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**Supplementary material**

# Wild populations of Sydney rock oysters differ in their proteomic responses to elevated carbon dioxide

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## Two-dimensional electrophoresis and protein digestion and identification

*Two-dimensional electrophoresis*

Oysters were shucked and 500 μL of haemolymph was harvested from the pericardial cavity using a micropipette. The haemolymph was immediately mixed with 1.3 mL of Tri-Reagent LS (Sigma–Aldrich, Sydney, NSW, Australia). This was followed by a three-phase separation as per the manufacturer's protocol. Then 100 μL of bromochloropropane was added for 15 min then centrifugation for 15 min at 12 000*g* (4°C) before the resulting colourless aqueous (RNA) phase was removed. DNA was removed by adding 300 μL of 100% ethanol for 3 min followed by centrifugation at 2000*g* for 5 min (4°C), after which the DNA pellet was discarded. Proteins were then precipitated from the remaining aqueous solution by adding three volumes of ice-cold acetone, standing samples at room temperature for 10 min and centrifugation for 10 min at 12 000*g* (4°C). The resulting protein pellet was washed four times in 1 mL of 0.3-M guanidine hydrochloride in 95% ethanol (v : v) for 10 min per wash. The pellet was then air-dried at room temperature before resuspension in 50 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; (CHAPS), 50 mM dithiothreitol; (DTT)).

The concentrations of total protein in the resuspended pellets were determined using Amersham 2-DE Quant Kits according to a modification manufacturer's instructions (GE Healthcare, Amersham,

UK), as follows. Two microlitres of each sample was added in triplicate to wells of a 96-well microtitre plate, followed by 10 μL of Cu solution, 40 μL of Milli Q water and 100 μL of colour reagent. The plate was then left to incubate at room temperature for 20 min. Absorbance was measured at 490 nm on a spectrophotometer (xMark, Bio-Rad, Hercules, CA, USA) and protein concentrations were interpolated from a standard curve generated with bovine serum albumin. Five randomly selected oysters were quantified twice for quality assurance purposes. Haemolymph from five randomly selected oysters per treatment per tray was pooled with the remaining five oysters per tray kept for later use. Hence, for each of the three populations of oysters (CB, FC, HR), there were $n = 3$ replicate trays per $pCO_2$ treatment. Each pooled haemolymph sample contained 150 μg of protein based on the relative protein concentrations of each oyster.

Two-dimensional electrophoresis was performed on each pooled haemolymph sample according to the method of Thompson *et al*. (2011). Isoelectrofocussing (IEF) was undertaken using immobilised pH linear gradient gel strips (7 cm, pH 4–7; GE Healthcare) on an IPGphor IEF system (GE Healthcare). IPG strips were passively rehydrated overnight with 150 μg of extracted proteins in 125 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.002% bromophenol blue and 0.5% carrier ampholytes; GE Healthcare). IEF was then performed at 100 V for 2 h, 500 V for 20 min, a gradient up to 5000 V for 2 h and 5000 V for 2 h. Prior to second-dimension electrophoresis, gel strips were reduced for 20 min (1% DTT in equilibration solution: 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol) and alkylated for 20 min (2.5% iodoacetamide in equilibration solution). Second-dimension separation was undertaken by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with TGX precast (Bio-Rad) 12% TRIS-HCl polyacrylamide gels (1.5 M TRIS-HCl, 10% SDS, 12% acrylamide) in a Mini PROTEAN system (Bio-Rad). Gels were stained with Lava Purple (The Gel Co., San Francisco, CA, USA) and visualised on a Pharos UV scanner (Bio-Rad). PDQuest proteomic analysis software (Bio-Rad) was used to determine the relative concentrations (relative fluorescence intensities) of individual proteins on each 2-DE gel.

*Protein digestion and identification*

Protein spots that displayed significant differences in their relative concentration or presence between at least pairs of treatments were targeted for identification. These spots were picked from a fresh set of gels (stained with Coomassie for visualisation) and digested with trypsin. Each gel plug was washed three times for 10 min with 100-mM ammonium bicarbonate ($NH_4HCO_3$). Gel plugs were destained in 50% acetonitrile (ACN) : 50 mM $NH_4HCO_3$ (v : v) then dehydrated in 100% ACN for 5 min and air-dried. They were then reduced with 100 mM DTT in 100 mM $NH_4HCO_3$ at 56°C for 1 h then alkylated with 55-mM iodoacetamide in 100 mM $NH_4HCO_3$ (45 min at room temperature in the dark) before further washing and dehydration. For trypsin digestion, 30 μL of trypsin solution (12.5 ng $\mu L^{-1}$ in 50 mM $NH_4HCO_3$, Promega, Sydney, Australia) was added to each gel plug (30 min

at 4°C). More trypsin was added to cover the gel plugs as required before they were incubated overnight at 37°C. Gel plugs were then washed twice in 50 : 2% formic acid (v : v) for 30 min to extract tryptic peptides. The resulting supernatants (50–60 μL) containing peptides were concentrated to 12 μL in a vacuum centrifuge then centrifuged for 10 min at 20 800*g* to remove microparticles.

Tryptic peptides were analysed by nanoflow liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an LTQ-XL ion-trap mass spectrometer (Thermo, Carlsbad, CA, USA) according to (Andon *et al*. 2003). Reversed phase columns were packed in-house (~7 cm, 100-μm internal diameter) using 100 Å, 5 mM Zorbax C18 resin (Agilent Technologies, Santa Clara, CA, USA) in a fused silica capillary using an integrated electrospray tip. A 1.8-kV electrospray voltage was applied by a liquid junction upstream of the C18 column. A Surveyor autosampler (Thermo) was used to inject samples onto the column followed by an initial wash step with buffer A (5% (v/v) ACN, 0.1% (v/v) formic acid) for 10 min at 1 μL $min^{-1}$. Peptides were subsequently eluted from the C18 column with 0–50% Buffer B (95% (v/v) ACN, 0.1% (v/v) formic acid) over 58 min at 500 nL $min^{-1}$, followed by 50–95% Buffer B over 5 min at 500 nL $min^{-1}$. The column eluate was directed into a nanospray ionisation source of the mass spectrometer. Spectra were scanned over the range 400–1500 amu. Automated peak recognition, dynamic exclusion, and tandem mass spectrometry of the top six most-intense precursor ions at 35% normalisation collision energy were performed using Xcalibur software (ver. 2.06) (Thermo).

Raw mass spectrometry data files were converted to mzXML format and they were searched against a database containing 55 000 peptide sequences from the Pacific oyster (*Crassostrea gigas*) (plus common human and trypsin peptide contaminants) downloaded on 2 December 2012 from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov). Searches were performed with Global Proteome Machine (GPM) software ver. 2.1.1 of the X!Tandem algorithm, (www.thegpm.org) (Craig and Beavis 2003, 2004). A reversed sequence database was also assessed to evaluate the false discovery rate. GPM search parameters included MS and MS/MS tolerances of ±2 and ±0.4 Da, tolerance of up to three missed tryptic cleavages and K/R-P cleavages. Fixed modifications were set for carbamidomethylation of cysteine and variable modifications were set for oxidation of methionine. Only peptides that had $\log(e)^{+}$ values of less than –10 and yielded at least four spectral counts were retained for further analysis. Using these criteria, no reverse database peptide identifications were detected. Identified peptides were assigned a biological function based on functional annotations for the homologous sequences in the National Centre for Biotechnology Information database.

**Table S1. Putative identifications and associated biological function of differentially expressed protein spots ($P < 0.05$, SAM) between oyster populations at each $pCO_2$ exposure**

HR, Hastings River; CB, Cromarty Bay; FC, Fenninghams Creek. Arrow in fold change indicates an increase or decrease in protein expression between first and second site/exposure listed, i.e. spot 1405 is 2.6-fold higher in Hastings River after exposure to 856 µatm $pCO_2$

| Spot number | $pCO_2$ exposure | Accession/ID | Biological grouping | Fold | *P* |
|---|---|---|---|---|---|
| Hastings River | | | | | |
| 1405 | 380 / 856 | gi\|405963233\| Phosphoglycerate kinase 1 | Energy metabolism | ↑2.6 | 0.007 |
| 2609 | 380 / 856 | gi\|405972882\| T-complex protein 1 subunit beta | Cellular stress response (Chaperone) | ↑2.5 | 0.02 |
| 2609 | | gi\|405974443\| Adenosylhomocysteinase A | Energy metabolism | | |
| 2609 | | gi\|405963233\| Phosphoglycerate kinase 1 | Energy metabolism | | |
| 3208 | 380 / 856 | gi\|405960104\| Tenascin-X | Extracellular matrix | ↑2.9 | 0.04 |
| 3510 | 380 / 856 | gi\|405974443\| Adenosylhomocysteinase A | Energy metabolism | ↑2.1 | 0.04 |
| 3611 | 380 / 856 | gi\|405956520\| Putative aminopeptidase W07G4.4 | Protein synthesis | ↑2.3 | 0.04 |
| 5308 | 380 / 856 | gi\|405959361\| Tubulin beta chain | Cytoskeletal | ↑3.7 | 0.02 |
| 7107 | 380 / 856 | gi\|405952153\| Heterogeneous nuclear ribonucleoprotein H | Protein synthesis | ↑2.5 | 0.05 |
| 7211 | 380 / 856 | gi\|405973339\| Actin-2 | Cytoskeletal | ↑6.6 | 0.02 |
| Fenninghams Creek | | | | | |
| 1406 | 856 / 1500 | gi\|405975242\| Calponin-2 | Cytoskeletal | ↓3.1 | 0.04 |
| 1406 | | gi\|48476117\| Isocitrate dehydrogenase | Energy Metabolism | | |
| 1406 | | gi\|405959695\| Clathrin heavy chain 1 | Cytoskeletal | | |
| 4605 | 380 / 1500 | gi\|405975071\| \| Coronin-1B | Cytoskeletal | ↑21 | 0.001 |
| 4605 | 380 / 856 | gi\|405975071\| Coronin-1B | Cytoskeletal | ↑14 | 0.009 |
| 5514 | 380 / 856 | gi\|405969654\| Sodium/potassium-transporting ATPase subunit alpha | Energy metabolism | ↓2.9 | 0.03 |
| 7210 | 380 / 856 | gi\|405964567\| Tubulin beta chain | Cytoskeletal | ↑2.2 | 0.007 |
| 7210 | | gi\|405965637\| \| Tubulin alpha-1C chain | Cytoskeletal | | |
| 7210 | | gi\|405974703\| \| ATP synthase subunit alpha, mitochondrial | Energy metabolism | | |
| 7618 | 380 / 1500 | gi\|18565104\| Actin | Cytoskeletal | ↑3.2 | 0.02 |
| Cromarty Bay | | | | | |
| 2208 | 380 / 856 | gi\|405959361\| Tubulin beta chain | Cytoskeletal | ↑2 | 0.02 |
| 2208 | | gi\|405965638\| Tubulin alpha-1C chain | Cytoskeletal | | |
| 3608 | 380 / 1500 | gi\|405970867\| Enolase | Energy metabolism | ↑2.3 | 0.04 |
| 3608 | | gi\|405960104\| Tenascin-X | Extracellular matrix | | |
| 6308 | 380 / 856 | gi\|18565104\| Actin | Cytoskeletal | ↓2.4 | 0.005 |
| 6810 | 856 / 1500 | gi\|4838561\| AF144646_1 heat shock protein 70 | Cellular stress response (Chaperone) | ↑2 | 0.007 |

| Spot number | $pCO_2$ exposure | Accession/ID | Biological grouping | Fold | *P* |
|---|---|---|---|---|---|
| 6810 | | gi\|405974349\| Extracellular superoxide dismutase [Cu-Zn] | Cellular stress response | | |
| 7406 | 380 / 856 | gi\|405974534\| Actin | Cytoskeletal | ↑2.7 | 0.04 |
| 7618 | 380 / 1500 | gi\|18565104\| Actin | Cytoskeletal | ↑2.2 | 0.04 |
| 7711 | 856 / 1500 | gi\|405968607\| 78 kDa glucose-regulated protein | Cellular stress response (Chaperone) | ↑10 | 0.03 |
| 8610 | 856 / 1500 | gi\|405974790\| \| ATP synthase subunit beta, mitochondrial | Energy metabolism | ↑5.3 | 0.02 |
| 8610 | | gi\|405964567\| Tubulin beta chain | Cytoskeletal | | |
| 8806 | 856 / 1500 | gi\|405960567\| Low-density lipoprotein receptor-related protein 6 | Cellular stress response (Chaperone) | ↑25 | 0.04 |

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