Fiscal Year:	FY 2019	**Task Last Updated:**	FY 08/30/2019
PI Name:	Boothby, Thomas Ph.D.		
Project Title:	Using Water Bears to Identify Biological Countermeasures to Stress During Multigenerational Spaceflight		
Division Name:	Space Biology		
Program/Discipline:			
Program/Discipline--Element/Subdiscipline:			
Joint Agency Name:		**TechPort:**	No
Human Research Program Elements:	None		
Human Research Program Risks:	None		
Space Biology Element:	(1) Cell & Molecular Biology (2) Animal Biology: Invertebrate		
Space Biology Cross-Element Discipline:	(1) Reproductive Biology (2) Developmental Biology		
Space Biology Special Category:	None		
PI Email:	Thomas.Boothby@uwyo.edu	**Fax:**	FY
PI Organization Type:	UNIVERSITY	**Phone:**	
Organization Name:	University of Wyoming		
PI Address 1:	1000 E. University Ave., Department #3944		
PI Address 2:			
PI Web Page:			
City:	Laramie	**State:**	WY
Zip Code:	82071	**Congressional District:**	1
Comments:	NOTE: Previously at University of North Carolina until fall 2019.		
Project Type:	FLIGHT	**Solicitation:**	2014 Space Biology Flight NNH14ZTT001N
Start Date:	11/01/2014	**End Date:**	08/26/2019
No. of Post Docs:	1	**No. of PhD Degrees:**	
No. of PhD Candidates:		**No. of Master' Degrees:**	
No. of Master's Candidates:		**No. of Bachelor's Degrees:**	
No. of Bachelor's Candidates:	1	**Monitoring Center:**	NASA ARC
Contact Monitor:	Sato, Kevin	**Contact Phone:**	650-604-1104
Contact Email:	kevin.y.sato@nasa.gov		
Flight Program:	ISS		
Flight Assignment:	NOTE: End date changed to 8/26/2019 due to PI move to University of Wyoming; new grant 80NSSC20K0283 awarded (Ed., 7/24/2020) NOTE: Extended to 9/02/2020 per F. Hernandez/ARC (Ed., 10/12/18) NOTE: Extended to 10/31/2018 per F. Hernandez/ARC (Ed., 12/6/17)		
Key Personnel Changes/Previous PI:	September 2016 report: Kiera Patanella, an undergraduate at the University of North Carolina at Chapel Hill working on this project, has graduated and obtained her bachelors degree in Biology. Cody Weyhrich, an undergraduate at the University of North Carolina at Chapel Hill, has started working on this project as of 8/1/2016.		
COI Name (Institution):	Goldstein, Bob Ph.D. (University of North Carolina)		
Grant/Contract No.:	NNX15AB44G		
Performance Goal No.:			
Performance Goal Text:			

Task Description:

For most organisms the stresses associated with spaceflight induce a variety of detrimental effects. To foster a safe and productive long-term human presence in space, therapies and countermeasures to spaceflight-induced stress should be developed. Tardigrades (water bears) are polyextremophiles that have evolved to tolerate multiple extreme environments, which are restrictive to most life. In 2007 tardigrades were shown to survive and reproduce normally during an 11-day low Earth orbit on the Foton-M3 Capsule. We speculate that mechanisms tardigrades have evolved to withstand extreme environments on Earth may, as a side-effect, confer protection against the stresses of spaceflight. This makes tardigrades a uniquely valuable system for studying responses to spaceflight. We have sequenced the genome of the tardigrades Hypsibius dujardini, as well as developed and validated experimental and computational approaches for measuring the effect of different environmental conditions on tardigrade gene expression – allowing us to identify mechanisms used by tardigrades to protect themselves from different stresses. We have also developed a reverse genetic approach, RNA interference, for tardigrades that allows us to directly investigate the role of a gene in conferring tolerance to an environment. We will use these approaches to study tardigrades' initial, as well as multigenerational, response to spaceflight and use RNA interference to test the functionality of the genes identified in our study. Next-generation transcriptome sequencing will be conducted on tardigrades cultures kept 0 generations (founding generation) and 4 generations onboard the International Space Station (ISS). Differential expression analysis will be conducted to compare ISS spaceflight timepoints, ground controls, and tardigrades exposed to other extreme stresses (e.g., desiccation, freezing). This approach will allow us to identify potential mediators of stress tolerance, which will serve as candidates for functional RNA interference experiments. Understanding how tardigrades tolerate spaceflight will better guide future research into countermeasures and therapies for humans exposed to the stresses of prolonged space travel. This proposal's strengths are: the use of an organism that is suited to studying mechanisms of multigenerational tolerance of extreme environments and that has an established RNA interference method for confirming the function of genes identified in our study, our Preliminary Results that validate our proposed approach and technical capabilities as well as the uniqueness and suitability of tardigrades that will allow us to conduct this study. The participants for this study are comprised of experts in tardigrades' stress response and have considerable experience with next-generation sequencing and analysis of non-model organisms. The proposed experiments directly address recommendation AH16 of the Decadal Survey and are in line with recommendation OCB-5 and CMM-5 of NASA's Multigenerational and Developmental Biology of Invertebrates Research Emphasis as well as NASA's Fundamental Space Biology Plan 2010-2020 goals. Completion of our proposal will identify genes required for tardigrades to survive multigenerational spaceflight and will be a key step towards developing countermeasures and therapies for stresses associated with prolonged human exposure to space environments.

Rationale for HRP Directed Research:

Research Impact/Earth Benefits:

Along with using mechanisms of stress tolerance to counteract detrimental effects of space travel, data from our proposed experiments could be used in the long term toward solving serious problems in the field of human health. Utilizing mechanisms that allow tardigrades to stabilize their cellular proteins and nucleic acids has been proposed as an option for the dry storage and stabilization of vaccines and other biomaterials (Guo et al., 2000; Wolkers et al., 2001; Puhlev et al., 2001). Because current techniques for vaccine production, distribution, and storage nearly always require a constant cold chain (e.g., -80 and 20 degrees C freezers), these processes are extremely expensive. Some estimates put cold chain costs at around 80% of the total cost of vaccination (Chen et al., 2011). By generating additional stress response datasets, such as response to microgravity, freezing, irradiation, and hypoxia, we will increase our ability and that of other researchers to identify specific mediators of desiccation tolerance, which will then be applied to this and similar problems.
Additionally, a better understanding of mechanisms of stress tolerance could lead to the development of drought and/or freeze tolerant crops.

Guo, N., Puhlev, I., Brown, D. R., Mansbridge, J., & Levine, F. (2000). Trehalose expression confers desiccation tolerance on human cells. Nature biotechnology, 18(2), 168-171.

Wolkers, W. F., Walker, N. J., Tablin, F., & Crowe, J. H. (2001). Human platelets loaded with trehalose survive freeze-drying. Cryobiology, 42(2), 79-87.

Puhlev, I., Guo, N., Brown, D. R., & Levine, F. (2001). Desiccation tolerance in human cells. Cryobiology, 42(3), 207-217.

Chen, X. et al. (2011). Improving the reach of vaccines to low-resource regions, with a needle-free vaccine delivery device and long-term thermostabilization. J. Controlled Release 152, 349–355.

REPORTING FROM AUGUST 2019; PRINCIPAL INVESTIGATOR MOVED TO UNIVERSITY OF WYOMING IN FALL 2019 and NEW GRANT 80NSSC20K0283 AWARDED (Ed., 7/24/2020)
The bulk of our research on NASA Grant NNX15AB44G that was conducted at UNC (the University of North Carolina) can be broken down into two categories: 1.) ground controls and experiments, and 2.) testing and validation for our flight experiment. Below are summaries of our research in both of these categories. Ground Controls and Experiments

Identification and functional assays to identify mediators of tardigrade desiccation tolerance: To prepare for comparison of changes in gene expression manifested by tardigrades (water bears) exposed to multigenerational spaceflight with ground-based stresses we have begun gathering transcriptomic datasets from terrestrial stresses. In addition we have performed functional experiments to assess which candidate genes from our transcriptomic datasets are functional mediators of desiccation (drying) tolerance.

Our major findings from these endeavors have been published in Boothby et al., 2017 (Boothby TC, Pielak GJ. Intrinsically disordered proteins and desiccation tolerance: Elucidating functional and mechanistic underpinnings of anhydrobiosis. Bioessays. 2017 Nov;39(11):700119. Epub 2017 Sep 13. https:// ; PubMed PMID: 28901557 ; Ed. Note 9/9/19: reported in August 2018 FY2018 Task Book report Bibliography). These results will be summarized here briefly since detailed descriptions of the experiments, methods, and results are presented in publication.

To identify genes that might play a role in tardigrade desiccation tolerance, we extracted and sequenced RNA from tardigrades that had either been left unstressed in culture or desiccated. Comparison of transcript levels coming from each predicted gene was conducted and genes ranked based on fold change (how much expression of the gene increased during drying) and overall abundance (how many transcripts per million transcripts were coming from a particular

gene).

The main takeaway from this comparison was that a class of tardigrade specific genes known as Cytosolic Abundant Heat Soluble (CAHS) genes are upregulated heavily during desiccation. We performed RNA interference experiments in tardigrades to reduce the level of expression of these genes and found that the animals no longer robustly survived drying when CAHS genes were targeted. We also found that expressing these genes in bacteria and yeast (which normally do not have these genes) led to up to two orders of magnitude increases (100X) in desiccation tolerance. Amazingly, when purified CAHS proteins were found to protect biological material (the enzyme lactate dehydrogenase) about an order of magnitude (10X) better than current FDA approved excipient trehalose and serum albumin.

Finally, we correlate the protective capabilities of these CAHS proteins to their ability to form vitrified (glass-like) solids, as opposed to crystalline solids. These finding may be of interest to NASA, as this presents an avenue for stabilizing and protecting biological material in a dry state without refrigeration. This might be useful for prolonged storage of biomaterials on the ISS or other spaceflight missions where freezer and refrigeration space is limited or logistically difficult.

Exploring cross-tolerance between desiccation and freeze tolerances in tardigrades:

Tardigrades survive an amazing number of abiotic stresses, and in some cases the severity of these stresses is well beyond that tardigrades would ever experience in nature (e.g., temperatures close to absolute zero, thousands of gray of radiation, the vacuum of outer space). The question therefore arises--how did tardigrades evolve tolerance to stresses they have never experienced? One hypothesis is that as tardigrades moved onto land from the ocean (where they originally evolved) they developed desiccation tolerance in response to their new, dryer, conditions and as a by-product became tolerant to other stresses. If this hypothesis is correct, then the mediators that tardigrades use to survive desiccation should in theory be the same mediators they use to survive other stresses. To assess if this is true we performed transcriptome sequencing on tardigrades that had been frozen, and compared this data to our previous datasets (desiccated and unstressed).

Surprisingly, we found that changes in gene expression between desiccated and frozen tardigrades are highly divergent. In fact, either stress condition is more similar to unstressed conditions than they are to each other. Most telling, we observed that expression of CAHS genes was not influenced by freezing conditions, and furthermore RNA interference targeting these genes did not result in statistical decreases in survival in tardigrades exposed to freezing conditions.

These results are presented in detail in Boothby et al., 2017 (Boothby TC, Pielak GJ. Intrinsically disordered proteins and desiccation tolerance: Elucidating functional and mechanistic underpinnings of anhydrobiosis. Bioessays. 2017 Nov;39(11):700119. Epub 2017 Sep 13. https:// ; PubMed PMID: 28901557 ; Ed. Note 9/9/19: reported in August 2018 FY2018 Task Book report Bibliography).

We are now delving more into our frozen transcriptome to identify functional mediators of freeze tolerance in tardigrades using a similar approach to the one taken for our desiccation study.

Understanding to commonalities and differences between how tardigrades survive freezing and desiccation is an important facet of our overall strategy for this project, as spaceflight induced changes in gene response will ultimately be compared to ground-based stress responses. Comparing changes in gene expression for ground-based stresses now will help us understand the overlap with spaceflight induced changes later.

How do tardigrade CAHS proteins mediate desiccation tolerance?

To better understand of CAHS proteins protect tardigrades and other biological material and cells from the harmful effects of drying, and how these proteins might protect biological materials from other stresses (included spaceflight), we have been characterizing the biochemical and biophysical nature of these proteins.

Task Progress:

We have discovered that these proteins behave in a very peculiar way. At room temperature and at concentrations greater than or equal to 30 g/L these proteins form reversible hydrogels. We have characterized the gel state of these proteins via cone plate rheometry as well as scanning electron microscopy. Both techniques clearly demonstrate that these proteins have classic gel-like behavior and morphology. In retrospect it makes sense that these proteins form hydrogels, as hydrogels are known to form vitrified solids when dried (see above).

We were curious if the gel state of these proteins is important for their protective capabilities. To probe this, we used 19-F NMR to look at the folded state of a test protein, SH3. SH3 is a 'metastable' protein, meaning that normally (in solution) SH3 is in a folded state ~50% of the time and in an unfolded state about ~50% of the time. This is easily measured using 19-F NMR. We first tested SH3 in solution, and as expected two clear peaks (folded and unfolded) were present. We then co-incubated SH3 with a CAHS protein (at increasing concentrations) and looked at the levels of folded and unfolded protein. We found that CAHS proteins had no noticeable effect on SH3 folding below 30 g/L. However, above 30 g/L of CAHS protein, there was a reduction in the level of unfolded SH3 protein, and a corresponding increase in folded SH3. Interestingly, 30 g/L is the concentration determined by cone plate rheometer as the gel point for these proteins. The hydrogels that CAHS proteins form are reverse and heat dependent. Therefore, we tested whether heating to induce the breakdown of the hydrogel influenced SH3 folding. We found when heated from 19C to 42C the CAHS proteins went back into solution (the gel state vanished) and there was a return of an SH3 unfolded population. Cooling this solution back down to 19C resulted in re-gelling of the CAHS proteins and a corresponding disappearance of the SH3 unfolded species and an increase in the SH3 folded species. Therefore, it appears that there is a strong correlation between the gelled state of CAHS proteins and their ability to stabilize proteins in a folded state.

We are now characterizing the sequence features of CAHS proteins at allow them to form gels. These studies are being conducted by making mutant versions of the proteins and testing their ability to form gels and protect biological materials.

Testing and Validation for Flight Experiment

The bulk of this effort has been made in preparing for and performing our Science Validation Test (SVT-1).

The main goal of SVT-1 was to compare the efficacy of culturing tardigrades (Hypsibius exemplasris) using the CellMax and PI Start Kit (PISK).

Syringes containing ~500 tardigrades or concentrated algae were prepared using the same stock cultures. Syringes were frozen at -80 degrees Celsius and either stored at this temperature or shipped on dry ice to NASA Ames.

On May 10th, tardigrades were injected into 3 PISK bioreactors (at NASA Ames) and 3 CellMax Bioreactors (at UNC).

Temperature control for PISK bioreactors was carried out using integrated temperature control – which varied between ~15 – 17 degrees Celsius. Oxygenation for PISK bioreactors was carried out via gas dosing using medical grade air.

Temperature control for CellMax bioreactors was carried out by placing the CellMax system in a controlled temperature chamber. The initial temperature used was 15 degrees Celsius, but was changed to 16 degrees Celsius to more closely mirror PISK temperatures. Oxygenation was not controlled, but rather relied on the passive transport of oxygen across the CellMax systems permeable tubing.

Every 7 days, up to day 21, subsamples (300 ul) were extracted from both PISK and CellMax bioreactors using syringes. These samples were stored at -80 degrees Celsius. At day 14, fresh algae (food source) was injected into each PISK and CellMax bioreactor. At day 28, the experiment ended and whole bioreactors were detached and frozen at -80 degrees Celsius. PISK subsample syringes and bioreactors were returned frozen on dry ice to the Principal Investigator's (PI) lab at UNC.

Upon receipt at the PI’s lab, samples were transferred from their shipping unit to a -80 degree freezer.

To compare the viability of culturing using the PISK, all samples were thawed. For each subsample (300 ul) the entire volume of thawed sample was analyzed by direct observation using a dissecting microscope. Total animal counts were taken and densities calculated. Similarly, for bioreactors, the entire contents of the bioreactor was thawed and transferred to a 15 mL tube. Three 50 ul samples were taken from each tube and total animal counts made and densities calculated.

The remaining bioreactor contents was fixed with RNAlater and processed for total RNA extraction.

Tardigrade Densities and Total Counts

Prior to testing our definition for success was achieving Day 28 animal densities in the PISK bioreactors that were above or within 20% of the densities from our CellMax bioreactors. Our average PISK animal density at Day 28 was 357.8 animals/mL where as our CellMax Day 28 animal density was slightly lower at 282.2 animals/mL. By our original definition, the PISK successfully competed with the CellMax system with regards to effectively culturing tardigrades over a 28 day period.

REPORTING IN AUGUST 2018

We have been preparing for our flight experiment. This has mostly manifested itself in the development of an SRD (Science Requirements Document), protocols, and coordinating with the engineering team who are doing some final tests on our bioreactor setup. Our next step will be to actually get our animals in the hardware and do ground tests.

Bibliography Type:	Description: (Last Updated: 07/24/2020)
Abstracts for Journals and Proceedings	Boothby TC, Piszkiewicz S, Mehta A, Brozena A, Tapia H, Koshland D, Holehouse A, Pappu R, Goldstein B, Pielak G. "Gelation and Vitrification of Tardigrade IDPs." 62nd Annual Meeting of the Biophysical Society, San Francisco, CA, February 17-21, 2018. Biophysical Journal. 2018 Feb 2;114(3 Suppl):560a-561a. https:// , Feb-2018
Articles in Peer-reviewed Journals	Piszkiewicz S, Gunn KH, Warmuth O, Propst A, Mehta A, Nguyen KH, Kuhlman E, Guseman AJ, Stadmiller SS, Boothby TC, Neher SB, Pielak GJ. "Protecting activity of desiccated enzymes." Protein Sci. 2019 May;28(5):941-51. Epub 2019 Mar 30. https:// ; PubMed PMID: 30868674; PubMed Central PMCID: PMC6459994 , May-2019
Articles in Peer-reviewed Journals	Boothby TC. "Desiccation of Hypsibius exemplaris." Cold Spring Harb Protoc. 2018 Nov;18(11):871-3. https:// ; PubMed PMID: 30385670 , Nov-2018
Articles in Peer-reviewed Journals	Boothby TC. "Total RNA extraction from tardigrades." Cold Spring Harb Protoc. 2018 Nov 1;2018(11):905-7. https:// ; PubMed PMID: 30385675 , Nov-2018
Articles in Peer-reviewed Journals	Boothby TC, Pielak GJ. "Intrinsically disordered proteins and desiccation tolerance: Elucidating functional and mechanistic underpinnings of anhydrobiosis." Bioessays. 2017 Nov;39(11):700119. Epub 2017 Sep 13. https:// ; PubMed PMID: 28901557 , Nov-2017
Articles in Peer-reviewed Journals	Boothby TC. "Mechanisms and evolution of resistance to environmental extremes in animals." Evodevo. 2019 Nov 18;10:30. Review. https:// ; PMID: 31827759; PMCID: PMC6862762 , Nov-2019
Journal/Magazine covers	Piszkiewicz S, Gunn KH, Warmuth O, Propst A, Mehta A, Nguyen KH, Kuhlman E, Guseman AJ, Stadmiller SS, Boothby TC, Neher SB, Pielak GJ. "Cover in journal Protein Science for article, 'Protecting activity of desiccated enzymes.' " Protein Sci. 2019 May;28(5):941-51. https:// ; PubMed PMID: 30868674 , May-2019