Evaluating the Effects of Stressors on Immune Function during Simulated Dives in Marine Mammals

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LONG-TERM GOALS

The overall goal is to investigate the response of marine mammal immune cells to simulated dives and evaluate the effects of stressors on this response. The study will investigate both innate and acquired immune function in both belugas and seals in response to challenges associated with diving (i.e. pressure) both with and without the presence of an additional stressor.

OBJECTIVES

The objectives of this effort are: 1) To investigate the effects of simulated dive exposures on cellular immune function in beluga whales 2) To evaluate the effects of simulated dive exposures on cellular immune function following a known stressor event 3) To collect biological samples from wild belugas to compare with aquarium whales and 4) To compare the effects of simulated dive exposures on cellular immune function in seals between stranding (stressor) and release (healthy).

APPROACH

To fullfill objectives 1 and 2 blood samples will be utilized that were collected under a prior ONR effort (N00014-11-1-0437) from four belugas (*Delphinapterus leucas*) residing at the Mystic Aquarium, Mystic, CT. Baseline samples were obtained under positive behavioral reinforcement, minimizing the potential of confounding sampling stress. In addition samples were obtained before and after out of water examination (OWE). Several measures of immune function including phagocytosis, neutrophil and lymphocyte activation and lymphocyte proliferation will be measured in both baseline and OWE samples, with and without *in vitro* pressure exposures, and compared between conditions. The results will be compared with those obtained from human blood samples in order to describe possible evidence of dive adaptation of the marine mammal immune system.

To fullfill objective 3, blood samples will be obtained and utilized from wild belugas during live capture-release health assessments. Hormone values and lymphocyte function assays with and without *in vitro* pressure exposures will be conducted on these samples. The results of this aim will be compared with those obtained from aquarium animals in objectives 1 and 2.

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For objective 4, blood samples will be collected from stranded pinnipeds which are admitted to the Marine Mammal & Turtle Stranding Program at Mystic Aquarium, Mystic, CT. Pinniped species will include harbor seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*) and harp seals (*Phoca groenlandica*). Blood samples will be obtained at the time of admit ('stressed') and again at pre-release ('healthy'), for both hormone analysis and immune function with and without pressure. Results will be compared between the two conditions.

Tracy Romano (P.I) Tracy Romano is the P.I. for the proposed project. She is the primary mentor of Laura Thompson, the graduate student who will be carrying out the proposed project for her PhD thesis. She will oversee progress on all aspects of this project, review methodologies and results and work closely with Laura Thompson on writing up this work for scientific publication and presentation at scientific meetings. Biweekly meetings will be conducted (or more as needed) between her and Laura Thompson.

Laura Thompson is a PhD student at the University of Connecticut (UCONN), Department of Marine Biosciences. Her research focus is impact of stressors on marine mammals particularly as it relates to diving physiology and immune function. Laura will be involved in all aspects of the project including sample collection, archiving, method development, analysis as well as publication in peer reviewed journals and presentation of results at scientific meetings and public forums. Her PhD thesis will result from this work.

WORK COMPLETED (July 1 – September 30, 2013)

Specific Aim 1
Baseline blood samples to evaluate the cellular response to simulated dive exposures will be obtained from beluga whales resident at the Mystic Aquarium. Cells of the immune system will be exposed to increased pressure (simulated dives) using a stainless steel benchtop pressure chamber and mineral oil. Neutrophil phagocytosis and expression of CD11b will be subsequently evaluated; blood will also be used to archive white blood cells for subsequent lymphocyte proliferation and IL2 receptor assays. Plasma catecholamines (norepinephrine, epinephrine and dopamine) will be measured via High Performance Liquid Chromatography in our laboratory and cortisol will be measured via EIA at the AHDC Endocrinology Lab at Cornell University. In addition, human blood samples will be purchased and exposed to the same dive conditions for evaluation of immune function. Results will be compared between belugas and humans in order to identify potential dive adaptation of immune cells. Baseline blood sample collection (n=4) and immune function testing has been ongoing in belugas. Human samples (n=2) have been purchased and immune function tests in response to pressure have been completed.

Specific Aim 2
As part of a prior ONR effort, blood samples were obtained from belugas prior to and at 30 min during an OWE. Immune function will be measured in response to simulated dives, and plasma hormones will be monitored as per objective 1. Some of the immune function studies in response to pressure as well as hormone analysis have been completed to date.

Additionally, baseline blood samples will be used to investigate the effects of individual hormones on immune function during pressure exposures. Isoproterenol (a beta adrenergic receptor agonist) and hydrocortisone (cortisol) will be purchased and used *in vitro* to investigate the effects of individual hormones on the response of immune cells to simulated dives.

Specific Aim 3

Established collaborations will be used to obtain blood samples from wild beluga whales in Alaska during live capture/release studies in Bristol Bay. Initial processing, including isolation of white blood cells, will occur in the field and samples will be shipped back in liquid nitrogen for analysis of hormones (as per aim 1) and immune function. Due to assay requirements, frozen cells can be used only to assess lymphocyte proliferation and activation. Hormone levels and immune function measurements will be compared to specific aims 1 (baseline measurements in aquarium animals) and 2 (stressor conditions in aquarium animals). Archived samples were obtained from 9 belugas during prior live capture-release health assessments. Lymphocyte function assays in response to pressure for these 9 whales have been completed.

Specific Aim 4

Blood (both archived and fresh) will be obtained from stranded pinnipeds admitted to the Mystic Aquarium Marine Mammal and Sea Turtle Stranding Program, providing a unique opportunity to follow individuals over the course of rehabilitation and back to health for release into the wild. Immune function parameters to be measured are neutrophil phagocytosis, neutrophil expression of CD11b, lymphocyte proliferation and T cell expression of the IL2 receptor. In addition, plasma catecholamines will be measured by high performance liquid chromatography and plasma cortisol will be measured at the AHDC Endocrinology lab at Cornell University.

Pressure Exposures

A pressure chamber will be used for the dive simulation. Briefly, four ml of blood will be added to the pressure chamber through a top loading port, and over-laid with a thin layer of mineral oil. Mineral will then be pumped into the chamber by hand using a hydraulic pump, rated to 40 000 psi, in order to pressurize the sample. A pressure gauge will be used to monitor the rate of compression and decompression, as well as to maintain the sample at the desired pressure. At the conclusion of a pressure excursion, pressure will be released by hand by loosening valve connections between the pressure chamber and oil pump. Blood samples will then be removed using a sterile transfer pipette and aliquoted into tubes for subsequent assay. Immune function measures include neutrophil phagocytosis, neutrophil expression of CD11b, lymphocyte proliferation and T cell expression of the IL2 receptor.

For dive profiles three durations will be used; a single 30 minute dive, a single 5 minute dive, and two 5 minute dives with a 1 min ‘rest’ period. Target depths include 1360 m (2000 psi) and 680 m (1000 psi). Compression and decompression will occur over a period of either 2 minutes or 15 seconds.

RESULTS

Objectives 1, 2, and 3

Immune Function

No significant differences in control measures of immune function were detected between baseline and OWE conditions in aquarium belugas. Some differences however were observed in the response of immune cells to simulated pressure exposures to 2000 psi. Figure 1 shows the MFI for granulocyte phagocytosis for 30 minute exposures to 2000 psi with 2 minutes of compression and decompression, measured in control and pressure exposed samples for belugas and humans. During the pressure exposure (dive) both baseline and OWE samples in belugas showed a decrease in phagocytosis as

compared with controls, while humans show a slight increase. Following a further 20 minute recovery however, baseline beluga samples exposed to pressure return to control values, while OWE samples suggest an increase in phagocytosis similar to that observed in humans. These similarities become more apparent when the % change in MFI from control values is compared between conditions (Figure 2).

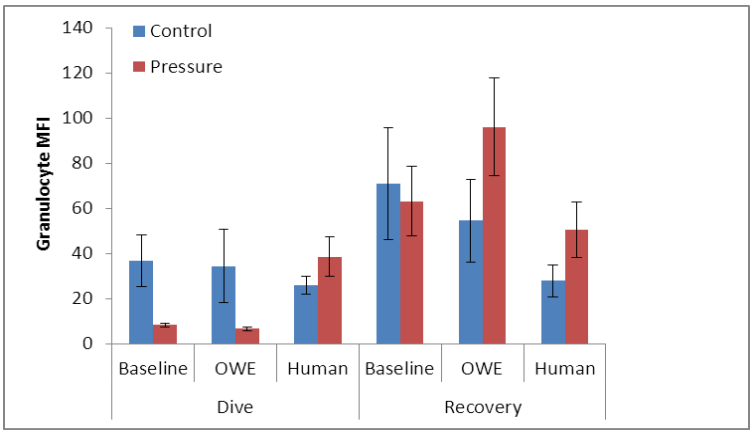

Figure 1 : Granulocyte MFI for Phagocytosis in measured in belugas and humans following exposure to 2000 psi for 30 minutes with 2 minutes of compression and decompression.

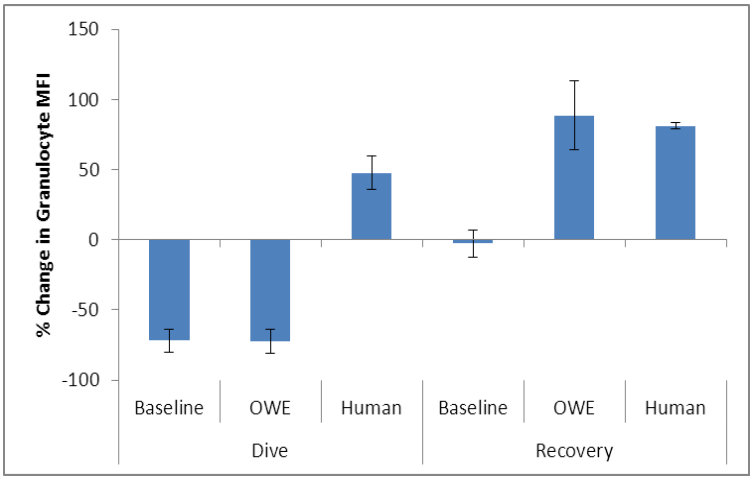

Figure 2 : The calculated % change in granulocyte MFI between control and pressure exposed samples for belugas and humans following exposure to 2000 psi for 30 minutes with 2 minutes of compression and decompression.

White blood cells from wild belugas were only available for lymphocyte function assays, and results showed some differences in the cellular response to pressure exposures as compared with aquarium animals (Figure 3). For baseline conditions, aquarium animals displayed significant decreases in lymphocyte proliferation following both 30 minute and repeated 5 minute exposures to 2000 psi with 2 minutes of compression and decompression. Similarly, significant decreases in proliferation were detected for all exposures for OWE samples as well. Wild belugas however, displayed slight increases in proliferation following all pressure exposures. The increase observed for the wild animals was also significantly different in magnitude from aquarium animals (Figure 4).

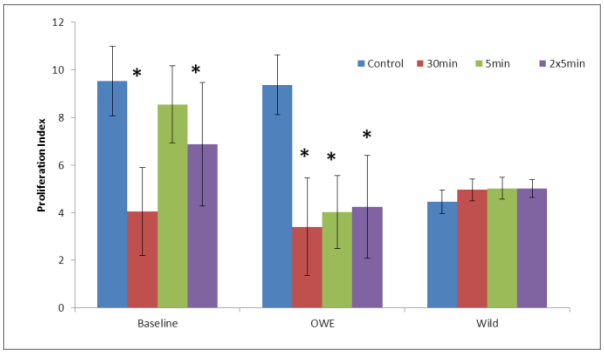

Figure 3: Proliferation Indices for lymphocytes exposed to 2000 psi with 2 minutes of compression and decompression for 30 minute, 5 minute and repeated 5 minute exposures. Asterisks indicate where proliferation indices for a pressure exposure were significantly different from controls (α=0.05).

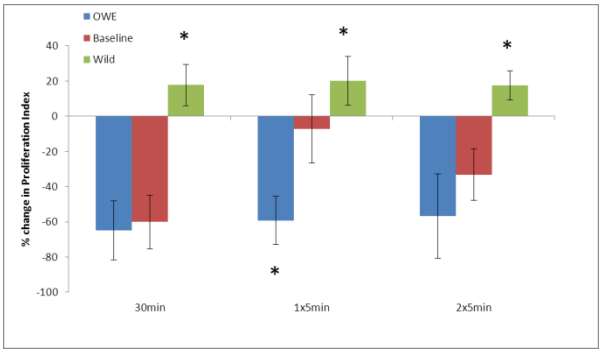

Figure 4: Calculated % change in proliferation indices for lymphocytes exposed to 2000 psi with 2 minutes of compression and decompression for 30 minute, 5 minute and repeated 5 minute exposures. Significantly different responses are indicated with an asterisk (α=0.05).

IMPACT/APPLICATIONS

This project has direct relevance to ONR's interest in the effects of sound on marine mammals and physiology (diving and stress). There is growing interest in evaluating the effects of sound, and other anthropogenic activities on marine mammal behavior and health. In order to do this, it is necessary to study the marine mammal immune system and how the immune system is adapted to the natural challenges of living in an aquatic environment, including the challenges associated with diving. Changes in pressure, temperature and availability of oxygen to tissues and cells are known to alter immune function in humans and such alterations play important roles in the development of dive related pathologies, including decompression sickness (DCS). As more reports surface describing DCS-like emboli in stranded or by caught marine mammals, the question arises as to whether human activities can alter the response of the marine mammal immune system to natural challenges associated with diving and render them more susceptible to disease and injury. This study relates to both diving physiology interests and stress physiology interests by evaluating first the response of immune cells to changes in pressure (i.e. simulated dives) and then evaluating how additional stressors may alter that response.

This study falls under the Office of Naval Research goal of understanding the effects of sound on marine mammals through physiology, diving and stress by specifically addressing the questions of how the marine mammal immune system is adapted to maintain health during diving, and whether a stressor, such as sound, can interrupt that adaptation. This study is the first to look at immune function in response to diving in marine mammals, and is an important step towards understanding the dynamic balance between marine mammal health and their unique environmental challenges. The results of this study can begin to provide insight as to the implications of non-auditory effects of noise on individual and population health in marine mammals. Moreover, this is the first study to investigate immune function in relation to natural environmental challenges associated with diving and potential cumulative effects associated with a stressor.

RELATED PROJECTS

Title: Investigation of the Physiological Responses of Belugas to "Stressors" to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health
PI: Tracy Romano, PhD Mystic Aquarium, a division of Sea Research Foundation
An ONR effort investigating the neuroimmunological responses of belugas to "stressor".