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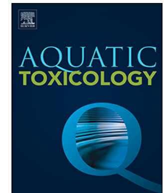

Impacts of the combined exposure to seawater acidification and arsenic on the proteome of *Crassostrea angulata* and *Crassostrea gigas*

Anthony Moreira[a], Etelvina Figueira[a], Nélia C. Mestre[b], Denise Schrama[c], Amadeu M.V.M. Soares[a], Rosa Freitas[a,*], Maria João Bebianno[b]

[a] Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, Aveiro, Portugal
[b] CIMA, Universidade do Algarve, Campus de Gambelas, Faro, Portugal
[c] CCMAR, Universidade do Algarve, Campus de Gambelas, Faro, Portugal

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ABSTRACT

Proteomic analysis was performed to compare the effects of Arsenic (As), seawater acidification (Low pH) and the combination of both stressors (Low pH + As) on *Crassostrea angulata* and *Crassostrea gigas* juveniles in the context of global environmental change. This study aimed to elucidate if two closely related *Crassostrea* species respond similarly to these environmental stressors, considering both single and combined exposures, to infer if the simultaneous exposure to both stressors induced a differentiated response. Identification of the most important differentially expressed proteins between conditions revealed marked differences in the response of each species towards single and combined exposures, evidencing species-related differences towards each experimental condition. Moreover, protein alterations observed in the combined exposure (Low pH + As) were substantially different from those observed in single exposures. Identified proteins and their putative biological functions revealed an array of modes of action in each condition. Among the most important, those involved in cellular structure (Actin, Atlastin, Severin, Gelsolin, Coronin) and extracellular matrix modulation (Ependymin, Tight junction ZO-1, Neprilysin) were strongly regulated, although in different exposure conditions and species. Data also revealed differences regarding metabolic modulation capacity (ATP β, Enolase, Aconitate hydratase) and oxidative stress response (Aldehyde dehydrogenase, Lactoylglutathione, Retinal dehydrogenase) of each species, which also depended on single or combined exposures, illustrating a different response capacity of both oyster species to the presence of multiple stressors. Interestingly, alterations of piRNA abundance in *C. angulata* suggested genome reconfiguration in response to multiple stressors, likely an important mode of action related to adaptive evolution mechanisms previously unknown to oyster species, which requires further investigation. The present findings provide a deeper insight into the complexity of *C. angulata* and *C. gigas* responses to environmental stress at the proteome level, evidencing different capacities to endure abiotic changes, with relevance regarding the ecophysiological fitness of each species and competitive advantages in a changing environment.

1. Introduction

The uptake of carbon dioxide (CO_2) by the world's ocean surface waters is altering global seawater chemistry. Dissolution of CO_2 in seawater leads to a net increase of carbonic acid (H_2CO_3), hydrogen (H^+) and bicarbonate (HCO_3^-) ion concentrations, thus increasing seawater acidity ($pH = -\log [H^+]$). These reactions are followed by a decrease of carbonate ion (CO_3^{2-}) concentration and lower saturation states of aragonite (ΩAr) and calcite (ΩCa). In short, this physico-chemical phenomenon is termed ocean acidification (OA) (Fabry et al., 2008). The International Panel for Climate Change (IPCC) projected pCO_2 levels in surface oceanic waters to reach between 490 and 1370 µatm (0.06 to 0.32 pH unit drop), depending on the atmospheric emission scenario (IPCC, 2014), while other authors suggest that pH in the ocean will decrease up to 0.5 units by year 2100 (Caldeira and Wickett, 2005; Raven et al., 2005). However, the impacts of OA are expected to be amplified in nearshore habitats (estuaries, bays, mangroves), where diel and seasonal pH and pCO_2 fluctuations are common features (Baumann and Smith, 2018; Miller et al., 2009; Tomanek et al., 2011). In such ecosystems, OA may further exacerbate the higher amplitude of pH fluctuations (Ringwood and Keppler, 2002), and increased pCO_2 levels (Melzner et al., 2012) compared to oceanic waters,

* Corresponding author at: Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.
E-mail address: rosafreitas@ua.pt (R. Freitas).

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thus presenting increased environmental stress to inhabiting biota.

Nonetheless, changes in seawater physico-chemical characteristics due to increased pCO_2 in seawater are not occurring in isolation, given the concurrent threat of other stressors, especially considering anthropogenic pollution (Roberts et al., 2013). In fact, recent research demonstrated that OA may alter the bioavailability of inorganic contaminants such as metal(loid)s (Zeng et al., 2015), by increasing the relative amount of free metal form, given that pH influences the adsorption properties of these elements with dissolved organic matter, carbonate ion, sulphide, and iron (oxy)hydroxide minerals. OA is therefore expected to increase metal bioavailability, thus potentially increasing element uptake by organisms (Martín-Torre et al., 2017). Such interactions become most relevant in highly dynamic ecosystems such as estuaries, which function as major sinks for contaminants. The contaminants accumulate particularly in the sediment compartment, but tend to be remobilized into the liquid phase during sediment resuspension events (e.g. waves, storms, tidal currents, bioturbation, dredging), during which contaminants concentrations may exponentially increase in the water column (Harley et al., 2006; Mamindy-Pajany et al., 2013; Robins et al., 2016). Arsenic (As) is a highly toxic metalloid known to accumulate in estuarine sediments (e.g. 24.7 mg kg^{-1} d.w.; Costa et al., 2008) and to re-enter the water phase during (bio)turbation events (De Gieter et al., 2005; Masson et al., 2007; Mamindy-Pajany et al., 2013) (e.g. 260 mg kg^{-1} in suspended particulate matter; Ereira et al., 2015), as well as during extreme weather events (Henke, 2009; Galloway et al., 2017).

These issues raise the question on the impacts of increased As exposure concentrations to biota, and to its toxicology when interacting with climate change-related phenomena like OA.

The current understanding of the complex interaction of climate change-associated stressors like OA and contaminants such as As is still in its infancy, and the need to improve the predictive power on the impacts of hazardous substances in aquatic ecosystems is becoming increasingly urgent considering the eminent threat of climate change (Niinemets et al., 2017). Accordingly, the combined effects of multiple stressors on marine organisms is a growing field of research, and the interaction between OA and contaminants has gained increasing attention (Freitas et al., 2016; Nardi et al., 2017, among others).

The sessile nature, calcification and accumulation capacity have made marine bivalve species preferential models to study the interactive effects of OA and pollutants. Among marine bivalves the Ostreidae family includes some of the most extensively studied species (Tomanek et al., 2011; Ivanina and Sokolova, 2013; Matoo et al., 2013; Moreira et al., 2016). *Crassostrea gigas* (Thun.) and *C. angulata* (Lam.) are the most important cultured oyster species at a global level (Buestel et al., 2009; Hsiao et al., 2016), and hold major ecological (Grabowski et al., 2012) and socio-economic importance (FAO, 2015). However, these species present different ecophysiological traits (e.g. growth, filtration rate, energy metabolism) despite occupying the same ecological niche (Goulletquer et al., 1999; Soletchnik et al., 2002; Li et al., 2017), thus raising the question on how competitive advantages may define species resilience in a changing environment.

Proteomic approaches to evaluate environmental stress give powerful insights into the modes of action (MoA) involved in stress adaptation of marine organisms in response to external stimuli. Moreover, proteomics allows comparisons between closely related congeners and discriminate if different environmental stressors induce the same stress response (reviewed in Tomanek, 2015). Recent studies applied proteomics to assess closely related *Mytilus* congeners (Tomanek et al., 2011), and different breeding lines of *Saccostrea glomerata* (Thompson et al., 2016) in response to different environmental stressors (e.g. thermal, osmotic and hypercapnic stress). However, none of them focused on the combined effects of OA and As exposure.

Hence, the present study aimed to compare the response of two closely related oyster species, *C. angulata* and *C. gigas*, after laboratory exposures to As, OA (Low pH), and the combined effects of both stressors (Low pH + As), bringing new insights on the ability of these species to respond to a changing environment. To achieve this objective, a proteomic approach was applied to compare protein expression profiles after 28 days of exposure.

2. Materials and methods

2.1. Experimental setup

Juvenile *C. angulata* and *C. gigas* specimens were obtained from aquaculture producers in the Sado estuary – Portugal (SW Europe) in April 2017. Similar-sized oysters were selected for laboratory exposures (2.6–3.8 cm height, 1.9–2.3 cm length). Oysters were acclimated to laboratory conditions for 6 weeks in separate 400 L tanks (one per species) in recirculated artificial seawater (Tropic Marine Sea Salt) set at 30 ± 1 salinity, 17 °C (Hailea), UV filtration (TMC Vecton[2]), protein skimming (Deltec), and a total recirculation rate of 3000 L h^{-1} (Eheim). During the acclimation period seawater was partially renewed every 2 days (30%) and completely renewed every week. After the first week of acclimation, oysters were fed live microalgae (*Isochrysis galbana* and *Chaetoceros calcitrans*) 5 days per week, at a daily ration of ca. 10^9 cells L^{-1} day $^{-1}$ (T-*Iso* equivalents cells).

After the acclimation period, oysters were transferred to 20 L aquaria (10 oysters per aquarium) in a triplicate design. Aquaria were filled with fresh seawater and used to simulate 4 separate conditions: control (CTL), As exposure (As), seawater acidification (Low pH) and the combination of seawater acidification and As exposure (Low pH + As).

For As exposure, a stock solution of sodium arsenate (Na_3AsO_4) (CAS no. 10048-95-0) was directly spiked into aquaria to achieve a final concentration of 500 μg As L^{-1}. Arsenic exposure concentration was chosen considering: i) previous studies that showed sublethal effects of As to adult oysters (Zhang et al., 2015a; Moreira et al., 2016); median effect concentrations to oyster embryo-larval development (Martin et al., 1981; Mamindy-Pajany et al., 2013; Moreira et al., 2018); iii) data on As concentrations in the water column from suspended particulate matter (260 mg kg^{-1}, Ereira et al., 2015), and considering that OA increases the bioavailability of elements (Martín-Torre et al., 2017; Zeng et al., 2015).

Seawater acidification was achieved by pH manipulation using a pH stat system (Aquamedic) targeting a 0.4 pH unit decrease from CTL conditions (pH = 8.0) to pH 7.6. Seawater pH of acidified conditions was gradually lowered 0.1 pH units per day until target pH level was achieved to prevent an abrupt change in seawater acidity. The chosen pH levels were based on: i) the average pH in the Sado Estuary sampling area (Amaral and Costa, 1999); ii) present global oceanic seawater pH and worst case scenario projections of pH decrease by year 2100 (Caldeira and Wickett, 2005; Raven et al., 2005); and pCO_2 levels (ca. 600 and 1650 μatm) within those projected for estuarine systems under present and future acidification conditions (Tomanek et al., 2011; Melzner et al., 2012). To achieve this, independent pH probes were used to constantly monitor pH in every aquarium in all acidified conditions. Probes were linked to a central computer (Aquamedic) that enabled to automatically switch on or off a dedicated solenoid valve for each aquarium, enabling for CO_2 gas to flow through glass diffusers and maintain targeted pH levels. Each pH electrode measurement was crosschecked with an independent probe (Hanna Instruments) at least every two days, and the pH stat system electrodes recalibrated if necessary.

Experimental exposures were performed during 28 days. Maintenance procedures included daily faecal debris removal (ca. 5% water change), feeding (*I. galbana* and *C. calcitrans* at 10^9 T-*Iso* equivalent cells L^{-1}) at least 5 days per week, As concentration replenishment, pH monitoring and total weekly water renewals. Prior to complete water renewals pH, salinity and temperature were annotated (Hanna Instruments), and seawater samples collected from each

Table 1
Experimental conditions (mean ± SD; n = 4). pH, total alkalinity (Ta), partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate ions concentrations (CO_3^{2-}), saturation states of calcite (ΩCal) and aragonite (ΩAg), calculated with CO2SYS software (Robins et al., 2016) (Temperature 19.7 °C ± 0.4 and Salinity 31 ± 1).

Condition		pH	Ta (μmol kg^{-1})	pCO2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^- (μmol kg^{-1})	ΩAr	ΩCa
C. angulata	CTL	8.01 ± 0.03	2046 ± 54	576 ± 34	1776 ± 49	110.2 ± 5.2	2.7 ± 0.1	1.8 ± 0.09
	As	7.99 ± 0.02	2103 ± 41	599 ± 28	1830 ± 37	110.2 ± 4.9	2.7 ± 0.1	1.7 ± 0.08
	Low pH	7.59 ± 0.03	2087 ± 61	1633 ± 110	1970 ± 56	46.4 ± 3.6	1.1 ± 0.08	0.7 ± 0.05
	Low pH + As	7.59 ± 0.02	2129 ± 44	1734 ± 99	2015 ± 42	46.4 ± 2.2	1.1 ± 0.05	0.7 ± 0.03
C. gigas	CTL	7.99 ± 0.02	2001 ± 58	586 ± 21	1743 ± 49	102.8 ± 4.7	2.5 ± 0.1	1.6 ± 0.07
	As	7.98 ± 0.04	1996 ± 59	577 ± 13	1739 ± 46	102.8 ± 5.9	2.5 ± 0.1	1.6 ± 0.09
	Low pH	7.57 ± 0.01	2040 ± 64	1732 ± 84	1933 ± 63	42.7 ± 1.7	1.1 ± 0.04	0.7 ± 0.03
	Low pH + As	7.58 ± 0.02	2027 ± 62	1654 ± 62	1920 ± 55	43.8 ± 3.2	1.1 ± 0.08	0.7 ± 0.05

aquarium to determine total alkalinity (TA). TA was determined by potentiometric titration (Gran, 1952) for each aquarium every week and data obtained were plotted together with pH, temperature and salinity measurements corresponding to the time of each sample collection in CO2SYS Calc software (Robbins et al., 2010) to further characterize seawater carbonate system parameters using K1 and K2 CO_2 dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and $KHSO_4$ from Dickson (1990) (Table 1).

After 28 days exposure period, oysters were immediately frozen in liquid nitrogen, and stored at -80 °C for further analysis.

2.2. Arsenic concentrations in oysters' soft tissue

Total As concentration in oysters was quantified in whole soft tissue homogenates of 3 oysters from each experimental condition replicate, including in CTL following the protocol described in Freitas et al. (2012). Briefly, each specimen's soft tissue was homogenised using a mortar and pestle under liquid nitrogen. Known weight aliquots (ca. 0.3 g) from each oyster were digested overnight in Teflon bombs with analytical grade HNO_3 and H_2O_2 (30% w/w) (Chem-Lab NV). The digested samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS) and total As determined based on IV- 71 A standard (Inorganic Ventures). Calibration curve was verified with standard reference material (NIST SRM 1643) (calculated measure of trueness over 90%). The recovery percentage of the digestion procedure was verified by parallel digestion of reference standard material TORT-3 (Lobster Hepatopancreas, NRC Canada) and recovery percentages determined for As (103.7%). Results were expressed in μg g^{-1} wet weight (w.w.).

2.3. Protein extract preparation

Oysters' whole soft tissue were used for protein extractions. For each species, the soft tissue of two oysters per condition replicate were pooled together, and 3 individual samples (n = 3) per experimental condition were analysed. Samples were weighed and homogenised (1:3 w/v) in 10 mM HEPES buffer, 250 mM sucrose, 1 mM DTT, 1 mM Na_2EDTA, 1 mM PMSF, and protease inhibitor (Sigma Aldrich Tablets) with an Ultra-Turrax homogenizer on ice (4 °C). Homogenates were centrifuged at 15 000 *g* (4 °C) for 2 h, and the cytosolic fraction (supernatant) collected, separated in aliquots and stored (-80 °C) or immediately used for protein concentration determination following the Bradford method using BSA as standard (Bradford, 1976).

Posteriorly, supernatant volume corresponding to 150 μg protein from each sample was precipitated in 10% TCA in acetone solution at 1:9 (v/v) for 2 h at -20 °C and centrifuged at 10000 *g* for 30 min (4 °C). After this procedure the pellet containing precipitated protein was washed with ice-cold acetone. Washing procedure was repeated three times, and the pellet left to dry for 3–5 min. The pellet containing dried protein extracts of each sample were reconstituted in 300 μL lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (w/v) pharmalyte, 65 Mm DTT, protease inhibitor (Sigma Aldrich Tablets), and traces of bromophenol blue) for 30 min, centrifuged at 14 000 *g* for 10 min. Samples were immediately used or stored at −80 °C for further analysis.

2.4. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed twice for each sample, in order to obtain 6 gel images representative of each condition. For the first-dimension protein separation by isoelectric focusing (IEF), the sample (150 μg protein in 300 μL lysis buffer) was loaded into 24 cm ceramic immobilized pH gradient (IPG) holders (GE Healthcare). IPG strips (GE Healthcare Immobiline DryStrip pH 4–7, 18 cm) were carefully introduced over each sample. The IPG ceramic holders were sealed with dedicated mineral oil (GE Healthcare DryStrip Cover Fluid) and lids, holding each sample and the IPG strip inside, were placed. IEF was performed on an Ettan IPGphor 3 System (GE Healthcare) set to develop the following programme: 6:30 h at 0 V (rehydration); 6:30 h at 50 V (fast); 1 h at 1000 V (linear); 1 h at 4000 V (fast); 1 h at 8000 V (linear); 1 h 8000 V (fast). After IEF, IPG strips containing separated proteins by their isoelectric point, were removed from the ceramic holders and stored at -80 °C.

Before second-dimension protein separation, IPG strips were equilibrated by consecutive emersion in two SDS equilibration buffers (6 M urea, 75 mM Tris-HCl pH 8.8; 29.3% glycerol; 4% SDS) for 15 min each: the first SDS equilibration buffer contained 1% (w/v) DTT, 0.1 mM EDTA, and a trace amount of bromophenol blue; the second SDS equilibration buffer contained 4% (w/v) iodoacetamide, 0.1 mM EDTA, and a trace amount of bromophenol blue. After equilibration, IPG strips were carefully placed on 10% (v/v) polyacrylamide gels (made from 40% Acrylamide/Bis Solution 37.5:1, Biorad) containing 0.1% SDS (w/v), sealed using 0.5% agarose, and run under denaturing conditions in an Ettan Dalt 6 (GE Healthcare) refrigerated at 20 °C following a two-step program: 15 mA and 5 w per gel (120 V) for 30 min; plus 60 mA and 17 W per gel (500 V) for ca. 5 h (until the front die reached the end of the gel). After second dimension separation, gels were fixed overnight in 40% (v/v) ethanol and 10% (v/v) acetic acid. Gels were silver stained following Blum et al. (1987). After staining, gels were scanned with a densitometer (Bio-Rad, GS-800).

A total of 6 gel images were obtained representative of each condition for each species (3 experimental replicates times 2 duplicates).

2.5. Data analysis

2.5.1. Arsenic concentrations in oysters' soft tissue

Statistical analysis was performed to verify the existence of significant differences concerning accumulated As concentrations among conditions using permutational analysis of variance (PERMANOVA) with PRIMER v6 software (Anderson et al., 2008). Briefly, hypotheses testing was performed on Euclidean distances similarity matrix constructed based on As concentration data of each sample. "Experimental

condition” and “species” were defined as the fixed factors. One-way hierarchical designs considering 9999 permutations were followed to test the null hypothesis: H_0’ = for each species no significant differences existed in total accumulated As among conditions; H_0” = for each condition no significant differences existed in total accumulated As between species. Significant differences ($p \leq 0.05$) among conditions were represented with different letters, differences between species were represented with an asterisk.

2.5.2. 2-DE proteomic data analysis

Gel image (2-DE map) analysis and statistics were performed using PDQuest software version 8.0.1 (Bio-Rad). Master gels representative of each condition were constructed based on 6 gels (3 experimental replicates times 2 duplicates). Protein spot intensity of each 2-DE map was normalized by total density of each gel image. Each protein spot assignment and matching between gels were verified using the software’s matching and group consensus tools. Reproducibility of the 2-DE process was verified and accepted for mean coefficients of variation among gels representative of the same condition over 70%. Analysis followed pairwise comparisons between all conditions by overlaying master gels’ 2-DE maps. The analysis was performed separate for each species.

Protein expression was considered differentially altered between conditions whenever the intersection of quantitative (2-fold or higher) and statistically different ($p \leq 0.05$, Student’s *t*-test) spot intensity changes were observed. Differentially expressed protein spots were ranked by highest fold change in each condition, and the most important proteins (top 10 increased or decreased expression levels) for each condition were selected for excision and mass spectrometry analysis.

2.6. Protein identification by mass spectrometry

In-gel protein digestion was performed as outlined by Shevchenko et al. (2006). Before MS analysis, samples were resuspended in 10 μL of formic acid 0.3% (v/v) and 0.5 μL of sample was hand-spotted onto a MALDI target plate (384-spot ground steel plate). Posteriorly, 1 μL of a 7 mg/mL solution of α-cyano-4-hydroxycinnamic acid matrix in 50% (vol/vol) acetonitrile in aqueous trifluoroacetic acid 0.1% (vol/vol) was added and allowed to dry. Mass spectra were acquired on an Ultraflex II MALDI TOF mass spectrometer (Bruker) operated in positive ion mode using a reflectron (*m/z* range of 600–3500). A total of 500 spectra were acquired for each sample at a laser frequency of 50 Hz. External calibration was performed with $[M+H]^+$ monoisotopic peaks of bradykinin 1–7 (*m/z* 757.3992), angiotensin II (*m/z* 1046.5418), angiotensin I (*m/z* 1296.6848) substance P (*m/z* 1758.9326), ACTH clip 1–17 (*m/z* 2093.0862), ACTH18–39 (*m/z* 2465.1983) and somatostatin 28 (*m/z* 3147.4710). Peptide mass fingerprints (PMF) were searched via MASCOT search engine set for the following parameters: (i) NCBInr *Crassostrea* (138572 sequences; 81064928 residues); (ii) molecular weight of protein: all; (iii) two missed cleavage; (iv) fixed modifications: carbamidomethylation (C); (v) variable modifications: oxidation of methionine and (vi) peptide tolerance up to 100 ppm. The significance threshold was set to a minimum of 95% ($p \leq 0.05$). Protein matching was considered successful when protein identification score was located out of the random region (Mascot score ≥ 64).

3. Results and discussion

3.1. Arsenic accumulation in oyster tissues

Overall, the average concentration of As in oysters ranged from ca 1.0 to 5.4 μg g^{-1} w.w. in both species (Fig. 1) (equivalent to ca. 5 and 27 μg g^{-1} dry weight (d.w.) (Zhang et al., 2013). Residual As concentrations (ca. 1 μg g^{-1} w.w.) were observed in both oyster species (CTL), evidencing As contamination levels in the estuary where oysters were collected (Costa et al., 2009). In the present study, the highest As concentrations (5.4 μg g^{-1} w.w. = ca. 27 μg g^{-1} d.w.) accumulated in oyster tissues after exposures were equivalent to reported values in oysters collected in other estuarine systems e.g. up to 26.7 μg g^{-1} d.w. in *C. gigas* in France (Kohlmeyer et al., 2002) and 25.4 μg g^{-1} d.w. in *C. virginica* from the U.S.A. (Valette-Silver et al., 1999).

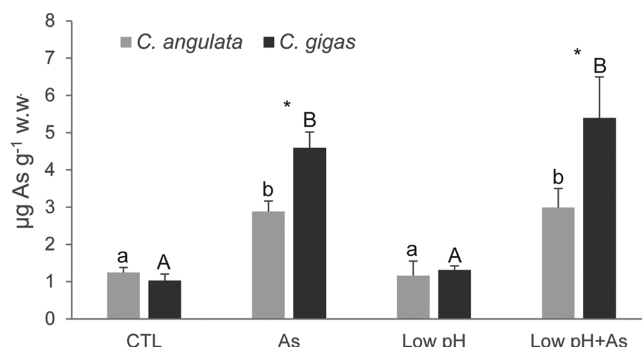

Fig. 1. Arsenic concentrations (mean + SD; n = 9) in juvenile *C. angulata* and *C. gigas* whole soft tissues unexposed (CTL) and exposed for 28 days to As, Low pH and Low pH + As. Significant differences in As concentrations between conditions are represented with different letters: lower-case letters for *C. angulata* and upper-case letters for *C. gigas*. Significant differences between species in each condition are represented with *.

No significant differences were observed concerning As concentration between unexposed (CTL) and exposed oysters at Low pH for either species (Fig. 1), while As concentrations were significantly higher in both As exposures (As; Low pH + As) than in CTL. No significant differences were observed in accumulated As concentrations between oysters exposed to As and those exposed to Low pH + As in either species (Fig. 1). These results show that seawater acidification did not influence oysters’ uptake/excretion dynamics of As. A previous study conducted by Moreira et al. (2016) also demonstrated that As accumulation patterns of fully grown *C. gigas* and *C. angulata* year recruits was not influenced by seawater acidification.

Between-species comparison of As concentrations in oyster tissues within each experimental condition showed that *C. gigas* accumulated significantly higher amounts of As than *C. angulata* (in both As and Low pH + As conditions). Factors such as higher filtration/metabolic rates in *C. gigas* (Heral et al., 1986; Goulletquer et al., 1999) and likely higher metal tolerance in *C. angulata* (Cross et al., 2014) could explain different accumulation capacities observed between species, highlighting previously described ecophysiological differences between these closely related congeners (His, 1972; Heral et al., 1986; Soletchnik et al., 2002; Moreira et al., 2016 among others).

3.2. Protein changes in Crassostrea angulata and Crassostrea gigas

Proteomic analysis of *C. angulata* showed differentially expressed protein profiles between all tested conditions (Fig. 2). Protein identification through MS allowed to identify several proteins with significantly altered expression levels among conditions, which play biological roles related to metabolism (ATP synthase subunit β), cytoskeletal structure (Actin, Coronin 1-B; Severin; Gelsolin) cellular stress response (Retinal dehydrogenase, Lactoylglutathione lyase and Alpha crystallin b chain), cell signalling (progesterone-induced-blocking factor 1; piRNA biogenesis protein) proteolysis (aminopeptidase W07G4.4), cell differentiation/apoptosis (MYCBP-associated protein), cell to cell adherence related proteins (Ependymin, Tight junction protein ZO-1), transport (V-type proton ATPase subunit B), as well as two uncharacterized proteins (KIAA1109-like isoform X5; LOC105343084 isoform X1) (Table 2 and Fig. 2).

Proteomic analysis of *C. gigas* also showed differentially expressed protein profiles between all tested conditions (Fig. 2). MS analysis

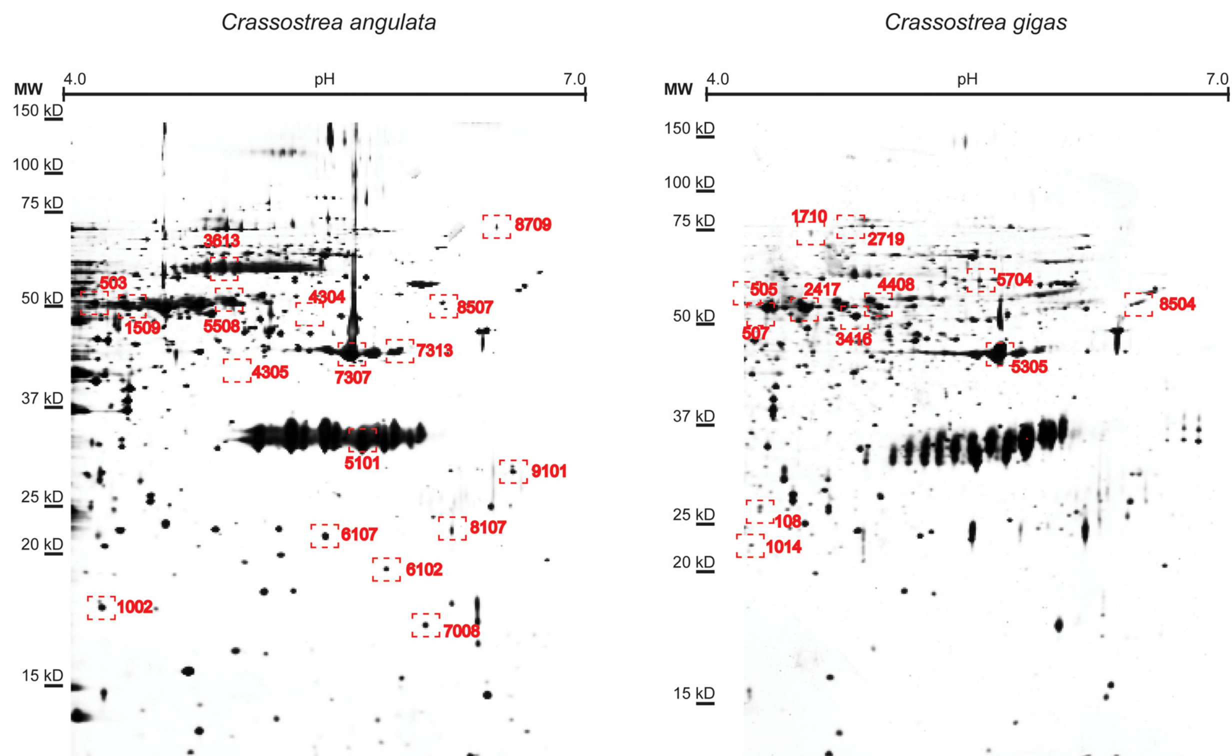

Fig. 2. Protein expression profile of juvenile *C. angulata* (left) and *C. gigas* (right) whole soft tissue by 2-DE. Differentially expressed proteins identified by MALDI TOF MS are highlighted in each representative gel image.

allowed to identify significantly altered proteins with biological functions related to metabolism (Enolase; Aconitate hydratase), cytoskeletal structure (Actin; Atlastine), extracellular structure (Neprilysin) and cellular stress response (Heme-binding protein 2; Arginine-tRNA-protein transferase 1; Alpha crystallin a chain; Atypical serine-protein kinase ATM; Aldehyde dehydrogenase; and Retinal dehydrogenase) (Table 2 and Fig. 2). One uncharacterized protein LOC105317411 (annotated in the *C. gigas* genome) was also identified, but its molecular and biological roles remain unknown.

Exposure of oysters to different scenarios of environmental stress such as As exposure (As), seawater acidification (Low pH), and simultaneous exposure to seawater acidification and As (Low pH + As) allowed to observe different protein expression profiles compared to control (CTL), but also among all stress conditions providing a deeper insight into the mechanisms involved in the responses of both species to different environmental stimuli.

3.3. *Crassostrea angulata*

3.3.1. Protein changes under As exposure

C. angulata exposed to As presented higher expression levels of cytoskeleton-related proteins compared to levels observed in CTL, namely Actin (Actin), Severin (Severin) and Gelsolin-like protein 2 (Gelsolin) (Table 2). Alterations in the expression levels of cytoskeletal proteins is a common response observed in marine organisms exposed to environmental stress (Tomanek, 2014). Altered levels of Actin have also been reported in other oyster species (*S. cucculata* and *S. glomerata*) exposed to different types of contaminants (Muralidharan et al., 2012; Thompson et al., 2012; Khondee et al., 2016; Melwani et al., 2016), and could be indicative of cytoskeleton rearrangement to prevent oxidative damage (Dailianis et al., 2009).

Additionally, Severin and Gelsolin (both assigned to the Gelsolin superfamily) that play regulatory roles on Actin filament assembly and disassembly processes, are key proteins in cytoskeleton structure maintenance and remodelling (Silacci et al., 2004). The expression levels of these proteins were increased in As-exposed *C. angulata* relative to CTL (and to Low pH), and further corroborate the role of Actin dynamics in *C. angulata* stress response to As. Other studies have described altered levels of Severin and Gelsolin-like proteins in bivalves experiencing environmental stress. For instance, increased expression of Severin has been described in *C. gigas* under thermal and hypercapnic stress (Dineshram et al., 2015; Harney et al., 2016), and in *S. glomerata* exposed to highly polluted sites (Melwani et al., 2016). Likewise, altered abundance of Gelsolin-like proteins was described in *S. cucculata* exposed to TBT (Khondee et al., 2016), and *Pecten maximus* under hypoxia (Artigaud et al., 2015). In addition the importance of cytoskeletal proteins on Actin dynamics regulation, several other biological functions have been linked to Gelsolin superfamily proteins, which include modulation of cell motility, cortical shape changes, apoptosis and phagocytosis (Silacci et al., 2004; Li et al., 2009). Considering these studies, and the present findings showing increased expression of Actin, Severin and Gelsolin in *C. angulata* exposed to As, results suggest eminent cytoskeleton remodelling and could also be indicative of a pro-apoptotic cellular status in *C. angulata* exposed to As.

Lactoylglutathione lyase (Lgl) levels were significantly lower in *C. angulata* exposed to As compared to the remaining conditions including CTL (Table 2). Other studies have shown a negative correlation between Lgl and pollutant exposure, including in *Macoma balthica* exposed to As (Regoli et al., 1998), and in *S. glomerata* exposed to high-impacted sites (PAHs, PCBs, TBT, Pb and Zn) (Melwani et al., 2016). This enzyme is important in the glyoxalase system by participating in detoxification reactions of reactive α-ketoaldehydes (glycolytic by-products) using glutathione as cofactor (Regoli and Giuliani, 2014). Provided that Lgl regulation depends on cellular redox status (Birkenmeier et al., 2010), it is possible that the decrease of Lgl abundance indicates cellular redox imbalance induced by As exposure.

Decreased expression of Progesterone-induced blocking factor (Pibf), was observed in *C. angulata* exposed to As compared to CTL (also compared to Low pH + As) (Table 1). In mammals, Pibf is involved in immunomodulation, cytotoxic control, cell cycle regulation, cytokine

Table 2
Differentially expressed proteins identified in *C. angulata* or *C. gigas* by MALDI TOF MS. Observed molecular weight (MW) and isoelectric point (pI) are provided. Fold change of spot density between tested groups (CTL; As ; Low pH; and Low pH + As) are expressed after pairwise comparisons that showed significant differences ($p \leq 0.05$).

Crassostrea angulata

Spot no.	GenBank ass. no.	Protein name	Mascot score	MW(kDa)/pI	Putative biological function(s)	Fold change
503	XP_011429993.1	Putative aminopeptidase W07G4.4	64	51.8/4.3	Proteolysis	3.3 ↑ in Low pH + As vs. Low pH 7.1 ↑ in Low pH + As vs. As
1002	XP_011447841.1	MYCBP-associated protein-like isoform X5	64	18.5/4.4	Cell differentiation and apoptosis	9.1 ↑ Low pH + As vs. As
1509	XP_011436817.1	Retinal dehydrogenase 1	108	59.5/4.5	Regeneration	2.3 ↑ in Low pH vs. CTL
3613	XP_011429476.1	Coronin-1B isoform X5	118	64.0/4.9	Cytoskeleton	2.6 ↑ in Low pH + As vs. CTL
4304	EKC22157.1	Severin	108	55.1/5.2	Cytoskeleton	4.3 ↑ in As vs. CTL 4.8 ↑ in As vs. Low pH
4305	EKC30581.1	Progesterone-induced-blocking factor 1 (Pibf)	100	38.6/5.0	Immunomodulation	5.0 ↑ CTL vs. As 10.0 ↑ in Low pH + As vs. As
5101	XP_022328428.1	Uncharacterized protein KIAA1109-like isoform X5	64	34.0/5.5	Lipid metabolism	2.8 ↑ in As vs Low pH 4.2 ↑ in As vs. Low pH + As
5508	EKC36437.1	V-type proton ATPase subunit B	73	53.3/5.1	Transport	3.3 ↑ in Low pH vs. Low pH + As
6102	EKC27629.1	Alpha-crystallin B chain	111	17.3/5.6	Molecular chaperone	2.0 ↓ in As vs Low pH + As 3.0 ↓ in Low pH + As vs. CTL 4.3 ↓ in As vs Low pH 6.3 ↓ in As vs. CTL
6107	XP_011413901.1	Ependymin-related protein 1	114	22.0/5.3	Cell-matrix adhesion	4.4 ↑ in Low pH vs. Low pH + As
7008	XP_011422530.1	Lactoylglutathione lyase	118	17.8/5.7	Antioxidant defense	5.9 ↑ in Low pH + As vs. As 5.6 ↑ in CTL vs. As 5.9 ↑ in Low pH vs. As
7303	XP_011427051.1	Gelsolin-like protein 2	80	54.6/5.9	Cytoskeleton	2.6 ↑ in As vs. Low pH 4.1 ↑ in As vs. CTL
7307	NP_001295788.1	Actin	78	47.6/5.6	Cytoskeleton	4.7 ↑ in Low pH + As vs. CTL 5.9 ↑ in As vs. CTL
8107	XP_022321285.1	piRNA biogenesis protein EXD1-like	64	26.4/5.9	Cell signaling	6.7 ↓ in Low pH + As vs. CTL
8507	EKC39411.1	ATP synthase subunit beta. mitochondrial	64	65.5/5.8	Oxidative phosphorylation	2.8 ↓ in Low pH + As vs. CTL 5.2 ↓ in Low pH vs. CTL
8709	EKC34842.1	Tight junction protein ZO-1	88	91.1/6.0	Cell-Cell adherence	6.7 ↑ in CTL vs As 7.1 ↑ in Low pH vs. As 10.0 ↑ in Low pH + As vs. As
9101	XP_011448567.1	Uncharacterized protein LOC105343084 isoform X1	83	28.0/6.0	Unknown	2.5 ↑ in Low pH vs. Low pH + As

Crassostrea gigas

Spot no.	GenBank ass. no.	Protein name	Mascot score	MW(kDa)/pi	Putative biological function(s)	Fold change
108	EKC31868.1	Arginyl-tRNA–protein transferase 1	65	28.1/4.6	Stress signalling/apoptosis	4.5 ↓ in As vs. CTL
505	CGI_10013191	Atlastin-2-like isoform X4	64	60.9/4.4	ER Dynamics	3.1 ↑ in Low pH vs. CTL
507	XP_022316405.1	Neprilysin-2-like	64	54.6/4.5	Extracelular structure	2.4 ↑ Low pH vs. CTL
1014	XP_011448780.1	Alpha-crystallin A chain	90	23.5/4.5	Stress protein	7.1 ↓ in As vs. Low ph
1710	XP_011445349.1	Heme-binding protein 2	60	82.1/4.6	Redox balance Imune response	2.6 ↑ in As vs. CTL
2417	XP_011436817.1	Retinal dehydrogenase	129	59.5/4.5	Stress response	2.2 ↑ in Low pH vs Low pH + As
2719	XP_011411873.1	Aconitate hydratase isoform X2. cytoplasmic	90	91.8/4.7	Metabolism	2.7 ↑ in As vs Low pH
3416	XP_011436228.1	Enolase	87	55.6/4.9	Glycolitic process	2.8 ↑ in Low pH vs. Low pH + As
4408	XP_011450475.1	Aldehyde dehydrogenase. mitochondrial	94	59.5/4.8	Antioxidant	2.9 ↑ in Low pH + As vs Low pH
5305	EKC30048.1	Actin	119	45.7/5.4	Cytoskeleton	3.8 ↑ in Low pH vs. Low pH + As 4.0 ↑ in Low pH vs. As 4.1 ↑ in Low pH vs. CTL
5704	XP_019919028.1	Serine-protein kinase ATM	65	66.8/5.4	Cell cycle Stress response	2.2 ↑ in Low pH + As vs. CTL
8504	CGI_10008427	Uncharacterized LOC105317411	64	61.5/6.0	Unknown	2.9 ↓ in Low pH vs. CTL 2.3 ↓ Low pH vs. Low pH + As

balance and cell invasion (Laskarin et al., 2002; Szekeres-Bartho et al., 2005; Balassa et al., 2018). Moreover, Pibf is involved in cell signalling processes by interaction with the interleukin-4 binding system. Although the current knowledge on Interleukin signalling pathways in marine bivalves is still in its infancy (Malagoli, 2010; Li et al., 2014), interleukins are known effectors that modulate inflammatory response of organisms to pathogens, tissue injury and irritants (Liu et al., 2015). Considering this, the present data suggest that decreased expression levels of Pibf observed in *C. angulata* exposed to As were likely related to inflammatory response regulation and modulation of the interleukin-4 binding system. Because Pibf deficiency generally enables natural killer cell activity (Szekeres-Bartho et al., 2005), our data showing lower Pibf could also indicate a pro-apoptotic status of *C. angulata* exposed to As.

Expression levels of Alpha crystallin b chain (α-crystallin b), a member of the small heat shock protein family (sHsp) family were lower in *C. angulata* exposed to As compared to all other tested conditions (Table 2). Heat shock proteins are molecular chaperones

ubiquitous to all clades of life, which maintain correct folding profiles of native proteins, and are generally conceived to be induced in response to a variety of stress stimuli (Gupta et al., 2010). However, the Hsp response in bivalves can be highly dynamic, depending on tissue, time of exposure and type of Hsp (reviewed by Fabbri et al., 2008). For instance, Zhang et al. (2015b) observed increased levels of several sHsps in *C. gigas* exposed to thermal stress, whilst no alteration in the same proteins were observed in oysters exposed to hypoosmotic stress or aerial exposure. Park et al. (2016) also observed differential expression profiles of the gene expression of several Hsp families in response to different biofouling agents. Therefore, our results showing decreased expression of α-crystallin b could result from concerted regulation of Hsps in response to As exposure. Moreover, α-crystallin b depletion could be indicative a pro-apoptotic status according to Acunzo et al. (2012), and therefore could indicate a deleterious cellular status in *C. angulata* exposed to As, also in line with results from Pibf, Severin and Gelsolin previously described.

C. angulata exposed to As showed significantly lower Tight junction ZO-1 protein (ZO-1) levels than in all other conditions (Table 2). ZO-1 are scaffolding proteins integral in the link between the tight junction and the actin cytoskeleton, modulating cell to cell adherence processes. Different elements have been shown to cause tight junction restructuring and deficiency, for instance in mouse respiratory cells exposed to As (Sherwood et al., 2013) and in *Tegillarca granosa* clams exposed to Cd (Bao et al., 2016). Therefore, the present data suggest that As caused impairment of cell to cell adherence proteins in *C. angulata*.

Adding to differences observed between As exposure and CTL, data showed increased expression of Uncharacterized protein KIAA1109-like (spot no. 5001, Table 2) in *C. angulata* exposed to As compared to both acidification conditions (Low pH and Low pH + As). This protein is assigned to the Fragile site-associated protein (Fsa) domain. Abundance of this protein was higher in oysters exposed to As compared to Low pH (and to Low pH + As). Fsa's C-terminus shares high similarity with *lpd-3* gene (Kuo et al., 2006) that is related to lipid storage in nematode *Caenorhabditis elegans* (McKay et al., 2003). The present results on increased KIAA1109-like protein in *C. angulata* exposed to As could be related to alterations in lipid metabolism due to As as observed in other organisms (Carlson and Van Beneden, 2014; Wang et al., 2015).

3.3.2. Protein changes under Low pH

C. angulata exposed to Low pH presented lower abundance of ATP synthase subunit betta (ATP β) in comparison to CTL (Table 2). Other studies have also described ATP synthase subunit depression in marine invertebrates exposed to acidification, namely in coral (Moya et al., 2012), polychaete (Wäge et al., 2016) and bivalve (Hüning et al., 2013) species including *C. gigas* (Harney et al., 2016). ATP synthase is a protein complex situated in the inner mitochondrial membrane and is an important player in ATP synthesis in all living organisms. The decrease of ATP synthase subunit's expression has been discussed as a means of oxidative metabolism suppression (Moya et al., 2012). Metabolic arrest is characteristic of organisms incurring extreme stress (Guppy and Withers, 1999), likely resulting from decreased aerobic scope for ATP synthesis (Sokolova et al., 2012) and a possible indication of a shift to anaerobic metabolism (Hüning et al., 2013). Hence, the decrease of ATP synthase β expression levels observed in *C. angulata* in Low pH condition most likely indicate metabolic depression in response to acidification in this species.

Another protein, retinal dehydrogenase (Rdh), increased in *C. angulata* compared to CTL (Table 2). Thompson et al. (2015) also observed increased Rdh in *Saccostrea glomerata* exposed to high pCO_2. Rdhs catalyse the irreversible oxidation of retinal to retinoic acid (RA) (Gutierrez-Mazariegos et al., 2014). Thus, increased expression of Rdh potentially increases RA concentration in oyster tissues. The physiological effects of such alterations in invertebrates remain to be clarified although RA seems to be involved in several processes from organ formation to differentiation and regeneration (Albalat, 2009; Gutierrez-Mazariegos et al., 2014). The present data, showing increased expression of Rdh suggests a protection mechanism towards acidification.

3.3.3. Protein changes under Low pH + As

Coronin is an actin filament binding protein that participates in Actin dynamics, including filament disassembly, bundling, crosslinking and several other actin network reorganization processes (Rybakin and Clemen, 2005; Lin et al., 2010; Srivastava et al., 2015). In the present study, increased expression of Coronin suggests alterations of Actin dynamics related to cytoskeleton restructuring in *C. angulata* exposed to Low pH + As. Concomitantly, results also showed increased expression of Actin in the same condition, supporting the effective role of increased Coronin observed. Accordingly, other studies (Thompson et al., 2015, 2016) also observed simultaneous increase of both Coronin and Actin in *S. glomerata* exposed to acidification.

Alpha crystallin b (α-crystallin b) expression levels were lower in Low pH + As compared to CTL, similar to results observed in oysters exposed to As. However, the decrease of protein expression levels was 2-fold lower than that observed by As single exposure (Table 2). These findings could be related to the fact that Hsp expression can depend on oysters' energetic fitness. *C. gigas* also presented lower levels of Hsp70 and Hsp69 in close relation to depleted energetic reserves (Li et al., 2007).

Decreased expression levels of ATP synthase β observed in Low pH + As in relation to CTL (Table 2) evidenced energetic efficiency modulation. In line with results observed for Low pH single exposure previously discussed, lower ATP synthase β abundance was likely related to suppression of oxidative metabolism in *C. angulata* exposed to Low pH. However, the decrease of ATP β was lower (2.8 fold) in Low pH + As than in Low pH single exposure (5.2 fold) (Table 2), an indication that metabolic adjustment was differentially modulated under the combination of both stressors (Low pH + As).

piRNA biogenesis protein (piRNA) expression levels were lower (6.7-fold) in Low pH + As comparing to CTL (Table 2). piRNAs are involved in genome integrity maintenance via transposon regulation (Mani and Juliano, 2013; Iwasaki et al., 2015). Transposable elements (TEs) are mobile DNA sequences that can move within the genome, and are silenced by piRNAs (Luo and Lu, 2017). Researchers have recently proposed that stress-induced TE mobility via piRNA suppression allows for genome reconfiguration in response to extreme environmental stress (Ryan et al., 2016), enabling for heritable phenotypic variation (Piacentini et al., 2014). Considering this theory, and the fact that TEs are abundant in the oyster genome (Cross et al., 2014), the present data showing decreased expression levels of piRNA biogenesis protein in *C. angulata* exposed to Low pH + As could imply genome reconfiguration in response to multiple stressors, a novel observation concerning adaptive evolution mechanisms in oysters.

Apart from proteomic differences observed between Low pH + As and CTL, our results also revealed differences in protein abundance profiles between stress conditions (Table 2). For instance, abundance of Aminopeptidase W07G4.4 (Aminopeptidase) and MYCBP-associated protein (Mycbp) increased in Low pH + As compared to Low pH and/or As. On the other hand, *C. angulata* presented lower abundance of V-type proton ATPase subunit B (V-ATPase b), Ependymin-related protein 1 (Epend-1) and one uncharacterized protein (spot number 9101) in Low pH + As compared to levels in Low pH single exposure (Table 2).

Increased levels of Aminopeptidase have also been described in *S. glomerata* oysters exposed to seawater acidification (Thompson et al., 2016), although expression levels of the same Aminopeptidase were decreased in oysters from highly contaminated sites (Melwani et al., 2016). Aminopeptidase molecular function suggests a primary role in protein catabolism (Brooks and Isaac, 2004) and therefore, increased level of Aminopeptidase in *C. angulata* exposed to Low pH + As indicates higher protein turnover necessary to endure oxidative stress (Sokolova et al., 2012).

Expression levels of MYCBP-associated protein (Mycbp) were higher in Low pH + As compared to As single exposure. Mycbp is related to cell growth, proliferation and apoptosis by modulating *Myc* gene activity (Young et al., 2011). Additionally, recent studies showed that Mycbp is a positive modulator of the Hedgehog signalling pathway (Lin et al., 2014), which is conserved among metazoans (Ingham et al., 2011). The roles of Hedgehog post-natal signalling have been recently reviewed by Smelkinson (2017), and include tissue homeostasis maintenance and immunity, which can be activated by pathogenic agents, tissue injury and damaging agents. Considering this, the present results showing increased levels of Mycbp in *C. angulata* exposed to Low pH + As could be linked to Hedgehog signalling activation, as a response mechanism resulting from external stress stimuli for homeostatic maintenance. The fact that significant differences between Low pH + As and As conditions were observed for Mycbp, agrees with the present overall analysis showing that oysters exposed to As alone were experiencing a different kind of external aggression from that in Low pH + As.

Results showing increased levels of V-ATPase b protein in Low pH compared to Low pH + As are in line with previous studies that described V-ATPase b increase in other oyster species (*C. gigas* and *S. glomerata*) exposed to OA (Dineshram et al., 2012; Thompson et al., 2015). V-ATPases are ATP consuming ion channels that play roles in acid-base regulation (HCO_3^-), calcification, carbon concentrating processes, as well as excessive proton excretion in marine invertebrates exposed to OA (Parker et al., 2013; Ivanina and Sokolova, 2015; Tresguerres, 2016). Interestingly, oysters exposed to Low pH + As did not induce increased expression of V-ATPase b (Table 1), evidencing that As influenced oysters' response to acidification. This could be related to the energetic costs associated to V-ATPase,s activity (Tresguerres, 2016) and the possible preferential energy allocation towards As detoxification processes in Low pH + As condition. These results highlight different modes of action of *C. angulata* towards multiple stressors.

Ependymin related-protein 1 (Epend) expression levels were also increased in Low pH compared to Low pH + As (Table 2). Ependymin's physiological role is related to cell adhesion processes through cell-matrix contact formation. Generally, increased expression of these proteins reflects tissue remodelling in response to environmental stimuli as reported in *Littorina saxatilis* (Muraeva et al., 2016) and *C. gigas* (Zhao et al., 2012) under osmotic stress. Zhang et al. (2015b), observed a versatile behaviour of ependymin proteins in *C. gigas* exposed to different abiotic factors. These authors described increased expression of Ependymin in oysters exposed to thermal and osmotic stress, while the expression levels of the same proteins were lower under aerial exposure. The present results suggest that Low pH induced higher Epend to modulate cell adhesion, while in the presence of As (Low pH + As) *C. angulata* presented lower capacity to promote Epend biosynthesis with possible impacts on tissue structure modulation capacity.

Other proteins presenting altered abundance levels in single exposures (Low pH or As) did not present the same alteration in oysters exposed to the combination of both stressors (e.g. Rdh, Severin, Gelsolin, Pibf, ZO-1). No alteration of Rdh levels was observed in *C. angulata* exposed to Low pH + As, contrasting with increased expression of Rdh in Low pH single exposure, could be related to inhibition of this enzyme by metals (Luo et al., 2014; Meng et al., 2015; Bao et al., 2016). These data show that the combined effects of Low pH and As induce a different response from that of the isolated exposure to Low pH regarding Rdh, with likely implications at the physiological level.

Both Severin and Gelsolin, that increased in As single exposure, did not show alterations under Low pH + As, evidencing that multiple stressors induced a differentiated response capacity at the cytoskeletal level. Moreover, *C. angulata* exposed to Low pH + As presented the highest fold change of ZO-1 (10-fold lower, Table 1) and Pibf abundance (10-fold higher) compared to single As exposure, indicating a stronger modulation of cell to cell adherence capacity (ZO-1), and higher immunomodulation response (Pibf) in *C. angulata* exposed to multiple stressors.

3.4. *Crassostrea gigas*

3.4.1. Protein changes under As exposure

Differentially expressed proteins identified in *C. gigas* exposed to As in comparison to CTL were Heme-binding protein 2 (Heme-bp) and Arginyl-tRNA-protein transferase 1 (Ate-1) (Table 2).

Expression levels of Heme-binding protein 2 (Heme-bp), assigned to the SOUL heme-binding superfamily, were increased in *C. gigas* exposed to As. Other studies have described upregulated heme binding genes in marine invertebrates under environmental stress, including *C. gigas* infected by Ostreid herpesvirus-1 (He et al., 2015) and *Tigriopus japonicus* copepods exposed to Cu (Ki et al. 2009). Even though the current knowledge on the specific physiological roles of these proteins remains cryptic, their involvement in thiol/disulphide redox switches that modulate ion channel functioning, heme affinity and carbon monoxide metabolism appear consensual (Ragsdale and Yi, 2011). Moreover, different biological functions can be linked to this family of proteins across different clades of life, such as oxidative stress repair, apoptosis and immune response (Fortunato et al., 2016). Therefore, increased expression of Heme-bp suggests As induced stress response in *C. gigas*.

Arginine-tRNA-protein transferase 1 (Ate-1) expression levels were lower in *C. gigas* exposed to As in comparison to CTL (Table 2). Ate-1 mediates proteolysis by catalysing protein arginylation, a process involved in actin cytoskeleton regulation (Kashina, 2014). Additionally, Kumar et al. (2016) recently studied Ate-1 and arginylation processes during stress response signalling towards oxidative, thermal, osmotic and metal stress. Among several findings, these authors suggested that Ate-1 depletion may increase cellular resistance to several stressors (e.g. H_2O_2, $CdCl_2$, ultra violet radiation and others) and supress apoptosis (Kumar et al., 2016). Considering these findings, decreased abundance of Ate-1 in *C. gigas* exposed to As suggests increased stress resistance and apoptosis suppression. Interestingly, oysters exposed to the combination of Low pH + As did not present decreased Ate-1 levels, indicating either impairment of this process or a less deleterious oxidative status in *C. gigas* exposed to Low pH + As.

Significant differences were also observed between protein expression profiles of As exposed oysters compared to conditions other than CTL. Alpha-crystallin a chain (α-crystallin a), was significantly lower in As compared to Low pH. This difference could be related to the dynamic expression of sHsps (Morrow and Tanguay, 2012) as discussed for α-crystallin b in *C. angulata* (Section 3.4.1) and provide further evidence of concerted regulation of sHsps towards different types of stressors, and/or a evidencing a pro-apoptotic status as previously discussed.

Results also showed higher abundance of Aconitate hydratase (Achd) in *C. gigas* exposed to As in comparison to oysters exposed to Low pH (Table 2). This mitochondrial enzyme is an important player in the TCA cycle, and a potential oxidative stress biomarker (Bota et al., 2002). Khondee et al. (2016) also observed increased Achd abundance in *C. gigas* exposed to TBT and explained their results as a stimulation of the energy flow through the TCA cycle to fuel energetically demanding responses towards xenobiotic exposure. Because Achd is sensitive to oxidative stress (Cherkasov et al., 2007), its transcriptional upregulation has also been discussed as a compensation mechanism to counteract ROS-mediated self-inactivation in *Argopecten irradians* scallops under hypoxia (Ivanina et al., 2016). Hence, the present findings showing altered Achd abundance suggest relatively higher oxidative stress and increased energetic flow through the TCA cycle in *C. gigas* exposed to As compared to those exposed to Low pH.

3.4.2. Protein changes induced by Low pH

C. gigas exposed to Low pH presented increased expression levels of Actin, Atlastin and Neprilysin in comparison to oysters from CTL (Table 2). Increased Actin levels in oysters (*S. glomerata*) exposed to

acidification scenarios were described (Thompson et al., 2015, 2016), and discussed as a compensation mechanism to replace damaged proteins incurring oxidative damage. Actin, the predominant structural component of the cytoskeleton (Small, 1988) is among the most differentially expressed proteins in oyster species experiencing environmental stress (reviewed in Anderson et al., 2015). Hence, the present results show significantly higher abundance of Actin in oysters exposed to Low pH suggest cytoskeleton restructuring and a possible prooxidant status.

The present findings further revealed induction of Atlastin in *C. gigas* exposed to Low pH compared to unexposed oysters (CTL) (Table 2). Atlastin, has a role in shaping endoplasmic reticulum (ER) tubular network, by generating branched ER structures (Barlowe, 2009) and modulating proteoliposome/membrane fusion (Farhan and Hauri, 2009). The ER is key organelle in protein synthesis/folding, calcium storage, metabolism and many signalling processes (Görlach et al., 2006), but also a major source of reactive oxygen species (ROS) in invertebrates experiencing environmental stress (Tomanek, 2015). Therefore, the present results suggest reconfiguration of the ER by induction of Atlastin in oysters exposed to Low pH, with likely implications in all the above-stated cellular processes and modulation of ROS production rates.

Neprilysin was also increased in *C. gigas* exposed to Low pH compared to CTL (Table 2). In invertebrates the biological roles of Neprilysin have been related to neural stimulation (Turner et al., 2001), regeneration capacity (Sarras et al., 2002), extracellular matrix breakdown, gelatinolytic and fibrinolytic activities (Domínguez-Pérez et al., 2018). Given this, the present findings indicate that Neprilysin participates in extracellular restructuring and dynamics in *C. gigas* exposed to Low pH.

3.4.3. Protein changes under Low pH + As

Atypical serine-protein kinase ATM (ATM_Hsap) expression levels were higher in Low pH + As compared to CTL (Table 2). Protein kinases are important enzymes involved in protein phosphorylation regulation necessary for cell signal transduction processes in response to external stimuli (Epelboin et al., 2016). The recent assessment of *C. gigas* kinome revealed that Atypical protein kinases are mobilized under different biological processes, namely during embryonic development and environmental stress (Epelboin et al., 2016). For instance, these authors described ATM_Hsap regulation during *C. gigas* embryogenesis suggesting a role in cell-cycle progression. Additionally, some kinases appear to respond to stress (e.g. thermal, osmotic, metal and aerial exposure); they include atypical kinases (SMG1_Hsap and TRRAP_Hsap) that are increased in response to Zn (Epelboin et al., 2016). The present data provide further evidence of the involvement of protein kinases in environmental stress response, namely increased ATM_Hsap expression in response to the combined effects of Low pH + As, but the physiological effects of differential ATM_Hsap expression remain to be investigated.

Abundance levels of aldehyde dehydrogenase (Aldh) were higher in Low pH + As than in Low pH condition (Table 2). Aldh is an enzyme involved in aldehyde detoxification, a biproduct of ROS interaction with polyunsaturated fatty acids (Singh et al., 2013). Hence Aldh is an important enzyme in the oxidative stress response, by mitigating deleterious effects of aldehyde formation in processes such as lipid peroxidation (Marchitti et al., 2008; Singh et al., 2013). Several studies have demonstrated increased Aldh expression in bivalve species in response to environmental stress, including *S. cucculata* exposed to TBT (Khondee et al., 2016) and *M. galloprovincialis* under thermal stress (Tomanek, 2012). Apart from aldehyde detoxification, another role of Aldh is to provide reducing equivalents (NADPH) to increase reactive oxygen species (ROS) scavenging capacity by the glutathione system (Tomanek, 2014, 2015). Therefore, the present findings showing higher Aldh levels in *C. gigas* exposed to Low pH + As in comparison to Low pH, demonstrate that the combined exposure resulted in higher oxidative stress response in *C. gigas*.

Apart from differences in protein expression observed between Low pH + As and CTL, the present results also revealed differences in protein abundance profiles between stress conditions, namely Retinal dehydrogenase (Rdh) and Enolase (Table 2). Increased expression of Retinal dehydrogenase (Rdh) in *C. gigas* exposed to Low pH compared to Low pH + As was similar in *C. angulata* (previously described). These findings indicate that increased expression of Rdh could be a common mechanism in oyster species exposed to acidification, in line with results from Thompson et al. (2015), with likely physiological implications regarding tissue regeneration capacity (Albalat, 2009; Gutierrez-Mazariegos et al., 2014). However, induction of this protein was not observed under the combined effects of Low pH + As, evidencing that the presence of both stressors induced a different response, possibly because Rdh is inhibited by metals (Luo et al., 2014; Meng et al., 2015; Bao et al., 2016).

C. gigas presented higher expression levels of Enolase in Low pH exposure compared to Low pH + As (Table 2). Other studies have described increased expression of Enolase in bivalves, such as *Mytilus edulis* under thermal stress (Péden et al., 2016) and *S. cucculata* exposed to TBT (Khondee et al., 2016). Glycolysis stimulation by Enolase has been discussed as a mechanism to increase carbohydrate metabolism in bivalves experiencing energetically demanding conditions (Tomanek, 2014; Artigaud et al., 2015). Considering the concept of energy-limiting stress tolerance proposed by Sokolova et al. (2012), and the present data showing strong intra- and extracellular restructuring (Actin, Atlastine and Neprilysin) as well as induced regeneration capacity (Rdh) in *C. gigas* exposed to Low pH, it is likely that abundance levels of Enolase were increased to stimulate carbohydrate metabolism to fuel these energetically demanding stress responses. Significant differences of expression levels of Enolase between Low pH and Low pH + As only, indicate differences in energetic modulation in oysters exposed to single and combined exposures.

Other proteins, such as Actin, Atlastine, Neprilysin, Heme-bp, that were altered in single exposures (Low pH or As) were not altered in oysters exposed to the combination of both stressors (Table 2). Generally, none of these proteins presented changes in expression profiles in the combined exposure (Low pH + As), despite the observed changes in single exposures previously described, evidencing that the combined effects of different stressors induced a different proteomic response in *C. gigas*.

3.5. Species comparison

Both species presented differential expression of several proteins under As exposure, generally revealing a deleterious cellular status, but with different response patterns for each species. *C. angulata* showed cytoskeleton restructuring (Actin, Severin, Gelsolin), cellular redox imbalance (Lgl), inflammatory response and pro-apoptotic status (Pibf, α-crystallin b), alterations in lipid metabolism (uncharacterized KIAA1109-like), and impairment of cell-cell adherence (ZO-1) processes. On the other hand, *C. gigas* exposed to As presented pro-oxidant status (Heme_bp) and development of anti-apoptotic mechanisms (Ate_1), oxidative stress response and higher metabolic demand (Achd). These differences could at least partially result from lower accumulated As concentrations observed in *C. angulata* than in *C. gigas* (Section 3.1), and together illustrate different capacities of each species to accumulate As and subsequently have different cellular responses towards it.

Overall, protein expression profiles revealed a different proteomic response of each species to Low pH exposure, although increased Rdh expression was common for both species exposed to acidification. Results obtained for *C. angulata* exposed to Low pH suggest metabolic depression (ATP β) and enhanced regeneration capacity (Rdh) towards CTL. Moreover, higher acid-base regulation (V-ATPase b) capacity, and tissue remodelling (Epend) potential were observed in comparison to Low pH + As. On the other hand, results obtained for *C. gigas* indicate

major alterations of cellular and extracellular structure and dynamics (Actin, Atlastine, Neprilysin), as well as induction of Rdh. Additionally, increased carbohydrate metabolism (Enolase) in *C. gigas* exposed to Low pH compared to Low pH + As, highlight shifts in energetic efficiency to cope with multiple stressors. These findings highlight different metabolic strategies by each species to cope with seawater acidification. *C. angulata* presented supressed metabolism, while *C. gigas* increased energetic demand and concomitant carbohydrate metabolism stimulation to enable for cell and tissue remodulation.

Differentially expressed proteins identified in *C. angulata* exposed to Low pH + As indicate alterations in cytoskeleton structure (Actin, Coronin), metabolic depression (ATP β), increased protein turnover (Aminopeptidase) and homeostatic maintenance (Mycbp). Interestingly, several proteins that were differentially expressed in *C. angulata* exposed to single stressors (Low pH and As), showed overall lower change in expression levels in oysters exposed to Low pH + As (e.g. ATP β, Rdh, Pibf, ZO-1, Epend-1, Gelsolin, Severin and Lgl). Overall these findings demonstrate a clear difference in cellular response of *C. angulata* to single or combined exposures to Low pH and As.

C. gigas exposed to Low pH + As presented increased kinase signalling (ATM_Hsap) compared to CTL, and enhanced antioxidant capacity (Aldh) compared to Low pH. Moreover, several proteins that were altered in *C. gigas* exposed to single stressors (Low pH or As) did not present the same expression profiles in *C. gigas* exposed to Low pH + As (e.g. Rdh, Actin, Enolase, Neprilysin, Atlastin, Ate-1 and Heme-bp) evidencing different response capacity in the presence of multiple stressors.

Overall, data on protein changes towards Low pH + As evidenced different responses by each species. The present results showing higher accumulation of As in *C. gigas* could partially explain these differences. Interestingly, decreased expression of piRNA in *C. angulata* suggested genome reconfiguration in response to Low pH + As (as opposed to *C. gigas*), possibly induced as an adaptive mechanism towards extreme stress. These data add to recent findings by Li et al. (2017) that showed higher phenotypic plasticity of *C. angulata* compared to *C. gigas* towards global warming and suggested higher adaptive potential of the former species under environmental change.

4. Concluding remarks

The results obtained in the present study gave a deeper insight into the modes of action of two closely related oyster species in responses towards the combined OA and As exposure. Arsenic accumulation analysis revealed no influence of acidification on As uptake by each species. However, *C. gigas* tended to accumulate higher As concentrations than *C. angulata*.

As a corollary, both species induced cellular remodelling in response to external stimuli, observed by altered levels cytoskeleton related proteins, namely Actin and Atlastine (*C. gigas*); Actin, Severin, Coronin and Gelsolin (*C. angulata*). However, the conditions at which each species presented such alterations differed, with *C. gigas* presenting altered cytoskeleton proteins in Low pH exposures, while *C. angulata* showed most alterations under both Arsenic exposures (As and Low pH + As).

Different metabolic strategies in response to environmental stimuli could explain some of the differences observed between species. For instance, metabolic suppression in *C. angulata* (decreased ATP β) in both Low pH and Low pH + As exposures, contrasted with results for *C. gigas* that suggested increased carbohydrate metabolism (Enolase and Achd) to sustain energetically challenging conditions (Low pH and As), and also highlight species specific response strategies towards different stressors.

Proteomic analysis of oysters exposed to the combined exposure to Low pH + As revealed important differences in oysters' response capacity compared to that observed in single exposures, corroborating the working hypothesis that multiple stressors will further challenge oyster species in the environment.

The variety of modes of action observed and discussed in the present study, provide a glimpse into the multitude of responses that enable these species to thrive in highly dynamic estuarine systems. However, the present results also illustrate marked differences in response signatures of each species to each condition. Ultimately, these differences may define competitive advantages in a changing environment, with likely implications for populations of both species worldwide.

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