



2017

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## Recommended Citation

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**THE EFFECT OF ACUTE THERMAL STRESS ON GENE EXPRESSION LEVELS OF  
HSP70 IN THE ANTARCTIC NOTOTHENIOID, *Notothenia coriiceps***

By Eudiah L. Ochieng

Under the mentorship of Dr. Johanne M. Lewis

**ABSTRACT**

The waters of the West Antarctic Peninsula are known as one of the most cold, stable environments on earth and organisms living within, such as the fish *Notothenia coriiceps*, have become highly specialized over evolutionary time. However, water temperatures in this environment have been steadily warming over the past two decades due to global climate change. In addition to the challenges created by increasing temperatures *N. coriiceps* will also be faced with difficulty meeting oxygen demands to carry out metabolic processes due to the decrease solubility of oxygen in warmer environments. Heat stress proteins (Hsps) are critical molecules that assist in intracellular processes. Hsp genes are highly conservative and upregulated in all species. However, it has been shown that many Antarctic organisms are unable to mount a heat shock response (HSR) making them highly vulnerable to the effects of global warming. Previous studies have shown that Hsps are strongly upregulated in the red blood cells (RBCs) of most fish in response to thermal stress, but date the HSR has not been investigated in the red blood cells from Notothenioid fishes. As a point of departure, our study investigated the HSR *N. coriiceps* red blood cells. Blood was collect from fish exposed to either an elevated, but sub-lethal temperature (4°C; n=8) or ambient conditions (0.5°C). Our results show that transcript for HSP70 is expressed at detachable levels in *N. coriiceps* erythrocytes. Additional studies analyzing changes in relative mRNA expression as a result of thermal stress are in progress.

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April 2017  
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## Acknowledgements

I would like to acknowledge the following departments that made my research possible: Georgia Southern University Biology Department for providing laboratory space and resources and the Honors Programs for the undergraduate research experience. As well as the following individuals that provided assistance during my research: My thesis mentor, Dr. Johanne M. Lewis for guidance, edits, molecular troubleshooting, and overall support. I acknowledge the staff at the US Antarctic Research, Palmer Station and the masters and crew of the ARSV Laurence M Gould for their assistance with fish collection in the field. As well, a special thank-you to Dr. Kristin O' Brien (University of Alaska, Fairbanks) and her research team for providing the red blood cell samples from their 2013 Antarctic field season funded by her US National Science Foundation Grant (ANT-1043781) and for providing primer sequences.

## INTRODUCTION

The waters surrounding the West Antarctic Peninsula provide a stable cold environment for polar fish (-1.9 to 0.5° Celsius). As a result, over evolutionary time the organisms living in this environment have become highly adapted. The West Antarctic Peninsula and its surrounding water are one of the most rapidly warming areas of the world due to global climate change. The National Oceanic and Atmospheric Administration has documented an increasing yearly temperature change (+0.05 to +0.1°C) in Antarctica for the last two decades. Many of the fish living in this environment have upper lethal temperature limits of 6-8°C (Somero and DeVries 1967). These fishes appear to have lost the evolutionary conserved heat shock response (HSR), decreasing their ability to repair heat induced damage to their cells (Hofman et al. 2000, Buckley et al. 2004). In addition, warmer water temperatures will be accompanied by a decrease in the solubility of oxygen in the water meaning in addition to thermal stress, organisms will also be challenged with meeting their oxygen demands. As a result, these organisms will have to find ways to increase oxygen carrying capacity in order to meet their physiological demands.

The HSR is characterized by temperature-induced expression of genes for molecular chaperons which function in the refolding of proteins that have become denatured due to exposure to high temperatures. These proteins were named heat shock proteins (Hsps) as a result of their initial discovery in response to heat stress, however, they have since been found to be upregulated in response to many other environmental stressors (Lindquist 1986). Heat stress proteins (Hsps) are a family of proteins that play a crucial role in intracellular processes and are classified individually related to their

molecular weight in kiloDaltons (Clark and Peck 2009). Hsps are highly conservative and occur in every species; their highly conserved nature has facilitated their sequencing and comparisons in many organisms (Feder and Hofmann 1999). Hsp70, is a chaperone protein that assists in correct folding and stabilizing of cellular proteins, regulates removal of degraded proteins, and adaptively responses to thermal stress. An important chaperone, Hsc70, is expressed constitutively (under all conditions). The inducible form, Hsp70i, is expressed in response to external stimuli such as elevated temperatures. Although Hsps are often strongly upregulated, there is reduced expression of the inducible form of Hsp70 in Antarctic organisms. The majority of Antarctic fish cannot upregulate Hsp70 in response to external stress (Clark and Peck 2009). Studies have investigated constitutive and inducible forms have overlooked one important tissue, blood. Maintenance of healthy erythrocytes is essential for maintaining oxygen carrying capacity for fishes. Studies have shown that nucleated erythrocytes of fish outside of the Antarctic environment fish have a very strong HSR (Currie and Tufts 1997).

Previous studies have shown that Hsps serve as a responding molecular chaperon, repairing damage to proteins during heat stress. This process aids in maintaining viable RBCs in circulation to preserve oxygen carrying capacity. In warmer environments where environmental oxygen decreases this is particularly vital. Currently the effect of thermal stress on red blood cells of Notothenioid fish is unknown.

This study investigated the erythrocyte HSR in *Notothenia coriiceps*, a red-blooded Notothenioid species commonly found in the waters of the West Antarctic Peninsula. Specifically, we will measure changes in the relative mRNA expression, of the inducible form of the Hsp70 protein (HSP70) in response to elevated, but sub-lethal

temperatures. I hypothesize that in response to heat stress, *Notothenia coriiceps* have elevated levels of Hsp70, HSP70, which can be measured by an increase in relative mRNA expression of HSP70 in the thermal challenged fish.

## METHODS

### *Animals and Holding Conditions*

*Notothenia coriiceps* specimens (Richardson 1844) were collected (and provided by Kristin O' Brien, University of Alaska, Fairbanks) from Dallmann Bay (64°S, 62°W) and off the southwestern shore of Low Island (63°S, 62°W), Antarctica by baited fish traps and held in aerated, circulating seawater tanks aboard the ARSV Laurence M Gould research vessel at  $0\pm 0.5^{\circ}\text{C}$  until transferred to the US Antarctic research station, Palmer Station. At Palmer Station, fish were maintained in circulating seawater tanks at  $0\pm 0.5^{\circ}\text{C}$ . All experiments were conducted in accordance with IACUC regulations (University of Alaska, Fairbanks 247598-12).

### *Thermal Challenge*

*N. coriiceps* specimens for the thermal challenge treatment (N=7) were placed in two 700-L insulated current flowing seawater tanks at  $0.1\pm 0.5^{\circ}\text{C}$  (3 or 4 fish per tank). Specimens were held for 24 hours and then using 3-KW Electro Titanium inline heaters (Aqualogi, San Diego, CA, USA) placed in closed circuit to each tank, temperature per tank was increased  $0.5^{\circ}\text{C day}^{-1}$  for six days until tanks reached  $4^{\circ}\text{C}$ . Acclimated fish were

held at  $4 \pm 0.2$  °C whereas non-acclimated (control) fish (N=6) were held in similar tanks at  $0.1 \pm 0.5$  °C. Treatment groups were held at their respective temperatures for 22 days and then euthanized with a lethal dose of MS-222 (1:7500 in seawater). Blood was drawn from the caudal vessel (~3 ml), centrifuged at 10,000g for 5 minutes. The resulting packed red blood cells were separated from the plasma and stored at 80°C until analysis.

### *RNA Extraction*

To isolate RNA, red blood cells of *N. coriiceps* were homogenized in a 1.5 mL Eppendorf tube in TRIzol reagent with sterile, disposable pestles and a hand held homogenizer (100 mg RBCs: 1.2 mL TRIzol). Homogenates were used for total RNA extraction following the TRIzol manufacturer's protocol with modifications. Homogenized samples were left to incubate at room temperature (RT) for 5 minutes. Samples were extracted by adding 240 µl of chloroform to each sample and vigorously shaken for 15 seconds proceeded by 3 minute incubation at RT. Homogenates were centrifuged at 12,000 g for 15 minutes at 4°C and the supernatant was transferred to a new 1.5 mL Eppendorf tube. 600 µl of 100% isopropanol was added to each aqueous phase sample and incubated at RT for 10 minutes. Samples were centrifuged at 12,000 g for 10 minutes at 4°C. Resulting supernatant was removed from the tube, leaving only the RNA pellet, which was washed with 1.2 mL of 75% ethanol. Pellet and wash were vortex briefly then centrifuged at 7,500 g for 5 minutes at 4°C. Wash was discarded and pellet was air dried for 5- 10 minutes. Once dried, pellet was suspended in 20µl of RNase-free water and incubated in a heat block for 15 minutes at 55-60°C. RNA concentration was

measured on a Nanodrop UV spectrophotometer. All total RNA samples were stored at -80°C until analysis.

*cDNA Synthesis; and validation of primers with RT-PCR*

cDNA was synthesized from 2 ug of total RNA in a 20 ml reaction using random primers [200 ng (Invitrogen)] and RevertAid H- MuL V Reverse transcriptase [200 U/ml (Fisher Scientific)] with the manufacturer's 5X reaction buffer and RNase OUT [40 unit/ml (Invitrogen)] at 42°C for 60 minutes. Negative control reverse transcriptase (NRT) and no-template control (NTC) reactions were performed on a subset of RNA samples from each treatment. cDNA was diluted in nuclease-free water to a final volume of 40 ml. cDNA concentration was measured with NanoDrop UV spectrophotometer. Samples were stored at -20°C until further analysis.

PCR primers for the gene for interest (HSP70) and Gene specific primers for HSP70 and the housekeeping gene, (EF-1 $\alpha$ ,) were designed and provided by K.O'Brien and purchased from Integrated DNA Technologies (Table 1). Using these primers, RT-PCR amplifications were performed using a Mastercycler thermal-cycler in a 25  $\mu$ l reaction that contained 0.1  $\mu$ l Platinum®Taq (Life Technologies, Grand Island, NY), 0.5  $\mu$ l of cDNA, and 10  $\mu$ M of both forward and reverse primers. Duplicate reactions were completed on the thermal-cycler for 3 minutes at 94°C, followed by 38 cycles of 30 seconds at 94°C, 30 seconds at 58 °C, and 30 seconds at 72°C, and final extensions of 15 minutes a 72°C. Negative controls were included in each run and contained all components of the reaction omitting the cDNA template, which was replaced with

DEPC- treated nuclease-free water. Products were separated on 1% agarose gel with a 100 bp ladder to verify amplification of a single, appropriately sized target with no primer dimers.

Table 1. Oligonucleotide primer pairs used for quantifying transcript levels with qRT-PCR in *N. coriiceps* (primers designed by K. O'Brien) HSP70, heat shock protein 70kDa; EF-1 $\alpha$ , elongation factor 1 $\alpha$ .

GENE	PRIMER SEQUENCE	AMPLICON SIZE
HSP70	F - 5' ACCAGGTGGCTTTGAACCCT 3' R - 5' CCACCACTGGATCATCAATCTTT 3'	81bp
EF-1 $\alpha$	F - 5' CCTCGGTGTGAAGCAGCTC 3' R - 5' GTTGTCTCCGTGCCATCCA 3'	175bp

*Quantitative Reverse Transcription –Polymerase Chain Reaction Analysis (QRT-PCR)*

HSP70 and EF-1 $\alpha$  transcript expression levels were quantified by QRT-PCR on the QauntStudio 6 (Life Sciences Solutions) using SYBR Select Master Mix (Life Technologies). Amplification for was carried out in duplicate in a 12.5  $\mu$ l reaction containing 2 $\mu$ l template, 0.5  $\mu$ l primer (2.5  $\mu$ M), 6.25  $\mu$ l SYBR Green Master Mix (Fisher Scientific), and a volume of RNase-free water to bring total volume to 12.5  $\mu$ l. The real-time analysis program consisted of 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes and 40 cycles of (95 °C for 15 seconds and 60°C for 1 minute), followed by dissociation analysis, +1 % increases every 30 seconds from 60°C to 95°C. Pooled cDNA samples were taken from fish in both treatments (control and acute thermal exposure) were used for quality testing of each primer set. This included dissociation curve to confirm there was a single peak and amplification efficiency calculation

[ $E=10^{(-1/\text{slope})}$ ; Pfaffl 2001] based on 5-point fivefold dilution standard curves.

Qualification cycle (Cq) values obtained from the mastercycler were used to generate a standard curve and to calculate  $R^2$  values and amplification efficiency. Primers were checked for primer dimers via a melt curve analysis.

## RESULTS

Gel electrophoresis results from RT-PCR displayed successful amplification of product at appropriate amplicon size. Dark bands at approximately 50-100 bps for HSP70 and 100- 200 bps for EF-1 $\alpha$  were seen in gel imaging. RT-PCR resulted in a single product with no evidence of primer dimers. NTC lane for EF-1 $\alpha$  showed no amplification. NTC lane for HSP70 showed a possibility of contamination in the sample (Figure 1).

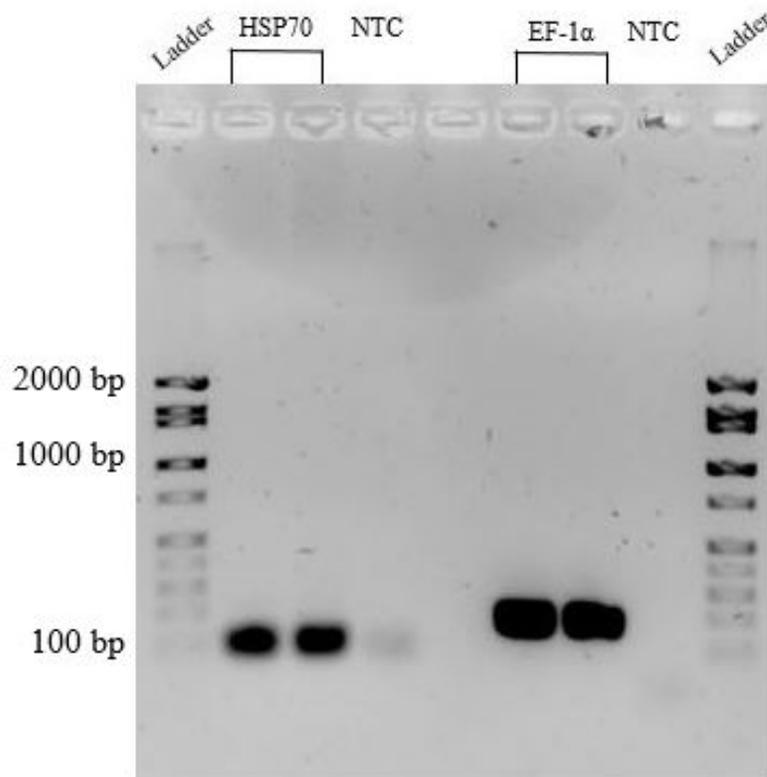


Figure 1. Representative gel electrophoresis (1% agarose) of RT-PCR results for HSP70 (lanes 2&3) with associated NTC (lane 4) with EF-1 $\alpha$  (lanes 6&7) with associated NTC (lane 8). A 100 bp ladder (lanes 1&9) was included for amplicon size comparison.

Analysis of EF-1 $\alpha$  has an  $r^2$  of 0.961 and efficiency of 117.701% (Figure 2). Analysis of HSP70 has an  $r^2$  of 0.395 and efficiency of 2,008.518% (Figure 3). Melt curve data displays all dilutions occurring at the same temperature at converging peaks (Figure 4).

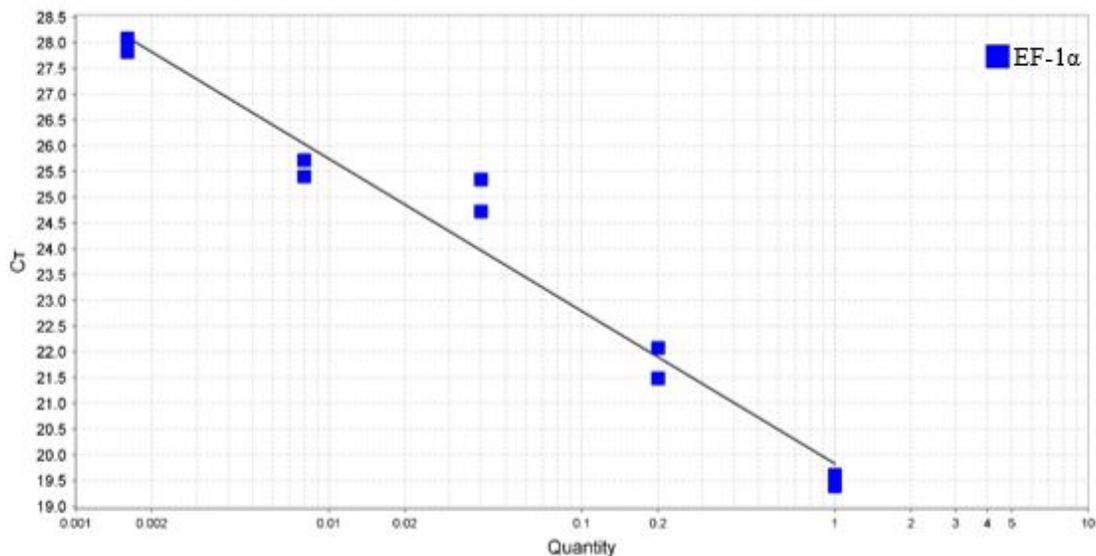


Figure 2. Standard Curve for EF-1 $\alpha$ . Serial dilutions ratios were 1:1, 1:5, 1:25, 1:125, and 1:625. EF-1 $\alpha$  standard curve has an  $r^2$  of 0.961 and efficiency of 117.701%.

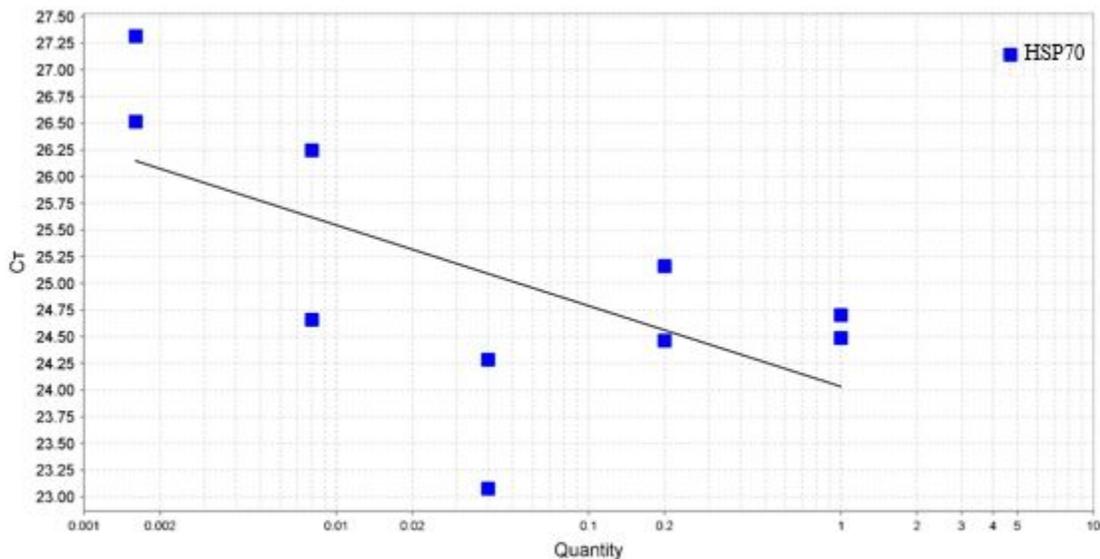


Figure 3. Standard Curve for Hsp70. Serial dilutions ratios were 1:1, 1:5, 1:25, 1:125, and 1:625. HSP70 standard curve has an  $r^2$  of 0.395 and efficiency of 2,008.518%.

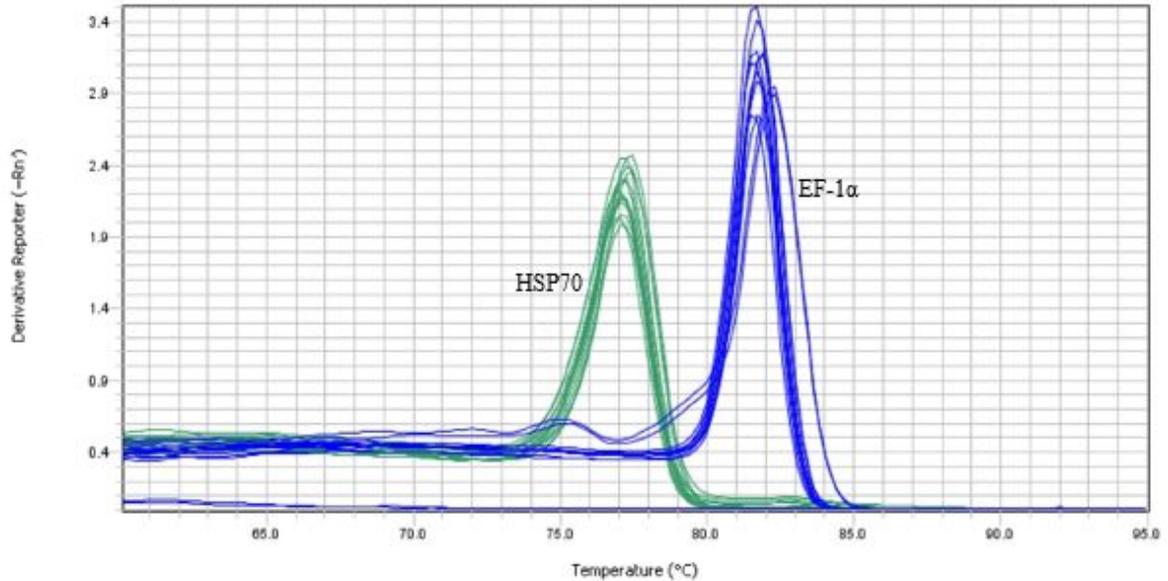


Figure 4. Melt Curves of EF-1 $\alpha$  and Hsp70. Primers targeted specific genes of interest.

## DISCUSSION

Notothenioid fishes are well adapted to Antarctic cold environments in the Southern Ocean. Recent climate changes pose a threat to their stable, cold environment and create challenges to carry out metabolic functions and maintenance of oxygen demands. Previous studies indicate a loss of heat shock response in gill tissues of Antarctic notothenioid fishes occurred during evolution at cold and constant environmental temperatures. Our study aimed to measure changes in mRNA expression of the inducible form of the HSP70 protein in *Notothenia coriiceps*, specifically in red blood cells, under acute thermal stress to study how the organism adapts and functions under harsh conditions.

RT-PCR results showed expression of HSP70 in erythrocytes of *Notothenia coriiceps*. Our study successfully validated the primer set for housekeeping gene, EF-1 $\alpha$  at strong  $r^2$  and within efficiency range. Validation of GOI, HSP70, was not successful. Lack of validation did not permit for quantification of change in expression of HSP70 transcript in response to heat stress. Possible methods to meet primer validation include trouble shooting of alter primer concentration, change in primer set, or annealing temperatures from 58 °C to 59.3 °C. There is uncertainty if fish red blood cells respond the same as other tissues, maintaining constant levels of Hsp70, or if the response is similar to other fish and upregulate gene expression with supporting evidence from Curie and Tufts discovery of increase Hsp70 synthesis in rainbow trout RBCS subjected to anoxia and thermal stress (1996).

qRT-PCR of Housekeeping gene, EF-1 $\alpha$ , suggest that gene expression is present at normal levels. The presence of HSP70*i* in RBCs suggest potential repair to external stress. The RBCs of *N. coriiceps* are responding to thermal stress and producing molecular proteins to repair damage to maintain depend for oxygen to carry out other metabolic processes vital to survival. If Antarctic fish have lost this response, then threats from global warming will affect fish ability to maintain adequate RBCs count which can affect the organism's ability to maintain homeostasis. Quantifying expression change of HSP70*i* will provide further information on how Antarctic fish's' ability to respond to threats at the organism level.

## CONCLUSION

The study was able to show the presence of the inducible form of Hsp70 in the Antarctic fish, *Notothenia coriiceps*. Results suggest a response to acute thermal stress. Future directions of study include measuring changes in mRNA expression of HSP70 in the constitutive form and other HSPs (Hsp60, Hsp90, etc.). Further studies measuring expression changes of Hsp70i will provide information of how the organism responds to threats of global warming.

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