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Insights from sodium into the impacts of elevated pCO_2 and temperature on bivalve shell formation

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ABSTRACT

Ocean acidification and warming are predicted to affect the ability of marine bivalves to build their shells, but little is known about the underlying mechanisms. Shell formation is an extremely complex process requiring a detailed understanding of biomineralization processes. Sodium incorporation into the shells would increase if bivalves rely on the exchange of Na^+/H^+ to maintain homeostasis for shell formation, thereby shedding new light on the acid-base and ionic regulation at the calcifying front. Here, we investigated the combined effects of seawater pH (8.1, 7.7 and 7.4) and temperature (16 and 22 °C) on the growth and sodium composition of the shells of the blue mussel, *Mytilus edulis*, and the Yesso scallop, *Patinopecten yessoensis*. Exposure of *M. edulis* to low pH (7.7 and 7.4) caused a significant decrease of shell formation, whereas a 6 °C warming significantly depressed the rate of shell growth in *P. yessoensis*. On the other hand, while the amount of Na incorporated into the shells of *P. yessoensis* did not increase in acidified seawater, an increase of Na/Ca_{shell} with decreasing pH was observed in *M. edulis*, the latter agreeing well with the aforementioned hypothesis. Moreover, a combined analysis of the shell growth and sodium content provides a more detailed understanding of shell formation processes. Under acidified conditions, mussels may maintain more alkaline conditions favorable for calcification, but a significant decrease of shell formation indicates that the mineralization processes are impaired. The opposite occurs in scallops; virtually unaffected shell growth implies that shell mineralization functions well. Finding of the present study may pave the way for deciphering the mechanisms underlying the impacts of ocean acidification and warming on bivalve shell formation.

1. Introduction

Rapidly increasing anthropogenic CO_2 emissions are not only resulting in ocean warming, but also ocean acidification (OA) and altered seawater carbonate chemistry (Doney et al., 2009). These changing environmental conditions may have widespread implications for marine biota, especially bivalve mollusks (Kroeker et al., 2010; Rodolfo-Metalpa et al., 2011; Hendriks et al., 2015). There is mounting evidence that OA can impair the capacity of marine bivalves to produce their shells, leading to reduced calcification and growth rates (Gazeau et al., 2013; Kroeker et al., 2013; Waldbusser et al., 2014; Milano et al., 2016) and, in extreme cases, decalcification and mortality (McClintock et al., 2009; Gazeau et al., 2013). Furthermore, the negative effects of OA on shell mineralization may have significant functional and ecological implications. Reduced shell mechanical properties may increase the susceptibility to predation and, ultimately, alter the predator-prey dynamics in marine ecosystems (Gaylord et al., 2011; Schalkhausser et al., 2013; Kroeker et al., 2014).

OA does not act in isolation but usually interacts with other environmental stressors, specifically temperature (Pörtner and Farrell, 2008; Kroeker et al., 2013). The interactive effects of OA and temperature, which can be antagonistic or synergistic, may shape highly variable responses of marine bivalves to near-future climate change scenarios. For example, elevated temperature, within the thermal tolerance window, may alleviate the detrimental effects of OA on bivalve shell formation (Parker et al., 2009; Ko et al., 2014; Li et al., 2016) and, conversely, may exacerbate these threats (Hiebenthal et al., 2013; Schalkhausser et al., 2013; Li et al., 2015). Evidently, predicting the impacts of elevated pCO_2 and temperature on marine bivalves is fraught with great difficulty given the complexity and diversity of the responses observed. Therefore, it becomes critical to gain a better understanding of the processes and mechanisms determining their sensitivity and resilience to ocean acidification and warming.

Biomineralization of bivalve shells takes place in the extrapallial fluid (EPF), a thin film of liquid between the outer mantle epithelium (OME) and the calcifying shell (Wheeler, 1992). Bivalve mollusks can elevate the pH of the EPF to facilitate inorganic $CaCO_3$ precipitation (Crenshaw and Neff, 1969). For example, the oyster *Crassostrea gigas* appears to alkalinize the EPF by ca. 0.23 pH unit during the growing season (Wada and Fujinuki, 1976). Over a tidal cycle, the formation of shell

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growth increment in the intertidal cockle *Cerastoderma edule* is favored by the raise of extrapallial pH during immersion (Richardson et al., 1981). In situ measurements of the EPF in *Arctica islandica* show that pH rises rapidly from the calcifying front toward to the OME (Stemmer, 2013), indicating active removal of protons generated during $CaCO_3$ precipitation. It is therefore conceivable that the acid-base homeostasis at the calcifying front is a key determinant of the formation of bivalve shells. OA has been shown to acidize the EPF and shift the pH toward lower values (Thomsen et al., 2010; Heinemann et al., 2012; Zittier et al., 2015). Presumably, bivalves exposed to high pCO_2 need to actively remove more protons from the EPF to maintain an elevated pH level and a sufficient $CaCO_3$ saturation state that favor shell deposition (Cyronak et al., 2015). However, the processes involved in the proton removal from the EPF are poorly understood, probably because continuous monitoring of the pH at the calcifying front remains an extremely challenging task.

Under acidified conditions, bivalves rely on the active proton equivalent ion exchange to prevent ongoing extracellular pH drops and maintain steady-state values (Pörtner et al., 2004; Fabry et al., 2008; Melzner et al., 2009; Parker et al., 2013). Support for the existence of such active transport processes comes from the significantly increased expression of genes involved in ion and acid-base regulation, for example in the OME of the pearl oyster *Pinctada fucata* exposed to elevated pCO_2 of 862 μatm (Li et al., 2016). Two membrane transporters, vacuolar type H^+-ATPase and Na^+/H^+ exchanger, have been identified as being responsible for active removal of more H^+ ions (Li et al., 2016). In the latter process, the Na^+/K^+-ATPase likely plays a key role through creating ionic and electrochemical gradients used by secondary active ion transporters specifically the Na^+/H^+ exchanger (Melzner et al., 2009). For example, the rate of Na^+/H^+ exchange relies on the Na^+ gradient between the extracellular fluid and the ambient water which is actively built up by the Na^+/K^+-ATPase (Fabry et al., 2008; Parker et al., 2013). Since the activity of Na^+/K^+-ATPase is highly sensitive to changes in extracellular pH caused by elevated pCO_2 (Pörtner et al., 2000; Lannig et al., 2010), the extracellular Na^+ concentration may be shifted toward higher or lower values. For example, an increase of extracellular Na^+ has previously been observed in *Mytilus edulis* exposed to higher CO_2 levels (Thomsen et al., 2010), whereas a reduction occurred in *C. gigas* (Lannig et al., 2010). Likewise, if bivalves actively alkalinize the EPF through the exchange of Na^+/H^+ when subjected to OA, an increase of Na^+ transported into the EPF could be anticipated. If this is the case, then one would also expect an increase of the sodium content in the shells because the amount of Na^+ incorporated into the shells is directly proportional to its corresponding level in the EPF (Lorens and Bender, 1980).

Here, we investigated the influence of elevated pCO_2 and temperature on the formation of bivalve shells. The blue mussel, *M. edulis*, and the Yesso scallop, *Patinopecten yessoensis* were selected as model species not only because of their ecological and economical significance but also because they inhabit distinctly different habitats which may shape differential growth responses. Furthermore, the sodium composition of the shells was analyzed with the aim of evaluating whether Na/Ca_{shell} holds the potential as a proxy of the acid-base and ionic regulation in the EPF. Specifically, it is expected that the amount of Na incorporated into the shells would increase with decreasing seawater pH when bivalves rely on the Na^+/H^+ exchanger to achieve the acid-base and ionic homeostasis. Finally, we demonstrated that an integrated approach of combining Na/Ca_{shell} with the examination of the rate of shell deposition may help decipher the mechanisms by which bivalves respond to current and ongoing ocean acidification and warming.

2. Materials and methods

2.1. Experimental setup

Specimens of the blue mussel, *Mytilus edulis* (24–32 mm shell length) were collected by hand from the rocky intertidal shore of Xinghai Bay (38°52′34.51″ N, 121°33′42.47″ E), Yellow Sea, China, while Yesso scallops, *Patinopecten yessoensis* (41–45 mm shell length) were collected from the shallow subtidal zone near Zhangzi Island (39°01′58.46″ N, 122°44′22.48″ E). The monthly average seawater temperature and pH provided by marine stations (Dalian Oceanic and Fishery Administration) nearest to the sample localities vary between 3.4 °C and 24.6 °C and 7.91 and 8.09 at Xinghai Bay, and between 4.6 °C and 23.2 °C and 8.04 and 8.13 at Zhangzi Island, respectively. Upon arrival in the laboratory, the bivalves were kept in 500 L tanks supplied with circulating water for two weeks and were fed daily with an equal mixture of microalgae, *Platymonas subcordiformis* and *Nitzschia closterium*. Water temperature, salinity, pH_T (total scale) and dissolved oxygen were maintained constant at 16 °C, 31, 8.0–8.1 and 7–8 mg/L.

To evaluate the influence of seawater pH and temperature, six separate, but identical, recirculating systems were established, as schematically illustrated by Xu et al. (2016). Each system comprised two exposure chambers (i.e. each species per chamber), a filter chamber, a temperature controlling chamber, and a gas mixing chamber and each held a total volume of ca. 480 L seawater. After acclimation to laboratory conditions, specimens of each species were randomized into six groups and assigned into each system at a density of 20 individuals per chamber. Afterwards, the bivalves were exposed to six combined treatments with different seawater pH (7.4, 7.7 and 8.1) and different temperature (16 and 22 °C) regimes. No replicate systems were performed for each treatment in the present experiment. The acidified conditions mimicked near-future climate change scenarios, i.e., pH 7.7 projected for the year 2100 and 7.4 projected for 2300 (Caldeira and Wickett, 2003). The temperature of 16 °C represents the annual mean sea surface temperature in the Yellow Sea (Lin et al., 2005), and 22 °C corresponds to an additional warming of ca. 5.1 °C projected for the end of the 21st century (Sokolov et al., 2009). During the subsequent two weeks, they were acclimated to the experimental conditions. No mortality was observed.

To identify newly formed shell portions during the experiment, bivalves were exposed to a 150 mg/L calcein solution for 3 h (Thébault et al., 2006). Calcein is a fluorescent dye which can be incorporated into biogenic calcium carbonate and fluoresce under blue light (Kaehler and McQuaid, 1999). Afterwards, bivalves were reared under controlled conditions for five weeks. Throughout the duration of the experiment, 50% of the tank water was renewed every two days to maintain water quality. To minimize potential influence of high phytoplankton biomass on seawater carbonate chemistry, bivalves were fed every two days with an equal mixture of *P. subcordiformis* and *N. closterium* at a concentration of ca. 40,000 cells/mL.

2.2. Chemical analysis of the water samples

Temperature and pH were monitored every day. Water samples for chemical analysis were taken on a weekly basis. Total alkalinity (TA) was determined by means of an alkalinity titrator. Water temperature, salinity, pH and TA were used to calculate the carbonate system parameters using the CO2SYS software program (Pierrot et al., 2006). A detailed overview on experimental conditions is provided in Table 1. The concentration of Ca^{2+} and Na^+ of the water samples were determined by means of a Thermo Electron IRIS Intrepid II XSP inductively coupled plasma – atomic emission spectrometer (ICP-AES). Analytical precision was better than 1% (1RSD) for Ca^{2+} and Na^+. Accuracy which was determined by a multi-element standard solution (National Research Center for Certified References Materials, China; GNM-M25748-2013) and was better than 5% for all elements.

2.3. Shell preparation and analysis

Five specimens of each species from each treatment were randomly selected for analysis. Soft tissues were removed immediately after collection. All specimens were carefully cleaned with tap water and then air-dried. The left valve of each specimen was mounted on a Plexiglass