


# Freshwater fishes maintain multi-trait phenotypic stability across an environmental gradient in aqueous calcium

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## Abstract

Reductions in a limiting nutrient might be expected to necessitate compromises in the functional traits that depend on that nutrient; yet populations existing in locations with low levels of such nutrients often do not show the expected degradation of functional traits. Indeed, logperch (*Percina caprodes*), pumpkinseed sunfish (*Lepomis gibbosus*) and yellow perch (*Perca flavescens*) residing in low-calcium water in the Upper St. Lawrence River were all previously found to maintain levels of scale calcium comparable to those of conspecific populations in high-calcium water. Yet it remains possible that the maintenance of one functional trait (i.e., scale calcium) under nutrient-limited (i.e., low calcium) conditions could come at the expense of maintaining other functional traits that depend on the same nutrient. The present study therefore examines other calcium-dependent traits, specifically skeletal element sizes and bone densities in the same fish species in the same area. Using radiographs of 101 fish from the three species across four locations (two in high-calcium water and two in low-calcium water), this new work documents multi-trait “homeostasis” along the gradient of water calcium. That is, no effect of calcium regime (low-calcium vs. high-calcium) was detected on any of the measured variables. Further, effect sizes for the skeletal traits were very low – lower even than effect sizes previously documented for scale calcium. These results thus show that native fishes maintain phenotypic stability across a suite of functional traits linked to calcium regulation, perhaps pointing to an “organismal-level homeostasis” scenario rather than a “trait-level homeostasis” scenario.

## KEYWORDS

environmental gradients, freshwater ecosystems, functional traits, homeostasis, skeletal traits, water chemistry

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## 1 | INTRODUCTION

Environmental gradients generate spatial variation in natural selection that favours different trait values in populations found at different positions along the gradient (Endler, 1986; MacColl, 2011; Siepielski *et al.*, 2017). In the absence of overwhelming constraints, the outcome will be adaptive population divergence that tends to improve whole organism performance in populations across the gradient (Hendry, 2017; Schluter, 2000). For instance, Darwin's finches occupying environments with different seed types in Galápagos tend to evolve beak sizes and shapes that maximize their ability to consume the local seed types (Schluter & Grant, 1984). Similarly, fish populations found in habitats with different temperatures typically show corresponding divergence in the temperatures at which they manifest maximal growth (Jensen *et al.*, 2000). In these and many other classic examples of local adaptation (Schluter, 2000), the environmental gradient is driven by variation in biotic variables such as the types of resources (Schluter & Grant, 1984) or predators (Langerhans *et al.*, 2004; Lardner, 2000; Van Buskirk, 2002), or the gradient is driven by variation in abiotic variables such as temperature or light (Jensen *et al.*, 2000; Pigliucci *et al.*, 2003). When the environmental gradient is driven by variation in the amount of an abiotic variable such as a limiting nutrient required for survival, growth or reproduction, we might expect adaptive divergence to be – simultaneously – more important (Brady, Bolnick, Barrett, *et al.*, 2019) and more difficult (Hoffmann & Merilä, 1999). The key question in such cases becomes: how do organisms maintain performance across both high and low levels of nutrients?

A typical approach to answering this question examines variation in a single trait for a single species across a limiting environmental gradient (Grether, 2005; Grether *et al.*, 1999); yet such an approach has two major limitations. First, this univariate approach might overlook trade-offs, where the maintenance of performance through stability for one functional trait might come at the cost of the maintenance of performance for other functional traits (Agrawal, 2001; Agrawal *et al.*, 2010; Reznick *et al.*, 2000). Second, the single-species approach can miss how different evolutionary lineages might achieve, or fail to achieve, the maintenance of performance (*via* functional traits) in similar or different ways across an environmental gradient (Langerhans & DeWitt, 2004). As such, the study of multiple lineages across the same environmental gradient facilitates our understanding of shared phenotypic or genetic solutions to a common environmental challenge. The typical approach to such assessment is to examine different lineages of a single species occupying the same environmental contrast (e.g., high predation vs. low predation; benthic vs. limnetic) in multiple locations (Langerhans, 2018; Oke *et al.*, 2017). A limitation of this design is that variation among “replicates” might reflect non-parallelism in the environments, and therefore non-parallelism in selection (Stuart *et al.*, 2017). A less common but potentially more powerful approach, the one used here, examines multiple traits in multiple species across the same environmental gradient in the same locations (Härer *et al.*, 2018; Rosenblum *et al.*, 2017). Specifically, the present study examines multiple calcium-dependent traits (scale

calcium, skeletal element sizes and bone density) in three species of fish in the same locations across a water calcium gradient.

Calcium is an essential element involved in many physiological functions, including muscle contraction, intracellular messaging and reproduction (Crichton, 2008; Loewen *et al.*, 2016). In teleost fishes, c. 99% of the whole body fraction of calcium is stored in biomineralized structures such as otoliths, scales, fins and bones (Flik *et al.*, 1986). Although fish can acquire calcium from their diet, this source of calcium is minimal. Instead, fish mainly acquire calcium directly from the surrounding water *via* their gills (Simkiss & Wilbur, 1989); and so low-calcium water should – in the absence of compensatory adaptations – limit fish distributions and abundances. Indeed, low calcium levels in laboratory experiments are known to compromise various aspects of fish performance, such as growth, feeding and survival (Baldwin *et al.*, 2012; Flik *et al.*, 1986).

A number of fish species are nevertheless capable of adapting to severe calcium gradients. Examples include anadromous fishes that move from high-calcium marine conditions to low-calcium freshwater conditions as they mature – and catadromous fishes that make the same transition as juveniles (Hendry *et al.*, 2004). These transitions appear to be accomplished partly by plastic changes in gill osmoregulation that adjust ion homeostasis in response to changes in the environment (Evans *et al.*, 2005; McCormick, 2001). This marine-to-freshwater transition involves many environmental changes, including multiple ions beyond calcium and the developmental requirements of specific life-history stages. Further, anadromous or catadromous lifestyles also require that populations adapt to accommodate trade-offs between marine and freshwater environments, which is particularly conspicuous when anadromous populations become landlocked, releasing them from this trade-off (Palkovacs *et al.*, 2008; Pearse *et al.*, 2009). As a consequence of these complexities, marine-to-freshwater transitions are not a particularly good model for studying adaptation to calcium *per se*.

More appropriate models for understanding adaptation to low calcium specifically can be found in the comparison of conspecific populations in freshwater environments with different calcium levels. For example, threespine stickleback (*Gasterosteus aculeatus*) populations exist in many low-calcium freshwater environments, demonstrating their ability to adapt to those conditions. Interestingly, however, such adaptation usually involves the reduction in calcium-rich bony structures, especially spines, plates and the pelvis (Bell & Ortí, 1994; Klepaker & Østbye, 2008; Lescak *et al.*, 2015, but also see Reimchen *et al.*, 2013). Further, populations and individuals that do not show reductions in those structures can show trade-offs with other important functions, such as growth (Spence *et al.*, 2012). In short, adaptation to low-calcium water in stickleback might be difficult and costly – at least for some structures and functions. Further, some species appear incapable of generating self-sustaining populations in low-calcium water, such as Ponto-Caspian invaders in eastern North America (Astorg *et al.*, 2021; Iacarella & Ricciardi, 2015; Jones & Ricciardi, 2005; Palmer & Ricciardi, 2005). These species originating from the Ponto-Caspian region (Black, Caspian and Azov Seas) have been invading the Great Lakes (and the St. Lawrence River) *via* ship

ballast water (Ricciardi & MacIsaac, 2000). Importantly, these invaders – which do not have an evolutionary history of exposure to low-calcium water – are restricted to high-calcium water in their invasive range (Iacarella & Ricciardi, 2015; Jones & Ricciardi, 2005; Palmer & Ricciardi, 2005). Further, experimental work confirmed that these limited distributions of invasive species are specifically due to low levels of dissolved calcium (Baldwin *et al.*, 2012; Iacarella & Ricciardi, 2015). How then are native fishes coping across the same gradient?

## 1.1 | The present study

Studies demonstrating the importance of low-calcium water in limiting the performance, and thus spread, of aquatic invasive species have mainly focused on the confluence of high-calcium water from the Upper St. Lawrence River and low-calcium water from the Ottawa River (Figure 1). Although the community shift between these water regimes is dramatic for invasive species, native species are found at high abundance in both regimes (Astorg *et al.*, 2021), implying an evolved ability to achieve high performance even in low-calcium conditions. In previous work, three native fish species - logperch (*Percina caprodes*), pumpkinseed sunfish (*Lepomis gibbosus*) and yellow perch (*Perca flavescens*) - maintained homeostasis in scale calcium concentrations in nature across the dramatic water calcium gradient (Sanderson, Derry, & Hendry, 2021). Yet it remains possible that maintaining scale calcium under calcium-limited conditions might come at the cost of other functional traits that depend on calcium. Therefore, in the present paper, variation across the gradient in other functional traits is assessed: specifically, the size of important skeletal elements

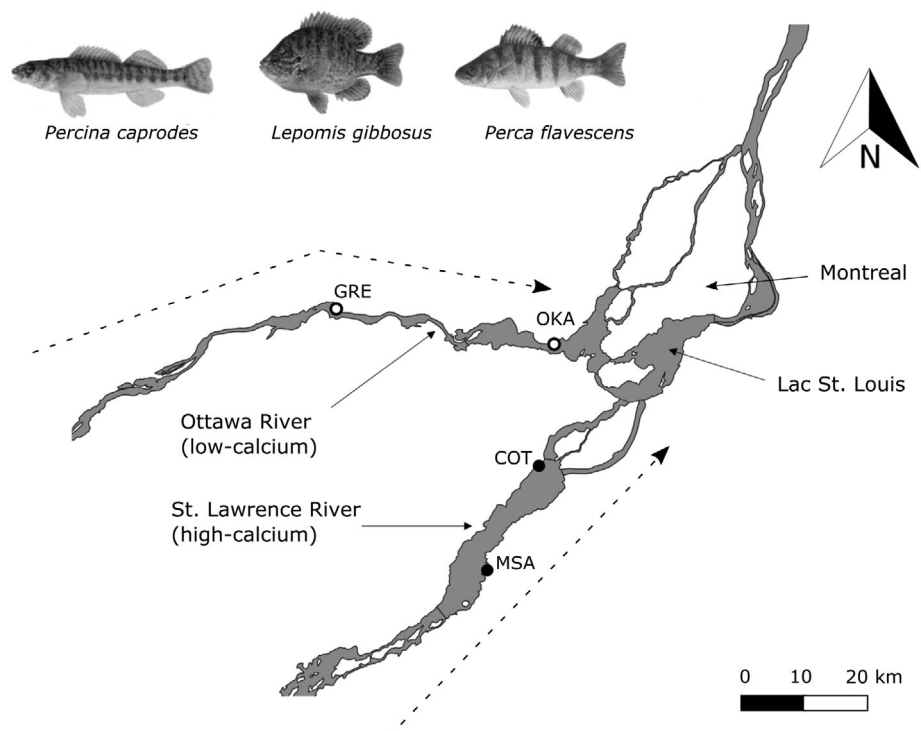
(vertebral number and size, neural spine length and dorsal spine length) and bone density at three different locations in the skeleton (parasphenoid, vertebrae and hypural).

Discussion of the results will consider two alternative scenarios. The “trait-level homeostasis” scenario predicts that fishes maintain homeostasis across the environmental gradient only for some functional traits, thus implying trade-offs when attempting to simultaneously optimize multiple traits. Alternatively, the “organismal-level homeostasis” scenario predicts that fish maintain homeostasis across the environmental gradient for all functional traits. In the latter case, the search for trade-offs – if they exist – would need to be extended to even more traits and functions – and would ideally include common-garden assessments that can separate genetic from plastic influences. Continued failure to uncover trade-offs even then would perhaps indicate the intriguing possibility that the lack of trade-offs in the present is the result of performance and fitness costs already having been paid during selection and adaptation in the past.

## 2 | MATERIALS AND METHODS

### 2.1 | Study system

Lake St. Louis, QC, Canada (45° 22' N, 73° 81' W) is a continuous freshwater ecosystem at the confluence of the Upper St. Lawrence River and the Ottawa River, and it is characterized by strong environmental gradients between the north and south shores (Figure 1). These gradients are created by “low-calcium” water (conductivity 70.5  $\mu\text{S cm}^{-1}$ ; dissolved calcium 9.8  $\text{mg l}^{-1}$ ) that enters lac St. Louis



**FIGURE 1** Map of study system, including lac St. Louis at the confluence of the Ottawa River and the St. Lawrence River, modified from Sanderson, Derry and Hendry (2021). High-calcium water from the Great Lakes flows (flow direction is indicated by dashed arrow) from the southeast and low-calcium water from the Ottawa River flows from the west, with two water types meeting at lac St. Louis. Sites of fish and water collection are indicated by circles, where low-calcium sites are indicated in black (MSA and COT), high-calcium sites white (GRE and OKA)

from the Ottawa River along its north shore, contrasting with “high-calcium” water (conductivity  $363.0 \mu\text{S cm}^{-1}$ ; dissolved calcium  $46.1 \text{ mg l}^{-1}$ ) from the Upper St. Lawrence River that enters along its south shore. The calcium gradients in this system are sufficiently strong to determine the distributions of invasive fishes and invertebrates from the Ponto-Caspian that cannot persist in low-calcium habitats (Astorg *et al.*, 2021, 2022; Iacarella & Ricciardi, 2015; Jones & Ricciardi, 2005; Palmer & Ricciardi, 2005). As such, the gradient is likely a source of selection (Baldwin *et al.*, 2012; Flik *et al.*, 1986) on biomineralized structures in aquatic organisms, including native freshwater fishes (Sanderson, Derry, & Hendry, 2021).

## 2.2 | Sample collection

Three native species were studied: logperch, pumpkinseed sunfish and yellow perch (Bernatchez & Giroux, 2012). These species are common throughout the environmental gradient and mostly occupy benthic habitat. Fieldwork was conducted in the summer of 2018 when the three species were collected at four different sites that maximized the environmental gradient, including two sites in low-calcium water (GRE, OKA) and two sites in high-calcium water (MSA, COT) (Figure 1; Table 1). The fish were collected using a seine net (114 cm deep by 407 cm long, 5 mm mesh) deployed from shore to about 1 m water depth. The target fish species were euthanized using an overdose of buffered tricaine methanesulfonate (MS222) at a concentration of  $250\text{--}500 \text{ mg l}^{-1}$ . Non-target species were released immediately. After euthanization, the fish were suspended by their tails to limit the amount of bending during transportation. Each fish was then frozen individually in a  $-20^\circ\text{C}$  freezer. Fish collection and handling followed McGill University Animal Use Protocol #2016-7831. Physico-chemical water measurements (temperature, conductivity, dissolved oxygen, pH, dissolved organic carbon, total nitrogen and total phosphorus) were taken at each sampling site on a single occasion (Table 1), and detailed methods are described in Sanderson, Derry, and Hendry (2021). The measurements were taken on a single occasion because previous work provides ample evidence that ecological differences between the two water bodies (especially differences in calcium) remain relatively stable (Astorg *et al.*, 2022; Derry *et al.*, 2013; Environnement et changements climatiques Canada, 2019, 2020; Kestrup & Ricciardi, 2010). Although intra-annual variation does exist in water calcium concentrations, the

difference between the two water bodies always remains greater than  $19.2 \text{ mg l}^{-1}$  (Sanderson, Derry, & Hendry, 2021).

## 2.3 | Data collection

In addition to the already-collected data on scale calcium (Sanderson, Derry, & Hendry, 2021), additional functional traits were measured using 2D radiographs. Specifically, lateral X-ray images of each individual fish were taken using a custom-built digital X-ray unit comprising a micro-focus X-ray source (Hamamatsu L6731-01, Hamamatsu Corporation, Bridgewater, NJ, USA) and a digital X-ray detector (PaxScan 2520E, Kodex Inc., Nutley, NJ, USA) housed in a lead-shielded cabinet (Beckmann *et al.*, 2015; Riesch *et al.*, 2018). All further data collection was then performed in Image J version 1.52 (Schneider *et al.*, 2012).

### 2.3.1 | Skeleton element sizes

Fifteen univariate skeletal element sizes were measured on the 2D radiographs. First, the area of three different vertebrae at each of three different locations in the vertebrae column (head region, mid region and tail region) (Figure 2) was measured. Second, the length of nine neural spines was measured: three neural spines in each of the just-mentioned regions (Figure 2). Finally, the length of three dorsal spines was measured: the first three dorsal spines at the first dorsal fin (Figure 2).

### 2.3.2 | Bone density

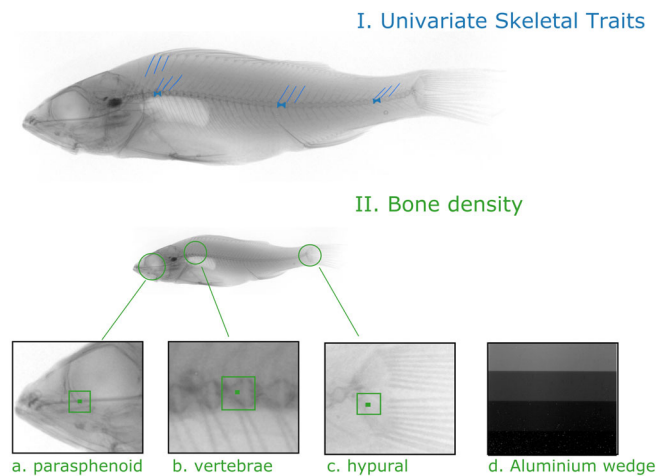
Bone density was estimated using a proxy based on millimetre aluminium equivalents (mmAeq) (Nackaerts *et al.*, 2007). In each radiograph, an aluminium step wedge with three different densities (1, 2 and 3 mmAeq) (Figure 2) was included. These references were used to estimate bone density in mmAeq for three different bones: parasphenoid, vertebrae and hypural (Figure 2). To standardize measurements, a three-by-three pixel square was used for each area of interest and its mean grey value was obtained in ImageJ. The mean grey value for each bone of interest was corrected with that of the reference aluminium wedges to obtain mmAeq as per Nackaerts *et al.* (2007).

**TABLE 1** Physico-chemical properties of the study sites recorded during summer 2018

Site	Water type	Cond. ( $\mu\text{S cm}^{-1}$ )	DOC ( $\text{mg l}^{-1}$ )	$\text{Ca}^{2+}$ ( $\text{mg l}^{-1}$ )	pH	DO (%)	Temp ( $^\circ\text{C}$ )	TP ( $\mu\text{g l}^{-1}$ )	TN ( $\text{mg l}^{-1}$ )
GRE	Low- $\text{Ca}^{2+}$	70.5	19.70	15.7	7.51	98.6	21.3	64.32	0.59
OKA	Low- $\text{Ca}^{2+}$	96.8	13.52	12.8	8.83	71.7	26.0	76.67	0.77
COT	High- $\text{Ca}^{2+}$	263.8	4.01	45.3	8.80	119.7	26.0	11.34	0.33
MSA	High- $\text{Ca}^{2+}$	363.0	6.04	46.1	8.87	61.9	26.5	15.40	0.40

Note: Tables modified Sanderson, Derry, and Hendry (2021).

Abbreviations:  $\text{Ca}^{2+}$ , dissolved calcium ions; Cond., conductivity; DO, dissolved oxygen; DOC, dissolved organic carbon; Temp, temperature; TN, total nitrogen; TP, total phosphorus.



**FIGURE 2** Traits measured from radiographs (example of *yellow perch*). I. Univariate skeletal element sizes: number of vertebrae, area of three vertebrae, length of neural spines, and length of dorsal spines. II. Bone density: a. parasphenoid, b. vertebrae, c. hypural. Panel d. is the aluminium step wedge used to approximate mmAleq.

### 2.3.3 | Scale calcium

Sanderson, Derry and Hendry (2021) removed 30 scales from the left flank at the mid-lateral region of every fish and dried the scales overnight at 80°C (Jeziorski & Yan, 2006; Metz *et al.*, 2014). The dried scales were then dissolved in 60 – 100 µl of 65%–69% nitric acid for 24 h, and analysed with ICP-OES (inductively coupled plasma optical emission spectrometry) using a Thermo Fisher Scientific iCAP 6500 Duo avec Autosampler CETAC ASX-520 (made in England) to obtain scale calcium concentrations. For more details, refer to Sanderson, Derry and Hendry (2021), where an initial analysis is presented. Those authors present data from two sampling years (2017 and 2018); here, only the 2018 data are presented to compare the same fish across all trait types (scale calcium, bone density, and skeletal element sizes).

## 2.4 | Data analyses

For all analyses, body size (standard length) was log-10 transformed to better meet the assumptions of a Gaussian distribution (Bland & Altman, 1996; LaBarbera, 1989). Moreover, in all the models, body size was included as a covariate to correct for any allometry. Analyses were performed in several different statistics packages – as noted later.

To enable comparisons of effect sizes across traits in each analysis, the trait difference between low-calcium vs. high-calcium populations was estimated as *partial*  $\eta^2$  and Cohen's *d*. First, *partial*  $\eta^2$  measures the proportion of variance explained by a given explanatory factor relative to the unexplained variation (Langerhans & DeWitt, 2004). It is a common effect size measure used in studies quantifying the importance of trait adaptation to different environments (Chacin *et al.*, 2015; Sanderson, Beausoleil, *et al.*, 2021), including in fishes (Langerhans & DeWitt, 2004; Riesch *et al.*, 2016; Tobler

*et al.*, 2011), and it provides the basis for comparison to extensive meta-analyses of trait effects in ecology and evolution [e.g., (Møller & Jennions, 2002)]. Second, Cohen's *d* is the difference between means in S.D. units (Cohen, 1988), here quantified using pooled S.D.s for populations within a calcium regime. Cohen's *d* was calculated for each trait using LSM (least square means) and S.E. from the multivariate analysis of covariance (MANCOVA) models described later. Thus, the Cohen's *d* values reported represent the average magnitude of the difference between traits observed between populations living in low-calcium or high-calcium water in relation to within-population S.D. values. Cohen's *d* is a common effect size measure in many ecological and evolutionary studies, including previous work on adaptation in fishes (Langerhans, 2018), and it thus allows general statements about the magnitude of any effects documented for traits between the two environments. As per Langerhans (2018), Cohen's  $d \geq 1.0$  is treated as a moderate effect and Cohen's  $d \geq 2.0$  is treated as a strong effect. A negative Cohen's *d* value indicates an increase in low-calcium water compared to high-calcium water, whereas a positive value indicates a decrease in low-calcium water compared to high-calcium water.

The data were first analysed with MANCOVAs implemented in SAS version 8.0. (MANCOVA analyses with random effects have been optimized in SAS, which is currently the standard for their implementation). Fixed effects included water regime (low-calcium or high-calcium water), species and standard length. The random effect was site nested within each regime. Separate analyses were conducted for the three data types to ensure the best possible assessment of each class of functional trait. For skeletal element sizes, the 15 log-transformed univariate skeletal element sizes were used as response variables. For bone density, the density in mmAleq of the three measured bones (parasphenoid, vertebrae and hypural) were used as response variables. For scale calcium, a single measurement per fish based on 30 pooled scales from a given fish was used as the response variable (Sanderson, Derry, & Hendry, 2021). This last analysis was done with ANCOVA of the same model structure as the above MANCOVAs – also conducted in SAS for consistency with the MANCOVAs.

Because scale calcium is directly comparable to water calcium (ppm), an additional effect size measure could be calculated as “proportional sensitivity”: the ratio of proportional change between sites in scale calcium relative to water calcium (Sanderson, Derry, & Hendry, 2021). This measure of “proportional sensitivity” compares the ratio of proportional change between two variables across the same sites:

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

where  $y_1$  and  $y_2$  are the predicted estimates of scale calcium concentrations obtained from fixed values of water calcium  $x_1$  and  $x_2$  in a linear mixed effect model with the fixed effects of water regime (low-calcium or high-calcium water), species, body size as a covariate and a random effect of site. This LMM was performed in R version 3.6.1 (R Core Team, 2021) using nlme package (Pinheiro *et al.*, 2019).



### 3 | RESULTS

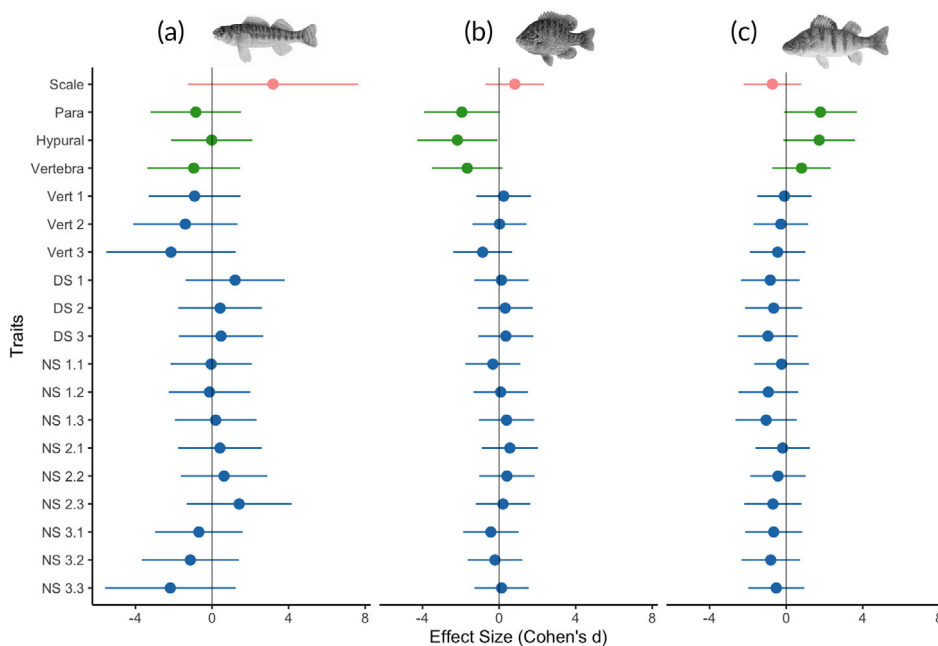
Radiographs were analyzed from a total of 101 fish, with sample sizes distributed as evenly as possible (given differential availability across the samples) over the three species (yellow perch, logperch and pumpkinseed sunfish) across the four different sites (Table 2). The main outcome was that no noteworthy relationships were detected between any of the three calcium-dependent trait types (skeletal element sizes, bone density and scale calcium) and water regime (low-calcium vs. high-calcium) (Figures 3 and 4). Moreover, this result generally held for all three species examined (Figures 3 and 4; Table 3). We now further detail results for each trait type.

#### 3.1 | Skeletal element sizes

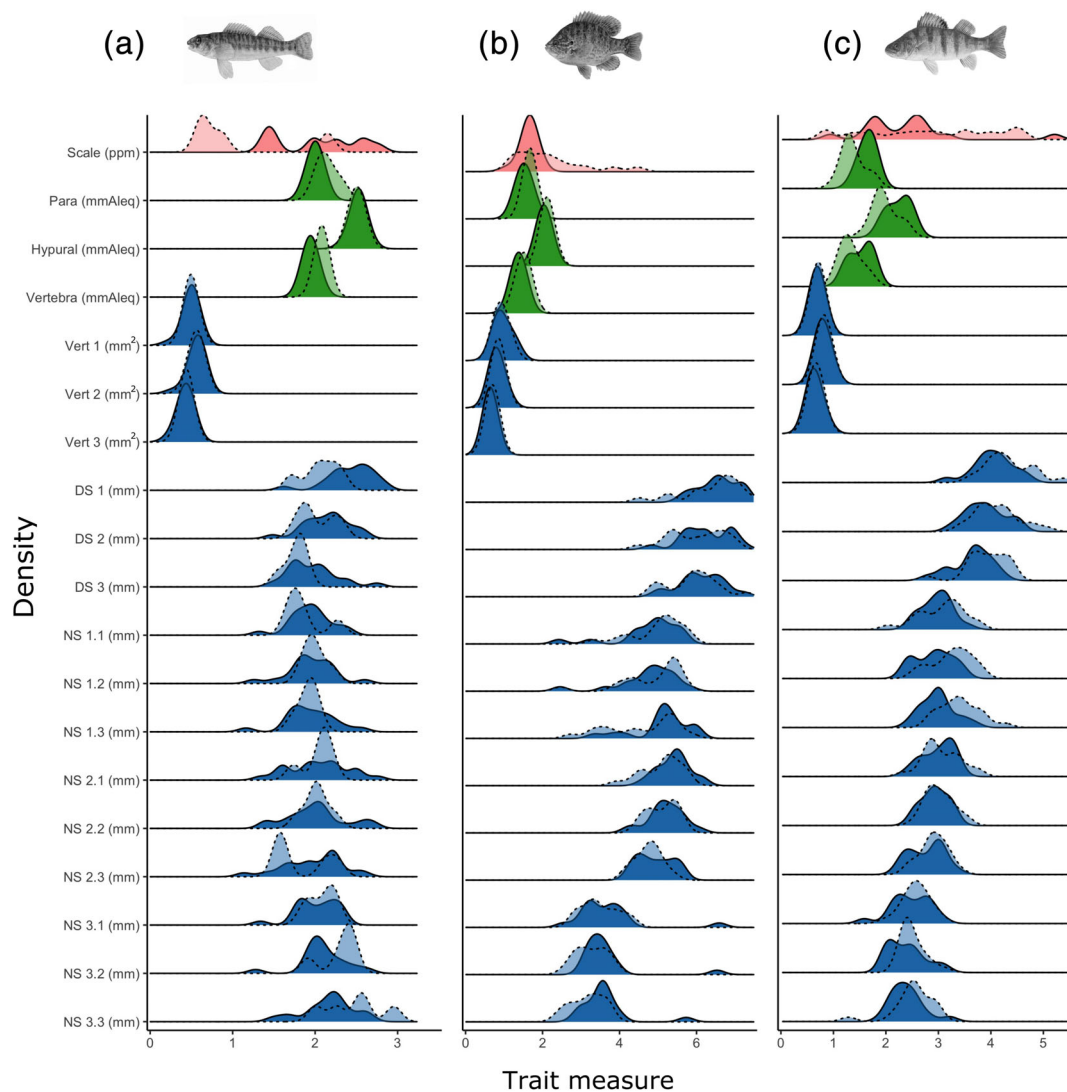
A consistent relationship was not evident between the sizes of skeletal elements (vertebral area, dorsal spine length and neural spine length) and water regime (low-calcium vs. high-calcium water). For instance, MANCOVA (see *P*-values in Table 3) did not reveal a significant effect of water regime overall (no main effect), nor any species-specific effects (no regime-by-species interaction). Beyond statistical significance, effect sizes (Table 3) for water regime and the regime-by-species interaction were very low (0.18 and 0.25, respectively) in comparison to the *partial*  $\eta^2$  for species and standard length (0.91 and 0.90, respectively). Further, Cohen's *d* values for the individual skeletal elements were typically very low

Species	Water regime	Site	Latitude (DD)	Longitude (DD)	N	Total N
<i>Percina caprodes</i>	High-Ca <sup>2+</sup>	COT	45.253611	-74.211944	10	25
<i>P. caprodes</i>	Low-Ca <sup>2+</sup>	GRE	45.629803	-74.607868	0	
<i>P. caprodes</i>	High-Ca <sup>2+</sup>	MSA	45.096041	-74.413554	10	38
<i>P. caprodes</i>	Low-Ca <sup>2+</sup>	OKA	45.459560	-74.087574	5	
<i>Lepomis gibbosus</i>	High-Ca <sup>2+</sup>	COT	45.253611	-74.211944	8	
<i>L. gibbosus</i>	Low-Ca <sup>2+</sup>	GRE	45.629803	-74.607868	10	38
<i>L. gibbosus</i>	High-Ca <sup>2+</sup>	MSA	45.096041	-74.413554	10	
<i>L. gibbosus</i>	Low-Ca <sup>2+</sup>	OKA	45.459560	-74.087574	10	
<i>Perca flavescens</i>	High-Ca <sup>2+</sup>	COT	45.253611	-74.211944	8	101
<i>P. flavescens</i>	Low-Ca <sup>2+</sup>	GRE	45.629803	-74.607868	10	
<i>P. flavescens</i>	High-Ca <sup>2+</sup>	MSA	45.096041	-74.413554	10	
<i>P. flavescens</i>	Low-Ca <sup>2+</sup>	OKA	45.459560	-74.087574	10	

**TABLE 2** Sample sizes (number of fish analysed “N”) and geographic coordinates per species for each sample site



**FIGURE 3** Standardized effect sizes (difference between high-calcium and low-calcium sites in SD units for each trait) for each species. Panel (a) is logperch, panel (b) is pumpkinseed sunfish and panel (c) is yellow perch. Colours indicate the different set of traits: pink is scale calcium, green is bone density, and blue is skeletal traits. Error bars are 1 S.E. *Scale* is scale calcium, *Para* is parasphenoid bone density, *Hypural* is hypural bone density, *Vertebrae*, is vertebrae bone density, *Vert1-3* is vertebrae area, *DS1-3* is dorsal spine length and *NS1.1-3.3* is neural spine length.



**FIGURE 4** Density plots of all different sets of traits (scale calcium, 3 bone density traits, and 15 skeletal element sizes traits) for each species. Panel (a) is logperch, panel (b) is pumpkinseed sunfish, and panel (c) is yellow perch. Colours indicates the different sets of traits: pink is scale calcium, green is bone density, and blue is skeletal traits. Light colours with dotted lines are low-calcium sites and dark colours with solid lines are high-calcium sites. *Scale* is scale calcium, *Para* is parasphenoid bone density, *Hypural* is hypural bone density, *Vertebrae*, is vertebrae bone density, *Vert1-3* is vertebrae area, *DS1-3* is dorsal spine length, and *NS1.1-3.3* is neural spine length. Units for each trait are indicated in parenthesis on the y-axis. All traits were allometrically adjusted for each species using the formula  $M_{std} = M_o (\overline{SL}/SL_o)^b$ , where  $M_o$  and  $SL_o$  are the observed trait length (not log transformed) and standard length (not log transformed), respectively,  $\overline{SL}$  is the grand mean of all standard lengths, and  $b$  is the slope of an ANCOVA of the form  $M \sim \overline{SL} + Site$ , where  $M$  and  $\overline{SL}$  are log transformed (Hendry & Taylor, 2004; Reist, 1986).

(Langerhans, 2018) between the two water regimes (average Cohen's  $d = -0.26$ ; Figure 3). Indeed, the average difference in skeletal elements was in the opposite direction of the adaptive prediction (Figure 4).

### 3.2 | Bone density

Similar to skeletal element sizes, no consistent relationship was evident between the densities of three focal bones (hypural, vertebrae and parasphenoid) and water regime. For instance, the MANCOVA (see  $P$ -values in Table 3) did not reveal a significant

effect of water regime overall (no main effect), nor any species-specific effects (no regime-by-species interaction). Beyond statistical significance, effect sizes (Table 3) for water regime and the regime-by-species interaction were relatively low (0.23 and 0.35, respectively) compared to the *partial*  $\eta^2$  for species and standard length (0.56 and 0.66, respectively). Cohen's  $d$  values for the bone density estimates were also very low (Langerhans, 2018) between the two water regimes (average Cohen's  $d = -0.37$ ; Figure 3). Again, the average difference in skeletal elements was often in the opposite direction of the adaptive prediction, exemplified here by pumpkinseed sunfish which tended to exhibit greater, not lower, bone density in the low-calcium water regime (Figure 4).

**TABLE 3** Results for different sets of traits from nested MANCOVAs

	Partial $\eta^2$	F	P
Univariate skeletal element sizes			
Species	0.91	32.41	<b>&lt;0.001***</b>
Regime	0.18	1.09	0.36
Standard length (log)	0.90	19.83	<b>&lt;0.001***</b>
Species-by-regime	0.25	1.06	0.39
Bone density			
Species	0.56	2.93	<b>&lt;0.05*</b>
Regime	0.23	1.49	0.23
Standard length (log)	0.66	10.16	<b>&lt;0.001***</b>
Species-by-regime	0.35	1.02	0.40
Scale calcium			
Species		4.28	0.07
Regime		2.90	0.13
Standard length (log)		4.72	0.03
Species-by-regime		3.10	0.11

Note: Bold values represents as statistically significant  $p < 0.05$ . Significance bold codes: \*\*\* $P < 0.001$  and \* $P < 0.05$ .

### 3.3 | Scale calcium

In the first (LMM) analysis of these data (Sanderson, Derry, & Hendry, 2021), no consistent effect of water regime on scale calcium overall (no main effect) was detected, nor was any suggestion of species-specific effects (no interaction). That analysis also showed that, for a given increase in water calcium, the proportional change in scale calcium was smaller (average 15% of the change in water calcium – meaning that for a given proportional increase in water calcium across the environmental gradient, we recorded a much smaller proportional change in scale calcium). Re-analysing these data with ANCOVA to enable direct comparison to skeletal traits (above) also failed to find any main effect of water regime or a species-by-regime interaction (Table 3). Further, the average Cohen's  $d$  effect size was 1.01 for scale calcium (Figure 3). Thus, in comparison to this already-small effect size for scale calcium reported previously, even smaller effect sizes were present for the new traits (skeletal element sizes and bone density) reported in the present study (Figures 3 and 4).

## 4 | DISCUSSION

Does the maintenance of one calcium-dependent functional trait come at the cost of maintaining other calcium-dependent functional traits? In previous work (Sanderson, Derry, & Hendry, 2021), three native fish species (logperch, pumpkinseed sunfish and yellow perch) were found to maintain scale calcium across a steep gradient in water calcium, suggesting that adaptive divergence among populations has maintained “homeostasis” in this important functional trait. Yet – based on those data alone – it was not possible to distinguish

between “trait-level homeostasis” and “organismal-level homeostasis.” It is now possible to report that all three species also maintain skeletal element sizes and bone densities across the same water calcium gradient. This additional outcome suggests that native fishes deploy calcium regulation mechanisms that allow the uptake and deposition of sufficient calcium to maintain several broad classes of calcium-dependent traits. Stated another way, the maintenance of high calcium in scales when water calcium is low does not trade-off with the development of skeletal structures. In short, these fishes at least show “multi-trait homeostasis” across the calcium gradient, which might also indicate “organismal-level homeostasis” (see below).

This maintenance of multiple functional phenotypes across an environmental gradient of resources necessary for those phenotypes is suggestive of “countergradient variation,” where adaptive population divergence compensates for local environmental effects, thus dampening phenotypic differences in nature (Conover & Schultz, 1995; Levins, 1968). Such countergradient patterns can involve several mechanisms, including phenotypic buffering, genetic compensation, plasticity or a combination of these mechanisms (Brady, Bolnick, Angert, et al., 2019; Conover et al., 2009; Grether, 2005; Reusch, 2014). Distinguishing among these possible mechanisms of homeostasis would require common-garden rearing experiments with different populations of each species raised under a range of water calcium levels. For the time being, it seems profitable to at least discuss various alternative explanations for the lack of trade-offs between investment in multiple calcium-dependent structures even when calcium levels are very low are.

A default expectation for why calcium-dependent traits were not compromised in low-calcium water might be that even the “low” calcium levels in this system were not sufficiently limiting (i.e., they were not “too low”), and therefore would not require any specific adaptive adjustments. Two lines of evidence argue against this explanation. First, concentrations of calcium in the Ottawa River water are similar to low-calcium treatment levels shown in experimental settings to have detrimental effects on fish (Baldwin et al., 2012; Blanksma et al., 2009; Iacarella & Ricciardi, 2015; Metz et al., 2014). Second, aquatic invasive species (round gobies, *Neogobius melanostomus*; exotic amphipods, *Echinogammarus ischnus*; and zebra mussels, *Dreissena polymorpha*) have been unable to colonize Ottawa River sites (Astorg et al., 2022; Iacarella & Ricciardi, 2015; Jones & Ricciardi, 2005; Morissette et al., 2018; Palmer & Ricciardi, 2005) apparently due to the low levels of dissolved calcium (Baldwin et al., 2012; Iacarella & Ricciardi, 2015). Both lines of evidence suggest that the native fishes in the present study deploy some sort of an adaptive response (whether genetic or plastic) that maintains a suite of calcium-dependent functional traits even when calcium availability should otherwise make it difficult to maintain those traits. If we accept this argument that calcium levels were low enough to require adaptive responses, we can next discuss the fact that these fishes were able to show this compensation across multiple calcium-dependent traits such that no trade-off was evident between those traits.



#### 4.1 | Whither the trade-off?

In a situation of multiple competing demands (here scales and skeletons) for a potentially limiting resource (here calcium), we might expect trade-offs such that maintaining homeostasis in some functional traits would reduce functionality of other traits. Studies of threespine stickleback provide an illustrative example. Stickleback have pelvic spines (a defence against vertebrate predators) that are often reduced – and are sometimes absent – in populations living in low-calcium conditions (Bell *et al.*, 1993; but also see Reimchen *et al.*, 2013). Here, then, is a clear indication that low-calcium water can limit (or at least select against) the production of some calcium-dependent structures. Stickleback also have rows of lateral defensive plates composed of dermal bone, which are often reduced in size and/or number in freshwater populations (Spence *et al.*, 2012). Further, stickleback from freshwater populations having a full complement of lateral plates can exhibit lower growth than fish from populations having fewer plates when they are both tested in low-calcium water (Spence *et al.*, 2012). In this case, maintaining a full complement of calcium-dependent plates in low-calcium water here leads to a trade-off with growth rate, generating trait-level homeostasis but not organismal-level homeostasis. In short, stickleback demonstrate adaptation to low-calcium water (under certain conditions) – but also show trade-offs among traits that reflect current costs of that adaptation.

Contrasting with this work on stickleback, no trade-offs were observed between calcium-dependent structures in the three fish species examined here; yet it remains possible that undetected trade-offs might still be present. Stated another way, the present demonstration of multi-trait homeostasis might still be insufficient to infer organismal-level homeostasis. Most obviously, not all calcium-dependent traits have been assessed. Other traits that might be affected by low levels of dissolved calcium include otoliths, plasma  $\text{Ca}^{2+}$  levels (Flik *et al.*, 1986; Shephard, 1981), scale thickness (Long *et al.*, 1996; McHenry *et al.*, 1995; Wainwright & Lauder, 2016), growth (Arendt *et al.*, 2001; Flik *et al.*, 1986; Flik & Verbost, 1993) and expression of epithelial calcium channels (Gibbons *et al.*, 2016). Regardless, it is also possible that fish in low-calcium water might simply be more effective or efficient overall at calcium uptake, mobilization, deposition and use. Even so, we might still expect trade-offs with some other class of traits or functions, such as the acquisition of other limiting nutrients or the performance of other functions (such as growth rate or reproduction). Here, then, is the general problem of inferring the absence of trade-offs in organismal performance (and thus organismal-level homeostasis): it is always possible that some other hidden trait or function is paying the cost.

Three hypotheses have thus far been considered to explain the lack of observed trade-offs for fish living in low-calcium water: (a) calcium levels were not sufficiently low to be stressful (and so, no trade-off would be expected), (b) trade-offs might occur with other (unmeasured) calcium-dependent structures or functions or (c) trade-offs might occur with other (not directly calcium dependent) classes of traits or functions. Yet the failure to find trade-offs when they would

be expected is such a common feature of many natural systems (Hereford, 2009; Jennions *et al.*, 2001; Leroi *et al.*, 1994) that we should also consider more general explanations. In particular, the costs of adaptation to a limiting nutrient might be absent in the present because those costs have been paid *via* selective mortality in the past. This explanation is not commonly invoked (as opposed to those discussed earlier) and therefore warrants further explanation.

#### 4.2 | Perhaps the lack of current trade-offs can be explained by past selection

When an environment changes to become more stressful (*e.g.*, colonizing an environment with limited resources, like calcium), selection for improved tolerance to that stressor is expected to be “hard” (as opposed to “soft”) – that is, selection will increase mortality rates (review: Brady, Bolnick, Angert, *et al.*, 2019). This increased mortality represents a cost of selection in the form of “genetic load” that stays high until the population adapts to the new conditions. This form of genetic load can be thought of as the number of selective deaths that must occur for the population to fully adapt to the new conditions. Once the population adapts and increases in abundance, however, it has paid those costs of selection (removing maladaptive alleles) and now has no difficulty inhabiting the conditions that were originally so stressful. This logic can be extended to provide another explanation for the lack of trade-offs in the present study system and more generally – as now explained.

Adaptation to low-calcium water might have been extremely difficult at first – generating substantial mortality and strong selection. This expectation is supported by experiments that expose naïve fish to low-calcium water (Baldwin *et al.*, 2012; Iacarella & Ricciardi, 2015) and by the failure of Ponto-Caspian invaders to colonize low-calcium water in eastern North America (Iacarella & Ricciardi, 2015; Jones & Ricciardi, 2005; Palmer & Ricciardi, 2005). During this period when adaptation was difficult, trade-offs would be expected. During the initial period of intensive adaptation, however, selection would also tend to remove individuals that showed the strongest trade-offs – or that suffered the most from those trade-offs. Once the population passed through this difficult initial period and became reasonably well adapted to the originally stressful conditions, the result could be a locally adapted populations able to maintain homeostasis across multiple traits – perhaps even at the whole-organism level – without incurring large costs in the present (because they were paid *via* selective deaths in the past).

#### 4.3 | A possible application to invasion biology

The above logic does not imply that the cost of selection is high each time a high-calcium fish population encounters low-calcium water. Instead, a long history of native fishes occupying a diversity of calcium environments has probably maintained a pool of standing variation that facilitates rapid adaptation to new calcium conditions without

paying excessive costs of selection each time. Instead, the costs paid during rapid adaptation in such a situation would presumably be lower than the cost paid the first time that adaptation to those conditions took place – that is, the first time a given high-calcium fish lineage successfully colonized low-calcium conditions. Subsequently, alleles suitable for adaptation to low-calcium conditions might persist within the species as a whole – even for populations not typically occupying low-calcium water. Indeed, this situation characterizes the ability of marine threespine stickleback to adapt repeatedly and rapidly to new freshwater habitats *via* standing genetic variation in the marine population that persists *via* gene flow from freshwater populations (Roberts Kingman *et al.*, 2021; Roesti *et al.*, 2014; Schluter & Conte, 2009).

We can see considerable value in applying these ideas to systems where some fishes can occupy a broad diversity of habitats without obvious costs, whereas other fishes cannot. Indeed, although all three native species (logperch, pumpkinseed sunfish and yellow perch) maintained homeostasis in multiple calcium-dependent traits (scale calcium, skeletal element sizes and bone density) across a steep gradient in water calcium, invasive species appear unable to do so in the same locations. For example, round gobies are not found in the Ottawa River and are rare in the plume of the Ottawa River water after it joins the St. Lawrence River (Astorg *et al.*, 2022). This adaptive failure might reflect a lack of past adaptation to low-calcium conditions given that round gobies and other Ponto-Caspian invaders live exclusively in high-calcium environments (Astorg *et al.*, 2021, 2022; Iacarella & Ricciardi, 2015; Jones & Ricciardi, 2005; Palmer & Ricciardi, 2005) – and therefore might not have yet - in their past history - paid the costs of adaptation to low-calcium water. This possibility suggests an interesting way of assessing and monitoring invasion risk into the Ottawa River. In particular, the native species might be used to identify specific genes mediating the spread of invasive species. For instance, genome sequencing of native fish across the calcium gradient could identify specific genes – and allelic variants – that contribute to the maintenance of multi-trait homeostasis. The resulting candidate genes then could be sequenced in invasive species to assess if genetic variation is rare or missing. If so, these specific genes might be responsible for limiting the spread of invasive species into low-calcium waters. Further, variation in those genes could be monitored to assess if any potentially problematic adaptive variants are arising in those genes, which could then kick off the beginning of adaptation by the invaders to low-calcium water.

#### AUTHORS CONTRIBUTIONS

S.S. led conceptualization, data curation, formal analyses, investigation, methodology, validation, visualization, writing and editing; L.A. contributed to methodology and fieldwork; G.E.H. led geometric morphometric analyses; S.B.C. contributed to methodology; R.B.L. performed analyses and developed protocols; A.M.D. contributed to conceptualization, funding acquisition, resources and supervision; A.P.H. contributed to conceptualization, funding acquisition, methodology, resources and supervision, and led project administration, reviewing and editing.

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#### ETHICAL STATEMENT

Fish collection and handling followed a McGill University animal care protocol #2016-7831.

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