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Response of larval barnacle proteome to CO_2-driven seawater acidification

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ABSTRACT

The majority of benthic marine invertebrates have a complex life cycle, during which the pelagic larvae select a suitable substrate, attach to it, and then metamorphose into benthic adults. Anthropogenic ocean acidification (OA) is postulated to affect larval metamorphic success through an altered protein expression pattern (proteome structure) and post-translational modifications. To test this hypothesis, larvae of an economically and ecologically important barnacle species *Balanus amphitrite*, were cultured from nauplius to the cyprid stage in the present (control) and in the projected elevated concentrations of CO_2 for the year 2100 (the OA treatment). Cyprid response to OA was analyzed at the total proteome level as well as two protein post-translational modification (phosphorylation and glycosylation) levels using a 2-DE based proteomic approach. The cyprid proteome showed OA-driven changes. Proteins that were differentially up or down regulated by OA come from three major groups, namely those related to energy-metabolism, respiration, and molecular chaperones, illustrating a potential strategy that the barnacle larvae may employ to tolerate OA stress. The differentially expressed proteins were tentatively identified as OA-responsive, effectively creating unique protein expression signatures for OA scenario of 2100. This study showed the promise of using a sentinel and non-model species to examine the impact of OA at the proteome level.

1. Introduction

As a result of the dissolving of anthropogenic atmospheric CO_2 into the surface oceans over the past two centuries (Sabine et al., 2004), the carbonate chemistry equilibrium has shifted toward a higher hydrogen ion concentration and a lower carbonate (CO_3^{2-}) ion concentration, in a process commonly known as ocean acidification (OA) (Feely et al., 2004). The current global average surface seawater pH is around 8.1 units, a drop of 0.1 units compared to pre-industrial levels (Raven et al., 2005), and it is projected to drop by 0.3 to 0.5 pH units within this century (Caldeira and Wickett, 2003; Solomon, 2007). Such a drastic decrease in pH is unprecedented to contemporary marine organisms, which have evolved in a relatively chemically-static marine environment over the past 20 to 30 Ma (Pearson and Palmer, 2000).

Barnacles are representative organisms in studies of the ecology of intertidal shores and of the larval development of intertidal benthic invertebrates (Gosselin and Qian, 1996; Holm et al., 2000; Leslie et al., 2005). A growing amount of empirical evidence suggested that they are generally robust to OA, albeit discrepancy in sensitivity has been observed between certain populations and species. A pilot companion experiment showed that larval attachment and metamorphic success was compromised with increasing pCO_2 (under pH 7.9, 7.6 and 7.3 regimes) in the larval barnacle *Balanus amphitrite* (Lane et al., unpublished data). Another study on the same species by McDonald et al. (2009) showed that their larval development until the cyprid stage as well as the percentage of nauplius larvae survived to metamorphosis were not affected by rearing the larvae under the level of OA condition down to a pH of 7.4 units. In addition, Thomsen et al. (2010) showed that settlement of barnacles, such as *Balanus improvisus*, was abundant between summer and autumn in a natural CO_2-rich coastal habitat with a pH value that can be lower than pH 7.5 (but see Findlay et al., 2009a,b, 2010a,b,c for opposite responses such as compromised adult survival, delayed embryonic development as well as compromised post-larval growth and calcification in a few other barnacle species). These observations suggested that CO_2 responses and thus sensitivity may vary between barnacles depends on taxonomic group, life history and habitat patterns, as well as degree of the OA stress (Widdicombe and Spicer, 2008; Dupont and Thorndyke, 2009; Lannig et al., 2010).

To cope with OA stress, barnacles could adjust the expression patterns of proteins as short-term adaptation. This is a common strategy adopted by organisms to tolerate abiotic stressors (known as 'plastic proteome responses') (Lopez et al., 2001; González-Riopedre et al., 2007). For example, a marine snail has two ecotypes that result from proteome variation elicited by different habitats (Martínez-Fernández et al., 2008). Recently, it has been observed that plastic proteome stress responses include the post-translational modification (PTM) of proteins following their synthesis (Mann and Jensen, 2003). Altogether, dynamics in protein expression and the state of PTM provide parallel, overlapping, or complementary levels of regulation in cellular functions (Scroggins and Neckers, 2007; Tomanek, 2010).

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One type of PTM that is better studied in marine invertebrate larvae is protein phosphorylation, which is postulated to play a central role in the transduction of cellular signals to modulate and coordinate a spectrum of biological processes, including larval metamorphosis onset (Thiyagarajan et al., 2009; Wong et al., 2010; Zhang et al., 2010a) and responses to environmental shifts (e.g. Burlando et al., 2006; Ulrich and Marsh, 2009). On the other hand, glycosylation of specific proteins appears to regulate stress response in higher organisms (Hart, 1992; Henle et al., 1993). However, a large scale analysis of proteome and protein post-translational modification response to environmental stressors is rare in marine invertebrates.

The field of proteomics, specifically two dimensional gel electrophoresis (2-DE)-based proteomics, has been increasingly applied to study proteome responses in many non-model species (those without a completely sequenced genome) to various environmental stresses (Apraiz et al., 2006; Chora et al., 2008). Despite the lack of genomic data, the wealth of molecular information contained in these organisms can be characterized using the concept of protein expression signatures (PES) (Shepard and Bradley, 2000). The PES of an organism consists of the set of proteins expressed at any given time, some of which may represent a particular stress response or be associated with a transient developmental stage (Bradley et al., 2002). By establishing the PES for animals exposed to various stressors, proteomics can potentially be used to study the stress response of a non-model species at the molecular level. In this respect, proteomics enables testing hypothesis surrounding the molecular basis for stress responses in these organisms (Knigge et al., 2004).

Most marine invertebrates are non-model species and have a biphasic life cycle, where the fitness of the pelagic larval stage directly impacts the success of the benthic adult stage (reviewed in Pechenik, 2006). During development, larvae are confronted with various stressors through which they must overcome in addition to finding an appropriate substrate, attaching, and then finally metamorphosing into their adult form (reviewed in Thiyagarajan, 2010). Larvae of many species are capable of physiological and behavioral adaptation to various environmental stressors, including OA (reviewed in Dupont and Thorndyke, 2009). Therefore, larval forms, especially those responsible for the settlement (attachment and metamorphosis) process, may show more profound and more readily detectable proteome responses to environmental stressors compared to the adult form due to their rapid development, greater susceptibility and the complex developmental reprogramming in the proteome during such a larval–juvenile transition (Thiyagarajan and Qian, 2008; Thiyagarajan et al., 2009; Thiyagarajan, 2010). Clearly, an examination of protein expression patterns would help better understand the molecular basis of OA stress responses in larvae during attachment and metamorphosis.

It has been suggested that the precisely orchestrated expression of larval proteins is crucial to environmental cue recognition, settlement signal conveying, as well as preliminary preparation of the larvae for subsequent juvenile tissue production (Thiyagarajan and Qian, 2008; Thiyagarajan et al., 2009; Zhang et al., 2010b). The expression of proteins that provide essential functions related to larval attachment and metamorphosis may change in response to an environmental stressor. In this respect, exposure to stressful environmental conditions such as OA may elicit changes in the regulation of protein expression, corresponding to specific PES, indicative of the molecular preparations for attachment and/or biochemical responses to OA stress. In this study, larvae of the barnacle *B. amphitrite* were used as model to examine the above hypothesis at the total protein level as well as two PTM levels (phosphoprotein level and glycoprotein level) using 2-DE based proteomic approach. This species is used because it has been a representative organism in the study of ecology (Rittschof et al., 1992) and biofouling research (Hung et al., 2007). In addition, its larval development, survival and metamorphic success in response to OA have been recently examined in detail (McDonald et al., 2009; Lane et al., unpublished data).

2. Materials and methods

2.1. Study organism

Adults of the barnacle *B. amphitrite* (Cirripedia, Balanidae) (=*Amphibalanus amphitrite*) were scrapped off from the concrete posts at a pier of Pak Sha Wan, Kowloon, Hong Kong (22°21′45″ N, 114°15′35″E) on 29 March 2010. Adults (about 100 to 200 individuals) were induced to release their larvae by the aerial exposure method (Thiyagarajan et al., 2003). The newly hatched stage I nauplii larvae developed into stage II within 30 min and were used in the following experiment.

2.2. Experimental design

The swimming stage II nauplii larvae were reared to the competent larval stage (i.e. the stage exploring hard substrate before attaching and metamorphosing onto it), called cyprids (Supplementary Fig. S1), in the control and the OA treatment aquaria using optimized culturing techniques (Thiyagarajan and Qian, 2008). The control and the treatment represented the current (pH 8.1) and the projected levels of OA for 2100 (pH 7.6), respectively (Caldeira and Wickett, 2003). The nominal value of pH 7.6 was selected because it is also within the environmentally relevant range that can be encountered by the barnacle *B. amphitrite*. Surface coastal waters close to Pak Sha Wan (the adult barnacle collection site) had a pH range from 7.3 to 8.6 units from 2005 to 2009 (Environmental Protection Department, Hong Kong — Marine water quality data, Port Shelter, sample station PM6, at http://epic.epd.gov.hk/ca/uid/marinehistorical/p/1). In addition, it is also suggested that intertidal organisms encounter frequent pH fluctuation lower than 7.5 units (Marchant et al., 2006). Hence, pH 7.6 should be an appropriate level to examine the fundamental mechanisms that the barnacle larvae employ to tolerate environmental pH variation in the natural intertidal settings, as well as to unravel the potential strategy they adopt to tolerate OA stress, at the proteome level.

In this study, the pH values were used as a proxy for the changes in seawater carbonate system (CO_2, HCO_3^-, CO_3^{2-}, pH) in response to the dissolving CO_2 (Dickson et al., 2007). There were 3 replicate culture tanks for control and treatment. The carbonate system in each treatment tank was adjusted by bubbling CO_2-enriched air, while each control tank was bubbled with ambient air. During the entire larval culture period, temperature, salinity and pH were measured 2 to 3 times per day. The pH was measured using a pH probe (Mettler-Toledo SG2). TA levels of each tank at a time point were calculated using the Gran Plot method (Gran, 1952; Brewer et al., 1986), and validated against certified seawater reference materials (Batch 98, A. G. Dickson, Scripps Institution of Oceanography). Carbon chemistry of each tank was calculated using the program CO2SYS developed by Lewis and Wallace, 1998 by inputting pH, TA, temperature and salinity of the culturing seawater immediately before and approximately a few hours after seawater change. The following parameters were used in the CO2SYS program: seawater pH was in total scale, carbonate dissociation constant from Roy et al. (1993), KSO_4 constant from Dickson (1990), and concentrations of silicate and phosphate for FSW were both set as default (i.e. 0.0 μmol kg SW^{-1}) at the salinity level corresponding to each of the culture tank.

Roughly 10,000 nauplii larvae were introduced into each of the 6 culture tanks (<2 larva mL^{-1}). More than 90% nauplii larvae developed into the cyprid stage, irrespective of pH treatments, in 4 days. At the end of the culture period, cyprids were sieved out using a 240 μm mesh and immediately fixed for 2-DE work.

2.3. Sample preparation for 2-dimensional gel electrophoresis (2-DE)

Cyprids were washed with Milli-Q water and then lyzed in 2-DE buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, and

2% Bio-Lyte 3/10 ampholyte (Thiyagarajan and Qian, 2008). There were 6 samples in total (the control or the OA treatment × 3 replicate tanks per group). Each sample tube contained around 500 cyprids. The cyprid larval proteins were then solubilized with a sonicator (Branson Sonifier 150) on ice to prevent protein denaturing. After centrifugation for 20 min at 16,000 *g*, the supernatant was collected and stored at −80 °C until use. The protein concentration was then quantified with the 2D quant kit (GE Healthcare Life Sciences, Uppsala, Sweden).

2.4. 2-DE

The 2-DE preparation and the subsequent separation of proteins were carried out according to our optimized larval proteomic protocol (Thiyagarajan and Qian, 2008). In brief, cyprid proteins were purified using the 2-D clean up kit (Bio-Rad) and rehydrated in rehydration buffer consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte, 3/10 ampholyte, and 1% Bromophenol blue. Sample buffer (200 μL containing 120 μg protein) was applied to 11 cm IPG strips (Bio-Rad), pH 3–10 (linear), overnight for active rehydration at 50 V and then subjected to IEF using a Protean IEF Cell (Bio-Rrad). Focusing conditions were as follows: 250 V for 20 min, followed by a linear gradient from 250 V to 8000 V over 2.5 h, and at 8000 V for a total of 60,000 V h. The maximum current did not exceed 50 μA per gel. After IEF, 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 buffer 2 (same as buffer 1 but containing 2.5% iodoacetamide instead of DTT). For the second dimension separation, the equilibrated IPG strips were inserted on top of the 12.5% Criterion Tris–HCl gels (Bio-Rad) and sealed with 0.5% w/v agarose. The running buffer was standard Laemmli buffer for SDS-PAGE (modified using 0.2% w/v SDS). The gels were run at room temperature at 200 V until the bromophenol blue front reached the bottom of the gel. After electrophoresis, the 2D gels were fixed overnight in 50% methanol and 10% acetic acid to remove SDS.

2.5. Multiplex staining of 2-DE gels

A series of fluorescent stains were applied to each 2D gel following the procedure of Wu et al. (2005) (hereafter referred to as the 'multiplexed proteomics platform'): the gels were first stained with the Pro-Q Diamond phosphoprotein stain (Pro-Q DPS; Molecular Probes, Eugene, OR, USA), then with the Pro-Q Emerald 488 glycoprotein stain (Pro-Q EGS stain; Molecular Probes), and finally post-stained with the Sypro-ruby total protein stain (Molecular Probes). All staining and destaining procedures were performed according to the manufacturer instructions, with the following optimization: For phosphoprotein staining, the gels were incubated in Pro-Q DPS stain in darkness for 3 h. For glycoprotein staining, the gels were extensively washed with 3% acetic acid (7 times, each 20 min) following the oxidation of the carbohydrate moieties of the glycoproteins to remove the oxidizing reagent thoroughly. The gels were then stained with Pro-Q EGS stain in darkness for 2.5 h followed by destaining with 3% acetic acid (3 times, each 45 min) before image acquisition. For quality control, specificity of the two Pro-Q stains was validated by the Peppermint (Molecular Probes) or the Candycane (Molecular Probes) molecular weight markers, which contained two phosphoproteins and four glycoproteins respectively as positive controls.

2.6. Proteome analyses

Two different gel image analysis packages, namely the Redfin Solo program (Ludesi, Sweden) and the PDQuest software (ver. 8.0; Bio-Rad), were used to analyze the total proteome gels as well as the PTM (phosphoproteome and glycoproteome) gels, respectively. Spots that displayed significant difference according to the Student's *t*-test ($p<0.05$) and with 1.5-times or greater change in the mean normalized volume with respect to the control were considered differentially expressed at the total protein level. The spot analyses in this study assumed normal distribution of spot volumes in replicate gels within each group (control or OA treatment). This is a common approach adopted in many 2-DE based proteomics studies (e.g. Maurer et al. 2005; Zhang et al., 2010a). For PTM gels, the normalized volume of the differentially expressed phosphoprotein and glycoprotein spots was divided by the normalized volume of their respective total protein spots. This ratiometric comparison was used to determine the relative phosphorylation or glycosylation state of each total protein spot (Thiyagarajan et al., 2009; Zhang et al., 2010a).

2.7. Identification of differentially expressed proteins

After multiplexed staining and image analysis, the gels were stained with the colloidal Coomassie G-250 dye to enable visualization of the proteome (Candiano et al., 2004). Differentially expressed spots were picked, subjected to in-gel trypsin digestion and peptide purification with Ziptip (Millipore) (Thiyagarajan and Qian, 2008). Protein identification was then performed by tandem mass spectrometry analysis using ABI 4800 MALDI TOF/TOF™ MS Analyzer (Applied Biosystems, USA).

Combined PMF and MS/MS information obtained from the MS process was subjected to searching against the NCBI non-redundant database with entities restricted to metazoan or other-metazoan sequences using the GPS Explorer algorithm, ver. 3.6 (Applied Biosystems) and in-house MASCOT database ver. 2.2 (Matrix Science). Mass spectrum searches were performed using mass tolerance settings of ±75 ppm for PMF and ±0.2 Da for the MS/MS spectra. For PMF, it was assumed that peptides are monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. MASCOT scores greater than 69 ($p<0.05$) and ion score of minimum one peptide greater than 50 ($p<0.05$) or three peptides greater than 20 ($p<0.05$) were considered significant. In addition, the MS/MS spectra of the unmatched protein spots were also subjected to searching against the on-line NCBI non-redundant and invertebrate EST database, using the on-line MASCOT search engine. The scoring criteria was revised accordingly in that MASCOT scores greater than 40 ($p<0.05$) were considered significant. The matched transcripts were then subjected to in silico calculation of the translated amino acid properties, namely molecular weight (M_W) and isoelectric point (p*I*), and further homology searched against proteins in the GenBank database using protein BLAST (BLASTp) search. Blastp score greater than 100 with an E-value lower than E^{-10} was considered significant.

3. Results and discussion

3.1. OA and carbonate chemistry variability

The pH levels in the experiment tanks (i.e. pH 8.1 and pH 7.6, respectively) simulating the current and the near future (year 2100) OA scenarios maintained relatively stable carbonate chemistry parameters across the 4-day larval culture period. The measured and calculated carbonate chemistry parameters before and after the seawater change (in the 3rd day of the culture period) remained similar in the control and the treatment, respectively (Supplementary Fig. S2). We recognized that alkalinity decreased during the experiment, with an accompanied drift in seawater pCO_2. However, this difference is small relative to the separation between the control and the OA treatment group throughout the entire larval culture period. In addition, seawater from the same source was used for the culture tanks in both groups. In this regard, the introduced/bubbled

CO_2 is essentially responsible for the observed changes in the larval proteome.

3.2. Larval proteome structure

Our proteome data revealed about 566 total protein spots in the cyprids larval proteome (Fig. 1 and Supplementary Fig. S3), which is consistent with previous studies (Thiyagarajan and Qian, 2008; Thiyagarajan et al., 2009). Approximately 34% of the protein spots were phosphorylated, consistent with the about 30% phosphorylated proteins in the barnacle cyprid proteome as reported in Thiyagarajan et al. (2009). Besides, approximately 23% spots were glycosylated, in contrast to studies in higher organisms, where up to 50% of the proteins are glycosylated (e.g. in human; Taniguchi et al., 2006). The correlation coefficients between replicate gels within a group (i.e. control versus control or treatment versus treatment) ranged from about 0.7 to 0.9 for the total proteins, phosphoproteins, and glycoproteins (data not shown). On the other hand, correlation analyses of each gel pair between the opposite groups (i.e. control versus treatment) yielded a correlation coefficient from 0.6 to 0.9 at the different proteome levels. These correlation results illustrate that the inter- and intra-group biological variability at the different proteome levels was low (Challapalli et al., 2004; Bland et al., 2006; Sun et al., 2007). Being a dominant species in the intertidal region, it is postulated that the barnacle larvae have evolved mechanisms to cope with short-term fluctuations in temperature, salinity, and pH (Pane and Barry, 2007; Widdicombe and Spicer, 2008). In this regard, dramatic changes in the cyprid proteome are expected in response to

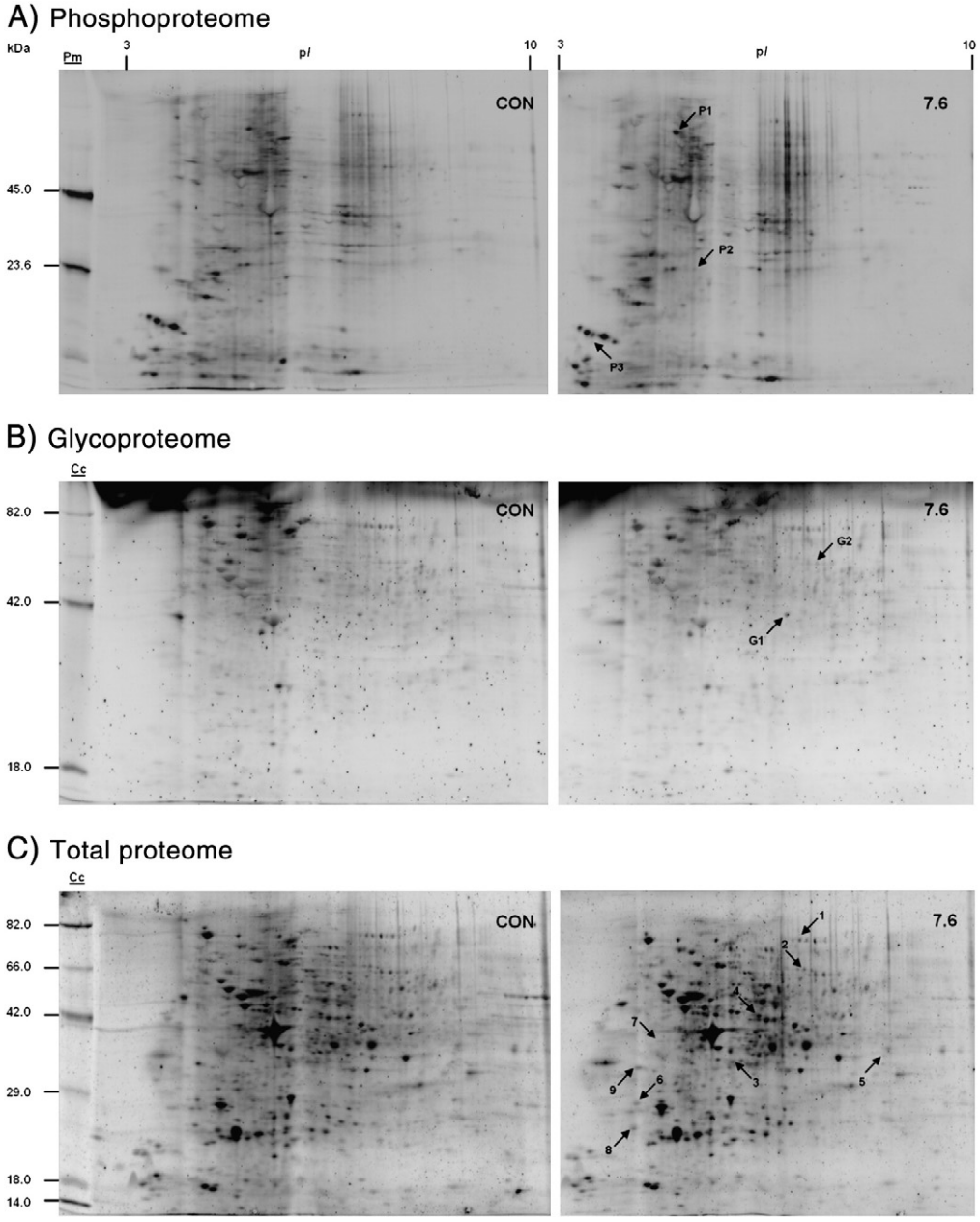

Fig. 1. Representative multiplexed 2D gels of barnacle cyprids, *B. amphitrite*, obtained from 3 replicates at each pH. This multiplexed staining procedure examined the effects of the near future level of OA stress on the phosphoproteome, glycoproteome, as well as the total proteome of barnacle cyprids. The 2D gels were first stained with the Pro-Q DPS phosphoprotein-specific dye, followed by the Pro-Q EGS glycoprotein-specific dye, and lastly stained with the Sypro-ruby total protein stain. The differentially regulated total protein spots (spot nos. 1–9) as well as the OA-responsive phosphoprotein (spot P1) and glycoprotein spots (spots G1 and G2) were respectively magnified in Figs. 2 and 3 to enhance clarity. 'Pm' denotes the Peppermint phosphoprotein marker, whereas 'Cc' denotes the Candycane glycoprotein marker. Only 3 out of 4 glycoprotein marker lanes were positively stained by the Pro-Q EGS stain since the α2-Macroglobulin (a glycoprotein) has a Mw of 180 kDa, which is outside the resolving range of 12.5% Tris–HCl gels.

the near future level of OA stress. Results of our proteome analysis support this postulation, however, only a small number of proteins responded to OA treatment at the total protein and the two PTM levels. As a result, spot-by-spot analysis of 2D gels was conducted to explore these selective regulations.

3.3. Global protein expression response to seawater acidification

Comparative analysis of the total proteome gels between the control and the OA treatment by univariate methods (quantitative and qualitative detection, and Student's test using the Redfin software) showed that the global protein expression pattern is slightly but significantly affected by OA stress. Importantly, the trend observed here is in good agreement with the notion of proteome plasticity in that the barnacle larval proteome responded to OA stress by selectively regulating protein expression. In light of this, the 2-DE based proteomics is a promising tool to test hypotheses regarding ecological or environmental effects of protein expression changes.

In total, 9 proteins were differentially expressed 1.5-fold or more in OA treatment compared to the control (Fig. 2). From the MALDI-TOF/TOF analyses using the combined PMF MS/MS strategies, 7 of them were tentatively identified (Table 1). The remaining 2 proteins could not be identified owing to the lack of sequenced genomic information in barnacles. The identified proteins can be grouped as follows: a) 4 proteins that were significantly up-regulated compared to the control, and b) 3 proteins that decreased in expression compared to the control. There was a discrepancy between the theoretical M_W and p*I* and those obtained experimentally in gel. The PMF and MS/MS results suggested that this could be due to the low sequence coverage of the analyzed peptide fragments in the database, which is common in non-model species (Kültz et al., 2007). Yet, validation of the differentially expressed proteins was not performed in the current study as the immunoblotting and gene transcript quantification methods up-to-date are deemed immature for barnacles. As a consequence, the interpretation of these results requires a cautious approach.

3.4. Up-regulated proteins in response to seawater acidification

The up-regulated OA-responsive proteins comprise 4 spots. Spot 1 was identified as the protein elongation factor 2 (EF2), which is essential in the polypeptide elongation steps during protein production (Qiu et al., 2008). This implies that such an up-regulation might facilitate the production of stress response proteins. Nevertheless, the present study did not observe significant up-regulation of other translation-related proteins, namely those responsible for initiation and termination of translation (Holcik and Sonenberg, 2005). In this regard, it is speculated that the EF2 gene product may be selectively regulated for alternative functions. Indirect evidences were provided by Wang et al. (2011) on the acute stress response of shrimps challenged by extreme pH levels as well as cadmium stress, by which a marked increase in EF2 gene expression was observed. Taken together with the present report of the enhanced expression of EF2 under OA stress, these observations encourage the re-examination of EF2's role in cellular stress and/or growth response in future studies, while the possibility of selective regulation of this protein in translation control should not be neglected.

Two hemoglobin beta chain-like proteins showed significant up-regulation (spots 2 and 3) in cyprids reared under OA stress. The physiological function of hemoglobin is well-documented in vertebrates as an important respiratory protein for oxygen (O_2) transportation and CO_2 removal (Hardison, 1998). Essentially, the extracellular hemoglobin-like protein (i.e. hemoglobin in the hemolymph) has been reported in the Cirripedia, presumably as polymeric structure of varying sizes (Weber and Vinogradov, 2001). Hemoglobin exchanges O_2 with CO_2 and H^+ from peripheral tissues during cellular respiration. This process obeys the Bohr effect, which describes that such an exchange is achieved in a concentration-dependent manner (i.e. O_2 loading by hemoglobin is the greatest in respiratory tissues and the high CO_2 and acidity produced during cellular respiration in exercising muscles decrease O_2 affinity in hemoglobin when H^+ and CO_2 are bound, and this facilitates the release of O_2 to the tissue with the removal of the gaseous wastes) (Weber and Vinogradov, 2001). As proposed by a recent model, exposure to the increasing extracellular CO_2 and acidity brought about by OA may have aberrantly compromised the capacity for O_2 loading in the gaseous exchange interface (Widdicombe and Spicer, 2008). In this regard, respiration could be an emerging physiological 'weak link' to OA (Todgham and Hofmann, 2009) and the up-regulation of hemoglobin-like proteins could be a compensatory response for the reduced O_2 affinity for hemoglobin under OA condition.

The remaining up-regulated spot in the OA treatment matches the long chain specific acyl CoA dehydrogenase (spot 4), one of the first enzymes required for energy production via fatty acid oxidation in mitochondria (Bartlett and Eaton, 2004; Athenstaedt and Daum, 2006). Under environmental stress cells allocate energy to generate stress response proteins in order to maintain homeostasis that is essential to cellular function. Thus, the up-regulation of this energy-related protein is expected to serve this considered purpose, thereby fueling the enhanced expression of hemoglobin-like proteins and EF2-like protein under low pH. However, as triacylglycerol (TAG) is the major type of internal reserve in barnacle cyprids (Tremblay et al., 2007; Thiyagarajan, 2010), enhanced oxidation of fatty acids constituents in TAG may have adverse consequences to larval settlement, as many laboratory and field studies attested that the physiological quality of barnacle cyprids, which is usually measured in terms of TAG levels, dictates settlement success (Tremblay et al., 2007; Thiyagarajan, 2010).

3.5. Down-regulated proteins in response to seawater acidification

Spot 5 which was identified as an EH-domain containing protein appear to be a regulator for endocytosis (Mayer, 1999). Its lowered expression under the OA treatment illustrated the possibility that this process may be sensitive to extracellular acidification. Spot 6 matches cathepsin L-like protein, which belongs to the family of cysteine proteases related to cell matrix protein degradation upon release from lysosomes in higher eukaryotes (Obermajer et al., 2008; Diez, 2010). Spot 7 matches the heat shock protein 83 (hsp83), a member of the molecular chaperone family which plays a central role in modulating the proper folding of other proteins under environmental challenges, such as heat stress (Mathew and Morimoto, 1998). The noticeable suppression of this hsp-like protein under the OA treatment is consistent with previous studies from other groups. In one such study, the mRNA expression levels of one and seven heat shock protein genes were subtly but significantly repressed in purple sea urchin, *Strongylocentrotus purpuratus*, larvae exposed to OA conditions of pH 7.96 and 7.88 respectively (Todgham and Hofmann, 2009), while in another study, exposure of red sea urchin, *Strongylocentrotus franciscanus*, larvae to a comparable level of OA stress followed by an additional 1-h actual heat stress resulted in a lowered and delayed maximal expression level of hsp70, another important member of the molecular chaperone family (O'Donnell et al., 2009). In agreement with these observations, the reduced expression of hsp83 in larval response to low pH agrees with the growing view that OA exerts a suppressive effect on molecular chaperone expression (Hofmann and Todgham, 2010). This could have ecological consequences whereby the barnacle cyprids may be more susceptible to stress-induced protein damage caused by additional environmental stressors in the intertidal zone, in the near future OA scenario (year 2100).

Lastly, spot 8 and spot 9, which were down-regulated by 2.5 fold and 2.2 fold respectively under OA condition, match two hypothetical proteins (Table 1). However, the paucity of genomic information prohibits further interpretation of their function.

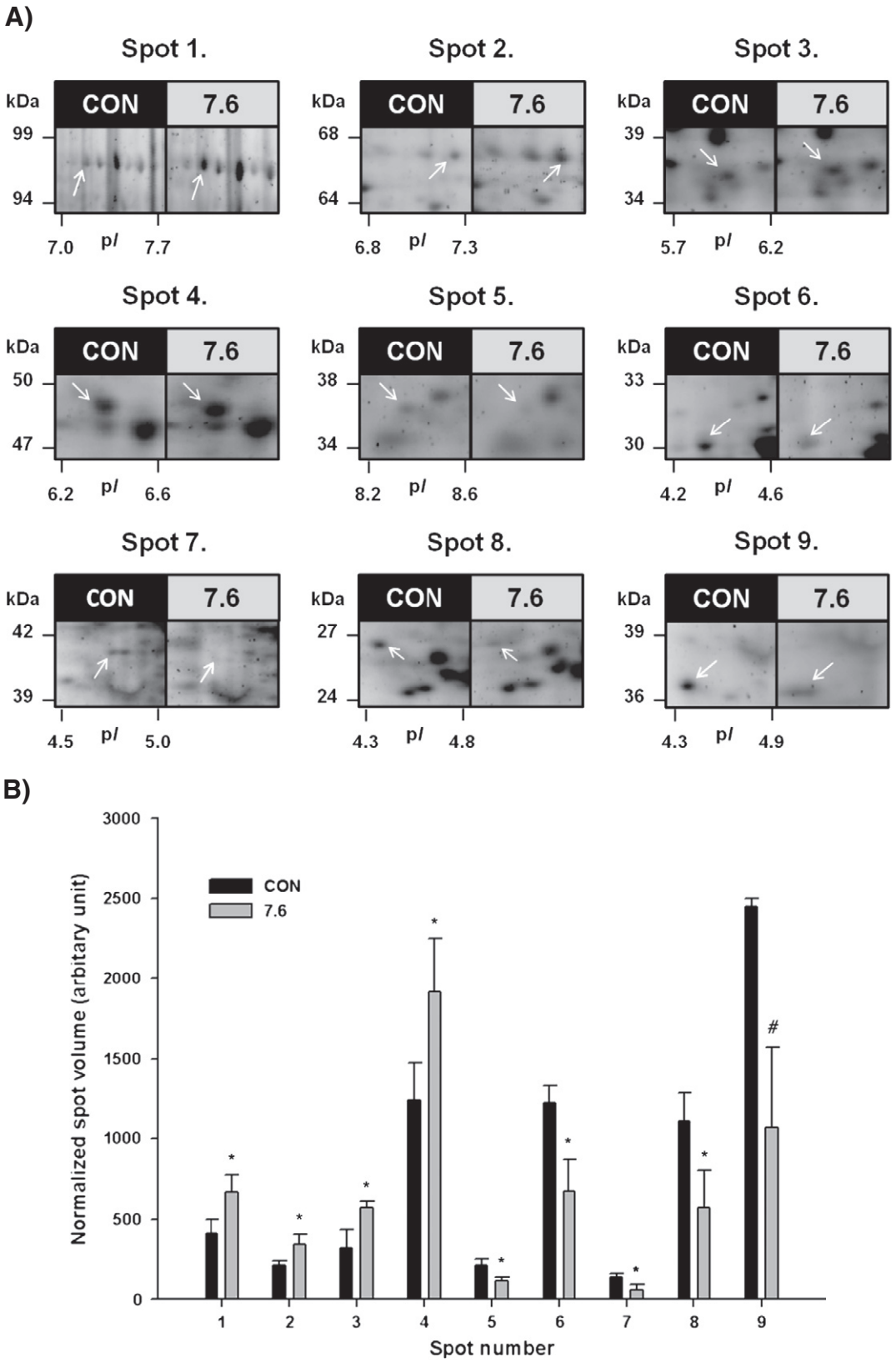

Fig. 2. A magnified view of the tentatively identified spots from barnacle cyprids, *B. amphitrite* at the total protein level. (A). The arrows mark the spots whose intensity changed considerably in the OA treatment (pH 7.6), using a threshold of 1.5 or more fold changes with statistical significance (Student's *t*-test, $p<0.05$), with respect to the control (B). Statistical representation of the changes in expression level of each identified proteins from biological triplicates (± 1 S.D.). Asterisk indicates statistical difference with a p-value <0.05, and hash indicates p-value <0.01. Refer to Table 1 for their protein identity.

Table 1
Identified proteins responsive to OA stress (pH 7.6) in the barnacle cyprids *B. amphitrite* at the total protein level as well as the PTM levels (phosphorylation level or glycosylation level), respectively.

Spot no.[a]	Putative identification[b]	Fold change 7.6/CON[c]	MASCOT protein score (C.I.)[d]	MASCOT MS/MS score (C.I.)[e]	MASCOT peptides identified by MS/MS[f]	MP[g]	Seq cov. (%)[h]	Momology to protein (Genbank)[i]	Theoretical[j] Mw	Theoretical[j] pI	Experimental[k] Mw	Experimental[k] pI
Total proteins												
1	Elongation factor 2	1.6*	95 (100)	95 (100)	AHLPVNESFGFTADLR	7	12%	148646395 *Penaeus monodon*	95.0	6.17	97.0	7.20
2	Hemoglobin subunit beta	1.6*	75 (93.2)	55 (98.1)	LLGNVLVVVLAR	5	36%	294459577 *Bos taurus*	16.0	6.36	66.0	7.25
3	Hemoglobin subunit beta-C	1.8*	110 (99.9)	67 (99.9)	LLGBVLVVVLAR	8	54%	122549 *Ammotragus lervia*	15.7	11.57	37.5	6.00
4	Long-chain specific acyl-CoA dehydrogenase	1.5*	130 (100)	107 (100)	AQDTAELFFEDVR	10	26%	6978431 *Rattus norvegicus*	48.2	7.63	49.0	6.40
5	EH-domain-containing protein 1	−1.9*	75 (93.8)	52 (95.4)	FGNAFLNR	13	23%	72113270 *Strongylocentrotus purpuratus*	62.5	6.89	36.0	8.40
6	Cathepsin L	−1.7*	/	205 (191/2E^{-47})[e]	DQGQCGSCWAFSSTGALEGQHFR	1	17%*	159792912 *Apostichopus japonicus*	14.1	5.19	30.0	4.40
7	Heat shock protein 83	−2.4*	260 (100)	56 (100) 66 (100) 118 (100)	HSQFIGYPIK ELISNASDALDKIR NPDDISQEEYGEFYK	8	29%	37963505 *Drosophila willistoni*	38.3	4.74	40.0	4.70
8	Contig CAGA42356.fwd	−2.5*	/	60[e#]	RNIILVVVILN	1	4%*	223792153 *Alvinella pompejana*	26.2	7.68	27.0	4.40
9	NMD3 family protein	−2.2#	/	48 (959/0.0)[e]	IADIILVR	1	1%*	170584344 *Brugia malayi*	58.8	6.48	37.0	4.40
Phosphoprotein												
P1	Heat shock protein 90	1.6*	171 (100)	63 (99.7)	DQVSNSAFVER	15	20%	14041148 *Dendronephthya kluzingeri*	84.6	4.80	85.0	5.30
Glycoproteins												
G1	Citrate synthase 1	2.3*	234 (100)	97 (100) 98 (100)	SGQVVPGYGHAVLR GLVTETSVLDPEEGIR	4	10%	225713236 Lepeophtheirus salmonis	52.6	7.25	45.0	7.05
G2	Transketolase isoform 2	2.2#	186 (100)	75 (100) 100 (100)	KIDNDLEGHPTPR LDNLVAIFDVNR	8	11%	115944249 *Strongylocentrotus purpuratus*	67.4	6.09	65.5	7.51

a) Spot number as assigned in Figs. 2 and 3.
b) Name of the putatively identified protein.
c) Differences in protein expression by dividing the mean normalized spot volume in pH 7.6 group by that in CON. All identified spots shows statistical difference (Student's *t*-test $p<0.05$: indicated by *); spot 9 and G2 both have a $p<0.01$ (indicated by #).
d) The in-house MASCOT protein score (confidence interval in parentheses) obtained through searching against the NCBI non-redundant database. Spots that were identified solely based on MS/MS were indicated by /.
e) and f) Peptide selected for MS/MS and corresponding ion score (s). For spots that were identified solely based on MS/MS, the score present here was obtained through the online MASCOT engine, searching against the NCBInr and invertebrate EST database with the MS/MS spectra (all spots shows a C.I. >95% and MASCOT score >40 is considered significant). The corresponding blastp score for the in silico translated EST transcript was shown in parentheses (blastp score >100 with an E-value <E^{-10} is considered significant).
g) Number of matched peptides (MP).
h) Peptide fragment sequence coverage (%). The percentage values that were indicated by * represent only the MS/MS sequence coverage.
i) Genbank accession number.
j) The theoretical MW and pI value of the matched protein or that derived by in sillico calculation of amino acid properties during database search by online MASCOT.
k) The experimental MW and pI values estimated from the 2D gel pattern.

3.6. Proteome phosphorylation response to seawater acidification

Phosphorylation of 3 proteins showed significant changes (Figs. 1 and 3). However, only the up-regulated phosphoprotein (i.e. spot P1) could be identified, and its identity matches the heat shock protein 90 (hsp90) (Table 1). The total protein counterpart of this hsp90, however, did not exhibit significant change, as opposed to the other down-regulated hsp83-like protein in the low pH treatment (spot 7 in Section 3.5). Such a specific increase in the phosphorylation state of hsp90 together with the particular decrease in hsp83 expression at the total protein level under the OA treatment suggests that the function of the respective molecular chaperones in barnacle cyprids may involve complementary levels of regulation. Apart from these, a relationship between hsp90 phosphorylation and its chaperone activity had been observed by Wandinger et al. (2006). Their study showed that deletion of a hsp90 dephosphorylase gene had led to compromised maturation and activation of all hsp90-client proteins tested in vivo in yeast cells, illustrating that hsp90 dephosphorylation may have the effect of modulating the chaperone function of hsp90. Thus, the observed increase in hsp90 phosphorylation is possibly a reflection of a compromised or deranged chaperone function. This change may be detrimental to the recovery of denatured proteins under stress or the folding of nascent proteins produced in the cyprids for settlement under OA conditions.

3.7. Proteome glycosylation response to seawater acidification

The expression levels of 2 putative glycoprotein spots were up-regulated under the OA treatment (spots G1 and G2 in Figs. 1 and 3). They match citrate synthase (spot G1) and transketolase (spot G2) with a fold difference of 2.3 ($p<0.05$) and 2.2 ($p<0.01$), respectively (Table 1). These spots exhibited changes only in their relative glycosylation status but their total protein level remained largely unchanged (Fig. 3). Transketolase is a key enzyme implicated in the non-oxidative pentose phosphate pathway (nO-PPP); this is a central compensatory machinery for the recycling of ribose-5-phosphate into glucose-6-phosphate, a common precursor for both oxidative PPP and glycolysis (then citric acid cycle) to produce NADPH or pyruvate (then ATP) in response to shifts in energy metabolism, such as those observed under stress (Alexander-Kaufman and Harper, 2009). On the other hand, the citrate synthase-like protein also showed increased glycosylation level at the OA treatment. This mitochondrial enzyme catalyzes the first step of the citrate acid cycle to produce ATP (Wiegand and Remington, 1986), and it is also tightly linked to the glycolytic and the PPP pathway. The precise mechanism by which the increased glycosylation mediate the activity of these 2 enzymes remains undetermined. Nevertheless, together with the observed increased expression of the fatty acid metabolism-related acyl-CoA dehydrogenase-like protein under the low pH treatment (spot 4 in Section 3.3), the increased glycosylation of these two major bioenergetics enzymes has generated the hypothesis that OA induces shifts in fatty acid, NADPH and/or ATP turnover in the barnacle cyprids during settlement. As a consequence of these changes, energy could then be allocated to fuel the enhanced production of hemoglobin-like proteins and the EF2 protein for potential stress response in OA condition (see Section 3.3).

Nevertheless, such an alternate use of energy may inevitably translate into a compromised attachment and metamorphic success in this species of barnacle. This is caused by a mismatch between the finite energy reserve and the cyprid settlement capacity with potentially adverse effects on the subsequent attachment and metamorphosis (Tremblay et al., 2007; Thiyagarajan, 2010). A recent study seems to lend support to this model. Lane et al. (unpublished data) observed that larval attachment and metamorphic success was increasingly compromised down three different levels of OA stress, namely pH 7.9, 7.6 and 7.3, respectively in cyprids of the same barnacle species used in the current study, *B. amphitrite*, with respect to the control (pH 8.1). The stress response under OA thus bears an energetic cost, which may partly translate into the reduced attachment and metamorphic success with increasing levels of OA stress.

3.8. Proposed protein expression signature for seawater acidification stress

Shepard and Bradley, 2000 proposed that the set of proteins that are representative enough to distinguish a life stage or an experimental condition, such as the degree of OA experienced by the cyprids, could be referred to as protein expression signatures (PES). Thus, based on the proteome data in hand, the identified proteins could be used to constitute the PES for barnacle larvae reared under pH 7.6 OA stress (Fig. 2): the up-regulated set comprising 2 hemoglobin-like proteins, 1 elongation factor 2-like protein, and 1 fatty acid oxidation-associated enzyme together with the down-regulated spots, namely the hsp-like protein, the protease, and the endocytosis-related protein combine to form candidates for the PES to provide an overview of the level of OA stress the barnacle cyprid are expected to experience in 2100.

3.9. Synthesis: proteome plasticity and energy metabolism during OA stress

Exposure to OA conditions may result in a physiological shift in energy homeostasis, depends on the strategies that a given species adopted to enhance stress-tolerance and promote survival (Widdicombe and Spicer, 2008; Hofmann and Todgham, 2010). A common response harnessed by marine invertebrates is metabolic depression (Fabry et al., 2008), such as the response to OA stress in adults of crabs (Metzger et al., 2007), squid (Rosa and Seibel, 2008), as well as in coral larvae (Nakamura et al., 2011). Although in many cases the suppression of metabolism could be adaptive for survival under short-term stress (Guppy and Withers, 1999), it can also manifest in long-term acclamatory response. For example, a compromised metabolic rate has been observed in adult and juvenile mussels *Mytilus galloprovincialis* exposed to long-term OA stress (Michaelidis et al., 2005). It has been estimated that protein translation alone could consume up to 50% cellular energy (Holcik and Sonenberg, 2005). By suppressing cellular metabolism through down-regulating the expression of relevant gene products, the energy can then be allocated to potentially synthesize stress response proteins (Gracey et al., 2008; Place et al., 2008) or be saved to 'wait out' periods of OA stress (Fabry et al., 2008). Observations in sea urchin larval response to OA lend support to this model. Widespread down-regulation of gene transcription has been observed in two species of urchin larvae under various levels of OA stress. Both studies observed broad-scale suppression of gene expression from the biomineralization and the energy metabolic pathways (Todgham and Hofmann, 2009; O'Donnell et al., 2010). Several genes encoding the ions and acid–base regulatory proteins were also specifically up-regulated in the pluteus stage red urchin larvae in O'Donnell's study.

In contrast to the common strategy of bulk protein synthesis depression, the stress response of barnacle cyprids during attachment may involve a distinct strategy. Barnacle cyprids are specialized for attachment in that many presumptive juvenile structures are generated before attachment and metamorphosis (Hadfield et al., 2001). Characterized by their 'active' habitat selection behavior, these 'streamlined' features enable the larvae to complete attachment and metamorphosis relatively rapidly (compared to that in insects and amphibians) once a correct settlement cue is perceived (Tremblay et al., 2007; Thiyagarajan, 2010). Considering that larval attachment itself as an 'active' process involving the orchestrated expression of many proteins within the body and their interaction with the environment (Thiyagarajan and Qian, 2008; Zhang et al., 2010b), it could be argued that a global depression of protein synthesis under the energetic expenses of stress, if adopted, would hamper the metabolic processes for replacing essential cellular

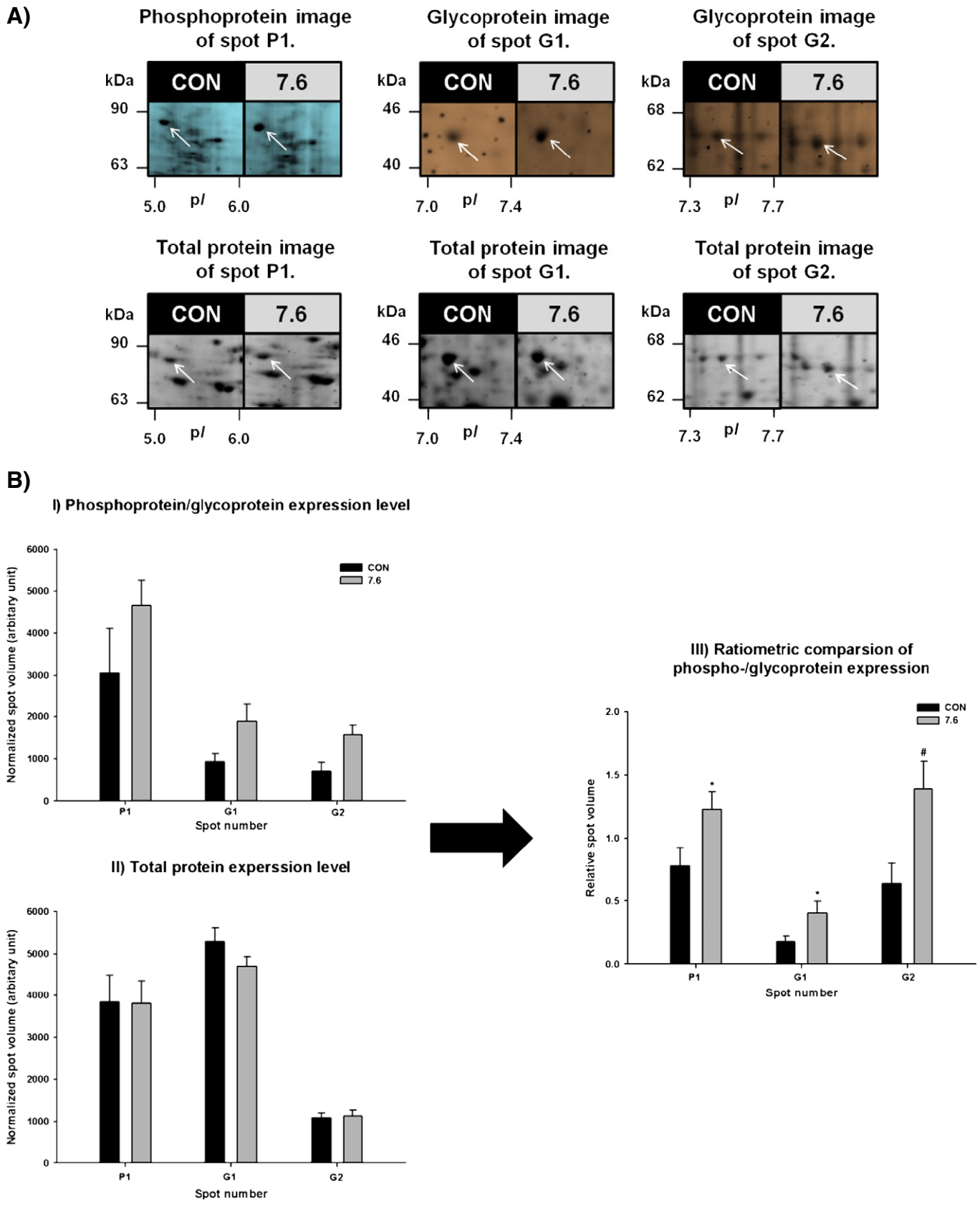

Fig. 3. A magnified view of the tentatively identified phosphoprotein and glycoproteins from barnacle cyprid larvae, *B. amphitrite*. (A). The arrows mark the respective identified PTM spots whose intensity changed considerably in pH 7.6, using a threshold of 1.5 or more fold changes with statistical significance ($p<0.05$), compared to the control. The two unidentified phosphoprotein spots (P2 and P3; Fig. 1) were not presented. The Pro-Q DPS and EGS gels were false-colored as blue and red, respectively for better illustration. (B). Statistical representation of the expression level of the phosphoprotein P1 and glycoprotein spots G1 and G2, respectively, at the post-translational level (BI), at the total protein level (BII), and the relative expression level (BIII) after ratiometric comparison (the respective normalized PTM spot volume divided by that of the total protein counterpart). Each bar presents the mean expression level from biological triplicates (± 1 S.D.). Asterisk indicates statistical change ($p<0.05$), and hash indicates p-value<0.01.

macromolecules during settlement. The results of this study support the above observation. The expression of most proteins in the barnacle cyprids showed no significant changes in response to OA stress, except for those few rate-determining enzymes involved in energy metabolism. These selective changes could indeed reflect a trade-off between various components of the physiological mechanisms, all directed toward the more effective integration and better exploitation of energy for settlement. Probably such a shift in energy usage was the result of a need to fuel the increased expression of the hemoglobin-like proteins and the EF2 protein in response to the OA stress.

It should be noted that strategies permitting marine invertebrates to tolerate OA stress are probably more diverse and complicated than expected. Opposite responses such as increased metabolism were displayed in the mussels *Mytilus edulis* as a result of long-term exposure to OA stress (Thomsen and Melzner, 2010). Wood et al. (2008) illustrated a similar increase in the metabolic rate of the brittle stars *Amphiura filiformis*, fueled by consuming their arms as a potential alternative energy source. On the other hand, a shift in energy metabolism intermediates was observed in different tissues of the adult oysters *Crassostrea gigas* (Lannig et al., 2010). Furthermore, an increase in the expression of two important genes/proteins implicated in aerobic metabolism (i.e. glyceraldehyde-3-phosphate dehydrogenase and mitochondrial malate dehydrogenase) was up-regulated in the amphipods *Gammarus locusta* (Hauton et al., 2009) and the adult oysters *C. gigas* respectively under comparable levels of OA stress (Tomanek et al., 2011). Altogether, these observations may indicate that in some of the OA-exposed animals they could tolerate the stress by reprioritizing the energy use and/or increasing metabolic supply for cellular stress processes via stress-induced up-regulation of genes whose products are related to energy metabolism (as opposed to metabolic depression). The ability to respond physiologically to OA encompasses a concerted regulation of gene/protein expression (i.e. the transcriptome/the proteome). Current systems biology research is underway to examine the physiological and metabolic fates of gene and protein expression under OA stress in marine invertebrates and their larvae (Hofmann and Todgham, 2010; Tomanek, 2010). As molecular biology tools are sensitive to detect subtle changes in gene/protein expression as well as early/sub-lethal responses to stress, the future avenue of research could incorporate more physiological end-point measurements to the 'omics' studies explore whether a change in the basic molecular and cellular mechanism would lead to changes up a biological hierarchy. By doing so, they would contribute to provide a more holistic view of OA effect on the physiology and metabolism in marine invertebrates.

4. Conclusions

Our choice of the relatively acute OA stress (i.e. 4-day exposure) was mainly meant to match the normal developmental phase length of the barnacle cyprids (Thiyagarajan et al., 2003), with a research focus on the critical, larval–juvenile transition (i.e. the short metamorphosis window). As an initial endeavor to describe the strategy/molecular repertoire that operate in intertidal organisms (i.e. the barnacle species in this study) to consider potential cellular mechanisms of stress tolerance during attachment of these species in the future, a longer-term exposure would have been less meaningful and of lower relevance to the life history of these barnacles at this current avenue of research. An extended culture of the cyprids could further deplete their reserve and compromise their performance, thereby introducing the potentially confounding age-related effects (Pechenik, 2006). A further experiment encompassing prolonged/chronic OA exposure (e.g. a multi-generation CO_2-pertubation experiment) is required before the current proteome data can be fully extrapolated to predict the sensitivity of this larval barnacle species to OA conditions that is projected to occur by the end of this century. This is because several previous studies examining the responses of other abiotic stressors on marine organism have shown that gene expression patterns could be different in the course of a long-term exposure study, before a new physiological equilibrium is reached (Gracey et al., 2001, 2004; Deigweiher et al., 2008). Nevertheless, the present study has fulfilled the considered research goal.

Rearing larvae under the near future level of OA stress (pH 7.6) for 4 days seemed to exert only modest effect on the cyprid proteome, as reflected by the relatively low number of proteins being differential regulated at the total protein as well as the two PTM levels (phosphorylation and glycosylation) compared to the control. The results of correlation analyses between as well as within the control and the OA treatment seem to support these spot-oriented observations.

The current proteome analysis demonstrated larval proteome plasticity under OA stress by regulating energy usage, possibly for activating proteins to cope with OA stress. Certain biological process seemed to be significantly affected by the OA treatment, including the molecular chaperone-associated defense. A possible energetic consequence of barnacle cyprid response to OA stress would involve a channeling of the reserved finite energy for settlement. This alteration in energy metabolism may translate into a reduction in attachment and metamorphic success under low pH as the level of energy reserves can determine the physiological condition of barnacle cyprids, thereby their habitat selection ability, and ultimately their metamorphosis (reviewed by Thiyagarajan, 2010).

The present multiplex proteomic study detected proteins that have not been reported as OA-responsive before. This study is a first attempt to explore the proteome dynamics together with PTM changes in a sentinel and non-model larval species. As there is an urgent need to better forecast the potential widespread effects of OA on marine ecosystems, the current 2-DE based larval proteomics approach shows promise for future comparative proteomics studies on the physiological plasticity of different non-model species of marine invertebrate larvae in response to OA stress. Such efforts would facilitate the forecast of the tolerances of different taxa in the face of rapidly spreading OA stress from southern oceans to tropical seas.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cbd.2011.07.001.

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