Ocean acidification can interact with ontogeny to determine the trace element composition of bivalve shell

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Scientific Significance Statement

The trace element composition of the calcium carbonate (CaCO₃) structures of organisms, such as bivalve shells or fish otoliths, can provide information on the physical and chemical properties of the water in which it is formed. Ocean acidification, from increases in anthropogenic CO₂, changes the ocean's carbonate chemistry; however, it is unclear how it might influence trace elemental incorporation into carbonate structures. This study provides evidence that 13 out of 17 elements tested in Greenshell™ mussels are affected by pCO₂, but that it differed by shell age. Our results suggest that elements in young shells are stable under different pCO₂ levels and can be used to determine dispersal histories, but older shell could be used to reconstruct carbonate chemistry.

Abstract

We sought to determine how pCO₂ will affect the incorporation of trace elements into bivalve shell. This was to validate that under high pCO₂ conditions reconstruction of animal movements is still viable; and to investigate potential trace element proxies for ocean carbonate chemistry. Here, we examined shell of the bivalve Perna canaliculus formed under current CO₂ (pCO₂ = 400 μatm) conditions and those predicted to exist in 2100 (pCO₂ = 1050 μatm). Seventeen trace element:calcium ratios were examined at two locations within shells. Elements that are typically most useful in determining connectivity patterns (e.g., Sr, Mn, Ba, Mg, B) were not affected by pCO₂ in shell produced early in individual’s lives. This suggests that the effects of ocean acidification on dispersal signatures may be dampened. However, cobalt, nickel, and titanium levels were influenced by pCO₂ consistently across shells suggesting their role as potential indicators of CO₂ level.

The trace elemental composition of biogenic calcium carbonate (CaCO₃) structures such as shells or otoliths reflect the physicochemical properties of the water in which they are formed (Beer et al. 2011; Poulain et al. 2015). Therefore, the trace elemental composition of CaCO₃ structures can provide valuable information on the water mass in which they were produced. Currently, there are two primary areas in which this information is applied: population connectivity and paleoclimate reconstructions. Variations in multiple trace elements in bivalve shells, gastropod statoliths, and fish otoliths have been used to infer dispersal histories, population connectivity and stock structure, a technique known as trace elemental fingerprinting (Gomes et al. 2016; Kroll et al. 2016). Among climate researchers, there is also interest in the use of the elemental composition of CaCO₃ structures as proxies to reconstruct environmental conditions such as temperature, salinity or pH at their time and location of formation (Poulain et al. 2015, Immenhauser et al. 2016).

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Ocean acidification (OA) is the decrease in ocean pH, due to the uptake of anthropogenic CO₂ from the atmosphere. Once dissolved into seawater, CO₂ increases the concentration of bicarbonate ions ([HCO₃⁻]) and lowers the pH and concentration of the carbonate ion ([CO₃²⁻]) (Gattuso and Hansson 2011). In addition to this change in the carbonate equilibrium reaction, the availability of ions will also be affected (Millero et al. 2009). As the incorporation of trace elements into biogenic CaCO₃ is affected by both the availability of ions and a number of other environmental and physiological processes (Beer et al. 2011), the composition of these carbonate structures is likely to be affected by changes in oceanic pH (Hoffmann et al. 2012; Frieder et al. 2014, Levin et al. 2015).

Understanding how OA affects the trace elemental composition of carbonate structures will provide valuable information for both trace elemental fingerprinting and climate reconstructions. If the trace elemental composition of shell material is to be used to reconstruct the pH history of the oceans, any proxies that are used should have a defined and stable relationship with pH (Yu et al. 2007; Henehan et al. 2013). For trace elemental fingerprinting, understanding how OA will affect trace elemental incorporation into carbonate structures will allow us to ensure this technique remains viable under future conditions. Currently, differences in shell chemistry caused by physicochemical properties such as temperature, salinity, and the availability of elements in the surrounding environment are used to assign shells to their origin (Becker et al. 2007; Kroll et al. 2016). If the effects of pH on the composition of shell material are unknown the possibility that any differences observed are erroneously attributed to other environmental variables exists. In addition, many estuarine and coastal environments experience seasonal or diel changes in pH (Law et al. 2017), which could complicate reconstructions of both climactic conditions and larval dispersal without a solid understanding of how this variation influences shell chemistry.

The possibility also exists for these largely separate applications of CaCO₃ trace elemental composition to be combined. The suite of trace elements used in trace elemental fingerprinting studies does not frequently overlap with the elements used in climate reconstructions (Dunphy et al. 2011; Hahn et al. 2012; Poulain et al. 2015; Norrie et al. 2016). If changes in pH influence elements, which are not generally used in trace elemental fingerprinting, it may be possible to use trace elemental fingerprinting techniques to determine the location at which a CaCO₃ structure formed and simultaneously infer the environmental conditions at this location using a complementary suite of elements. This could potentially reduce the need to deploy expensive monitoring equipment at a number of locations and examining the ecological consequences of sequential exposure to varying pH conditions at different life history stages (Frieder et al. 2014).

To date the majority of research has focused on the impact of OA on the incorporation of trace elements into foraminiferal tests (Yu et al. 2007, Henehan et al. 2013). Bivalves, however, are more frequently used in trace elemental fingerprinting studies (Dunphy et al. 2011; Gomes et al. 2016). The aim of this study was, therefore, to answer the question “how will predicted future ocean Perna canaliculus formed under current CO₂ (pCO₂) affect the incorporation of trace elements into the shell of Greenshell™ mussels Perna canaliculus?” We explore implications of pCO₂ on both elements that have been used for elucidating dispersal histories and those elements used to reconstruct geological history.

Methods
Animal spawning and husbandry
To minimize genetic influences, a single full-sibling family of Greenshell™ mussels was examined. Individual male and female mussels were randomly selected from unrelated F2 families raised on long-line aquaculture facilities in the Marlborough Sounds (South Island, New Zealand) as part of the Cawthron Perna canaliculus selective breeding program. Thermal shock was used to induce spawning; eggs were diluted to 1000 mL⁻¹ in either 400 μatm CO₂ (ambient) or 1050 μatm CO₂ (elevated) seawater and fertilized with sperm at 200 egg⁻¹ (Ragg et al. 2010). Prior to transfer to 160 L conical incubation tanks containing 5 μm-filtered seawater and 12 μM EDTA at 16 °C, maintained at 400 μatm CO₂ (ambient) or 1050 μatm CO₂ (elevated) using gentle aeration (air or CO₂ enriched via a gas mixer/analyser (WMA-5; PP Systems, Annesbury, MA). Carbonate conditions in each of the tanks was monitored weekly throughout the course of the experiment (Supporting Information). After 48 h incubation, the embryos had formed the prodissocothen shell, entering the feeding veliger stage. Larvae were transferred to the continuous-flow culture system described by Ragg et al. (2010). Triplicate 2.5 L control tanks with 200 larvae mL⁻¹ received filtered seawater enriched with dietary microalgae (Tisochrysis lutea + Chaetoceros calcitrans, 40 cells μL⁻¹). After 3 weeks, pediveligers were offered coir string as a settlement substrate and allowed to metamorphose. Juveniles were harvested after 3 months, rinsed in deionized water, snap frozen, and stored (−20 °C) prior to analysis.

Laboratory methods
Methods used were adapted from Norrie et al. (2016). Mussel were defrosted, the shells were split open, and the flesh was removed using stainless steel forceps. Twenty seven individuals raised at 400 μatm CO₂ and 32 raised at 1050 μatm CO₂ were examined. We selected one valve from each individual at random and fixed it to a glass microscope slide using double sided adhesive tape. To determine the elemental composition of shell material, we performed laser ablation inductively coupled plasma mass spectroscopy (LA-ICP-MS) using a New Wave deep ultra violet (193 nm) laser ablation system (Elemental Scientific Industries, Omaha, Nebraska, USA).
Laser power was 45% and the every 20 spots for standardization and calibration purposes. We analyzed NIST610 and NIST612 standards corrected data by subtracting background average counts within shell material (Strasser et al. 2008). We background reduce the chances of laser burn through to lower layers mine the elemental composition of the shell material to (rie et al. 2016). We used only the next 10 s of data to deter- tion (Tables S1 and S2).

To remove contaminants, we used a pre-ablation tech- nique in which the first 5 s of the laser dwell time were not included in the data reduction process (Marr et al. 2011; Nor- rie et al. 2016). We used only the next 10 s of data to deter- mine the elemental composition of the shell material to reduce the chances of laser burn through to lower layers within shell material (Strasser et al. 2008). We background corrected data by subtracting background average counts from the ablation average counts. We then converted back- ground corrected counts to trace element:calcium (TE:Ca) ratios and standardized them using the most recently pub- lished NIST610 values (Jochum et al. 2005). Internal precision was calculated from the NIST612 standard. Finally, TE:Ca ratios were converted to μmol:mol ratios, which were used for all statistical analyses.

**Statistical analyses**

To determine whether differences existed in the incorpora- tion of trace elements into *P. canaliculus* shell under the two pCO2 conditions we performed univariate statistical analyses on each of the elemental ratios analyzed. All statistical ana- lyses were performed in R version 3.3.3 (R core team). The data did not fit the assumptions of normality; thus, we per- formed a log transformation on all elemental ratios which improved normality. We used a general linear model (GLM) to examine the effects of the location analyzed within the shell (location), the pCO2 conditions to which they were exposed (treatment) and any interaction effects. Due to the nested design of this experiment tank was included as a ran- dom effect in the GLM. Post hoc t tests were performed, with a FDR p value adjustment being performed due to multiple comparisons.

**Results**

The results of the GLMs indicated that, for 10 of the 17 ele- mental ratios examined (B:Ca; Mg:Ca; V:Ca; Mn:Ca; Cu:Ca; Sr:Ca; Y:Ca; Ba:Ca; Pb:Ca; U:Ca), there was a significant inter- action effect between the location analyzed within shells and the CO2 treatment to which they were exposed (Table 1). The GLM showed a significant main effect of location within the shell for four TE:Ca ratios (Zn:Ca; Li:Ca; Al:Ca; Co:Ca) and a significant main effect of pCO2 for another three TE:Ca ratios (Co:Ca; Ni:Ca; Ti:Ca) (Table 1). The magnitude of differences observed in TE:Ca ratios was highly specific to the element analyzed, the location within shell, and the treatment to which they were exposed. No significant differences were found to exist for Y:Ca ratios after the application of FDR comparisons for multiple corrections.

The results of the post hoc pairwise comparisons showed that Ba:Ca, Cu:Ca, Mn:Ca, and V:Ca ratios differed signi- ficantly at the two locations analyzed in shell grown under both ambient and elevated CO2 conditions (Figs. 2A–D, 3A; Table 1). Ba:Ca, Cu:Ca, and Mn:Ca ratios were significantly higher at the shell edge than at the umbo of animals grown under both elevated and ambient pCO2. The effect of CO2 level on the concentration of Ba:Ca, Cu:Ca, and Mn:Ca was dependent on the location within the shell, with higher ratios at the shell edge under ambient pCO2 and higher ratios at the umbo under elevated pCO2. V:Ca ratios followed the opposite trend with significantly higher V:Ca ratios at the umbo than...
Table 1. Mean (μmol/mol ± SEM) of the TE:Ca ratios within shell raised under ambient and predicted future pH conditions. *p* values from the GLM are shown.

| Location within shell | pCO2 (μatm) | TE:Ca Ratio | Li:Ca | B:Ca | Mg:Ca | Al:Ca | Ti:Ca | V:Ca | Mn:Ca | Co:Ca | Ni:Ca | Cu:Ca | Zn:Ca | Sr:Ca | U:Ca | Ba:Ca | Pb:Ca | U:Ca |
|----------------------|------------|-------------|-------|------|-------|-------|-------|------|-------|-------|-------|-------|-------|-------|------|-------|-------|------|
| Edge                 | Ambient    | (p = 0.01) | 2.017 | 222.66 | 15.46 | 7.65 | 3.42  | 39.14 | 0.285 | 27.63  | 1.807 | 2.835 | 45.79 | 3.62  | 40.45 | 0.353 | 8.45  | 0.013 | 0.248 | 0.102 |
|                      | Elevated   | (p = 0.1)  | 2.06  | 147.06 | 11.64 | 10.22 | 3.27  | 44.73 | 1.25  | 16.218 | 3.969 | 14.59 | 5.32  | 37.23 | 0.145 | 6.109 | 0.025 | 0.172 | 0.333 | 0.033 |
| Umbo                 | Ambient    | (p = 0.10) | 1.296 | 132.05 | 78.02 | 24.17 | 16.52 | 40.67 | 1.146 | 4.14  | 7.514 | 5.666 | 3.04  | 35.84 | 0.204 | 4.44  | 0.017 | 0.279 | 0.232 | 0.055 |
|                      | Elevated   | (p = 0.10) | 0.999 | 134.17 | 131.38 | 13.52 | 7.325 | 60.90 | 0.983 | 9.968  | 0.778 | 6.15  | 13.40 | 39.77 | 0.187 | 4.092 | 1.182 | 0.425 | 0.027 | 0.208 |

1Indicates significant treatment main effects were observed.
2Indicates that significant interactions were shown to exist between location and treatment by the GLM.
3Indicates that significant main effect of location within the shell was observed.

Discussion

The results of this study show that the incorporation of all but three of elements analyzed were in some way affected by the CO2 concentrations in CO2 was most pronounced in shell produced under CO2 conditions (Figs. 2M, 3B; Table 1). Al:Ca and Zn:Ca ratios differed significantly between the two locations analyzed in shell produced under CO2 conditions (Fig. 2M, 3B; Table 1). The GLMs indicated that Al:Ca, Zn:Ca, Li:Ca, and Co:Ca ratios differed significantly at the umbo under both CO2 conditions (Fig. 2M, 3B; Table 1). The L:Ca ratios also showed significantly higher at the edge than the umbo of shell produced under both CO2 conditions (Fig. 2M, 3B; Table 1). The GLMs indicated that Al:Ca, Zn:Ca, Li:Ca, and Co:Ca ratios differed significantly at the umbo under both CO2 conditions (Fig. 2M, 3B; Table 1). For Pb:Ca ratios, differences were only found between the two locations analyzed in shell produced under elevated CO2 conditions (Figs. 2E, 3C; Table 1). Exposure to high CO2 conditions effectively homogenized significant differences in these TE:Ca ratios across the shell.

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Fig. 2. Mean TE:Ca (±SEM) ratios at the two locations analyzed within *P. canaliculus* shell for each of the elements for which significant interaction or main effects were found. Note: superscript a indicates that GLMs showed significant interaction effects, b indicates a significant location main effect, and c indicates a significant pCO2 treatment main effect as shown.
this study indicate that the processes, which result in the incorporation of elements into bivalve CaCO₃ under OA conditions will be highly element specific.

The within shell differences observed for many elements in shells from both CO₂ treatments show that even in the absence of OA, stress elements are not incorporated consistently as animals age. It is possible that these changes in elemental incorporation are related to ontogenetic changes in the CaCO₃ polymorph produced (Weiss et al. 2002). It has also been suggested that growth rates may affect the incorporation of elements into bivalve shell (Strasser et al. 2008) which may explain why the TE:Ca ratios differed between shell produced early in life, when growth rates are faster, differed from that deposited later.

If using TE:Ca ratios in CaCO₃ structures to reconstruct environmental conditions it is important that the elements used have a direct relationship with pH and are not affected by biological factors. Although it has previously been suggested that U:Ca ratios in bivalve shell will serve as useful proxies of seawater pH (Frieder et al. 2014, Levin et al. 2015), this study showed biological changes may modify the relationship between pH and U:Ca ratios. In this study Co:Ca, Ni:Ca, and Ti:Ca ratios were shown to clearly and consistently differ across the shell between the two CO₂ treatments to which they were exposed. This suggests that these elements may serve as useful proxies for environmental pH. Nickel and cobalt show similarities which are likely to be responsible for pH changes being reflected in shell chemistry. In seawater, both of these elements are dominated by only two species, the free ion (Ni²⁺ and Co²⁺), with the carbonate forms (NiCO₃ and CoCO₃) making up most of the remainder of these elements (Millero et al. 2009). Under the predicted future pH conditions used in this study, the portion of these elements in the carbonate forms is predicted to fall by approximately half (Millero et al. 2009). This fall is reflected in the results of this study, where the mean Ni:Ca and Co:Ca ratios in shells from treatment tanks were 58% and 48%, respectively, of those from control tanks. The behavior of Ti in response to pH changes is not well known due to low concentrations in seawater.

As only one environmental variable was manipulated in this study, it is important to establish that other conditions such as temperature or pollution do not play a greater role or interact with pH to influence the incorporation of these elements into bivalve shell. This is particularly the case as OA is unlikely to occur in isolation from other anthropogenic stressors (Byrne and Przeslawski 2013).

With regard to climate proxies, these results highlight that caution should be taken when selecting both elemental proxies and individuals for analysis. If comparing different individuals, care should be taken to ensure that they are analyzed in the same location within shells to ensure that ontogenetic differences in elemental incorporation are not misinterpreted as being due to changes in environmental conditions. Individuals
of a similar size should also be selected to reduce the possibility of biological changes influencing results.

Few elements were affected in the early shell material, which is used to reconstruct dispersal, by the CO2 conditions to which they were exposed. This implies that reconstructing the dispersal trajectory and larval origin of individuals will remain largely unaffected by OA. The three elements that did differ in early stage shell of animals exposed to different CO2 conditions (Co:Ca Ni:Ca, Ti:Ca, and U:Ca) have not previously been useful in assigning individuals to geographical locations or have provided only small increases in classification when included in a discriminant function analysis (Dunphy et al. 2011; Fodrie et al. 2011). When using trace elements to reconstruct dispersal trajectories, it is important that temporal stability in reference signals exists (Cathey et al. 2014). In the natural environment daily and seasonal swings in pH may occur (Law et al. 2017). These results show that these swings are unlikely to affect the early shell material and will not affect the temporal stability of these reference signals, although other seasonal changes may cause temporal instability.

Finally, the results of this study provide essential information that may allow the two primary applications in the field of trace elemental analysis of carbonate structures to be combined and open an exciting new avenue of research. The stability in the TE:Ca ratios at the umbo, which are generally used in trace elemental fingerprinting studies indicate that regardless of the pH in which the carbonate structure was deposited, its formation location will likely be able to be inferred. The TE:Ca ratios that are influenced by pH of seawater can then be examined to determine the carbonate chemistry of the water at which the carbonate structure was produced. It should be noted, however, that in the growing field of stable isotope analysis, the same ratios may be used reconstruct the properties of water masses (primarily nutrient dynamics) and estimate connectivity (Selleslagh et al. 2015). This suggests the response of stable isotope ratios to different environmental stressors and biology should be investigated in the future. In addition it has been shown that OA may have intergenerational (Parker et al. 2015), which will become a consideration over timescales longer than were investigated in this study. Nonetheless, provided the reference atlas of trace elemental fingerprints remains stable over time (Cathey et al. 2014), this may reduce the need for constant in situ sampling of seawater carbonate chemistry to determine how anthropogenic CO2 emissions are affecting the world’s oceans. Indeed, as multiple stressors are likely to interact to affect marine organisms (Byrne and Przeslawski 2013), it may be possible to reconstruct different environmental parameters using different TE:Ca ratios. This may allow individual exposure to varying environmental conditions at early life history stages to be inferred and the ecological consequences of this exposure to be better understood.

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