Project report (April 2011)

Variation in heat-shock responses of marine invertebrates of sub-Antarctic Marion Island: effects of acute and acclimatory temperature exposures

Heat shock proteins (HSP), or stress proteins, are likely to play a significant role in thermal tolerance of near-shore marine invertebrates (Tomanek 2010). Although a few studies have shown that stress proteins belonging to the family HSP70 are up-regulated after heat exposures in marine invertebrates (e.g. Chapple et al. 1998; Tomanek and Somero 1999), data for Antarctic and sub-Antarctic species are very limited (e.g. La Terza et al. 2001, but see Clark et al. 2008). To our knowledge, no studies have investigated variation in HSP70 response to acclimation in Antarctic marine invertebrates.

Our existing project aimed at quantifying the magnitude of plasticity in upper thermal tolerance of two near-shore crustaceans from sub-Antarctic Marion Island, the isopod *Exosphaeroma gigas* and the amphipod *Hyale hirtipalma*. The two additional objectives within the framework of this project were to (1) to assess the variation in the HSP70 response induced by acclimation and (2) to measure differences in HSP70 expression after acute exposures at 25°C across acclimation treatments. The results of this study should have significant implications for predicting the effect of global climate change on these species physiology and geographic distributions.

Methods

Several hundred individuals of the amphipod *H. hirtipalma* and isopod *E. gigas* were collected on Marion Island (Trypot beach, 46º53'09''S; 37º52'37''E) in April 2010 and maintained in temperature-regulated, oxygenated tanks at 7°C for 3-4 days to allow habituation to new conditions. For each species, individuals were split in 3 acclimation (ACC) treatments: 3, 7 and 11°C for 5 days with a photoperiod maintained at 12:12h (see picture 1). Filtered, fresh sea-water and food items were given every 2 days. Salinity and dissolved oxygen levels in the tanks were monitored throughout the acclimation periods using appropriate meters (YSI 30 Salinity Conductivity and Temperature Meter; Lutron Dissolved Oxygen Meter DO-5510). To assess acclimation effects on upper temperature limits, a static ULT_{50} protocol was followed for each acclimation group using standard protocols.

Briefly, five to ten individuals from each acclimation group were placed in a small container filled with filtered sea water, preheated to the required experimental temperature. Individuals were exposed for 2h to pre-determined test-temperatures (TT) maintained by water-circulating baths (Grant GP 200 R4). A wide range of TT was employed, with treatments added based on the organism responses to preliminary trials. Sea water was aerated using

air pumps during the exposures and, salinity, conductivity and dissolved oxygen monitored before and after each experiment. Individuals were then placed at respective ACC temperatures where individual responses were checked after 10 min and 1h. The number of dead and live individuals was determined to score percentage mortality, and each individual body mass and sex recorded. All experiments were replicated three times.

For HSP assessments, 10 individuals of each species were used in the following treatments: (1) field fresh (directly collected from the field); (2) acclimated at 3, 7 or 11°C; (3) acclimated at 3, 7 or 11°C and then exposed to 2h 25°C treatment and (4) acclimated at 3, 7 or 11°C and then exposed to 2h 7°C treatment (representing controls for (3)). After each treatment, individuals were snap frozen and kept at -40°C until later HSP assays in Stellenbosch University.

Relative levels of HSP70s are determined using western analysis following standard protocols (e.g. Tomanek and Somero 2002). Briefly, the steps are (1) isolation of proteins from each acclimation-temperature treatment and control group, (2) normalization of samples using a protein assay, (3) preparation of loading samples and gels, (4) transfer of proteins from gel to membrane and western blotting with HSP70 antibodies and (5) visualization and quantification of proteins. A protein extraction and Western blotting protocol has been developed following Sørensen et al. (1999) and Karl et al. (2009), and with the assistance from the Magic Lab, Division of Molecular Biology and Human Genetics, Department Biomedical Sciences, Faculty of Health Sciences, University of Stellenbosch (Appendix 1).

Results to date and next phase

Results of the post-acclimation ULT trials provided contrasting results between the amphipod and isopod species. For both species, and for all acclimations, temperature had a significant effect on survival (typical sigmoid shape curve). However, acclimation had an effect on ULTs for the isopod species but not for the amphipod species (figure 1 and 2). The $ULT_{50,}$ representing the temperature at which 50% of the individuals survived, was 26.4°C for the amphipods. For the isopods, the ULT_{50} for acclimations at 3, 7 and 11°C were 25, 26.4 and 29.5°C, respectively, indicating that higher acclimation temperatures improved the temperature tolerance of this species. Given the treatments utilized in this study, these data indicate that the isopod is more plastic to moderate temperature treatments compared to the amphipod.

Because HSP70 expression had not been determined before for these species, no specific antibodies were known. So far, HSP assessments have revealed that several primary antibodies used successfully in other arthropods do not work adequately for our Marion Island species (i.e. HSP70 Antibody (Cell Signalling #D69), Mouse monoclonal [C92F3A] and HSP70 (Abcam #47455)). However, positive results (successful antibody binding to

HSP70) were found for the isopod samples using the protocol described in Appendix 1, but not for the amphipod species. The next phase will now include a semi-quantitative Western blotting protocol, using protein staining with Ponceau S solution (Sigma # P3504) as a loading control for all the samples (see Romero-Calvo et al. 2010). This technique will provide quantitative differences in HSP70 expression between treatment groups. In regards to the amphipod samples, despite several attempts to obtain targeted protein bands using a range of sampling conditions and antibody concentrations, alternative antibodies are now being investigated based on additional published literature.

References

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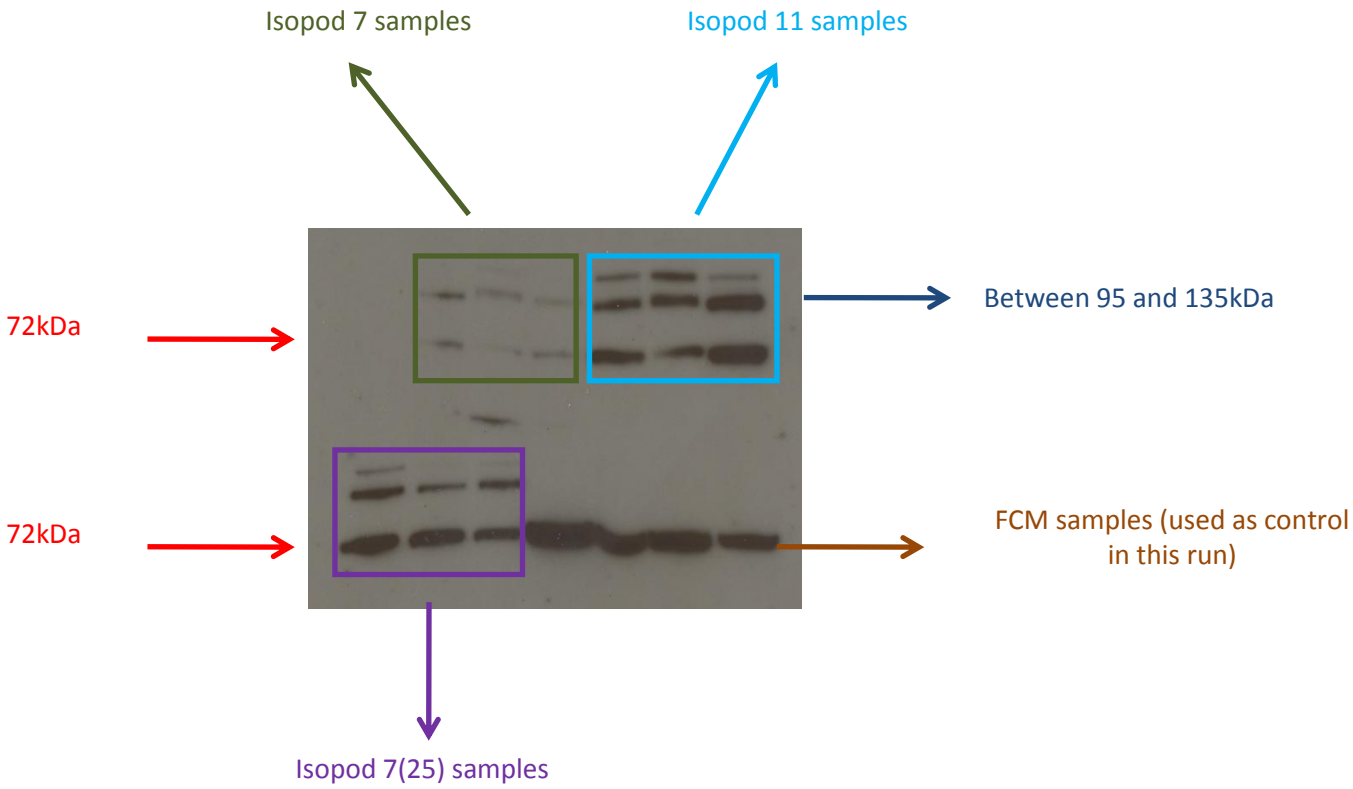
Picture 1. Isopods and amphipods maintained in an incubator set acclimation temperature (3, 7 or 11°C), Physiological Ecology Laboratory, Marion Island.



Picture 2. HSP 70 western blot bands for isopod samples. Isopod 7 and 11 samples represent the acclimations at 7 and 11°C. Isopod 7(25) represent the isopods acclimated at 7°C but exposed to 25°C for 2h. The bottom right bands are the positive control at a size of approximately 72kDa. Red arrows indicate protein marker at 72kDa. False codling moth (FCM) samples were used as a positive control.

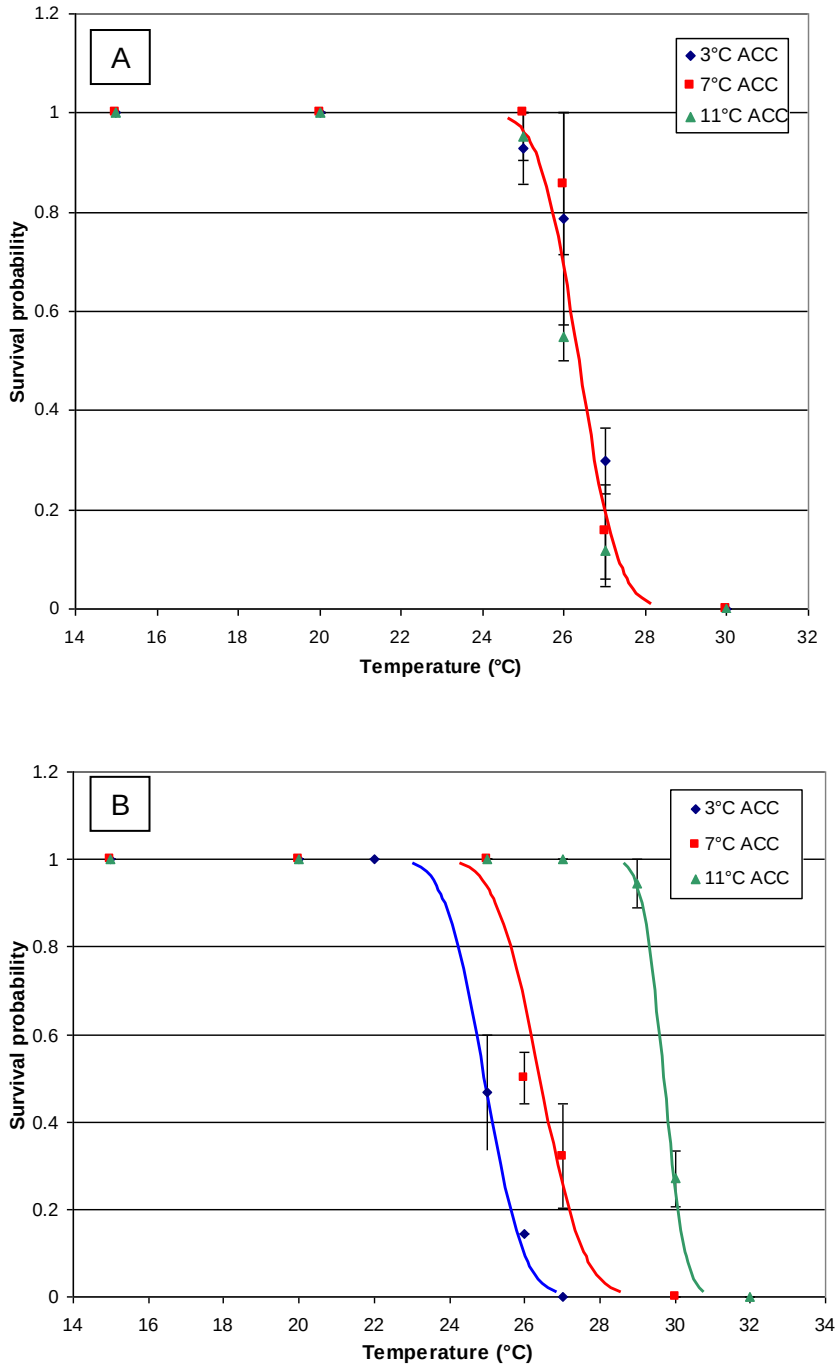


Figure 1. Measured survivorship of (A) the amphipod *Hyale hirtipalma* and (B) the isopod *Exosphaeroma gigas* as a function of temperature. Logit linear regressions are fitted for each 5-day acclimation: cold acclimation in blue (3°C), sea water temperature in red (7°C) and warm acclimation in green (11°C).

Appendix 1. Protein extraction and Western blotting protocol

Protein samples were obtained by homogenising individuals in a cold phosphate buffered saline containing PEFAbloc and an antiprotease cocktail (pepstatin A, leupeptin, benzamidine, sodium metabisulfite). The homogenate was centrifuged at 4 °C at 11,000*g* (13,000 rpm) for 30 min and the supernatant was transferred to a new microfuge tube. Protein supernatants were quantified using a BCA assay kit (Pierce #23227), as per the manufacturers' instructions, on a Nanodrop spectrophotometer (ND1000, Nanodrop Technologies). Samples were prepared for sodium-dodecyl-sulphate polyacryamide gel electrophoresis (SDS-PAGE) by adding equal parts protein supernatant and SDS loading buffer, followed by denaturing at 95 °C for 5 min. Prepared samples were frozen at -80 °C until further analysis with Western blotting. Precast gels (7.5% resolving gel, 4 % stacking gel, Ready Gel Tris-HCl, BioRad 161-1100) were loaded with protein marker (SpectraTM Multicolor Broad Range Protein Ladder, Fermentas #SM1841), HeLa cytoplasmic lysate (Abcam # 29546) and 6 samples (50 µl protein in each sample) and run in SDS Running buffer at 100V for 75 min in BioRad Mini Protean(R) Tetra Cell (PowerPac Basic). Proteins were transferred onto a PVDF membrane using the Invitrogen iBlot transfer system and transfer stacks (Invitrogen #IB401001, Life Technologies, Israel). The membrane was incubated overnight using the Western Card system (Invitrogen #WP1001) in Invitrogen BenchPro™ 4100 (Life Technologies, Israel; Invitrogen BenchPro(TM) 4100 Card processing station). The membrane was blocked in 5% milk-TBTS for 60 min, incubated with 1:2500 primary antibody (Sigma # H5147) in 5% milk-TBST for 900 min and 1:10 000 HRP-conjugated rabbit anti-mouse IgG secondary antibody (Abcam # 6728) in 5% milk-TBST for 60 min, with washing steps performed between each incubation. The membrane was incubated in Super Signal West Chemiluminescent substrate (Pierce #34079, Thermo Scientific) and exposed to film (Amersham Hyperfilm MP) for 30 s. The film was developed in an automatic autoradiography film processor (Hyperprocessor; Amersham pharmacia biotech).