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Stress Gene Expression in H. dujardini

A Major Qualifying Project Report

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Why Tardigrades?

Members of the phylum tardigrada, also known as tardigrades or more commonly as “water bears” are polyextremeophilic animals found in virtually every moist habitat on the planet, both semi-terrestrial and in large bodies of water. Tardigrades are unique among the vast majority of animals due in part to their ability to survive a large number of adverse environmental exposures such as excessive heat and cold, immersion in various protic and aprotic organic solvents, vacuum pressure, and exposure to direct full spectrum solar radiation. These tolerances have been shown to increase dramatically when the animal are in a state of almost complete desiccation (Horikawa et al., 2006; Hengherr et al., 2009; Jonsson et al., 2008; Jonsson & Schill 2007; Wright 2001). These, along with other morphological and developmental properties make tardigrades a desirable model for astrobiological research, or the study of and search for life in extraterrestrial environments and condition

Morphology and Development

The tardigrade *Hypsibius dujardini* is a small animal that is part of the phylum Tardigrada, a subset of the superphylum Ecdysozoa. Adult tardigrades possess four pairs of legs terminating either in claws or sucker type appendages and consist of a segmented body (four segments not including the head) that are surrounded by a thick cuticle that is periodically molted during the animals growth and development (Gabriel et al., 2007). The tardigrade genome is one of the shortest to be found among invertebrates, *H. dujardini* having a genome size of approximately 75 Mb and other tardigrade species possessing genomes varying in size from around 80 to 800 Mb (Gabriel et at., 2007). Tardigrades may either be carnivorous, feeding off of smaller organisms such as rotifers, or herbivorous as in the case of *H. dujardini*, feeding on several species of green algae such as *Chlorococcum* and *Botrydiopsis* (Horikawa et al., 2008). Reproduction may be either sexual or parthenogenic, however parthenogenic reproduction is observed in the majority of species including *Hypsibius dujardini* (Gabriel et al., 2007).

Tardigrade populations may range in size from 0.5mm to approximately 1.5mm once fully developed, however within species the cell number tends to be constant. The cleavage pattern of cells during development as well as the cell number are highly conserved between individuals as determined by Gabriel et. al., 2007). Development to reproductive maturity occurs very rapidly in this species, with the generation time being approximately 13 to 14 days in a culture raised at room temperature (Gabriel et.al., 2007). *H. dujardini* is also one of several tardigrades that possess a clear cuticle surrounding their bodies.

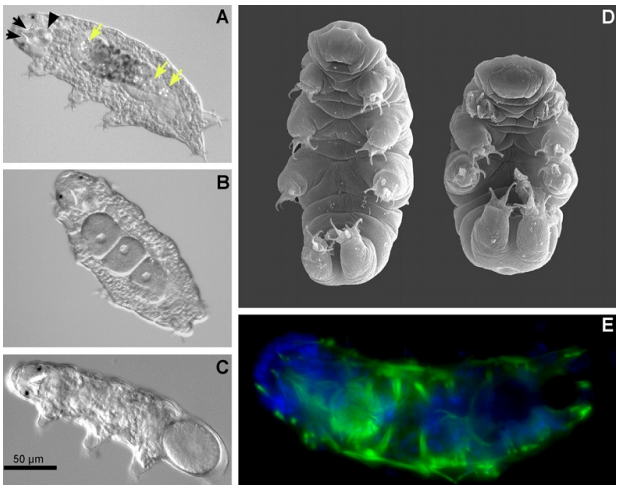

Figure 1: Figure 1. Adult morphology of Hypsibius Dujardini. (A) The midgut is shown to bemorphologically distinct by the presence of algae (dark area). Eyespots are indicated by the black arrowheads. (B) Parthenogenic production of embryos. Three oocytes have been produced by this specimen. (C) Tardigrades lay eggs as they molt, leaving the exuvia to serve as a developmental shell for the embryos. (D) SEM image of two adult H. Dujardini, approximately 500um long. (E) DAPI and Phalloidin stained individual. Musculature and pharynx are shown as green while nuclei are stained blue. Reproduced from Garbriel et al., 2007.

These physical properties make tardigrades a good candidate organism for astrobiological research. The small size and easily stereotyped developmental patterns can help to pinpoint important effects on events in development, therefore providing a highly conserved timeline to be used when studying a large group of individuals. The clear cuticle makes observation of internal morphology and staining under a light microscope much more simple. Another aspect of these animals that make them great candidates for laboratory research is a compact genome, estimated to be approximately 75 Mb (Gabriel et al., 2007). This allows for very fast gene sequencing, mapping, and augmentation. The general hardiness of the animals as well as their propensity to produce viable offspring in laboratory also makes the cultivation of large sample populations relatively simple.

Tardigrades are Widely Distributed

Tardigrades are highly prolific and may be found living in virtually every moist environment on the planet. The animals may inhabit freshwater, marine, or semi-aquatic terrestrial environments (Blaxter et al., 2004). Semi-aquatic terrestrial organisms are often found living in leaf litter or on mosses and lichens while aquatic specimens are generally founds in littoral regions of lakes and oceans. All species require a film of water coating their bodies in order to remain in an active metabolic state. Less than desirable conditions such as dehydration, food scarcity, and other adverse environmental factors cause the species to form a second protective cuticle layer and contract into what is known as a tun state (Halberg et al., 2009).

Tardigrades are Polyextremeophiles

Tardigrades have been shown to be incredibly resistant to conditions including extreme temperatures, vacuum pressure, exposure to varying levels of ultraviolet radiation, and immersion in various protic and aprotic solvents, in particular once they have entered cryptobiosis and formed a tun (Horikawa et

al., 2006; Hengherr et al., 2009; Jonsson et al., 2008; Jonsson & Schill 2007; Wright 2001). During the time that they are desiccated and exposed to these conditions the animals enter a state that has been experimentally shown to be virtually ametabolic.

It has also been shown through a number of studies on lifespan and fecundity that the amount of time spent in this state has no bearing on the total lifespan or the reproductive viability of the animals (Hengherr et al., 2008; Bertolani et al., 2004). This type of stasis has been termed the "sleeping beauty" model due to the fact that while in this state, the animals may be considered virtually ageless and maintain reproductive viability even given long periods of dehydration and/or stress.

When exposed to large doses of ultraviolet radiation most organisms rapidly acquire large numbers of deleterious mutations in their genomes, most often resulting in death or sterility. Exposure to freezing temperatures generally causes ice crystals to form in both the cytosol and intercellular space, shearing proteins and membranes which results in massive cell degradation. Immersion in solvents can displace water and disrupt intracellular osmotic pressure or dissolve cellular components. Drying of cells generally results in rapid cell death and large amounts of DNA damage (Neumann et al., 2009). The survival of tardigrades under these generally adverse and often fatal environmental conditions points to unique molecular mechanisms for the maintenance and/or repair of DNA, RNA and proteins due to the deleterious effects that the aforementioned conditions have on the integrity and stability of these structures.

Cells May Be Partially Protected By a Process of Water Replacement

A number of hypotheses have been proposed to explain the stability of proteins and nucleic acids under adverse conditions. The most well known is the water replacement hypothesis. This idea is based on two concomitant processes: the removal of body water by desiccation and the replacement of the water

of solvation around proteins, membranes, nucleic acids, and other vital cell components with polyhydroxyl compounds, specifically non-reducing sugars such as trehalose (Neumann, 2006). It is believed that these compounds undergo a process known as vitrification, forming glass structures which protect the structure and integrity of these macromolecules as well as other necessary components including cell walls and various organelles. This seems unlikely as a principle candidate for cryptobiotic survival however. Experimental data has shown only 1.6% dry-mass trehalose accumulation in the tardigrade *Adorybiotus coronifer*, implying that trehalose accumulation may supplement, but not drive, cryptobiotic survival in tardigrades (Liang et at., 1997).

Heat Shock Proteins are Implicated in Cryptobiotic Survival

During drying, many cells exhibit a method for compensating for the lack of hydrogen bonding to water. This takes the form of new hydrogen bond formation with other proteins, membranes, and molecules. While this can help to retain the structure of the cell as a whole, the protein-protein interaction implicated in this mechanism can often lead to irreversible conformational changes in the proteins themselves, often resulting in enzymatic inactivity (Carpenter et al., 1987).

Although complete desiccation is normally fatal to most organisms, it has been shown that certain stress proteins are rapidly transcribed under water stress in species that can survive desiccation. Heat shock proteins are some of the most common implicated in this (Pareek et at., 1995; de Jong et al., 1998; Clegg et al., 1994). Heat shock proteins in unstressed cells perform a number of important functions which may mirror those that are used advantageously in stress conditions. These include help with the correct folding, assembly, localization, secretion, regulation, and degradation of various proteins throughout the cell (Gethig & Sambrook, 1992; Gethig, 1997). It is believed that heat-shock proteins may function to mediate the damaging protein-protein interactions that occur while cells are compensating for the lack of hydrogen bonds to water by helping to prevent aggregation or

unfolding/misfolding (Schill et at., 2004). Hsps have also been implicated in preventing oxidative damage to cryptobiotic cells (Plumier et al., 1995).

Hsp70 Is a Large Family of Heat Shock Proteins

Hsp70 isoforms are the most abundant heat-shock proteins yet discovered although they also contain come of the most highly conserved sequences yet identified. The functions of Hsp70 include inhibiting protein aggregation as well as helping to fold newly synthesized proteins (Kiang & Tsokos, 1998; Fink, 1999). It has also been shown to interact with cell membranes and may hold a role in the regulation of transcription by binding transcription factors (Wickner et al., 1991). Schill et al. have quantified the mRNA expression of three isoforms of Hsp70 in a different tardigrade species, *Milnesium tardigradum*, and discovered a positive correlation between Hsp70 isoform 2 mRNA expression and the induction of and restoration from the tun state.

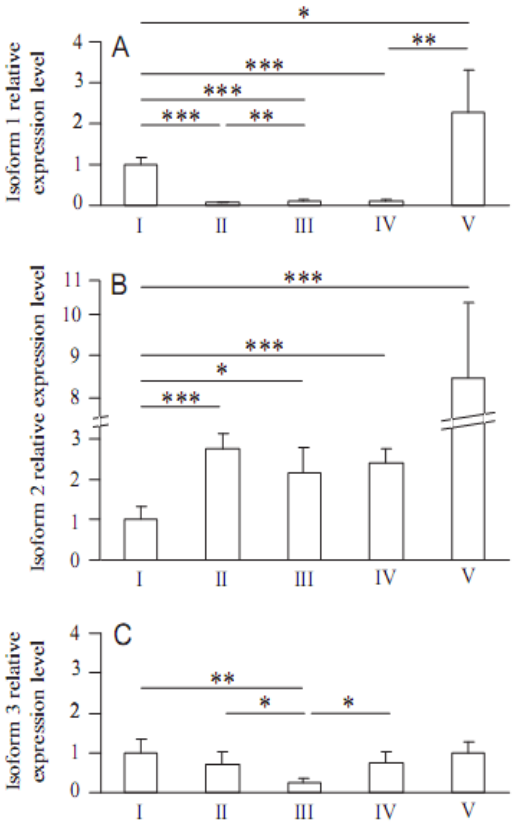

Figure 3. Hsp70 expression at the different life cycle stages (I–V) of the tardigrade Milnesium tardigradum: (A) hsp70 isoform 1, (B) hsp70 isoform 2 and (C) hsp70 isoform 3. The mRNA copy number was calculated based on the beta-actin housekeeping gene as described in the Materials and methods. Relative expression levels refer to stage I=100%=1.0. Results are presented as means± S.D.The stages are as follows: I, active before cryptobiosis; II, transitional before cryptobiosis; III, cryptobiotic; IV, transitional after cryptobiosis; V, active after cryptobiosis. Reproduced from Schill et al., 2004.

Tardigrades Would be Good Candidates for Model Organisms

These properties possessed by tardigrades make them attractive model organisms for genetic studies in a number of disciplines. In particular, the field of astrobiology is particularly well suited to take advantage of this type of organism. This is due primarily to the fact that tardigrades have been experimentally shown to survive conditions similar to those that exist in space (freezing, vacuum pressure, solar radiation etc.). These animals have recently been exposed to such conditions on board the Biopan-6 orbital platform. This platform was placed in low earth orbit for a period of ten days during the European Space Agency's FOTON-M3 mission in December of 2007. The animals were exposed to filtered and direct solar radiation (upwards of 7000 kJ/m^2) as well as vacuum pressure and temperatures approaching 0 K. Animals exposed to only vacuum pressure survived remarkably well, while those exposed to combined vacuum pressure as well as full solar radiation had significantly reduced survival. This was, however, the first time any animal had been conclusively proven to possess the mechanisms necessary for survival in such conditions (Jonsson set al., 2005; Jonsson et al., 2008; Rebecchi et al., 2009).

In particular, *H. dujardini* is an attractive species of tardigrade to use as a model organism. It has a generation time of approximately 13-14 days at room temperature, lays several eggs at a time (6 to 8), has a relatively fast lifespan, and possesses a clear outer cuticle. These properties allow for fast cultivation of large populations and easy observation of morphology, making it a prime candidate for morphological sand developmental studies (Ammermann & Bosse, 1968).

Purpose

In the previous experiment performed by Schill et. al., mRNA levels were used to indirectly implicate the expression of Hsp70 in a different species, *M. tardigradum*. The purpose of these experiments is to

quantify the expression of Hsp70 in *H. dujardini* under desiccating conditions by looking protein levels. This is an attempt to implicate the regulation and expression of these proteins in cryptobiotic entry, tolerance, and restoration of function in a new model organism.

Materials and Methods

Tardigrade Culture

The animals were reared in petri dishes with a thin layer of 1.5% agar, as the animals appeared to have difficulty with locomotion on plastic. The green algae c*hlorococcum* was used as a food source and substrate. Dishes were stored in a sealed box in the dark with an open beaker of 30% glycerol to maintain a high relative humidity of 98% and prevent evaporation. Plates were kept active for up to three weeks before the population grew too large. At this point the animals and any eggs were picked onto new plates.

Desiccation Conditions

Animals were desiccated on plates coated with a thin layer of 1.5% agar. A normal culture plate was taken and the excess water and algae decanted off. The plates were then left in a closed box adjusted to 98% relative humidity with an open beaker of 30% glycerol for 48 hours in order to ensue gradual and correct entry into the tun state. At this time the plate was placed in a desiccator over silica gel to maintain 0% relative humidity for at least 24 hours to ensure total desiccation.

Desiccation Controls

Controls were run to account for any small difference in protein amounts that might be attributable to animal death before entering, during or after exiting the state of anhydrobiosis. The experiment was run in triplicate. A total of 45 animals were picked onto new 1.5% agar plates and desiccated according to

the previous procedure. The tubes were then transferred to a desiccation chamber and left open for another 24 hours to ensure total desiccation. The animals were then rehydrated in Deer Park water. PCR tubes were filled with water and sealed for 1 hour, after which time the water was pipetted into petri dishes (also filled with water) to check animal survival. Live animals were removed to avoid double counting. Animals were checked again at 24 and 48 hours after rehydration.

Heat Shock

Active tardigrades were exposed to heat shock to check the inducibility of Hsp70 expression. Approximately 100 (1 plate) of animals were picked into a PCR tube filled with Deer Park bottled water and placed in a heat block at 37 C for 90 minutes before being prepped for analysis as described in the sample preparation section.

Sample Preparation for Detection

For protein analysis large plates of tardigrades were gently washed with dH2O to removed excess *chlorococcum.* The animals were then separated from the agar by pressurized blasts of water from a pipette and funneled into centrifuge tubes. After spinning at 3,000*g* for 2 minutes the excess water was decanted off. The samples were centrifuged a second time in a microfuge tube (2 minutes at 10,000*g*) and the excess water again pipetted off. 200 ul of loading buffer were added to the tubes which were subsequently boiled in a block at 100C for 15 minutes. The samples were loaded into 30% acrylamide-bisacrylamide gels and run until entry at 90V before being resolved at 120V.

Sample Preparation for Quantification

Full plates were washed with dH2O and the animals pipetted into microfuge tubes. The tubes were centrifuged at 10,000*g* for 3 minutes and the excess water aspirated off. The tubes were quickly frozen

in liquid nitrogen and 100ul of IP buffer (IPB) was added. The samples were sonicated 3 times using a Sonicator 3000 with an output of 6 and a duty cycle of 10 seconds. Tubes were then centrifuged at 4C and 14,000*g* for 10 minutes. The supernatant was collected and saved. A Bradford standard curve was constructed and used to determine total net protein levels in each sample. The samples were then subjected to a western blot as described below but with constant protein levels being loaded.

Western Blotting

Gels were transferred to PDVF membranes running for 1 hour at 200mA. The membranes were presoaked in 100% methanol for 3 minutes and washed with water before being placed in the transfer apparatus with a transfer buffer containing 20% methanol. The membranes were then checked using Ponceau stain before washing in water followed by TBST (0.02M Tris-base, 0.15M NaCl, 0.05% Tween 20, pH 7.4) 3 times for 5 minutes. The membranes were blocked with a solution of 5% milk for 1 hour and incubated with the primary antibody (Hsp70 mouse anti-human, *Sigma*) overnight in a cold room at 4C. The next day the primary was poured off and the membranes were incubated with the secondary antibody for 1 hour. After being washed 3X with TBST for 10 minutes the membranes were incubated in a 1:1 mix of development solution and hydrogen peroxide for 2 minutes and developed with an appropriate exposure time. Runs with a synchronized number of animals were developed concurrently to obtain comparable results.

Densitometric Analysis

Developed gel films were scanned and imported to ImageJ, an open source image analysis tool. A threshold level for detection was chosen based on visual inspection of the film. Two equal areas in each lane each lane containing the bands of interest were selected for analysis, and plots were obtained of

the optical density (based on pixel count and intensity) versus vertical position. Using these plots, the area under each curve (which is directly proportional to the band intensity) was determined. The relationship between these areas mirrors that of protein levels in the organism.

Results and Discussion

Model System

A successful system for the rearing of high volumes of H. dujardini in the laboratory was developed (see *Materials and Methods*). The animals maintained their fecundity, laying approximately 6-8 eggs per clutch per week, as well as their lifespan (approximately 14 days at room temperature)**.** The animals were also assayed for viability upon recovering from desiccation, showing a mean recovery rate among 3 trial groups of 45 animals to be 94% (see Table 1).

TOTAL	Animals	1 Hour	24 Hours	48 Hours
1	45	4	36	41
2	45	4	41	42
3	45	3	42	44
Average		3.67	39.67	42.33

TRIAL 1	Animals	1	24	48
1	15	2	9	14
2	15	1	14	14
3	15	1	13	13
Average		1.33	12	13.67
TRIAL 2	**Animals**	**1**	**24**	**48**
1	15	3	13	14
2	15	1	14	14
3	15	0	14	14
Average		1.33	13.67	14
TRIAL 3	**Animals**	**1**	**24**	**48**
1	15	0	12	14
2	15	0	15	15
3	15	3	15	15
Average		1	14	14.67

Table 1: Desiccation Trial Results. Average survivability 48 hours after dehydration was 94% among 3 trial groups of 45 animals.

This was performed to ensure that the desiccation procedure was not generally fatal to the animals, which would result in significant protein degradation during the 24-48 hour periods in 0% relative humidity.

A system of obtaining various qualities of whole tardigrade lysate was also developed along with successful sample preparations for proteomic analysis via separation by SDS-PAGE and subsequent probing. The hardy nature of the tardigrade cuticle made this more difficult than initially expected; too strong a method of lysis appeared to have a detrimental effect on protein viability, with blots showing significantly less total protein when analyzed with Ponceau stain. These attempted methods involved varying concentrations of Triton X-100 in a standard loading buffer followed by a simple boiling preparation for 10 minutes at 100C. While a less harsh boiling preparation in running buffer without the detergent was adequate to detect Hsp70 in small amounts, a preparation performed via a well adjusted series of sonication steps greatly improved total protein yield as seen by visual inspection of Ponceau stained blots and was determined to be more suitable for quantification of specific protein levels in the whole animal lysate.

Hsp70 Detection in H. dujardini

Hsp70 was confirmed to be present in both active and dehydrated tardigrades (see Figures 1 & 2). Initial preparations with a constant number of animals (both dehydrated and active) and concurrent exposure reinforced the hypothesis that Hsp70 is more heavily expressed in dehydrated animals than in active ones (see figure). However, due to the insufficiently stable protein levels obtained from a standard boiling preparation a more delicate sonication procedure was necessary for densitometric quantification of Hsp70 levels.

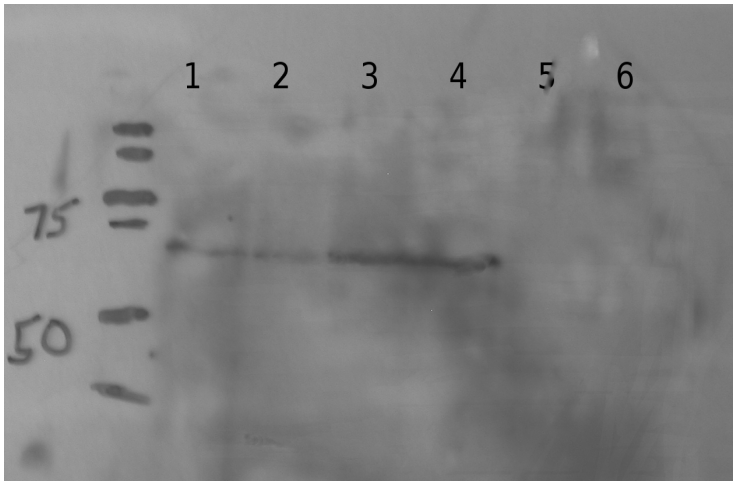

Figure 1: Hsp70 detection in one large plate of active animals. Lanes from left to right - 1 &2: 20ul active animal boiling preparation, 3 & 4: 40ul active animal boiling preparation, 5 & 6: 40ul chlorococcum boiling preparation as a control.

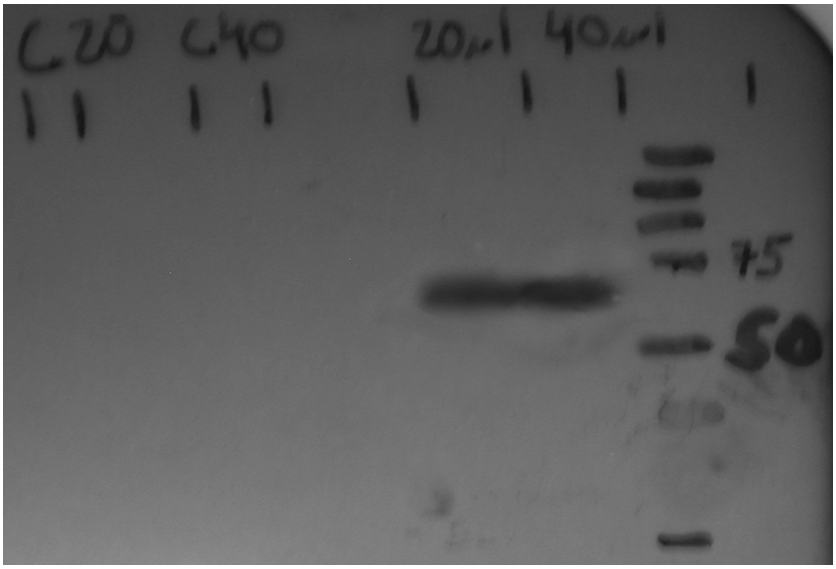

Figure 2: Detection of Hsp70 in dehydrated animals. Lanes from left to right – 1: 20ul chlorococcum control boil preparation, 2: 40ul chlorococcum control boil preparation, 3: 20ul full plate tun boil preparation, 4: 40ul full plate tun boil preparation.

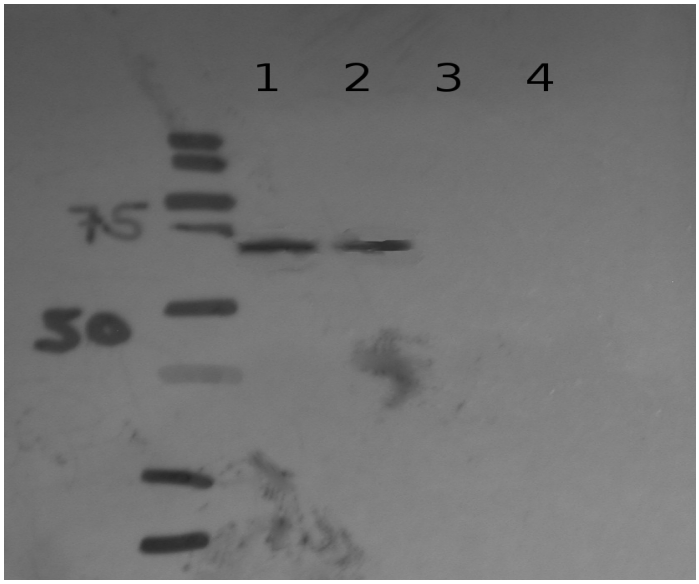

Figure 3: 100 animal preparation of active animals. Lanes – 1: 40ul active boil preparation, 2: 20ul active boil preparation, 3: 40ul chlorococcum control boil preparation, 4: 20ul chlorococcum control boil preparation

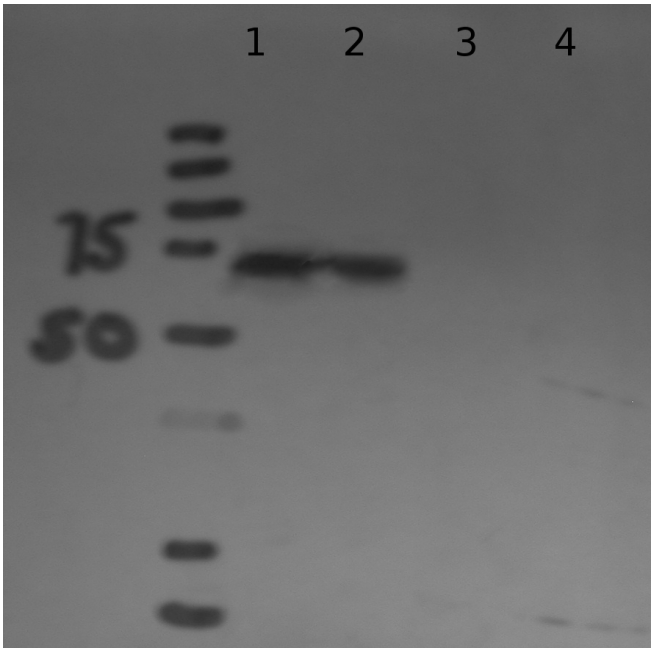

Figure 4: 100 animal preparations of tuns. Lanes – 1: 40ul tun state boiling preparation, 2: 20ul tun state boiling preparation, 3: 40 ul chlorococcum control boiling preparation, 4: 20ul chlorococcum control boiling preparation.

Hsp70 Quantification in h. dujardini

Analysis of the constant total protein western blot (see Figure 5) by densitometry using ImageJ software reveals a marked increase in the expression of Hsp70 in dehydrated animals, confirming the initial hypothesis and suggesting strongly that heat shock proteins are implicated in anhydrobiotic survival of tardigrades (see Table 2 & Figure 6). The analysis reveals that dehydrated animals produce approximately 1.47 times as much Hsp70 than active animals.

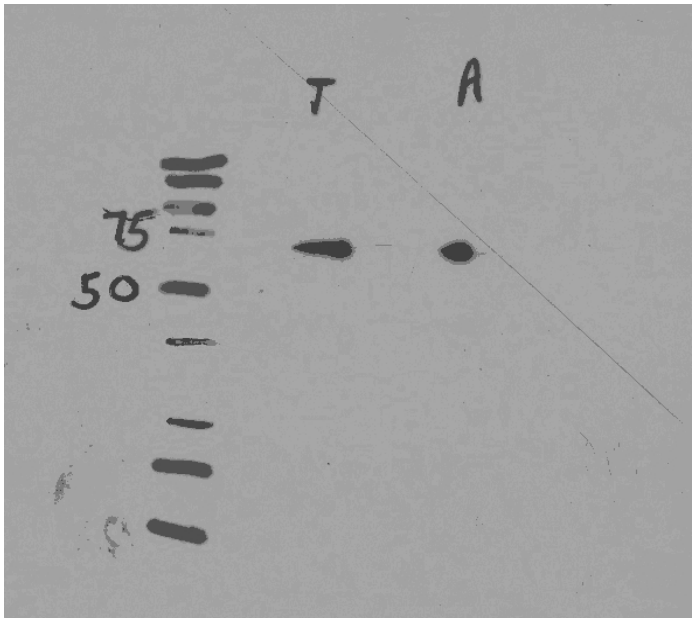

Figure 5 Constant protein level western blot of active (A) and dehydrated (T) animals.

Lane	Area Under Curve
T	216.05
A	147.19

Table 2 Results of analysis of area under OD curve using ImageJ.

Further Work

In order to fully understand the role that Hsp70 plays in cryptobiotic survival and stress recovery, more work is needed. In particular, it will be necessary to show localization of the protein before and after entering anhydrobiosis via antibody staining, as well as to determine the specific interactions that Hsp70 has with other tardigrade proteins or cellular structures.

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