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Description/rationale of the project

The aim of the project was to use a high throughput non-targeted tissue proteomics approach to study how marine organisms respond to environment stress. In this sense the project was essentially a proof-of-concept. The project examined the effect of an acute stress that is evoked in a crustacean species under extreme environmental conditions. This condition, idiopathic muscle necrosis (IMS), is well-documented in Norway lobsters (*Nephrops norvegicus*) in response to both lowered salinity and aerial exposure, and can also be induced by the procedures of trawl capture and post-capture handling (Stentiford and Neil, 2000: J Fish Dis 23, 251-263). So far, IMN in Norway lobsters has been described using only biochemical and histopathological measures (Ridgway et al., 2007: J Fish Dis 30, 279-292). However, the underlying mechanisms of IMN expression are unknown. Hypotheses for underlying mechanisms include a form of apoptosis, and/or immune suppression. Therefore, the objective of this work was to use an untargeted proteomics approach to obtain a better understanding of the molecular mechanisms involved in the onset of IMN.

Activities completed

Methodology - *Nephrops* were caught by otter trawl in the Clyde Sea Area on the 10th May 2013 and recovered in on-board tanks for at least 6 hours before being transported (for 1 hour) to in-house seawater tanks at a commercial facility. Animals were further recovered for 2 days before being sampled. A total of 6 muscle samples from 6 *Nephrops* that were visually non-necrotic (necrosis index 0) were sampled. Furthermore, 12 muscle samples were sampled from 6 animals that showed signs of IMN (necrosis index 6-8). Six of these samples were from visually non-necrotic muscle tissue and 6 samples were from muscle blocks that were visually necrotic. Muscle samples were dissected and immediately placed in liquid nitrogen before being stored at -80°C. Protein extracts were obtained following the Filter Aided Sample Preparation (FASP) method (Wiśniewski et al., 2009), which encompasses the analysis of complex extracts in a single chromatographic run allowing for increased reproducibility. Protein extracts were separated on a Dionex Ultimate 3000 RSLS nano flow system. A 5 µl sample of each extract was loaded in 0.1% formic acid and acetonitrile (98:2) onto a Dionex 100 µm x 2 cm, 5 µm C18 nano trap column at a flowrate of 5µl/min. Elution was performed on an Acclaim PepMap C18 nano column 75 µm x 50 cm, 2 µm, 100 Å with a linear gradient of solvent A, 0.1% formic acid and acetonitrile (98:2) against solvent B, 0.1% formic acid and acetonitrile (20:80) starting at 1% B for 5 minutes rising to 50%B at 360 minutes. The sample was analyzed in an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). The MS was operated in a data-dependent mode (top 40) to switch between MS and MS/MS acquisition and parent ions were fragmented by collision-induced dissociation (CID). Data files were searched against the IPI crustacea database, which contained 410 reviewed and 69,287 un-reviewed sequences (05/06/13) using SEQUEST, with the enzyme specified as trypsin. A fixed modification of carbamidomethylation was set and oxidation of methionine and proline as variable modifications were selected. Mass error windows of 10 ppm and 0.8 Da were allowed for MS and MS/MS, respectively. Data processing was carried out using Proteome Discoverer and data analysis was conducted in Cytoscape. Statistical p-value analysis was performed using the t-test analysis across all samples. Fold changes were calculated for individual proteins across all samples by averaging the total ion counts per condition.

Results - Proteins detected and identified using this protocol were mainly proteins integral to the membrane, nucleus and also cytoplasm (Supplementary Fig. 1) while the main biological processes associated with these proteins were proteolysis, oxidation-reduction processes, translation and signal transduction (Supplementary Fig. 2). After statistical analysis, a total of 90 proteins were found to be significantly associated with the necrosis process (p value ≤ 0.05 and fold change ≥ 1.5). Frequency, mean average and key information on the significant proteins is given in Supplementary Table 1. In general terms, the main differences indicated that during late necrosis (visually affected tissue) there was a general down-regulation of transcription factors, ATP binding and proteins with ATPase activity. Furthermore, when we compared early versus late necrosis it was also observed that there was a down-regulation of key components for the assembly and functioning of striated muscles (titin), other proteins involved in muscle contraction (myosin 7B) and proteins involved in cell activation, energy homeostasis and skeletal muscle regeneration. Compared to the non-necrotic condition, in early necrosis there was a down-regulation of cell adhesion and motor activity proteins, and interestingly also a down-regulation of a protein that in other species has been found to have an anti-apoptotic function (Uniprot accession number Q8WWQ0) and an up-regulation of proteins coupled to apoptotic pathways (Uniprot accession number P05067). In conclusion, this work has served as a successful proof-of concept that tissue proteomics can give a deeper understanding of and a novel insight into the physiological responses of marine organisms to stressful conditions, even though the current protein databases for such species are not as comprehensive as in other animal species such as humans.

Cost expenditure summary

The funds obtained for this project were mainly spent on mass spectrometer instrument time (£2759.79). A total of 18 samples were run and each sample was run for 6 hours (total time 108 hours in sampling time for testing samples plus 36 hours in running time for blank samples). The remainder of the funding was spent in consumables (£240.21).

Table 2. Cost summary report showing how the funding was spent.

Heading Name	Description	Supplier Name	£ Actuals
BIOCHEMICALS	Sodium Dodecyl Sulfate, Reagent PLUS	Sigma-Aldrich CO LTD	11.81
ORGANIC CHEMICALS	DL-Dithiothreitol:DL-Dithiothreitol	Sigma-Aldrich CO LTD	80.01
ORGANIC CHEMICALS	Urea ACS Reagent	Sigma-Aldrich CO LTD	13.40
BIOCHEMICALS	Formaldehyde MB Reagent	Sigma-Aldrich CO LTD	12.92
ORGANIC CHEMICALS	V5111 Seq Grade Modified Trypsin	Promega	84.00
ORGANIC CHEMICALS	1429609 Perchloric acid	Sigma-Aldrich CO LTD	28.08
ORGANIC CHEMICALS	Acetic Acid, ACS Reagent	Sigma-Aldrich CO LTD	9.99
INSTRUMENT TIME	Instrument time (LTQ Orbitrap MS 108 hours of sample time)	University Glasgow	2,759.79
		TOTAL EXPENDITURE	**3,000.00**

Interactions with MASTS community

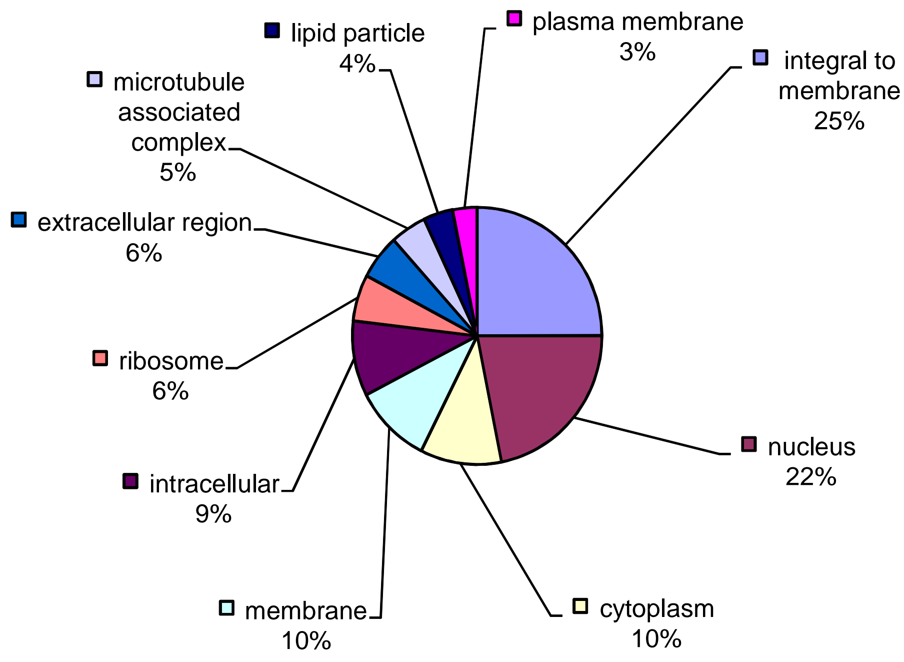
The results obtained in this project were presented in an oral presentation on the MASTS Annual Meeting in Heriot-Watt University, September 2013.

Outputs produced and expected

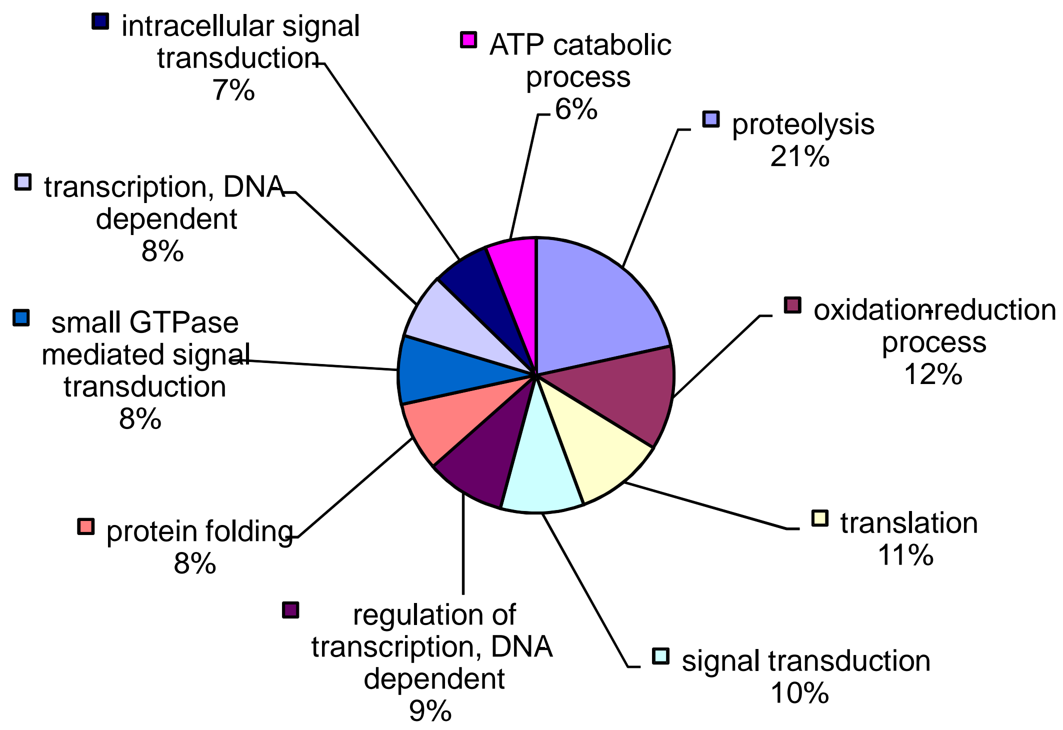
It is expected that this work will be prepared for publication in the next three months and that a publication will be submitted during 2014.

Future plans for building on the grant

The current project has established that the proteomic approach can provide a sensitive early indication that a marine animal is responding to an environmental stress. The grant holders are therefore planning to submit a grant application to BBSRC during the year 2014 in which we will propose to use this proteomic approach to elucidate further physiological responses of crustacean species towards a range of different environmental conditions that are potentially stressful.



Supplementary Fig. 1. Cellular components of proteins detected and identified in this trial according to their GO annotations.



Supplementary Fig. 2. Biological associated processes of proteins detected and identified in this trial according to their GO annotations.