



Ca²⁺ levels in *Daphnia* hemolymph may explain occurrences of daphniid species along recent Ca gradients in Canadian soft-water lakes.

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ABSTRACT

Calcium levels are declining in eastern North American and western European lakes. This widespread issue is affecting the composition of crustacean zooplankton communities, as the presence and abundance of several calcium-rich daphniid species are declining, while two other daphniids, *D. catawba* and *D. ambigua*, that apparently tolerate low calcium environments, are prospering. The physiological basis for low calcium tolerance of these daphniids is unknown. In this study the presence of one Ca-rich (*D. pulicaria*) and one Ca-poor (*D. ambigua*) daphniid species in Canadian Shield lakes is assessed in relation to lake water Ca levels. The occurrence of *D. ambigua* was independent of Ca levels in Ontario lakes, whereas *D. pulicaria* was more likely to occur in lakes with relatively more Ca. In the laboratory, *D. ambigua* maintained lower levels of hemolymph Ca²⁺ across a range of low Ca levels (0.7 to 7 mg l⁻¹) compared with *D. pulicaria*. The hemolymph pH remained steady across this Ca gradient in *D. ambigua* while it was significantly more acidic in *D. pulicaria* in the two lowest Ca treatments. While Ca²⁺ uptake was observed adjacent to the surface of *D. ambigua* individuals, Ca²⁺ loss was observed for *D. pulicaria* assayed under moderately high Ca levels. Based on these observations we propose that *D. ambigua* is able to survive in low Ca lakes by maintaining low free ionic Ca²⁺ levels in the hemolymph which minimizes the Ca gradient across the body wall in low Ca water thus limiting overall Ca loss and facilitating Ca²⁺ uptake.

1. Introduction

Remarkable changes are occurring in the major ion chemistry of lakes in eastern North America and western Europe where calcium levels (Ca: ionic and combined) are declining (Hessen et al., 2017; Jeziorski et al., 2008). Ca decline is most prevalent in freshwaters draining watersheds with thin soils, overlying granitic terrain in areas that have received many decades of atmospheric acid deposition linked mainly to SO₂ emissions. In these lakes, Ca levels are currently declining for three reasons. Since roughly the middle of the 20th century, acid deposition rates have exceeded mineral weathering rates in these regions, thus soil Ca levels necessarily fell (Likens and Buso, 2012). This initially raised Ca export to downstream waters, but such increases were not sustainable so long as acid deposition rates exceeded mineral weathering rates. Secondly, logging followed by afforestation further lowered soil Ca (Jeziorski et al., 2008). Tree biomass is often > 1% Ca; logging typically removes this Ca pool from the watershed, and once removed newly growing trees remove even more exchangeable Ca from

the soil (Reid and Watmough, 2016). Finally, recent reductions in acid deposition rates, through government regulatory efforts to curb acid rain, are lowering the rate of export of the residual exchangeable Ca pool from the soil, lowering downstream levels in lakes. The importance of these mechanisms varies with location, but whatever the site-specific mix, there is no doubt that Ca levels have declined dramatically in Canadian Shield lakes over the last 4 decades, to levels < 1.5 mg/L in many lakes, and this is threatening Ca-rich biota including daphniids (Cairns and Yan, 2009).

Daphnia (Anomopoda, Branchiopoda, Crustacea) species are arguably the most important filter-feeding zooplankton in lakes throughout the world, as they play a major role in the dynamics of the food web. Daphniids feed on bacteria, protozoans and especially on phytoplankton while serving as prey to many species of fishes (Hambricht and Hall, 1992) and predatory invertebrates (Miner et al., 2012; Blumenshine and Hambricht, 2003). Their effect on phytoplankton levels in lakes depends on their abundance and body size with larger species having a greater impact and outcompeting their smaller

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congeners (Kreutzer and Lampert, 1999). A comprehension of how and why calcium decline impacts daphniids must necessarily include an understanding of basal physiological processes that regulate somatic ion and water levels, but, for the most part, we lack that information for daphniids.

Only a few studies have examined the ion and osmo-regulatory physiology of *Daphnia* and these have only considered salinity tolerance. For example, Elphick and colleagues noted that *Daphnia magna* and *Daphnia pulex* are particularly sensitive to NaCl (Elphick et al., 2011). In contrast, *Daphnia exilis* can tolerate relatively high levels of salinity up to 8 g/L and responds to salinity challenges as an osmoconformer (Heine-Fuster et al., 2010). As a result, *Daphnia exilis* has successfully invaded Chilean and American lakes that receive heavy loads of anthropogenic NaCl (Hairston et al., 1999; Márquez-García et al., 2009). The most rigorous study of the ion-regulatory physiology of *Daphnia* suggests that *Daphnia magna* possesses a $2\text{Na}^+/\text{1H}^+$ antiporter unique to crustaceans. This antiporter is shared by Na^+ and Ca^{2+} , supporting ion uptake from dilute freshwater habitats (Ahearn and Franco, 1990; Glover and Wood, 2005). Through this mechanism, *Daphnia* can sequester both sodium and calcium from their environment. The site of ion uptake in *Daphnia magna* has not been directly shown; however, the thoracic appendages possess a pair of gill-like structures which contain putative ionocytes that stain with the silver nitrate – nitric acid test, indicative of the accumulation of chloride ions (Kikuchi, 1983). These studies are useful for specific *Daphnia* species, but do not provide the underlying fundamental physiological explanation for interspecific differences in low-Ca tolerance.

Some daphniids can survive and reproduce in soft water containing as little as 0.1 to 0.5 mg/L Ca; however, their brood size is significantly reduced and maturation time increased at Ca levels < 1.5 mg/L (Ashforth and Yan, 2008; Cairns and Yan, 2009). In addition, a decline in daphniid abundance correlated to low Ca levels in Ontario shield lakes has been documented (Desellars et al., 2011). Total body Ca levels differ among daphniid species (Jeziorski and Yan, 2006), and between different Cladoceran and Copepod species with the Cladocerans having relatively higher total body concentrations (Jeziorski and Yan, 2006; Yan et al., 1989). The majority of the total body Ca is in the carapace, which in crustaceans is hardened by the deposition of calcium carbonate (Neufeld and Cameron, 1993). It is not clear why daphniids have such high levels of calcium relative to other cladocerans and copepods.

In an effort to begin to understand the fundamental ion and osmo-regulatory physiology of *Daphnia* species endemic to the Canadian Shield lakes, an area of widespread Ca decline, we measured and compared levels of major inorganic cations in the hemolymph of three *Daphnia* species under relatively high Ca conditions. We then compared hemolymph pH and Ca^{2+} levels in daphniids reared over a Ca gradient representative of current Ca levels in Canadian Shield lakes. We also measured Ca^{2+} gradients near the surface of daphniids using the scanning ion-selective electrode technique. These measurements were coupled with a previous field survey documenting the occurrence of two of the species in lakes on the Canadian Shield in Ontario with different Ca levels. Our intent was to determine if levels of inorganic cations in the hemolymph of *Daphnia* can be used to predict their relative tolerance to Ca decline, given that total body Ca concentration of the species appears to be an unreliable predictor of their low Ca tolerance in nature (Azan et al., 2015).

2. Methods

2.1. Box plots of the distribution of lake water Ca levels in the presence and absence of two *Daphnia* species

Our lake survey was conducted in the region commonly known as Muskoka, the tertiary watershed formally identified as 2EB by Cox (1978). This is a 5635 km² watershed containing over 1600 lakes located on the eastern side of Georgian Bay of Lake Huron. It is the major

geographic focus of terrestrial (Reid and Watmough, 2016) and aquatic (Jeziorski et al., 2008) research on environmental calcium decline in Ontario.

We employed a stratified-random sampling design to sample 311 lakes within the watershed in 2005 and 2006. Details of the lake selection and stratification protocols and lake and zooplankton sampling protocols are provided by Cairns and Yan (2009), Weisz and Yan (2010) and Kelly et al. (2013). In brief each lake was visited once in the summer between June and August. The lakes were typically nutrient poor (median total phosphorus of 7.8 µg/L), with slightly brown (median dissolved organic carbon of 5.3 mg/L), low conductivity (median = 23.3 µS cm⁻¹), and slightly acidic (median pH = 6.2) waters. The median lake area was 25 ha. All lakes with recent invasions by *Bythotrephes*, a macroinvertebrate zooplanktivore with large impacts on daphniid assemblages (Yan et al., 1989) were removed, as were lakes with conductivity of > 50 µS cm⁻¹, as this was indicative of lakes affected by road salt. The resulting number of lakes for our analysis was 250.

Pelagic zooplankton were collected at a deep, mid-lake station with a 30 cm diameter, 65 µm mesh conical tow net, using two, subsequently-pooled, vertical hauls from 1 m above bottom to the surface. Samples were preserved in the field with a 5.5% buffered, sucrose formalin solution. A minimum of 250 animals, including the daphniids, were subsequently identified and counted, subsampling to ensure that no one species formed > 10% of the count. Subsamples were generated with a Folsom plankton splitter, and count protocol followed Yan et al. (2008). Kelly et al. (2013) present a detailed description of the zooplankton communities of these lakes.

Vertical oxygen and temperature profiles were generated at the sampling station, and thermal strata were delineated. A composite epilimnetic + metalimnetic water sample was generated for subsequent major ion, including Ca analyses, at the Ontario Ministry of the Environment and Climate Change's (MOECC) Dorset Environmental Science Centre, following standard MOECC procedures (Ontario Ministry of the Environment, 2015).

2.2. Experimental animals for measurements of hemolymph ion levels

Two separate studies were conducted, the first used individuals of *Daphnia magna*, *Daphnia pulex*, and *Daphnia ambigua* that were obtained from the Field Laboratory for the Assessment of Multiple Ecological Stressors (FLAMES), Queens University's zooplankton ecotoxicology field lab located at the MOECC's Dorset Environmental Science Centre in Dorset, Ontario, Canada. For the first study, cultures were held in 8 L aquaria containing aerated dechlorinated Toronto municipal tap water (DCT, approximate composition, [Ion] µmol l⁻¹: [Na^+] 590; [Cl^-] 920; [Ca^{2+}] 760 or 30.4 mg/L; [K^+] 43; pH 7.35). The aquaria were held at room temperature (RT, ~21 °C), exposed to a 12/12 h, light/dark regime and individuals were fed with a mixture of green algae. The hemolymph [Ca^{2+}], [Na^+], [K^+] and pH of the daphniids were measured using ion-selective microelectrodes (ISMEs). Results of this study are presented in Fig. 2.

Based on results of the first study, a subsequent, second study was undertaken on 7 day old individuals of *D. pulex* and *D. ambigua* cultured at the FLAMES under controlled conditions in Conviron culture chambers at 20 °C, and a 12/12 h light/dark regime. All individuals were sampled in the inter-moult phase. Ten neonates (< 24 h old) were set in 300 mL containers in FLAMES medium (Celis-Salgado et al., 2008) spiked with four different levels of Ca in mg/L (0.7, 1.0, 2.5, 7.0), in three replicates per concentration. The daphniids were fed 1.5 mg of carbon/L/day of the green alga *Pseudokirchneriella subcapitata* cultured in Bold Basal Medium (Canadian Phycological Culture Collection, University of Waterloo) at 20 °C. The algae were settled and re-suspended in the corresponding calcium treatment previous to feeding. The Ca chemical analysis of the four calcium treatments is provided in Tables 1. The hemolymph [Ca^{2+}] and pH of the daphniids were

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