

**1 Magnesium (Mg/Ca,  $\delta^{26}\text{Mg}$ ), boron (B/Ca,  $\delta^{11}\text{B}$ ), and calcium ( $\text{Ca}^{2+}$ )**  
**2 geochemistry of *Arctica islandica* and *Crassostrea virginica***  
**3 extrapallial fluid and shell under ocean acidification**

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17 **Abstract.** The geochemistry of biogenic carbonates has long been used as proxies to record changing seawater parameters.  
18 However, the effect of ocean acidification (OA) on seawater chemistry and organism physiology could impact isotopic  
19 signatures and how elements are incorporated into the shell. In this study, we investigated the geochemistry of three  
20 reservoirs important for biomineralization - seawater, the extrapallial fluid (EPF), and the shell - in two bivalve species,  
21 *Crassostrea virginica* and *Arctica islandica*. Additionally, we examined the effects of three ocean acidification conditions  
22 (ambient: 500 ppm  $\text{CO}_2$ , moderate: 900 ppm  $\text{CO}_2$ , and high: 2800 ppm  $\text{CO}_2$ ) on the geochemistry of the same three  
23 reservoirs for *C. virginica*. We present data on calcification rates, EPF pH, measured elemental ratios (Mg/Ca, B/Ca), and  
24 isotopic signatures ( $\delta^{26}\text{Mg}$ ,  $\delta^{11}\text{B}$ ). In both species, comparisons of seawater and EPF Mg/Ca and B/Ca,  $\text{Ca}^{2+}$ , and  $\delta^{26}\text{Mg}$   
25 indicate that the EPF has a distinct composition that differs from seawater. Shell  $\delta^{11}\text{B}$  did not faithfully record seawater pH  
26 and  $\delta^{11}\text{B}$ -calculated pH values were consistently higher than pH measurements of the EPF with microelectrodes, indicating  
27 that the shell  $\delta^{11}\text{B}$  may reflect a localized environment within the entire EPF reservoir. In *C. virginica*, EPF Mg/Ca and B/Ca,  
28 as well as absolute concentrations of  $\text{Mg}^{2+}$ , B, and  $\text{Ca}^{2+}$ , were all significantly affected by ocean acidification, indicating that  
29 OA affects the physiological pathways regulating or storing these ions, an observation that complicates their use as proxies.  
30 Reduction in EPF  $\text{Ca}^{2+}$  may represent an additional mechanism underlying reduction in calcification in *C. virginica* in  
31 response to seawater acidification. The complexity of dynamics of EPF chemistry suggest boron proxies in these two

mollusc species are not straightforwardly related to seawater pH, but ocean acidification does lead to both a decrease in microelectrode pH and boron-isotope-based pH, potentially showing applicability of boron isotopes in recording physiological changes. Collectively, our findings show that bivalves have high physiological control over the internal calcifying fluid, which presents a challenge to using boron isotopes for reconstructing seawater pH.

## 1 Introduction

The elemental geochemistry of marine biogenic carbonate shells is widely used to track and reconstruct environmental change (e.g. Broecker and Peng, 1982; Elderfield et al., 2006). The incorporation of elements within the skeleton of marine calcifiers has been shown to be correlated with different environmental parameters, such as temperature (Dunbar et al., 1994; Alibert and McCulloch 1997) and pH (e.g. Hemming and Hanson, 1992; Hönisch et al., 2004; McCulloch et al., 2018). However, elemental and isotopic signatures of biogenic carbonate deviate from inorganic carbonate grown under the same conditions, complicating the use and interpretation of these theoretical models for paleo-reconstructions (e.g. Urey, 1951; Craig, 1953; Weiner and Dove, 2003). “Vital effects” are the physiological processes that alter the geochemistry of biominerals and consequently offset the environmental signal incorporated in biogenic carbonates, which includes the different biomineralization strategies that can modify the chemistry of the calcification fluid (Urey, 1951; Weiner and Dove, 2003). For organisms to calcify, a semi-isolated calcification space will be, to varying degrees, separated from seawater for supersaturation to be achieved in support of calcification (Weiner and Dove, 2003). The geochemistry of the calcification fluid can be altered due to isolation from the parent fluid as well as the modulation of the calcification fluid chemistry via methods of passive or active ion transport to the site of calcification (Weiner and Dove 2003; McCulloch et al., 2017; Sutton et al., 2018; Liu et al., 2020). A mechanistic understanding of such vital effects is desirable for the accurate interpretation of geochemical proxies preserved in the shells of these organisms.

Bivalve extrapallial fluid (EPF) is an internal fluid reservoir physically semi-separated from seawater that circulates in the pallial cavity, between the mantle organ and shell (Wilbur and Saleuddin, 1983). Seawater enters the pallial cavity when valves are open, then the internal hemolymph fluid circulates within the organs of the mollusc and finally can also be transported across the mantle to the EPF (Zhao et al., 2018). Bivalve mollusc shell calcification is thought to occur at the interface of the EPF and growing shell where the ions for calcification interact with organic matrices, such as polypeptide molecules and proteins within the EPF that act as a scaffolding template for nucleation and are important in the calcification process (e.g. Crenshaw, 1972; Wilbur and Bernhardt, 1984; Addadi, 2006; Checa 2018). Unlike the calcifying fluid reservoirs in most organisms, bivalve EPF has a large enough volume that it can be directly sampled, allowing for direct measurements of the reservoir to compare with seawater geochemistry and elucidate *in situ* changes in EPF chemistry. A foundational study by Crenshaw (1972) found that, in three mollusc species, the EPF calcification fluid had a different chemical composition and pH from seawater and hemolymph fluid (Crenshaw et al., 1972). A previous study on the king scallop, *Pecten maximus*, by Cameron et al. (2019) showed that EPF pH was lower than seawater and also depended on

64 seawater  $p\text{CO}_2$  and temperature. Additionally, Ramesh et al., (2017) used a microelectrode approach to show that pH and  
65  $[\text{CO}_3^{2-}]$  were elevated proximal to the growing shell in larval *Mytilus edulis* shells. This result using microelectrode suggests  
66 a potential difference in pH between the bulk EPF and the pH close to the site of calcification. In the quahog *Arctica*  
67 *islandica*, Stemmer et al. (2019) reported synchronous short-term fluctuations in EPF  $\text{Ca}^{2+}$  and the pH at the outer mantle  
68 epithelium surface, providing further support that the extrapallial fluid of molluscs is a discrete fluid under biological  
69 control. Understanding the elemental composition and isotope signatures of mollusc internal fluid reservoirs, mechanisms of  
70 calcification, and ion transport to the site of calcification is critical to understanding these vital effects. It may also give  
71 insight into the sensitivity of bivalves to  $p\text{CO}_2$ -induced ocean acidification, a major environmental challenge for bivalves,  
72 which are typically amongst the more sensitive group of marine calcifier species to acidification (Ries et al., 2009; Kroecker  
73 et al., 2011; Gazeau et al., 2013; Stewart-Sinclair et al., 2020).

74 Molluscs have long been recognized as valuable archives for climate reconstructions, given their annual resolution  
75 growth bands, long lifespans, and wide geographic distributions (Gibson et al., 2001; Peharda et al., 2021). Several  
76 different elemental systems like boron (B) and magnesium ( $\text{Mg}^{2+}$ ) have the potential to give valuable information about the  
77 seawater bivalves precipitate their shells in or even in internal calcification fluid they precipitate their shells from. For  
78 example, shell B/Ca has been shown to be correlated to EPF pH in bivalves such as *M. edulis* (Heinemann et al., 2012) and  
79 *Mercenaria mercenaria* (Ulrich et al., 2021), and can potentially be useful in understanding the internal carbonate chemistry  
80 within the calcification fluid. Shell  $\delta^{11}\text{B}$  is used as a proxy for seawater pH in foraminifera (Foster and Rae, 2016) and  
81 calcification fluid pH in corals (McCulloch et al., 2017; Eagle et al., 2022), but seems to be offset from theoretical pH  
82 calculations in bivalves like *M. edulis* (e.g. Heinemann et al., 2012; Liu et al., 2020), *M. mercenaria* (Liu et al., 2020), and  
83 *Crassostrea virginica* (Liu et al., 2020). Shell Mg/Ca is used as a temperature proxy in bivalves (Wanamaker et al., 2008;  
84 Schöne et al., 2011), however molluscs can regulate and actively exclude  $\text{Mg}^{2+}$  from their shells (e.g. Lorens and Bender,  
85 1977; Planchon et al., 2013), showing that biological regulation of the internal fluids for shell formation can have a strong  
86 influence on Mg-based geochemical proxies. Furthermore, Mg isotope analyses can potentially inform the  $\text{Mg}^{2+}$  transport  
87 process in molluscs. Although few Mg isotopic studies on molluscs have been done, a study by Planchon et al. (2013)  
88 investigated the  $\delta^{26}\text{Mg}$  of *Ruditapes philippinarum* tissues, shell, and EPF and found that seawater and EPF Mg isotopic  
89 signatures were similar, suggesting that seawater is the source of  $\text{Mg}^{2+}$  ions within the EPF. Additionally, they found that Mg  
90 isotopic signatures of some specimens deviated from inorganically precipitated aragonite, suggesting an ability to  
91 physiologically alter or regulate  $\text{Mg}^{2+}$  within the EPF (Planchon et al., 2013).

92 Marine calcifiers are thought to be particularly sensitive to ocean acidification because of lowered saturation state  
93 of calcite ( $\Omega_{\text{calcite}}$ ) and availability of carbonate ions they need to precipitate their shells (Orr et al., 2005). However, marine  
94 calcifiers can exhibit extremely different calcification responses to ocean acidification (e.g. Ries et al., 2011; Kroecker et al.,  
95 2013). Bivalves show similar variable responses to ocean acidification (Gazeau et al., 2013). A study conducted by  
96 Waldbusser et al. (2015) found that juvenile *Crassostrea gigas* and *Mytilus galloprovincialis* had developmental and growth  
97 sensitivities to decreasing seawater  $\Omega_{\text{calcite}}$ . Furthermore, a study by Fitzer et al. (2016) found that *M. edulis* shell

crystallography was affected by ocean acidification, compromising the organism's shell. Additionally, studies have found that exposure to ocean acidification conditions could affect trace element uptake in bivalve shells (Norrie et al., 2018; Zhao et al., 2020). The effects of ocean acidification on bivalve shell geochemistry is of particular consequence for paleoclimate reconstructions due to primary or secondary effects such as calcification or physiological impairment. (e.g. Michaelidis et al., 2005; Waldbusser et al., 2015; Norrie et al., 2018; Zhao et al., 2020).

Few taxa have been studied using combined geochemical tracer work to determine the chemistry of calcification fluid pools and sources of ions to the calcification front. To date, one study has investigated the B/Ca and  $\delta^{11}\text{B}$  of shell and EPF of the bivalve *M. edulis* (Heinemann et al., 2012). Mollusc extrapallial fluid is an attractive target to investigate geochemical vital effects because not only can it be probed with electrodes, but it can also be extracted and analyzed. In this study, we investigate the  $\delta^{11}\text{B}$ , B/Ca,  $\delta^{26}\text{Mg}$ , and Mg/Ca of extracted EPF and aragonite shell of the quahog, *A. islandica*, and the calcite shell of the eastern oyster, *C. virginica*. This allows for the investigation of the tripartite fractionation between seawater, EPF, and shell. Individuals were kept in controlled laboratory experiments, with EPF pH determined with microelectrodes, and other physiological parameters, such as calcification rate, determined by conventional methods (Downey-Wall et al., 2020). Additionally, in order to examine if elemental ratios and isotopic signatures can be impacted under ocean acidification, specimens of *C. virginica* were also cultured in three different treatments of  $p\text{CO}_2$ : ambient, moderate and high ocean acidification conditions. Geochemical analysis of the seawater, shell, and EPF thereby allow novel insights into the transport of ions from seawater to the EPF, and the fractionation of isotopes and elements between the EPF and shell for both species. Additionally, how the same analyses can change for *C. virginica* under acidified conditions.

## 2 Materials and Methods

### 2.1 Experimental Conditions

Adult *A. islandica* specimens were collected from Beals Island, Maine, USA (44° 31' 11"N, 67° 36' 54"W) in March 2018, transferred to Northeastern University's Marine Science Center, and maintained in the lab until March 2019. The average *A. islandica* shell length was  $5.8 \pm 0.5$  (n=6). For *A. islandica*, seawater was maintained at a pH of  $7.93 \pm 0.09$ , temperature of  $9 \pm 1$  °C, and salinity of 35 in the control conditions (Cameron 2020).

A detailed explanation of the collection and culturing of *C. virginica* is outlined in Downey-Wall et al. (2020). Adult *C. virginica* specimens were collected from three intertidal sites on Plum Island Sound, Massachusetts, USA (Site 1, 42°45'6" N, 70°50'13" W; Site 2, 42°43'31" N, 70°51'18" W; 42°40'43" N, 70°48'49" W) in April 2017 and transferred to Northeastern University's Marine Science Center. The average *C. virginica* shell length was  $9.2 \pm 2.4$  cm (n=53). Specimens were acclimated to laboratory conditions for 33 days and then transferred to experimental tanks. Seawater salinity and temperature were monitored and maintained throughout the experiment. *Crassostrea virginica* seawater was maintained at a

temperature of  $18.2 \pm 1$  °C, and salinity of 31. *C. virginica* were exposed to control (mean  $p\text{CO}_2 \pm \text{SE} = 570 \pm 14$  ppm;  $\Omega_{\text{calcite}} = 2.95 \pm 0.30$  ), moderate OA ( $990 \pm 29$  ppm,  $\Omega_{\text{calcite}} = 1.93 \pm 0.32$ ), or high OA ( $2912 \pm 59$  ppm,  $\Omega_{\text{calcite}} = 0.75 \pm 0.09$ ) treatments. Target  $p\text{CO}_2$  treatment was achieved by mixing compressed  $p\text{CO}_2$  and compressed ambient air using solenoid-valve-controlled mass flow controllers at flow rates that target  $p\text{CO}_2$  conditions. The treated seawater was introduced to the flow-through aquaria at a rate of  $150 \text{ mL min}^{-1}$ . For the acidification experiment, tank salinity, temperature, and DIC and TA were measured for the duration of the experiment and used to calculate pH (total scale),  $\Omega_{\text{calcite}}$ ,  $[\text{CO}_3^{2-}]$ ,  $[\text{HCO}_3^-]$ ,  $[\text{CO}_2]$ , and  $p\text{CO}_2$  of each tank using CO2SYS version 2.1 (Pierrot et al. 2011; Downey-Wall et al. 2020). Measured and calculated seawater parameters are reported in Table 1. Oysters were fed 1% Shellfish Diet 1800® twice daily following best practices outlined in Helm and Bourne (2004).

**Table 1.** Seawater carbonate chemistry parameters (pH, DIC, TA,  $\Omega$ ,  $\delta^{11}\text{B}$ -calculated EPF pH, and  $\Delta\text{pH}$ ) and seawater geochemical parameters (Mg/Ca,  $\delta^{26}\text{Mg}$ , B/Ca,  $\delta^{11}\text{B}$ ) for both *C. virginica* and *A. islandica* under control conditions and *C. virginica* for OA conditions. Parameters that were unable to be not measured due to insufficient sample size or unable to be calculated are marked with ‘n/d.’

|                                        | Control<br><i>A. islandica</i> | Control<br><i>C. virginica</i> | Moderate OA<br><i>C. virginica</i> | High OA<br><i>C. virginica</i> |
|----------------------------------------|--------------------------------|--------------------------------|------------------------------------|--------------------------------|
| Measured seawater parameters           |                                |                                |                                    |                                |
| pH (total scale)                       | $7.93 \pm 0.09$                | $8.01 \pm 0.08$                | $7.75 \pm 0.07$                    | $7.29 \pm 0.11$                |
| DIC ( $\mu\text{mol/kg}$ )             | n/d                            | $1966 \pm 44$                  | $1998 \pm 212$                     | $2177 \pm 160$                 |
| TA ( $\mu\text{mol/kg}$ )              | n/d                            | $2120 \pm 46$                  | $2120 \pm 42$                      | $1511 \pm 40$                  |
| Mg/Ca (mol/mol)                        | $5.13 \pm 0.07$                | $5.15 \pm 0.07$                | $5.23 \pm 0.06$                    | $5.12 \pm 0.03$                |
| $\delta^{26}\text{Mg}$ (‰)             | $-0.82 \pm 0.06$ ‰             | $-0.77 \pm 0.01$               | $-0.82 \pm 0.03$                   | $-0.76 \pm 0.09$               |
| B/Ca (mol/mol)                         | $41.75 \pm 1.52$               | $41.66 \pm 1.07$               | $43.08 \pm 2.9$                    | $42.11 \pm 1.8$                |
| $\delta^{11}\text{B}$ (‰)              | $39.88 \pm 0.13$               | $40.29 \pm 0.33$               | $39.39 \pm 0.33$                   | $39.82 \pm 0.33$               |
| Calculated seawater parameters         |                                |                                |                                    |                                |
| $p\text{CO}_2$ (ppm)                   | n/d                            | $570 \pm 90$                   | $990 \pm 173$                      | $2912 \pm 373$                 |
| $[\text{CO}_3^{2-}]$ ( $\mu\text{M}$ ) | n/d                            | $120 \pm 12$                   | $79 \pm 13$                        | $31 \pm 4$                     |
| $\Omega_{\text{Calcite}}$              | n/d                            | $2.95 \pm 0.30$                | $1.93 \pm 0.32$                    | $0.75 \pm 0.09$                |

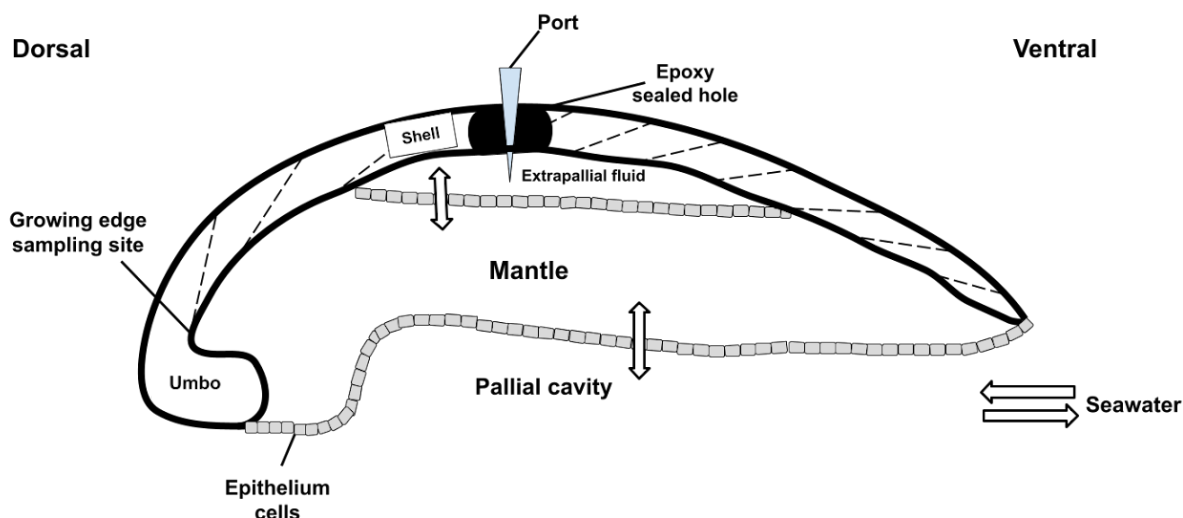
|                                                                 |                 |                 |                 |                 |
|-----------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| $\Omega_{\text{Aragonite}}$                                     | n/d             | $1.89 \pm 0.19$ | $1.24 \pm 0.21$ | $0.48 \pm 0.06$ |
| $\delta^{11}\text{B}$ -calculated pH<br>(total scale)           | $7.76 \pm 0.07$ | $8.12 \pm 0.09$ | $8.06 \pm 0.10$ | $8.01 \pm 0.08$ |
| $\Delta\text{pH}_{\text{SW}} - \delta^{11}\text{B}_{\text{pH}}$ | 0.17            | 0.64            | 0.77            | 0.88            |

## 142 2.2 Calcification rate measurements

143 Net calcification rate for *C. virginica* specimen (n=35) was calculated in Downey-Wall et al. (2020) using the  
144 buoyant weighing technique. Buoyant weight was measured by submerging oysters in a 27.65 liter tank (48 cm long, 24 cm  
145 wide and 24 cm deep) filled with seawater. Specimens were placed on a bottom-loading scale (Cole Parmer Symmetry S-PT  
146 413E, precision= 0.001 g) and weighed three times. At the end of the experiment, an empirical linear relationship was  
147 created between buoyant weight and dry shell weight of shucked oysters following the same methodology in Ries et al.  
148 (2009). The residual mean squared error (RMSE) for the dry weight/buoyant weight model was 1.939 mg. Calcification was  
149 calculated as the difference in calculated dry weight at the start and end of the experiment over the number of days. This  
150 number was then divided by the initial weight and multiplied by 100 to get the percent change in calcification.

## 151 2.3 Extrapallial fluid sampling

152 Sampling of the extrapallial fluid (EPF) for both species was previously described in Downey-Wall et al. (2020).  
153 Briefly, a hole was drilled onto the shell to expose the EPF cavity, a port was inserted and sealed with epoxy to directly  
154 sample the EPF with a syringe and prevent seawater intrusion (Figure 1). Oysters recovered for 4 days before being  
155 transferred to experimental tanks for acclimation before the experiment. To sample the EPF, oysters were removed from the  
156 tanks and EPF was extracted by inserting a sterile 5 mL syringe with a flexible 18-gauge polypropylene tip through the port.  
157 EPF samples were stored in 2 mL microcentrifuge tubes and refrigerated at 6°C for further analysis. EPF pH (Total scale)  
158 was measured directly after extraction using a micro-pH probe. EPF measurements were collected at the end of the  
159 experiment, on day 71, for *C. virginica* and day 14 for *A. islandica*. EPF pH diel variability was also explored by measuring  
160 EPF pH at 6 timepoints to produce time series for both species in a 24-hour period.



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162 **Figure 1.** Schematic of a bivalve cross section from the dorsal to the ventral sides. Seawater enters the pallial cavity and ions  
 163 can diffuse or be transported across the mantle organ to the extrapallial fluid space. A hole was drilled through the top of the  
 164 shell into the extrapallial fluid space and sealed with epoxy following port insertion. The shell was drilled on the inner side  
 165 of the growing edge of the shell to sample new growth.

## 166 2.4 Shell sampling

167 Following EPF extraction, bivalves were shucked and cleaned in 90% ethanol. The cleaned shells were dried at  
 168 room temperature for 48 hours and sealed in plastic bags for analysis. Whole shells were cut into cross sections from the  
 169 hinge to the margin using a circular saw while being rinsed with ethanol during sectioning to prevent mineralogical changes  
 170 from heat exposure. For skeletal geochemical and elemental ratio analysis, the inner (lamellar) layer of the oyster shell was  
 171 gently shaved with a diamond-tipped Dremel tool. Care was taken to ensure sampling the most recently deposited material  
 172 right near the growing edge of the bivalve, located below the umbo of the oyster shell (Figure 1). Cross sections showed  
 173 growth bands and aided in sampling the newest growth under experimental conditions. For *A. islandica*, we wanted to  
 174 compare ambient conditions, so new growth was not necessary to sample. About 5 mg of ground powder was stored in  
 175 sealed microcentrifuge tubes.

## 176 2.4 Elemental ratio analysis

177 For the shells, about 2.5 mg of powder was sub-sampled from each specimen shell and oxidatively cleaned with a  
178 0.3 % hydrogen peroxide in 0.1 N sodium hydroxide solution to remove organic matter as described in Barker et al. (2003).  
179 Carbonate samples were dissolved in 1 N double-distilled HCl. Elemental ratios were measured on a Thermo Fisher  
180 Scientific Element XR HR-ICP-MS at the PSO (Plouzané, France) after Ca analyses on an Agilent ICP-AES Varian 710 at  
181 the University of California, Los Angeles (UCLA, Los Angeles, USA). Data quality and external reproducibility were  
182 maintained and quantified via repeated measurements of international standard JC<sub>P</sub>-1 during a particular session (Gutjahr et  
183 al., 2021). Typical measured concentrations of procedural blanks for the trace element analyses for sessions in which  
184 samples are diluted to 30 ppm Ca are  $^7\text{Li} < 3\%$ ,  $^{11}\text{B} < 4\%$ ,  $^{25}\text{Mg} < 0.1\%$ ,  $^{87}\text{Sr} < 0.1\%$ , and  $^{43}\text{Ca} < 0.1\%$ . Typical analytical  
185 uncertainties on the X/Ca elemental ratios are 0.3  $\mu\text{mol/mol}$  for Li/Ca, 21  $\mu\text{mol/mol}$  for B/Ca, 0.09 mmol/mol for Mg/Ca,  
186 and 0.01 mmol/mol for Sr/Ca (2 SD,  $n = 28$ ).

187 For EPF and seawater samples, 10  $\mu\text{L}$  of sample was added to 490  $\mu\text{L}$  of a solution of 0.1 N  $\text{HNO}_3$ /0.3 M HF.  
188 Mono-elemental solution of indium was added to reach a concentration of 1 ppb to monitor any matrix effect or drift of the  
189 instrument during a particular session. Standards were prepared by diluting an in-house seawater standard spiked with  
190 indium. International standards NRC-NASS-6 was used to ensure quality of the data.

## 191 2.5 Boron isotope analyses

192 Boron purification for the different samples was achieved via microdistillation following the method described in  
193 Guillermic et al. (2021) and originally developed by Gaillardet et al. (2001) and modified for Ca-rich matrix by Wang et al.  
194 (2010). Approximately 2.5-3.0 mg of oxidatively cleaned shell powders were dissolved in 1N HCl. For the EPF, 25  $\mu\text{L}$  of  
195 EPF was added to 40  $\mu\text{L}$  of 1N HCl. For the seawater, 50  $\mu\text{L}$  of concentrated HCl was added to 450  $\mu\text{L}$  of seawater. 60 $\mu\text{L}$  of  
196 each of the solutions was loaded for microdistillation. Boron isotopes were analyzed at the Pôle Spectrométrie Océan (PSO),  
197 Plouzané, on a Thermo Neptune inductively coupled plasma mass spectrometry (MC-ICP-MS) equipped with 10<sup>11</sup> Ohm  
198 Faraday cup.

199 The certified boron isotope liquid standard ERM<sup>®</sup> AE120 ( $\delta^{11}\text{B} = -20.2 \pm 0.6 \text{ ‰}$ , Vogl et al., 2011) was used to  
200 monitor reproducibility and drift during each session. Samples measured for boron isotopes in carbonates were typically run  
201 at 80 ppb B (~30 ng B per <0.5 mL), whereas samples of EPF and seawater were typically run at 150-200 ppb B (~150 ng B  
202 per mL). Sensitivity on  $^{11}\text{B}$  was 10 mV/ppb B (e.g., 10 mV for 1 ppb B) in wet plasma at 50  $\mu\text{L/min}$  sample aspiration rate.  
203 Procedural boron blanks ranged from 0.3 to 0.4 ng B and the acid blank during analyses was measured at 3 mV on the  $^{11}\text{B}$ ,  
204 indicating a total blank contribution of <2% of the sample signal with no memory effect within and across sessions. External  
205 reproducibility was ensured by the measurements of carbonate standard microdistilled at the same time as the samples.  
206 Results for the isotopic composition of the JC<sub>P</sub>-1 is  $\delta^{11}\text{B} = 24.67 \pm 0.28 \text{ ‰}$  (2 SE,  $n=41$ ), within error of published values  
207 ( $24.36 \pm 0.45 \text{ ‰}$ , 2SD, Gutjahr et al., 2021).

## 208 2.6 Magnesium isotope analyses

209 Carbonate samples were dissolved in 0.1 N buffered acetic acid ammonium hydroxide solution over four hours in a  
210 sonicator. Samples were then centrifuged and aliquots of the supernatant were transferred into cleaned 15 mL centrifuge  
211 tubes. Aliquots of the bulk supernatants were then diluted ~30-fold and calcium and magnesium were separated and purified  
212 in different runs via a Thermo-Dionex ICS-5000+ ion chromatograph equipped with a fraction collector according to  
213 established methods outlined by Husson et al. (2015). EPF samples contained organics that obscured elution profiles, thus  
214 limiting the elemental yield and purification. Therefore, samples were digested on a hot plate in hydrogen peroxide and nitric  
215 acid to remove organics prior purification. Seawater and EPF samples were purified through the Thermo-Dionex ICS-5000+  
216 ion chromatograph using another elution method than for carbonate samples. Seawater and carbonate standards were also  
217 purified at the same time to ensure quality of the method.

218 Samples were then dried and then rehydrated in a solution of 2% nitric acid. Magnesium isotopic ratios were  
219 measured at Princeton University using a Thermo Neptune+ (MC-ICP-MS) spectrometer according to methods outlined in  
220 Higgins et al. (2018) and Ahm et al. (2021). Samples were introduced via an ESI Apex-IR sample introduction system.  
221 Magnesium isotope ratios ( $^{26}\text{Mg}/^{24}\text{Mg}$ ) were measured in low resolution mode, with every sample bracketed by the analysis  
222 of standards. Results are reported relative to the Dead Sea Magnesium-3 standard (DSM-3). Long term external precision on  
223 magnesium isotope results at the Higgins Lab (Princeton) was determined through repeated measurements of the  
224 Cambridge-1 standard ( $-2.59 \pm 0.07\text{‰}$ , 2 SD,  $n = 19$ ) and modern seawater ( $-0.82 \pm 0.14 \text{‰}$ , 2 SD,  $n = 21$ ) and is reported  
225 in Ahm et al. (2021). Measured standards during the analytical session are given for the Cambridge-1 standard ( $-2.60 \pm 0.20$   
226  $\text{‰}$ , 2 SD,  $n = 2$ ) and for modern seawater ( $-0.82 \pm 0.06 \text{‰}$ , 2 SD,  $n=2$ ).

## 227 2.7 Calculation of boron proxies and EPF carbonate chemistry

228 The use of boron proxies to reconstruct pH and  $[\text{CO}_3^{2-}]$  of the precipitating solution (i.e., the organism's calcifying  
229 fluid) is based upon boron speciation and fractionation in seawater (Hemming and Hanson, 1992; Hönisch et al., 2004). In  
230 seawater-type solutions, the speciation of boric acid  $[\text{B}(\text{OH})_3]$  and borate ion  $[\text{B}(\text{OH})_4^-]$  varies as a function of pH (Hemming  
231 and Hanson 1992). In addition to the pH dependence of their relative abundances, the boron proxy also relies upon the large  
232 isotopic fractionation between the two boron species (Klochko et al., 2006, Nir et al., 2015). A key assumption of the proxy  
233 is that boron, in the form of borate ion, is the predominant form incorporated into the crystal lattice of calcite via carbonate  
234 ion substitution during the precipitation of calcium carbonate (Hemming and Hanson 1992). The  $\delta^{11}\text{B}$  of the carbonate  
235 ( $\delta^{11}\text{B}_{\text{CaCO}_3}$ ) should then, in theory, reflect the boron isotopic composition of the borate ion ( $\delta^{11}\text{B}_{\text{B}(\text{OH})_4^-}$ ) in the bivalve  
236 calcifying fluid (extrapallial fluid), which in turn reflects pH of the calcifying (extrapallial) fluid.

237 The boron isotopic signature of the shell ( $\delta^{11}\text{B}_{\text{carb}}$ ) was used to calculate pH of the calcifying fluid ( $\text{pH}_{\text{CF}}$ ) using the  
238 following equation (Hemming and Hanson, 1992; Zeebe and Wolf-Gladrow, 2001):

239

$$\text{pH}_{\text{cf}} = \text{pK}_{\text{B}} - \log \left( \frac{\delta^{11}\text{B}_{\text{sw}} - \delta^{11}\text{B}_{\text{carb}}}{\delta^{11}\text{B}_{\text{sw}} - \alpha * \delta^{11}\text{B}_{\text{carb}} - \varepsilon} \right) \quad \text{eq. 1}$$

241

242 In equation 1,  $\text{pK}_{\text{B}}$  is the dissociation constant,  $\delta^{11}\text{B}_{\text{sw}}$  represents the measured boron isotopic composition of seawater,  
 243  $\delta^{11}\text{B}_{\text{carb}}$  represents the boron isotopic composition of the shell, and  $\alpha/\varepsilon$  represents the boron isotopic fractionation factor/  
 244 fractionation between boric acid and borate ion (Klochko et al. 2006).

245

246 The saturation state of calcite ( $\Omega_{\text{calcite}}$ ) and aragonite ( $\Omega_{\text{aragonite}}$ ) of the EPF for each species were calculated using  
 247 temperature, salinity, pressure, measured EPF  $\text{Ca}^{2+}$ , measured EPF  $\text{Mg}^{2+}$ , pH either from microelectrode pH or  
 248  $\delta^{11}\text{B}$ -calculated pH, and literature values of DIC (3000 for *A. islandica* from Stemmer et al. 2019, and 4200 for *C. virginica*  
 249 from McNally et al., 2022). The saturation states were calculated using Seacarb with maximum input of  $\text{Mg}^{2+}$  allowed by  
 250 the code for samples presenting higher EPF  $\text{Mg}^{2+}$  than the limit allowed by the code (Raitzsch et al., 2021). Those saturation  
 251 state values are limited by the fact that no direct measurements of EPF DIC was performed during this study, and a range of  
 252  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  values were measured in the EPF, resulting in a range of calculated saturation states. The apparent partition  
 253 coefficient calculated as the ratio of E/Ca for the mineral over the E/Ca for seawater.

## 254 2.8 Statistical analysis

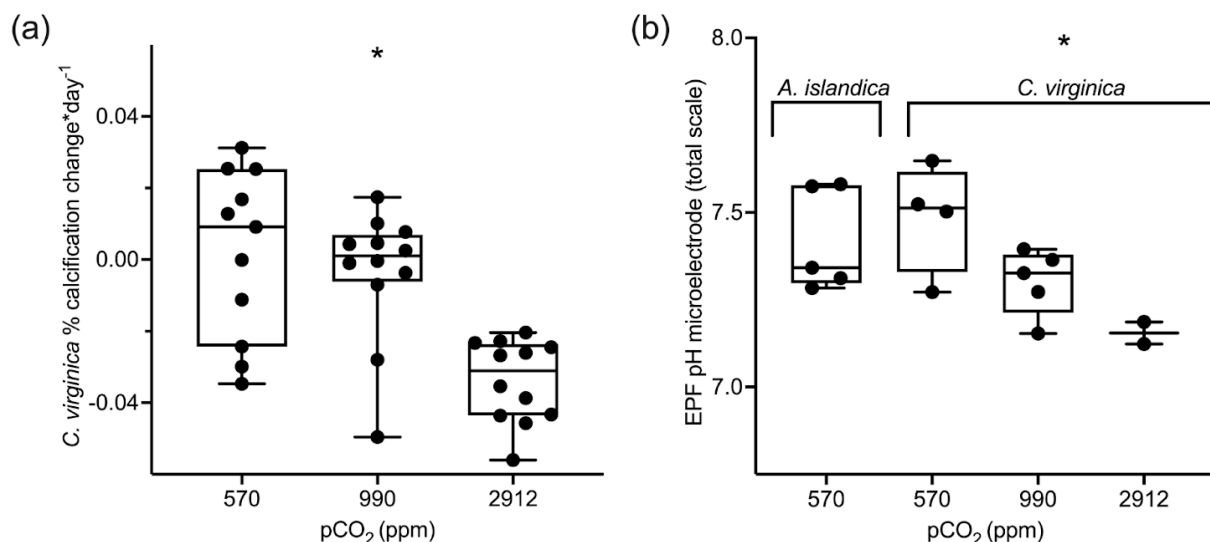
255 All statistical tests were performed and data graphed using GraphPad Prism software version 9 (GraphPad Software  
 256 Inc.; San Diego, CA, USA). Prior to statistical analyses, a Shapiro-Wilks test was run to determine normality and a  
 257 Brown-Forsythe test was used to determine heterogeneity of variance of residuals. Only two comparative t-test data did not  
 258 meet requirements, so a nonparametric Mann-Whitney u test was run in place of a t-test. T-test and Mann-Whitney u tests  
 259 were performed in order to test whether there was a difference between seawater and EPF geochemical parameters and  
 260 between the EPF of both species under ambient conditions. A one-way ANOVA with pH as a three level factor was used to  
 261 test whether pH had a significant effect on our geochemical data. ANOVA and t-test significance was achieved if the p-value  
 262 was less than 0.05. Regression analysis was performed on GraphPad Prism and significance was denoted if the slope of the  
 263 regression was statistically non-zero.

## 264 3 Results

### 265 3.1 Culturing experiment, calcification rates, seawater and EPF chemistry

266 *Crassostrea virginica* specimens were previously cultured in experimental tanks with seawater that was  
 267 continuously bubbled with gas mixtures comprising three  $p\text{CO}_2$  levels: 400 ppm, 900 ppm, 2800 ppm (Downey-Wall et al.,  
 268 2020). The tank seawater saturation states of calcite ( $\Omega_{\text{calcite}}$ ) was calculated for *C. virginica* under the ocean acidification  
 269 experiment and not *A. islandica*. As seawater  $p\text{CO}_2$  increased, seawater  $\Omega_{\text{calcite}}$  decreased. Only the highest  $p\text{CO}_2$  treatment

270 produced calcite saturation states  $\Omega_{\text{calcite}} < 1$ , which does not favor calcification (Table 1). Similarly to  $\Omega_{\text{calcite}}$ , calcification  
 271 rates were also only measured for the *C. virginica* OA experiment.  $p\text{CO}_2$  treatment had a significant effect on *C. virginica*  
 272 calcification, with the percent change in calcification per day decreasing with increasing  $p\text{CO}_2$ . There was also variability in  
 273 calcification between specimens within each treatment (Fig 2a).  
 274



275

f02

276 **Figure 2.** (a) Box plots showing percent calcification change over the experiment for *C. virginica* for each treatment. (b)  
 277 Averaged microelectrode EPF pH for *A. islandica* under control conditions and *C. virginica* for OA treatments. Stars denote  
 278 a statistically significant ANOVA (at significance  $p < 0.05$ ).  
 279

280

280 In this study, we present unpublished EPF pH microelectrode data for *A. islandica* cultured at a single control  
 281 condition (400 ppm  $p\text{CO}_2$ ) and we present published EPF microelectrode data for the *C. virginica* acidification experiment of  
 282 Downey-Wall et al. (2020). At control seawater conditions the EPF pH of *A. islandica* was 7.41, compared to 7.48 for *C.*  
 283 *virginica*. The EPF pH of both species were not statistically different (t-test  $p > 0.05$ ) and the average EPF pH of both species  
 284 was well under seawater pH (Fig 2b). Additionally  $p\text{CO}_2$  treatment also had a significant effect on *C. virginica* EPF pH  
 285 (ANOVA  $p$ -value  $< 0.05$ ), with microelectrode measure EPF pH decreasing as  $p\text{CO}_2$  increased (Fig 2b). Downey-Wall et al.  
 286 (2020) also report that  $p\text{CO}_2$  treatment had a significant effect on EPF pH (linear model,  $p < 0.05$ ) and that at the highest  $p\text{CO}_2$   
 287 treatment, EPF pH was significantly lower than seawater pH (post hoc  $p$ -value  $< 0.05$  see Downey-Wall et al., 2020). We  
 288 report the change in pH ( $\Delta\text{pH}$ ) for both species as the (seawater pH - EPF pH). The  $\Delta\text{pH}$  for *A. islandica* was 0.52 and was  
 289 similar to the control condition  $\Delta\text{pH}$  for *C. virginica* which was 0.53 (Table 2). Under OA treatments,  $\Delta\text{pH}$  for *C. virginica*

decreased with decreasing seawater pH. The  $\Delta\text{pH}$  for the control treatment was 0.53, the moderate OA treatment was 0.46, and the high OA treatment was 0.08.

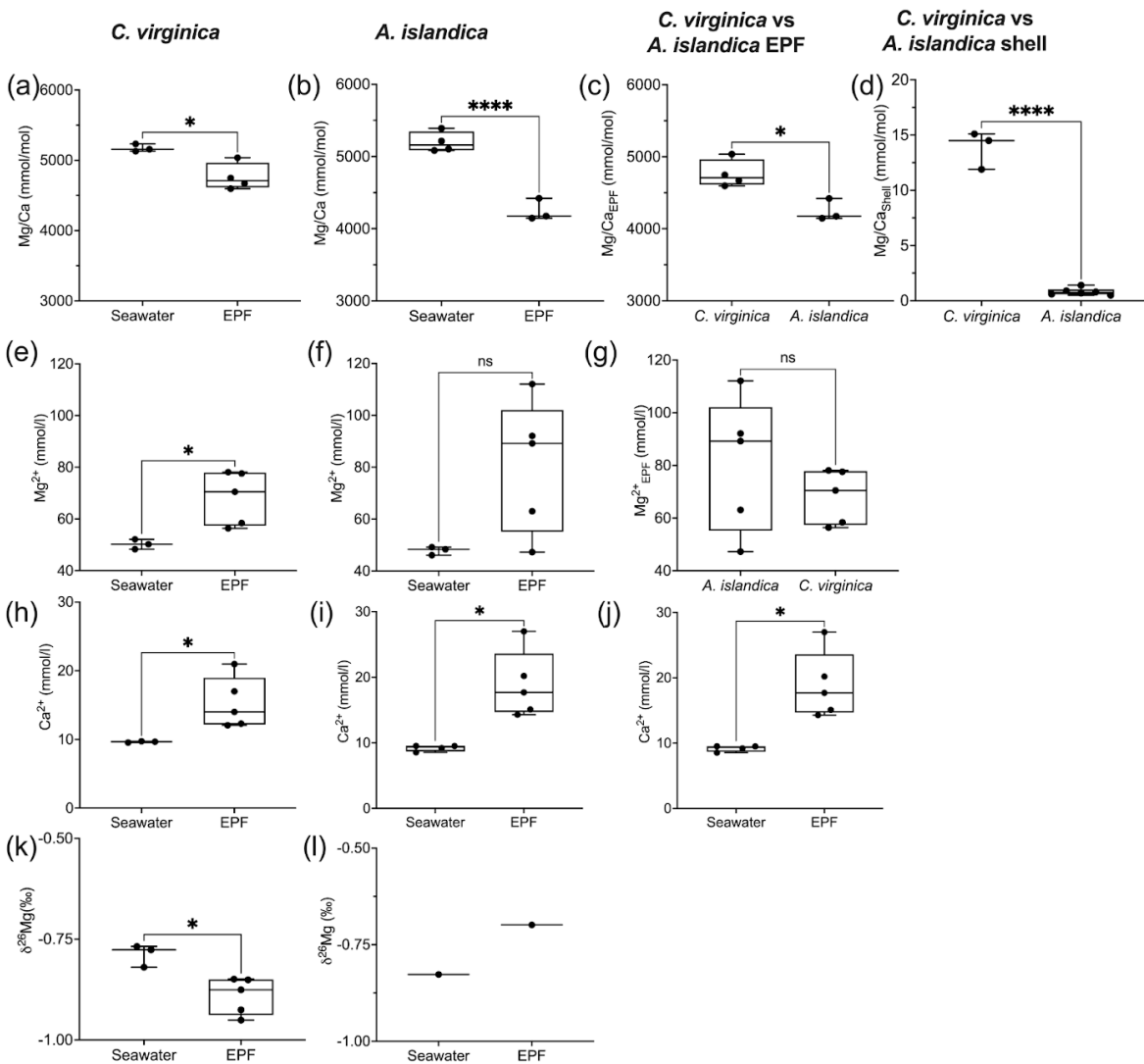
**Table 2.** Measured extrapallial fluid (EPF) carbonate chemistry parameters (pH, DIC, TA,  $\Omega$ ,  $\delta^{11}\text{B}$ -calculated EPF pH, and  $\Delta\text{pH}$ ) and shell geochemical parameters (Mg/Ca,  $\delta^{26}\text{Mg}$ , B/Ca,  $\delta^{11}\text{B}$ ) for both *C. virginica* and *A. islandica* under control conditions and *C. virginica* for OA conditions. Parameters that were unable to be not measured due to insufficient sample size or unable to be calculated are marked with ‘n/d.’

|                                   | Control<br><i>A. islandica</i> | Control<br><i>C. virginica</i> | Moderate OA<br><i>C. virginica</i> | High OA<br><i>C. virginica</i> |
|-----------------------------------|--------------------------------|--------------------------------|------------------------------------|--------------------------------|
| EPF geochemistry                  |                                |                                |                                    |                                |
| EPF pH                            | $7.41 \pm 0.14$                | $7.48 \pm 0.15$                | $7.29 \pm 0.10$                    | $7.21 \pm 0.10$                |
| $\Delta\text{pH}_{\text{SW-EPF}}$ | 0.52                           | 0.53                           | 0.46                               | 0.08                           |
| Mg/Ca                             | $4.25 \pm 0.67$                | $4.55 \pm 0.50$                | $5.73 \pm 0.34$                    | $5.58 \pm 0.46$                |
| $\delta^{26}\text{Mg}$            | $-0.69 \pm 0.1$                | $-0.88 \pm 0.06$               | $-0.87 \pm 0.07$                   | $-0.9 \pm 0.1$                 |
| B/Ca                              | $31.17 \pm 4.87$               | $33.66 \pm 2.81$               | $42.22 \pm 3.33$                   | $43.26 \pm 2.82$               |
| $\delta^{11}\text{B}$             | $39.5 \pm 0.4$                 | $39.3 \pm 1.0$                 | $38.9 \pm 0.4$                     | n/d                            |
| Shell geochemistry                |                                |                                |                                    |                                |
| Mg/Ca                             | $0.8 \pm 0.2$                  | $13.8 \pm 1.7$                 | $13.4 \pm 2.3$                     | $12.3 \pm 1.5$                 |
| $\delta^{26}\text{Mg}$            | n/d                            | $-3.2 \pm 0.1$                 | $-3.1 \pm 0.1$                     | $-3.0 \pm 0.2$                 |
| B/Ca                              | $57 \pm 17$                    | $114 \pm 22$                   | $125 \pm 11$                       | $124 \pm 9$                    |
| $\delta^{11}\text{B}$             | $15.2 \pm 0.4$                 | $18.3 \pm 0.5$                 | $16.9 \pm 0.5$                     | $16.8 \pm 0.3$                 |

### 3.2 Comparison of *A. islandica* and *C. virginica* geochemistry of seawater, EPF, and bivalve shell

There was a significant decrease in EPF Mg/Ca compared to seawater Mg/Ca for both *A. islandica* and *C. virginica* (t-test,  $n=2$ ,  $p\text{-value}<0.05$ ; Fig 3a-b). The Mg/Ca of *C. virginica* EPF was  $4.55 \pm 0.50$  mol/mol and significantly higher than *A. islandica* EPF which was  $4.25 \pm 0.67$  mol/mol (Fig 3d; Table 2). For both species, the low EPF Mg/Ca versus seawater

Mg/Ca was driven by higher  $\text{Ca}^{2+}$  concentrations in the EPF relative to seawater (Fig 3h-i). Considering the elemental concentrations alone, instead of as a ratio, there was no significant difference in EPF  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  concentrations between species (Fig 3g and 3j). Shell Mg/Ca for the calcitic *C. virginica* was  $13.8 \pm 1.7$  mmol/mol and significantly higher than the aragonitic *A. islandica* shell which was  $0.8 \pm 0.02$  mmol/mol, in line with shell polymorph mineralogy. The apparent partition coefficient ( $K_{\text{Mg}}$ ) between the seawater and the shell was 0.003 in *C. virginica* and 0.002 in *A. islandica* (Table 3).  $K_{\text{Mg}}$  between EPF and shell was 0.003 in *C. virginica* and 0.002 in *A. islandica*.  $K_{\text{Mg}}$  between seawater and the EPF is 0.9 for *C. virginica* and 0.8 for *A. islandica* (Table 3). *C. virginica* seawater and EPF  $\delta^{26}\text{Mg}$  were  $-0.77 \pm 0.01$  ‰ and  $-0.88 \pm 0.06$  ‰, respectively and displayed a significant decrease in EPF  $\delta^{26}\text{Mg}$  compared to seawater for *C. virginica* (t-test,  $n_1=3$   $n_2=5$ , p-value < 0.05; Fig 3k-l). For *A. islandica*, seawater and EPF  $\delta^{26}\text{Mg}$  were  $-0.82 \pm 0.06$  ‰ and  $-0.69 \pm 0.01$  ‰, respectively, but no statistical analysis could be done between the two reservoirs owing to the small sample size (Table 1 and 2). The average shell  $\delta^{26}\text{Mg}$  for *C. virginica* was  $-3.2 \pm 0.1$  ‰, but *A. islandica* shell  $\delta^{26}\text{Mg}$  could not be analyzed because of low shell  $[\text{Mg}^{2+}]$  content and limited sample material.



f 03

314

315 **Figure 3.** Box plots of Mg/Ca comparing seawater and extrapallial fluid for (a) *C. virginica* and (b) *A. islandica*, (c)  
 316 comparing EPF Mg/Ca between species, and (d) shell Mg/Ca between species. Box plots of [Mg<sup>2+</sup>] comparing seawater and  
 317 extrapallial fluid for (e) *C. virginica* and (f) *A. islandica*, (g) comparing EPF [Mg<sup>2+</sup>] between species. Box plots of [Ca]  
 318 comparing seawater and extrapallial fluid for (h) *C. virginica* and (i) *A. islandica*, (j) comparing EPF [Ca] between species.  
 319 Box plots of δ<sup>26</sup>Mg comparing seawater and extrapallial fluid for (k) *C. virginica* and (l) *A. islandica*. Stars denote  
 320 statistically different means and 'ns' signify non significant mean differences in a pairwise t-test or Mann-Whitney u test (at  
 321 significance p < 0.05). No comparison was tested on (l) due to limited sample size.

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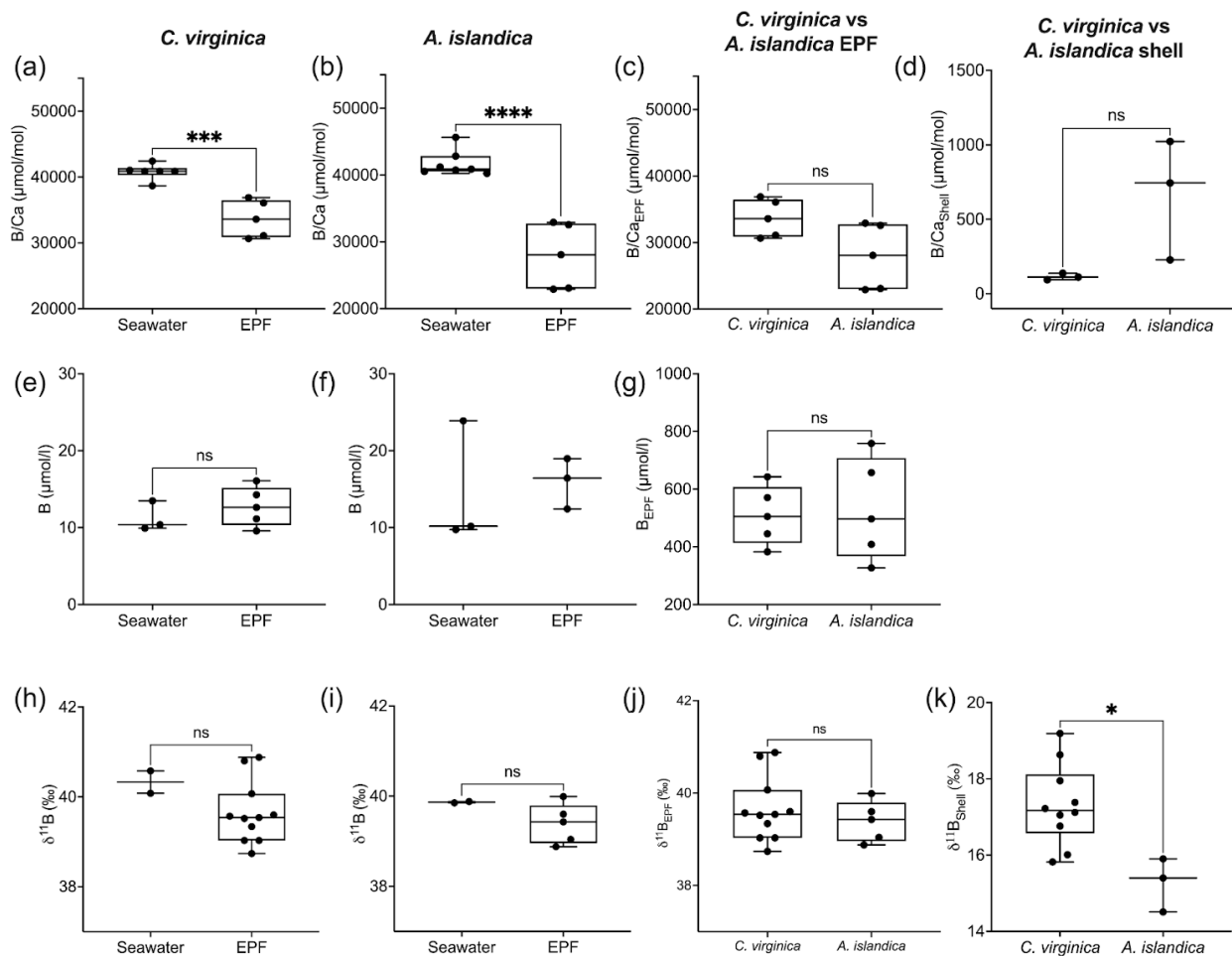
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325

| <i>A. islandica</i> |        | <i>C. virginica</i> |       |       |       |
|---------------------|--------|---------------------|-------|-------|-------|
|                     |        | $p\text{CO}_2$      | 400   | 900   | 28000 |
| $K_{\text{Mg}}$     | 0.0002 |                     | 0.003 | 0.002 | 0.002 |
| $K_{\text{B}}$      | 0.001  |                     | 0.003 | 0.003 | 0.003 |

**Table 3.** Partition coefficients between seawater and the mineral for Mg/Ca and B/Ca.

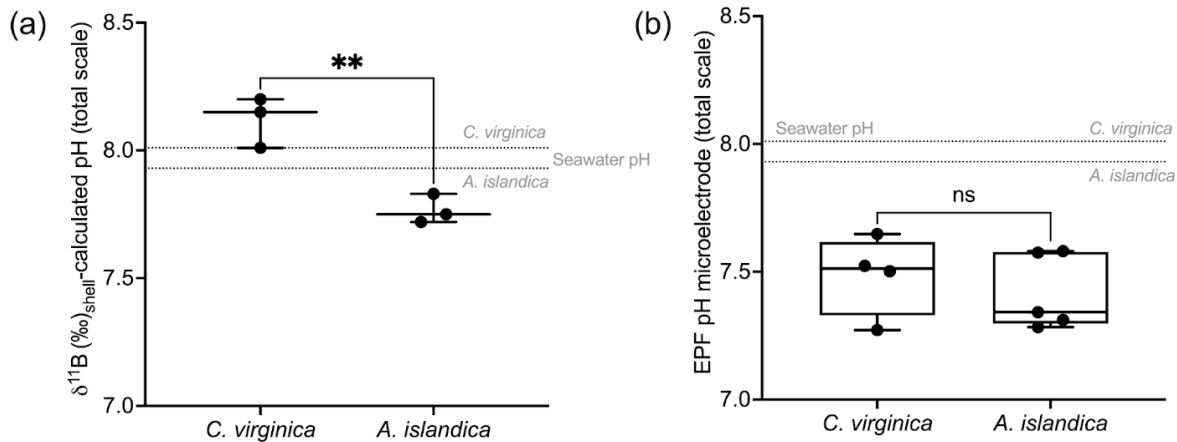
*Arctica islandica* EPF B/Ca was  $27.91 \pm 4.87$  mmol/mol and was significantly lower than seawater B/Ca which was  $41.75 \pm 1.52$  mmol/mol (t-test,  $n_1=7$   $n_2=5$ ,  $p\text{-value}<0.05$ , Fig 4a). *C. virginica* EPF B/Ca was  $41.66 \pm 1.07$  mmol/mol and was significantly lower than seawater B/Ca which was  $33.66 \pm 2.81$  mmol/mol (t-test,  $n_1=6$   $n_2=5$ ,  $p\text{-value}<0.05$  Fig 4b) The boron concentration was not significantly different between seawater and EPF for both *C. virginica* and *A. islandica* (Fig 4e-f). There was no significant difference in shell or EPF B/Ca between *C. virginica* and *A. islandica* (Fig 4c-d). The apparent partition coefficient ( $K_{\text{B}}$ ) between the seawater and the shell was 0.003 in *C. virginica* and 0.001 in *A. islandica*.  $K_{\text{B}}$  between EPF and shell was 0.003 in *C. virginica* and 0.002 in *A. islandica*.  $K_{\text{B}}$  between seawater and the EPF is 0.8 in *C. virginica* and 0.7 for *A. islandica* (Table 3). There was no significant difference in  $\delta^{11}\text{B}$  between seawater and EPF for both species in the control condition (Fig 4h-l). There was also no significant difference in EPF  $\delta^{11}\text{B}$  between species (Fig 4j); however, there was a significant difference in shell  $\delta^{11}\text{B}$  between *C. virginica* and *A. islandica* (t-test,  $n_1=10$   $n_2=3$ ,  $p\text{-value}<0.05$ , Fig 4k). Under control conditions, shell  $\delta^{11}\text{B}$  was measured to be  $15.26 \pm 0.41\text{‰}$  (2 SD,  $n=3$ ) for *C. virginica* and  $18.34 \pm 0.59\text{‰}$  (2 SD,  $n=3$ ) for *A. islandica*.



f 04

339

340 **Figure 4.** Box plots of B/Ca comparing seawater and extrapallial fluid for (a) *C. virginica* and (b) *A. islandica*, (c)  
 341 comparing EPF B/Ca between species, and (d) shell B/Ca between species. Box plots of [B] comparing seawater and  
 342 extrapallial fluid for (e) *C. virginica* and (f) *A. islandica*, (g) comparing EPF [B] between species. Box plots of δ¹¹B  
 343 comparing seawater and extrapallial fluid for (h) *C. virginica* and (i) *A. islandica*, (j) comparing EPF δ¹¹B between species,  
 344 and (k) shell δ¹¹B between species. Stars denote statistically different means and 'ns' signify non significant mean  
 345 differences in a pairwise t-test or Mann-Whitney u test (at significance  $p < 0.05$ ).



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**Figure 5.** (a) Box plot of  $\delta^{11}\text{B}$ -calculated pH for *C. virginica* and *A. islandica*. (b) Box plot of measured microelectrode pH for *C. virginica* and *A. islandica*. The grey line shows seawater pH for *C. virginica* and *A. islandica*. Stars denote statistically different means and 'ns' signify non significant mean differences in a pairwise t-test (at significance  $p < 0.05$ ).

The control condition  $\delta^{11}\text{B}$ -calculated EPF pH for *C. virginica* was  $8.12 \pm 0.08$  ‰ (2 SD,  $n=3$ ) and for *A. islandica* was  $7.93 \pm 0.09$  ‰ (2 SD,  $n=3$ ), which yielded a statistically significant difference between the two species (t-test,  $n_1=3$ ,  $n_2=3$ ,  $p\text{-value}<0.05$ , Fig 5a). For *C. virginica*, the  $\delta^{11}\text{B}$ -calculated EPF was 0.1 pH units higher than the seawater pH and 0.6 lower than measured EPF pH. Conversely, the *A. islandica*  $\delta^{11}\text{B}$ -calculated EPF was 0.1 pH units lower than the seawater pH and 0.3 higher than the measured EPF pH (Fig 59).

356

|                                                                | Control<br><i>A. islandica</i><br>( $\Omega$ aragonite) | Control<br><i>C. virginica</i><br>( $\Omega$ calcite) | Moderate OA<br><i>C. virginica</i><br>( $\Omega$ calcite) | High OA<br><i>C. virginica</i><br>( $\Omega$ calcite) |
|----------------------------------------------------------------|---------------------------------------------------------|-------------------------------------------------------|-----------------------------------------------------------|-------------------------------------------------------|
| $\Omega$ using EPF pH<br>(range)                               | 1.7 (1.0-3.8)                                           | 3.7 (1.3-11.4)                                        | 1.1 (0.5-2)                                               | 0.9 (0.5-1.2)                                         |
| $\Omega$ using $\delta^{11}\text{B}$ -calculated pH<br>(range) | 3.8 (2.9-6.7)                                           | 15.4 (6.7-37)                                         | 6.1 (3-11.7)                                              | 6.5 (3.4-9.7)                                         |

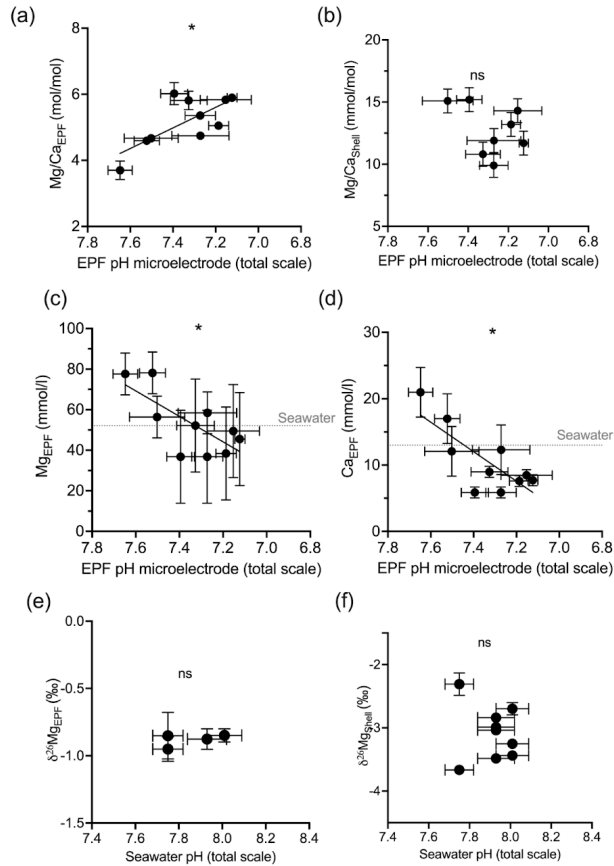
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**Table 4.** Table of calculated saturation state ( $\Omega$ ) with respect to calcite (*C. virginica*) or aragonite (*A. islandica*) for the average EPF pH value based on microelectrode measurements or  $\delta^{11}\text{B}$ -calculated EPF pH.

In Table 4, the EPF aragonite saturation state ( $\Omega_{\text{aragonite}}$ ) for *A. islandica* and EPF calcite saturation state ( $\Omega_{\text{calcite}}$ ) for *C. virginica* were calculated using the averaged measured EPF pH and averaged  $\delta^{11}\text{B}$ -calculated EPF pH, averaged measured  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ , and literature values of DIC (3000  $\mu\text{mol/L}$  for *A. islandica* taken from Stemmer et al. (2019) and 4200  $\mu\text{mol/L}$  for *C. virginica* from McNally et al. (2022). Under control conditions, the *A. islandica*  $\Omega_{\text{aragonite}}$  and *C. virginica*  $\Omega_{\text{calcite}}$  that was calculated using  $\delta^{11}\text{B}$ -calculated EPF pH and measured EPF pH (Table 4). Under the ocean acidification experiment, EPF  $\Omega_{\text{calcite}}$  decreased with decreasing seawater pH when using either EPF pH or  $\delta^{11}\text{B}$ -calculated EPF pH to calculate EPF  $\Omega_{\text{calcite}}$ . There were large differences in *A. islandica*  $\Omega_{\text{aragonite}}$  and *C. virginica*  $\Omega_{\text{calcite}}$  when using either EPF pH ( $\Omega_{\text{aragonite}}=1.7$  and  $\Omega_{\text{calcite}}=3.7$ ) or the  $\delta^{11}\text{B}$ -calculated pH ( $\Omega_{\text{aragonite}}=3.8$  and  $\Omega_{\text{calcite}}=15.4$ ).

### **3.3 *C. virginica* ocean acidification experiment geochemistry**

In the *C. virginica* acidification experiment, EPF but not shell  $\text{Mg/Ca}$  was found to increase as EPF pH decreased (regression,  $n=10$ ,  $p\text{-value}<0.05$ ; Fig 6a-b). OA treatment had a significant effect on shell  $\text{Mg/Ca}$  (ANOVA,  $n=10$ ,  $p\text{-value}<0.05$ , Fig 6a-b). The concentration of both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the EPF decreased with decreasing EPF pH (regression,  $n=10$ ,  $p\text{-value}<0.05$ ; Fig 6c-d). However, when binning by seawater pH treatments, only the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  of the ambient condition was significantly elevated compared to the moderate and high ocean acidification treatments (Tukey HSD,  $n_1=4$ ,  $n_2=3$ ,  $p<0.05$ , Fig 6c-d). The EPF and shell  $\delta^{26}\text{Mg}$  did not change as a function of EPF or seawater pH (Fig 6e-f and 5e-f).



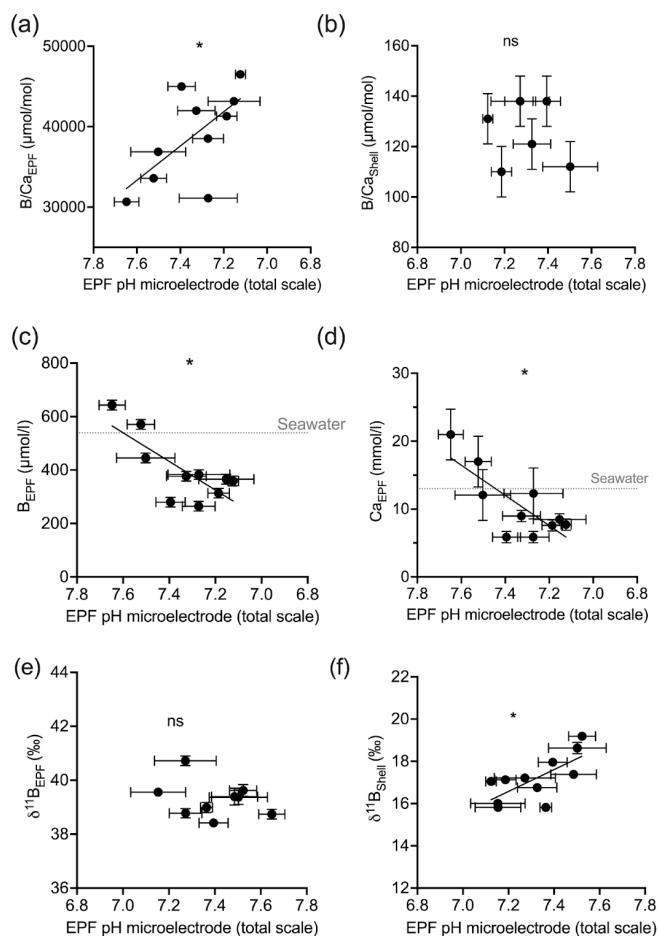
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376 **Figure 6.** Scatter plots showing *C. virginica* individual specimen (a) EPF Mg/Ca and (b) shell Mg/Ca across corresponding  
 377 microelectrode pH. Additionally, scatter plots (c) EPF  $Mg^{2+}$ , (d) EPF  $Ca^{2+}$ , (e) EPF  $\delta^{26}Mg$ , and (f) shell  $\delta^{26}Mg$  across  
 378 microelectrode EPF pH. Dotted gray lines on (c) and (d) show the average  $Mg^{2+}$  and  $Ca^{2+}$  seawater concentration,  
 379 respectively. Stars denote statistically significantly nonzero regression slopes and ‘ns’ signify non significant regressions (at  
 380 significance  $p < 0.05$ ).

381 Under OA conditions, EPF B/Ca but not shell B/Ca was found to increase as seawater pH decreased (ANOVA  
 382  $p$ -value $<0.05$ , compare Fig 7a-b). The EPF but not shell B/Ca was found to increase as EPF pH decreased (regression  
 383  $p$ -value $< 0.05$ , Fig 7a-b). The boron concentration of the EPF, but not the shell, significantly decreased with decreasing EPF  
 384 pH (regression  $p$ -value $< 0.05$ , Fig 7c). The EPF B concentration increased with increasing seawater pH (ANOVA  $p$ -value $<$

0.05, Fig 7c); however, shell boron concentrations did not significantly change with seawater pH. Due to small EPF sample volume, EPF for the oysters in the lowest seawater pH treatment was not measured for  $\delta^{11}\text{B}$ . There was a significant difference in mean EPF  $\delta^{11}\text{B}$  between the control pH treatment which was 39.39 ‰ and moderate pH treatment which was 38.92 ‰ (t-test,  $n_1=11$   $n_2=7$ ,  $p\text{-value}<0.05$ , Fig 7e-f). The difference between seawater  $\delta^{11}\text{B}$  and EPF  $\delta^{11}\text{B}$  was 0.91 ‰ for the control treatment and decreased to 0.47 ‰ for the moderate pH treatment. Shell  $\delta^{11}\text{B}$ , but not EPF  $\delta^{11}\text{B}$ , significantly decreased with decreasing EPF pH (regression  $p\text{-value}<0.05$ , Fig 7e-f).

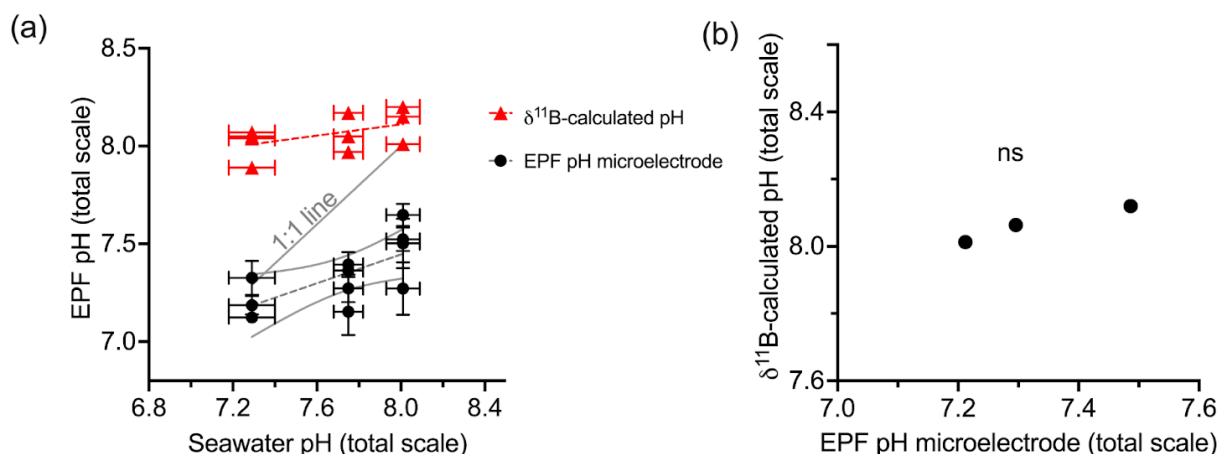


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f 07

**Figure 7.** Scatter plots showing *C. virginica* individual specimen (a) EPF B/Ca and (b) shell B/Ca across corresponding microelectrode EPF pH. Additionally, scatter plots of (c) EPF B, (d) EPF  $\text{Ca}^{2+}$ , (e) EPF  $\delta^{11}\text{B}$ , and (f) shell  $\delta^{11}\text{B}$  across microelectrode EPF pH. Dotted gray lines on (c) and (d) show the average B and  $\text{Ca}^{2+}$  seawater concentration, respectively. Stars denote statistically significantly nonzero regression slopes and ‘ns’ signify non significant regressions (at significance  $p < 0.05$ ).

Fig 8a shows the measured EPF pH, the  $\delta^{11}\text{B}$ -calculated EPF, and seawater to EPF 1:1 pH line graphed across the *C. virginica* acidification experiment. The slope of the measured microelectrode EPF pH versus seawater pH linear regression was 0.36, and lies below the seawater to EPF 1:1 pH line, but intersects the seawater to EPF 1:1 pH line at lowest pH/highest  $p\text{CO}_2$  culture conditions (Fig 8). Conversely, the slope of the  $\delta^{11}\text{B}$ -calculated EPF pH versus seawater pH linear regression was 0.14, lies above the seawater to EPF 1:1 pH line, but intersected the seawater to EPF 1:1 pH line at higher culture pH conditions (Fig 8).



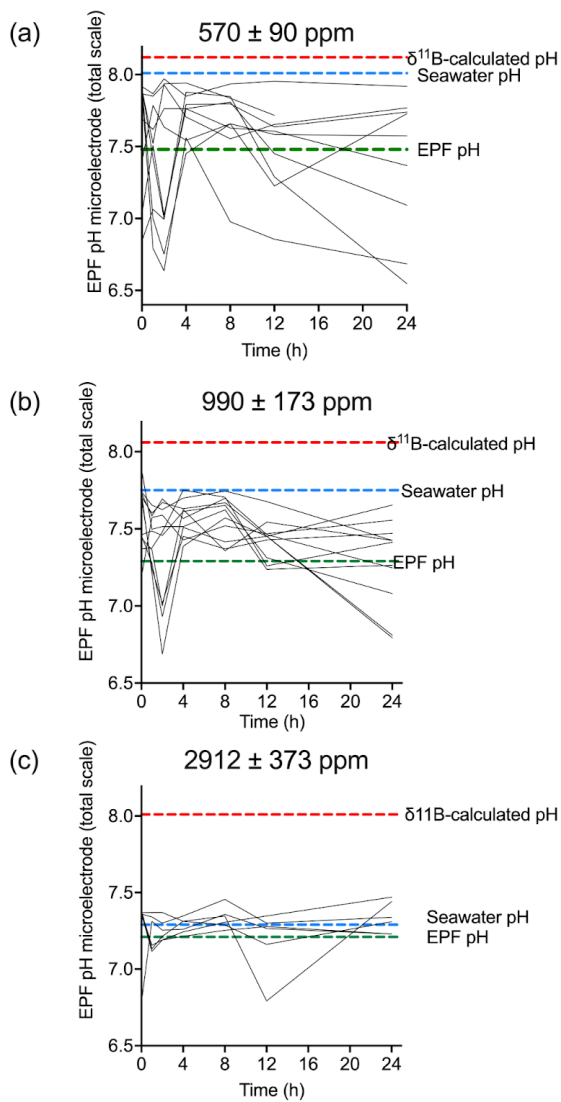
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404

**Figure 8.** (a) Scatter plot of  $\delta^{11}\text{B}$ -calculated pH and microelectrode EPF pH across seawater pH treatments. The gray line shows the 1:1 seawater to EPF pH line. The  $\delta^{11}\text{B}$ -calculated pH regression line had a slope of 0.14. The microelectrode EPF pH line had a slope of 0.36. (b) shows the averaged  $\delta^{11}\text{B}$ -calculated pH versus microelectrode EPF pH. The 'ns' signifies a non significant regression (at significance  $p < 0.05$ ).

409

For the *C. virginica* acidification experiment, Downey-Wall et al., (2020) measured the EPF pH of individual specimens in each acidification treatment over a 24-hour period ( $n_{\text{total}}=108$  and  $n=6$  per time point per treatment). Fig 9 shows how the EPF pH for each individual fluctuated over 24 hours. Ambient treatment EPF pH ranged from 6.63-7.94, moderate OA treatment ranged from 6.68-7.88, and high OA treatment ranged from 6.78-7.47. The control treatment EPF pH of individuals did intersect the averaged seawater pH for the treatment tanks, however, the EPF pH in the moderate and high pH treatments fell below the corresponding average treatment seawater pH lines. For all treatments, the time series EPF pH lines fell below the corresponding treatment averaged  $\delta^{11}\text{B}$ -calculated EPF pH line.



f 09

417

418 **Figure 9.** Time series (in hours) of microelectrode EPF pH over a 24 hour period for (a) control (n=10) (b) moderate (n=11)  
 419 and (c) high  $p\text{CO}_2$  treatments (n=6). Each line represents the microelectrode EPF pH for each individual specimen measured  
 420 in that treatment. The red dotted line shows the corresponding average  $\delta^{11}\text{B}$ -calculated pH for the treatment, the blue dotted  
 421 line shows the average seawater pH for the treatment, and the green dotted line shows average EPF pH.

422

## 423 4. Discussion

### 424 4.1 Comparison of *A. islandica* and *C. virginica* $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$ geochemistry of seawater, EPF, and bivalve shell

425 This study examined tripartite element and isotope fractionation between different reservoirs involved in the  
426 biomineralization of two bivalves species, aragonitic *A. islandica* and calcitic *C. virginica*. Marine bivalves source ions for  
427 internal fluids from seawater and previous studies by Crenshaw (1972) have highlighted that the extrapallial fluid is  
428 chemically different from seawater. The ions sourced from seawater are modulated either passively or actively across the  
429 outer mantle epithelium (OME) cells into the extrapallial cavity, where biomineralization occurs (Zhao et al., 2018). The  
430 exact mechanisms behind bivalve biomineralization is still a topic of active research and evidence has been put forth for  
431 several distinct pathways, primarily regulation of calcification constituents across the OME or transport of a precursor phase  
432 of  $\text{CaCO}_3$  to promote calcification (Addadi et al., 2003; Checa 2018).

433 In this study we found that the extrapallial fluid is chemically distinct from seawater. Here we show that, under  
434 ambient conditions, both the EPF  $\text{Mg}/\text{Ca}$  and  $\text{B}/\text{Ca}$  of both *C. virginica* and *A. islandica* were lower than that of seawater,  
435 indicating that the EPF has a distinct geochemical make up from seawater (Fig 3). This is consistent with the anatomical  
436 understanding in bivalves that the extrapallial fluid is semi-isolated from seawater and its geochemistry can be influenced by  
437 ion fluxes across the OME as well as other ion pathways (Crenshaw 1972; Sillanpaa et al., 2018; Stemmer et al., 2019).  
438 However, we also find that for both  $\text{Mg}/\text{Ca}$  and  $\text{B}/\text{Ca}$ , this result is driven by an increase in absolute  $\text{Ca}^{2+}$  in EPF, so we do  
439 not find evidence for dilution or concentration of the absolute  $\text{Mg}^{2+}$  or B in the EPF (Fig 3). Previous work on bivalves has  
440 shown that magnesium can inhibit calcite crystal nucleation and there is evidence for exclusion of  $\text{Mg}^{2+}$  from the EPF  
441 (Lorens and Bender, 1977). In line with other studies, we show that *C. virginica* and *A. islandica* have lower  $\text{Mg}/\text{Ca}$  in EPF  
442 than seawater (Lorens and Bender, 1977; Planchon et al., 2013); however, we note that the EPF  $\text{Mg}/\text{Ca}$  trend is driven by  
443 changes in EPF  $\text{Ca}^{2+}$ . *C. virginica* and *A. islandica* EPF  $\text{Mg}/\text{Ca}$  were significantly different, with lower EPF  $\text{Mg}/\text{Ca}$  for *A.*  
444 *islandica*, possibly due to different controls over EPF  $\text{Ca}^{2+}$  between both species. The partition coefficient between EPF and  
445 the shell was calculated to be 0.003 for *C. virginica* 0.0002 for *A. islandica*, which is consistent with previous studies on  
446 bivalves and with the  $\text{Mg}/\text{Ca}$  mineralogical difference between the calcite produced by *C. virginica* and the aragonite  
447 produced by *A. islandica* (Ulrich et al. 2021).

448 We found that the EPF  $\delta^{26}\text{Mg}$  of *C. virginica* was depleted compared to seawater  $\delta^{26}\text{Mg}$  (Fig 3). Our  $\delta^{26}\text{Mg}$  values  
449 for the EPF and shell were in line with previous work on bivalves (Planchon et al., 2013). Planchon et al. (2013) found a  
450  $-0.23 \pm 0.25 \text{ ‰}$  (2 SD,  $n=5$ ) difference between EPF and seawater in the aragonitic manila clam, *Ruditapes philippinarum*.  
451 Similarly, in the present study, a difference of  $-0.11 \pm 0.06 \text{ ‰}$  was observed for the calcitic *C. virginica*, but no  $\delta^{26}\text{Mg}$  data  
452 were collected for *A. islandica* due to sample limitation. Both Planchon et al. (2013) and the present study show depleted  
453 EPF  $\delta^{26}\text{Mg}$  relative to seawater  $\delta^{26}\text{Mg}$ , indicating a potential biological modulation of EPF  $\text{Mg}^{2+}$  which has been previously  
454 attributed to heavier isotopes being incorporated into soft tissues or magnesium fixation within organic molecules (Planchon  
455 et al., 2013). However, it is important to note that the difference between EPF and seawater  $\delta^{26}\text{Mg}$  is low and the  $\delta^{26}\text{Mg}$

fractionation between the shell and seawater (2.43‰) was slightly larger than but still in line with inorganic calcite precipitation studies (Mavromatis et al., 2013; Saulnier et al., 2012).

#### 4.2 Comparison of *A. islandica* and *C. virginica* EPF pH and boron geochemistry of seawater, EPF, and bivalve shell

The boron isotopes and B/Ca proxies have been used as paleo-pH and  $\text{CO}_3^{2-}$  proxies, recording changes in seawater carbonate chemistry in the shells of foraminifera (e.g. Hemming and Hanson 1992; Sanyal et al., 2001; Foster and Rae, 2016). In different taxa however, there is evidence that these proxies monitor changes in the carbonate chemistry of the internal calcifying fluid, which may be different from seawater geochemistry (e.g. Allison and Finch 2010; Cornwall et al., 2017; Sutton et al., 2018; Guillermic et al., 2021). In the present study, we constrained the B/Ca and  $\delta^{11}\text{B}$  of the main reservoirs involved in the biomineralization (seawater, extrapallial fluid, and shell) of *C. virginica* and *A. islandica*.

We found an incongruence between seawater pH, measured EPF pH and  $\delta^{11}\text{B}$ -calculated pH. For both *C. virginica* and *A. islandica* microelectrode EPF pH was lower than seawater pH. These findings are similar to previous work on bivalves which also show that the EPF pH is lower than seawater pH (Crenshaw 1972; Heinemann et al., 2012; Stemmer et al., 2019; Cameron et al. 2019). Microelectrode EPF pH between species was found to not be significantly different, indicating a similar downregulation in pH compared to seawater. However, our  $\delta^{11}\text{B}$ -based EPF pH was different between species (Fig 5). Using boron isotope systematics, this translated to a  $\delta^{11}\text{B}$ -calculated EPF pH of  $7.76 \pm 0.07$  for *A. islandica* and  $8.12 \pm 0.09$  for *C. virginica*. Although boron isotopes have been shown to probe the internal calcification fluid of certain taxa, like corals (e.g. Allison and Finch 2010), our results show an incongruence between measured EPF pH and  $\delta^{11}\text{B}$ -calculated pH.

#### 4.3 *C. virginica* ocean acidification effects on $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$ geochemistry of seawater, EPF, and bivalve shell

In the complementary study by Downey-Wall et al. (2020), it was found that the *C. virginica* calcification rates decreased with seawater pH (Downey-Wall et al., 2020; Fig 2). The reduction of calcification under ocean acidification conditions is well documented in other seawater pH experiments on different bivalve species (e.g., Ries et al., 2009; Beniash et al., 2010; Waldbusser et al., 2011; Downey-Wall et al., 2020). This result is consequential as the shell is important in protecting the animal from predation, desiccation, and the effects of transient changes in seawater chemistry (Gosling et al., 2008). Under ocean acidification treatments, the average microelectrode EPF pH of *C. virginica* was lower than seawater pH. This is in line with other simulated ocean acidification studies that also found a decrease in EPF pH (Michaelidis et al., 2005; Thomsen et al., 2013; Zittier et al., 2015; Cameron et al., 2019; Downey-Wall et al., 2020). However, the change in pH between EPF and seawater pH ( $\Delta\text{pH}$ ) decreased with decreasing pH, resulting in an EPF pH that was closer to seawater pH under acidified conditions (Fig 8a, Fig. 9c).

Only *C. virginica* was cultured under ocean acidification (OA) treatments representing control, moderate, and high OA treatments. As mentioned above, the control experiment showed elevation of EPF  $\text{Ca}^{2+}$  and EPF  $\text{Mg}^{2+}$  relative to seawater. However, as EPF pH decreased, the EPF  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  significantly decreased as well (Fig 6). Ion transporters such as voltage gated  $\text{Ca}^{2+}$ -channels tend to also affect chemically similar ions like  $\text{Mg}^{2+}$  and a reduction of such a transporter could possibly explain the similar trends in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations under OA (Hess et al., 1986). Under OA

conditions, EPF  $\text{Ca}^{2+}$  decreased to concentrations that were similar to or below seawater  $\text{Ca}^{2+}$ , indicating a reduced ability of the organism to upregulate these ions under OA conditions. Previous studies have found a similar tight coupling between pH and  $\text{Ca}^{2+}$ . For example, Stemmer et al. (2019) found synchronous patterns between pH and  $\text{Ca}^{2+}$  dynamics in *A. islandica* that they explained to be the result of calcium-transporting ATPase, which exchanges protons and calcium ions across the mantle and has proven to be important for acid-base regulation and calcium transport in bivalves (Stemmer et al., 2019; Sillanpaa et al., 2018; Sillanpaa et al., 2020). Although calcium transporting ATPase could explain this increase in  $\text{Ca}^{2+}$  under ambient conditions, this transport mechanism may be reduced under acidified conditions, thereby impairing the bivalve's ability to regulate protons and calcium ions in the extrapallial fluid, rendering EPF  $\text{Ca}^{2+}$  and pH more similar to that of seawater.

Alternatively, the simultaneous reduction in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  under OA conditions could point to an ion storage mechanism. The reduction of both calcium and magnesium within the EPF under moderate and high OA treatments could possibly be linked to changes of storage and budgets of ions under stressful conditions (Mount et al., 2004; Johnstone et al., 2015; Wang et al. 2017). Further, several studies have highlighted significant changes in bivalve  $\text{Ca}^{2+}$  ion transport and storage in different extracellular and subcellular compartments associated with shell damage and repair under acidified conditions (Sillanpaa et al., 2016; Mount et al., 2004; Fitzer et al., 2016). Lastly, the EPF  $\text{Ca}^{2+}$  could simply reflect the balance between calcification and dissolution of the shell, as exemplified in a study on *C. virginica* conducted by Ries et al. (2016) which found that under similarly low saturation states, localized shell calcification was maintained despite net dissolution of the shell. Regardless of the exact mechanism, the reduction in extrapallial fluid  $\text{Ca}^{2+}$  under ocean acidification is a significant result that could impact the ability of bivalves to calcify by decreasing the  $\text{CaCO}_3$  saturation state of the EPF.

#### 4.4 *C. virginica* ocean acidification effects on boron geochemistry

Similarly to ambient conditions, the calculated  $\delta^{11}\text{B}$ -based pH for *C. virginica* is systematically higher than microelectrode EPF pH (Fig 8). Both  $\delta^{11}\text{B}$ -based pH and measured EPF pH record a decrease in pH under acidified conditions (regression  $p < 0.05$  for microelectrode pH). However, the offset between microelectrode EPF pH and the  $\delta^{11}\text{B}$ -calculated pH was 0.3 pH units and increased to 0.6 and 0.8 pH units for the moderate and high OA treatments, respectively (Fig 8). This demonstrates that, under OA conditions, the incongruence between  $\delta^{11}\text{B}$  based pH and measured EPF pH increases and potentially renders the seawater pH proxy impractical, even after species-specific empirical calibration. Under OA conditions, shell  $\delta^{11}\text{B}$  was not correlated with changes in seawater pH, but was significantly correlated to microelectrode pH (Fig 7f). These data indicate that microelectrode EPF pH does not fully resolve  $\delta^{11}\text{B}$  vital effects or discrepancies.

However it is important to note the differences in timescales associated with  $\delta^{11}\text{B}$ -calculated EPF pH and microelectrode pH. Our microelectrode pH measurements, although averaged across several time points, show snapshots in time and are variable due different behavioral scenarios such as open (feeding, high pH) and closed (respiring into a closed system, low pH) cycles. Conversely, the  $\delta^{11}\text{B}$  approach represents EPF pH integrated average EPF pH over the interval that the sampled shell was formed, which could range from days to weeks. Furthermore, the  $\delta^{11}\text{B}$  method will only record EPF pH at the site of calcification when the shell is forming, which can skew the archiving of the  $\delta^{11}\text{B}$  pH signal in the shell to

524 higher values because the crystal only forms when saturation states and calcification rates are higher. This potential bias is  
525 also consistent with our  $\delta^{11}\text{B}$ -calculated EPF pH data being higher than the microelectrode pH data, and similar to trends  
526 seen in corals (Cameron et al, 2022).

527 A possible explanation for the incongruence between  $\delta^{11}\text{B}$ -based pH and measured EPF pH arises from boron  
528 isotope systematics. The boron isotope proxy assumes that only the charged borate ion is incorporated as  $\text{BO}_4$  into the  
529 mineral but has been shown that boric acid can also be incorporated as  $\text{BO}_3$ , and NMR studies have shown the presence of  
530  $\text{BO}_3$  in the shells of different marine organisms (Rollion Bard et al., 2011; Cusack et al., 2015). However, the presence of  
531  $\text{BO}_3$  does not obviously translate to a strong bias in the  $\delta^{11}\text{B}$  signature of the mineral due to the potential re-coordination of  
532  $\text{BO}_4$  to  $\text{BO}_3$  within the crystal lattice (Klochko et al., 2009). A simple calculation shows that 14-17% boric acid incorporation  
533 could explain the observed difference between EPF pH and  $\delta^{11}\text{B}$ -calculated pH for *C. virginica*, which could very well  
534 explain the discrepancy. Alternatively, shell  $\delta^{11}\text{B}$  could also be affected by seawater or extrapallial fluid DIC, which bivalves  
535 are known to modulate under ambient and OA conditions (Crenshaw 1972, Stemmer et al., 2019). Gagnon et al. (2021)  
536 found that the shell  $\delta^{11}\text{B}$  of deep-water coral is independently sensitive to changes in seawater DIC as a result of diffusion of  
537 boric acid (Gagnon et al., 2021), though no similar studies have looked at the same effect in bivalves this mechanism is still  
538 possible. Taken together, these findings could explain the offset between  $\delta^{11}\text{B}$ -based pH and seawater or EPF pH.  
539 Nevertheless, this remains speculative as there is no further evidence of boric acid incorporation in these species.

540 The difference between microelectrode EPF pH and  $\delta^{11}\text{B}$ -based EPF pH implies that pH measured with boron  
541 isotopes probes a localized site of calcification rather than the entire EPF pool measured with microelectrode. A spatial and  
542 temporal study conducted by Stemmer et al. (2019) measured the EPF of *Arctica islandica* and showed highly dynamic  
543 changes in pH,  $\text{Ca}^{2+}$  and DIC from the surface of the shell to the outer mantle epithelium (OME), with localized environment  
544 at the OME reaching pH values up to 9.5. Due to this high variability, it is possible that the EPF microelectrode  
545 measurements in this study did not capture the full variability of the EPF. Stemmer et al. (2019) presented EPF pH values  
546 measured at the shell surface ranging [7.1-7.6] for *A. islandica*, comparable to the values measured from microelectrode in  
547 this study (Fig 9). Additionally, Stemmer et al. (2019) found large influxes of DIC which could not have been explained just  
548 from metabolic activity, but instead indicated intense DIC pumping and bursts of calcification. These findings are in line  
549 with the holistic view of biomineralization outlined in Checa (2018) and Johnstone (2015) which argue that crystal  
550 deposition is a series of periodic events under biological regulation. In our study, a time-series of microelectrode EPF pH  
551 shows that at no point, during ventilation and closed cycles, does the EPF pH reach the  $\delta^{11}\text{B}$ -calculated pH (Fig 9). The fact  
552 that microelectrode EPF pH is systematically lower than seawater pH for both of our bivalve species may reflect localized  
553 differences in pH associated with zones of calcification. The two environments (site of calcification and bulk EPF) can act  
554 distinctly, with low pH and high DIC EPF being a source of carbon for the site of calcification in the bulk EPF, and elevated  
555 pH of the site of calcification supporting the conversion of the DIC species to  $[\text{CO}_3^{2-}]$  in support of mineral precipitation.  
556 Further work would be needed to assess this highly dynamic and localized environment, however our study shows that boron  
557 isotopes may reflect the pH of the microenvironment where calcification occurs within the EPF, which has previously been

558 inferred by prior studies using non-geochemical approaches (Ramesh et al., 2017; Ramesh et al., 2018; Stemmer et al.,  
559 2019).

## 560 Conclusion

561 In this study, we used numerous approaches constraining the geochemical composition of and partitioning between  
562 the tripartite reservoirs of the bivalve mineralization system—seawater, EPF and shell. Comparisons of seawater and  
563 extrapallial fluid Mg/Ca and B/Ca,  $\text{Ca}^{2+}$ , and  $\delta^{26}\text{Mg}$  indicate that the EPF has a distinct composition that differs from  
564 seawater. Additionally, our OA experiments show that the EPF Mg/Ca and B/Ca, as well as absolute  $\text{Mg}^{2+}$ , B, and  $\text{Ca}^{2+}$ , all  
565 were significantly affected by  $\text{CO}_2$ -induced ocean acidification, demonstrating that the biological pathways regulating or  
566 storing these ions involved in calcification are impacted by ocean acidification. Decreased calcium ion concentration within  
567 the extrapallial fluid due to OA could impair calcification by lowering the saturation state of the EPF with respect to  $\text{CaCO}_3$ .  
568 Additionally, our results show that shell  $\delta^{11}\text{B}$  does not faithfully record seawater pH. However, shell  $\delta^{11}\text{B}$  is correlated with  
569 EPF pH, despite an offset from *in situ* microelectrode pH measurements. Both microelectrode pH and  $\delta^{11}\text{B}$ -calculated pH  
570 decreased with decreasing pH. However, the  $\delta^{11}\text{B}$ -calculated pH values were consistently higher than microelectrode pH  
571 measurements, indicating that the shell  $\delta^{11}\text{B}$  may reflect pH at a more localized site of calcification, rather than pH of the  
572 bulk EPF. Furthermore, the offset between the  $\delta^{11}\text{B}$ -calculated pH and microelectrode pH increased with decreasing pH  
573 under ocean acidification, indicating OA has a larger effect on bulk pH of the EPF measured via microelectrode than on site  
574 of calcification pH—the latter of which the bivalve may have more physiological control over to ensure continued  
575 calcification even under chemically unfavorable conditions. These complex dynamics of EPF chemistry suggest that boron  
576 proxies in these two bivalve species are not straightforwardly related to seawater pH, precluding utilization of those species  
577 for reconstructing the carbonate chemistry of seawater. Moreover, the  $\delta^{11}\text{B}$  proxy may not be suitable for reconstructing  
578 seawater pH for bivalves with high physiological control over their internal calcifying fluid and is further complicated under  
579 conditions of moderate and extreme ocean acidification, where  $\delta^{11}\text{B}$  EPF pH deviates further from bulk microelectrode pH,  
580 possibly due to the effect of DIC on shell  $\delta^{11}\text{B}$  or the tendency for shell  $\delta^{11}\text{B}$  to reflect EPF pH at the more localized site of  
581 calcification, rather than pH of the bulk EPF.

## 582 Author contribution

583 LPC, AD, JBR, and KL designed the experiments and carried them out. BAC, MG, and RAE developed the geochemical  
584 study. BAC and MG performed geochemical analysis with the help of JNS and JAH. BAC, MG, and RAE prepared the  
585 manuscript with contributions from all co-authors.

## 586 Competing interests

587 The authors declare that they have no conflict of interest.

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