JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v3i1.125

Proteome response to heat stress in the Antarctic clam *Laternula elliptica*

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Received: 13 January 2013 **Accepted:** 20 March 2013 **Available Online**: 30 March 2013

Abstract

The proteome can be regarded as a molecular phenotype, as changes in protein expression patterns have a direct effect on organismal physiology and fitness. The analysis of the proteome can therefore be an invaluable tool for our understanding of the mechanisms underlying phenotypic changes in response to environmental change. However, proteomic studies on thermal stress in marine species have mainly focused on heat shock protein expression, and little information is available for other components of the cellular stress response. This is particularly limiting for Antarctic species, which can lack the ability to induce heat shock protein expression in response to experimentally induced heat stress. The present study analysed changes in protein expression patterns in the Antarctic clam *Laternula elliptica* after exposure to elevated temperatures using two dimensional gel electrophoresis and mass spectrometry. Acute exposure to elevated temperatures had an effect in global protein expression patterns, suggesting that *L. elliptica* has the capacity to alter protein expression in response to heat stress. Changes in the expression of 14 proteins out of 264 analysed were observed in response to different levels of heat stress. Four of the 14 proteins had database matches and were identified as the cytoskeletal protein tubulin and associated chaperone TCP-1, and the enzymes enolase and aldehyde dehydrogenase, part of the minimal stress proteome and involved in redox regulation.

Keywords: Thermal stress; Proteomics; Two-dimensional gel electrophoresis; Antarctic invertebrates.

Abbreviations:

CSR, Cellular stress response; **HSPs**, heat shock proteins; **ANOVA**, analysis of variance; **AIC**, Akaike information criterion; **REML**, Restricted Maximum Likelihood; **FDR**, false discovery rate; **TCP-1**, T-complex polypeptide-1; **GST**, Glutathione s-transferase.

1. Introduction

Genomics has provided an invaluable toolkit to aid in the understanding of organism responses to environmental changes at all levels of biological organization, from molecular to organismal. In this respect, proteomic and transcriptomic analyses are complementary approaches. Whilst much variation in mRNA expression is biologically meaningful, protein expression is more likely to determine the phenotype of an organism [1]. In fact the proteome, the full complement of proteins expressed by the genome of a cell, a tissue or an organism at a specific time point [2], can be regarded as a molecular phenotype [3] as changes in protein expression patterns will have a direct effect on organismal physiology and fitness [4]. The analysis of the proteome can therefore be an invaluable method to advance our understanding of the mechanisms underlying phenotypic responses to environmental change in ecologically relevant species.

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However, the main thrust of discovery-driven genomics and particularly proteomics work is oriented towards well-known model species. In ecologically relevant non-model species, a greater number of investigations on the mechanisms underlying phenotypic change have focused on the transcriptome. Recent work has demonstrated that new hypotheses can be tested in proteomics studies in non-model organisms, opening the possibility of "addressing old problems, from a new perspective" (reviewed in [5]). The present paper demonstrates the application of an exploratory proteomics approach in the study of the physiologically important stress response in a non-model Antarctic species.

Non-lethal heat shock results in the activation of the cellular stress response (CSR), a proposed universal response which comprises a series of biochemical changes aimed at maintaining homeostasis [6]. This mechanism protects cells from sudden fluctuations in the environment, and ultimately reacts to the threat of macromolecular change [7]. Some of the best understood components of the CSR are heat shock proteins (HSPs), which play a housekeeping role in the normal functioning of the cell, but are best known for being important molecular mechanisms of stress tolerance [8]. Proteomic studies of thermal stress in marine organisms have thus mainly focused on the expression of HSPs, using 1 -DE or 2-DE (for a review of proteomic studies in the marine environment see [9]). The expression of the inducible HSPs in the presence of a thermal challenge has been identified in most species studied to date, leading to the proposal of a universal response. However, some Antarctic species have been found to be an exception (reviewed in [10]) and while others are able to induce HSP expression in response to elevated temperatures [11], the experimental induction temperature of the inducible form of HSP70 in these animals is in excess of +8°C, a temperature never experienced in the Antarctic marine environment. Whilst some other components of the CSR may be present in Antarctic species, information is currently very limited, and it is possible that other mechanisms differ. Exploratory proteomics approaches, which look at a large number of proteins at any one time of which no *a priori* knowledge is needed, will likely aid in the identification of such mechanisms.

In the Antarctic marine environment, much work has been conducted on the clam *Laternula elliptica* (King and Broderip), a keystone species of the Antarctic marine ecosystem [12,13]. As a highly abundant infaunal filter-feeder, it plays a significant role in benthopelagic coupling [13,14]. It is a relatively large mollusc, growing over 100 mm in shell length [15] and this, allied to its abundance and experimental tractability, makes it an attractive candidate for understanding Antarctic marine ectotherm responses to environmental perturbation. *L. elliptica* is also one of the most thermally sensitive species studied [16-18]. It suffers 50% failure in its ability to burrow, an essential biological activity, under acute exposure at 2–3 °C, and complete loss at 5°C. *L. elliptica* moves vertically in the sediment during normal activity cycles, and needs to rebury when ploughed from the sediment by ice disturbance, hence the ability to bury is considered an essential biological function [19]. The upper lethal temperature was estimated around 9°C when animals were warmed at a rate of 1–2°C per week [16]. Much of the work carried out to study the response to elevated temperatures in *L. elliptica* initially concentrated on physiological responses. This has identified the thermal limits and changes in metabolism in response to temperature and oxygen availability [16,17,20]. Other temperature related studies included investigations on burrowing capacity [19], seasonal energetics [21], lipid radical [22] and reactive oxygen species generation [23]. Molecular analyses included the study of HSP expression [11,24], and the study of antioxidant systems with the characterization of a glutathione s-transferase (GST) [25] and two peroxiredoxin genes [26]. More recently, a microarray was developed to study gene expression changes on individuals exposed acutely to elevated temperatures [27]. This, together with a 454 sequencing project [28] have significantly increased the genomic resources available for this species. At the protein level, an investigation of the effect of thermal stress on antioxidant defense systems using enzyme assays has been carried out [29]. To our knowledge, there are currently no studies on global patterns of protein expression in this species and the techniques used in the present study have not been applied to this, or any other Antarctic marine invertebrate.

In the present study, the proteome of the Antarctic clam *Laternula elliptica* was analysed by two dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) after exposure to elevated temperatures *in vivo*. Changes in protein expression patterns in response to different levels of heat stress were analysed with the aim of i) determining whether exposure to elevated temperatures has an effect of protein expression and ii) identifying proteins associated with the stress response, which may be candidates for stress biomarkers in this species, a representative example of Antarctic marine ectotherms.

2.Material and Methods

2.1. *Sample collection*

L. elliptica specimens were collected by scuba divers at a depth of 10-18m in February 2006 at North Cove, Rothera Point, Adelaide Island, Antarctic Peninsula (67°34'07"S, 68° 07'30"W) and shipped to the British Antarctic Survey facilities in Cambridge, UK. The animals were allowed to acclimate for four weeks in a closed water system at water temperature and salinity of 0±0.5°C and 34±1 p.p.t. respectively. A marine microalgae concentrate (Nannochloropsis, Reed Mariculture) was added to the water on a weekly basis. All necessary permits were obtained. This work was approved by the British Antarctic Survey ethics review committee and meets the requirements of UK legislation. All work on living organisms was conducted under the Antarctic Act (1994) Section 3 permit issued by the UK Foreign and Common-

wealth Office. No endangered or protected species were used.

2.2. *Heat shock experiments*

At the end of the acclimation period, a control and two heat shock experiments were performed at 0, 3 and 9°C as described in an earlier transcriptomic study [27]. Briefly, seawater temperature was raised gradually from 0˚C to 3˚C±0.5˚C or 9˚C±0.5˚C over a 12 h period, after which the animals were left in contact with a sand surface to allow them to burrow. The temperature was maintained at a constant value for 12 h. Treatment temperatures were selected based on previous studies [19] where *L. elliptica* was shown to suffer 50% failure in essential biological activities (burrowing) at 3˚C and survive only a few days at 9˚C. The ability to burrow is therefore a measure of the physiological status in this species, thus animals at 3°C were allowed to bury to determine whether differences observed at the organismal level could be detected at the molecular level. Immediately after the treatment, the number of buried animals was noted, individual animals were dissected and samples from mantle tissue were taken, snap frozen and stored at -80ºC. The procedure was repeated for a control group, for which seawater temperature was maintained constant at 0˚C±0.5˚C. Approximately 90% of the animals reburied at 0°C, 25% at 3°C and none at 9°C (n = 10, 20 and 10 for 0, 3 and 9°C respectively). Animals exposed to 3°C were divided into two groups named 3°C buried and 3°C not buried. No mortalities were recorded. The choice of mantle material was based on previous work which showed that this tissue was one of the most responsive to heat challenge [11] and it also allowed for direct comparisons with a previous gene expression analysis carried out in the same population, tissue and experimental condition [27].

2.3. *Experimental design for 2-DE*

Six 2-DE gels could be run at the same time in the electrophoresis chamber. This influenced the design of the experiment. Six independent biological replicates (i.e. six individual animals) were used per treatment, with the exception of the 3°C buried treatment where only five replicates were available (i.e. only five animals buried during the 3°C exposure period). A subset of all biological replicates was run more than once to generate technical replicates, which allows estimation of experimental variation due to the technique itself. Eight out of the total 23 animals across treatments were run on more than one gel to generate technical replicates. In order to determine whether there was a batch effect, replicates were run on the same day and on different days (i.e. same or different batches of six gels, the maximum allowed by the chamber). Animals 13, 15 and 19 were run on two different days, and animals 1, 7, 9, 17 and 23 were run twice within a day and again on a separate day, permitting one within day and two between day estimates.

2.4. *Preparation of protein mixtures*

Approximately 30 mg of mantle tissue was crushed in N_2 (l) with pestle and mortar, and the powder homogenised in 700 µl lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 3% DTT and 2% IPG ampholytes) with a sonicator (Branson digital sonicator 250). After centrifugation at 4ºC for 30 min (15000g), the pellet was discarded and the supernatant stored at -80ºC. Protein concentrations were measured with a 2-D Quant Kit (GE Healthcare) and a total of 90 µg (for analytical gels) or 300 µg (for preparative gels) of protein from each individual was cleaned using a 2-D Clean-Up Kit (GE Healthcare) to remove interfering substances (salt or charged detergents) for the first dimension isoelectric focusing (IEF).

2.5. *2-DE electrophoresis, silver staining and image analysis*

The first dimension, isoelectric focusing (IEF), was carried out on immobilized pH gradient strips (pH 3-10NL/24cm, GE Healthcare) with a horizontal electrophoresis apparatus (Ettan IPGphor, GE Healthcare). Strips were equilibrated in two steps in 10 ml equilibration buffer (6M urea, 2% (w/v) SDS, 75 mM Tris-HCl (pH 8.8), 30% glycerol and 0.001% (w/v) bromophenol blue). In the first step, the strips were washed in equilibration buffer containing 100 mg DTT for 15 min with light shaking. In the second step, the first equilibration buffer was decanted and the wash procedure repeated with an extra 10 ml equilibration buffer with 250 mg iodoacetamide replacing the DTT. After equilibration, strip were loaded along the top of the 12.5% DALT pre-cast polyacrylamide gels and transferred to an Ettan Dalt six Electrophoresis System (GE Healthcare) according to manufacturer instructions (for further details see [30]). Silver staining of the analytical gels was used to visualize protein spots using a modification of the protocol developed by Heukeshoven and Dernick [31]. For mass spectrometric analysis of candidate spots, gels were stained with a modified protocol [32], compatible with mass spectrometry.

Silver staining has a linear range up to two orders of magnitude in terms of quantitative response [33,34]. This limited dynamic range compared to fluorescently labeling methods is in part due to the well known "bleaching" effect of silver staining on spots for highly abundant proteins. After spot filtering, when saturated and negatively stained spots were eliminated, the difference between the weakest and strongest spots fell within this range, as observed in a previous study carried out under similar conditions [30]. Silver-stained gels were scanned to TIFF files using an Image Scanner (Amersham Pharmacia Biotech). Progenesis Samespots v2.0 software (Nonlinear Dynamics Ltd) was used for gel alignment, spot detection and spot volume measurement. Visual inspection was incorporated into the filtering process as detailed in [35]. The "lowest on boundary" method for background subtraction was applied, as recommended by Nonlinear Dynamics Ltd. For analysis, the total number of fea-

tures on the gel (including spots and artifacts) was reduced from over 1000 to 264 spots. In each treatment, only spots present in >50% of the animals, and all technical replicates in available, were selected. Absolute spot volumes were normalised using a modification of a method previously described for one channel microarray data [36]. The gel with the lowest median spot volume was selected as a baseline. A normalisation factor was calculated for each gel by dividing the mean spot volume in the baseline gel (i.e. for the total number of spots in the gel retained after filtering) by the mean spot volume of any gel to be normalised. Individual spot volumes within a gel were multiplied by the normalisation factor calculated for that gel. Normalised spot volumes were $\log_2$ transformed for further analysis. Normalisation removes variation arising from different overall staining intensities between gels and allows comparison of individual spot volumes between gels.

2.6. *Statistical analysis*

Data were analysed to identify individual spots for which volume showed statistically significant between treatments, as well as to test for an overall treatment effect on global protein expression. In addition, sources of variation were analysed. Statistical analysis was carried out using R version 2.8.1 [37]. Normalised spot volumes were used throughout the analysis. The $\log_2$ transformed normalised spot volumes gave acceptable fit to normality and homogeneous variance values.

2.7. *Treatment comparisons*

Comparisons between treatments were performed on a spot by spot basis and also using a global protein expression approach. A spot by spot analysis was performed to determine those spots with a statistically significant treatment effect. The characteristics of the dataset (unbalanced design with some animals replicated in multiple days) determined the statistical method applied. Two linear mixed effects (lme) models were fitted to the data for each spot and implemented with the lme package in R [38]. As multiple animals were sampled per treatment, and a number of them replicated over several days, *Animal* and *Day* were included as random factors. For the first model, *Treatment* was not included as a factor, and for the second model it was specified as a fixed factor whilst *Animal* and *Day* remained in the model as before. To test the null hypothesis that for each individual spot there are no differences in spot volume between treatments, the fit of the two models was compared for each spot using a likelihood ratio test implemented with the ANOVA (analysis of variance) function in R. The Akaike information criterion (AIC) and the log likelihood ratio and associated probability were used to compare the models. A p-value of less than 0.05 was considered statistically significant, revealing differences between treatments in the normalised spot volume of that particular spot. For those spots showing statistically significant differences between treatments, further comparisons between treatments were made in a pairwise manner by fitting an lme model to each spot using Restricted Maximum Likelihood (REML). A single model was considered in which *Treatment* was specified as fixed and *Day* and *Animal* as random factors. Since multiple tests were carried out, false discovery rate (FDR) corrections were applied to account for potential type I errors after Benjamini and Hochberg [39]. Fisher´s method for combining probabilities was also applied to the 264 p-values from each pairwise treatment comparison [40]. This method combines the *a priori* p-values of all the spots into a single p-value for the data as a whole, which is then compared with the chosen significance level α value. If the combined p-value is statistically significant, it can be concluded that for at least one of the spots in the list, the null hypothesis that all individual spots show no difference between treatments is false; the best candidates for showing a treatment effect being those with the lowest p-values.

An additional complementary approach to provide evidence of treatment effects on global expression patterns was to determine whether there is a correlation between treatments in patterns of protein expression compared with the control. A 2x2 contingency table was created with the number of observations for which spots were 1) up-regulated in both treatments compared to the control, 2) down-regulated in both treatments , 3) up-regulated at 3°C, then down-regulated at 9°C and 4) down-regulated at 3°C, then up-regulated at 9°C. Fisher's exact test was used to test for association in the 2x2 table.

2.8. *Analysis of technical variation*

Analysis of technical variation was carried in two ways. Firstly, to test for differences in between and within day technical variation, factorial two-way ANOVAs were carried out on normalised spot volume with the variables *Spot* and *Gel* as random factors for each animal for which technical replicates were available. *Spot* represents all selected spots within a gel and *Gel* represents technical replicates for a single animal. The interaction *Spot*Gel* thus measures the technical error. Separate ANOVAs were carried out to extract the within day and between day technical errors (i.e. the same animal run on different gels on the same day or on different gels on different days). The ratio of the mean square of the two measures of the technical error (within and between days) and associated probabilities were calculated. *P*-values less than 0.05 in a variance ratio test indicate statistically significant differences in the technical variation measured between and within days. Secondly, for all animals replicated within or between days, the coefficient of determination (r^2) of normalised spot volume was calculated for each replicated animal across spots between and within days. This measures the technical error between and within days. Values can be compared with those reported in previous studies.

2.9. *Mass Spectrometry*

Gel preparation and mass spectrometric analysis were carried out at the mass spectrometry facility at the Cambridge Center for Proteomics at Cambridge University. All protein spots showing statistically significant changes between treatments in *a priori* tests were manually excised from the gel and subsequent sample preparation was performed in a MassPrep Station (Micromass). Briefly, proteins were reduced and alkylated with dithiothreitol (DTT) and iodoacetamide (IAA) and subjected to enzymatic digestion with porcine trypsin (Promega). After digestion, 10 µl of supernatant was pipetted into a sample vial and analysed by LC-MS/MS. All LC-MS/MS experiments were performed using an Eksigent NanoLC-1D Plus (Eksigent Technologies, Dublin) HPLC system and an LTQ Orbitrap Classic mass spectrometer (ThermoFisher). Separation of peptides was performed by reverse phase chromatography with the conditions described in [41]. The Orbitrap was set to perform in data dependent acquisition (DDA, Top3) mode where all m/z values of eluting ions were subjected to a survey scan in the Orbitrap mass analyzer, set at a resolution of 7500 over an m/z range 360-1500. Peptide ions with charge states of 2^+ and 3^+ were then automatically isolated and fragmented in the LTQ linear ion trap by collision-induced dissociation and MS/MS spectra were acquired. Resulting MS/MS spectra were processed using Bioworks Browser (version 3.3.1 SP1, ThermoFisher) and resulting mgf files were submitted to the Mascot search algorithm (version 2.2, Matrix Science) and searched against the NCBI (all entries) database with the following parameter settings: a fixed modification of carbamidomethyl cysteine, a variable methionine oxidation and two miscleavages. Average atomic masses were used in the searches and a tolerance of 0.8 Da for fragment ions and 2.0 Da for precursor ions was allowed. Putatively identified proteins were examined and only protein matches with two or more unique and statistically significant peptide matches were accepted. When different isoforms/homologies were identified, these were ranked according to their protein score and the match with the highest score was accepted. Observed molecular weights (MW) based on the spot position on the gel were used for confirmation with a 10 kDa tolerance. To further validate the identifications, all peptide sequences from each spot were used to search against a local *L. elliptica* database previously generated using 454 pyrosequencing (peptide against nucleotide database) [28]. Those contigs that had a match to one or more peptides were then identified using sequence similarity searching [42] against the NCBI nr database.

3. Results

3.1. *Analysis of technical variation*

Results of the analysis of within and between day technical variation are shown in Table 1. Apart from animal A1 there is no clear evidence that the mean square for between day replicates is significantly greater than that within days. For both of A9 and A17, one comparison is statistically significant and one is not. Unexpectedly, for A7, technical variation measured within days is greater than that measured between days, though this is not statistically significant. There is therefore no indication that technical variation is consistently higher between days, but appears to be sample dependent (i.e. not batch or treatment dependent). The coefficient of determination (r^2) for normalised spot volume for each animal for which technical replicates were run ranges from 0.97 to 0.92 for between day technical error, and from 0.97 to 0.95 for within day technical error.

Table 1. Comparisons of between and within technical error using ANOVA analyses of normalized spot volume with Spot and Gel as random factors. The Spot*Gel interaction for within day technical replicates (MSwithin) and between day technical replicates (MSbetween) is indicated for each animal. Degrees of freedom (df), F ratios for between divided by within Spot*Gel mean square values and associated probabilities (P) are also shown.

Animal	MS_{within}	$MS_{between}$	df	F ratio	*p-value*
A1	0.15	0.35	263	2.26	<0.001
		0.40	263	2.59	<0.001
A7	0.53	0.36	263	0.68	0.999
		0.42	263	0.79	0.972
A9	0.42	0.56	263	1.32	0.012
		0.39	263	0.93	0.722
A17	0.28	0.27	263	0.97	0.597
		0.39	263	1.38	0.005
A23	0.22	0.23	263	1.06	0.319
		0.23	263	1.06	0.319

3.2. *Comparisons of spot volumes between treatments*

Mean protein expression levels in treatment groups containing six animals each (five in the case of 3˚C buried) were compared. Of the 264 spots that passed quality screening, 14 (Figure 1) showed statistically significant differences in expression between at least two of the treatments at a p-value less than 0.05 in *a priori* tests (Table 2). The AIC for both models, log likelihood ratios and associated p-values are indicated for each spot. The AIC [43] balances the number of parameters in the model, against the maximised likelihood for the model, and can be used to determine the simplest model that best explains the data. Given a dataset and several competing models to explain it, the model with the lowest AIC can be considered the best. For the spots shown, AIC values are lower when the treatment effect is included in the model. Thus the model including treatment effect can be considered the preferred explanation for the data. After FDR corrections, none of the 14 spots showing statistically significant differences between treatments in *a priori* tests remain statistically significant. However, Fisher´s method for combining probabilities is statistically significant (p=0.04) for

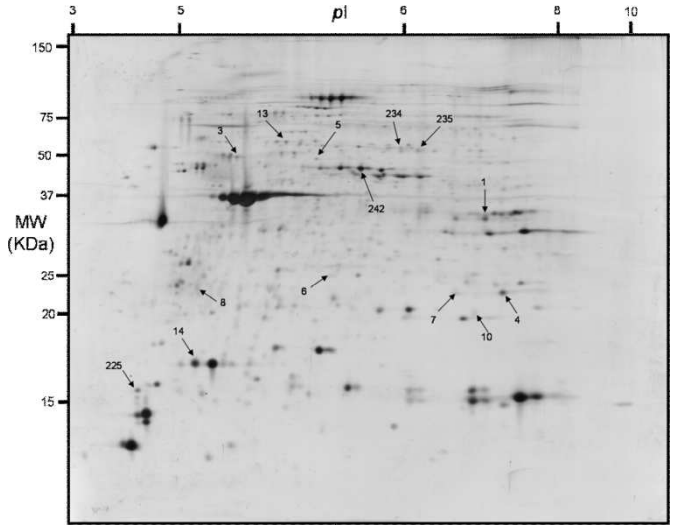

Figure 1. Representative 2DE gel from *L. elliptica* mantle tissue exposed to control conditions (0°C). The isoelectric point (pI) and molecular weight (MW) in kilodaltons are indicated on the horizontal and vertical axes respectively. Arrows indicate the 14 spots that showed significant differences between at least two of the temperature treatments (3°C and 9°C) or the treatments and the control.

Table 2. Results from the lme tests for model comparisons showing spot numbers for spots with expression levels significantly different between treatments are given, followed by the Akaike information criterion (AIC) for models 1 (no fixed effect) and 2 (treatment as a fixed effect), log likelihood ratios (L. Ratio) and associated *a priori* p-values.

Spot	AIC 1	AIC 2	L. Ratio	*p-value*
1	68.56	66.27	8.28	0.041
3	85.14	79.50	11.64	0.009
4	118.06	116.02	8.04	0.045
5	71.65	67.44	10.21	0.017
6	86.79	83.49	9.29	0.026
7	91.37	87.70	9.67	0.022
8	91.04	88.61	8.43	0.038
10	81.29	78.46	8.82	0.032
13	74.00	71.68	8.32	0.040
14	63.74	60.32	9.42	0.024
225	115.29	112.91	8.38	0.039
234	83.74	81.03	8.71	0.034
235	43.73	40.95	8.78	0.032
242	46.87	42.22	10.65	0.014

one of the pairwise treatment comparisons suggesting that at least one of the 264 null hypotheses of no treatment effect is false. The best candidates for further analysis would be the spots with the lowest *p*-values. Some of these will be false positives but this should not be of great concern in exploratory research, as they would be eliminated by repeat experimentation [44].

REML statistics (*t*-value) and associated probabilities from comparisons between treatments are shown in table 2. Mean fold changes in protein expression of each treatment in relation to the control, and to each other, are also shown for all spots showing statistically significant changes in expression between any of the treatments in *a priori* tests. More spots show statistically significant changes in their level of expression between 9˚C and all other temperature treatments than between any of the treatments and the control (Table 3). The two 3˚C treatments (buried and not buried) show the lowest number of proteins with statistically significant changes in expression between them.

In addition to differences in the expression of individual spots between treatments, the analysis using Fisher's exact test suggests that there is a correlation between the treatments across spots in the direction of changes in expression in relation to the control (P<0.001 one tailed Fisher´s exact test). That is, spots are up-regulated (or down-regulated) compared to the control in all treatments more often than this is expected by chance, assuming random variation in direction of expression.

3.3. *Protein identifications by MS/MS*

Protein identifications by mass spectrometry were attempted only on the 14 spots showing statistically significant differences between treatments prior to corrections, as the identification of all spots in the gel was beyond the scope of this study. Of these, four were identified by MS/MS (28.6%). Results are shown in table 4. All spots are found approximately within their theoretical MW (±10 kDa). The four spots are identified as alpha tubulin, aldehyde dehydrogenase, T-complex polypeptide-1 (TCP-1) alpha subunit and enolase.

4. Discussion

In the present exploratory study, changes in the expression of 14 proteins out of 264 analysed in *L. elliptica* exposed to different levels of heat stress were observed to be statistically significant in *a priori* tests. Although these do not remain statistically significant after application of the FDR method, Fisher's combined probability test and application of the Akaike information criterion provide evidence of a treatment effect. In addition, there is a statistically significant correlation between the treatments in the up- and down-regulation of spots in the treated animals compared to the control, which also provides evidence of a treatment effect. Environmental changes are likely to cause coordinated changes in the up- or down-regulation of different proteins, and may be a means of discovering protein networks underlying physiological or adaptive response as observed in a 2-DE study of the effect of mutational change in yeast [45]. Four of the 14 proteins were identified by mass spectrometry. The potential significance of the observed changes is explained and candidates for further analysis are proposed. The coefficient of determination (r^2) for normalised spot volume for each animal for which technical replicates were run (0.92 to 0.97) was within the range reported in previous studies in bivalves [30,46], indicating that technical variation was consistent with expectations from 2-DE analysis.

Table 3. Mean fold changes (FC) in protein expression of each treatment in relation to the control, and to each other for *L. elliptica* mantle tissue. Results are shown for significantly up- or down- regulated spots between at least two of the treatments in *a priori* tests. Positive and negative values indicate up- and down-regulation respectively. Restricted Maximum Likelihood statistics (t-value) and associated probability (P) are also shown. Bold probabilities indicate significant differences between the treatments in the level of expression of a given spot (P<0.05). "B", buried; "NB", not buried.

	FC			FC			FC		
Spot	**3°CB/0°C**	**t-value**	**P**	**3°CNB/0°C**	**t-value**	**P**	**9°C/0°C**	**t-value**	**P**
1	-1.35	1.34	0.195	-1.12	0.54	0.594	1.35	1.41	0.176
3	1.31	1.26	0.223	1.36	1.50	0.151	-1.39	1.54	0.141
4	-2.10	2.12	**0.047**	-2.03	2.09	0.050	-1.06	0.18	0.862
5	1.41	2.02	0.058	1.56	2.69	**0.015**	1.06	0.32	0.751
6	1.16	0.72	0.481	1.59	2.24	**0.037**	1.75	2.61	**0.017**
7	-1.19	0.77	0.452	-1.15	0.65	0.522	1.54	1.90	0.072
8	1.94	2.87	**0.010**	1.59	2.04	0.055	1.59	1.96	0.065
10	1.59	2.36	**0.029**	1.70	2.77	**0.012**	1.67	2.55	**0.020**
13	-1.46	2.05	0.054	-1.17	0.89	0.383	-1.61	2.56	**0.019**
14	9.94	0.03	0.976	10.31	0.21	0.836	6.02	2.40	**0.027**
225	-1.89	1.88	0.075	-1.84	1.86	0.079	1.10	0.28	0.786
234	1.52	1.91	0.071	1.78	2.71	**0.014**	1.77	2.58	**0.018**
235	1.03	0.28	0.780	1.20	1.56	0.135	1.37	2.56	**0.019**
242	-1.19	1.28	0.216	1.17	1.21	0.242	1.24	1.60	0.127
	FC			**FC**			**FC**		
Spot	**3°CNB/3°CB**	**t-value**	**P**	**9°C/3°CB**	**t-value**	**P**	**9°C/3°CNB**	**t-value**	**P**
1	1.20	0.85	0.408	1.82	2.73	**0.013**	1.52	1.99	0.061
3	1.04	0.20	0.846	-1.82	2.89	**0.009**	-1.90	3.20	**0.005**
4	1.04	0.11	0.913	1.98	2.00	0.060	1.91	1.96	0.065
5	1.11	0.67	0.513	-1.33	1.75	0.097	-1.48	2.43	**0.025**
6	1.36	1.60	0.125	1.51	2.02	0.058	1.11	0.51	0.618
7	1.03	0.14	0.891	1.83	2.80	**0.011**	1.78	2.73	**0.013**
8	-1.23	0.94	0.357	-1.23	0.90	0.377	1.00	0.00	1.000
10	1.07	0.38	0.709	1.05	0.26	0.801	-1.02	0.11	0.916
13	1.24	1.29	0.213	-1.11	0.57	0.576	-1.38	1.84	0.081
14	1.04	0.17	0.865	-1.65	2.36	**0.029**	-1.71	2.66	**0.016**
225	1.03	0.10	0.925	2.08	2.21	**0.040**	2.02	2.20	**0.041**
234	1.17	0.76	0.457	1.16	0.71	0.486	1.00	0.01	0.989
235	1.16	1.35	0.192	1.32	2.42	**0.026**	1.14	1.16	0.261
242	1.39	2.59	**0.018**	1.47	2.94	**0.008**	1.06	0.45	0.656

In a previous study of transcriptomic responses to heat stress in *L. elliptica* exposed to the same 3°C treatment described herein, a total of 160 unique transcripts, 7.5% of the total number of clones retained for analysis, showed statistically significant changes in expression between treatments [27]. The reasons for the disparity between the transcriptome and proteome studies in the number of statistically significant changes in expression could be related to the subset of transcripts and proteins analysed. Only a subset of the transcriptome was printed on the array (8500 clones) and likewise, only a subset of the total proteome expressed at any one time can be separated under the same running and preparation conditions. It is possible that the set of transcripts on the arrays by chance showed relatively more differences between treatments than did the set of proteins visualised by 2-DE. In addition, it is likely that some changes occurring in mRNA expression do not result in changes at the corresponding protein level. Several studies have shown that predictions on protein expression levels based on changes in the corresponding mRNA abundance are not always met (reviewed in [5]). It is also possible that there is a time lag between the generation of mRNAs and subsequent protein production in response to changes in temperature. However, when taken as a proportion of the total number of proteins changing in expression between the treatments, the percentage of identified proteins (approximately 29%) was higher than that of identified transcripts (17% in the microarray and 454 sequencing work previously referred to).

A higher number of spots showing changes in expression in the 9˚C treated animals when compared to other treatments could be expected. At 9˚C, the critical temperature has been exceeded and the transfer to anaerobic metabolism has occurred [16], potentially resulting in different protein expression profiles and a larger set of proteins showing sta-

Table 4. Protein spots identified by MS/MS in *L. elliptica* mantle. Mascot protein score, number of peptides, % sequence cover, theoretical molecular weight (MW), sequences for retained peptides and allocated function are shown.

Spot number	Protein	Uniprot ID	Protein score	Peptides	Sequence cover (%)	Theoretical MW	Peptides	GO annotation
3	Tubulin, alpha 1c	Q6P8G7	686	17	31.4	50532	K.DVNAAIATIK.T	Microtubule based movement
							R.QLFHPEQLITGK.E	
							R.TIQFVDWCPTGFK.V	
							K.VGINYQPPTVVPGGDLAK.V	
							R.AVCMLSNTTAIAEAWAR.L	
							R.AFVHWYVGEGMEEGEFSEAR.E	
							R.FDGALNVDLTEFQTNLVPYPR.I	
5	Aldehyde dehydrogenase	A7XZK3	153	3	6.0	53544	K.ILGLIESGK.K	Oxidation reduction
							R.EEIFGPVQQILK.Y	
235	T-complex protein 1, alpha subunit	Q4AE76	269	5	7.6	59962	R.LLEVEHPAAK.V	Protein folding
242	Enolase	A8DU76	233	9	10.8	40012	R.ICDDELIIIK.G	Glycolysis
							R.TQNVMAASSIANIVK.S	
							K.IQIGMDVAASEFCK.D	
							R.AAVPSGASTGIYEALEMR.D	

tistically significant changes in expression. However, the differences in physiological performance (buried and not buried) reflected at the organismal level was not detected at the protein level (only one protein showed statistically significant differences between the 3˚C buried and not buried groups, table 3). Of the proteins identified, aldehyde dehydrogenase and the glycolytic enzyme enolase changed in their level of expression in *L. elliptica* exposed to elevated temperatures. Both proteins are part of the minimal stress proteome of cellular organisms, a set of proteins that participate in different aspects of the cellular stress response and are ubiquitously conserved in all three super kingdoms [6]. In the absence of stress, aldehyde dehydrogenase is an enzyme in the glycolytic pathway. However, under acute stress, it acts as an oxidoreductase, involved in redox regulation by detoxifying aldehydes. The induction of this protein may be necessary for generating reducing equivalents (NADH, NADPH) that are needed for cellular antioxidant systems, or to respond to the energetic requirements of protein degradation, protein chaperoning, and DNA repair [6]. Both are in accordance with the observed decrease in aerobic capacities [20] and the induction of antioxidant defenses at 3˚C [27].

Tubulin is a cytoskeletal protein and main constituent of microtubules, which consists of many isoforms [47,48]. The upregulation of tubulin in clams exposed to 3˚C is consistent with the induction of cytoskeletal protein transcripts observed in heat stressed *L. elliptica* [27]. It is also likely to be associated with the changes in the expression of the fourth protein identified, T-complex polypeptide-1 (TCP-1), a member of the TRiC family which functions as a chaperone in eukaryotes directing folding of cytoskeletal proteins (reviewed in [49]) and is key in the biogenesis of tubulin in the cytosol [50]. It is thus possible that the upregulation of TCP-1 responds to the demand for tubulin and, based on the gene expression data previously cited, other cytoskeletal proteins. The induction of TCP-1 is likely linked with the induction of a prefoldin transcript observed at the level of gene expression [27]. Prefoldin is a molecular chaperone complex which transfers target proteins to chaperonins, promoting folding of newly synthesised or denatured proteins. The biogenesis of the cytoskeletal proteins actin and tubulin involves the interaction of nascent chains of each of the two proteins with prefoldin and their subsequent transfer to the cytosolic chaperonin containing TCP-1 [50,51]. TCP-1 was highly upregulated during heat stress in the coral *Montastraea faveolata* [52]. Owing to the absence of inducible HSPs at environmentally relevant temperatures, TCP-1 deserves further attention as a potential heat stress induced chaperone in *L. elliptica*. Its role in other Antarctic species and potential use as a marker for thermal stress in the Antarctic marine environment awaits examination.

The number of identified proteins in this study using protein databases (4 out of 14 spots) reflects the amount of information available in public databases for *L. elliptica* (18 proteins at the time of writing), which has also been a feature in previous work in this species [27,28]. Identifications in the

present study were limited to highly conserved sequences. Searches for the 14 proteins showing *a priori* statistically significant differences between treatments against the 454 *L. elliptica* transcriptome did not result in further proteins being identified but confirmed the MASCOT results, increasing confidence in the identifications. Since the individuals used for 454 transcriptome analysis were not exposed to stress, it is possible that genes involved in the stress response are underrepresented. This would emphasise the desirability in transcriptomics studies of constructing databases derived from material exposed to contrasting experimental treatments. Identifying proteins associated with specific environmental stressors, may thus require the generation of further transcriptomic resources.

5. Conclusions

In summary, this exploratory study provides evidence for a treatment effect due to experimentally induced heat stress at the protein level in *L. elliptica*. Four candidate proteins have been identified, providing evidence of the activation of a cellular stress response in *L. elliptica* at 3°C, and a candidate spot (TCP-1) that deserves further attention as a potential stress marker in this species. Successful identifications will likely increase as genomic resources rapidly increase through the development of next generation sequencing projects. A strong statistically significant correlation in the pattern of expression of spots in the treated animals compared to the control was observed. This is an interesting approach, which might be investigated more generally where *a priori* values are not statistically significant, as a means of identifying treatment effects and coordinated regulation of protein networks underlying the physiological response.

Acknowledgements

Financial support was provided by the Marine Genomics Europe (MGE) Network (EU-FP6 contract No. GOCE-CT-2004-505403) and the BAS Q4 BIOREACH/ BIOFLAME core programme. MGE also funded a PhD fellowship for MT, and postdoctoral fellowship for APD. MAST and MSC were supported by the British Antarctic Survey Polar Science for Planet Earth Programme, Adaptation and Physiology Work Package. APD is currently supported by "I. Parga Pondal" fellowship and "Grupos de Referencia Competitiva" (code 2010/80) from Xunta de Galicia (Spain) and Fondos FEDER "*Unha maneira de facer Europa*". The authors would like to thank all members of the Rothera dive team for collecting animals.

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