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Non-additive effects of ocean acidification in combination with warming on the larval proteome of the Pacific oyster, *Crassostrea gigas*

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ABSTRACT

Increasing atmospheric carbon dioxide results in ocean acidification and warming, significantly impacting marine invertebrate larvae development. We investigated how ocean acidification in combination with warming affected D-veliger larvae of the Pacific oyster *Crassostrea gigas*. Larvae were reared for 40 h under either control (pH 8.1, 20 °C), acidified (pH 7.9, 20 °C), warm (pH 8.1, 22 °C) or warm acidified (pH 7.9, 22 °C) conditions. Larvae in acidified conditions were significantly smaller than in the control, but warm acidified conditions mitigated negative effects on size, and increased calcification. A proteomic approach employing two-dimensional electrophoresis (2-DE) was used to quantify proteins and relate their abundance to phenotypic traits. In total 12 differentially abundant spots were identified by nano-liquid chromatography-tandem mass spectrometry. These proteins had roles in metabolism, intra- and extra-cellular matrix formations, stress response, and as molecular chaperones. Seven spots responded to reduced pH, four to increased temperature, and six to acidification and warming. Reduced abundance of proteins such as ATP synthase and GAPDH, and increased abundance of superoxide dismutase, occurred when both pH and temperature changes were imposed, suggesting altered metabolism and enhanced oxidative stress. These results identify key proteins that may be involved in the acclimation of *C. gigas* larvae to ocean acidification and warming.

Significance: Increasing atmospheric CO_2 raises sea surface temperatures and results in ocean acidification, two climatic variables known to impact marine organisms. Larvae of calcifying species may be particularly at risk to such changing environmental conditions. The Pacific oyster *Crassostrea gigas* is ecologically and commercially important, and understanding its ability to acclimate to climate change will help to predict how aquaculture of this species is likely to be impacted. Modest, yet realistic changes in pH and/or temperature may be more informative of how populations will respond to contemporary climate change. We showed that concurrent acidification and warming mitigates the negative effects of pH alone on size of larvae, but proteomic analysis reveals altered patterns of metabolism and an increase in oxidative stress suggesting non-additive effects of the interaction between pH and temperature on protein abundance. Thus, even small changes in climate may influence development, with potential consequences later in life.

1. Introduction

Today's oceans are undergoing widespread alteration as a result of the changing global climate [1]. Elevated atmospheric CO_2 leads to increasing sea surface temperatures [2] and acidification of marine environments [3,4], with the oceans acting as a sink for more than a third of all anthropogenic carbon emissions [5]. End-of-the-century projections for atmospheric pCO_2 range from 600 to 1000 ppm (corresponding roughly to a decrease of 0.2–0.5 pH units) and an increase in sea surface temperatures of 0.6 °C to 2.0 °C, depending on the scenario adopted [1]. A direct consequence of this ocean acidification (OA) will be a reduction in the saturation state for carbonate ions [6], which will make the calcification mechanisms employed by many marine organisms more energetically costly [7], potentially putting them at a disadvantage compared to organisms that do not produce calcified structures [8]. Concurrent warming and acidification can result in synergistic negative effects on calcification [9] and other biological processes, although responses are likely to vary between taxa.

Larval stages of molluscs may be particularly at risk of acidification in warming marine environments [10], as their shells are extremely fragile [11], and the high rate of biocalcification required to

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form early skeletal structures makes them highly sensitive to reductions in the saturation state of carbonate ions [12]. The effects of changing environmental conditions on larvae are important to understand as these early life stages often represent a bottleneck for populations struggling to survive in the changing marine environment [13,14]. Reductions of 0.5 pH units often results in reduced survival of marine molluscs, as shown for *Mercenaria mercenaria*, *Argopecten irradians* [15] and *Crassostrea gigas* [16], while a pH decrease of 0.7 units can lead to abnormal development and high levels of mortality, as shown in *C. gigas* [17] and *Mytilus galloprovincialis* [18]. Increasing temperatures compound problems caused by acidification, increasing mortality and rates of abnormality and reducing size when both are considered concurrently, as shown in both the Sydney rock oyster *Saccostrea glomerata* [19] and *C. gigas* [20] during the first 48 h of development.

Prolonged exposure to pH reductions of 05–0.7 pH units will likely elicit strong stress responses in many organisms (for a review, see [21]), but reductions in pH by 0.2–0.3 pH units (corresponding to future pCO_2 levels of 600–750 ppm) are considered more useful for understanding how organisms will respond to future changes in pH, according to the European Commission's *Guide for best practices for ocean acidification research* [22]. Although populations will typically have many generations to adapt to these average changes in pH and temperature [23], coastal ecosystems also demonstrate temporal and regional variations in pH and temperature meaning many populations already face conditions predicted for the end-of-the-century at certain points in time or in parts of their range [24]. Therefore identifying how organisms acclimate to these changes is a crucial step in understanding how populations might respond to global climate change. Even small changes in pH can influence growth and development: a reduction of 0.2 pH units resulted in significantly slower growth in both *C. gigas* and the Sydney rock oyster *S. glomerata* during the first 48 h of development [20], while a reduction of 0.3 pH units reduced growth and calcification in *C. gigas* during the first three days of development [25]. On the other hand, small increases in temperature may result in faster rates of growth and development, offsetting negative effects of acidification [26–28]. Differences in growth and the timing of development may have important consequences for population dynamics and demography [29], and developmental plasticity is a key source of variation in adult phenotypes, directly influencing evolution of populations and species [30,31]. For these reasons, investigation of smaller changes in pH over single generations may provide a more realistic indication of how organisms will respond to ocean acidification.

As well as directly investigating the effects of environmental change on the developmental plasticity of phenotypes, it is now possible to use molecular '-omics' approaches to reveal the molecular signals underlying the response of development traits to small changes in pH and temperature, even when there are few outward signs of phenotypic change [32]. Proteomic techniques such as two-dimensional gel electrophoresis (2-DE) can help to identify the mechanisms underlying developmental plasticity [33] without a priori expectations. Identification of differentially accumulated proteins highlights potential candidates involved in acclimatory or adaptive responses. In this study we characterize the effects of acidification and warming, both separately and together, on protein abundance in larvae of the Pacific oyster *C. gigas* 40 h post-fertilization, at which point the vast majority of larvae have reached veliger stage and are calcifying [25]. *C. gigas* is a bivalve of commercial significance and is also an important ecosystem engineer [34]. The 2-DE methodology presented has already been validated in organs [35,36] as well as in oocytes of this species [37], and is used here to identify major changes in abundant and soluble larval proteins in response to near future change in pH and temperature, in order to identify key proteins involved in developmentally plastic phenotypes.

2. Materials and methods

2.1. Conditioning of adults and larval rearing

Thirty mature *C. gigas* aged two years were transferred to the Ifremer laboratory in Plouzané, France, in July 2013 and were kept, unfed, in constantly flowing filtered sterile sea water (FSSW) from the bay of Brest at 19.7 ± 0.3 °C and pH 8.07 ± 0.04 units (reflective of natural conditions for the time of year) for 5 weeks prior to the spawning. Sex was determined under a light microscope and fertilisation was carried out with oocytes from five females combined and fertilized with the sperm from four males, and following a standard protocol [38]. Oocytes and sperm were periodically mixed, and after 90 min eggs were split equally among 12 open-flow 5 L tanks in one of four treatments (3 replicates per treatment): 'control' (median temperature and median pH ± upper/lower bound = 19.9 ± 0.4 °C, pH 8.06 ± 0.06), 'acidified' (19.8 ± 0.3 °C, pH 7.87 ± 0.05), 'warm' (21.7 ± 0.5 °C, pH 8.09 ± 0.05) and 'warm acidified' (21.8 ± 0.5 °C, pH 7.87 ± 0.03). To create these conditions, a large header tank (100 L) was filled with filtered and UV-sterilized sea water that had been warmed to 22 °C. This water was then pumped directly to the three 'warm' treatment tanks and to two other header tanks (50 L), one in which water was cooled to 20 °C via heat exchange, the other in which pH was reduced by bubbling CO_2 through a CO_2 reactor (JBL GmbH & Co. KG, Neuhofen, Germany), maintained by negative feedback. These header tanks pumped water to the three 'control' and three 'warm acidified' treatment tanks respectively. Finally, water from the 'warm acidified' header tank was also pumped to a fourth header tank (50 L) where the water was cooled to 20 °C by a second heat exchange, before being pumped to the three 'acidified' treatment tanks. Flow rate to experimental tanks was maintained at 80 ml min^{-1}, and the outflow passed through a 20 μm mesh filter to keep larvae in tanks. Larvae were kept in suspension and free from outflow filters by constantly bubbling air from the bottom of the tanks over the filters. An overview of the installation is shown in Supplementary Fig. 1. Temperature and pH in each treatment tank were measured at four times during the 40-hour experiment using a WTW pH 340i fitted with a WTW SenTix 41 pH electrode (WTW GmbH, Weilheim, Germany); total alkalinity was determined from bicarbonate titration of triplicated samples collected at three times from a large header tank (analyses performed by Labocea laboratories, Brest, France); and salinity measurements were taken before and after the experiment using a WTW LF 340-B conductivity salinometer (WTW GmbH, Weilheim, Germany). Values for dissolved inorganic carbon, partial pressure of carbon dioxide, concentrations of bicarbonate and carbonate ions, and saturation states of calcite and aragonite were calculated using the CO_2SYS program [39] based on constants from Cai and Wang [40] fitted to the Total pH scale; mean conditions during the experiment are presented in Supplementary Table 1.

Larvae were allowed to develop for 40 h post-fertilization before counts and sampling, because preliminary experiments showed that in all treatments, only D-larvae were present in experimental tanks at this time. For morphological measurements, approximately 10,000 individuals were collected by passing FSSW through a 40 μm mesh, and aspirating the larvae into a tube containing 1 ml of absolute ethanol:phosphate buffered saline (PBS) solution 1:1 (v/v). After four days at 4 °C, samples were transferred to a 70% ethanol solution and stored at 4 °C. For protein analysis, approximately 4 L of water from each tank were passed through a 40 μm mesh, and the remaining larvae (mean ± SE estimated at 160,000 ± 16,000 per tank) were recovered into 2 ml tubes with the minimal amount of sea water possible (<1 ml) to minimize salting-out of proteins during lysis [37]. 1 ml of lysis buffer [41] was added to the tube and samples were snap frozen in liquid nitrogen and stored at −80 °C until extraction.