADIENT TRAIT VARIATION AND PLASTICITY

Alternatively (to parallelism), we might expect species to adapt to the environmental gradient through countergradient trait variation or plasticity. The following section provides information about both countergradient trait variation and plasticity, and how these topics relate to my thesis.

The theory of natural selection suggests that populations should locally adapt to their environmental conditions through interaction of genotype and environment. This local adaptation results in resident genotypes having higher relative fitness in their home range as opposed to non-resident genotypes (Hereford, 2009; Derry *et al*., 2019). However, these expectations hold true only if no other forces or constraints are present (Kawecki and Ebert, 2004). Many different factors, such as gene flow, interact with natural selection and, thus, hinder local adaptation (Barton and Partridge, 2000; Lenormand, 2002; Garant *et al*., 2007). Alternatively, adaptive phenotypic plasticity can be used to achieve improved fitness without any underlying genetic differentiation (Kawecki and Ebert, 2004). Phenotypic plasticity is defined as the ability of a genotype to change the expressed phenotype in response to different environments (Agrawal, 2001; West-Eberhard, 2005). Phenotypic plasticity is favoured over local adapation under conditions of high gene flow between environmentally-heterogeneous environments (Lind and Johansson, 2007, 2011; Thibert-Plante and Hendry, 2011; Sultan and Spencer, 2017), and in habitats with temporally-variable environmental conditions (Stomp *et al*., 2008; Lande, 2009; Schoeppner and Relyea, 2009a).

Although phenotypic plasticity is often viewed as an alternative outcome to local adaptation, it can also be maladaptive. Indeed, in some instances, phenotypic plasticity can be a maladaptive by-product of environmental influences on physiology (Kawecki and Ebert, 2004; Grether, 2005). In such cases, "maladaptive" refers to a condition that is less than the optimal fitness (Derry *et al*., 2019). If phenotypic plasticity is indeed maladaptive, the forces of selection will counteract it, and this process can result in reduced phenotypic differences between environmentally contrasting habitats. In this case, selection will be divergent at the genetic level but not necessarily at the phenotypic level (Kawecki and Ebert, 2004). This phenomenon was termed countergradient variation by Conover & Schultz (1995).

Countergradient variation was first observed by Levins when he studied *Drosophila melanogaster* along an altitude gradient (Levins, 1968, 2002). In the mountains of Puerto Rico, flies from the cooler highlands were slightly larger than those from the warmer lowlands. However, when raised in a common laboratory environment, the observed pattern was reversed (Levins, 1968). Levins originally described countergradient variation as a phenomenon that occurs when genotypes are distributed in way that counteracts the environmental gradient (Conover and Schultz, 1995), resulting in reduced phenotypic differences between habitats with contrasting environmental conditions. Thus, countergradient variation can conceal genetic differentiation and give an impression of a lack of local adaptation. At first, countergradient variation contradicted the well-established principals of cogradient variation, where genetic and phenotypic variation would mirror the environmental gradient (Conover *et al.*, 2009). Countergradient variation often occurs when genetic and environmental influences have opposing effects on the phenotype (Conover *et al.*, 2009), which is indicative of local adaptation.

Extensive work has documented cases of countergradient responses to climate variation (Berven *et al.*, 1979; Conover and Present, 1990; Blanckenhorn *et al.*, 1995). However, most cases of countergradient variation have been demonstrated in altitudinal (Levins, 1968; Falconer, 1981; Berven, 1982; Smith *et al.*, 1994), latitudinal (Dehnel, 1955; Gerard and Du Bois, 1988) or temperature (Chapin and Chapin, 1981; Blanckenhorn, 1991) gradients. An exception is a study that detected counter-gradient plasticity in calcium uptake of freshwater amphipods across natural ion gradients using a common garden laboratory experiment (Derry *et al.*, 2013). Based on the literature, there is a need for more research on countergradient trait variation as a response to different patterns of environmental heterogeneity to understand its ubiquity in nature as an adaptive response to environmental challenges and spatial environmental heterogeneity on landscapes. My thesis will contribute to filling this knowledge gap by studying an ionic gradient in a spatially continuous ecosystem where phenotypic plasticity might be predicted to prevail over local adaptation.

ST. LAWRENCE SYSTEM

To investigate my research questions, I will use the naturally occurring environmental gradient in the Upper St. Lawrence River around Lac St. Louis. The section below provides information about the ecological and environmental importance of the system.

The Upper St. Lawrence River at Lac St. Louis, Montreal, Qc, Canada (45°N, 73°W) is a useful ecosystem to address questions of potential adaptation to a spatially heterogeneous environment as it is characterized by strong environmental gradients between the north and south shores. This spatial variation is created by ion-rich water (305μS cm^{-1}) from the St. Lawrence River along the south shore and ion-poor water from the Ottawa River (80μS cm^{-1}) along the north shore. The specific conductivity in the system is positively correlated with dissolved calcium ions (*Fig. A2*) where the Ottawa River has low dissolved calcium ions (8.3 mg L^{-1}) and the St. Lawrence River has high dissolved calcium ions

(36.3 ml L^{-1}). From aerial photographs, one can clearly see the two different water bodies coming together: the brown, humic-colored water of the Ottawa River and the green, relatively clear water of the St. Lawrence River. In fact, the two water bodies have significantly different concentrations of dissolved organic carbon (DOC; 3.64 mg L^{-1} vs 19.70 mg L^{-1}) which give them their colour.

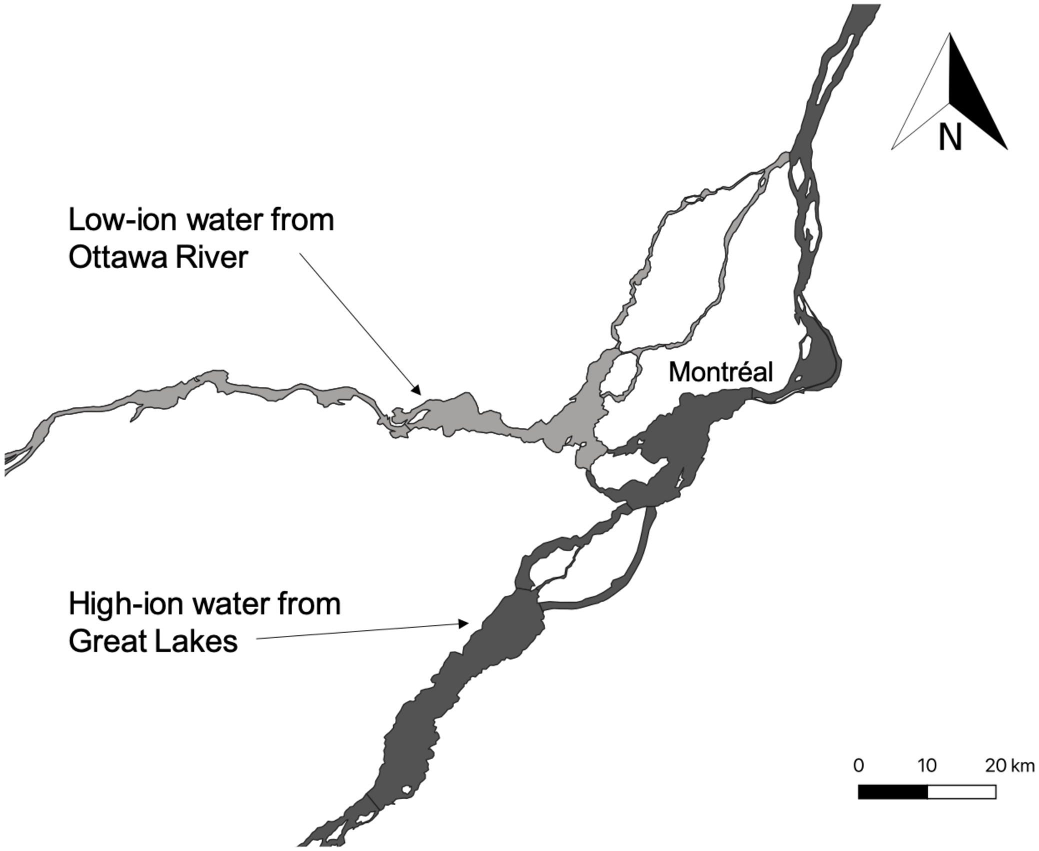

Fig. 1. Map of study system (including Lac St. Louis, Ottawa River and St. Lawrence River), Québec, Canada, with ion-rich water from the Great Lakes coming from the southwest and ion-poor water from the Ottawa River coming from the west.

The St. Lawrence River contains 25% of the world's freshwater (Warwick and Dodson, 1999). The river flows through Lake Ontario, into three fluvial lakes (Lake St. Francis, Lake St. Louis and Lake St. Pierre) and into the Gulf of the St. Lawrence, where it merges with the Atlantic Ocean. As the St. Lawrence flows through these fluvial lakes, it is extremely important in terms of discharge and economy. It is one of the most important bodies of freshwater in North America, and it passes through the most industrialized regions of North America (Carignan and Lorrain, 2000). The river supplies 46% of

Quebecers with drinking water, it supplies an important number of industry, and it serves as the main transport route toward the inland through its multiple channels (Warwick and Dodson, 1999). The Great Lake-St. Lawrence system is also a major driver of both the Canadian and U.S. economy supplying 31% of employment (Kavcic, 2016).

The tremendous amount of activity in the St. Lawrence River makes its ecosystem vulnerable to multiple stressors such as degradation of its water quality, shoreline erosion, decline in plant and animal populations and modification of the flow pattern (*Working Group on the State of the St. Lawrence Monitoring, 2014*). Although progress has occurred on many fronts, the system continues to experience runoff from intensive farming, dredging for channels and flow regulation. A more recent threat to the native fauna and flora of the St. Lawrence system was the introduction of exotic species through ballast water (Niimi and Reid, 2003). In fact, the Upper St. Lawrence River is now home to multiple exotic species from the Ponto-Caspian such as the round goby, *Neogobius melanostomus,* (Iacarella and Ricciardi, 2015), the exotic amphipod, *Echinogammarus ischnus,* (Palmer and Ricciardi, 2005) and zebra mussels, *Dreissena polymorpha* (Iacarella and Ricciardi, 2015) which are all known to compete with native species. The distribution of these exotic species is delineated by the natural ion gradient where exotics are only present in the ion-rich waters of the St. Lawrence river and not the ion-poor waters associated to the Ottawa river (Astorg *et al.* in review).

Although native species are found throughout the environmental gradient, previous work has shown that some native species differ in terms of their morphology between the two types of water. For example, lake sturgeon *(Acipenser fulvescens)* from Lac des Deux Montagnes, in the Ottawa River, differ morphologically from those of the St. Lawrence river (Guénette *et al.*, 1992). Additionally, native amphipods, *Gammarus fasciaus*, from ion-poor water are smaller and have less calcification compared to those from ion-rich water (Derry *et al.*, 2013).

Evidently, the high spatial environmental variation found in the St. Lawrence system has important effects on the morphology and distribution of both native and invasive species. The combination of both this environmental variation and the multiple stressors affecting the ecosystem, makes the St. Lawrence river an important system to study. It is crucial to understand how native species are affected by environmental gradients, and whether they are locally adapted. Adaptive mechanisms such as local adaptation and countergradient plasticity are important for determining species distributions and abundance, and in the case of the St. Lawrence River, may explain native species persistence in face of exotic invasion along the ion gradient.

KNOWLEDGE GAP AND OBJECTIVES

The goal of my research was to compare scalar calcium concentrations (hereafter scalar calcium) across an environmental gradient for three species at shared locations across a large, spatially continuous aquatic ecosystem. Given then the widespread occurrence of parallelism in fishes (Oke *et al*., 2016), we predicted that multiple fish species would show parallel responses in scalar calcium at specific locations along an environmental gradient in aqueous ions and other abiotic and biotic factors, but that these responses would depend on the environmental conditions of the location along the environmental gradient.

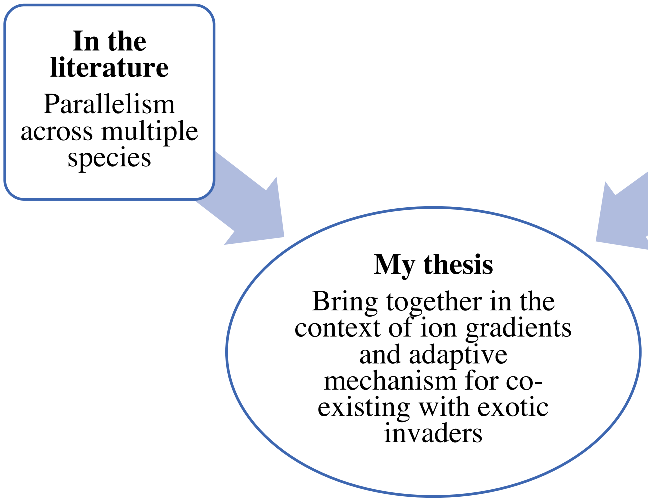

Fig. 2. Conceptual diagram depicting how my thesis will bridge the knowledge gap in the literature.

My study contributes to our knowledge of parallel adaptive responses between fish species, especially in continuous ecosystems where phenotypic plasticity is often prevalent. More importantly, my work has important implications in the Upper St. Lawrence system as the Ottawa River may serve as a natural refuge for native species in the face of invasive round gobies.

MANUSCRIPT

FISH MAINTAIN A NARROW RANGE OF SCALE CALCIUM ACROSS A WIDE RANGE OF CALCIUM AVAILABILITY

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KEYWORDS
Convergence, countergradient variation, ecophysiology, phenotypic buffering, and uninvaded refuges

SUMMARY

1. Heterogeneous environments can promote phenotypic divergence among conspecific populations as a result of local adaptation or phenotypic plasticity. However, each process can be modified by various constraints, such as high gene flow, limited genetic variation, temporal fluctuations, or developmental constraints. We focus on the constraint that might be imposed when some populations are found in locations likely made stressful owing to low levels of an essential nutrient.
2. We use fish scales to investigate phenotypic divergence in response to spatial variation in a potentially limiting nutrient (calcium). Scales are important to fish through their multiple functional consequences, including protection against predators, influence on swimming efficiency, and potential for calcium storage. Calcium is an essential element of scales that serves as the foundation for biomineralization and an important contributor to strength and flexibility.
3. If scalar calcium simply reflects calcium availability, we would expect higher levels of scalar calcium in fish from calcium-rich water, as compared to fish from calcium-poor water. To test this “passive response” hypothesis, we collected three fish species (pumpkinseed, logperch, and yellow perch) at multiple sites across a dissolved calcium gradient in the Upper St. Lawrence River. Scalar calcium concentrations were analyzed using atomic absorption and Inductively Coupled Plasma Optimal Spectrometry (ICP-OES).
4. Contradicting the “passive response" hypothesis, we did not detect strong or consistent relationships between scalar calcium concentrations and water calcium concentrations. Instead, all three species maintained a relatively narrow range of calcium in their scales despite the wide environmental gradient. We propose an “active homeostasis” hypothesis, wherein fish from

calcium-poor water are better able to uptake, mobilize, and deposit calcium than are fish from calcium-rich water.

5. We further suggest that the inability of some invasive fish to colonize calcium-poor water might reflect a calcium-rich history that would not necessitate the evolution of such homeostatic ability.

INTRODUCTION

Heterogeneous environments and limiting environmental conditions pose important challenges for natural populations who are continuously adapting to increase fitness in their local habitat (DeFaveri and Merilä, 2014). Adaptive responses in populations are often the outcome of a balance between the diversifying influence of selection and the constraining effects of various forces, including gene flow (Garant *et al.*, 2007; DeFaveri and Merilä, 2014). When selection is strong enough to overcome environmental and genetic constraints, heterogeneous environments can promote adaptive population divergence by differential selective pressures (Schluter, 2000; Nosil and Schluter, 2011), thus promoting local adaptation (Kawecki and Ebert, 2004; Hereford, 2009). Local adaptation is most often observed in discrete habitats where there is often lower gene flow and where populations can theoretically evolve more efficiently toward their local optimum, but local adaptation can also potentially exists in continuous habitats with relatively higher gene flow (Endler, 1973; Lenormand, 2002). Alternatively, continuous heterogeneous environments with high gene flow can promote phenotypic plasticity over local adaptation (Lande, 2009; Schoeppner and Relyea, 2009b; Molina-Montenegro *et al.*, 2012; Sassenhagen *et al.*, 2015). Phenotypic plasticity is the capacity of an organism to change its phenotype in response to biotic or abiotic environmental factors (West-Eberhard, 2003).

Local adaptation and/or phenotypic plasticity might be limited in improving local fitness when environmental gradients are in a key limiting nutrient. A limiting nutrient is determined by the mass balance between essential elements such as C, P, and N (Wetzel, 2001). Although general patterns exist such as nitrogen being the key limiting nutrient in coastal systems and phosphorus the key limiting nutrient in freshwater systems (Howarth and Marino, 2006), other nutrients can be limiting in aquatic systems. For example, silicate has been recognized as a key limiting nutrient which determines the growth rate and biomass of diatoms and other phytoplankton (Kennington *et al.*, 1999; Wassmann *et al.*, 1999; Wu and Chou, 2003). In other cases, low concentrations of dissolved calcium ions can limit the growth rate of freshwater organisms such as many crustaceans or fish (Cairns and Yan, 2009; Baldwin *et al*., 2012). Limiting environments can affect population sizes and thus lead to population extinction (Hoffmann and Parson, 1987). Alternatively, intense natural selection in limiting environments can favour expression of characters that are not favoured under "normal" conditions and influence expression of phenotypic variation (Hoffmann and Parson, 1987).

Different populations experiencing the same environmental pressures are expected to respond in a similar adaptive manner (Langerhans & DeWitt, 2004), whether through adaptive genetic divergence or phenotypic plasticity. When this phenomenon occurs, it is commonly referred to as parallelism (the term I will use) or convergence, and – when genetically based – parallel or convergent evolution. Parallel evolution is most commonly studied by comparing multiple populations of a same species in both similar and different habitats (Oke *et al*., 2016). The main downfall of this approach is that it is not always possible to distinguish between potential causes of "non-parallelism" (Oke et al. 2019). There are also multiple examples of parallelism between different species that share a similar environment (Langerhans and DeWitt, 2004). In such cases, it can be difficult to untangle unique histories from environmental responses (Langerhans and DeWitt, 2004). Few studies have evaluated parallelism across multiple species in a single "location" within the same continuous habitat (Rosenblum *et al*., 2017). Yet this approach is important because assessing parallelism across an environmental gradient, when the different "replicates" (population pairs or species) are in different locations, runs the risk of mistaking spatial variation in the nature of the environmental gradient for non-parallelism in response to a common environmental gradient (Oke et al. 2019). Exploring the adaptive responses of multiple species at single locations along environmental gradients within continuous habitats, can therefore allow replicated variation in factors that influence adaptive evolution and plasticity, as well as reduces analytical noise.

A limited number of studies comparing separate lineages in the same locations (habitats) have been performed. For example, Rosenblum et al. (2017) quantified the degree of parallel evolution in three different lizard species and two arthropods (*Sceloporus cowlesi, Aspidoscleis inornata, Holbrookia maculata, Habronattus ustulatus, and Ammobaenetes arenicolus*) in the white sands of New Mexico. They found that all phenotypes converged in the same direction, but at different magnitudes (Rosenblum *et al*., 2017). Similarly, Oke *et al*. (2019) quantified the amount of parallelism in run timing (breeding migration timing) in even-year and odd-year pink salmon. Mature breeding pink salmon die at age two, resulting in two completely reproductively isolated populations (even-year and odd-year) that share the same habitat (Oke *et al*. 2019). As demonstrated by the examples above, most studies of parallel adaptive signals have been done in fragmented landscapes with discrete habitats across populations (Hanski *et al*.,

2011) and species (Derry and Arnott, 2007) but no studies to our knowledge have explored adaptive parallel responses between species in spatially-continuous environments where there is high potential for strong intraspecific gene flow and adaptive phenotypic plasticity.

Scale physiology and calcium ions

Fish scales serve as an adaptive trait that can vary both intra- and inter-specifically as a response to environmental gradients and provide a useful system for exploring parallel adaptive responses between fish species in continuous, environmentally heterogeneous aquatic ecosystems. More generally, fish are known to have strong associations between form and function (Webb, 1984; Langerhans and Reznick, 2010), such that many species have strong signals of local adaptation in ecological tolerances (Tobler *et al.*, 2011; McDonnell and Chapman, 2015) and morphology (Ehlinger and Wilson, 1988; Berner *et al.*, 2008). However, fish scales are relatively understudied as structures and traits (Flik and Verbost, 1993; Arendt *et al.*, 2001; Metz *et al.*, 2014), and yet, are useful from a parallel evolution perspective because they are shared by all bony fishes (Helfman *et al.*, 2009). The functional roles of scales are diverse, yet underexplored, despite knowing that they serve multiple purposes in the survival of fish (Arendt *et al.*, 2001). Examples include predator protection by acting as a protective layer (Reimchen, 1988; Bereiter-Hahn and Zylberberg, 1993), in swimming performance by reducing drag by breaking the boundary layer (Aleyev, 1977) and as a calcium and phosphorus pool by maintaining homeostasis (Bereiter-Hahn and Zylberberg, 1993). Scales are also an important site of calcium metabolism and deposition (Crichton, 2008).

Organisms selectively take up or extract elements such as calcium from their surrounding environment to incorporate them into biological structures and function (Loewen *et al.*, 2016). Calcium is an essential element involved in many physiological functions such as muscle fiber contraction, intracellular messaging and reproduction (Crichton, 2008). Calcium also serves as the foundation for biomineralization (Crichton, 2008). Although fish can uptake calcium from their diet, this source of calcium is rather minimal and they mostly acquire calcium directly from the surrounding water (Simkiss and Wilbur, 1989). Calcium is deposited in the bones, scales and skin (Lall and Lewis-McCrea, 2007). Around 99% of the whole-body fraction of calcium is stored in biomineralized structures such as otoliths, scales, and bones (Flik *et al.*, 1986). Fish use their scales and bone as an internal calcium reservoir where they can store calcium for times of need (Dacke, 1979). In such times, calcium can be taken from scales and bones and be used in other cellular processes (Dacke, 1979). Based on the physiological properties of scales, we would expect the calcium concentration of fish scales to vary environmentally based on the available calcium ions in the water. We refer to this hypothesis as the "passive response" hypothesis as opposed to a "active homeostasis" hypothesis where fish actively maintain scalar calcium concentrations throughout the environmental gradient. No other studies that we are aware of to date have compared inter- and intra-specific variation in the scalar calcium of fishes in nature, which is key for the survival, fitness, and function of these aquatic organisms.

Objectives

Our goal was to compare scalar calcium across an environmental gradient for three species at shared locations across a large, spatially continuous aquatic ecosystem. Given the widespread occurrence of parallelism in fishes (Oke *et al*., 2016), we predicted that multiple fish species would show parallel responses in scalar calcium at specific locations along an environmental gradient in aqueous ions and other abiotic and biotic factors, but that these responses would depend on the environmental conditions of the location along the environmental gradient. More specifically, we predicted high calcium concentrations at locations with high aqueous ion concentrations, and lower calcium concentrations at locations with lower aqueous ion concentrations ("passive response" hypothesis). Our study contributes to our knowledge of parallel adaptive responses between fish species, especially in continuous ecosystems where phenotypic plasticity is often prevalent.

METHODS

Study System

The Upper St. Lawrence River and Lower Ottawa River, QC, Canada (45°N, 73°W) is a useful ecosystem to address questions of potential adaptation to a spatially heterogeneous environment as it is characterized by strong environmental gradients between the north and south shores. Central to our study system is Lac St. Louis which receives ion-poor water from the Ottawa River (80µS cm^{-1}) along its north shore and ion-rich water from the St. Lawrence River along its south shore (305µS cm^{-1}). Specific conductivity was positively correlated ($R^2 = 0.8$) with dissolved calcium ion concentrations in the system (Fig. A2) where the Ottawa River has low dissolved calcium ions (8.3 mg L^{-1}) and the St. Lawrence River has high dissolved calcium ions (36.3 ml L^{-1}). In addition to conductivity and dissolved aqueous calcium, the two water bodies have other different environmental conditions, such as different concentrations of dissolved organic carbon (DOC; 3.64 mg L^{-1} vs 19.70 mg L^{-1}) and differences in food webs (Astorg *et al*. in review). Moreover, most invasive species are restricted to only part of the environmental gradient. Their distribution is delineated by the natural ion gradient where exotics are only present in the ion-rich waters of the St. Lawrence River and not the ion-poor waters associated to the Ottawa River (Astorg *et al*. in review). The Upper St. Lawrence River is home to multiple exotic species from the Ponto-Caspian such as the round goby (*Neogobius melanostomus)*, (Iacarella and Ricciardi, 2015), the exotic amphipod (*Echinogammarus ischnus*), (Palmer and Ricciardi, 2005) and zebra mussels (*Dreissena polymorpha*) (Iacarella and Ricciardi, 2015), which are all known to compete with and/or prey on native species. The current distribution of the invasive species suggests that the Ottawa River serves as a natural uninvaded refuge for native species. Therefore, the rich- and poor-ion water masses of the Upper St. Lawrence River form an important continuous environmental gradient for several abiotic and biotic factors to which aquatic species might adapt to.

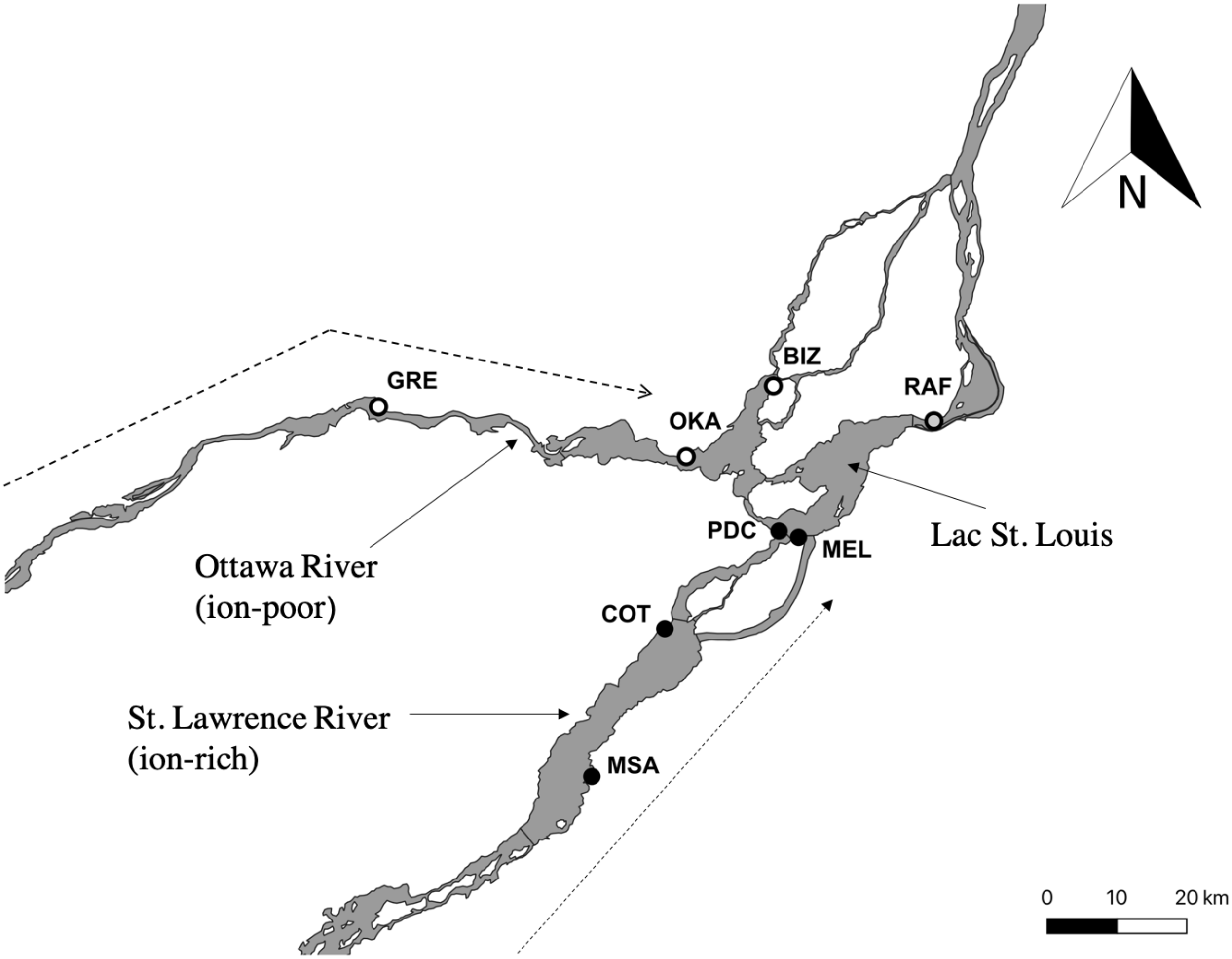

Fig. 3. Map of study system (including Lac St. Louis, the Ottawa River and the St. Lawrence River) in Québec, Canada. Ion-rich water from the Great Lakes comes from the southwest and ion-poor water from the Ottawa River comes from the west. Sites of fish and water sample collection are indicated by circles, where ion-rich sites are black (MSA, COT, MEL, and PDC), ion-poor sites are white (GRE, OKA and BIZ), and the mixing zone site is gray (RAF). Water flows from west to east into Lac St. Louis which then drains into the St. Lawrence River indicated by the dashed arrows.

Sample collection

To answer our questions, we selected three different native fish species: logperch (*Percina caprodes*), pumpkinseed (*Lepomis gibbosus*) and yellow perch (*Perca flavescens*) (Bernatchez and Giroux, 2012). These specific species were selected because they are found throughout the environmental gradient, are fairly common throughout the area, and all occupy a mainly benthic habitat. In the summer of 2017, we collected fish across five different sites (PDC, MEL, RAF, BIZ and GRE). When visually examining our results (Fig. 4), we noticed a possible relationship (Table 4) between water calcium and scalar calcium. Thus, in the summer of 2018, we expanded our sampling to sites further up the environmental gradient (GRE, OKA, COT and MSA). In total, 286 fish were collected from eight sites across the three species. All specimens were collected using a seine net (114cm per 407cm) deployed from shore (about 1m water depth). The three target species were euthanized using Tricaine Methanesulfonate (MS222) at a concentration of 250-500 mg L^{-1}. Non-target species were released immediately upon identification. Immediately after euthanasia, each fish was hung by its tail on a hook and put into a cooler for transportation to a laboratory in the Redpath Museum at McGill University, Montreal, QC, Canada, where they were transferred to a -20°C freezer. After 24 hours, the fish were taken off the hooks and transferred into plastic bags for storage.

Table 2. Sample sizes (number of fish analyzed "N") and geographic coordinates per species per year for each sample site.

Species	Water Type	Site	Latitude (DD)	Longitude (DD)	N	Year
Percina caprodes	Ion poor	BIZ	45.516119	-73.897533	14	2017
Percina caprodes	Ion poor	GRE	45.629803	-74.607868	14	2017
Percina caprodes	Ion rich	MEL	45.319210	-73.628341	12	2017

Percina caprodes	Ion rich	PDC	45.333996	-73.961851	13	2017
Percina caprodes	Mixed	RAF	45.415151	-73.628341	6	2017
Lepomis gibbosus	Ion poor	BIZ	45.516119	-73.897533	3	2017
Lepomis gibbosus	Ion poor	GRE	45.629803	-74.607868	13	2017
Lepomis gibbosus	Ion rich	MEL	45.319210	-73.628341	12	2017
Lepomis gibbosus	Ion rich	PDC	45.333996	-73.961851	15	2017
Lepomis gibbosus	Mixed	RAF	45.415151	-73.628341	13	2017
Perca flavescens	Ion poor	BIZ	45.516119	-73.897533	13	2017
Perca flavescens	Ion poor	GRE	45.629803	-74.607868	14	2017
Perca flavescens	Ion rich	MEL	45.319210	-73.628341	14	2017
Perca flavescens	Ion rich	PDC	45.333996	-73.961851	13	2017
Perca flavescens	Mixed	RAF	45.415151	-73.628341	14	2017
Percina caprodes	Ion rich	COT	45.253611	-74.211944	10	2018
Percina caprodes	Ion poor	GRE	45.629803	-74.607868	0	2018
Percina caprodes	Ion rich	MSA	45.096041	-74.413554	10	2018
Percina caprodes	Ion poor	OKA	45.459560	-74.087574	5	2018
Lepomis gibbosus	Ion rich	COT	45.253611	-74.211944	10	2018
Lepomis gibbosus	Ion poor	GRE	45.629803	-74.607868	10	2018
Lepomis gibbosus	Ion rich	MSA	45.096041	-74.413554	10	2018
Lepomis gibbosus	Ion poor	OKA	45.459560	-74.087574	9	2018
Perca flavescens	Ion rich	COT	45.253611	-74.211944	10	2018
Perca flavescens	Ion poor	GRE	45.629803	-74.607868	9	2018
Perca flavescens	Ion rich	MSA	45.096041	-74.413554	10	2018
Perca flavescens	Ion poor	OKA	45.459560	-74.087574	10	2018

Physico-chemical water measurements (temperature, °C; specific conductivity, µS cm^{-1}; dissolved oxygen, %; pH) were taken at each sampling site with a YSI series-pro multi-parameter sonde (model 10102030; Yellow Springs Inc., Yellow Springs, Ohio, USA). Dissolved calcium was measured using a Perkin Elmer Analyst 100 atomic absorption spectrophotometer at the McGill University Geotop lab, Montreal, QC, Canada. Water samples were collected in acid washed bottles and stored at 4°C until analyses for water chemistry at the GRIL (Groupe de Recherche Interuniversité en Limnologie) laboratory at Université du Québec à Montréal, Montréal, QC, Canada. Dissolved organic carbon (DOC) concentrations were measured in water samples filtered through of 0.45 μm filters (surfactant-free membrane filters) after acidification (phosphoric acid 5%) followed by sodium persulphate oxidation using a 1010 TOC analyser (O.I. Analytical, College Station, TX, U.S.A.). Total phosphorus (TP) was measured spectrophotometrically on the same instrument by the molybdenum blue method after persulphate digestion (Griesbach and Peters, 1991).

Table 3. Physico-chemical properties of the study sites recorded during summer 2017-2018, where conductivity is specific conductivity, DOC is dissolved organic carbon, Ca^{2+} is dissolved calcium ions, DO is dissolved oxygen, Temp is temperature, TP is total phosphorus and TN is total nitrogen.

Site	Water type	Conductivity (μS cm^{-1})	DOC (mg L^{-1})	Ca^{2+} (mg L^{-1})	pH	DO (%)	Temp (°C)	TP (μg L^{-1})	TN (mg L^{-1})
GRE	Ion-poor	70.5	19.70	15.7	7.51	98.6	21.3	64.32	0.59
OKA	Ion-poor	96.8	13.52	12.8	8.83	71.7	26.0	76.67	0.77
BIZ	Ion-poor	80.7	6.79	9.8	7.59	89.3	20.0	42.94	0.50
RAF	Mixed	199.4	7.37	22.5	8.08	88.2	19.9	35.28	0.46
PDC	Ion-rich	300.3	3.64	44.0	8.40	98	18.9	18.60	0.41
MEL	Ion-rich	307.3	19.62	37.6	8.50	108.5	20.8	66.41	0.44
COT	Ion-rich	263.8	4.01	45.3	8.80	119.7	26.0	11.34	0.33
MSA	Ion-rich	363.0	6.04	46.1	8.87	61.9	26.5	15.40	0.40

Mineral analyses

We removed 30 scales from the left flank at the lateral line of every fish and dried them overnight at 80°C (Jeziorski and Yan, 2006; Metz *et al.*, 2014) for mineral analysis. The dried scales were dissolved in 60-100μL of 65-69% nitric acid for 24h. 2017 samples were diluted in 4-8 mL of nanopure water and analysed by Atomic Absorption using a Perkin Elmer Analyst 100 atomic absorption spectrophotometer at McGill University Geotop (Research Centre on the Dynamics of the Earth System). Scales from 2018 samples were dried overnight and dissolved in 500 μL of nitric acid and diluted in 10 mL of nanopure water. 2018 samples were analysed with ICP-OES (Inductively Coupled Plasma Optimal Emission Spectrometry) using a Thermo Fisher Scientific iCAP 6500 Duo avec Autosampler CETAC ASX-520 to get both scalar calcium and phosphorus concentrations.

Data Analyses

Our main goal was to investigate the relationship between scalar calcium and environmental ion concentrations in nature, thus informing whether fish adjusted calcium deposition in their scales in response to the environmental ion gradient. Data from each summer (2017 and 2018) were analyzed separately because they represented two independent tests of the same question and because they involved different spatial scales and scalar calcium methodology (Fig. 3, Table 2). For all analyses, scalar calcium and body size (total length) data were log-transformed to better meet the assumptions of a Gaussian distribution (LaBarbera, 1989; Bland and Altman, 1996).

We examined the extent to which scalar calcium varied along the ionic gradient by analyzing our data in linear mixed effect models where scalar calcium was the response variable. Fixed effects included water calcium, species (logperch, LP; pumpkinseed fish, PS; yellow perch, YP) and body size (L_s). The random effect was sampling site in a given year. The significance of each fixed effect was tested by comparing the log-likelihood ratios of the full model described above to a model lacking each fixed effect (Zuur *et al*., 2009). Post-hoc analyses were performed to determine which species were different from each other.

Given that the species-by-water calcium interaction was sometimes significant (Table 4) in the full models described above, we also constructed species-specific models. Such species-specific models (Table A1) allowed us to determine the relationship between scalar calcium and water calcium for each species, and if these species have a parallel response.

To determine not only the significance (as above) but also the importance of the water calcium gradient to scalar calcium, we used an effect size measure based on elasticity (Link and Doherty, 2002). If fish are passively up-taking calcium ions from the water and depositing them in their scales ("passive response"), then a proportional equivalence should be observed between increasing water calcium and increasing scalar calcium. That is, for a given proportional increase in water calcium, we should observe a similar proportional increase in scalar calcium – and this should be evident as a ratio of proportional change of 1. Alternatively, if fish are regulating their calcium uptake within a specific range despite highly variable calcium availability in the environment ("active homeostasis"), we should observe that a given proportional increase in water calcium should lead to a smaller (if any) proportional increase in scalar calcium: i.e., a ratio of proportional change of less than 1. Thus, we calculated the ratio of the proportional change in scalar calcium to the proportional increase in water calcium based on the following equation modified from (Link and Doherty, 2002):

$$ratio\ of\ proportional\ changes = \frac{|ln(y_2) - ln(y_1)|}{|ln(x_2) - ln(x_1)|}$$

where y_1 and y_2 are the predicted estimates of scalar calcium concentrations obtained from fixed values of water calcium, x_1 and x_2, in the linear mixed effect models described above.

All statistical analyses were performed in R version 3.6.1 (R Core Team, 2019). All linear models were performed with *lme* using REML estimation in the *nlme* package (Pinheiro *et al.*, 2019). We assumed a Gaussian error distribution which we confirmed by visual inspection of the residuals of the model and QQ plots (Zuur *et al.*, 2009). We obtain R^2 values using the *r.squaredGLMM* function in the *MuMIn* package (Barton, 2016). Post-hoc comparisons were obtained using the *pairs* function in the *emmeans* package (Lenth, 2019).

RESULTS

A total of 183 fish were collected in the summer of 2017 and 103 fish in the summer of 2018. In 2017, logperch were mean ± S.D. 74.91 ± 11.05 mm L_s, pumpkinseed were 79.41 ± 25.21 mm L_s, and yellow perch were 97.22 ± 13.20 mm L_s. In 2018, logperch were mean ± S.D. 54.28 ± 8.20 mm L_s, pumpkinseed were 59.88 ± 20.34 mm L_s, and yellow perch were 64.18 ± 20.66 mm L_s (Fig. A1).

We did not observe a consistent relationship between scalar calcium and water calcium. Fitted values of the 2017 dataset, seem to suggest that species in ion-poor water have lower scalar calcium – but the trend is non-significant (Fig. 4; panel b; Table 4). Moreover, when we expand sampling along the ionic gradient to include sites at the extremes of the gradient (2018 dataset), we no longer observe any hint of a general relationship between scalar calcium and water calcium (Fig. 4; panel d).

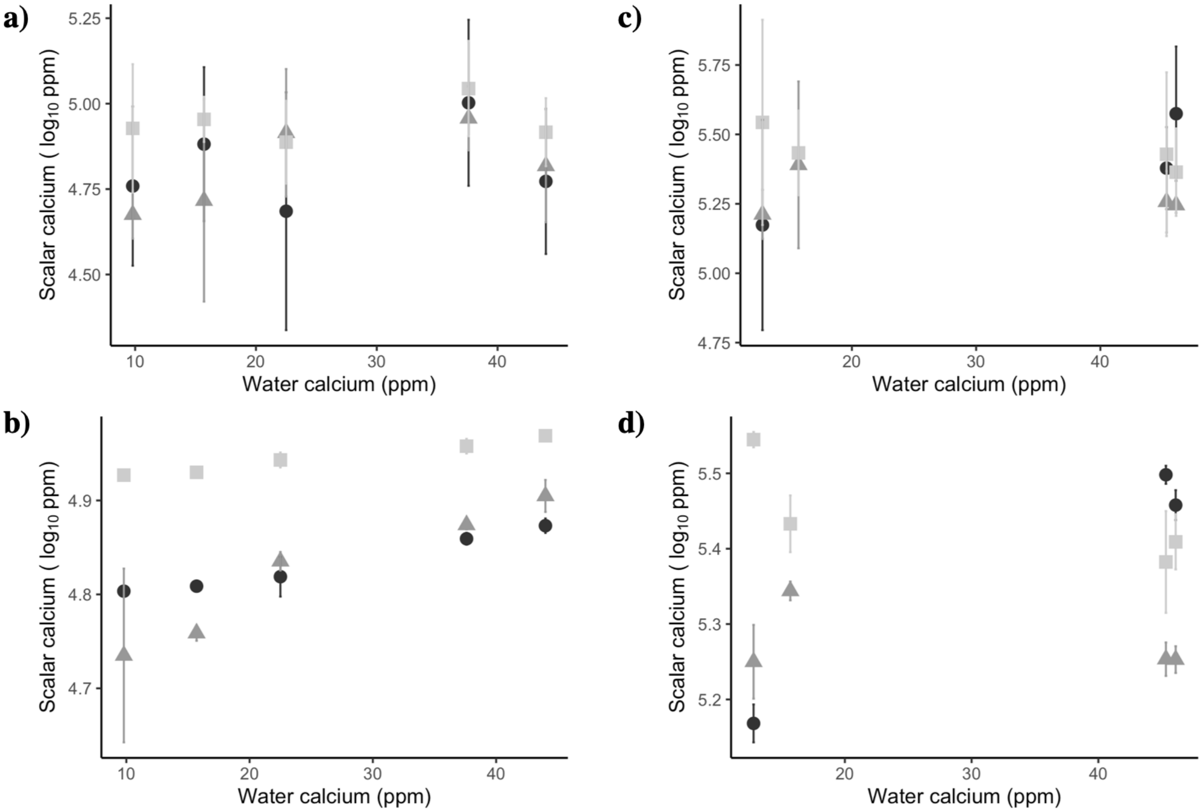

Fig. 4. Mean scalar calcium (log$_{10}$ transformed) of each species at every site along the water calcium gradient for each year: a) 2017 raw means (± standard deviation), b) 2017 fitted means from the linear mixed effect model (± 95% confidence interval), c) 2018 raw means (± standard deviation), and d) 2018 fitted means from the linear mixed effect model (± 95% confidence interval). Pumpkinseed fish are represented by triangles, logperch by circles and yellow perch by squares.

Overall, we conclude that water calcium was not a significant predictor of scalar calcium. In the 2017 dataset, water calcium was not a significant predictor of scalar calcium (P=0.53), whereas it was a significant predictor in the 2018 dataset (P=0.008).

Post-hoc analyses of scalar calcium from the linear mixed models described above suggest that in both years pumpkinseed and yellow perch differ from each other (2017, P=0.002; 2018, P=0.002), logperch and pumpkinseed do not differ from each other (2017, P=0.99; 2018, P=0.65, and logperch and yellow perch differ in 2017, but not in 2018 (2017, P=0.004; 2018, P=0.14) (Fig. A3). Given that the species-by-water calcium interaction was sometimes significant (Table 4), we also constructed species-specific models. In all but one instance, water calcium was not a significant predictor of scalar calcium (Table A1); the exception being 2018 logperch. However, in that case, we had small sample sizes and were lacking any samples for one of the sites (GRE) (Table 2).

Table 4. Linear mixed effect models predicting scalar calcium concentrations of logperch (LP), pumpkinseed (PS) and yellow perch (YP) along the ionic gradient. Scalar calcium concentrations and body size were $\log_{10}$ transformed before model fitting. Significance of effects was estimated using a log-likelihood ratio test.

Effect	**Estimate (±s.e.)**	**d.f.**	**LLR**	**p-value**
2017 (marginal R^2=0.09, conditional R^2=0.20)[a]				
Intercept	5.15 (0.35)	173		
SpeciesPS	-0.07 (0.09)	173		
SpeciesYP	0.15 (0.08)	173	15.24	**0.004**
WaterCa	0.002 (0.003)	3	2.22	0.528
Body size	-0.19 (0.18)	173	1.61	0.281
SpeciesPS * waterCa	0.003 (0.003)	173		
SpeciesYP * waterCa	-0.0008 (0.003)	173	1.29	0.522
	0.12			
Random (repeatability)[b]				
Site	0.073		7.008	**0.008**
Residual	0.195			
2018 (marginal R^2=0.22, conditional R^2=0.22)[a]				
Intercept	5.94 (0.38)	94		
SpeciesPS	0.19 (0.16)	94		
SpeciesYP	0.53 (0.16)	94	22.81	**0.0004**
WaterCa	0.01 (0.003)	2	11.77	**0.008**
Body size	-0.54 (0.21)	94	6.71	**0.01**
SpeciesPS * waterCa	-0.008 (0.004)	94		

SpeciesYP * waterCa	-0.013 (0.004)	94	10.22	**0.006**
Random (repeatability)	$1.06e^{-9}$			
Site	$6.98e^{-6}$		$2.41e^{-8}$	0.10
Residual	0.22			

[a] Marginal R^2 describes the proportion of total variance explained by the fixed effects in the model. Conditional R^2 described the proportion of total variance explained by the fixed effects and the random effect together.

[b] Repeatability was estimated as the proportion of the remaining variance (not explained by the fixed effects).

When comparing the proportional relationship between water calcium and scalar calcium (Table 5) we obtain an average 15% increase (or decrease in certain species) in scalar calcium in response to water calcium. This result indicates that fish are regulating their scalar calcium within a much narrower range than the range of calcium availability in the water.

Table 5. Proportional changes in scalar calcium concentrations in relation to changes in water calcium concentrations. Proportions were obtained using the modified equation for elasticity presented above.

	2017	2018
All species	0.10	0.04
Logperch	0.14	0.38
Yellow perch	0.05	0.17
Pumpkinseed	0.18	0.16

DISCUSSION

Given the importance of calcium and scales in fishes, we sought to understand how fish respond to a wide environmental gradient in calcium concentration. Our results suggest that all three studied species (logperch, pumpkinseed, and yellow perch) maintain a relatively similar concentration of calcium in their scales across the water calcium gradient of the Upper St. Lawrence River system. Specifically, relationships between scalar calcium and water calcium were – even if statistically significant in a few instances – inconsistent and weak in both years for models considering all species together (Fig. 4; Table 4). Moreover, species-specific models revealed statistical significance in only one instance (log perch in 2018), and all relationships were again relatively weak (Table A1). Thus, our results clearly indicate that the studied species maintain scalar calcium concentrations within a relatively stable range across a dramatic gradient in water calcium concentration supporting our "active homeostasis" hypothesis. This outcome means that fish from calcium-poor water must be able to acquire relatively more calcium from their environment compared to fish from calcium-rich water, suggesting that some minimal level of scalar calcium is important for all species. At the other end of the spectrum, fish in calcium-rich environments clearly do not continue to deposit more and more calcium in their scales, suggesting a possible cost to having too much calcium in scales. These two possibilities will be unpacked further in the discussion. Overall, we advance a hypothesis for "active homeostasis" where multiple populations of multiple species maintain a similar concentration of scalar calcium in a continuous environment where there is a high potential for gene flow and adaptive plasticity. Our study is unique in which we investigate the potential parallelism in a functionally relevant trait, fish scales, across multiple species in a continuous environmental gradient. Our findings are especially relevant to the natural refuge provided by Ottawa River for native species in face of a potential invader, the round goby, which has already successfully invaded the St. Lawrence system (Kornis *et al*., 2013).

Why do fish in calcium-poor environments maintain relatively "high" scalar calcium?

Our finding that all three species did not show lower scalar calcium levels in calcium-poor water, suggests that selection favors enhanced calcium uptake and deposition under those conditions. One reason might be that fish maintain relatively high levels of scalar calcium as a storage reservoir for future use (Arendt *et al*., 2001). Indeed, calcium can be resorbed form the scales and mobilized into its ionic form to be used in other functions such as metabolic demands and physiological activity (Metz *et al*., 2014). Another reason to maintain relatively high levels of scalar calcium might be for protection from predators. Indeed, higher concentrations of scalar calcium result in thicker scales which should, in turn, result in increased protection from puncture (Arendt *et al*., 2001). For instance, predation has been suggested to select for thicker plates and longer spines in stickleback, even in calcium-poor environments (Bell *et al*., 1993). For instance, Bell et al. (1993) found that, in calcium-poor environments, stickleback populations did not have pelvic reduction when predation was high but did have pelvic reduction when predation was low. In our study system, predation pressure is likely high throughout both the high and ion-poor waters as it is a contiguous system where native predators are found in both types of water (Astorg *et al.* in review). Indeed, logperch, pumpkinseed, and yellow perch in these waters are known to be important prey items for many predators such as *Micropterus dolomieu* (small mouth bass), *Esox lucius* (northern pike), *Sander canadensis* (sauger), and *Esox masquinongy* (muskellunge) (Bernatchez and Giroux, 2012). Thus, the species that we studied are likely under strong predator-induced selection to maintain high scalar calcium regardless of water calcium levels.

Why do fish in calcium-rich environments not deposit even more calcium in their scales?

If fish in calcium-poor water experience natural selection for improved calcium uptake and deposition, then why do fish in calcium-rich water not simply deposit even more calcium in their scales? The fact that they do not suggests that having too much calcium in scales has negative effects on the scales, or on some other traits. Although the functional properties of scales are not fully understood, they have been argued to play a role in swimming efficiency, amongst other functions. In fact, scales (and scale thickness) contribute to skin stiffness, which in turn influences swimming efficiency (McHenry *et al.*, 1995; Long *et al.*, 1996). In particular, skin stiffness determines the translation efficiency of muscle contraction to forward movement. Alternatively, maintaining high levels of scalar calcium may trade-off with some other important function, such as growth or reproduction. For instance, it has been shown in multiple species of fish, including pumpkinseeds, that a trade-off exists between scale strength, which is correlated with scalar calcium, and body growth (Flik *et al.*, 1986; Flik and Verbost, 1993; Arendt *et al.*, 2001). Moreover, Flik (1993) found that adding growth hormones to water with low calcium concentrations caused fish to decrease the amount of calcium found in their scales. The scale-growth trade-off may imply that past a certain level of scalar calcium concentrations; it may be more profitable to invest additional calcium in increasing growth. Finally, scalar calcium concentrations could be trading-off with metabolic demand. In fact, calcium can simply be re-allocated to metabolic functions. For example, growing individuals might require higher metabolic calcium levels to meet increased metabolic rates (McCarthy, 2000).

How do fish maintain a narrow range of scalar calcium throughout a wide environmental gradient?

Fish could be maintaining relatively constant scalar calcium concentrations across a wide environmental range of water calcium through either plasticity, genetic compensation, differential gene expression, or a combination of these processes. Based on previous research investigating calcium physiology in fish, we suggest that differential gene expression is the most likely reason (Shephard, 1981;

Rotllant *et al*., 2005; Guerreiro *et al*., 2007). One line of evidence comes from studies where plastic responses to water calcium did not yield outcomes consistent with our field observations. For instance, fish held in varying concentrations of water calcium maintain their plasma Ca^{2+} levels, but do not maintain scalar calcium concentrations (Shephard, 1981; Flik *et al*., 1986). In fact, when exposed to low levels of water calcium, fish (both tilapia and zebrafish) decrease calcium concentrations in their scales (Flik *et al*., 1986; Metz *et al*., 2014). However, these are short-term studies. To fully test the potential mechanism of developmental plasticity, one would have to raise fish in varying levels of water calcium concentration. A second line of evidence comes from studies pointing to a strong genetic basis for calcium uptake, regulation, and deposition. Calcium ion transport across the branchial membrane is mediated through Ca^{2+} ATPase (Perry and Flik, 1988; Verbost *et al*., 1994; Marshall *et al*., 1995). The activity level of this enzyme has been found to be correlated with the amount of plasma calcium levels (Shephard, 1981), which may be an indication of adaptation. Moreover, in teleost, calcium is regulated through PTH hormones and related proteins (Rotllant *et al*., 2005). Teleost have two forms of PTH hormones, two forms of PTHrP proteins, and one PTH intermediate protein which are all encoded by separate genes (Guerreiro *et al*., 2007). However, no studies have yet directly tested for local adaptation in calcium transport, regulation, or deposition among conspecific fish populations.

Overall, then, we postulate that the plastic effect of low water calcium in reducing scalar calcium is counteracted through genetic compensation that increases calcium uptake, regulation, and deposition. The outcome, then, looks like countergradient variation (Levins, 2002; Derry et al., 2013), where the plastic effect of low water calcium in reducing scalar calcium is counteracted by genetic changes that improve calcium uptake, regulation, and deposition in scales. Such local adaptation seems possible in our system given that our upstream-most sites are very far from calcium-rich water, such that gene flow from such populations must be low, and that the few studies conducted of fish across the gradient show some evidence of divergence in a number of traits. For example, lake sturgeon (Acipenser fulvescens) from Lac des Deux Montagnes and Lac St. Louis were found to differ morphologically where sturgeon from the Lac des Deux Montagnes had higher and longer heads, than those from Lac St. Louis (Guénette et al., 1992). Moreover, studies of other organisms across the same gradient have confirmed genetic divergence in some phenotypic traits and a countergradient plastic response in some life-history traits. For instance, Derry et al. (2013) found strong plastic effects on postmoult calcification in amphipods (more rapid in ion-rich water) and genetic differences in multiple life-history traits. The combination of our results and those from Derry et al. (2013) provide evidence for a parallel response across multiple taxa in the calcium gradient observed in the Upper St. Lawrence River.

Further research will be necessary to confirm the mechanism behind the observed pattern where fish from varying levels of calcium water concentration seem to maintain stable scalar calcium concentrations. For example, plasticity could be tested by reciprocal transplant experiments or common garden experiments. If fish maintain scalar calcium concentrations through a plastic response, we would expect all individuals (or populations) to maintain stable scalar calcium concentrations when exposed to calcium-poor water. Alternatively, genetic compensation leading to countergradient variation would be inferred if fish from calcium-poor water populations maintain higher scalar calcium than do fish from calcium-rich water populations when both are raised in common (especially poor) calcium water. Finally, genetic analyses, and gene expression analysis, would allow us to determine if fish from different types of water have different genes or if they are expressed at different levels.

Implications

All of the native fish species we studied were capable of maintaining high scalar calcium despite calcium-poor water, and all of them were abundant in such water (Fig. 4, Table 2). This finding suggests that a particular scalar calcium level is important to fish – not too little, not too much – and that all of the studied native fishes could achieve this level, perhaps through differential gene expression for the plastic effect of growing in calcium-poor water. And yet not all fish can do this. Invasive round gobies, for example, are not found in the Ottawa River, and are very rare in the plume of Ottawa River water after it joins the St. Lawrence River (Iacarella and Ricciardi, 2015). These gobies also show low foraging, growth, and survival rates when held in calcium-poor water in the laboratory (Baldwin *et al*., 2012; Iacarella and Ricciardi, 2015). Perhaps, then, invasive gobies and possibly other fishes are unable to evolve differential gene expression for these negative effects, thus limiting the range of their expansion. This putative adaptive failure could reflect high gene flow constraining local adaptation, low genetic variation in calcium-relevant genes, simply insufficient time for adaptation, or some combination of these factors. We therefore suggest that studies of important physiological traits expressed in native fishes across environmental gradients that invasive fish cannot tolerate can be a guide to identifying the specific factors, and perhaps genes, that mediate the spread of aquatic invasive species.

GENERAL CONCLUSION

SUMMARY

My thesis set out to investigate potential adaptation across multiple species to ionic gradients in continuous environments. My work suggests a physiological importance of calcium concentrations in fish scales, where native fish species maintain a narrow level of scalar calcium concentrations throughout a wide environmental range. I postulate that this "active homeostasis" is achieved through differential gene expression between populations in calcium-rich and calcium-poor water. Combining my results with those of previous research on the St. Lawrence system (Derry *et al.* 2013) suggests a potential parallel response to ion gradients across multiple taxa, such as fish and amphipods. The results of my work have important conservation implications since the naturally occurring ion gradient delineates the habitat range of the invasive round goby that has not yet adapted to the ion-poor water of the Ottawa River.

FUTURE WORK

Fish in the Upper St. Lawrence River system offer a unique opportunity to improve our understanding of how ionic gradients function as a dispersal barrier for an invasive species. I am currently undertaking two other projects which will further our understanding of the morphological and genetic basis of adaptation in fish to ionic gradients.

Skeletal morphology and bone density

Skeletal growth is often limited by the availability of nutrients and minerals such as calcium (Lall and Lewis-McCrea, 2007). As stressed previously, calcium is the primary component of calcification of skeletal structure and is crucial to normal growth and development in most vertebrates. Calcium restrictions have been shown to have effects on fish skeletal development (Flik *et al.*, 1986). To better understand how calcium-poor water might affect native fish species in the Upper St. Lawrence River system, I am currently investigating variation in skeletal morphology and bone density between populations in calcium-poor and calcium-rich water. Using X-ray images of the same specimens used in the current study, I am analyzing variation in vertebrae number, vertebrae area, neural spine length, dorsal spine length, and bone density. These new results will solidify our understanding of fish adaptation to the calcium gradient in the Upper St. Lawrence River.

Genetics of adaptation to ionic gradients

In collaboration with Dr. Madlen Stange, we are interested in detecting potential genetic adaption to ion-poor water in native fish species which might be absent in invasive species. To investigate our question, we are applying whole-genome pool sequencing to comparatively investigate structural (inversions, insertions, and deletions) and functional variation (mutations and allele frequency distributions) among genomes of native yellow perch and logperch as well as the invasive round goby. Each pool is composed of 50 individuals where we have two pools per habitat (ion-rich and ion-poor) per species. We will use the threespine stickleback (*Gasterosteus aculeatus*) (Jones *et al*., 2012; Glazer *et al*., 2015) genome for the computational pipeline which is the closest related species with a published genome. We will detect potential genetic adaptation by identifying highly variable loci by means of functional and structural analyses. We will investigate signatures of selection by comparing genome-wide F_{ST} values and allele frequency distributions. The results from this work will further our understanding of adaptation to ion gradients in fish in the Upper St. Lawrence River system. More importantly, this work will identify the genetic basis that could be preventing the round goby from expanding its range into the Ottawa River.

FURTHER IMPLICATIONS

Understanding the effects of calcium-poor water on fish is of broad importance as calcium concentrations are currently falling throughout boreal lakes in eastern North America and western Europe (Stoddard et al., 1999). In fact, calcium ions are being depleted in areas that have experienced acid rain and extensive logging resulting in a novel stressor in these ecosystems: very low amounts of dissolved calcium. Although the mechanisms behind this calcium decline are well known (acidic deposition, reduction in atmospheric calcium inputs, calcium loss from forest biomass harvesting) the consequent effects on aquatic biota are not as well studied. Previous work has highlighted the effects of calcium-poor water on zooplankton communities (Jeziorski and Yan, 2006; Cairns and Yan, 2009). For example, sediment cores have revealed a shift in zooplankton communities where larger species are not found in calcium-poor water (Jeziorski and Yan, 2006). Such sifts in zooplankton community composition are likely to have effects on other trophic levels including planktivorous fish. We suggest that there is an urgent need to better understand the effects of calcium depletion in boreal lake on native fish species.

APPENDIX

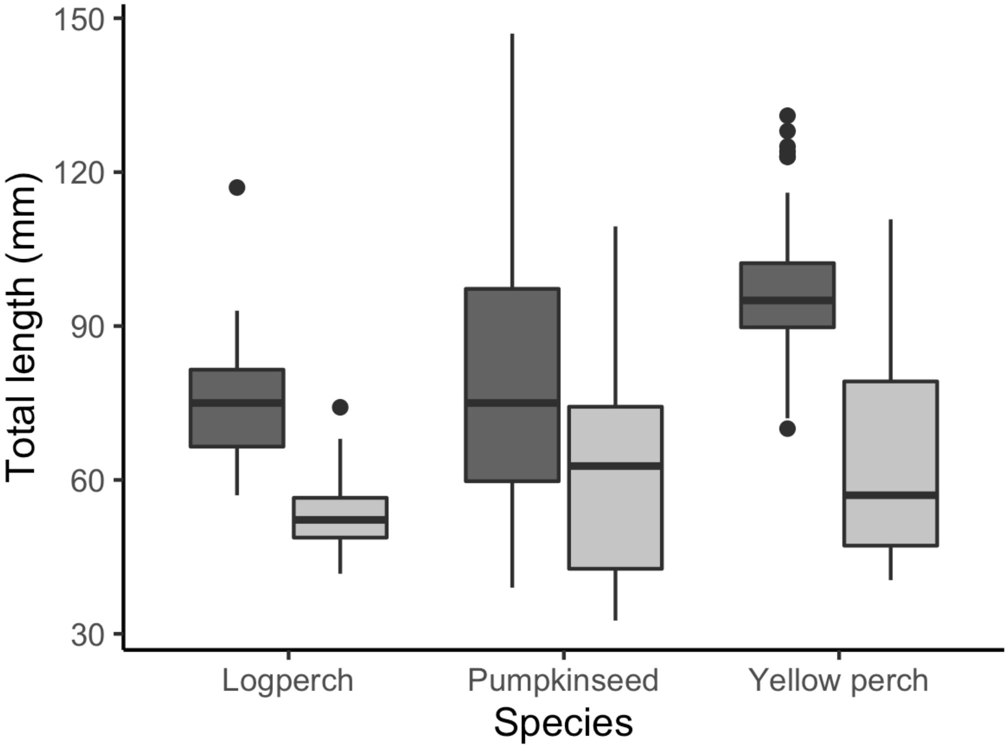

Fig. A1. Boxplot of total length for the three study specie (logperch, pumpkinseed, and yellow perch) where the box represents the 25th percentile to the 75th percentile range, the center line depicts the median and the lower and upper whiskers respectively show the smallest value within 1.5 times the interquartile range and the largest value within 1.5 times the interquartile range. Dark gray boxes are fish from 2017 and light gray boxes are fish from 2018.

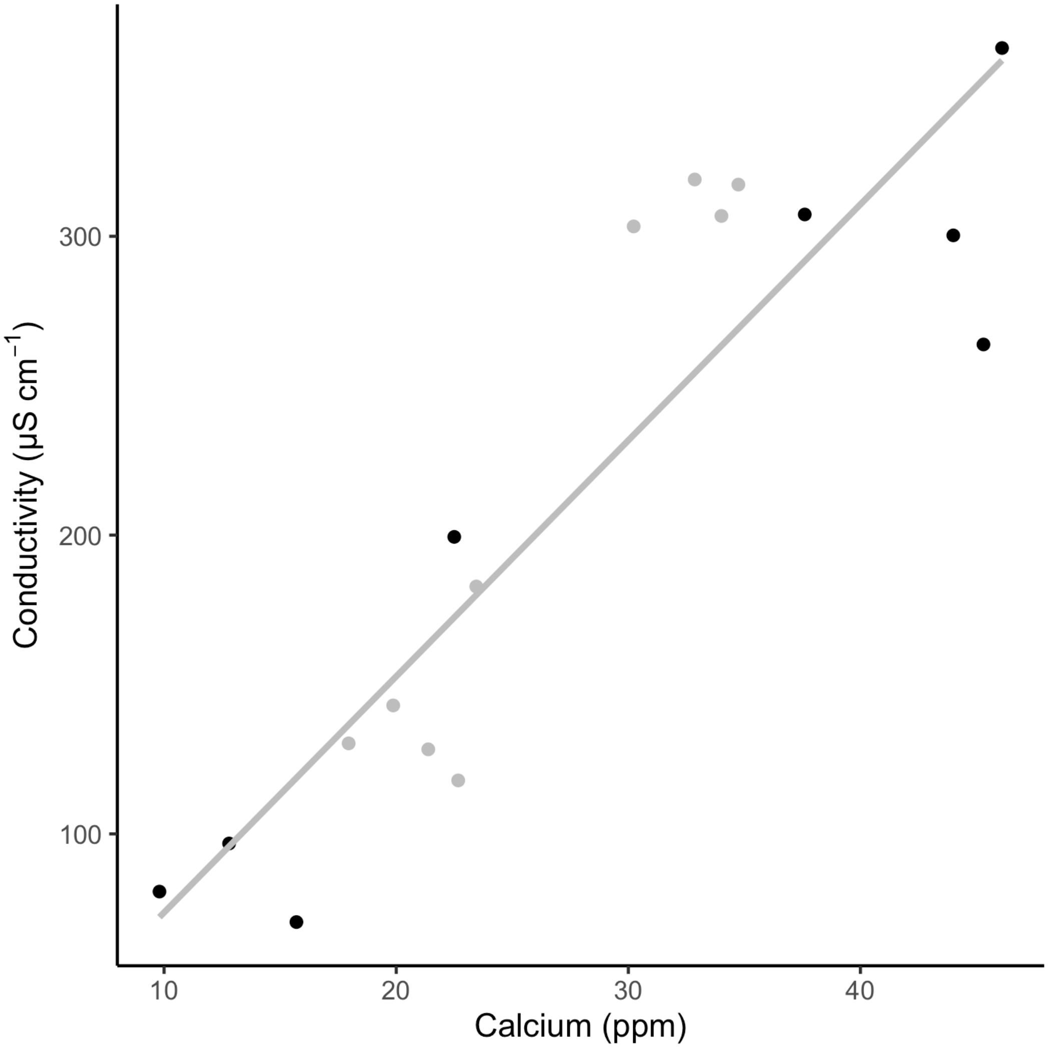

Fig. A2. Linear relationship between specific conductivity (µS cm^{-1}) and dissolved calcium ions (ppm) in the study system where the R^2 is 0.81 and the p-value is 9.069e-7. Black symbols indicate the eight study sites and gray symbols indicate additional sites in the study system from Astorg *et al*. (in review).

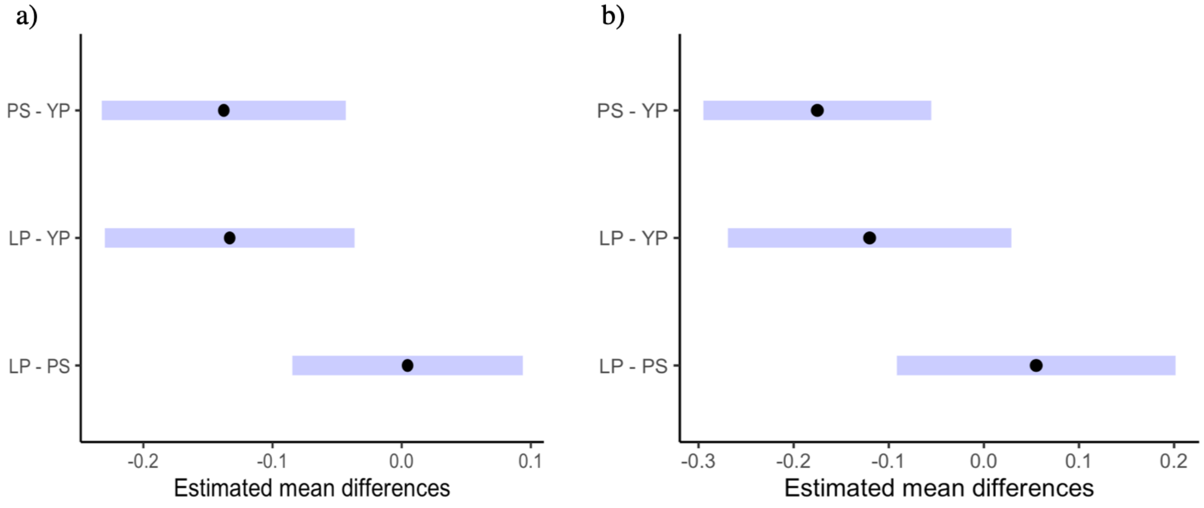

Fig. A3. Estimated mean differences of pairwise comparison of each species from post-hoc analyses of a linear mixed model where the response variable is scalar calcium, the fixed effects are water calcium, species (logperch, LP; pumpkinseed fish, PS; yellow perch, YP) and body size (L_s), and the random effect is sampling sites: a) 2017 and b) 2018.

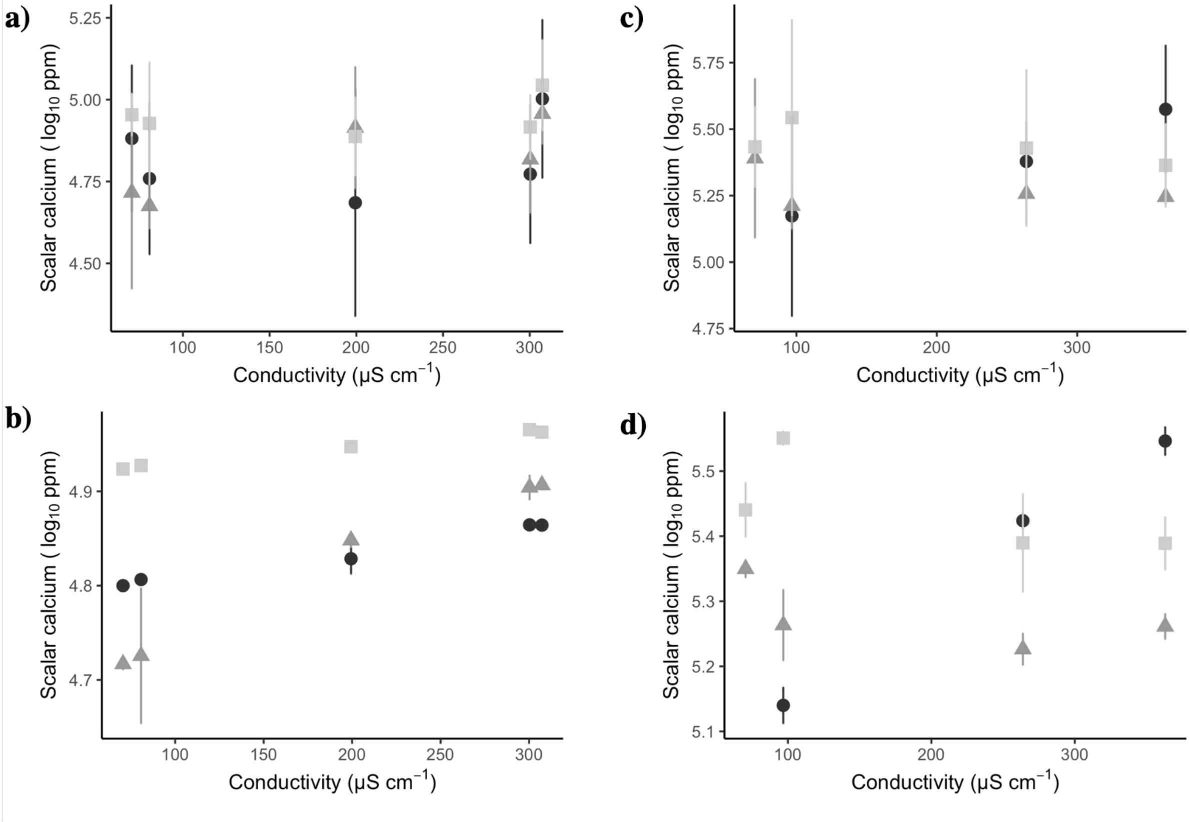

Fig. A4. Mean scalar calcium concentrations (log$_{10}$ transformed) of species at every site along the specific conductivity gradient for each year: a) 2017 raw means (± standard deviation), b) 2017 fitted means from linear mixed effect model (± 95% confidence interval), c) 2018 raw means (± standard deviation, and d) 2018 fitted means from linear mixed effect model (± 95% confidence interval). Pumpkinseed fish are represented by gray triangles, logperch by dark circles and yellow perch by light squares.

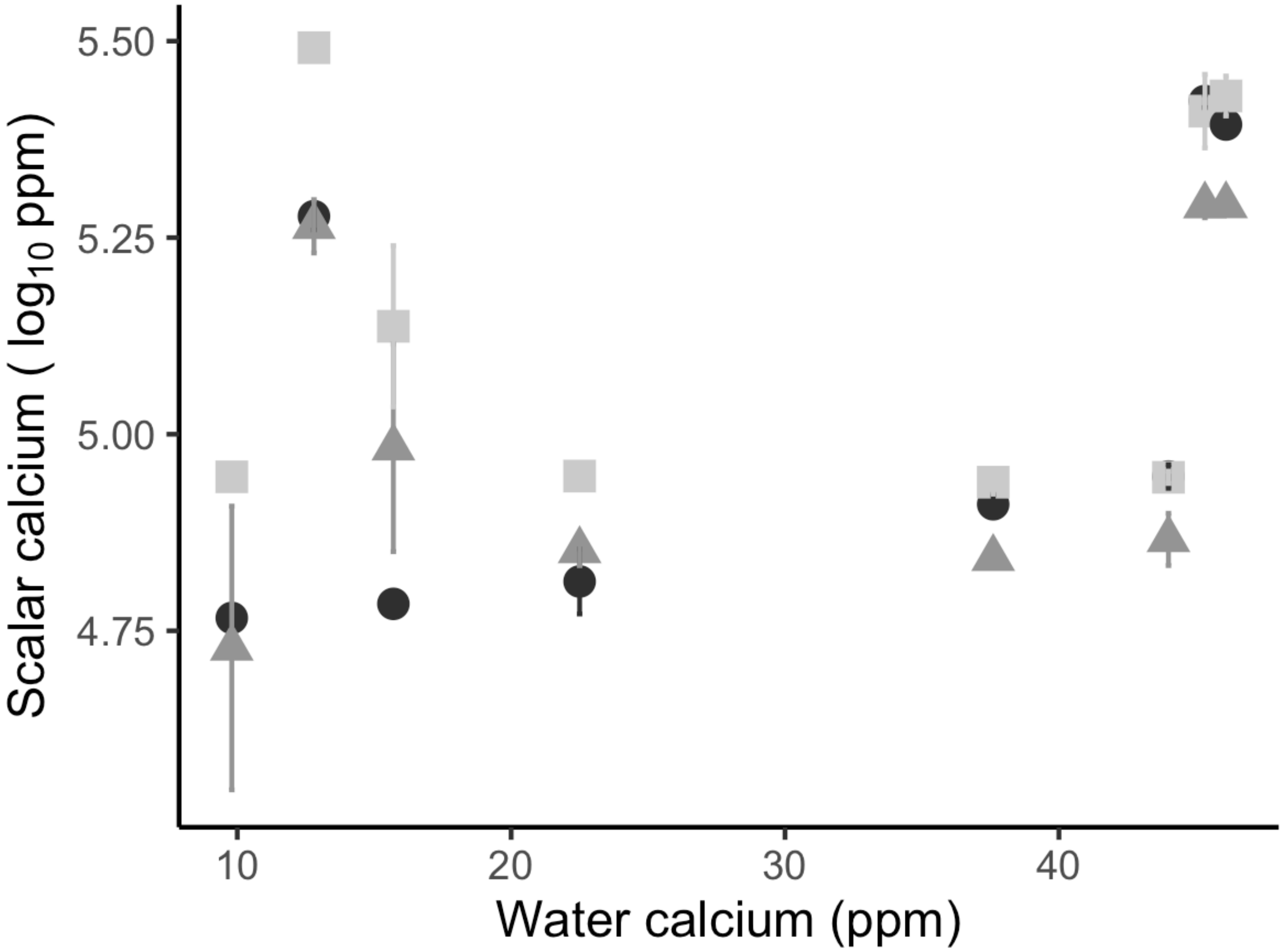

Fig. A5. Mean scalar calcium concentrations ($\log_{10}$ transformed) of species at every site along the specific conductivity gradient for years 2017 and 2018 combined. Fitted means from linear mixed effect model (± 95% confidence interval), where $\log_{10}$ transformed scalar calcium concentrations was the response variable, water calcium, species, body size ($\log_{10}$ transformed) and year were all fixed effects (with a water calcium- species interaction), and site was a random factor. Pumpkinseed fish are represented by gray triangles, logperch by dark circles and yellow perch by light squares.

Table A1. Linear mixed effect model predicting scalar calcium concentrations of logperch, pumpkinseed fish and yellow perch along an ionic gradient in the St. Lawrence system. Scalar calcium concentrations and body size were $\log_{10}$ transformed before model fitting. Significance of effects were estimated using a log-likelihood ratio test.

Effect	**Estimate**	**d.f.**	**LLR**	**p-value**
2017 logperch (marginal R^2=0.19, conditional R^2=0.44)[a]				
Intercept	0.765 (0.981)	53		
Water calcium	0.006 (0.005)	3	1.95	0.16
Body size (L_s)	2.07 (0.05)	53	14.19	**0.0002**
Random (repeatability)[b]	0.31			
Site	0.141		5.94	**0.01**
Residual	0.211			
2017 yellow perch (marginal R^2=0.02, conditional R^2=0.2)[a]				
Intercept	5.46 (0.584)	62		
Water Calcium	0.0009 (0.002)	3	0.25	0.62
Body size (L_s)	-0.27 (0.29)	62		
Random (repeatability)[b]	0.18		0.72	0.40
Site	0.06		4.11	**0.04**
Residual	0.13			
2017 pumpkinseed (marginal R^2=0.14, conditional R^2=0.23)[a]				
Intercept	5.62 (0.44)	50		
Water calcium	0.003 (0.003)	3	1.42	0.23
Body size (L_s)	-0.47 (0.22)	50	5.23	**0.02**
Random (repeatability)[b]	0.12			
Site	0.07		1.31	0.25
Residual	0.19			

2018 logperch (marginal R^2=0.16, conditional R^2=0.49)[a]				
Intercept	6.64 (1.95)	21		
Water calcium	0.01 (0.008)	1	3.9	**0.05**
Body size (L_s)	-0.963 (1.070)	21	0.55	0.70
Random (repeatability)[b]	0.39			
Site	0.19		1.5	0.22
Residual	0.24			
2018 yellow perch (marginal R^2=0.19, conditional R^2=0.19)[a]				
Intercept	6.89 (0.52)	34		
Water calcium	-0.002 (0.002)	2	0.51	0.47
Body size (L_s)	-0.78 (0.30)	34	6.87	**0.01**
Random (repeatability)[b]	$3.97e^{-8}$			
Site	$47.38e^{-06}$		$5.01e^{-9}$	0.10
Residual	0.24			
2018 pumpkinseed (marginal R^2=0.01, conditional R^2=019)[a]				
Intercept	5.36 (0.51)	34		
Water calcium	-0.001 (0.004)	2	0.21	0.65
Body size (L_s)	-0.03 (0.33)	34	1.02	0.32
Random (repeatability)[b]	0.2			
Site	0.08		0.64	0.43
Residual	0.16			

[a] Marginal R^2 describes the proportion of total variance explained by the fixed effects in the model. Conditional R^2 described the proportion of total variance explained by the fixed effects and the random effect together.

[b] Repeatability was estimated as the proportion of the remaining variance (not explained by the fixed effects).

Table A2. Linear mixed effect model predicting scalar calcium concentrations of logperch, pumpkinseed fish and yellow perch along an ionic gradient in the St. Lawrence system. Scalar calcium concentrations and body size were $\log_{10}$ transformed before model fitting. Significance of effects were estimated using a log-likelihood ratio test.

Effect	**Estimate**	**d.f.**	**LLR**	**p-value**
2017 and 2018 (marginal R^2=0.58, conditional R^2=0.61)[a]				
Intercept	5.43 (0.24)	272		
SpeciesPS	0.02 (0.08)	272		
SpeciesYP	0.28 (0.07)	272	34.70	**<0.0001**
Water calcium	0.005 (0.002)	6	8.73	**0.03**
Body size (L_s)	-0.38 (0.13)	272	8.73	**0.003**
Year	0.42 (0.05)	272	53.71	**< 0.0001**
SpeciesPS:cond	-0.002 (0.002)	272		
SpeciesYP:cond	-0.005 (0.002)	272	6.52	**0.04**
Random effect (repeatability)[b]	0.05			
Site	0.05		7.04	**0.008**
Residual	0.21			

[a] Marginal R^2 describes the proportion of total variance explained by the fixed effects in the model. Conditional R^2 described the proportion of total variance explained by the fixed effects and the random effect together.

[b] Repeatability was estimated as the proportion of the remaining variance (not explained by the fixed effects).

Table A3. Partial R^2 calculated by removing the effect of interest from the model

	2017		2018	
	R^2marginal	R^2conditional	R^2marginal	R^2conditional
Full model	0.09	0.20	0.22	0.22
Species	0.02	0.12	0.04	0.04
Water Calcium	0.07	0.17	0.14	0.15
Interaction (species*water calcium)	0.09	0.19	0.15	0.15

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