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ORIGINAL PAPER

Elevated CO_2 alters larval proteome and its phosphorylation status in the commercial oyster, *Crassostrea hongkongensis*

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Abstract Ocean acidification (OA) is beginning to have noticeable negative impact on calcification rate, shell structure and physiological energy budgeting of several marine organisms; these alter the growth of many economically important shellfish including oysters. Early life stages of oysters may be particularly vulnerable to OA-driven low pH conditions because their shell is made up of the highly soluble form of calcium carbonate ($CaCO_3$) mineral, aragonite. Our long-term CO_2 perturbation experiment showed that larval shell growth rate of the oyster species *Crassostrea hongkongensis* was significantly reduced at pH < 7.9 compared to the control (8.2). To gain new insights into the underlying mechanisms of low-pH-induced delays in larval growth, we have examined the effect of pH on the protein expression pattern, including protein phosphorylation status at the pediveliger larval stage. Using two-dimensional electrophoresis and mass spectrometry, we demonstrated that the larval proteome was significantly altered by the two low pH treatments (7.9 and 7.6) compared to the control pH (8.2). Generally, the number of expressed proteins and their phosphorylation level decreased with low pH. Proteins involved in larval energy metabolism and calcification appeared to be down-regulated in response to low pH, whereas cell motility and production of cytoskeletal proteins were increased. This study on larval growth coupled with proteome change is the first step toward the search for novel Protein Expression Signatures indicative of low pH, which may help in understanding the mechanisms involved in low pH tolerance.

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Introduction

Many benthic marine invertebrates produce pelagic larval stages that are specialized and adapted for dispersal and habitat selection. At the end of pelagic development, larvae attain competence to attach on hard substrata and metamorphose into adults. The time to acquire competence is dependent on larval history and environmental variables such as temperature, pH, salinity and food availability (Marshall and Morgan 2011). Oysters follow this general phenomenon (Tamburri et al. 1992; O'Connor et al. 2007; Rico-Villa et al. 2009, 2010). After reaching competence, larvae can delay metamorphosis until they find a suitable habitat and favorable environmental condition (Pechenik 1999). However, prolonged pelagic phases or delayed metamorphosis not only requires increased investment of energy resources (Mos et al. 2011) but also exposes larvae to predators longer than usual, which ultimately leads to decreased metamorphic success and juvenile survival (Thiyagarajan et al. 2007; Onitsuka et al. 2010). In the near future, larvae may be forced to take longer time to reach competence and to delay metamorphosis because of anthropogenically induced ocean acidification (OA) process (Dupont and Thorndyke 2009; Gibson et al. 2011; Van Colen et al. 2012). Average surface seawater pH has already decreased from 8.2 to 8.1 due to the absorption of anthropogenic CO_2 since the start of the industrial revolution (Feely et al. 2009). As CO_2 dissolves in seawater, it

increases the amount of hydrogen ions (H^+), which effectively reduces the carbonate ion concentration and saturation levels of calcium carbonate ($CaCO_3$) minerals used by calcifying larvae to build shells. Among marine invertebrate larval forms, oyster larvae are highly vulnerable to decreased pH and OA process because they may depend on seawater carbonate ion concentration and the saturation states of $CaCO_3$ minerals to produce and maintain their shells. Moreover, oyster larval shells are made up of aragonite (Weiss et al. 2002) which is about 50 % more soluble than the adult calcite shells (Guinotte and Fabry 2008; Miller et al. 2009).

Being an estuarine and intertidal organism, oysters have developed physiological plasticity to cope with harsh and variable environmental conditions including low pH (Melzner et al. 2009; Amaral et al. 2011). For example, larvae of the Suminoe oyster and the Portuguese oyster were able to cope with and metamorphose normally in low pH (~7.8 and 7.5, respectively) (Miller et al. 2009; Thiyagarajan and Ko 2012). On the other hand, numerous oyster larval species showed developmental delay, decreased calcification and altered shell ultrastructure in response to low pH (Gazeau et al. 2007; Kurihara et al. 2007; Talmage and Gobler 2009; Watson et al. 2009; Gazeau et al. 2010; Parker et al. 2010; Welladsen et al. 2010; Tomanek 2011; Dineshram et al. 2012; Timmins-Schiffman et al. 2012). In some of these oysters, delayed larval growth resulted in poor recruitment (Parker et al. 2010; Barton et al. 2012). Therefore, it is intriguing to understand how larvae of different commercially important oyster species will cope with low pH, which is essential for coastal zone management, aquaculture and prediction of coastal climate change impacts (Miller et al. 2009; Talmage and Gobler 2010; Byrne 2011). One important oyster species that needs urgent attention is the subtropical white meat oyster, *Crassostrea hongkongensis*. This species has been under intensive cultivation in southern China for many years (Lam and Morton 2004). We hypothesize that low pH would significantly decrease their larval shell growth rate and lengthen the time to reach the metamorphosis or pediveliger stage in *C. hongkongensis*.

Despite larval feeding history including delayed growth and calcification, once larvae reached the competence stage (e.g., pediveliger stage in case of oysters), metamorphosis is accomplished. It is during this critical period, larvae are expected to display remarkable proteome plasticity and differential expression of genes to accomplish the daunting task of metamorphosis (Thiyagarajan 2010; Huan et al. 2012). Larvae spending extra resources to cope with low pH will likely display differentially expressed proteome either at total expressed protein level or at post translational modification (PTM) level. This hypothesis will be tested in this study. Phosphorylation is one of the key reversible PTMs that regulate enzymatic activity, subcellular localization, complex formation and degradation of proteins (Mumby and Brekken 2005). Most importantly, PTM has been implicated in signal transduction pathway and metamorphosis in several larval species (Chandramouli et al. 2011). It appears that the study on the global proteome and changes in protein phosphorylation status in response to environmental stress might provide new insights to understand the molecular basis of acclimation and tolerance to low pH (Tomanek 2011).

Recent studies on gene expression and transcriptome shed new light on the mechanisms of pH acclimation in marine invertebrate larvae. In several sea urchin larval species (*Lytechinus pictus* and *Strongylocentrotus purpuratus*), calcification- and energy metabolism–related genes were significantly down-regulated in response to low pH (Todgham and Hofmann 2009; O'Donnell et al. 2010; Stumpp et al. 2011). On the other hand, the up-regulation of developmental and calcification-related genes showed molecular plasticity in the sea urchin (*Paracentrotus lividus*) larvae, potentially helping them to develop and calcify normally in low pH conditions, despite a delay in development (Martin et al. 2011). Similarly, a significant change in gene expression pattern and molecular plasticity in response to low pH was found in coral *Acropora millepora* (Kaniewska et al. 2012). Since most plasticity and acclimation responses to physiological processes are executed by proteins rather than gene expression (mRNA), information on global protein expression pattern is necessary to gain new insights into mechanisms underlying performance at low pH (Hofmann et al. 2008).

Proteomic technologies such as two-dimensional electrophoresis (2-DE)-based analysis have proven to be helpful in identifying a large number of proteins whose expression changes under low pH conditions, especially when using non-model species (Tomanek 2011; Tomanek et al. 2011; Wong et al. 2011; Dineshram et al. 2012; Sun et al. 2012). Using similar 2-DE approach, we have investigated how the proteome changes when larval oysters (*C. hongkongensis*) were exposed to elevated CO_2 and low pH using comparative proteomic analysis by 2-DE and MALDI-TOF/TOF MS. The main purpose of this study was to analyze whether larval performance at low seawater pH levels is correlated to large protein changes, possibly as a compensatory response.

Materials and methods

Experimental facilities

This experiment was conducted in an oyster hatchery cum research station (South China Sea Institute of Oceonology,

Chinese Academy of Sciences, China) located near Zhanjiang (Guangdong province, China) between July and August 2010 using the Hong Kong's native commercial oyster, *Crassostrea hongkongensis*. Adult *C. hongkongensis* brood stocks were obtained from an oyster farm population in Zhanjiang, China. Seawater pumped from the nearby estuary was treated with 3–5 ppm of EDTA to remove heavy metals in a seawater storage tank. This natural seawater was diluted to 15 ppt (salinity) with groundwater to meet the typical estuarine conditions used in the experiment. After 2 weeks of acclimation and conditioning at 29 °C with ambient pH (NBS) 8.2 ± 0.2 in the brood stock tank, sperm and eggs were obtained by "strip spawning" from two males and eight females aged >2 years and of 10–12 cm shell lengths. Fertilization was carried out by mixing sperm and egg following a ratio of 5:1 (Havenhand and Schlegel 2009). More than 90 % of fertilization success was observed after 30 min. Embryos seen after fertilization were sieved out using 25-μm mesh to rinse excess sperm and cultured in 450-L black fiberglass-reinforced plastic tank for 24 h to obtain D-shaped trochophore larvae according to the hatchery procedure (Breese and Malouf 1975). Homogeneous batch cultures of D-shaped veliger larvae were used for the following CO_2 (pH) perturbation experiment.

Experimental design

Three pH treatments with six replicate cultures per treatment were used (18 culture tanks in total at pH 8.2, pH 7.9 or pH 7.6). Experimental pH values were selected based on EPOCA guidelines for ocean acidification research (Riebesell et al. 2010). Accordingly, ambient pH and carbonate system parameters used in the experimental station (oyster hatchery where this study was conducted) were used as baseline value and control, that is, pH 8.2. On this slightly elevated ambient pH control condition, the two low pH treatment conditions, pH 7.9 and pH 7.6, were selected to represent pCO_2 value projected for the year 2100 and 2300 (or beyond), 1,000 and 3,000 μatm, respectively (Zeebe et al. 2008). Each polypropylene culture tank had ~80 L of 1 μm filtered seawater at 15 ppt salinity. The required CO_2 concentration in the bubbled air was obtained by mixing 99.9 % CO_2 and outdoor natural air using gas flow rotameters (Cole-Parmer, USA). The required pH levels were obtained by constantly bubbling CO_2-enriched ambient air directly into culture tanks and routinely checked using a Mettler-Toledo pH meter. Culture tanks were kept at room temperature (~29 °C). Salinity, temperature, pH (NBS scale) and total alkalinity were measured once every 4 days. However, the pH was monitored twice or thrice per day to maintain treatment conditions. Total alkalinity was measured in triplicate using potentiometric titration and Gran plot method (Dickson 1981) and calibrated against seawater reference materials (Batch 103, A.G. Dickson, Scripps Institution of Oceanography). The groundwater used to reduce the salinity had high level of hardness due to limestone-rich soil. Therefore, the diluted (salinity 15 ppt) seawater used in this experiment had relatively high total alkalinity (3,000 μmol kg^{-1}). Carbonate system parameters such as pCO_2 and $CaCO_3$ mineral saturation states ($\Omega_{aragonite}$ and $\Omega_{calcite}$) were obtained using the co2sys.xls spreadsheet (Pelletier et al. 2005) and dissociation constants K_1 and K_2 for estuarine waters (Millero et al. 2006).

Aliquots of D-shaped larvae were randomly assigned (~10–15 larvae ml^{-1}) into each culture tanks. Larvae were maintained in these culture tanks until they developed a visible eyespot. During the entire culture period, larvae were fed ad libitum with an active (i.e., log growth phase) culture of mixed *Isochrysis galbana*, *Chaetoceros* sp. and *Chlorella* sp. microalgae, which were all isolated from local South China Sea coastal water. The final concentration of microalgae in the tank was 5–10 × 10^6 cells ml^{-1} (Rico-Villa et al. 2006). Once every 3–5 days, larvae were sampled and reintroduced into pH-adjusted seawater. During every seawater change (on Day 5, 10, 14, 20, 25, 29, 30 and 32 from post-fertilization), larvae from each tank were sampled and fixed in $CaCO_3$-buffered 10 % formalin for subsequent larval shell size (diameter) measurement using a digital camera mounted on a compound microscope with Image J® software (Schneider et al. 2012). Larval shell height was calculated from the measured distance between the umbo and the posterior edge of the shell. For each sample, 15–20 larvae were randomly measured per sample per replicate. Mean larval growth rates were obtained from the slopes of linearly fitted lines using the least squares regression analysis. We compared the growth rates among different pH treatments using analysis of covariance (ANCOVA) (Zar 1999).

Under these pH treatment and larval culture conditions, significant number (>25 % regardless of treatment conditions) of larvae developed to the pediveliger stage with eyespot on Day 29, 30 and 32 at pH 8.2 (control), pH 7.9 and pH 7.6, respectively. Pediveliger larvae with similar shell size, sieved out using 250-μm mesh, were washed twice with double-distilled water and then snap frozen in liquid nitrogen prior to −80 °C storage for proteomic analysis.

Two-dimensional electrophoresis (2-DE) procedure

Fixed samples were thawed in room temperature and then were immersed in the standard 2-DE sample buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 40 mM DTT, 2 % Bio-Lyte 3/10 ampholyte, 1X protease and 1X phosphatase inhibitor cocktail) to facilitate larval cell lysis and protein

extraction without significant interference from protease enzymes (Görg et al. 2004). Solubilization of larval proteins was enhanced through sonication for about 1-min continuous pulse, followed by 2 min on ice (Branson Sonifier 150). The sonication cycle was repeated six times. Solubilized proteins were separated by centrifugation (14,000 rpm for 20 min at 15 °C) and quantified by the modified Bradford method (Ramagli 1999).

Larval proteins solubilized in the lysis buffer were separated in two dimensions using the optimized larvae 2-DE protocol (Thiyagarajan and Qian 2008). Exactly 300 μg of proteins dissolved in the rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 40 mM DTT, 0.2 % Bio-Lyte, 3/10 ampholyte and 1 % bromophenol blue) was used for separation in two dimensions. For the separation according to charge, proteins were applied to 17-cm ReadyStrip IPG strips (Bio-rad), pH 3–10 (linear), using an overnight active rehydration procedure and then subjected to isoelectric focusing (IEF) using a Protean IEF Cell (BIO-RAD Laboratories Inc.). IEF focusing conditions were as follows: 250 V for 20 min and 1,000 V for 2.5 h with a gradient of 10,000 V for a total of 40,000 Vh. After IEF separation, the IPG strips were equilibrated for 20 min in equilibration buffer 1 (6 M urea, 2 % SDS, 0.05 M Tris–HCl (pH 8.8), 50 % glycerol and 2 % w/v 1,4-DTT) followed by 20 min in the equilibration buffer 2 (same as buffer 1 but had 2.5 % iodoacetamide instead of DTT). For the separation according to size, the equilibrated IPG strips were placed on the top of 12.5 % sodium dodecyl sulfate–polyacrylamide gels (18 cm × 18 cm). The gels were run with Tris–glycine–SDS (TGS) buffer for SDS–PAGE at 20 °C. Global expression pattern of phosphoproteins and total proteins in each 2-DE gels were visualized using two fluorescence stains, the Pro-Q Diamond phosphoprotein stain (Pro-Q DPS; Molecular Probes, Eugene, OR) for phosphoproteins and the Sypro ruby (Molecular probes) total protein stain (Stasyk et al. 2005). Gels were scanned and imaged using the fluorescent Molecular Imager FX (Bio-Rad Laboratories, Inc.). The fluorescent stained gels were additionally stained with colloidal Coomassie Brilliant Blue (CBB), called the Blue silver staining procedure (Candiano et al. 2004; Wang et al. 2007), to visualize the protein spots being picked for further MS analysis.

Larval proteome (2-DE gel) analysis

Protein spots on each gel images were comparatively analyzed using the PD Quest software (version 8.0; Bio-Rad Laboratories, Inc.). The software modeled protein spots using three-dimensional Gaussian distribution and determined the spot intensities after raw image correction and background subtraction. The software-detected spots and matches (within and between treatments) were verified by manual inspection. The reproducibility of the gels among the biological replicates was further checked by correlation coefficient using the scatter plot. After normalizing the spot intensities using total spot density values, spots were analyzed using both qualitative and quantitative methods in the PD Quest software.

Differentially expressed proteins were identified using the fold-change method as well as the Student's *t* test program in the PD Quest software, which compared the normalized spot intensities of the control replicate groups with the low pH treatment replicate groups. The differentially expressed proteins were then subjected to analysis of variance (Beranova-Giorgianni 2003) to detect significant differences among pH treatments. If ANOVA results were significant, Dunnett's test was used to compare the expression level of each protein between the pH treatment groups and the control pH group. Three-way Venn diagram was used to illustrate the total number of proteins and the number of differentially expressed proteins in each pH treatment. Principal component analysis (PCA) was used to estimate similarities in the global protein expression patterns among the pH groups. These multivariate PCA proteome analyses were based on the Euclidean distances calculated using log (X + 1)-transformed data subjected to 9,999 permutations using the Primer software according to the description by Meunier et al. (2005). In addition, proteome data were analyzed using a TMEV heat map using the normalized spot volumes of all the differentially expressed protein spots (Saeed et al. 2006).

Protein identification using MALDI-TOF/TOF mass spectrometer

Twenty abundant spots that were differentially expressed among the three pH treatments were identified using the mass spectrometry (Shevchenko et al. 1996). Excised protein spots were washed with double-distilled water (H_2O) and then with H_2O/acetonitrile (ACN) (1:1 v/v). Proteins in the gel spots were first rehydrated and then digested by adding 10 μl of 20 ng/μl sequencing grade trypsin (Promega) in 20 mM NH_4HCO_3 buffer. Extracted peptides were cleaned up with ZipTip (Millipore), and then their mass/charge ratio was analyzed using MALDI-TOF/TOF mass spectrometer (ABI 4800 Proteomics Analyzer, Applied Biosystems). PMF and MS/MS information obtained from the MALDI-MS analysis was used to identify proteins from the NCBI non-redundant database using the GPS Explorer algorithm, ver. 3.6 (Applied Biosystems), and MASCOT database ver. 2.2 (Matrix Science). The mass tolerance settings of ±75 ppm for PMF and ±0.2 Da for the MS/MS spectra were used for mass spectrum search. MASCOT scores larger than 90 and ion score of minimum one peptide greater than 50 were used to

determine the statistical significance ($p < 0.05$) of a PMF and MS/MS match.

Results

Measured and calculated carbonate chemistry parameters in the larval culture tanks are shown in Table 1. Total alkalinity values were statistically similar among the three pH treatments (Table 1) and represented normal commercial culturing conditions in the study area. Within each treatment condition, the average pH, temperature, salinity and carbonate system variables were constant and stable throughout the experiment with minor fluctuations (see Fig. 1). Importantly, the pH, carbonate ion concentration, pCO_2 and saturation state of $CaCO_3$ minerals (aragonite and calcite) were all significantly different among treatments. For example, the aragonite saturation state of seawater was below one in the pH treatment 7.6, which was the goal of the experiment. This relatively stable and well-controlled CO_2 perturbation system allowed us to study the pH effect on oyster larval growth and proteome profile.

Larval growth response to pH

The majority of larvae (~62 %) in the control pH group (pH 8.2) reached the competent pediveliger stage, with the visible eyespot and foot, 28 day postfertilization. However, only ~30 % of larvae in the pH 7.9 and 7.6 groups reached the competent pediveliger stage on Day 30 and 32, respectively (Fig. 2a). During the entire experimental period, we did not observe any pH-specific mortalities or abnormalities in larval growth. Regardless of pH, larval shell size increased linearly with age and time (culture day). However, pH significantly affected the mean specific growth rate (Fig. 2b). Analysis of covariance detected significant differences among slopes ($F_{2,97} = 3.88$, $p < 0.05$). Although there was an apparent delayed growth response to pH 7.6 (Fig. 2a, b), the mean shell size of the pediveliger larvae did not differ significantly among pH treatments ($F_{2,15} = 4.96$, $p > 0.05$). The pediveliger larvae with similar shell size (about 280 μm) were considered to be an ideal sample for the comparative proteomic analysis, although they were collected at different time during their development. These similar-size group samples with the same developmental stage were necessary for comparative proteome study without interference of co-variables such as delayed growth due to low pH effect (Pörtner et al. 2010; Martin et al. 2011; Stumpp et al. 2011).

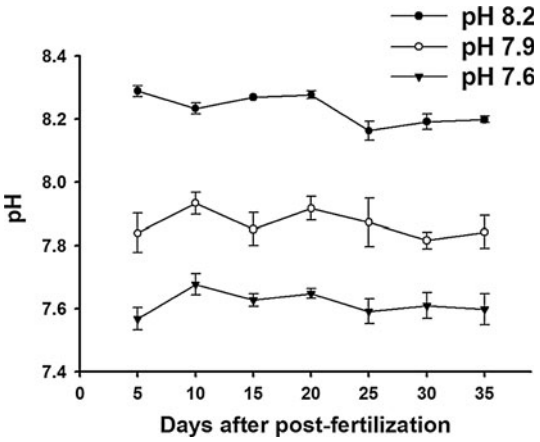

Fig. 1 Fluctuation of pH (NBS scale) during the experimental period. *Each bar* represents the mean ± SD of five or six replicate cultures

Larval proteome and phosphoproteome response to pH

The global expression pattern of oyster larval proteins at the end of the pediveliger stage with eyespot was resolved on 2-DE gels using 17-cm, pH 3–10, linear IPG (1st dimension) and 12 % SDS–PAGE (2nd dimension). A representative oyster larval proteome map, obtained from a control pH sample, is shown in Fig. 3. By using mass spectrometry-compatible Sypro Ruby staining for total protein, more than 350 distinctly separated proteins were detected with molecular weight (MW) ranging from 14 to 100 kDa and the pI values (isoelectric point) ranging from 4 to 9. Consequently, larval proteome (total as well as their phosphoproteome) response to pH was comparatively analyzed using 2-DE approach.

Table 1 Measured and calculated carbonate system components (mean ± SD, $n = 5$) in oyster larval culture tanks for the ambient (control) and the two low pH treatments at the time of sample collection for proteomic analysis (i.e., 29 day post-fertilization)

pH treatments	Calculated parameters				Measured parameters	
	$^{*}pCO_2$ (μatm)	$^{ns}HCO_3^-$ (μmol kg^{-1})	$^{*}CO_3^{2-}$ (μmol kg^{-1})*	$^{*}\Omega_{Ar}$	*pH (NBS)	^{ns}TA (μmol/kg^{-1})
8.2 (control)	657 ± 42	2,693 ± 203	228 ± 36	3.99 ± 0.68	8.22 ± 0.02	3,189 ± 270
7.9	1,697 ± 244	2,859 ± 269	100 ± 18	1.76 ± 0.34	7.87 ± 0.04	3,078 ± 302
7.6	3,581 ± 765	2,958 ± 300	50 ± 8	0.87 ± 0.15	7.60 ± 0.02	3,068 ± 311

Salinity was 15 ± 1 ppt and temperature was 29 ± 1 °C. Ω_{Ar}: aragonite saturation state, *TA* total alkalinity; * treatments differed significantly according to ANOVA and Tukey's test; *ns* no significant difference among pH treatments according to ANOVA

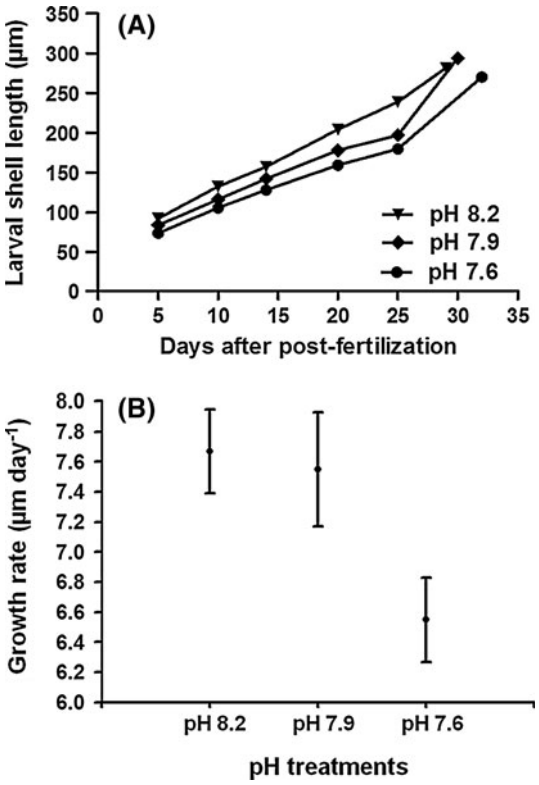

Fig. 2 **a** Relationship between larval shell size (µm) and culture day at three pH treatment conditions (pH 8.2, 7.9 and 7.6). In each pH treatment, larvae were cultured from D-shaped stage to pediveliger stage. There were 20–30 larval size measurements per replicate cultures. **b** Effects of pH on average daily larval shell growth rate (µm day^{-1}). *Each bar* represents the mean ± SD of five replicate cultures

Representative 2-DE images showing the proteomic response of oyster larvae to pH are shown in Fig. 4. Three randomly chosen replicate culture samples per pH treatment were analyzed. Plasticity of total proteome and phosphoproteome structure to pH was qualitatively and semiquantitatively examined using three biological replicates per pH treatment. The mean (of three replicates) number of total proteins, phosphoproteins and overlap of differentially expressed proteins among the three pH groups is shown using a Venn diagram analysis (Fig. 5). The number of expressed total proteins decreased in response to the two low pH levels (pH 7.9 and 7.6). Similarly, 20 phosphoproteins were changed in response to the two low pH treatments; however, only one phosphoprotein showing differential down-regulation was matched and identified with its total protein counterpart. Here, the differentially expressed proteins were identified using the fold-change and the Student's *t* test approach.

Additionally, multivariate statistical analyses such as correlation and principal component analysis (PCA) were used to compare global protein expression pattern in response to pH. The PCA plots showed that the total proteome of oyster larvae exposed to pH 8.2, 7.9 and 7.6 was distinctive. Total proteome structure of the control pH 8.2 group appears to be much closer to pH 7.9 (correlation coefficient = 0.78) than to pH 7.6 (correlation coefficient = 0.68). On a PCA plot, both total proteome (Fig. 6a) and phosphoproteome (Fig. 6b) of the two low pH treatment groups clustered far apart from the control pH group, confirming the significance of distinctive and unique proteome expression patterns under low pH. The two PCA components, PC1 and PC2, collectively accounted for 50 % of the variability in the data. For the total proteome dataset (Fig. 6a), PC1 separated the control and the two low pH treatments, but there was not much variability between the two low pH treatments. However, PC2 clearly separated the two low pH groups. For the phosphoprotein dataset (Fig. 6b), both PC components accounted for 70 % of variability and separated all the three pH samples far apart. Comparatively thus, there was a clear and unique low pH-responsive phosphoprotein expression pattern.

Differentially expressed proteins in response to pH

Based on this PCA analysis and visual examination of 2-DE images, a total of 20 abundantly and differentially expressed proteins had been subjected to further statistical analysis and also identified using MALDI-TOF/TOF mass spectrometry and MASCOT database search algorithm. Due to low protein sequence information, only 13 proteins were successfully identified with high confidence (see Table 2, protein spots were marked in Fig. 3). The expression pattern of these differentially expressed proteins was visualized and statistically analyzed using a heat map. The heat map presentation of a hierarchical cluster of the 13 proteins that showed significantly different (ANOVA results, $p \leq 0.05$) relative abundances between the pH treatment groups is shown in Fig. 7. The heat map broadly divided all the samples into two groups, the control pH 8.2 and the low pH groups subsequently split into pH 7.9 and pH 7.6 subgroups. Notably, all the three replicate samples within each pH treatment groups clustered together. Identification details of these 13 proteins are shown in Table 2. Some of these proteins were identified with high confidence owing to high PMF score and good tandem MS fragmentation, for example, calmodulin. Among these 13 differentially expressed proteins, seven proteins were found to be down-regulated in both pH 7.9 and 7.6 (Fig. 8).

The majority of these tentatively identified proteins were structural and cytoskeletal proteins (53 %), enzymes involved in energy metabolisms (27 %) and calcification-related proteins (7 %) (Fig. 9). Expression of the calcification-specific protein, the calmodulin (spot number 0005), was significantly decreased up to twofold in the pH 7.9 treatment, and its expression was slightly up-regulated at pH 7.6 (Fig. 8). Expression of energy-related proteins also followed a similar trend, that is, decreased in response to

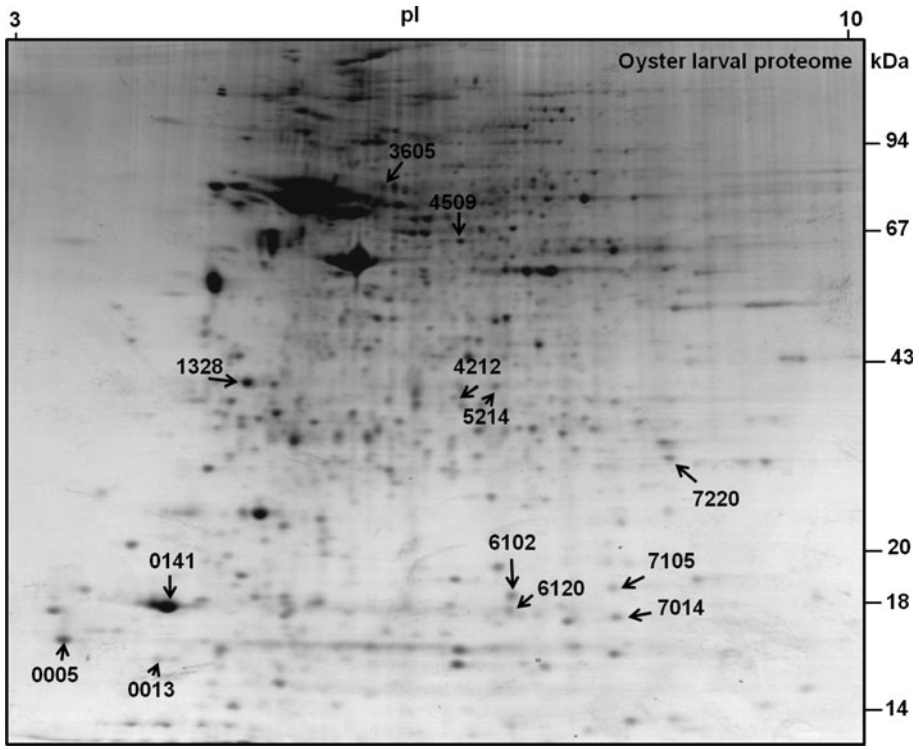

Fig. 3 Proteome map of the pediveliger larvae in the commercial oyster, *Crassostrea hongkongensis*. Larvae were cultured at ambient pH (8.2) at 15 ppt salinity and 28 °C. Larval proteins were separated by two-dimensional electrophoresis (2-DE). The first dimension was run on a pH 3–10 IPG strip (17 cm); the second dimension was run on a 12.5 % SDS–PAGE gel. Total amount of protein loaded was 300 µg. Separated proteins were visualized by staining gels with colloidal Coomassie Blue G-250 stain. Protein spots that were abundantly and differentially expressed due to pH treatment are indicated with *arrows* (one-way ANOVA, $p < 0.05$). These marked proteins were analyzed using MALDI-TOF/TOF as reported in Table 2

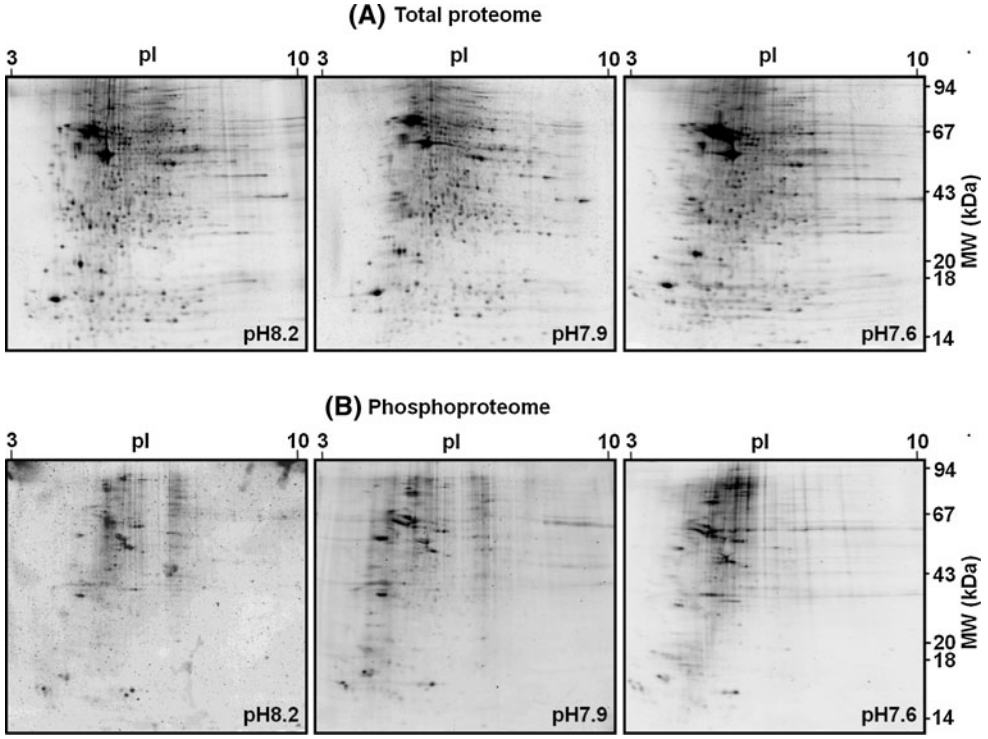

Fig. 4 Effects of pH on (**a**) total proteome and (**b**) phosphoproteome structure of the pediveliger larva in the commercial oyster, *Crassostrea hongkongensis*. Representative gel images of larval proteomes analyzed by two-dimensional gel electrophoresis (2-DE) are shown. The *upper panel* shows 2-DE gels stained with total protein stain, SYPRO® Ruby. *The lower panel* shows the images of the same 2-DE gel stained (after removing the SYPRO Ruby) with phosphoprotein-specific dye, Pro-Q Diamond

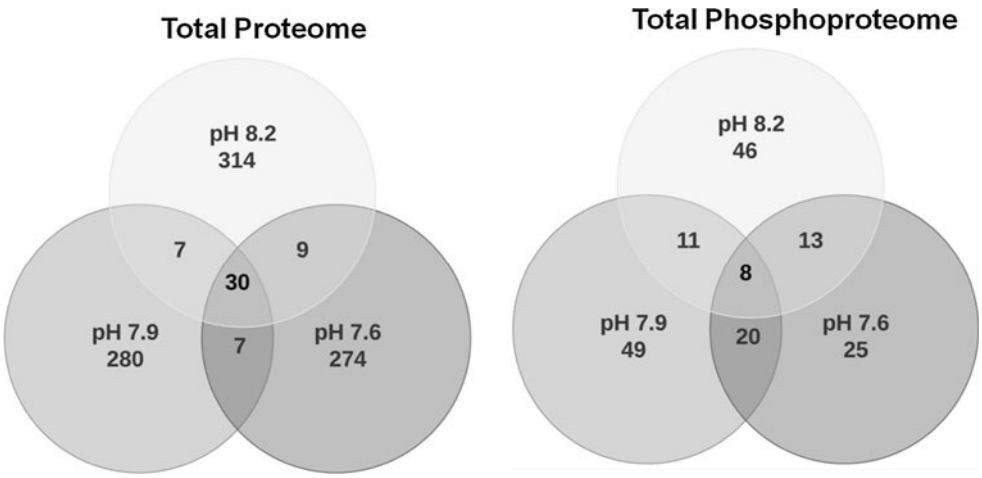

Fig. 5 Three-way Venn diagram showing the effect of pH on the total number of proteins and phosphoproteins apparent on the 2-DE proteome maps of the pediveliger larva in the commercial oyster, *Crassostrea hongkongensis*. The overlap of differentially expressed proteins among the three pH treatments was also shown in the diagram

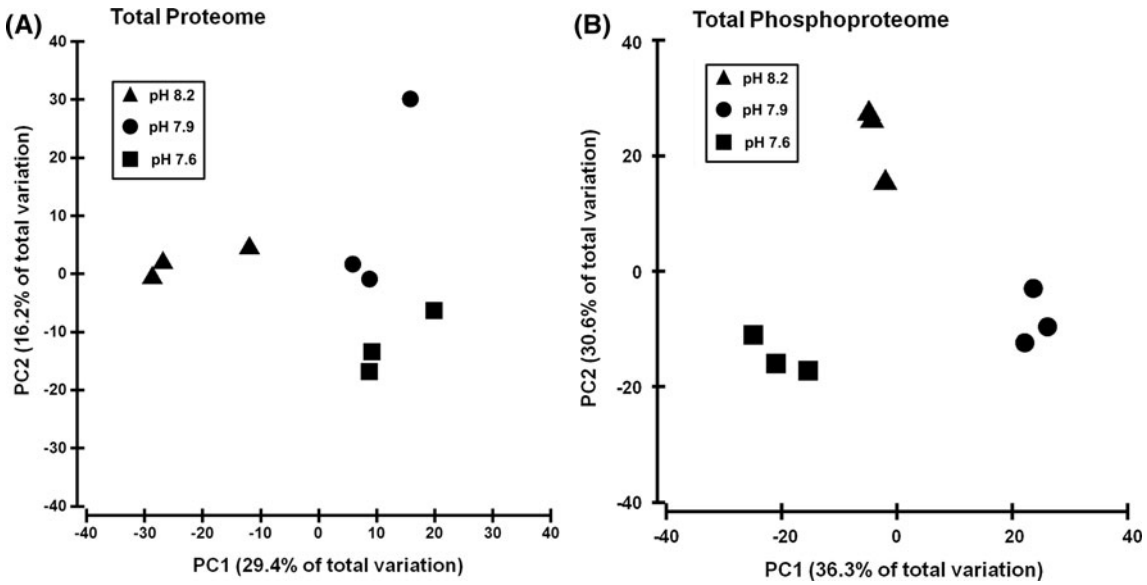

Fig. 6 **a**, **b** Principal component analysis (PCA) showing the effect of pH on proteome structure of the pediveliger larva in the commercial oyster, *Crassostrea hongkongensis*. PCA of total protein (**a**) and total phosphoprotein (**b**) confirmed the significance of proteome expression pattern among the pH treatments, and protein spots from the same treatment levels shared the same area on the plot. Euclidian distances summed over spots, and Ward's method for all protein spots in gels stained with Pro-Q Diamond and Sypro Ruby was used for PCA analysis

low pH. On the other hand, proteins related to structure, general metabolism and cytoskeleton were found to be differentially expressed in pH treatments showing either up- or down-regulation. The phosphorylation status of the 14-3-3 phosphoprotein was not significantly affected by pH; however, its total expression was significantly decreased in response to the two low pH treatments (Fig. 10).

Discussion

Larval growth response to pH

When exposed to low pH, the time to develop from D-shaped larvae to the competent pediveliger stage increased by 1 day at pH 7.9 and 2 days at pH 7.6 when compared to larvae in pH 8.2. In oysters, larval growth rate is positively correlated with CO_3^{2-} concentration but not with seawater pH or Ω_{arg}. For instance, the larval growth rate and calcium ion incorporation in the *C. gigas* larvae were significantly decreased only when the concentrations of CO_3^{2-} ions reached below 50 μmol kg^{-1} at pH 7.4 (Gazeau et al. 2011). Similarly, *C. hongkongensis* larvae showed a decreased mean specific growth rate when the concentrations of CO_3^{2-} ions reached below 50 μmol kg^{-1} at pH 7.6. Therefore, the seawater used in this study with slightly elevated total alkalinity may not have profound influence on the observed negligible effect of low pH on larval development. Furthermore, unlike previous studies (Kurihara 2008; Parker et al. 2009; Gazeau et al. 2011),

Table 2 Identification of selected proteins that are abundantly and differentially expressed in the pediveliger larvae of the oyster (*Crassostrea hongkongensis*) in response to control, pH 8.2, and the two low pH treatments, pH 7.9 and pH 7.6

Spot[a]	Putative identification[b]	Total protein score with e-value (C.I.)[c]	Peptide count/ Protein coverage[d]	MS/MS score with e-value (C.I.)[e]	MASCOT peptides identified by MS/MS[f]	Homology to protein (NCBI accession no. and species name)[g]	Theo. Mw/pI[h]	Expt. Mw/pI[i]
0005	Calmodulin	431 (100) 7.4×10^{-38}	6/45	136 (100) 86 (100) 162 (100)	VFDKDGNGFISAAELR EAFSLFDKDGDGTITTK EADLDGDGQVNYEEFVR	gil156915032 *Crassostrea gigas*	15.6/4.2	15.6/4.2
0013	Cytochrome c oxidase	190 (100)*	2/11	98 (100) 5.5×10^{-6} 91 (100) 4×10^{-5}	NNYAQFDGWELR NNNDYALAIR	EH646712 *Crassostrea virginica*	22.7/5.4	15.4/4.6
0141	Myosin essential light chain	354 (100) 3.7×10^{-30}	7/42	94 (100) 107 (100) 98 (100)	CVGLNPTLEIVR TFDREGQGYISGAELR LTDDQVDEIIR	gil40642994 *Crassostrea gigas*	18.2/4.5	18/4.5
1328	14-3-3 protein	296 (100)*	4/15	112 (100) 1.7×10^{-7} 85 (100) 9.5×10^{-5} 59 (100) 0.072 41 (100) 3.3	KLVENYKPLTNEER LVENYKPLTNEER QVIASAYR NANDSMAPTHPIR	HS111832 *Crassostrea gigas*	26.7/5.8	38/5.3
4212	ATPase	125 (100)*	3/13	37 (100) 8.3 52 (100) 0.14 37 (100) 10	IQSSNLLNQSR LSLISQQMIPELR TILYGANPNR	FQ660948 *Crassostrea gigas*	30.2/6.3	36/7.0
3605	Alpha tubulin	321 7.4×10^{-27}	9/26	81 (100) 144 (100) 43 (100)	AVFVDLEPTVVDEVR QLFHPEQLITGKEDAANNYAR NLDIERPTYTNLNR	gil115843650 *Strongylocentrotus purpuratus*	51.0/4.9	85/6.3
4509	Tektin	154 3.7×10^{-10}	14/33	44 (100) 15 (100)	TLEQTTEQIR TNRPNVELCRDPVQYR	gil94068377 *Saccostrea kegaki*	42.1/6.2	68/6.9
5214	Electron-transfer-flavoprotein, beta polypeptide	92 0.0006	7/17	69 (100)	HSMNPFDEIAVEEAVR	gil72040095 *Strongylocentrotus purpuratus*	49.1/5.5	35/7.7

Table 2 continued

Spot[a]	Putative identification[b]	Total protein score with e-value (C.I.)[c]	Peptide count/ Protein coverage[d]	MS/MS score with e-value (C.I.)[e]	MASCOT peptides identified by MS/MS[f]	Homology to protein (NCBI accession no. and species name)[g]	Theo. Mw/pI[h]	Expt. Mw/pI[i]
6102	Transgelin	238 (100)*	4/12	50 (100)	SICNYLR	HS165544	29.0/8.7	19/7.8
				0.67	QEGRNPVQFQK	*Crassostrea gigas*		
				26 (100)	EFEFQSGDLWEVR			
				1.1×10^{2}	EFEFQSGDLWEVRK			
				107 (100)				
				6.2×10^{-7}				
				55 (100)				
				0.09				
6120	Histone 3	238 (100)*	1/4	77 (100)	EIAQDFKTDLR	FY000232	27.4/10.4	17.5/7.8
				2.6×10^{-5}		*Bombyx mori*		
7014	Universal stress protein	337 (100)*	3/12	86 (100)	SIGGSPGEVICQVAK	HS171908	24.6/9.6	17.5/8.9
				9.4×10^{-5}	SIGGSPGEVICQVAK DENAQLIVTGTR	*Crassostrea gigas*		
				34 (100)				
				3.8×10^{-10}	DENAQLIVTGTR			
				117 (100)				
				3.3×10^{-8}				
7105	Transgelin	134 (100) *	3/10	38 (100)	SICNYLR	HS165544	29.0/8.7	18/8.9
				1.9×10^{-8}	SICNYLRDGVALCK	*Crassostrea gigas*		
				65 (100)	QEGRNPVQFQK			
				30 (100)				
				5.9×10^{-27}				
7220	Rieske apoprotein	337 (100)	3/45	67 (100)	AEQAVDLLSLR	gil260424007	28.8/9.3	28/9.4
		5.9×10^{-27}		95 (100)	KGPAPLNLTVAPHK	*Crassostrea gigas*		
				62 (100)	AEQAVDLLSLRDPQHDSDR			

Identified spots were marked on the proteome image in Fig. 3

* The protein is identified by the NCBI EST invertebrate database

[a] The assigned spot number

[b] Name of the putatively identified protein

[c] The in-house MASCOT total protein score obtained through searching against the NCBI non-redundant and NCBI EST invertebrate database (MASCOT score >69 is considered significant)

[d] Number of matched peptides and protein coverage

[e] The ion score(s) of the peptide(s) identified by MS/MS with their corresponding expectation value; ion scores of minimum one peptide >50 ($p < 0.05$) are considered significant

[f] The peptide(s) identified by MS/MS

[g] GenBank accession number

[h] The theoretical MW and pI value of the matched protein

[i] The experimental MW and pI values estimated from the 2D gel pattern

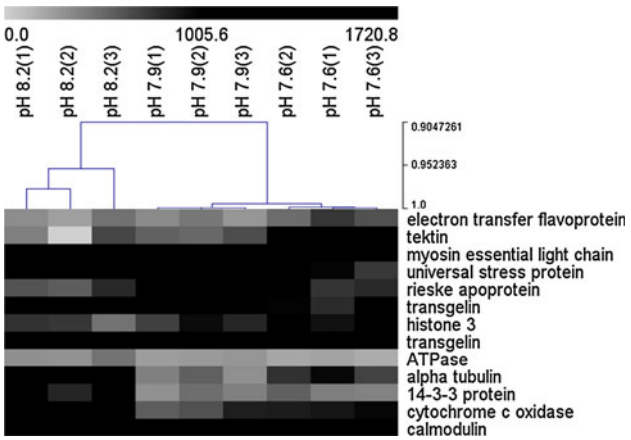

Fig. 7 Heat map of hierarchical clustering with TIGR MeV software identified expression patterns of proteins displaying up- or down-regulation. Clustering of treatments using Pearson's correlation coefficient revealed that control treatments remained distinct from the two low pH treatments. The expression pattern using color density graded from *black* to *white* based on normalized spot intensity values

fertilization success and trochophore development to low pH was not examined in this study. Contrarily, only matured trochophore stage or D-shaped larval stage was exposed to low pH. According to our results, D-shaped larval forms and subsequent advanced developmental stages of *C. hongkongensis* appear to be tolerant to the CO_3^{2-} concentration >50 μmol kg^{-1}. Oyster larval response to pH is highly dependent on life stages, species and population (Parker et al. 2011, 2012; Thiyagarajan and Ko 2012).

2-DE proteomics and oyster larval proteome

For the past few years, the 2-DE-based proteomics approach has been routinely used in larval biology research to visualize the majority of soluble proteins under different experimental conditions and developmental periods in a variety of non-model marine invertebrates (Thiyagarajan 2010; Wong et al. 2011; Huan et al. 2012; Slattery et al. 2012). The 2-DE-based proteomics is simple to use, highly reproducible and robust (Chich et al. 2007). However, this traditional proteomics approach has several limitations, for example, low solubility of membrane and hydrophobic proteins in 2-DE lysis buffer, low detection sensitivity and narrow dynamic range (Beranova-Giorgianni 2003; Görg et al. 2004; López 2007). Nevertheless, a large number of studies have now shown that this 2-DE approach can provide novel information about proteins involved in different larval stages, for example, those vital for larval metamorphosis and those that are responsible for stress response (Thiyagarajan 2010; Chandramouli et al. 2011). This method is especially powerful when testing hypotheses about proteome changes in response to stressors, including pH (Tomanek 2011), pollutants (Amaral et al.

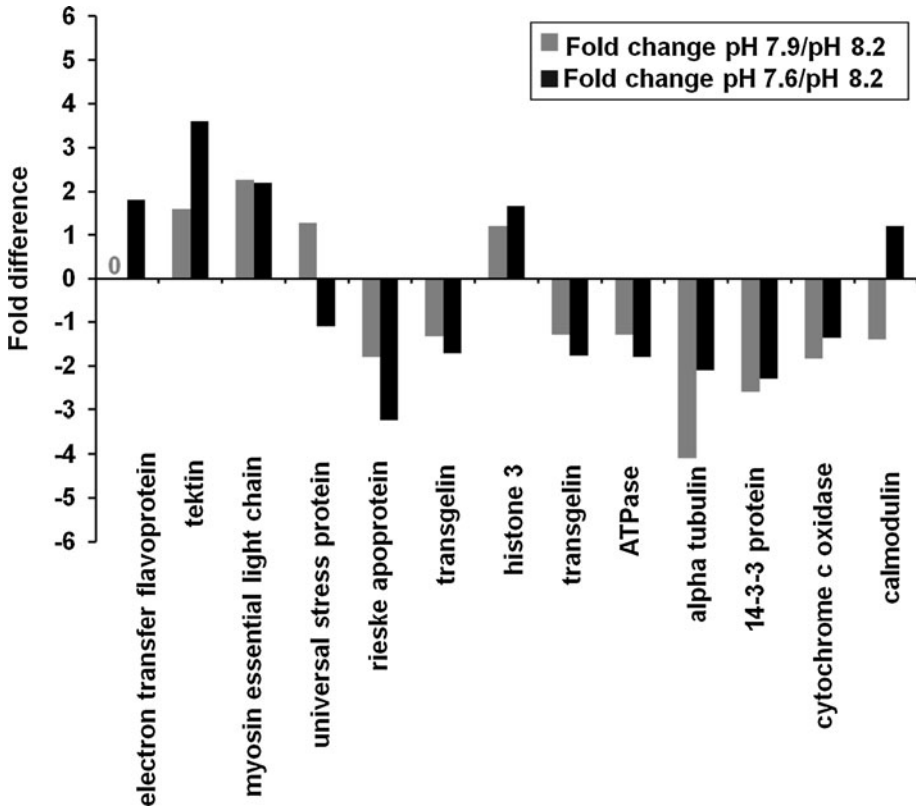

Fig. 8 Relative changes (fold change in relation to the control pH 8.2) in the expression level of 13 differentially expressed proteins in response to the two low pH treatments (pH 7.9 and 7.6) in the pediveliger larva of the commercial oyster, *Crassostrea hongkongensis*. Details of protein identification are given in Table 2, and spot location on the proteome map is shown in Fig. 3

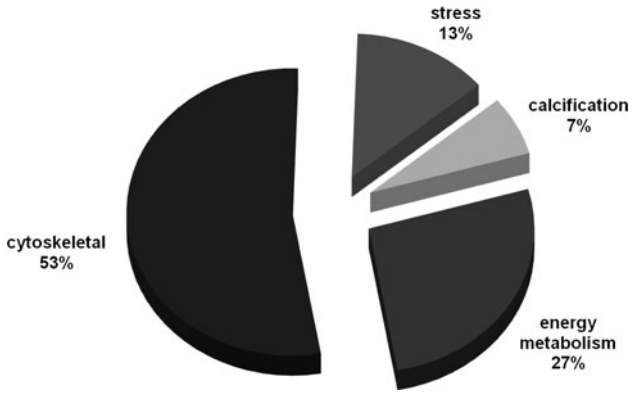

Fig. 9 Pie chart showing functional categories of identified and differentially expressed proteins in percentage

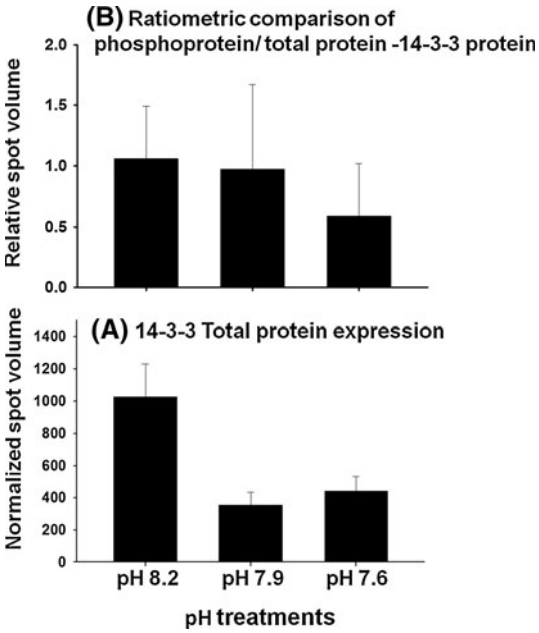

Fig. 10 Effects of pH on the phosphorylation status and the total expression level of 14-3-3 protein in the pediveliger larva of the commercial oyster, *Crassostrea hongkongensis*. *Each bar* represents the mean ± SD of three replicate gels. *Significantly ($p < 0.05$) different from control according to Dunnett's test

2011) as well as evolutionary hypotheses (Diz et al. 2012b). Therefore, application of the 2-DE approach in this study is the only technique that can analyze parallel quantitative proteome changes in response to pH from whole larvae containing large sets of complex protein mixtures.

The larval proteome of the oyster *C. hongkongensis* had about 350 SYPRO Ruby-stained proteins at the time of settlement (Fig. 3). Among them, over one-third were phosphorylated. Protein phosphorylation is one of the ubiquitous post translational modifications (PTMs) in animals, which is important for a variety of cellular functions such as signal transduction (Mumby and Brekken 2005). The number of total proteins and phosphoproteins detected in the oyster larval proteome is comparable to other marine larval proteomics studies. For instance, there were ~ 350 in corals (Deboer et al. 2007), ~250 in polychaete tubeworm (Zhang et al. 2010), ~400 in barnacles (Thiyagarajan et al. 2009; Wong et al. 2011), ~380 in oyster (Dineshram et al. 2012) and ~450 in mussels (López et al. 2005). Although the larvae of many species tend to express between 200 and 500 proteins at the time of their settlement, distribution of proteins on 2-DE map is highly species specific (Thiyagarajan et al. 2009). In the case of *C. hongkongensis*, expressed and visualized protein spots on 2-DE maps were widely and uniformly distributed on the entire range of pI and MW (Figs. 3, 4). Such a clearly defined proteome profile is a prerequisite for competitive proteome analysis, quantification of protein expression and hypothesis testing (Diz et al. 2012a).

Oyster larval proteome and phosphoproteome response to pH

This study showed the application of 2-DE with MALDI-TOF/TOF–MS/MS analysis in revealing both global protein expression pattern and differentially regulated proteins that are associated with low pH tolerance in the *C. hongkongensis*. Unlike the Pacific oyster (*C. gigas*) (Kurihara 2008), larvae of *C. hongkongensis* appear to be less impacted by low pH. Larval shell growth rate was significantly reduced along with a developmental delay at pH 7.9 and 7.6 (Fig. 2). The majority of larvae in all experimental treatments, while development was slowed in low pH treatments, were able to survive and attain competency, so it is therefore likely that physiological changes that are potentially observable in the larval proteomes may help larvae to cope with the low pH stress.

Being an estuarine coastal species, *C. hongkongensis* may have the ability to acclimate and tolerate a wide range of natural environmental conditions. Therefore, we have anticipated a high level of proteome plasticity in this species with wide range of low pH. Our proteome data support the above hypothesis. The applied near-future low (7.9) to extreme (7.6) pH stress altered the global expression pattern of proteins in the pediveliger stage, but such changes were moderate (see Fig. 4). Less than 10 % of proteins (about 52) changed their expression, and among them only 13 proteins displayed highly significant alterations in response to low pH treatments (Fig. 7). On the other hand, a large proportion of proteins changed their phosphorylation status in response to low pH treatments. There was a significant difference in total number of phosphoproteins detected between the control and the two low pH treatments (Fig. 5). These results support the hypothesis that the

oyster larval proteome may reflect the physiological plasticity that helps to cope with the tested two low pH levels. Similarly, there were no major and persistent changes in proteome structure of the barnacle larvae exposed to pH 7.6 when compared at similar size and developmental (cypris) stage (Wong et al. 2011). In contrast, exposure of the Pacific oyster larvae (*C. gigas*) to pH 7.5 resulted in significant reduction in global protein expression with a decrease or loss of 18 % of the expressed proteins than in the control pH 8.0 (Dineshram et al. 2012).

Post translational modification (PTM), for example, phosphorylation, of proteins regulates a variety of cellular functions such as enzymatic activity, signal transduction, larval development and metamorphosis (Mezhoud et al. 2008; Zhang et al. 2010). Interestingly, the total number of phosphoproteins and/or phosphorylation status slightly increased at pH 7.9; however, phosphorylation intensity of many proteins was markedly decreased at pH 7.6. This study does not provide an adequate explanation for such increasing or decreasing PTM response to pH. A previous larval phosphoproteome study, however, indicated that rapid phosphorylation of proteins in response to environmental signals is essential for transforming pelagic larval forms to benthic juveniles (larval settlement) (Thiyagarajan et al. 2009). Decreased phosphorylation in low pH, therefore, may lead to poor larval recruitment in oysters. However, this hypothesis is yet to be tested.

All the detected and identified differentially expressed proteins in the oyster larvae appear to play a key role in triggering a biochemical compensatory mechanism as part of a short-term adaptive response to low pH. Among these 13 differentially expressed proteins, collectively referred to as a "Protein Expression Signature (PES)," for low pH, the expression of 14-3-3 protein has specific significance. The expression of the highly conserved regulatory 14-3-3 protein is essential for signal transduction, apoptosis, metabolism regulation, cell cycle control and osmoregulation (Fu et al. 2000). Besides these functions, its down-regulation during pH treatments could alter the expression and function of other proteins such as calmodulin, ATPase and cytoskeletal proteins (Kültz et al. 2001; Comparot et al. 2003). Because of these diversified functions, its down-regulation in response to low pH may have contributed to delayed larval growth in multiple ways. The expression response of the calcification-associated protein, calmodulin, was also pH dependent (Figs. 7, 8). Its expression was significantly down-regulated at moderate low pH (7.9) but was slightly up-regulated at the extreme low pH (7.6) compared to control. Calmodulin is a calcium-dependent activator protein in several metabolic processes and plays a key role in calcium uptake, motility functions and calcium metabolism in a variety of species including oysters (Li et al. 2006; Yan et al. 2007; Suwa et al. 2010). Down-regulation of calmodulin under pH 7.9 might have contributed to the reduced larval calcification rate (Bibby et al. 2008; Dineshram et al. 2012). It is possible that a different energy allocation strategy, that is, more energy channeled to the production of more calcification-related proteins, is adopted by oyster larvae to tolerate extreme low pH conditions such as pH 7.6. This hypothetical compensatory physiological mechanism, however, has to be tested in future investigations. Similarly, expression of calmodulin gene was significantly down-regulated under low pH in corals (Kaniewska et al. 2012), and it was suggested that low pH could disrupt calcium homeostasis (Hidalgo 2005).

Expression of energy-related proteins such as cytochrome C oxidase, rieske apoprotein and ATPase all followed similar trends, showing down-regulation in response to low pH. This observation is not surprising because metabolic depression due to down-regulation of energy-related genes is one of the well-known and common symptoms of low pH (Kaniewska et al. 2012). Overall, larvae under low pH appear to be undergoing energy budget compensation (Ross et al. 2011). However, expression of several structural and cytoskeleton proteins was up-regulated in response to low pH (Figs. 7, 8). For example, the expression of tektin which is one of the essential cytoskeletal proteins present in cilia and flagellar microtubules was 1.6-fold up-regulated at pH 7.9 and over 3.6-fold at pH 7.6. Similarly, the cytoskeletal components associated with the mollusk shell formation (Weiss et al. 2002; Nakamura et al. 2011), the myosin light chain and histone 3 proteins, were up-regulated in response to low pH. The up-regulation of these proteins indicates positive structural alterations including cytoskeleton components (Kaniewska et al. 2012) in oysters, potentially as a compensatory response to low pH.

Limitations of 2-DE proteomics

2-DE-based proteomics has been widely used and strongly recommended for comparative total proteome analysis as well as for characterization of posttranslationally modified proteins, but its application in non-model species is limited by our inability to easily identify proteins using mass spectrometer and subsequent validation of their identity using Western blot analysis (Tomanek 2006; Johnson et al. 2007; Rabilloud and Lelong 2011; Sanchez et al. 2011; Diz et al. 2012a; Rodrigues et al. 2012; Slattery et al. 2012). Although there are few recent bioinformatics and data analysis tools to resolve protein identification issues using cross-species comparison (Carpentier et al. 2008; Turse et al. 2010), confident identification of proteins resolved on 2-DE gels is still highly challenging for species without complete genome information (Casado-Vela et al. 2011).

Despite these limitations, in this study, we have demonstrated that 2-DE-based proteomics approach can be used to quantitatively describe protein changes during acclimation at low pH in this commercially important oyster larval species.

Conclusion

This study successfully used the conventional and highly reproducible 2-DE-based proteomics approach to reveal proteome level changes in response to low pH (ocean acidification). Using MALDI-TOF/TOF MS analysis, proteins that were differentially expressed in response to low pH were identified. Low pH significantly slowed the larval shell growth through the end of pelagic growth period. The concurrent analysis of their proteome structure, at similar developmental stage and shell size, suggested that the proteome of the *C. hongkongensis* larvae is plastic, allowing the expression of calcification, cytoskeleton and energy-related proteins when exposed to lower pH. Although this study identified a set of proteins (i.e., differentially expressed proteins in each pH treatment) as a "Protein Expression Signature (PES)" for low pH, functional validation of these PESs in future studies will provide new insights into their role in low pH research, for comparisons between stressors and species, and/or as biomarkers for ocean acidification (OA).

Future molecular ocean acidification research projects should explore these possibilities using competent larval stages as models. We predict that oysters will play a central role in OA research because of their (1) commercial importance, (2) wide distribution range with relatively long larval life history and most importantly (3) complete genome information. Furthermore, our large-scale and long-term acclimation experiment in a commercial hatchery setting suggests that larval shell growth rate will be significantly reduced at projected carbonate chemistry conditions for the year >2100 in the oyster species (*C. hongkongensis*), a species that supports the livelihoods of millions of people in South China.

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