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The Combined Influence of Diving Physiology and Stressors on Immune Cell Function in a Deep Diving Monodontid and Three Shallow Diving Phocid Species

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Laura Anne Thompson, PhD

University of Connecticut, 2014

Abstract

Marine mammals possess adaptations for repetitive and extended diving to great depths without suffering the ill effects seen in humans [e.g. decompression sickness (DCS)] which involve altered immune activity. In recent decades, DCS-like symptoms in marine mammals have increased concerns about marine mammal health and whether anthropogenic activities can interfere with adaptive dive responses, increasing susceptibility to dive related pathologies. The purpose of this work was to address these concerns by: 1) evaluating the *in vitro* response of marine mammal immune cells to increased pressure, 2) comparing the response of cells between baseline and stressor conditions, and 3) developing a non –invasive means of monitoring cortisol in belugas (*Delphinapterus leucas*). Blood samples were obtained from belugas during baseline and stressor (e.g. out of water examination, wild chase and capture) conditions, as well as from stranded harbor seals (*Phoca vitulina*), harp seals (*Phoca groenlandica*) and grey seals (*Halichoerus grypus*) at the time of admit to rehabilitation and again pre-release. Catecholamines and cortisol were measured to demonstrate a physiological stress response. Phagocytosis, lymphocyte proliferation and cell activation were compared between pressure exposed and non-exposed cells for each condition, between different pressure profiles and between conditions using mixed generalized linear models (α=0.05). The response of cells to pressure varied 1) between species, with baseline beluga samples and admit phocid samples

showing opposite patterns of change than humans, 2) with stressor condition as responses differing from baseline but resembling human responses were detected for all stressor conditions in belugas, and 3) with exposure characteristics, with deeper exposures resulting in larger changes in phagocytosis but smaller changes in IL2R expression than shallower exposures. Blow (exhaled breath condensate) was also collected from belugas and validated as a matrix for monitoring cortisol using a commercial enzyme immunoassay. Changes in cortisol were observable in blow following known stressor conditions, supporting use of blow sampling for future endocrinology, dive physiology, health and conservation studies. This work validates non-invasive methodology for monitoring stress responses in cetaceans and provides the first evidence suggesting that anthropogenic stressors may impact marine mammal health by altering the relationship between dive behavior and immune function.

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Chapter 1 INTRODUCTION

General Introduction

Marine mammals have evolved specific physiological and behavioral adaptations to the unique requirements of their entirely aquatic environment, and in turn play important ecological roles in shaping marine communities and ecosystems (Bowen, 1997; Katona and Whitehead, 1988). Marine mammals are top level consumers and have top down effects on shaping biological communities. Declines in populations of large baleen whales in the North Pacific and Bering Sea have been implicated in prey switching in killer whales (*Orcinus orca*) which has contributed to the decline of smaller marine mammal species such as sea otters, seals and sea lions (Springer *et al.,* 2003). In the Southern Ocean, the impact of whaling on large baleen whales has led to greater availability of krill as a prey species for smaller whales, seals and penguins. This increase in food source has played an important role in increasing populations of seals and birds (Katona and Whitehead, 1988). Benthic-feeding species may shape communities by perturbing the environment and creating bare patches, thus increasing potential for re-colonization and spatial biodiversity (Katona and Whitehead, 1988). In addition, carcasses of large whales fall to the sea floor providing a food source for specialized deep sea biota (Smith and Baco, 2003). These 'whale falls' may also provide important stepping stones for the dispersal of fauna normally found at deep sea vents and seeps (Smith and Baco, 2003).

Among the specialized adaptations marine mammals have acquired are those which allow some species to make repeated or prolonged dives to great depths without suffering the ill-effects of compression/decompression which are seen in humans and other terrestrial mammals. In recent years however, there have been several reports of emboli and lesions in stranded and by-caught animals which resemble those seen in human decompression sickness (Jepson *et al.,* 2003; Fernandez *et al.,* 2005). Gas bubbles which likely arose from rapid decompression of saturated

tissues have been reported in stranded dolphins (Dennison *et al.,* 2012) as well as several species collected as by-catch in fishing operations, including harbor seals (*Phoca vitulina*), harbor porpoise (*Phocoena phocoena*), common dolphin (*Delphinus delphis*) and white sided dolphin (*Lagenorhyncus acutus*) (Moore *et al.,* 2009). In addition, there have been increasing reports of stranding and emerging diseases (Gulland and Hall, 2007). As a result, questions about the role anthropogenic activities could play in facilitating such conditions have increased. One concern is that human activities could serve as stressors, eliciting a physiological stress response that could alter normal physiology and compromise protective adaptations (Wright *et al.,* 2007; Talpalar and Grossman, 2005). One mechanism through which anthropogenic stressors could impact health is through modulation of the immune system (Romano *et al.,* 2004; Wright *et al.,* 2011). In order to understand this relationship, it is first necessary to examine the normal response of immune cells to diving. Once this is understood, the potential for additional stressors to impact or alter immune function can be investigated.

Potential stressors for animals in aquaria could include transport between facilities, noise, and social dynamics. Additional stressors faced by free ranging animals could include oil and other pollutants, increase shipping traffic, changing water temperatures and decreased ice coverage, and changing prey distribution and availability. Monitoring the health status of marine mammals in the face of such stressors is important in order to provide the best possible care and maintenance of marine mammals in aquaria and for assessing the health status of populations in the wild. In addition, marine mammals are excellent candidates for sentinels of emerging disease, pollutants and persistent contaminants, as well as changing environmental conditions such as climate change (Reddy *et al.,* 2001; Bossart, 2011).

Monitoring marine mammal health has important implications for human health. As apex predators with large fat (blubber) stores many marine mammal species have been used as sentinels of environmental contaminant loads and potential health threats to humans (Ross, 2000; Reddy *et al.,* 2001). For example, harbor seals have been used as indicators of environmental contamination of polychlorinated biphenyls (PCB's), dioxins and furans at several locations along the west coast of Canada and the United States (Ross *et al.,* 2004). The authors note that blubber accumulations measured reflect all physical, chemical and biological interactions and alterations to the pollutants, which are important considerations for assessing potential health risks.

Emerging diseases can be zoonotic, capable of passing to humans through direct contact or consumption, or indicative of an environmental risk in coastal environments that are shared by human populations. Climate change can have many direct and indirect effects on marine mammals, particularly arctic species including loss of feeding, resting or predator escape habitat, expansion of pathogen range and increased pathogenicity, changes in predator-prey interactions and increased exposure to human activities and noise (Burek *et al.,* 2008). Each of these conditions serves as a potential stressor resulting in altered neuro-endocrine activity. Monitoring these changes can provide insight as to the impacts of such conditions (i.e., subsequent changes in physiology and immune function) on the health of marine mammal populations. In addition, through comparative studies, models for prevention and treatment of ischemia and reperfusion injury (Zenteno-Savin *et al.,* 2002) as well as decompression and hypothermic related pathologies can be developed. Thus, learning how marine mammals avoid dive related injury and disease has potential application in human medical research. For example, preventative therapies

or treatments for decompression or hypoxia/reperfusion related injuries targeting specific aspects of immune function could be modelling after adaptation of the marine mammal immune system.

Immune Function

The immune system protects the body from invasion by foreign organisms and development of disease, and is also responsible for inflammation and wound healing. It can be broken down into two branches; 1) innate and 2) adaptive. Both of these branches rely on proper function of multiple subsets of leukocytes.

Present from birth, the innate immune system provides nonspecific defenses that are not based on prior exposures. Granulocytes are large cells that contain multiple granules (hence the name). These cells are highly mobile, and play an important role in inflammatory processes and the clearing of infection through processes called phagocytosis and respiratory burst. Phagocytosis is a process by which cells engulf foreign particles of debris. The production of oxidizing radicals and a process known as respiratory burst then acts to destroy the potential pathogen. Monocytes are also responsible for a small degree of phagocytosis and respiratory burst.

The response of neutrophils to pathogens is not affected by previous exposure as with cells of the acquired immune system, discussed below. However, the response can be increased when cells are activated or primed as compared with a resting state. Agents which result in activation or priming do not cause an immune response themselves (Downey, 1995), but prepare cells to mount a greater response when challenged with antigen. One marker of activation in neutrophils is increased expression of an adhesion complex known as Macrophage 1 antigen (MAC1). This protein complex is a heterodimer composed of an α and β unit, which are termed cluster of differentiation 11b (CD11b) and CD18, respectively. Expression and interaction of this complex

with endothelium or an invading pathogen facilitates extravasation (Orr *et al.*, 2007), phagocytosis (Graham *et al.,* 1989) and has been implicated in tissue damage (Orr *et al.,* 2007)

In contrast to innate immune function, the adaptive, or acquired, immune response develops through exposure to potential health threats and is responsible for immunological memory, which is the basis for vaccinations. Adaptive immunity can be further divided into i) humoral immunity, involving the production of antibodies by B lymphocytes, and ii) cell mediated immunity which primarily involves the activity of T lymphocytes. T lymphocytes themselves can be further subdivided by function and are involved in the destruction of infected cells (T cytotoxic cells) as well as the up and down regulation of immune function by T helper and T suppressor cells, respectively.

As with neutrophils, T lymphocytes can be activated early during an immune response. This activation requires binding of membrane receptors, clustering of membrane domains and changes in intracellular signaling and calcium flux. Activation of T lymphocytes involves production of the cell signaling molecule interleukin 2 (IL2), which acts as both an autocrine and paracrine signal affecting immune activity for many immune cell types. The IL2 receptor is a heterotrimer, made up of α, β and γ chains, the specific combination of which determine binding affinity (Tizard, 2000). T lymphocytes express high affinity receptors consisting of α chains (Tizard, 2000). Upon activation, IL2 is produced by T cells and it then binds to receptors on the same activated cell. This signal leads to increased production of IL2 as well as up-regulation in expression of the IL2 receptor in the membrane thus perpetuating the activation signal (Tizard, 2000).

Following cell activation, an important step in acquired immune responses is lymphocyte proliferation, or the rapid production of cells with antigen recognition ability (King and Stott, 2002). Nearly any possible pathogen may be recognized by T cells due to the expression of highly diverse receptors. However, in order to provide for this diversity, the absolute number of cells with each combination is very small. Thus in order to mount an effective response to a particular pathogen, the body must be able to rapidly produce cells capable of recognizing that pathogen. Because of immunologic memory, a second challenge by the same pathogen results in a faster response.

Influence of Stressors on Immune Function

Many factors can impact immune function including age, sex, environmental conditions and presence of a stressor. A stressor is loosely defined as a new and/or threatening stimulus that results in behavioral and physiological changes necessary to deal with the immediate threat and promote homeostasis. This stress response is facilitated by activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal axis and can influence the immune system via innervation of lymphoid organs, direct stimulation of cells close to nerve endings (Martin, 2009) or through release of hormones into the blood stream that bind with receptors on immune cells (Padgett and Glaser, 2003).

Following perception of a stressor, activation of the sympathetic nervous system results in stimulation of the adrenals (St Aubin and Dierauf, 2001). The first hormones to be released are catecholamines, such as epinephrine and norepinephrine, from the adrenal medulla. In addition, norepinephrine serves as a neurotransmitter and can be released directly from nerve endings to influence organs and cells of the immune system (Romano *et al.,* 2002).These hormones are short lived in the blood stream and are involved in the 'fight or flight' response (Borysenko and

Borysenko, 1982) preparing the body to deal with an immediate threat e.g., a predator. Essential behavioral activities, such as avoidance, and physiological responses such as increased heart rate are heightened, while non-essential behaviors such as feeding, grooming, or mating are abandoned (Romero and Butler, 2007).

In addition to sympathetic nervous activity, hypothalamic activity results in stimulation of the pituitary. Pituitary hormones, such as adrenocorticotropic hormone (ACTH) are released and stimulate corticosteroid secretion from the adrenal cortex (Borysenko and Borysenko, 1982; McEwen *et al.*, 1997; Dabhar, 2009). Two major corticosteroids are corticosterone and cortisol. The dominant corticosteroid produced varies between species (Reeder and Kramer, 2005), with cortisol being the main glucocorticoid for mammals (Romero, 2004). One main role these hormones play in the stress response is facilitating gluconeogenesis for mobilization of energy stores.

The complex effects of neuroendocrine hormones on leukocyte function are determined by multiple factors including receptor type, stimulus, cell type and state of maturation or activation of the cell (Madden, 2003). Alpha and beta adrenergic receptors are expressed to varying degrees in the membrane of different immune cell types (Padgett and Glaser, 2003) and bind to epinephrine and norepinephrine with different affinities. Following binding, changes in intracellular signaling result in the modulation of cell function. Binding of beta adrenergic receptors, for example, stimulates production of cyclic AMP (Qiu *et al.*, 2005) which may lead to increased phagocytosis, degranulation and chemotaxis (Madden *et al.*, 1995), while T lymphocyte proliferation may be inhibited (Qiu *et al.*, 2005). Differences in cellular responses to the same challenge can be influenced by factors such as cell maturity and immunocompetence (Borysenko and Borysenko, 1982).

Glucocorticoids interact with cells by binding Type I and Type II adrenal steroid receptors (McEwan *et al.,* 1997). Romero (2004) reports that baseline levels of glucocorticoids interact with Type I receptors, whereas increased glucocorticoids associated with a stress response alter immune function through binding of Type II receptors. Immunosuppression is generally associated with increased glucocorticoids, with altered cytokine production, neutrophil superoxide production, leukocyte activation, macrophage function and lymphocyte proliferation among other functional parameters (Dabhar, 2009). However, exposure duration can also play a role in determining the effects on immune function. Acute exposures, lasting hours or less, are more commonly reported to enhance immune function as compared with the general immunosuppression associated with chronic stress lasting hours, days or longer (Padgett and Glaser, 2003). For examples, acute exposures to glucocorticoids can result in enhanced immune functions, particularly inflammatory processes involved in wound healing (Martin, 2009). A potential important mechanism through which immune responses can be enhanced during acute stress is the redistribution of leukocytes from the blood to tissues, in preparation for a potential insult (Dabhar, 2002).

Overall, the modulation of immune function that accompanies a stress response may be beneficial or pathological. Whereas enhanced immune function lends itself to greater ability to resist infections and heal wounds, it can also cause an increase in pro-inflammatory parameters and thus immunosuppression would be protective against tissue damage (Dabhar, 2009). These responses should generally promote survival, and enhance an animal's fitness (Breuner *et al.*, 2008) by reallocating resources to deal with the immediate threat. However, if these responses become prolonged they could exhaust energy stores and leave animals more susceptible to disease. Selye (1936) introduced the General Adaptation Syndrome to describe the different

stages of a stress response and the physiological and pathological effects. First is the alarm stage, which would encompass the 'flight or fight' response, followed by an adaptive phase where the physiological response has worked on resolving the immediate challenge. However, should exposure to the stressor be prolonged, energy stores become exhausted and physiological responses are no longer appropriate to deal with the challenge, marking the exhaustion phase.

Potential Effects of the Neuroendocrine Stress Response on Immune Function in Marine Mammals

The field of marine mammal immunology has been expanding for several decades and currently multiple protocols for evaluating immune function in marine mammals exist. These include immunophenotyping (Romano *et al.,* 1993*;* Romano *et al.,* 2004*;* De Guise *et al.,* 1997), measurements of phagocytosis, respiratory burst, natural killer cell activity, lymphocyte proliferation (De Swart *et al.,* 1993; Beineke *et al*., 2004; Spoon and Romano, 2012; Keogh *et al.,* 2012), immune cell gene expression (Mancia *et al*., 2007) and cytokine production (DiMolfetto-Landon *et al.,*1995; St Laurent *et al.,* 1999; St Laurent and Archambault, 2000; King and Stott, 2002; Funke *et al*., 2003; Sweat *et al.,* 2005).

Differences in hematology and immunology parameters have been reported between terrestrial mammals and marine mammals. For example, Medway and Geraci (1964) report high counts of eosinophils in a bottlenose dolphin (*Tursiops truncatus*), even in the absence of parasitism. Romano *et al.,* (1992) reported that while the MHC class II complex is expressed on only B and activated T cells in humans, it is found on the majority of peripheral blood lymphocytes in bottlenose dolphins (*Tursiops truncatus*). This could reflect phylogeny as similar results are seen in ungulates, a distant relative, or may indicate that dolphin T cells are continuously activated, perhaps as an adaptation to the marine environment. Much remains unknown however and there

are many challenges to studying the marine mammal immune system including obtaining samples and the lack of species specific reagents and antibodies.

Diving and the Immune Response

Diving is a challenging behavior that is important for marine mammals during foraging and travelling, as well as in avoidance of unpleasant stimuli. It is now well known that marine mammals possess behavioral and physiology adaptations that allow them to make repeated, prolonged dives to great depth, sometimes surfacing quite rapidly; protecting them from the dive related pathologies which are seen in human divers. A pronounced mammalian dive response results in extreme bradycardia as well as peripheral vasoconstriction and shunting of blood towards critical hypoxia sensitive organs such as the brain (Reviewed in Kooyman *et al.,* 1981; Butler, 1982; Kooyman, 1985; Butler and Jones, 1997). The degree of bradycardia varies and has been correlated with variability in dive characteristics such as length of the dive. This decrease in heart rate occurs, facilitated by vagal activity and an increase in parasympathetic activity (Butler, 1982), despite increases in catecholamines that mediate peripheral vasoconstriction via binding of α receptors (Hochachka *et al.,* 1995). Bound α receptors expressed by the spleen cause splenic contraction (Foster and Sheel, 2005), providing additional blood oxygen stores by releasing red blood cells (Hochachka *et al.,* 1995). Lung collapse may prevent the uptake of inert gases, aiding in protection against gas embolisms (Hooker *et al.,* 2012; Ridgway and Howard, 1979). Slowed ascent rates and increased heart rates following a dive can also help reduce nitrogen loads (Fahlman *et al.,* 2006). In addition, decreased metabolic rates and variable swim activity, such as alternating gliding and active swimming, are thought to aid in saving energy and prolong aerobic activity (Shaffer *et al.,* 1997; Williams *et al.,* 2000; Hastie *et al.,* 2006).

Cellular adaptations have received much less attention, yet several challenges associated with diving have been known to affect cell function. Over the course of a dive, pressure increases 1 atm for every 10 meters depth, temperature of the surrounding water decreases and oxygen stores become depleted. The main effects of pressure on cells are facilitated through changes in volume, membrane structure and fluidity, which are integral for cell function (reviewed in Macdonald, 1982; Somero, 1992; Kato and Hayashi, 1999; Pradillon and Gaill, 2007). Volume changes are important for protein interactions and enzymatic reactions. Integrins play important roles in cell function, including cell-cell communication. Philp (1990) studied the effects of pressure on platelets as model cells, and concluded that integrin activation can be altered under high pressure, possibly to due changes in protein form. Such changes in the configuration of membrane associated proteins, or protein denaturation can be an indirect effect of the altered lipid membrane (Kato and Hayashi, 1999). Likewise, alterations in membrane fluidity may then secondarily affect ion channels, passive permeability and receptors such as lymphocyte receptors (Macdonald, 1982). Mast cells act during inflammatory and allergic reactions by releasing products such as histamine through a process called exocytosis. Under increased pressure, the fusion of granule membrane and cell membrane is decreased thus limiting degranulation (Heineman *et al.*, 1987). In addition, progression of the cell cycle and cell division can be slowed (Macdonald, 1982).

Decreased temperatures have ordering effects on the components of cell membranes resulting in decreased fluidity, similar to what occurs under increased pressure. For example, during T cell activation membrane micro-domains and T cell receptors migrate and cluster together. Decreased fluidity occurring in response to decreased temperatures may lead for grouping of these membrane components and trigger intracellular signals (Magee *et al.*, 2005).

Oxygen too, is an important factor for cellular function during a dive. As oxygen stores become exhausted, some anaerobic metabolism can occur, but this pathway is not energy efficient and resources quickly become exhausted (Boutilier, 2001). Though there is evidence of anaerobic metabolism in marine mammals, the majority of dives are thought to occur within an animal's aerobic dive limit (ADL). Increased dive times are aided by increased oxygen stores facilitated by red blood cell release from the spleen and high muscle myoglobin and lack of oxygen is not the only challenge. Rapid replenishment of oxygen stores at the conclusion of a dive can result in oxidative stress and the production of reactive oxygen species (ROS) that can damage tissues and endothelium, thus triggering an inflammatory reaction (Kaminski *et al.,* 2002).

The comparative effects of pressure and chilling on platelet activation in humans and the northern elephant seal (*Mirounga angustirostris*), which is capable of diving to depths of 1500 m for 2 hours, was investigated by Field (2000). Pressures of 2800 psi (corresponding to a depth of approximately 2000 m) did not induce activation in the elephant seal platelets, yet chilling from 37°C to 4°C did. This activation was reversible, and cells reverted to resting state upon re-warming to 37°C. Human platelets displayed changes in shape indicative of activation when exposed to both pressure and temperature. These differences indicate that elephant seal platelets are better suited for handling exposure to pressure, and the author describes the membrane cholesterol content as a potential mechanism.

Red blood cell metabolism of marine mammals during simulated exposures to pressure has been compared between shallow and deep diving species, as well as with non-diving terrestrial mammals (Castellini *et al.,* 2001). Glycolytic activity, the means by which red cells derive energy, was calculated at a ratio of lactate production: glucose utilization. In general, marine mammal red blood cells exhibited an increase or no change in lactate production, and tendency

towards positive changes in lactate production: glucose use ratios when exposed to 2000 psi for two hours, while the response of cells from terrestrial mammals was much more variable. Among the marine mammals, the largest changes occurred in shallow diving phocid species such as harbor seals, suggesting a greater sensitivity to the effects of diving than in species which regularly make deep dives.

In addition the erythrocytes from deeper diving species, such as elephant seals, have different fatty acid chain and cholesterol content in their membranes as compared to terrestrial mammals such as cows, horses and dogs (Williams *et al.,* 2001). Membrane composition for these terrestrial animals was found to be more similar to shallow diving marine mammals. Williams *et al.* (2001) also report that the membranes of deep diving species are more ordered and show greater sensitivity to pressure as compared to shallow divers or terrestrial mammals, indicating that membrane order is an important factor in cellular dive adaptation.

In humans, changes in cellular immune function contribute to the development of dive related pathologies. Inflammatory processes play an important role in the development of decompression sickness (DCS). Upon decompression, inert gas bubbles can form if tissues become supersaturated and ascent occurs rapidly. These bubbles may trigger immune activity by causing endothelial damage, denaturation of host proteins or by acting as a foreign surface for cells to interact with (Barack and Katz, 2005). The presence of bubbles does not lead unequivocally to development of symptoms, and 'silent bubbles' have been reported among human divers (Barack and Katz, 2005). It is unknown whether such 'silent bubbles' occur, and to what extent, in naturally diving marine mammals. The occurrence of nitrogen supersaturation and development of decompression sickness has been reported in breath hold diving humans (Wong, 2006; Schipke *et al.,* 2006) and gas bubbles may form under particular circumstances in

marine mammals (Hooker *et al.,* 2012). Tissue nitrogen loads have been estimated to reach up to 300% supersaturation for the deep diving sperm whale (Jepson *et al.,* 2003) and 200% for bottlenose dolphins, beaked whales and blue whales (Houser *et al,* 2001). It is possible that the occurrence of bubbles is fairly common in diving marine mammals. If this is the case, it is possible that adaptive non-responsiveness of the immune response is the mechanism by which these animals avoid the development of injury (Fahlman *et al.,* 2006).

In recent years, the occurrence of pathologies similar to those seen in human divers, as well as increasing emerging diseases and marine mammal strandings has raised concern that anthropogenic stressors may compromise natural adaptations, alter immune function and ultimately impact the health of individuals and marine mammal populations. The mechanism by which anthropogenic activity impacts marine mammal health may be behavioral or physiological. Altered dive behavior, suggestive of avoidance behavior, in response to human activity has been reported for hooded seal pups (*Cystophora cystata*) (Kvadsheim *et al.*, 2010) as well as bottlenose dolphins (Constantine *et al.,* 2004). Humpback whales (*Megaptera novaeangliae*) also display altered behaviors, including greater likelihood of diving and increased dive durations in the presence of whale watching boats (Corkeron, 1995; Schaffar *et al.,* 2009). In addition, higher baseline glucocorticoids associated with ocean noise have been reported in fecal samples from North Atlantic right whales (*Eubalaena glacialis*). A startle response occurring during a dive may be abnormal due to physiological adjustments which occur during this behavior (Talpalar and Grossman, 2005) and this in turn can result in augmentation of the stress response during a dive. It is unknown how any of these behavioral or physiological changes affect immune function.

The purpose of this research was to first investigate potential adaptation of marine mammal immune cells to challenges associated with diving. To do this, several aspects of immune function were measured, *in vitro*, in marine mammals and humans, with and without exposure to simulated dives (i.e., increased pressure exposures). The second aim was to evaluate the combined effect of additional stressors on the response of marine mammal immune cells to simulated dives. One advantage of studying marine mammal populations in aquaria is that confounding factors such as diet, water quality etc., can be controlled. In addition animals can be trained to participate in sampling session. Together, the ability to control external factors and work with trained animals allows not only for baseline samples to be drawn with greatly reduced handling stress, but also can provide controlled stressor situations which provide valuable physiological information and can eventually be compared with studies of wild populations. For this study, a thirty minute out of water examination was targeted as a known stressor event. Samples were also taken from wild belugas during a live capture health assessment for comparison with aquarium belugas, with the process of chase and capture serving as the stressor. In addition, samples were obtained from pinnipeds which stranded along the coast of New England at the time of admit to Mystic Aquarium's Seal Rescue Clinic and again at release. Finally, because of the invasive and potentially stressful nature of blood sampling particularly for wild animals, blow was investigated as a potential alternative matrix for monitoring changes in the stress hormone cortisol as a potential indicator of stress in cetaceans. This non-invasive methodology could be an efficient way of collecting biological information and facilitating future studies on endocrinology and dive physiology.

Chapter 2 INVESTIGATION OF GRANULOCYTE AND MONOCYTE FUNCTION DURING DIVING IN BELUGAS (*Delphinapterus Leucas*) USING *IN VITRO* PRESSURE EXCURSIONS

Abstract

The response of marine mammal immune cells to diving is unknown, but changes in immune function in humans are associated with dive related injuries and disease. This study evaluated granulocyte and monocyte phagocytosis, and granulocyte activation in belugas (n=4) and humans (n=4), with and without *in vitro* pressure exposures. Exposures were to 2000 psi (1360m) or 1000 psi (680m) with either 2 minutes or 15 seconds of compression and decompression for each target depth. Propidium-iodide labeled, killed *Staphylococcus aureus* was used to measure phagocytic activity, whereas granulocyte activation was monitored by measuring expression of CD11b using a commercially available mouse-anti-canine CD11b antibody. Results for both tests were read using flow cytometry. Comparisons were run between pressure exposed cells and controls (non- pressure exposed), belugas and humans, and between different dives using mixed generalized linear models (α=0.05). The effects of pressure differed by depth, compression/decompression rates, and length of exposures for both belugas and humans. Overall, belugas showed decreased phagocytosis following pressure exposures, with values returning to control levels or increasing following recovery. Humans displayed smaller decreases or increases in phagocytosis following pressure exposures. Few changes were observed in CD11b expression but some differing patterns of response were observed between belugas and humans. Pressure induced changes in granulocyte and monocyte function in belugas could serve a protective function against dive-related pathologies and differences in the response between humans and belugas might reflect degrees of dive adaptation.

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Introduction

The non-specific activity of the innate immune system is the first line of defense against pathogens, and plays an important role in wound healing. Innate immune responses aid in clearing infection and promotion of wound healing, yet abnormal cellular activity can lead to disease if 1) pathogens are not removed and destroyed, or 2) an inappropriate response occurs resulting in tissue damage. In humans, alterations in immune function resulting from the challenges associated with diving can lead to increased incidents of dive related injury and disease (Brenner *et al.*, 1999). For example, development of decompression sickness (DCS) is associated with immune activity and inflammatory damage in particular, resulting directly or indirectly from the formation of gas bubbles in blood and tissues (Ward *et al.,* 1987; Nyquist *et al.,* 2004; Barack and Katz, 2005). Symptoms are variable, with some resembling anaphylaxis and complement activation (Montcalm-Smith *et al.,* 2007) suggesting involvement of the immune system. Activation of the alternate complement pathway, involving binding of CR3, has been reported to be indicative of sensitivity to DCS development (Ward *et al.,* 1987). In addition, aggregation of leukocytes in tissue such as the lung and liver, accompanied by cell activation are a marker of DCS in pigs (Nyquist *et al.,* 2004). Less dramatic symptoms of mild DCS include pruritus or itching of the skin related to an inflammatory response.

It has long been thought that marine mammals are not subject to decompression related injuries. However, estimated tissue nitrogen super saturation (Houser *et al.,* 2001; Jepson *et al.,* 2003), spinal injuries possibly due to dysbaric osteonecrosis (Moore and Early, 2004; Hellier *et al.,* 2011) and reports of emboli, gas bubbles, and injury resembling DCS (Jepson *et al.,* 2003; Fernandez *et al.,* 2005; Dennison *et al.,* 2012; Moore *et al.,* 2009), suggest that marine mammals may normally face conditions that result in DCS in people, but have developed

protective adaptation to avoid it. DCS can occur during breath hold dives in people if the characteristics of the dive allow gas build-up in tissues without appropriate washout (Paulev, 1967; Ferrigno and Lundgren, 2003; Wong, 2006). One mechanism by which marine mammals can resist damage from bubbles may be a less reactive immune response (Fahlman *et al.,* 2006; Ward *et al.,* 1987)

The physiological and behavioral adaptations that allow marine mammals to make extended and repetitive dives to great depths without suffering injury have been the subject of great interest. Dive studies, however, are limited to relatively few species, and cellular adaptations have received little attention. Changes in pressure have been noted to affect multiple aspects of cell function (reviewed in Heineman *et al.,* 1987; Macdonald, 1982; Somero, 1992; Bartlett, 2002; Daniels and Grossman, 2003; Pradillon and Gaill, 2007). Many of these effects are likely mediated through changes in membrane characteristics (Kato and Hayashi, 1999; Macdonald, 1982) and reducing the volume in which molecules interact with each other (Bartlett, 2002). For example, ordering of membrane structures occurs under increased pressure resulting in reduced membrane fluidity as well as membrane volume (Macdonald, 1982; Somero, 1992). In addition, membrane related processes and membrane associated proteins are secondarily affected (Macdonald, 1982; Somero, 1992). Furthermore, gene expression and protein synthesis have been shown to be altered by high pressure (Pradillon and Gaill, 2007; Bartlett *et al.,* 1995). Components of the cytoskeleton can be reorganized and actin has been reported to undergo pressure induced de-polymerization (Haskin and Cameron, 1992). Whereas differences in the response of platelets and red blood cells to increased pressure have been noted between marine mammals and humans or other terrestrial species (Field, 2000; Castellini *et al.,* 2001; Williams

et al., 2001), there have been no published reports concerning potential impact of diving on immune function in marine mammals.

The purpose of this study was to investigate the functional response of granulocytes and monocytes to simulated diving in belugas (*Delphinapterus leucas*). Belugas have been reported to possess a repertoire of dive patterns (Martin *et al.,* 1998) and are capable of diving to depths of 900 m and more (Heide-Jorgensen *et al.,* 1998; Suydam *et al.,* 2001). Beluga Granulocyte and monocyte phagocytosis, as well as activation of granulocytes were measured with and without exposure to increased pressure *in vitro*. Adaptation of the response of these cells was assessed by comparing results with those measured in human blood samples, with the hypothesis that human cells would display altered function in response to changes in pressure but that beluga cells would not. The results of this study are intended as a first look at immune function in relation to diving in marine mammals and to elucidate adaptation to the unique requirements of their environment.

Methods

Animal Subjects and Samples

Blood samples were obtained from four belugas (two females ~30 years old and two males ~25 and 9 years old) resident at the Mystic Aquarium, Mystic, CT. Animals were trained to present the ventral aspect of the flukes for blood collection. Blood was drawn in 10 ml sodium heparin vacutainer™ tubes and placed on ice (Mystic Aquarium IACUC protocol No. 11001; UConn IACUC reciprocation No. R12-002). Beluga blood samples were processed within 3 hours of being collected. Human blood samples were purchased from Biological Specialty Corporation (www.biospecialty.com) and shipped on ice packs within 24 hours. Human blood samples were

processed within 3 hours of receipt. Both phagocytosis and granulocyte activation assays were performed on fresh whole blood samples. Remaining sample (10-20 ml) was centrifuged at 2000 x g and 10^{o}C for 10 minutes in order to isolate plasma and the white blood cell buffy coat for archiving. Plasma was removed and stored in 1.5 ml Sarstedt™ tubes, immediately placed on ice and transferred to -80^{o}C for storage. Buffy coats were mixed with an equal volume of freezing media (90% Fetal Bovine serum and 10% DMSO), slowly frozen at -80^{o}C over 24 hours and transferred to liquid nitrogen for future assays.

Simulated Pressure Excursions

Blood samples as well as the internal temperature of the pressure chamber were brought to 37^{o}C. Four ml of blood (with PI Staph) were added to the pressure chamber through a top loading port, and over-laid with a thin layer of mineral oil. The loading port was closed off and mineral oil pumped into the chamber by hand using a hydraulic pump, rated to 40,000 psi, in order to pressurize the sample. Mineral oil has been used in previous studies to pressurize biological samples (e.g. Field, 2000; Somero *et al.,* 1977*;* Curl and Jansen, 1950) and is not biologically reactive and so should not interfere with measurements of immune function targeted by this study. Control experiments were run exposing 4 ml of blood to mineral oil and comparing immune function measurements with blood not exposed to mineral oil with no significant changes.

A pressure gauge was 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 was released by hand by loosening valve connections between the pressure chamber and oil

pump. Blood samples were then removed using a sterile transfer pipette and aliquoted into FACS™ (BD Biosciences, San Jose, CA) tubes as per assay descriptions below.

Targeted dive profiles are summarized in Table 2-1. Three durations were used; a single 30 minute dive, a single 5 minute dive, and two 5 minute dives with a 1 min 'rest' period. Target simulated depths were 1360m (2000 psi) and 680m (1000 psi). Compression and decompression occurred gradually (G) over a period of either 2 minutes or rapidly (R) over 15 seconds.

Table 2-1: Dive profiles targeted for pressure exposures. Two depths were chosen with exposures lasting for 3 different durations. Compression and decompression were varied at 2 minutes (G) or 15 seconds (R).

Pressure	**Simulated Depth**	**Compression/ Decompression**	**Duration (min)**
2000psi	1360m	2min (2000G)	30 5 2x5
2000psi	1360m	15sec (2000R)	30 5 2x5
1000psi	680m	2min (1000G)	30 5 2x5
1000psi	680m	15sec (1000R)	30 5 2x5

Phagocytosis

Phagocytic activity was measured via flow cytometry using propidium iodide labeled *Staphylococcus aureus* (PI staph) based on the protocol from Spoon and Romano (2012). Slight modifications to this protocol were made to include simulated dive exposures.

For both belugas and humans, white blood cell counts were obtained using Trypan blue exclusion, and average cell counts were used to calculate the required volume of stock PI Staph ($4.8x10^9$ ml^{-1}) to obtain a bacteria: cell ratio of 25:1. One hundred µl of whole blood were added to FACS™ tubes for controls, with 4 ml of blood set aside to introduce to the pressure chamber as described above. Ten and 400 µl of PI Staph solution were added to control and 4 ml of blood, respectively. At the time the bacteria were added, the 4 ml of blood were introduced to the pressure chamber and all samples were then allowed to incubate for the duration of the simulated dive excursion. At the conclusion of the dive excursion, 100 µl of pressure exposed blood were aliquoted into FACS™ tubes. In order to stop cell activity, 10 µl of 10 mM N-ethylmaleimide were added immediately following the conclusion of each simulated dive excursion (dive period) or after an additional 20 minutes (recovery). Tubes were then incubated on ice until lysis (up to 1 hour). Two ml of lysis buffer (0.01M Tris; 0.001 M EDTA; 0.17M NH_4Cl solution; pH 7.4) were added to each tube and incubated for approximately 15 minutes to lyse red blood cells. Remaining white cells were then washed twice with 1 ml of 1x PBS and centrifuged at 220 x g for 3 minutes, before fixing in 250 µl of 1% paraformaldehyde. Tubes were stored at $4^{\circ}C$, in the dark, until analyzed by flow cytometry 24 hours later.

CD11b Phenotyping

Evaluation of CD11b expression was run simultaneously on the same samples as the phagocytosis assay and thus experimental cells were exposed to PI Staph. The presence of PI Staph in blood samples resulted in an increase in CD11b expression, thus to make control and pressure exposed samples comparable all tubes were incubated with PI Staph.

Following simulated dive excursions, 100 µl of either non-exposed or pressure exposed blood were aliquoted into FACS™ tubes, and 50 µl of Mouse Anti-Canine CD11b IgG1 (AbD Serotec, Raleigh, NC) diluted 1:5 in Hank's Balanced Salt Solution (HBSS) was added. Phorbol myristate acetate (PMA) stimulation was used to verify cross reactivity and behavior of this antibody and its target. Blank controls received 50 µl of HBSS, and negative controls received 50 µl of Mouse IgG (Sigma, St Louis, MO). Cells were incubated for a further 30 min at 37^{o}C, washed twice with 4^{o}C HBSS and centrifuged at 220 x g for 5 min. Cells were then incubated in the dark at 4^{o}C with a 1:10,000 dilution of FITC labeled goat anti-mouse IgG (Beckman Coulter, Miami, FL) for 30 min, washed twice with cold HBSS and placed on ice. Red blood cells were subsequently lysed in the same manner as reported above for the phagocytosis assay. Tubes were fixed in 250 µl of 1% paraformaldehyde and stored in the dark at 4^{o}C until analyzed by flow cytometry within 24 hours.

Flow Cytometry

Samples were read using a LSR flow cytometer (BD Biosciences, San Jose, CA) and cell quest software for analysis. Forward and side scatter plots were obtained from tubes containing cells only and used to gate the cell populations of interest. For phagocytosis data, both the granulocyte population, composed mostly of neutrophils, and monocyte population were gated for data

collection. Only the granulocyte population was targeted for data collection for expression of CD11b. The propidium iodide (phagocytosis) and FITC (CD11b) signals were read at emission wavelengths of 617 nm and 518 nm respectively and were detected in FL2 and FL1 channels respectively.

Within the gated populations, two measures of function were collected for each assay; 1) the percentage of gated cells which expressed either PI or FITC fluorescence and 2) the mean intensity of the fluorescence (MFI) expressed by the population, reflecting how many bacteria have been ingested or the degree of CD11b expression on average per cell. For phagocytosis 100,000 total events were collected (Spoon and Romano, 2012) and for CD11b expression 10,000 events within the gated granulocyte population were captured.

Statistics

Measures of immune function following pressure exposures were normalized to control values by division and comparisons were run to determine: 1) if pressure exposed cells functioned differently from non-exposed cells and 2 if beluga cells responded differently to pressure exposures than human cells. Mixed effects generalized linear models, with repeated measures, were run for the dive and recovery periods for each pressure exposure. Individuals were entered as a random factor in the models, with species (beluga or human) and treatment (control or pressure exposed) entered as fixed factors. For each exposure duration comparisons were also made between each dive profile. For all comparisons $\alpha=0.05$. However due to small sample sizes trends are also reported where $p<0.1$.

Results

Effects of Pressure on Phagocytosis

2000 psi with 2 minutes of Compression and Decompression (2000G) - Granulocytes

Significant effects of pressure on granulocyte phagocytosis were detected for belugas following all exposures to 2000G (Appendix A). Both the % of granulocytes undergoing phagocytosis (Figure 2-1) as well as the amount of phagocytosis occurring per cell (MFI; Figure 2-2) decreased significantly for the dive periods of the 30 minute ($p<0.001$) and single 5 minute ($p<0.001$) exposures. Belugas also displayed a significant increase in the % of cells performing phagocytosis following the recovery period of the repeated 5 minute exposures ($p=0.01$). This trend of increased activity was also observed for the recovery period of the 30 minute exposure ($p=0.092$). In contrast, the amount of activity occurring per cell displayed a decrease ($p=0.086$) during this period.

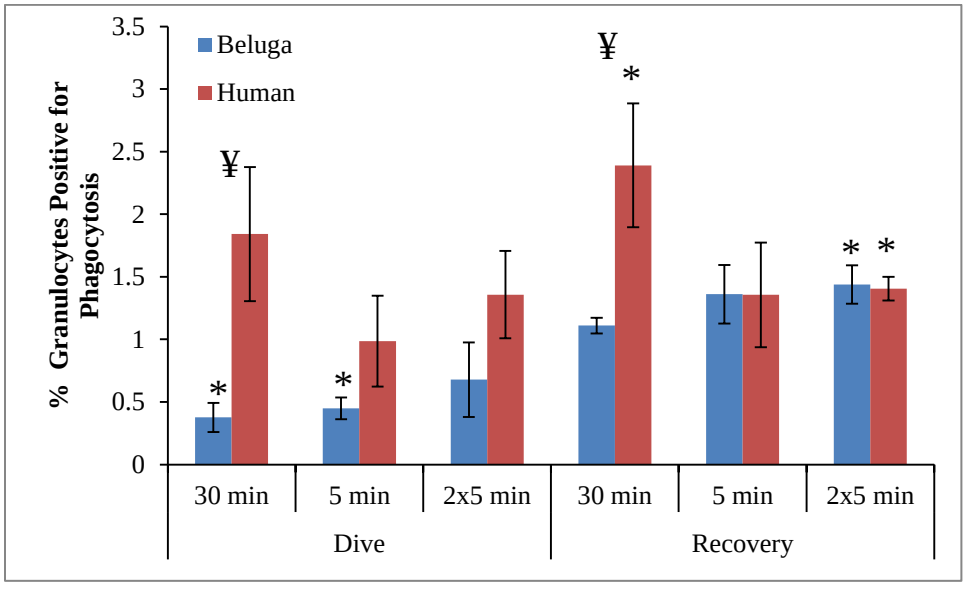

Figure 2-1: % Granulocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Significant differences between belugas and humans are indicated with ¥ ($p<0.05$).

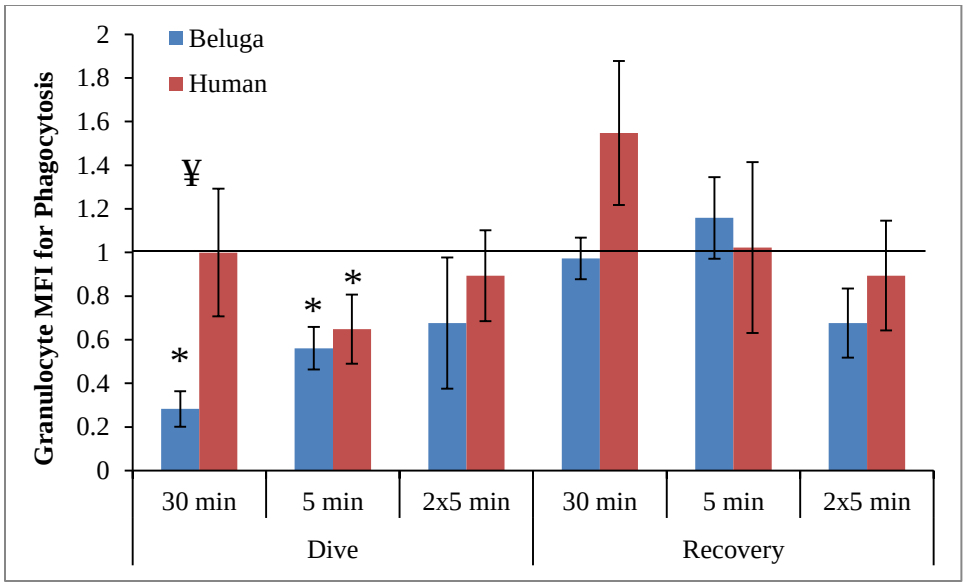

Figure 2-2 : Granulocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Significant differences between belugas and humans are indicated with ¥ ($p<0.05$).

Humans displayed significant pressure-induced changes in the % of granulocytes undergoing phagocytosis following the 30 minute and repeated 5 minute exposures (Appendix A). In both cases (Figure 2-1), a significant increase in activity was detected following the recovery periods (30 minute, $p=0.012$; 2x5 minute, $p<0.001$). A pattern of decreased MFI (Figure 2-2) was detected for the dive period of the single 5 minute exposure ($p=0.04$).

Humans displayed a significantly smaller change in granulocyte MFI for phagocytosis than belugas (Figure 2-2) for the dive period of the 30 minute exposure ($p=0.036$). However, humans displayed larger changes in the % of positive granulocytes than belugas following both the dive ($p=0.020$) and recovery ($p=0.025$) periods of the 30 minute exposure to 2000G (Figure 2-1).

2000 psi with 2 minutes of Compression and Decompression (2000G)-Monocytes

Beluga monocytes displayed significant pressure-induced changes in phagocytic activity following the single 5 minute exposures (Appendix A). Both the MFI ($p=0.003$) and the % of active cells ($p=0.002$) decreased significantly from controls during the dive period (Appendix B; Figures B-1, B-2). This pattern of decrease was also observed for the % of positive cells for the dive period of the 30 minute exposures ($p=0.099$). No significant changes were detected for the recovery periods of any pressure exposures.

Human monocytes displayed no significant changes in either measure of phagocytic activity for any pressure exposures to 2000G (Appendix A). However, an increase in the % of cells performing phagocytosis was observed for the recovery period of the 30 minute exposure ($p=0.037$). This change was significantly larger than the change measured in belugas ($p=0.041$).

1000 psi with 2 minutes of Compression and Decompression (1000G) - Granulocytes

Significant effects of pressure on granulocyte phagocytosis were detected for belugas following all exposures to 1000G (Appendix A). Both the % of granulocytes undergoing phagocytosis (Figure 2-3; $p=0.028$) and the amount of activity per cell (MFI; $p=0.007$) decreased during the dive period of the repeated 5 minute exposures. A pattern of decreased MFI (Appendix B; Figures B-3) was also observed for the dive period of the 30 minute exposure ($p=0.074$). Both measures of phagocytic activity increased for the recovery periods of the 30 minute (MFI, $p=0.055$; % Positive, $p<0.001$) and single 5 minute (MFI, $p=0.019$; % Positive, $p<0.001$).

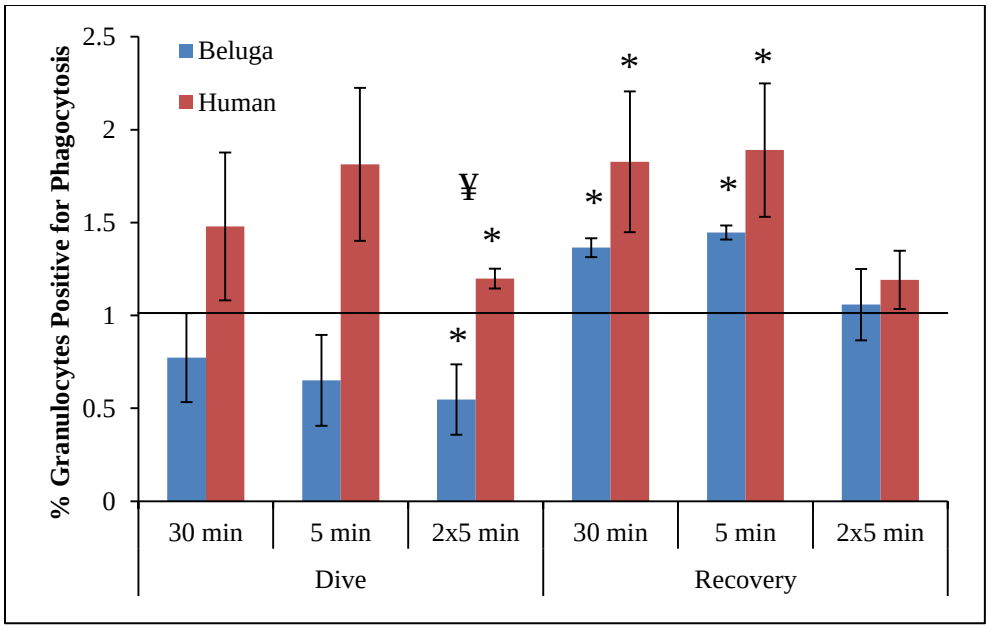

Figure 2-3: % Granulocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Significant differences between belugas and humans are indicated with ¥ ($p<0.05$).

In humans, the % of granulocytes undergoing phagocytosis changed significantly following each exposure to 1000G, while significant changes in the amount of activity per cell (MFI) displayed significant changes only following the repeated 5 minute exposures (Appendix A). The % of positive cells increase significantly during the dive period of the repeated 5 minute exposures (Figure 2-3; $p=0.001$), though this change was significantly smaller than the response in belugas cells ($p=0.006$) A similar pattern of increase is observed for the dive period of the single 5 minute exposure ($p=0.064$). In contrast, MFI (Appendix B; Figure B-3) decreased significantly during the dive period of the repeated minute exposures ($p<0.001$). During the recovery periods, significant increases in the % of granulocytes undergoing phagocytosis were detected following the 30 minute ($p=0.017$) and single 5 minute ($p=0.023$) exposures (Figure 2-3). A similar pattern of increase was observed in MFI for the recovery period of the 30 minute exposures ($p=0.052$).

1000 psi with 2 minutes of Compression and Decompression (1000G) - Monocytes

Changes in phagocytic function of beluga monocytes were observed for all duration exposures to 1000G (Appendix A). The only change occurring during a dive period was a decrease in MFI following the repeated 5 minute exposures (p=0.093). Both MFI and the % of monocytes undergoing phagocytosis displayed increases from controls following the recovery periods for the 30 minute (MFI, p=0.023; % Positive, p=0.024) and single 5 minute (MFI, p=0.069; % Positive, p=0.021) exposures (Appendix B; Figures B-4, B-5).

Human monocytes displayed significant pressure induced changes only for the 30 minute and single 5 minute exposures to 1000G (Appendix A). For dive periods an increase in the amount of phagocytosis occurring per cell (MFI) was observed following both duration exposures (30 minute, p=0.064; 5 minute, p=0.013). Similarly, an increase in the % of monocytes undergoing phagocytosis was detected for the dive period of the 5 minute exposure (p=0.001). The increase in monocyte MFI for humans was significantly different from the decrease observed in belugas for the dive period of the 5 minute exposure (p=0.016). Increased phagocytic activity was also observed for the recovery period of the 30 minute exposure (MFI, p=0.001; % Positive, p=0.063) and single 5 minute exposure (% Positive, p=0.012).

2000 psi with 15 seconds of Compression and Decompression (2000R) - Granulocytes

Phagocytic activity of beluga granulocytes displayed pressure-induced changes following the 30 minute and single5 minute exposures to 2000R (Appendix A). Both the amount of phagocytosis occurring per cell (Figure 2-4), as well as the % of cells performing phagocytosis (Appendix B; Figure B-6) decreased for the dive periods of both the 30 minute (MFI, p=0.023; % Positive, p=0.008) and 5 minute (MFI, p<0.001; % Positive, p=0.059) exposures. In contrast, both

measures of phagocytic activity displayed a significant increase for the recovery period of the single 5 minute exposure (MFI, $p<0.001$; % Positive, $p=0.014$).

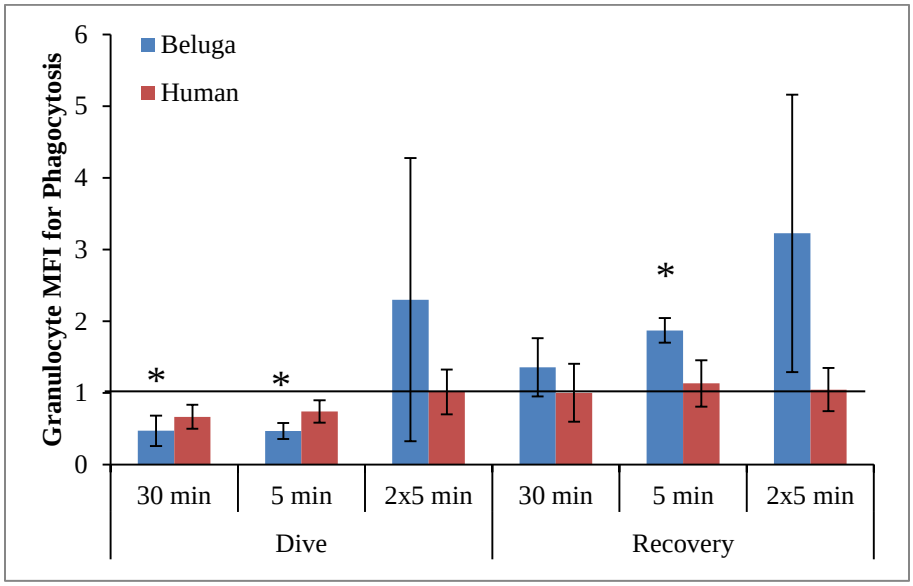

Figure 2-4: Granulocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

Human granulocytes displayed no significant changes in the amount of phagocytosis occurring per cell for the dive period following any pressure exposures to 2000R (Appendix A). Significant increases in the % of granulocytes performing phagocytosis however, were detected following the recovery periods of the single 5 minute ($p=0.001$) and repeated 5 minute ($p=0.018$) exposures (Appendix A and B; Figure B-6).

2000 psi with 15 seconds of Compression and Decompression (2000R) - Monocytes

Significant pressure induced changes in phagocytic activity of beluga monocytes were detected for the single 5 minute exposure (Appendix A). For the dive period of this exposure, only the amount of activity occurring per cell (MFI) showed a significant decrease ($p=0.002$), while

following the recovery period both the MFI ($p<0.001$) and % of cells undergoing phagocytosis ($p=0.008$) displayed significant increases (Appendix B; Figures B-7, B-8). Patterns of increased function were also observed for MFI following the recovery period of the repeated 5 minute exposure ($p=0.056$) and for the % of positive granulocytes following the recovery period of the 30 minute exposure ($p=0.053$).

The only significant changes in human monocyte phagocytosis following exposures to 2000R were for the 30 minute exposure (Appendix A). A significant decrease in MFI was detected during the dive period ($p=0.001$) while no change was detected following the recovery period (Appendix B; Figure B-7).

1000 psi with 15 seconds of Compression and Decompression (1000R) - Granulocytes

Beluga granulocytes displayed significant changes in phagocytic activity following the 30 minute and single 5 minute exposures to 1000R (Appendix A). No changes were detected for any dive period, however increased activity was detected in the MFI for the recovery period of the 5 minute exposure (Figure2-5; $p=0.011$) and in the % of granulocytes undergoing phagocytosis for the recovery period of both the 30 minute ($p=0.014$) and 5 minute ($p<0.001$) exposures (Appendix B; Figure B-9).

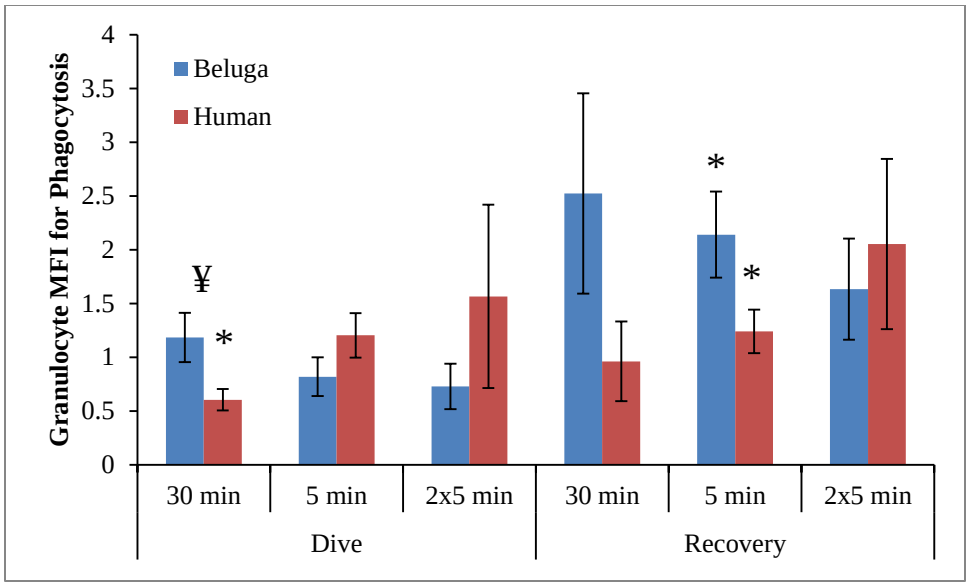

Figure 2-5 Granulocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

Similarly, changes in human granulocyte phagocytosis were only observed for the 30 minute and single 5 minute exposures to 1000R (Appendix A). A decrease in both MFI (Figure 2-5; p=0.001) and the % of cells undergoing phagocytosis (Appendix B; Figure B-9; p=0.029) occurred for the dive period of the 30 minute exposure. The change in MFI was significantly larger than that observed in belugas (p=0.039). In contrast, significant increases in the % of cells undergoing phagocytosis were detected for both the dive (p=0.002) and recovery periods (p=0.043) of the single 5 minute exposures (Appendix B; Figure B-9).

1000 psi with 15 seconds of Compression and Decompression (1000R) - Monocytes

Significant pressure induced changes in phagocytosis for beluga monocytes were detected following all exposures to 1000R (Appendix A). The amount of phagocytosis occurring per cell (MFI; Appendix B; Figure B-10) increased during the dive periods of both the 30 minute (p<0.001) and single 5 minute (p=0.087) exposures. Significant increases in the % of monocytes

undergoing phagocytosis were also detected for the dive period of the 30 minute ($p<0.001$) and single 5 minute ($p<0.001$) exposures (Appendix B; Figure B-11). Increased activity was also detected following the recovery periods of the 30 minute (MFI, $p<0.001$; % Positive, $p<0.001$), single 5 minute (MFI, $p=0.003$; % Positive, $p<0.001$) and repeated 5 minute (MFI, $p=0.012$) exposures. Patterns of increased function in the % of cells undergoing phagocytosis also occurred for both the dive ($p=0.017$) and recovery ($p=0.012$) periods of the repeated 5 minute exposures.

Human monocytes displayed pressure-induced changes in phagocytic activity following the 30 minute and single 5 minute exposures to 1000R (Appendix A). During the dive period of the 30 minute exposure, the amount of activity per cell (MFI) increase ($p=0.030$) while the % of active cells decreased ($p=0.047$). Opposite responses in these two measures were also observed for the single 5 minute exposure. For this exposure, MFI decreased ($p=0.077$) while the % of positive cells increased ($p=0.002$). This increase in the % of positive cells was also observed for the recovery period of the single 5 minute exposure ($p=0.068$). The change in monocyte MFI for humans was significantly larger than the changes in belugas for both the dive ($p<0.001$) and recovery ($p=0.016$). Results are shown in Appendix B; Figures B-10, B and 11.

Effects of Dive Exposure on Phagocytosis

Pressure exposure values, normalized over controls, were compared between dive profiles using mixed generalized linear models with repeated measures. Both depth and duration effects were investigated.

Granulocytes

For dive periods, beluga granulocytes displayed significantly different responses between all pressure exposures (Appendix C). For dive periods, both measures of phagocytosis displayed significantly different responses following exposures to 1000R than all other exposures for the 30 minute durations (MFI, Figure 2-6; % Positive, Appendix D; Figure D-1). Both measures of function also displayed significantly a significantly different response for the repeated 5 minute exposure to 1000R as compared with 1000G. The % of granulocytes undergoing phagocytosis displayed a significantly different change following exposure to 1000R as compared with all other exposures for the 5 minute durations (Appendix D; Figure D-1). Exposure to 1000R also resulted in a significantly different change in the amount of activity occurring per cell (MFI) for the 5 minute exposure as compared with 2000R (Figure 2-6).

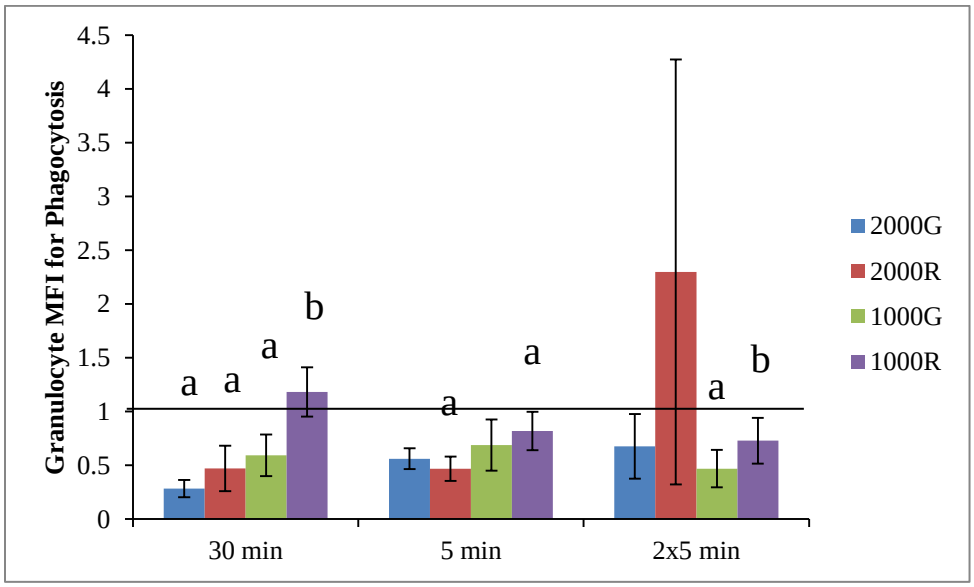

Figure 2-6: Granulocyte MFI for phagocytosis in beluga samples (n=4) measured following all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration, significant differences between dive profiles are indicated with letters ($p<0.05$).

Following recovery periods (Appendix D; Figures D-2, D-3), the response of granulocytes exposed to 1000R remains different from 1000psi for all durations (% positive cells), from 2000R for the 5 minute duration (% positive cells), and from 2000G for the 5 minute duration (MFI and % positive cells).

Significant effects of duration were detected only for granulocyte MFI (Appendix E). A significantly different response of cells was detected between the single and repeated 5 minute durations for 1000G exposures, and between the 30 minute and both the single and repeated 5 minute durations for 1000R exposures. Exposures lasting for the repeated 5 minute durations showed different responses in both measures of phagocytosis following the recovery period for 2000G as compared with 30 minute duration exposures. Additionally, the % of positive cells displayed a different change following the repeated 5 minute exposures to 1000G as compared with the 5 minute duration. Results are shown in Appendix F, Figures F-1 through F-4.

Monocytes

Exposures to 1000R resulted in changes in both measures of phagocytic activity that differed from all other exposures for the dive period of 30 minute durations (Appendix G; Figures G-1, G-2). This was also found for the single 5 minute duration, but only for the change in the % of positive monocytes. MFI results for 2000G exposures were significantly different from 1000R for the single and repeated 5 minute durations, and from 2000R for the 30 minute duration exposures (Appendix G; Figure G-1). Changes in the % of positive monocytes (Appendix G; Figure G-2) were significantly different between dives with 2 minutes of compression and decompression and dives with 15 seconds of compression and decompression (i.e. 2000Gvs2000R, 1000G vs. 1000R). Additionally, exposures to 1000G resulted in different

changes in the % of positive cells as compared with 2000G for the 30 minute and 5 minute durations.

The amount of phagocytosis occurring per cell (MFI) displayed different changes following the recovery period for exposures to 1000R as compared with all other exposures for the 30 minute exposures and from 2000G and 1000G for the repeated 5 minute durations (Appendix G; Figure G-3). The change in positive cells was also different was also different for 1000R as compared with all other exposures for the single 5 minute duration exposures (Appendix G; Figure G-4). The response to 2000G was different from both 2000R and 1000R for the recovery periods of the 5 minute duration (MFI, % positive), repeated 5 minute durations (MFI) and 30 minute duration (% positive).

Effects of exposure duration on the response of monocyte phagocytic activity were only detected in MFI for 1000R exposures (Appendix E). The response following the dive period of the 30 minute duration was significantly larger than the single 5 minute exposures (Appendix G; Figure G-5). The change in MFI for phagocytosis for the recovery period of the 30 minute duration to 1000R was also larger than the repeated 5 minute durations (Appendix G; Figure G-6).

Effects of Pressure Exposures on CD11b Expression

2000 psi with 2 minutes of Compression and Decompression (2000G)

Significant pressure induced changes in the expression of CD11b were detected for the repeated 5 minute exposures (Appendix H). Belugas displayed an increase in the % of cells expressing CD11b only following the 30 minute exposure (Appendix I; Figure I-1; $p=0.048$). Significant decreases in both the % of cells expressing CD11b and the relative expression per cell (MFI;

Figure 2-7) were detected for belugas following the repeated 5 minute exposures (MFI, $p<0.001$; % Positive, $p<0.001$).

Similarly, human granulocytes displayed significant decreases in MFI (Figure 2-7; $p=0.002$) and the % of cells expressing CD11b ($p=0.020$) following the repeated 5 minute exposures (Appendix I; Figure I-1).

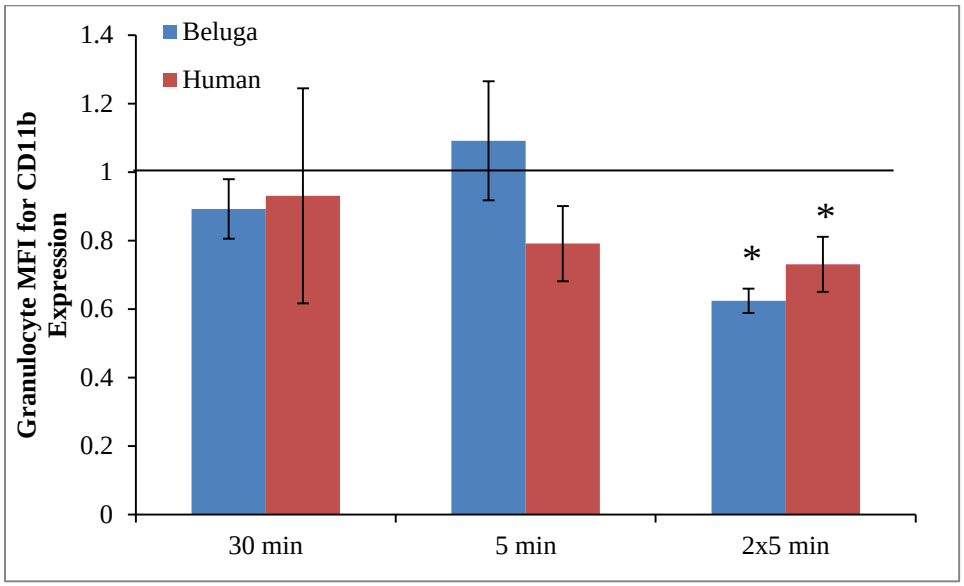

Figure 2-7: Granulocyte MFI for CD11b expression in beluga (n=4) and human (n=4) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$).

1000 psi with 2 minutes of Compression and Decompression (1000G)

No significant changes in either measure of CD11b expression were detected for either belugas or humans following exposures to 1000G (Appendix H).

2000 psi with 15 seconds of Compression and Decompression (2000R)

No significant changes in either measure of CD11b expression were detected for either belugas or humans following exposures to 2000R (Appendix H).

1000 psi with 15 seconds of Compression and Decompression (1000R)

Significant changes in CD11b expression were detected in belugas following all exposures to 1000R (Appendix H). Significant increases in the % of cells expressing CD11b, though not in the relative expression per cell (MFI), were detected for the 30 minute (p=0.032), single 5 minute (p=0.010) and repeated 5 minute (p=0.002) exposures (Figure 2-8).

In contrast to belugas, human granulocytes displayed a decrease in both the relative expression of CD11b per cell (Appendix I; Figure I-2; p=0.037) and the % of positive cells (Figure 2-8; p=0.034) only following the 30 minute exposure to 1000R.

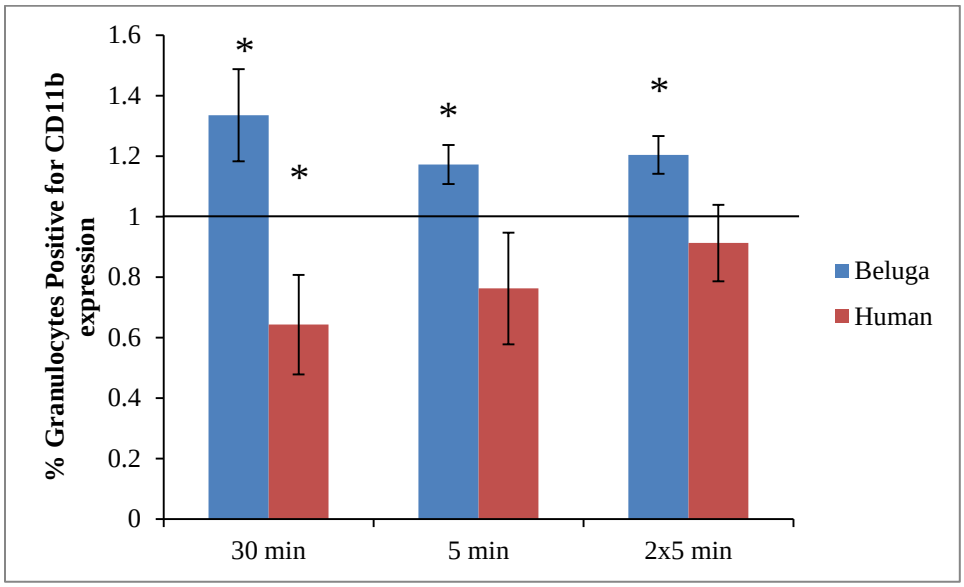

Figure 2-8: % Granulocytes positive for CD11b expression in beluga (n=4) and human (n=4) samples measured following exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

Effects of Dive Profile on CD11b Expression

Significantly different responses in both MFI and % positive cells were detected following exposure to 2000G as compared with all other exposures for the dive period of repeated 5 minute

durations (Figure 2-9 and 2-10). The % positive cells also differed between 1000R and 2000R for the single 5 minute exposure (Figure 2-10)

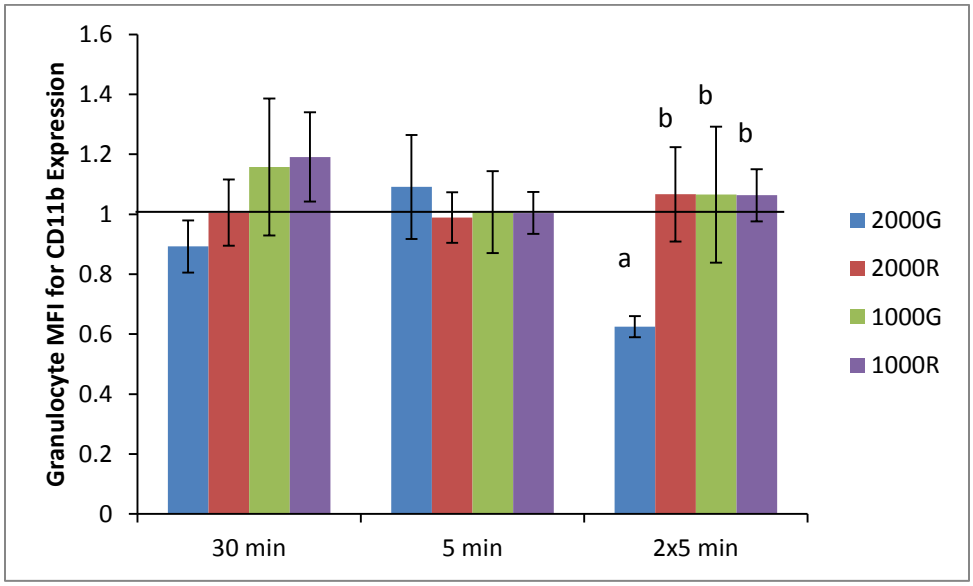

Figure 2-9: Granulocyte MFI for CD11b expression in beluga samples (n=4) measured following all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration, significant differences between dive profiles are indicated with letters ($p<0.05$).

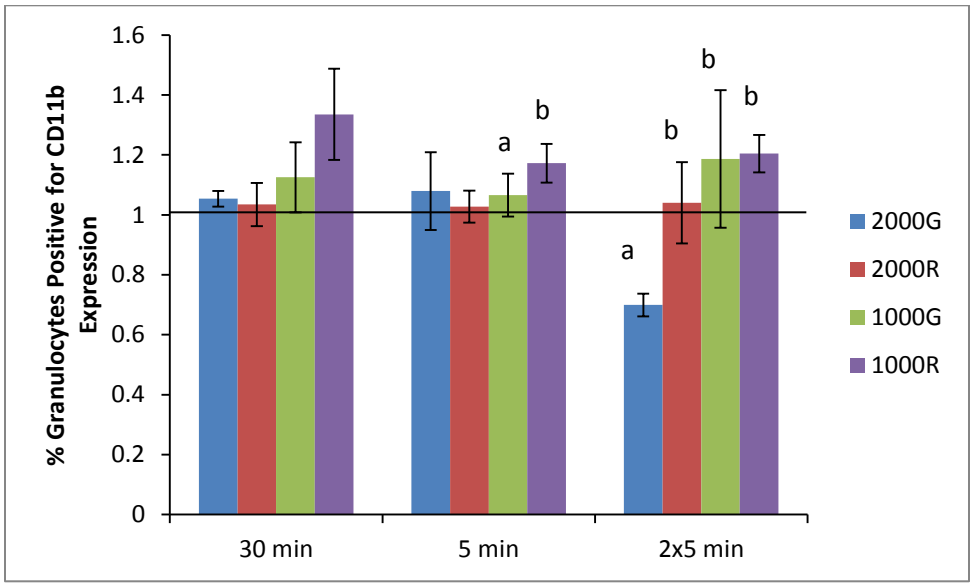

Figure 2-10:% Granulocytes positive for CD11b expression in beluga samples (n=4) measured following all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration, significant differences between dive profiles are indicated with letters ($p<0.05$).

Duration

Additionally, both measures were significantly different following repeated 5 minute exposures to 2000G as compared with the 30 and single 5 minute exposures (Appendix J; Figures J-1, J-2).

Discussion

Results of this study suggest that marine mammal immune cells can alter function during diving, and characteristics of a dive play a role in determining the response of immune cells. For most dive profiles beluga cells displayed either no change or decreased phagocytic activity, with exposures to 1000 psi with rapid compression/decompression being the exception. Function of pressure exposed cells either returned to control levels or increased following the 20 minute post dive recovery periods. These changes varied however (e.g. in magnitude) between dives with different rates of compression/decompression, different target depths, and different durations. In general, larger changes were observed for dives to 2000 psi as compared with 1000 psi and for exposures with 15 seconds of compression/decompression as compared with 2 minutes and with exposures to 1000 psi with rapid compression/decompression being the most different. In contrast, CD11b expression seemed to be less effected by simulated pressure exposures, though significant decreases in expression were detected for the repeated 5 minute dives to 2000 psi with 2 minutes of compression/decompression, and increases were observed following exposures to 1000 psi with rapid compression/decompression. Changes in expression were larger following exposures to 2000 psi with 2 minutes of compression and decompression than other exposures. In addition, changes in CD11b expression were different between exposures of different durations, particularly the single and repeated 5 minute exposures.

The decrease in phagocytosis observed immediately following decompression for many of the exposures was somewhat surprising, as it was expected that beluga immune cells would possess

adaptations which would allow 'normal' function to occur even during diving. Decreased immune function during diving, however, could be beneficial in reducing the likelihood of damage caused by aberrant immune activity or inflammatory processes. In humans, immune activity plays an important role in the development of decompression sickness (Ward *et al.*, 1987; Nyquist *et al.*, 2004; Barack and Katz, 2005). In particular, damage is facilitated by inflammatory processes initiated through interactions with bubbles or though endothelial damage or denaturation of host proteins (Barack and Katz, 2005). The increases in activity seen among the responses of human granulocytes may suggest an increase in reactivity of cells following a dive. In some cases, 'silent bubbles' can exist in a diver without eliciting an inflammatory response and resulting in disease (Barack and Katz, 2005). Errson *et al.*, (2002) report that studies which pre-tune the immune system by treatment with foreign proteins before a dive, lead to decreased incidence of DCS. Thus a less reactive immune system serves a protective function. To the best of our knowledge, tissue nitrogen saturation for diving belugas has not been reported. However, up to 300% nitrogen super saturation has been estimated for some deep diving species (Jepson *et al.*, 2003). It is possible that 'silent bubbles' exist and are more common in diving marine mammals than previously thought. Dennison *et al.*, (2012) found gas bubbles, arising from desaturation following interruption of normal dive behavior by stranding, can be asymptomatic in live-stranded dolphins. If this is the case, reduced reactivity of immune responses in marine mammals may be one mechanism by which they avoid development of decompression sickness and other dive related injuries. The decreases in granulocyte function observed in this study lend some support to this. The return of values to control levels following the recovery period suggests marine mammals cells can quickly recover from changes which occur during dives and may not be at greater risk of developing infection.

In contrast to the response of beluga cells to increased pressure exposures humans tended to show minimal change (following exposures to 2000 psi) or increased phagocytic activity (following exposures to 1000 psi). Shiratsuchi and Busson (2004) report that extracellular pressure, associated with inflammation for example, can modulate phagocytosis in human macrophages by altering intracellular pathways. It is not unreasonable then to see much larger changes in pressure also having an effect on human monocyte phagocytosis. In some cases, however, the relationship between belugas and humans is reversed. What is important about these trends is that samples drawn from belugas under baseline conditions seem to respond differently to changes in pressure than those obtained from humans. Belugas are capable of dives to depths greater than 1000 m (Heide-Jorgensen *et al.,* 1998; Suydam *et al.,* 2001). The deepest no-limit free dive in humans however reached only 249.6 m (herbertnitsch.com/world_records.html) with the deepest SCUBA dive reported at 318 m (scubarecords.com), though deeper dives can be achieved with special gas mixes and equipment. For example, exposure to 660 m has been achieved within a pressure chamber using Trimix (Logie and Baddeley, 1983). Thus, differences in the response of immune cells between humans and belugas may reflect different degrees of dive adaptation. Field (2000) reported that platelets from elephant seals and humans responded differently when exposed to 2800 psi pressure (2000 m). In addition, red blood cells from deep-diving, shallow-diving and terrestrial mammals have been reported to function differently (measured as glycolytic activity) in response to pressure exposures (Castellini *et al.,* 2001). For both platelets and erythrocytes, membrane cholesterol content has been reported to vary between marine and terrestrial mammals (Field, 2000; Williams *et al.,* 2001) and can be important in determining cell sensitivity to changes in pressure,

as cholesterol content is related to membrane fluidity. Thus future studies should consider investigating membrane composition of immune cells.

In most cases, no significant changes in either measure of CD11b expression were observed following pressure exposures, even when changes in phagocytic activity were detected. This may suggest that pressure may have a greater mechanical effect on immune cells, and physically alter phagocytosis by altering membrane structure and fluidity, rather than affecting the activation state of cells. Additionally, there are multiple pathways by which phagocytosis can occur. CD11b, also termed CR3, is a subunit of the adhesion complex known as macrophage 1 antigen (MAC1) and facilitates phagocytosis via the alternate pathway of complement activation by binding C3bi (Murphy *et al.,* 2008). Other pathways of phagocytosis were likely occurring in this study, and can be differentially affected by simulated pressure exposures. Thus, the phagocytosis results measured were cumulative of different pathways and it is perhaps not entirely unexpected that no apparent relationship was found between CD11b expression and phagocytosis.

Changes observed following pressure exposures varied with the duration of exposure, and comparisons of the response of immune cells between dive profiles revealed that altering depth and rates of compression and decompression can change the response of granulocytes and monocytes. Again, the patterns of response varied between species and with duration of exposure. For both belugas and humans, however, exposures to 1000 psi with rapid (15 second) compression/decompression appeared to be the most different from other exposures. This information may have important implications when considering that anthropogenic activities or changing environmental conditions could facilitate changes in dive behavior. While the rate of compression and decompression for these exposures is faster than any descent or ascent for

marine mammals, the results suggest that increasing descent or ascent rates has important implications for how immune cells respond to dive behavior. If a startle response at depth leads to a faster than normal ascent, unwanted immune activity may facilitate injury.

Behavioral changes, including changes in dive patterns can occur in response to human activities. Increased dive durations in the presence of boats have been reported for bottlenose dolphins (Constantine *et al.,* 2004) and belugas have been reported to show extreme avoidance of ships, such as ice breakers, though a lot of variability is noted in the response of these animals to human activities (Richardson and Wursig, 1997). Sivle *et al.,* (2012) reported changes in dive behavior of killer whales (*Orcinus orca*), long finned pilot whales (*Globicephela melas*) and sperm whales (*Physeter macrocephalus*) in response to mid and low frequency active sonar. Altered risk of bubble formation and DCS was predicted from these results (Kvadsheim *et al,* 2012). If dives are interrupted or animals are diving deeper or longer as avoidance behaviors, the result can be unwanted activity of immune cells which could potentially lead to injury or disease. The results of this work provide some evidence that changing the dive profile alters the response of immune cells. It is possible that the combination of dive characteristics that result in 'normal' changes to immune function is dynamic and that belugas are susceptible to dive related disease only under specific conditions. For example, a prolonged dive to greater than normal depth can pose a greater risk for an animal undergoing wound healing or fighting an infection than an uninjured healthy individual. Because this study looked at the effects of pressure, beluga samples were only obtained from belugas resident at the Mystic Aquarium in order to avoid any confounding effect of air shipment. This population consists of two males and two females of varying age, and due to the small numbers, differences between sexes and age groups could not be determined. The pressures used represent extreme deep dives, and while it would be

interesting and physiologically relevant to compare these results with exposures to much smaller pressures, this could not be done reliably with the pressure system available. Belugas are, however, capable of dives to over 1000 m, and certainly to 680m. Thus, the information gained from this study is relevant to physiological conditions experienced by belugas.

While the belugas in this study were either born in or housed in aquaria for the majority of their lives, it is possible that the response of wild belugas will be different due to previous diving experience. Dive experience and training have been shown to aid in development of dive capabilities (Lander *et al.*, 2003; MacArthur *et al.*, 2003; Ferretti and Costa, 2003) and result in acclimatization which reduces incidence and severity of decompression sickness (Su *et al.*, 2004). Additionally, this study used an entirely *in vitro* approach, which allowed us to focus on the effects of changes in pressure *per se* on immune function. However, there are many physiological changes which occur during diving that can also affect immune function, such as changes in oxygen availability or levels of circulating endocrine hormones that control the dive response. The temperature of the pressure chamber was monitored and held to $37^{o}C \pm 2^{o}C$ in order to avoid confounding effects of temperature changes on cell function, though the available equipment did not allow for the measurement of oxygen throughout the dive. Changes in oxygen availability within the chamber may have contributed to the measured results. Thus, future studies possibly combining dive behavior or tagging research with blood sampling would provide a more accurate picture of what occurs during natural diving. My results, however, demonstrate that beluga granulocytes and monocytes react differently to changes in pressure than human cells, and that this response can be modified by changing the depth, duration and rates of compression and decompression for the dive. The relationship between dive characteristics and immune function needs to be better described, but is important in understanding potential sub-

lethal impacts of anthropogenic activities on marine mammal health, and long term health trends for marine mammal species, not only in terms of increasing reports of DCS-like injury but also for populations whose foraging, resting or travelling behavior may be altered due to human activity.

Chapter 3 COMBINED EFFECTS OF INCREASED PRESSURE AND STRESSORS ON GRANULOCYTE AND MONOCYTE FUNCTION IN BELUGAS (*Delphinapterus leucas*)

Abstract

Beluga granulocytes and monocytes display pressure induced changes in function which vary with characteristics of exposure (e.g. depth, duration). The response of beluga cells to pressure differs from the response of human cells and may reflect dive adaption. In order to evaluate the potential for stressors to impact this response, this study measured pressure induced changes in granulocyte and monocyte function in belugas following: 1) a thirty minute out-of-water examination (OWE) (n=3) and 2) a period of mild chronic inflammation (n=2), and compared the results with baseline conditions in belugas (n=4) and humans (n=4). Phagocytic activity and granulocyte activation (CD11b expression) were measured following exposures to 2000 psi (1360m) and 1000 psi (680m). Catecholamines and cortisol were also measured for each condition. Results were compared using repeated measures ANOVA paired T-tests and mixed generalized linear models with repeated measures (α=0.05). Increased hormone values were measured during the OWE and inflammation conditions as compared with baseline. OWE and inflammation samples displayed decreased granulocyte phagocytosis following 2000 psi exposures, while increased monocyte phagocytosis, resembling the human response, was noted for inflammation samples. Decreased CD11b expression was observed but no significance was detected, though inflammation samples displayed greater changes than the OWE. Larger changes in immune function were noted for 1000 psi exposures in OWE and inflammation samples as compared with 2000psi. Differences between OWE, inflammation and baseline conditions in belugas suggests that the presence of additional stressors may alter the response of immune cells during diving and increase the potential for dive-related injury and disease in marine mammals.

Introduction

It has long been recognized that marine mammals possess physiological and behavioral adaptations which allow many species to make long, deep and repetitive dives. Among these adaptations is an extreme and dynamic mammalian dive response which includes pronounced bradycardia and vasoconstriction resulting in redirecting blood flow towards oxygen sensitive organs such as the brain (reviewed in Kooyman *et al.*, 1981). Uptake of inert gases, such as nitrogen, is prevented by lung collapse at depth (Ridgway and Howard, 1979). Additionally, energy is preserved and aerobic metabolic activity maintained through decreasing metabolic rates and alternating active swimming with periods of gliding (Shaffer *et al.*, 1997; Williams *et al.*, 2000; Hastie *et al.*, 2006).

Cellular responses, however, have not been studied extensively and there have been no published reports concerning the response of marine mammal immune cells to the challenges associated with diving such as changes in pressure and temperature. Breath hold diving and physiological responses, such as lung collapse, have been implicated in protecting marine mammals from developing decompression sickness, unlike human scuba divers who need to carry an air supply with them. However, DCS or DCS like emboli and symptoms can occur during breath hold diving if the characteristics of the dive do not allow appropriate gas washout (Paulev, 1967; Ferrigno and Lundgren, 2003; Wong, 2006). Tissue saturation of nitrogen has been estimated for several species of cetacean, including beaked whales and bottlenose dolphins (*Tursiops truncates*), and may reach 200-300 % (Houser *et al.*, 2001; Jepson *et al.*, 2003). Spinal injuries possibly due to dysbaric osteonecrosis, caused by bubble damage to bones have been reported in the sperm whale (*Physeter macrocephalus*) (Moore and Early, 2004) fin whale (*Balaenoptera physalus*) and humpback whale (*Megaptera novaeangliae*) (Hellier *et al.*, 2011). Thus it is

possible that diving marine mammals may contend with inert gas bubble/emboli formation, but that these are normally asymptomatic. One mechanism by which marine mammals may resist damage from bubbles may be a less reactive immune response (Fahlman *et al.,* 2006; Ward *et al.,* 1987).

In recent years there have been several reports of gas bubbles and associated injury in several marine mammal species including beaked whales which stranded in close proximity to naval sonar exercises (Jepson *et al.,* 2003; Fernandez *et al.,* 2005), stranded dolphins (Dennison *et al.,* 2012) and several species of marine mammals which became entangled in fishing gear at depth and were subsequently brought to the surface rapidly (Moore *et al.,* 2009). In each of these cases, rapid decompression has been a suggested etiology of bubble formation, but there is concern that human activities, such as sonar use, play a role, potentially by interrupting natural adaptation to environmental challenges, either through altering behavior or acting as a stressor. For example, deep diving beaked whales often perform short, shallow bounce dives following prolonged exposures to great depth, which have been suggested to aid in safe off gassing of high nitrogen loads (Tyack *et al.,* 2006). Avoidance behaviors, or a startle response, triggered by human activity may result in altered duration of dives or swim speeds, which may then increase the potential for bubble formation. Moreover, release of catecholamines or glucocorticoids occurs during a stress response. Romano *et al.,* (2004) reported increased epinephrine, norepinephrine and dopamine following exposure to loud sound in a bottlenose dolphin and beluga whale. These hormones can modulate immune function through adrenergic and adrenal steroid receptors expressed on cells (Madden *et al.,* 1995; McEwan *et al.,* 1997; Padgett and Glaser, 2003) and the effect on marine mammal health may be subtle, but cumulative over repeated exposures.

In a previous study we investigated the function of granulocytes and monocytes in response to increased pressure in the beluga (*Delphinapterus leucas*) during baseline (i.e. non stressor) conditions (Chapter 2). Belugas however are faced with many challenges in both the wild and aquaria, which may impact cell function during diving. For animals under human care, challenges may include noise, or may arise from social groupings and interactions. In the wild, stressors may stem from interactions with fisheries, ecotourism, climate change and changing sea ice cover, as well as noise in the ocean. The aim of this study was to investigate the effects of increased pressure on granulocyte and monocyte phagocytosis, and granulocyte activation in belugas during a 'stressor' condition (i.e. out of water examination) or physiological challenge (i.e. inflammation), and to evaluate differences in the effects as compared with baseline conditions. Granulocytes and monocytes are important cells of the innate immune system; the body's first line of defense. The response of these cells includes phagocytizing pathogens and cell debris, removing them from tissues or the blood. Activation of these cells can increase their responsiveness. However, inefficient or inappropriate responses can lead to disease and self-injury. Thus, this work aimed to investigate the potential impacts of multiple challenges on marine mammal health.

Methods

Subjects and Sample Collection

Baseline blood samples were collected through positive behavioral reinforcement from four belugas under human care at the Mystic Aquarium, Mystic, CT. Stressor samples were obtained from 3 animals following a thirty minute out of water examination (OWE) or from 2 animals presenting with clinical signs of inflammation. During the OWE, animals were stretchered and placed upon the exhibit beach for examination. Blood samples were drawn from the dorsal

aspect of the flukes following the 30 minute examination and prior to return of the animals back into the water. For the 2 whales presenting with inflammation, blood samples were obtained during follow-up health checks and were opportunistically used to further evaluate the potential role of a physiological challenge in altering immune function and the immune response to changes in pressure. All samples were collected in 10 ml sodium heparin vacutainers™. Human blood samples were purchased from Biological Specialty Corporation (www.biospecialty.com) and shipped on ice packs overnight to the Mystic Aquarium. All samples were processed within 24 hours of collection.

Immune function assays were run on fresh whole blood. From each sampling session, 10 to 20 ml of blood were centrifuged at 2000 x g and 10^{o}C for 10 minutes to separate blood components. In a sterile hood, plasma was removed and aliquoted into 1.5 ml Sarstedt™ tubes, placed immediately on ice and transferred to -80^{o}C. The white blood cell buffy coat was then removed using a sterile transfer pipette, mixed with an equal volume of freezing media (90 % fetal bovine serum and 10% DMSO) and stored in liquid nitrogen.

Simulated Pressure Excursions

For pressure excursions, 6 ml of fresh whole blood were set aside per each experiment. Control tubes received aliquots of 100 µl, and 4 ml of blood were kept to be introduced to the pressure chamber. Blood samples as well as the internal temperature of the pressure chamber were brought to 37^{o}C. Samples were added to the chamber through a top loading port, overlaid with a thin layer of mineral oil and closed off. Mineral oil was then pumped into the chamber by a hydraulic hand pump in order to pressurize the sample. Pressure was released by hand at the

conclusion of each excursion, and blood was removed and aliquoted into FALCON™ tubes as per assay descriptions below.

Targeted dive profiles are summarized in Table 3-1. Three durations were used; a single 30 minute dive, a single 5 minute dive, and two 5 minute dives with a 1 minute 'rest' period. Target depths were 1360m (2000 psi) and 680m (1000 psi). Compression and decompression of samples occurred over a period of 2 minutes.

Table 3- 1: Dive profiles targeted for pressure exposures. Two simulated depths were chosen with exposures lasting for 3 different durations. Compression and decompression occurred over a period of 2 minutes.

Pressure	Depth	Duration	Compression/ Decompression
2000psi	1360m	30min	2min
		5min	2min
		2x5min	2min
1000psi	680m	30min	2min
		5min	2min
		2x5min	2min

Phagocytosis

To measure phagocytosis, flow cytometry was used to detect uptake of propidium iodide labeled *Staphylococcus aureus* (PI Staph) in beluga and human granulocytes and monocytes. The protocol was slightly modified from Spoon and Romano (2012) in order to include simulated dive exposures (Chapter 2).

Stock PI Staph ($4.8x10^9$ ml^{-1}) was adjusted to obtain a bacteria/cell ratio of 25:1 based on average cell counts obtained using Trypan blue staining. Controls consisted of 100 µl of whole blood, with 4 ml of blood set aside to introduce to the pressure chamber as per above. Ten and 400 µl of PI Staph solution were added to control and 4 ml of blood, respectively. Bacteria were

added immediately before compression and all tubes were allowed to incubate for the duration of the exposure. Following decompression, pressure exposed blood was aliquoted in 100 µl into FALCON™ tubes and 10 µl of 10 mM N-ethylmaleimide was added to stop cell activity either immediately or after an additional 20 minute recovery period. Tubes were then incubated on ice until lysis.

CD11b Phenotyping

Simultaneous with the phagocytosis assay (i.e., in samples exposed to PI Staph), CD11b expression was measured as an indicator of granulocyte activation. Following simulated dive excursions, 100 µl of blood (non-exposed and pressure exposed) were aliquoted into FALCON™ tubes, and 50 µl of Mouse Anti-Canine CD11b antibody (AbD Serotec, Raleigh, NC) diluted 1:5 in HBSS were added. Blank and negative controls received 50 µl of HBSS and Mouse IgG (Sigma, St Louis, MO) respectively. Cells were incubated with the antibodies for 30 minutes at 37^{o}C, washed twice with cold HBSS and centrifuged at 220 x g for 5 minutes. Fifty µl of a 1:10,000 dilution of fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (Beckman Coulter, Miami, FL) were added to each tube for a further 30 minute incubation in the dark at 4^{o}C. Cells were washed twice with cold HBSS as per above and placed on ice until lysis.

Red Blood Cell Lysis

Cells were incubated for 15 minutes with 2 ml of lysis buffer (0.01M Tris; 0.001 M EDTA; 0.17M NH_4 CL solution; pH 7.4). Cells were washed twice with 1 ml of 1x PBS (pH 7.2) before fixing in 250 µl of 1% paraformaldehyde (pH 7.4). Tubes were stored in the dark at 4^{o}C for 24 hours before flow cytometric analysis.

Flow Cytometry

Results were read using a LSR flow cytometer (BD Biosciences, San Jose, CA) and Cell Quest software for analysis. Leukocyte populations were viewed on a density plot obtained from tubes containing cells only, and appropriate cell populations were gated for each analysis. Both granulocyte and monocyte populations were gated for the phagocytosis assay, whereas only the granulocyte population was targeted for expression of CD11b. The propidium iodide (phagocytosis) and FITC (CD11b) signals were read at emission wavelengths of 617nm and 518nm respectively. Two measures of each function were collected for each assay; 1) the percentage of gated cells which expressed either PI or FITC fluorescence and 2) the mean intensity of the fluorescence (MFI) expressed by the population, reflecting how many bacteria have been ingested or the degree of CD11b expression on average per cell. For phagocytosis 100,000 total events were captured and for CD11b expression 10,000 events within the gated granulocyte population were captured.

Hormone Analysis

Plasma concentrations of cortisol as well as the catecholamines epinephrine and norepinephrine were measured in blood samples to demonstrate a physiological change from baseline for stressor conditions. One ml of plasma was shipped to the AHDC Endocrinology Lab at Cornell University (Ithaca, NY) for determination of cortisol concentration. Catecholamines were measured in-house using a Waters (Milford, MA) High Performance Liquid Chromatography system (1515 isocratic pump, 717 auto sampler) with electrochemical detection (2465 electrochemical detector). The methodology for this has been detailed in "Plasma Catecholamines by HPLC" (Instruction Manual, June 2001, BIO-RAD, Hercules, CA). Two ml

of sodium plasma were thawed and added to 50 mg of acid washed alumina (BioRad Cat. 195-6055). Two ml of 0.1M H_3PO_4, Calibrator (BioRad) and high and low controls (BioRad) were also run. Two hundred µl of internal standard and 2 ml of 1M Tris buffer (pH 6.8) were added to each tube, mixed and placed on a rotor at 75% for 19 minutes. Samples were then centrifuged and washed twice with 1 ml water before the addition of 200 µl of 0.1M H_3PO_4. Samples were centrifuged at 2100 x g and 5^{o}C for 5 minutes after which the supernatant was transferred to autosamplier vials and loaded into the HPLC system for analysis.

Statistics

Hormones were compared between OWE and baseline conditions using repeated measures paired T-tests. Mixed generalized linear models with repeated measures were used to compare the response of cells to pressure with controls, between baseline and OWE conditions, and between dive profiles. Pressure exposure data was normalized over control values, and the resulting indices were compared with 1, as well as between conditions and dive profiles. Data for baseline conditions in belugas and humans have been reported in the previous section, and are used here for comparison with stressor conditions. No statistics could be run on data collected from inflammation samples due to the small sample size (n=2).

Results

Hormone Concentrations between Conditions

Catecholamine and cortisol concentrations for baseline, OWE and inflammation conditions are summarized in Table 3-2. A significant increase was detected only for OWE cortisol as compared with baseline cortisol (T=-5.794; p=0.029) though higher epinephrine values were also

observed. A slight increase in epinephrine was also observed for OWE and inflammation conditions, though this was not significant.

Table 3-2: Plasma catecholamines and cortisol (± SE) for belugas during baseline, OWE and inflammation conditions. Values which are significantly different from baseline ($p<0.05$) are indicated with an asterisk (*).

		Epinephrine pg ml-1	Norepinephrine pg ml-1	Cortisol µg dl-1
Belugas	**Baseline n=4**	<30	662.96 ± 110.5	1.57 ± 0.2
	OWE n=3	78.02 ± 43.4	757.06 ± 81.4	7.97 ± 1.2
	Inflammation n=2	<30	780.38	1.97

Immune Function Control Values between Conditions

Significant differences in immune function between baseline and OWE conditions were detected in control values for all measures of phagocytosis, and CD11b expression (Table 3-3). For phagocytosis, decreased control values were observed for OWE conditions, while inflammation samples showed general patterns of increased function. Patterns of increased CD11b expression in control samples were observed for both OWE and inflammation conditions, particularly for controls of the repeated 5 minute dives.

Table 3-3: Control values for phagocytosis and CD11b expression (±SE) for all simulated dive profiles. * indicates significant differences from baseline (α=0.05). Number of animals (n) for each condition are: baseline=4, OWE=3, inflammation n=2.

Phagocytosis							
Granulocytes			**30 min**	**5 min**	**2x5 min**	**F**	**p**
	MFI	Baseline	48.3±11.6	23.5±7.4	46.0±14.1	5.372	0.006
		OWE	34.2±16.2	12.1± 2.9	25.9±11.5		
		Inflammation	154.9	72.8	137.9		
	% Positive	Baseline	40.7±6.0	26.0±5.0	39.1±7.5	116.058	<0.001
		OWE	25.4±14.3	11.8±4.4	23.0±12.5		
		Inflammation	67.3	46.0	65.7		
Monocytes			30 min	5 min	2x5 min	F	p
	MFI	Baseline	162.9±61.9	51.07±9.93	94.46±19.14	6.258	0.003
		OWE	110.9±41.5	31.63±9.29	89.78±31.51		
		Inflammation	219.2	109.31	215.69		
	% Positive	Baseline	66.1±7.3	50.8±6.0	51.4±10.6	7.423	0.001
		OWE	53.6±15.2	34.5±15.5	52.8±13.9		
		Inflammation	74.4	55.4	74.0		

CD11b Expression		**30 min**	**5 min**	**2x5 min**	**F**	**p**
MFI	Baseline	33.0 ± 7.1	21.1±6.0	23.9±6.5	1.264	<0.001
	OWE	32.2±5.2	20.3±11.2	32.2±5.2		
	Inflammation	51.6	43.6	40.4		
% Positive	Baseline	75.7±6.9	55.7±5.8	59.6±8.9	6.855	0.002
	OWE	73.1±9.3	46.0±24.5	73.1±9.3		
	Inflammation	86.6	73.9	72.6		

Combined Effects of Diving and Stressors on Phagocytosis-OWE

2000 psi with 2 minutes of Compression and Decompression- Granulocytes

Significant changes phagocytic activity were detected following all pressure exposures (Appendix K). Following the dive period of the single exposures both the amount of phagocytosis occurring per cell (MFI; Figure 3-1; 30 minutes, $p<0.001$; 5 minutes, $p<0.001$) and the % of granulocytes undergoing phagocytosis (Appendix L; Figure L-1; 30 minutes, $p<0.009$; 5 minutes, $p=0.001$) displayed significant decreases from controls. A significant decrease in the % of positive granulocytes also occurred following the repeated 5 minute exposure ($p=0.003$). Following recovery periods, MFI increased significantly only following the 30 minute exposures (Figure 3-1; $p=0.011$).

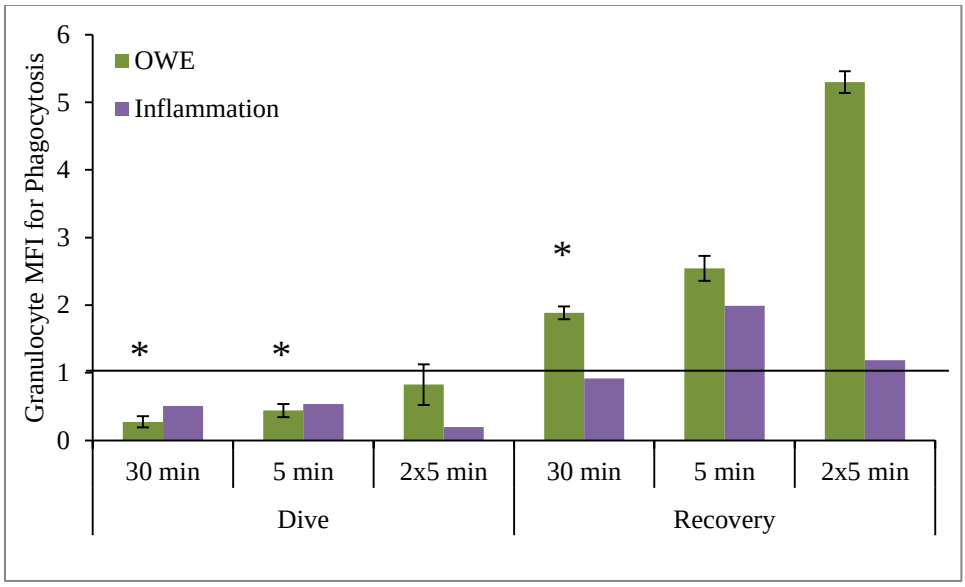

Figure 3-1: Granulocyte MFI for phagocytosis in OWE (n=3) and human (n=2) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

2000 psi with 2 minutes of Compression and Decompression- Monocytes

All pressure exposures resulted in significantly decreased monocyte phagocytosis in OWE samples (Appendix K). Both the % of monocytes undergoing phagocytosis and the amount of activity occurring per cell (MFI) decreased significantly for all pressure exposures ($p<0.001$ for all cases; Appendix L; Figures L-2, L-3). Following the recovery period for the 30 minute exposure, MFI displayed an increase ($p=0.051$) while the % of positive cells displayed a decrease ($p=0.072$). This pattern of decreasing % of monocytes undergoing phagocytosis was also observed for the recovery period of the 5 minute exposure ($p=0.053$).

Combined Effects of Diving and Stressors on CD11b Expression-OWE

No significant effects of pressure were detected for either measure of CD11b expression for any pressure exposures (Appendix M). However, patterns suggestive of an increase in expression were observed for the repeated 5 minute exposures (MFI, $p=0.079$); % positive, $p=0.096$). Results are displayed in Appendix N, Figures N-1 and N-2.

Combined Effects of Diving and Stressors on Phagocytosis-Inflammation

Effects of 2000 psi with 2 minutes of Compression and Decompression-Granulocyte

Decreased granulocyte MFI for phagocytosis was observed for the dive periods of all pressure exposures to 2000psi (Figure 3-1). These changes appear to return to control values during the recovery periods, except for the single 5 minute exposure for which an increase in MFI was observed.

The % of granulocytes undergoing phagocytosis (Appendix L; Figure L-1) decreased for all exposures to 2000 psi with the largest change occurring following the repeated 5 minute

exposure. Following the recovery periods, an increase in the % of positive granulocytes was observed only for the single 5 minute exposure.

Effects of 2000 psi with 2 minutes of Compression and Decompression- Monocytes

Decreased monocyte MFI for phagocytosis was observed for the dive periods of single 5 minute and repeated 5 minute exposures to 2000psi, while the % of monocytes undergoing phagocytosis increased for the 30 minute and 5 minute exposures (Appendix L; Figure L-2, L-3). Following recovery periods, an increase in both monocyte MFI and % positive cells was observed for the single 5 minute exposure. The % of positive monocytes also appears to decrease for the both the dive and recovery periods following the repeated 5 minute exposures (Appendix L; Figure L-3).

Effects of 1000 psi with 2 minutes of Compression and Decompression- Inflammation

For exposures to 1000 psi, decreases in both granulocyte MFI and the % of positive granulocyte were also observed for dive periods of all duration exposures (Figure 3-2). Monocytes also displayed decreased values of MFI for the dive periods of all exposures which values appeared to return to control levels for all recovery periods (Figure 3-2)

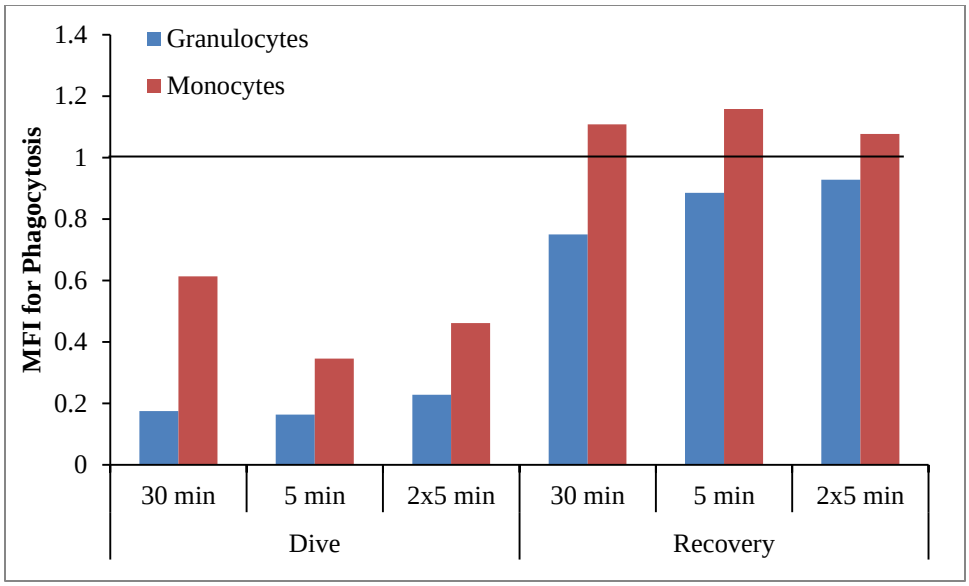

Figure 3-2: MFI for phagocytosis in inflammation samples (n=2) measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small samples size no statistics could be run for this data.

Effects of 2000 psi and 1000 psi on CD11b Expression

No apparent changes in either granulocyte MFI for the % of positive granulocytes for CD11b expression were observed for inflammation samples following exposures to 2000G (Appendix N; Figures N-1 and N-2). However, decreased granulocyte MFI for CD11b expression was observed for inflammation samples following the single 5 minute and repeated 5 minute exposures to 1000G (Figure 3-3).

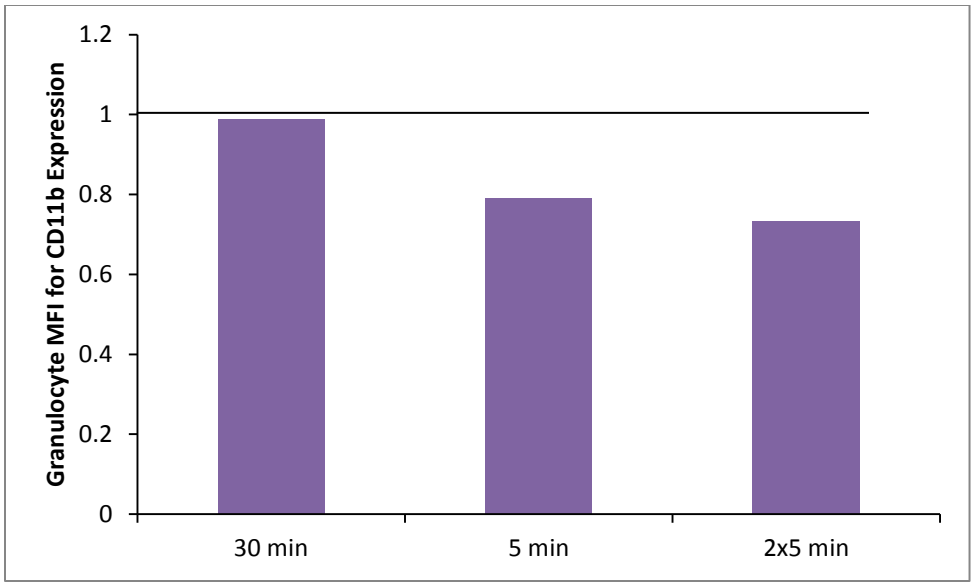

Figure 3-3: Granulocyte MFI for CD11b Expression in inflammation samples (n=2) measured following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small samples size no statistics could be run for this data.

Comparative Effects of Pressure on Baseline, Human and Stressor Samples

Effects of 2000 psi on Phagocytosis-Granulocytes

Significantly different granulocyte responses were detected for the 30 and single 5 minute pressure exposures to 2000 psi (Appendix O). The pressure-induced change in the amount of activity occurring per cell (MFI) was different between OWE conditions in belugas and humans for the dive period of the 30 minute exposure ($p=0.025$), and between OWE conditions and baseline conditions in aquarium animals for the recovery periods of the 30 minute ($p=0.001$) and repeated 5 minute ($p=0.09$) exposures (Figure 3-4).

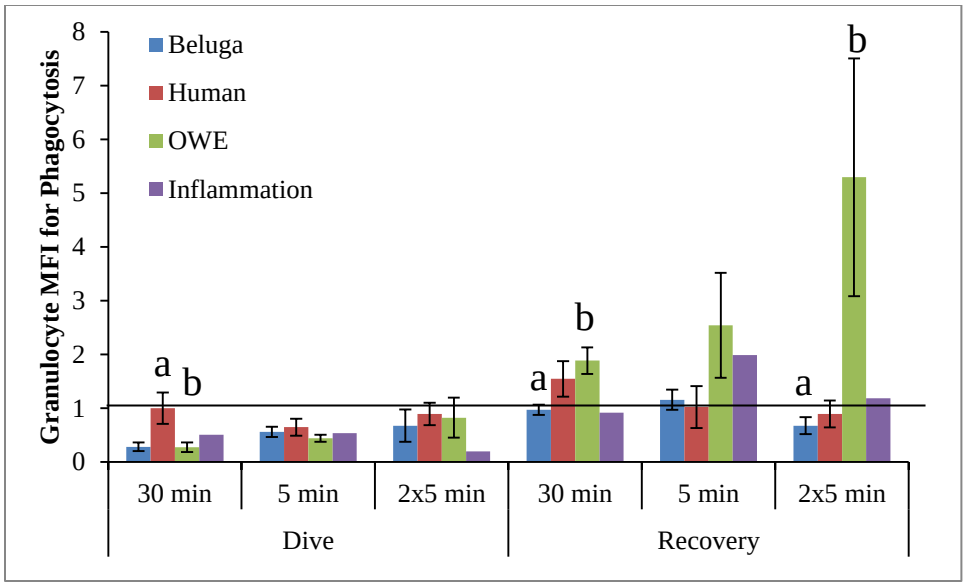

Figure 3-4: Granulocyte MFI for phagocytosis in belugas during baseline (n=4), OWE (n=3) and inflammation conditions (n=2) and human (n=4) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with letters ($p<0.05$).

Changes in the % of positive cells display similar patterns (Appendix P; Figure P-1), with OWE conditions in belugas differing from humans for dive period of the 30 minute ($p=0.012$) and repeated 5 minute ($p=0.017$), and from baseline conditions for the recovery period of the 30 minute exposure ($p=0.096$)

Inflammation samples appeared to show a greater decrease in granulocyte MFI than both baseline beluga and human samples for the dive period of the repeated 5 minute exposure (Figure 3-4).For the recovery periods, inflammation samples also display an apparently larger increase in granulocyte MFI for the single 5 minute exposure than both baseline and human exposures.

Effects of 2000 psi on Phagocytosis-Monocytes

Differences in the responses of monocytes to pressure were detected within all pressure exposures (Appendix O).

The % of positive monocytes (Appendix P; Figure P-2) displayed different changes between OWE conditions and baseline conditions in belugas for the dive period of the single 5 minute ($p=0.063$) and repeated 5 minute ($p=0.084$) exposures, and from humans following all duration exposures (30 minutes, $p=0.042$; 5 minutes, $p=0.006$, 2x5 minutes, $p=0.001$). OWE conditions in belugas also displayed different responses in monocyte MFI (Appendix P; Figure P-3) from humans following the dive period for the 30 minute (MFI, $p=0.075$) and 5 minute exposures ($p=0.079$). OWE conditions continued to show a different response to pressure as compared with baseline conditions in belugas for the recovery periods of the 30 minute (MFI, $p=0.039$; % Positive, $p=0.020$) and single 5 minute exposures (MFI, $p=0.041$; % Positive, $p=0.035$).

Inflammation samples (Appendix P; Figures P-2, P-3) appear to display much smaller changes in monocyte MFI than either baseline or OWE samples for belugas, and look more similar to human samples for the dive periods of the 30 minute and single 5 minute exposures. This pattern is also observed for the % of positive monocytes.

Effects of 1000 psi on Phagocytosis- Granulocytes

Inflammation samples display larger decreases in granulocyte MFI during the dive periods following all exposures to 1000 psi as compared with baseline beluga samples or humans (Figure 3-5). This pattern was also observed for the % of granulocytes undergoing phagocytosis (Appendix P; Figure P-4). No apparent differences in granulocyte responses were noted during

the recovery period of these exposures which differs from the increases observed in baseline samples (Figure 3-5; Appendix P; Figure P-4).

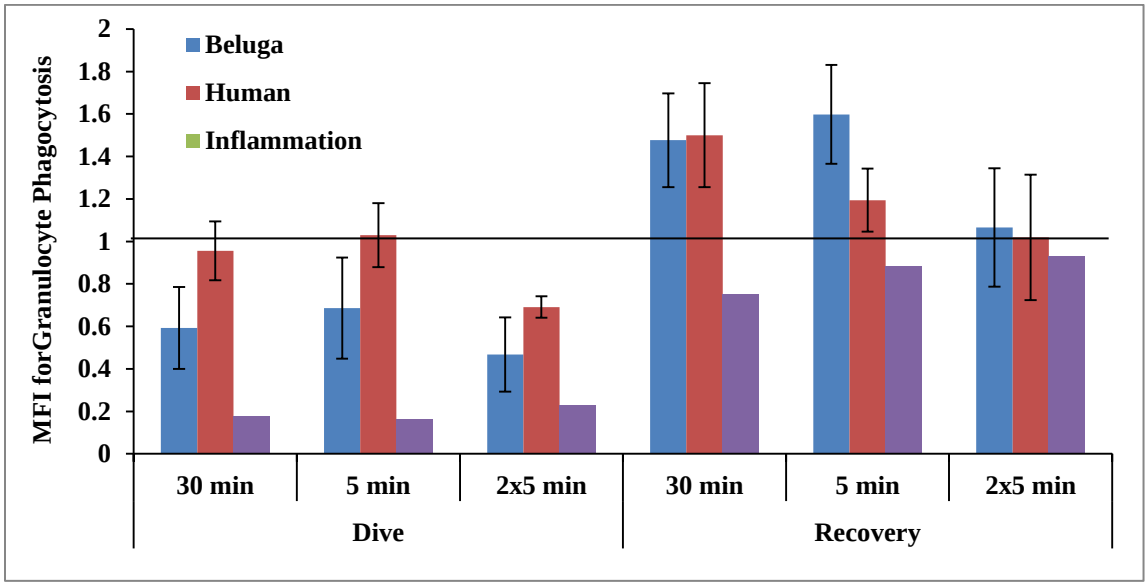

Figure 3-5: Granulocyte MFI for phagocytosis in belugas during baseline (n=4) and inflammation (n=2) conditions, and humans (n=4) measured following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small sample size no statistics could be run for inflammation data.

Effects of 1000 psi on Phagocytosis- Monocytes

Larger decreases in monocyte MFI for phagocytosis were observed in inflammation samples as compared with baseline beluga or human samples for the dive periods of all exposures to 1000 psi (Appendix P; Figure P-5).

Comparative Effects of Pressure Exposures on CD11b Expression

No significantly different responses in either granulocyte MFI or the % positive granulocytes for CD11b expression were detected following exposures to 2000 psi (Appendix Q). Inflammation

samples however, display slightly smaller changes in both measures of function following the repeated 5 minute exposures (Figure 3-6, Appendix R; Figure R-1).

Inflammation samples appear to show larger changes in granulocyte MFI for CD11b expression following the single 5 minute and repeated 5 minute exposures to 1000 psi (Figure 3-7).

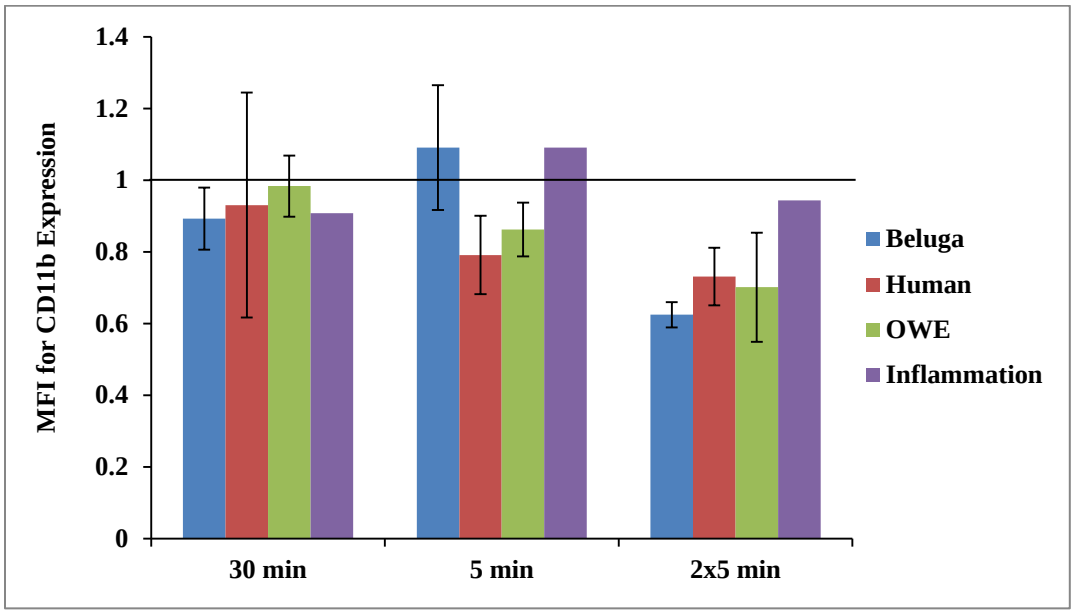

Figure 3-6: Granulocyte MFI for CD11b expression in belugas during baseline (n=4), OWE (n=3) and inflammation (n=2) conditions, following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Due to small sample size statistics could not be run for inflammation data.

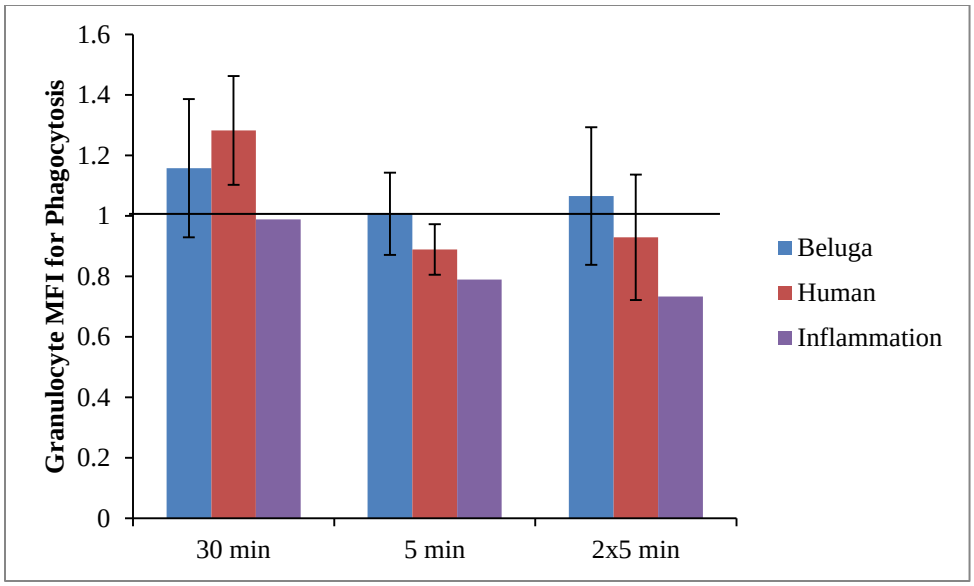

Figure 3-7: Granulocyte MFI for phagocytosis in belugas during baseline (n=4) and inflammation (n=2) conditions, and humans (n=4) following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Due to small sample size no statistics could be run for inflammation data.

Discussion

Hormone data support the categorization of the OWE as a stressor. Even though the increase in norepinephrine was not statistically significant, the increase in cortisol was, as expected. Schmitt *et al.,* (2010) reported baseline and OWE cortisol to be 2.2 ± .9 and 7.9 ±1.5 µg dl^{-1} respectively, and cortisol values obtained during this study were similar. Epinephrine was detectable only for the OWE, and not during most baseline or inflammation conditions.

Significant differences in control measures of phagocytosis and CD11b expression were also detected with patterns suggesting decreased phagocytosis during the OWE. Immunosuppression has generally been associated with increased cortisol in response to a stressor. In contrast, patterns of increased phagocytosis were observed in samples from inflammation conditions as compared with baseline. Patterns suggesting increased expression of CD11b were also observed

for both inflammation and OWE conditions. This is perhaps not surprising, as inflammatory processes result in priming, activation and increased phagocytic activity (Smith, 1994).

Phagocytosis data from OWE and inflammation conditions displayed general patterns of decreased phagocytic activity for the dive period of all exposures, with recovery periods for the 2000 psi exposures suggesting increased activity. No significant changes in CD11b expression were detected, which was similar to results from baseline conditions in belugas. However, larger changes were observed in both OWE and inflammation samples as compared with baseline samples, and in some cases (particularly monocytes) these changes were more similar to those observed in humans than in baseline conditions. In humans, dive-related changes in immune function have been linked to the development of injury and disease (Brenner *et al.,* 1999). If physiologically challenging conditions can alter the response of marine mammal cells such that it resembles the response of human cells, it is possible that under such conditions marine mammals become more susceptible to dive related pathologies. However, consequences of decreased immune function are not always negative, and conversely increased immune function is not always protective. What is of concern, is that responses occurring in belugas during stressor conditions are different from responses which occur under baseline conditions, suggesting potential consequences on animal health.

Changes in neuroendocrine hormones, such as norepinephrine, also occur during natural diving. Catecholamines play important regulatory roles in the dive response, for example controlling peripheral vasoconstriction (Hochachka *et al.,* 1995) and constriction of the spleen resulting in release of red blood cells and an increase in blood oxygen stores (Hance *et al.,* 1982; Hochacka *et al.,* 1995). Additionally, catecholamines may play a role in regulating post dive tachycardia which aids in replenishing oxygen stores (Elliott *et al.,* 2002). Thus, immune cells which are less

reactive to changes in these hormones, perhaps due to low binding affinity of receptors, may be protective and allow for normal immune responses to occur in the face of challenges such as repetitive diving. However, in the presence of an additional physiological challenge, changes in neuroendocrine hormones may be of abnormal magnitude (Talpalar and Grossman, 2005) thus altering the likelihood of receptor binding and cellular changes. Quantification of receptors on immune cells, as well as identification of high and low affinity receptors may be areas of interest for future research considering the effects of neuroendocrine hormones on immune function.

The *in vitro* approach used in this study allowed an investigation of the effects of pressure *per se* on immune function. Pressure changes during diving represent important physiological challenges, however combined influences of dive physiology and pressure changes could result in cellular responses which differ from those reported here. For example, changes in neuro-endocrine hormones during diving may mediate the effects of pressure such that significant changes in function reported here would no longer be significant. It is recognized that future studies using *in vivo* models are needed.

The impact of stressors on marine mammal health may also be indirect by influencing behavior, e.g. triggering avoidance responses and altering depth or duration of dives. The response of cells to increased pressure during baseline conditions show some variability between dive profiles to different depths, or with different rates of compression and decompression (Chapter 2). While samples were not available for dives with rapid compression and decompression for this study, the response of cells to 2000 psi for inflammation conditions showed some differences from the response of cells to 1000 psi exposures. These responses, in turn, showed some difference from baseline conditions, possibly suggesting that there are important trade-offs between dive

behavior and immune function, and that the relationship between behavior and health is dynamic.

Though these results are not conclusive as to the role stressors may play in altering the response of immune cells to challenges associated with diving, they do suggest that altered physiological states can lead to varied responses. Additionally, in some cases the response of cells from inflammation and OWE samples resembled the response of human cells rather than baseline beluga cells, supporting the idea that altered physiological states may effect on the ability of cells to respond 'normally' to the challenges associated with diving. Altering the effect of dives on immune cell function may result in a decrease ability of animals to respond to an infection or inflammation. Also, increased immune function may prepare a body to deal with an immediate threat such as a wound during an acute stressor. The activation of this response in conjunction with the effects of diving may lead to undesirable immune activity and increased susceptibility to dive related injury and disease. For example, increased activity during a stressor may lead to an increased responsiveness of marine mammal cells to otherwise 'silent' bubbles, leaving them more susceptible to development DCS or DCS-like injury. Again, this outcome may be influenced by characteristics of the dive, such as duration and depth.

Previously reported results (Chapter 2) suggested altered immune function during diving in belugas, as compared with humans, possibly reflecting dive-adaptation and protection against dive-related injury. The response of immune cells to diving also varied with dive characteristics such depth or duration. The results of this study suggest that health status of an animal (i.e. presence of infection, or additional stressor) can impact the response of immune cells to diving, with the implication that anthropogenic stressors could augment the normal adaptive responses to diving thus altering marine mammal susceptibility to dive-related injury.

More work, however, is needed to better describe the likely complicated relationship between diving, stress and immune function in marine mammals. Future studies should investigate membrane composition of immune cells and could also focus on other measures of immune function, such as radical oxide production which is important for destroying bacteria, but also in endothelial damage and facilitating inflammatory responses. Nonetheless, this work provides the first evidence that one mechanism through which anthropogenic activity could impact marine mammal health is by altering the response of immune cells during diving, either by acting as a stressor or altering behavior.

Chapter 4 LYMPHOCYTE ACTIVATION AND PROLIFERATION IN BELUGAS (*Delphinapterus leucas*) FOLLOWING PRESSURE EXPOSURES: POTENTIAL IMPACTS OF STRESSORS ON ADAPTIVE IMMUNE FUNCTION DURING DIVING

Abstract

Increased pressure, associated with diving, can alter immune functions performed by peripheral blood mononuclear cells (PBMC). The purpose of this study was to measure PBMC activation (IL2R expression) and Concanavalin A induced lymphocyte proliferation (BrdU incorporation) in belugas following *in vitro* pressure exposure during baseline and stressor conditions. Beluga blood samples (n=4) were obtained from animals at the Mystic Aquarium during baseline and stressor conditions, and from free ranging animals in Alaska. Human blood samples (n=4) (Biological Specialty Corporation) were run for comparison. *In vivo* catecholamines and cortisol were measured in belugas to characterize the neuroendocrine response. Comparison of cellular responses between controls and pressure exposed cells, between stressor conditions and species, as well as between dive profiles were run using mixed generalized linear models (α=0.05). Cortisol was significantly higher in wild belugas and stressor samples as compared with baseline for aquarium animals. Both IL2R expression and proliferation displayed significant pressure induced changes, and these responses varied between conditions in belugas, with characteristics of the pressure exposure and between species. Both belugas and humans displayed increased IL2R expression, while proliferation decreased for aquarium animals and increased for humans and wild belugas. Results suggest beluga PBMC function is altered during diving and changes may represent normal dive adaptation as the response differs from humans, a non-dive adapted mammal. In addition, characteristics of a dive (i.e., duration, depth) as well as stressors can alter the response of beluga cells, impacting the ability of animals to fight infection or resist dive related pathologies.

Introduction

During diving, marine mammals must contend with several environmental and physiological challenges, including changes in pressure. While a major concern with changes in pressure is the effect on air filled spaces, biological systems can also be affected at the cellular level, particularly by altering membrane characteristics (Somero, 1992; Kato and Hayashi, 1999; Pradillon and Gaill, 2007) and changing the volume of reactions (Bartlett, 2002). In the nervous system altered cell function may lead to tremors and convulsions (Bennett, 1982). Alterations in function of immune cells may lead to the development of infection if there is a failure to mount a response or the development of autoimmune disease and self-damage if inappropriate or augmented responses occur. Several immune functions performed by macrophages and lymphocytes have been found to be sensitive to changes in pressure (Hallenbeck and Andersen, 1982), including antigen processing and the production of immunoglobulins. Macdonald (1982) reports that procession of the cell cycle and cell division are inhibited by increased pressures. This would impact the process of proliferation and the production of clones with specific antibody recognition capabilities (Murphy *et al.*, 2008). In addition, denaturation of membrane associated proteins, including antigen receptors (Kato and Hayashi, 1999; Macdonald, 1982) may lead to decreased antigen binding and thus immune responses. T cell activation for example could be affected as it relies on binding and clustering of T cell receptors (TCR's) and co-stimulatory molecules in the membrane (Germain, 1997; Quintana, 2005; Murphy, 2008). Increased incidents of certain infections have also been reported in human divers as compared to non-divers, including dermatitis and external ear infections (Edmonds and Shilling, 1984).

There are however, many factors that can affect immune function including exposure to stressors. Following perception of a stressor, activity of the sympathetic nervous system and

hypothalamic pituitary adrenal axis results in the release of catecholamines and glucocorticoids from the adrenal glands. These hormones can exert influence on cell function by binding to specific receptors in the cell membrane. Catecholamines, such as norepinephrine and epinephrine bind with α and β adrenergic receptors (Madden *et al*., 1995) and glucocorticoids such as cortisol bind with Type I and Type II adrenal steroid receptors (McEwen *et al.,* 1997). These receptors have varied binding affinities for different hormones as well as expression in the membrane depending on cell type (Padgett and Glaser, 2003). In addition, norepinephrine serves as a neurotransmitter, effecting cells within close proximity to nerve endings (Padgett and Glaser, 2003).

The effects of a stress response on immune function are dependent on many factors including which receptors are bound, cell type, activation state of the cell and the stimulus of immune responses (Madden *et al.,* 1995; Madden, 2003) as well as the magnitude and duration of exposure (Dabhar, 2009; Martin, 2009). In humans, changes in blood components during dives, including those associated with immune responses may lessen with increased experience, lending evidence to the idea that stress impacts the dive response (Philp, 1974).

Marine mammals are faced with multiple stressors both in aquaria and the wild. For free ranging marine mammals, these stressors include direct anthropogenic activities including, shipping, sonar use and fishing, as well as the effects of changing environmental conditions associated with climate change (Fair and Becker, 2000). If a stress response associated with diving in humans can lead to changes in cell function, it is possible that stressors can impact the ability of marine mammal cells to function during diving. In recent decades, the occurrence of gas bubbles and injury resembling decompression sickness seen in people, have raised concern over the impacts of anthropogenic stressors on marine mammal health. It is unknown however how

marine mammal lymphocytes respond to the challenges of diving during 'normal' conditions, and whether additional stressors might affect this response.

This work was initiated in order to investigate the relationship between peripheral blood mononuclear cell (PBMC) activity, diving and stress in belugas (*Delphinapterus leucas*). Belugas are a mid-size odontocete with a wide range of diving behaviors and deep diving capabilities (Martin and Smith, 1999; Heide-Jorgensen *et al.,* 1998; Suydam *et al.,* 2001; Martin *et al.,* 1998; Hedrick *et al.,* 1991). Ultimately this work aimed to describe the functional response of beluga PBMC's to changes in pressure under baseline and stressor conditions, as compared with humans. Specifically, the aims of this work were to 1) measure T lymphocyte proliferation and PBMC activation [through expression of the IL2 receptor (IL2R)] in belugas in response to simulated dive excursions *in vitro* 2) to compare the response of beluga PBMC's to the response measured in humans and 3) to evaluate the combined effects of pressure and an additional stressor on beluga PBMC activity. It was hypothesized that beluga cells would continue functioning at control levels following pressure exposures during baseline conditions, while human cells would show inhibition of function. Stressor conditions in belugas were expected to result in decreased measures of PBMC activity following pressure exposures, similar to that observed in humans.

Methods

Baseline blood samples were obtained using positive behavioral reinforcement from four belugas resident at the Mystic Aquarium, Mystic, CT. Stressor samples were obtained at the conclusion of a thirty minute out of water examination (OWE, n=3), which has previously been shown to result in changes in adrenocorticotropic hormone (ACTH) and cortisol (Schmitt *et al.,* 2010). Additional samples, representing physiological challenge, were obtained from animals with

clinical signs of mild chronic inflammation (n=2). Blood samples were also obtained from belugas in the Nushagak and Wood river areas of Bristol Bay, AK (n=9) in accordance with NMFS Marine Mammal Research Permit No. 14245. In addition, human samples (n=4) were purchased from Biological Specialty Corporation Inc., for comparison with belugas.

For cell function assays, whole blood samples were collected in 10 ml sodium heparin vacutainers and centrifuged for 10 minutes at 2000 x g and 10^{o}C. Plasma was removed and 1 ml aliquots were stored in Sarstedt™ tubes at -80^{o}C for hormone analysis. The white blood cell buffy coat was aliquoted in sterile cryoviles, mixed with an equal volume of freezing media (90% fetal bovine serum and 10% DMSO). Buffy coats were stored at -80^{o}C for 24 hours and transferred to liquid nitrogen for storage until assay. Samples collected from Bristol Bay animals were initially processed in the field and plasma and buffy coat samples were shipped back to Mystic, CT in liquid nitrogen dry shippers.

Lymphocyte Proliferation

A colorimetric BrdU incorporation ELISA assay was purchased from Roche Applied Sciences (Indianapolis, IN) and used to measure concanavalin A (Con A) induced T lymphocyte proliferation. Buffy coats were thawed quickly at 37^{o}C and washed twice with RPMI 1640 (centrifuged for 5 minutes at 220 x g and 20^{o}C). The cell pellet was then brought up in 3 ml of RPMI 1640, which were then carefully overlaid on 3 ml of sterile histopaque 1077 and centrifuged for 30 minutes at 400 x g and 20^{o}C. The separated mononuclear cell layer was carefully removed and washed twice in Lymphocyte Proliferation Assay (LPA) media (RPMI 1640 with .1 µM non-essential amino acids, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin,

.292 mg ml^{-1} 1-glutamine, 1% 100mM sodium pyruvate, 1% 1M hepes, 10% FBS, 1% 0.01M 2-mercaptoehtanol).

Cell counts were obtained using Trypan blue exclusion and sample volume was adjusted to reach a target of 10^6 cells ml^{-1}. Control cells were set aside and the remaining cells were brought to 4 ml for simulated dive exposures. Following each dive, cells were recounted and final volume readjusted to reach 10^6 cells ml^{-1}.

One hundred µl of cell suspensions were aliquoted into BD Falcon™ 5 ml polystyrene round bottom tubes (BD Biosciences, San Jose, CA) and 100 µl of Con A working stock (5 µg ml^{-1}) was added for a final Con A concentration of 2.5 µg ml^{-1}, which had previously been determined to be optimal for belugas within the lab at Mystic Aquarium. Non stimulated controls did not receive any Con A. Plates were incubated for 72 hours in a 5% CO_2 incubator at 20°C. At 72 hours, 20 µl of BrdU was added to sample wells, as well as to non-stimulated BrdU controls and plates were returned to the incubator for a further 18 hours. After a total incubation of 90 hours, wells were pipetted to break up aggregates and plates were centrifuged for 10 minutes at 300 x g. Wells were then emptied and plates dried under a hair dryer for 15 minutes. Dry plates were then sealed in Ziploc bags and stored at 4°C until development. All plates were developed according to kit instructions within 1 week of drying.

Two hundred µl of FixDenat solution was added to each well and incubated for 30 minutes at room temperature. Solution was emptied from wells and 100 µl of anti-BrdU-POD (peroxidase conjugated monoclonal Fab fragments from mouse-mouse hybrid) were then added to each well. After a further 90 minute incubation, wells were emptied and washed three times with washing solution. One hundred µl of substrate solution was then added and color was allowed to develop

for 30 minutes. Twenty five µl of 1M H_2SO_4 was added to stop the reaction. Absorbance was read at 450 nm using an EL800 microplate reader (BioTek, Winooski, VT). Stimulation indices were calculated for each sample as the ratio of the optical density (OD) of stimulated cells to the OD of the control cells with BrdU but no stimulation.

Interleukin 2 Receptor (IL2R) Expression

Archived white blood cell samples were thawed quickly at 37^{o}C and washed twice with RPMI 1640. Final pellets were brought to a volume of 3 ml in RPMI 1640. Peripheral blood mononuclear cells (PBMC), including lymphocytes and monocytes, were isolated in the sample by Ficoll density gradient as per above. Cells counts were obtained using Trypan blue staining and sample volumes were adjusted to $2x10^6$ cells ml^{-1} with PBS. Two hundred µl of sample were set aside for controls. Remaining sample was adjusted to a volume of 4 ml for simulated dive exposures. Following decompression, cells were recounted and volume re-adjusted to a concentration of $2x10^6$ cells ml^{-1} in PBS.

A human IL2 biotinylated fluorokine kit was used to assess lymphocyte activation (R and D systems, Minneapolis, MN). Specificity testing as per manufacturer's instructions, as well as Con A stimulation tests were carried out on beluga samples. Twenty five µl of cell suspensions were aliquoted into Falcon™ tubes, and 10 µl of biotinylated IL2 cytokine was added. Negative controls received 10 µl of biotinylated soybean trypsin inhibitor (negative control). All tubes were incubated for 60 minutes at 4^{o}C after which 10 µl of avidin FITC were added. Tubes were then incubated for a further 30 minutes in the dark at 4^{o}C. Tubes were washed twice with 2 ml of RDF1 wash buffer (provided with the kit) and final cell pellets were re-suspended in 250 µl of 1% paraformaldehyde until flow cytometric analysis.

Flow Cytometry

Samples were read using an LSR flow cytometer (BD Biosciences, San Jose, CA). Peripheral blood mononuclear cells, including lymphocytes and monocytes, were gated using forward and side scatter plots generated using controls containing only cells. The avidin FITC was read at 518 nm in the FL1 channel. Two measures of IL2R expression were collected; 1) the percentage of gated cells which expressed IL2 R and 2) the mean intensity of the fluorescence (MFI) expressed by PBMCs, which reflects the relative amount of expression per cell i.e. increased MFI reflects an up regulation in expression. Ten thousand events within the gated population were targeted.

Simulated Dives

Simulated dives were carried out in a stainless steel pressure chamber. Four ml of cell suspension were added to the chamber and overlaid with a small layer of mineral oil. A hydraulic pump was used to pump mineral oil into the chamber until the desired pressure was reached. Simulated dive profiles are summarized in Table 4-1. Two pressures were targeted; 2000 psi (1360m) and 1000 psi (680m) with compression and decompression occurring over a period of either 2 minutes (G) or 15 seconds (R). Pressure excursions lasted for 30 minutes, 5 minutes or two repeated 5 minute periods with a one minute rest interval.

Hormone Analysis

One ml of archived plasma was shipped to the AHDC Endocrinological Lab at Cornell University (Ithaca, NY) for cortisol analysis using the Immulite® system (Schmitt *et al.,* 2010; Schwake *et al.,* 2013). Catecholamines were measured at the Mystic Aquarium using a Waters (Milford, MA) High Performance Liquid Chromatography system (1515 isocratic pump, 717 auto sampler) with 2465 electrochemical detector. Hormones were extracted using 50 mg of acid

washed alumina (BioRad Cat. 195-6055) and an internal standard was added to each sample being run. Details of this methodology are discussed in "Plasma Catecholamines by HPLC" (Instruction Manual, June 2001, BIO-RAD, Hercules, CA).

Table 4-1: Dive profiles targeted for pressure exposures. Two depths were chosen with exposures lasting for 3 different durations. Compression and decompression were also varied over two durations.

Pressure	Simulated Depth	Compression/ Decompression	Duration (min)
2000psi	1360m	2min (2000G)	30 5 2x5
2000psi	1360m	15sec (2000R)	30 5 2x5
1000psi	680m	2min (1000G)	30 5 2x5
1000psi	680m	15sec (1000R)	30 5 2x5

Statistics

Hormones and control values of immune function were compared between conditions in belugas using a mixed generalized linear model or ANOVA. Measures of immune function following pressure exposures were normalized to control values and compared to a value of 1 in order to

determine significant changes using mixed generalized linear models with repeated measures. In addition, these indices of immune function were compared between conditions, species and dive profiles. Significance was determine at α=0.05. Due to small sample sizes however $p<0.01$ are also reported as observed patterns.

Results

Hormones

Plasma hormone levels for baseline, OWE and inflammation condition in belugas have been reported previously (Chapters 1 and 2). Hormone results are summarized in Table 4-2 and significant differences were detected between conditions ($F=27.597$; $p<0.001$). Epinephrine levels were below the detectable range of the HPLC protocol (<30 pg ml^{-1}) for most baseline and inflammation beluga samples but were measured in wild belugas as well as during OWE conditions. Wild belugas had significantly higher epinephrine and norepinephrine than both baseline and OWE conditions for aquarium belugas. Though significant differences were not detected for cortisol, wild belugas also displayed higher plasma cortisol values than baseline and inflammation conditions for aquarium animals. Plasma cortisol was similar between OWE conditions and wild belugas.

Table 4-2: Plasma hormone concentrations (mean ± SE) for humans and belugas. ND= not detectable. Significant differences from wild animals are indicated by an asterisk (*). Significant differences from baseline are indicated by a double asterisk (**). p<0.05.

		Epinephrine pg ml^{-1}	Norepinephrine pg ml^{-1}	Cortisol µg dl^{-1}
Belugas	**Baseline n=4**	ND*	662.96 ± 110.5*	1.57 ± 0.2
	OWE n=3	78.02 ± 43.4*	757.056 ± 81.4*	7.97 ± 1.2**
	Inflammation n=2	ND	780.375	1.97
	Wild n=9	341.9 ± 48.68	1461.08 ± 240.48	6.43 ± 0.79

Controls

Significant differences in IL2R expression between baseline and OWE conditions were detected in MFI following the 5 minute (t=8.47; p=0.014) and repeated 5 minute (t=15.40; p=0.004) exposures, and for the % of positive PBMC for the 5 minute exposure (t=9.63; p=0.011). Controls values of IL2R expression for baseline samples tended to be higher than those measured for OWE and inflammation conditions, as well as wild animals. In addition controls from OWE conditions were smaller than both inflammation conditions and controls for wild belugas. Wild belugas also displayed significantly lower control indices of proliferation than either baseline (t=-4.185; p=0.002) or OWE conditions (t=-4.6; p=0.001) for aquarium belugas.

Effects of Pressure on Lymphocyte Proliferation

Mixed generalized linear model results are summarized in Appendix S.

2000 psi with 2 minute compression and decompression (2000G)

Significant pressure induced changes in lymphocyte proliferation were detected following all exposures to 2000 psi with 2 minutes of compression and decompression (Figure 4-1). Baseline and OWE samples displayed decreased proliferation the 30 minute (baseline, p<0.001;OWE,p<0.001) and repeated 5 minute exposures (baseline, p=0.032; OWE, p=0.024). OWE samples also displayed a pressure induced decrease in proliferation for the 5 minute exposures (p<0.00). In contrast, animals from Bristol Bay displayed increased proliferation following the repeated 5 minute exposures (p=0.045).

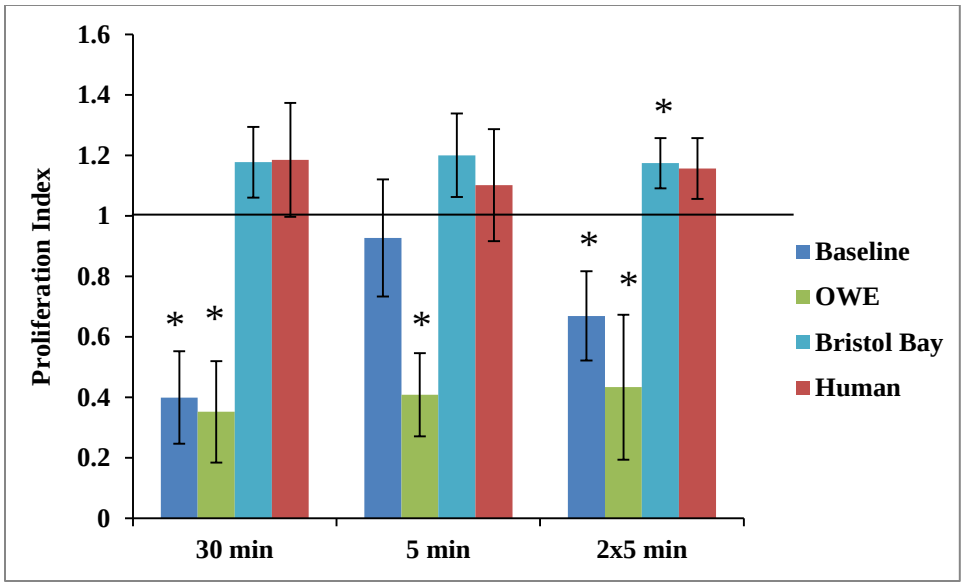

Figure 4-1: Lymphocyte proliferation in aquarium belugas during baseline (n=4) and OWE conditions (n=3), in wild belugas from Bristol Bay, AK (n=9) and humans (n=4) following exposures to 2000G. Data are normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

Comparative Effect of Condition on Lymphocyte Proliferation

Pressure induced changes in proliferation were also significantly different between conditions for each duration exposure (Appendix T). For both the 30 minute ($p<0.001$) and repeated 5 minute exposures ($p=0.014$) the response of beluga cells during baseline conditions was significantly different from humans (Figure 4-2). In addition humans displayed a significantly different response from OWE conditions in belugas for all durations (30 minutes, $p<0.001$; 5 minutes, $p=0.008$; 2x5 minutes, $p=0.012$). Bristol Bay animals displayed significantly different responses than aquarium animal baseline samples following the 30 minute ($p<0.001$) and repeated 5 minute exposures ($p=0.008$), and OWE samples for all duration exposures (30 minutes, $p<0.001$; 5 minutes, $p=0.001$; 2x5 minutes, $p=0.009$). In addition, aquarium belugas displayed significantly different responses during baseline and OWE conditions for the single 5 minute exposure ($p=0.042$).

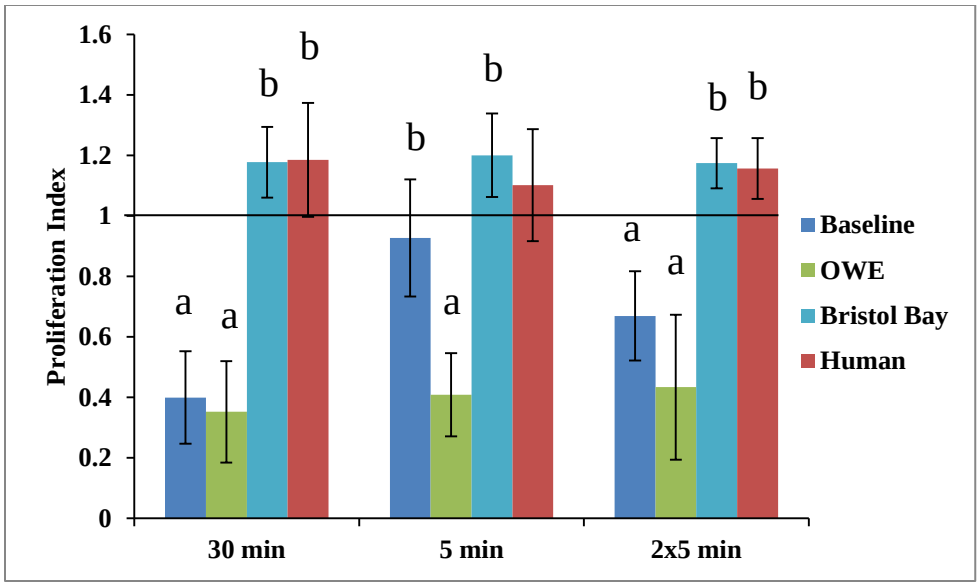

Figure 4-2: Lymphocyte proliferation in aquarium belugas during baseline (n=4) and OWE conditions (n=3), in wild belugas from Bristol Bay, AK (n=9) and humans (n=4) following exposures to 2000G. Data are normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with letters (p<0.05).

2000 psi with 15 second compression and decompression (2000R)

Significant decreases in proliferation were detected for baseline conditions in belugas following the 30 minute ($p<0.001$) and single 5 minutes ($p=0.013$) exposures (Appendix U; Figure U-1). The response of belugas was significantly different than humans for both the 30 minute ($p<0.001$) and single 5 minute ($p=0.020$) exposures (Appendix 20).

1000 psi

Samples were only available for 30 minute exposures to 1000 psi with both 2 minutes (1000G) and 15 seconds of compression and decompression (1000R). Aquarium belugas displayed a significant decrease in proliferation following exposure to 1000G ($p<0.001$) and this change was significantly larger than that observed in humans ($p<0.001$; Appendix U; Figure U-2). A similar decrease in beluga proliferation occurred following exposure to 1000R ($p=0.032$).

Effects of Dive Exposure

No significant differences in proliferation indices were detected between dive exposures or durations (Appendix V).

Effects of Pressure on Interleukin 2 Receptor Expression

For the purposes of this study, only the results of changes in the relative expression of IL2R per cell (MFI) are presented. Change in the % of positive cells followed similar patterns and results can be found in Appendices W and X, figures X-3 through X-8.

2000 psi with 2 minute compression and decompression (2000G)

Significant effects of pressure on the expression of IL2R on PBMC (MFI) were detected following all duration exposures (Appendix W). Humans displayed a significant increase in MFI following the 30 minute exposure ($p=0.041$), as did belugas from Bristol Bay ($p<0.001$; Figure 3). Baseline conditions in belugas ($p=0.031$) and humans (p-0.016) displayed increased expression for the single 5 minute exposure. Significant increases were measured for baseline ($p=0.005$) and OWE conditions ($p=0.030$) following the repeated 5 minute exposures (Figure 4-3).

The increase observed for the OWE samples was significantly larger than the change observed in both baseline conditions for aquarium belugas ($p=0.038$) and in Bristol Bay animals ($p=0.022$) for the repeated 5 minute exposures (Figure 4-3).

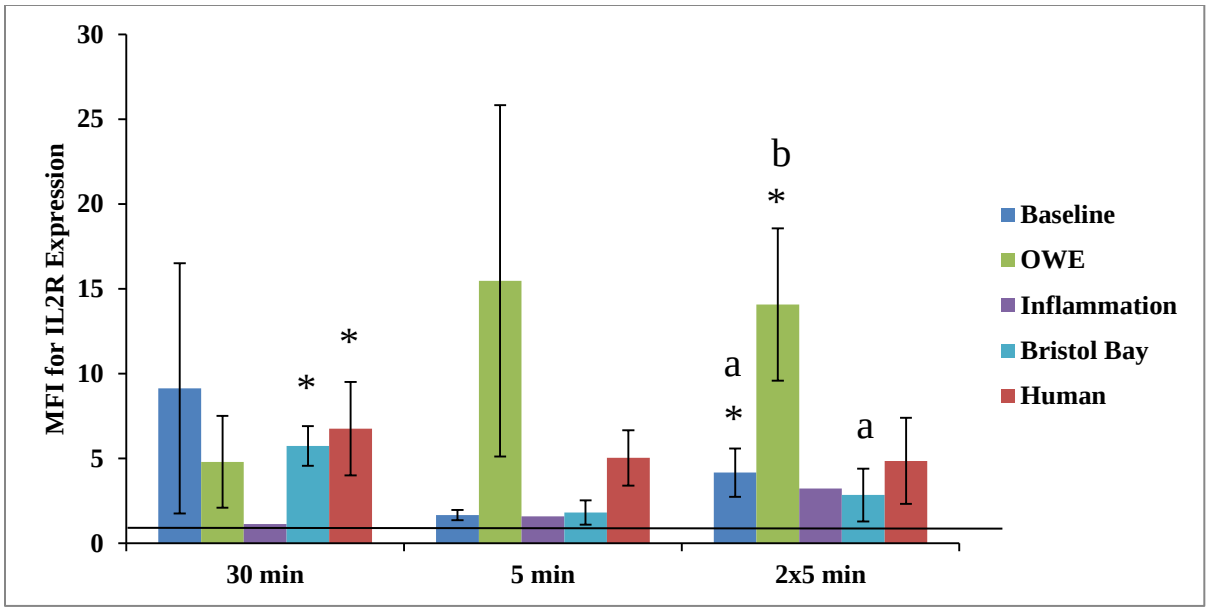

Figure 4-3: PBMC MFI in aquarium belugas during baseline (n=4), OWE (n=3 and inflammation conditions (n=2), in wild belugas from Bristol Bay, AK (n=9) and humans (n=4) following exposures to 2000G. Data are normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Significant differences between conditions are indicated with letters ($p<0.05$). Due to small samples size statistics could not be run for inflammation data.

2000 psi with 15 second compression and decompression (2000R)

Significant pressure induced changes in MFI were detected for all duration exposures (Appendix W). General patterns of increase were observed for both belugas and humans (Appendix X; Figure X-1). These changes were significant for belugas following all exposures (30 minutes, $p=0.019$; 5 minutes, $p=0.007$; 2x5 minutes, $p=0.031$), and for humans only following the 30 minute exposure ($p=0.011$).

1000 psi with 2 minute compression and decompression (1000psi)

Significant changes in MFI for IL2R expression were detected following all exposures (Appendix W). Significant increases in MFI were measured for baseline conditions in belugas following all duration exposures ($p<0.001$ for all exposures), and these changes were significantly larger than the change observed in humans (Figure 4-4). Inflammation samples

display a large increase in MFI following the 30 minute exposure, with smaller changes occurring following the single and repeated 5 minute exposures. During these shorter dives, the response measured in inflammation samples resembles that in humans (Figure4-4).

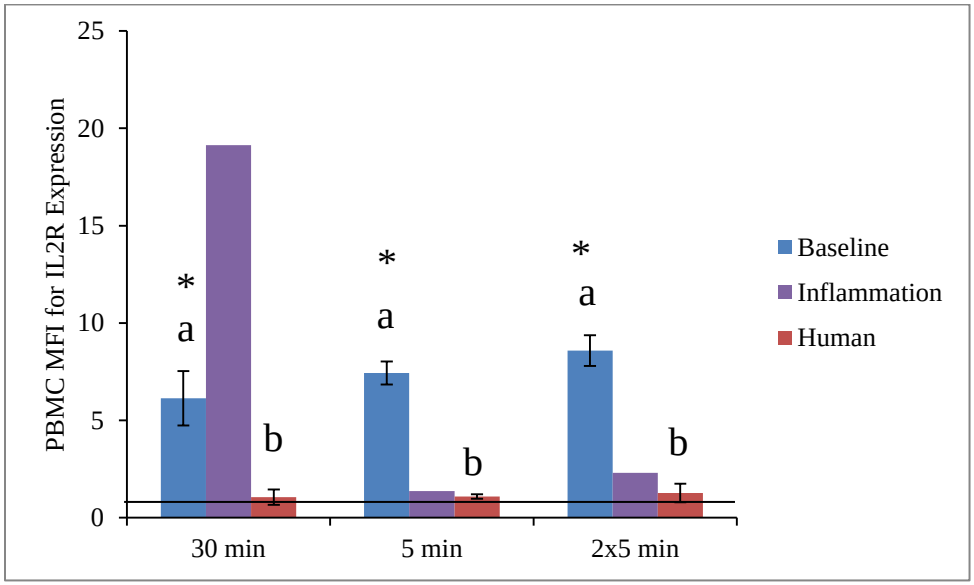

Figure 4-4: PBMC MFI in aquarium belugas during baseline (n=4) and inflammation conditions (n=2),and humans (n=4) following exposures to 1000G. Data are normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between conditions are indicated with letters (p<0.05). Due to small samples size statistics could not be run for inflammation data.

1000 psi with 15 seconds compression and decompression (1000R)

Significant pressure induced changes in MFI for IL2R expression were detected only during the 30 minute exposure (Appendix W), for which belugas displayed a significant increase in MFI (p=0.001) and this change was significantly larger than that observed in humans (Appendix X; Figure X-2).

Effects of Dive Profile IL2R expression

Pressure induced changes in MFI (the average IL2R expression per cell) were significantly different between dive profiles for the 5 minute and repeated 5 minute duration exposures

(Appendix Y). For both durations (Figure 4-5), exposures to 1000G resulted in larger changes that either 2000G (5 minute, $p<0.0001$; 2x5 minute, $p=0.003$) or 2000R (5 minute, $p<0.001$;2x5 minute, $p=0.002$). In addition, exposure to 2000G resulted in significantly smaller changes in MFI than exposure to 2000R for the 5 minute exposure ($p=0.036$). A different response to 1000G as compared with 1000R was also observed for the 30 minute exposures (Figure 4-5; $p=0.064$).

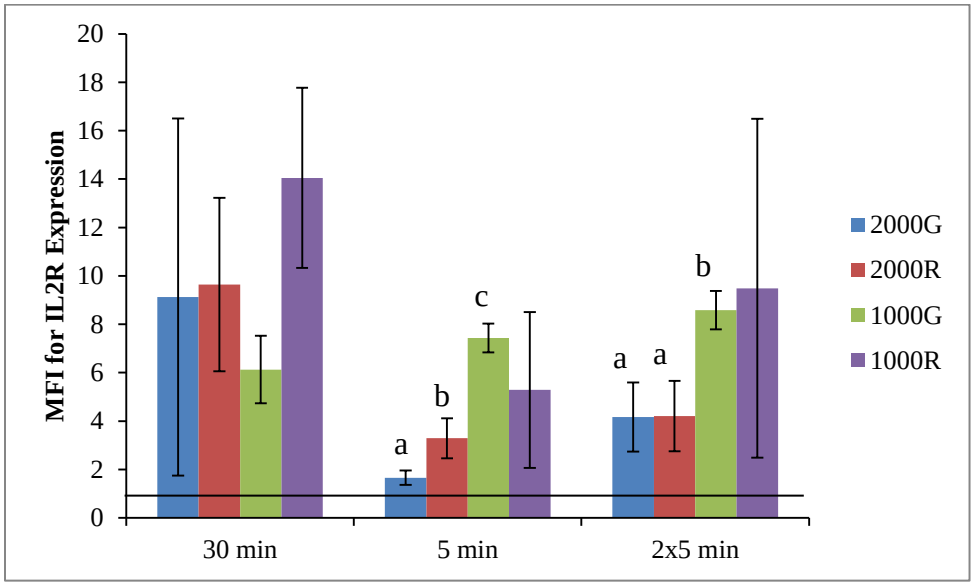

Figure 4-5: PBMC MFI in aquarium belugas during baseline conditions (n=4) following all pressure exposures. Data are normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with letters ($p<0.05$).

Duration

No significant differences were found between exposure durations for any dive profiles (Appendix Z). However, results suggest (Appendix AA; Figures AA-1)single 5 minute exposures result in smaller changes in MFI as compared with 30 minute exposures for 1000R ($p=0.098$) and 2000R ($p=0.064$).

Discussion

One of the natural challenges associated with diving is changing pressures, the effects of which can lead to cellular dysfunction in the central nervous system (Bennett, 1982; Macdonald, 1982), bone necrosis (McCallum and Harrison, 1982) and disease. Few studies have investigated the effects of pressure on marine mammal cells. Field (2000) found that elephant seal platelets responded differently than human platelets to increased pressure as well as decreased temperature. Cholesterol content of the platelet membranes was noted to be one mechanism of adaptation in the elephant seal. Castellini *et al.,* (2001) found different responses of red blood cells to changes in pressure between terrestrial mammals, shallow diving marine mammals and deep diving marine mammal species. In addition, granulocyte and monocyte phagocytosis in belugas also appears to be altered when cells are exposed to high pressures (Chapter 2). This study was the first to investigate the effects of pressure on PBMC function in belugas as compared with humans.

Previous work in our lab has characterized the OWE as a stressor, based on increased cortisol values. For this study, cortisol values were similar between OWE and wild belugas from Bristol Bay. Wild belugas also showed higher catecholamines than all conditions in aquarium animals. This is not unexpected. The Bristol Bay population of belugas are faced with many potential stressors including boat noise and seasonal subsistence hunting. In addition, these animals were followed, netted and restrained in shallow water for sampling and examination, and this process was expected to result in increased stress hormones. Some differences were noted in control measures of immune function between conditions, with general patterns suggesting decreased control values for stressor conditions and wild animals as compared with baseline conditions in aquarium belugas. Bristol Bay belugas also displayed significantly lower indices of proliferation than OWE and baseline conditions for aquarium animals. These findings are not unexpected as

increased catecholamines and cortisol have been linked with decreased measures of immune function (Webster-Marketon and Glaser, 2008; Nieman *et al.,* 1994; Qiu *et al.,* 2005; Dabhar, 2009).

Following pressure exposures, there was an overall pattern of increase for IL2R expression. Humans seemed to display smaller changes than baseline conditions in belugas for exposures to 1000 psi, and larger changes than baseline belugas for exposures to 2000 psi. In addition, larger changes for belugas were observed during OWE conditions as compared with baseline, while smaller changes were observed during inflammation conditions. In contrast, proliferation decreased in most cases, except for Bristol Bay animals and humans. The change in proliferation for Bristol Bay belugas was smaller however than aquarium animals.

Increases observed in measures of IL2R expression on human PBMC suggest that changes in pressure result in activation of human cells. A possible mechanism for this result is through altering membrane characteristics. T cell activation requires binding of the T cell receptor and an associated cluster of co stimulatory receptors. The receptors exist within the membrane as parts of mobile lipid rafts which aggregate as part of the activation signal. Aggregation of these rafts has been reported to occur in response to changes in membrane fluidity at decreased temperatures (Magee *et al.,* 2005). Increased pressure, like decreased temperature also has an ordering effect on cell membranes (Macdonald, 1982; Siebenaller and Garret, 2002) and thus may have led to signaling and increased IL2R expression. Increased IL2R expression is also indicative of monocyte activation, playing important roles in regulating cell differentiation and effector functions (Espinoza-Delgado *et al.,* 1995; Herrmann *et al.,* 1985). IFN γ released, in this case by pressure-activated T lymphocytes, may have influenced monocyte activation and up-regulation of IL2R expression (Herrmann *et al.,* 1985).

Interestingly this effect of increasing IL2R expression was also seen in beluga PBMC exposed to pressure. In some cases, the changes observed for belugas were larger than that observed for humans. This pattern of increase differs from general decreases observed in granulocyte phagocytosis for belugas (Chapters 2 and 3), thus suggesting that different cells respond differently to the effects of pressure. Additionally, while a decrease in immune function was hypothesized to be protective against the development of inflammatory damage associated with dive-related injury, activation of lymphocytes in belugas, may suggest that these cells are less involved in the development of such conditions. One role IL2R expression has in monocytes may be to regulate IL2 signaling in T lymphocytes (Toossi *et al.,* 1990; Herrmann *et al.,* 1985). Increased expression and binding within the monocyte population for example may mean less binding within the lymphocyte population thus preventing damaging lymphocyte responses.

One drawback of this study is that the pressure chamber used is not a sterile system, though it is washed and cleaned with 70% ethanol between usage, and it has been considered that the increase in IL2R expression observed in pressure exposed cells may be influenced by this. However, an increase was not always observed and proliferation indices decreased in aquarium animals following pressure excursions. Thus the effect of this potentially confounding factor was considered to be small.

Because the overall pattern of IL2R expression following pressure exposures was to increase, suggesting cellular activation, an increase in proliferative responses might have been expected. IL2 signaling is an important early step in proliferation for lymphocytes but does not induce proliferation among monocytes (Espinoza-Delgado *et al.,* 1995). Decreased proliferative responses have been reported in human lymphocytes stimulated with phytoheamagglutinin following exposure to increased pressure (Macdonald, 1982) and for this study decreased

proliferation following pressure exposures occurred in aquarium belugas, while no change was detected for humans.

Decreased proliferation can result from mechanical inhibition of cell division or altered ability of mitogen receptors to bind with the stimulus (Macdonald, 1982). Thus, even though results from this study suggest an activation of lymphocytes, it is possible that the pressures used resulted in a change in membranes or receptors that prevented a proliferative response. Additionally, these results could suggest a disconnect between the effects of pressure on early and later stages of activity in PBMC. Though beyond the capabilities of this work, it would be interesting to 1) repeat these studies at lower pressures or 2) see if beluga cells are able to recover after a certain period of time.

In general, changes in both IL2R expression on PBMC and lymphocyte proliferation were greater following the 30 minute exposures than shorter exposures. The relationship of the change in IL2R expression between belugas and humans appeared to vary with characteristics of the dive (i.e. for exposures to 1000 psi belugas displayed larger changes, while for exposures to 2000 psi humans displayed larger changes). Belugas also displayed larger changes in IL2R expression following 1000 psi exposures as compared with 2000 psi exposures for dives with 2 minutes of compression and decompression. These comparisons may suggest there is some limitation to dive adaptation in these cells, and thus a trade-off between dive behavior (depth, duration) and maintaining health. In addition these results may suggest that forced longer dives, perhaps as part of an avoidance response, could lead to unusual changes in PBMC activity. Alternatively, if the changes observed in baseline beluga samples represent protective adaptation then larger changes observed during longer dives may suggest plasticity to the response of PBMC, similar to that observed with other aspects of the dive response. For example, the degree to which bradycardia

occurs has been reported to be related to dive duration in Weddell seals (Kooyman *et al.,* 1980). Thus, there may be a balance between depth and duration of dives which is ideal for marine mammals, with dive-associated risks varying with dive patterns, conditions and species with different dive capabilities.

There were also some observable differences in the response of cells between conditions in belugas, suggesting that there is an intricate relationship between overall health, stress and dive ability in these animals. Despite similar hormone levels, pressure induced changes in IL2R expression for inflammation samples differ from baseline samples following the 30 minute exposure to 2000psi and for all exposures to 2000R. During this period, an active inflammatory response and the interaction between different cells of the immune system (e.g. granulocytes and lymphocytes) may have played a role in modulating the response of PBMC to pressure.

Both OWE and wild samples represent stressor conditions in belugas, and plasma cortisol was similar between the two conditions. However, both MFI and the % of positive PBMC for IL2R expression displayed larger changes in OWE samples as compared with Bristol Bay animals following the single and repeated 5 minute exposures. In addition, there was also a larger decrease in proliferation for OWE samples, as compared to the small increase observed in samples from the Bristol Bay animals. Not only are the magnitudes of changes different, but the direction of response is different. These differences may reflect different types of stress, as well as the perception of the stressor and experience of the animals. Free ranging animals from Bristol Bay will have had more opportunity to experience deep diving as compared with aquarium animals. Previous dive experience has been reported to refine dive capabilities and produce an acclimatization effect which reduces occurrence of decompression sickness (Lander *et al.,* 2003; MacArthur *et al.,* 2003; Ferretti and Costa, 2003; Su *et al.,* 2004) and thus this experience may

have resulted in cells from wild belugas being less responsive to pressure exposures. The nature of the stressors also differs between wild and aquarium belugas. The OWE was of short duration compared with the chase and restraint of wild animals. Wild animals also can have a greater stressor load as they face challenges associated with foraging, predation and hunting. In addition, there can be an effect of diet on the membrane composition of lymphocytes between the two groups which can alter cell sensitivity to pressure. Along with cholesterol, polyunsaturated fatty acids (PUFA) play a role in determining membrane fluidity, and dietary sources of PUFA's can be incorporated into cell membranes, including T cell micro-domains, thus affecting signaling and cell function (Switzer *et al.*, 2004 ; Fan *et al.*, 2003). Diets of free ranging whales may be more varied than in aquaria, and it would be interesting to investigate the role of different diets on lymphocyte membrane composition and the effects of pressure in these animals.

Whereas small sample sizes limit the statistical power of the results, this study provides the first evidence suggesting that anthropogenic activity (e.g., boat noise) could impact the ability for beluga PBMC to function normally during diving either through physiological stress responses, or by initiating changes in normal diving behavior. However, the relationship between the functional response of marine mammal immune cells to diving is complicated and more work needs to be done to better describe the conditions under which undesirable immune activity may occur. Such information is useful in trying to interpret growing reports of injuries in marine mammals that resemble dive related disease. Next steps of research should include *in vivo* measurements of dive related changes in immune function if possible (e.g. through behavioral training) in order to begin describing the combined effects of physiological adjustments and dive behavior. Such information is necessary to create a physiological understanding of dive adaption

among immune cells and is may prove useful in assessing the potential impact of anthropogenic activities on the health of marine mammal populations.

Chapter 5 EFFECTS OF PRESSURE ON IMMUNE FUNCTION IN STRANDED PINNIPEDS AND IMPLICATIONS FOR DIVE ABILITY

Abstract

The ability of marine mammals to cope with environmental challenges is a key determining factor in strandings and success of released rehabilitated animals. Dive ability is related to foraging activity and thus survival. Although dive adaptations have been well studied, it is unknown how the immune system responds to diving and whether the presence of additional stressors impacts immune function during diving. This study investigated the functional response of immune cells to dive simulations, i.e. increased pressure, from stranded phocids over the course of rehabilitation. Blood samples were drawn from harbor seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*) and harp seals (*Phoca groenlandica*) which stranded along the coast of southern New England, at the time of admit to the Mystic Aquarium, Mystic, CT and again after rehabilitation (pre-release). Immune function was measured *in vitro,* with and without exposure to 2000 psi (1360m) and epinephrine, norepinephrine and cortisol were measured *in vivo*. Within admit or release conditions, significant pressure induced changes in granulocyte and monocyte phagocytosis, granulocyte expression of CD11b and lymphocyte expression of the interleukin 2 receptor were detected. Stress hormones decreased between admit and release conditions. Only phagocytosis showed significantly different responses in both magnitude and direction to pressure between conditions. Results suggest 1) dive characteristics, specifically duration, affect the response of immune cells, and 2) different cell types respond differently to pressure, and 3) response varies with animal health. This is the first study describing the relationship between diving, immune function and stressors in phocids.

Introduction

A stress response involves neuroendocrine changes which in turn modulate physiology, behavior and immune function in order to maintain homeostasis in the face of threatening or new stimuli (Romero and Butler, 2007). There is increasing concern that anthropogenic stressors are impacting the health of marine mammal populations. Stress is a common factor in marine mammal strandings (Romano *et al.*, 1994), whether from an initial injury or disease, the stranding event itself, human interaction or handling for rehabilitation (Clark *et al.*, 2006; Bogomolni *et al.*, 2010). In some stranding cases, sudden death of animals has been reported without apparent injury or disease, and has been associated with an extreme stress response (Clark *et al.*, 2006; Cowan and Curran, 2008). Lesions on cardiac as well as skeletal muscle have been reported to be likely caused by the release of large quantities of catecholamines (Turnbull and Cowan, 1998). There are also many sub-lethal effects of a stress response which can affect individual health including impacts on the immune system. Inability to cope with natural challenges of the aquatic environment may play a role in driving marine mammals to come ashore, and is a major consideration when releasing animals after rehabilitation. For example, dive ability may be of concern since dive behavior is related to foraging success and therefore health and survival. Several differences have been noted in blood parameters between rehabilitated harbor seal pups (*Phoca vitulina*) and wild caught pups, including mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) which are important for developing increased oxygen stores necessary for diving (Lander *et al.*, 2003). These differences may be related to either the stress related to rehabilitation handling, or the confining environment of rehabilitation facilities which restrict diving and swimming behaviors. In addition, the stress associated with the stranding event itself, human handling and a novel environment could

potentially impact blood parameters and/or impact dive-related adaptations, thus affecting the capability of newly released animals to dive, forage and survive.

Diving ability among pinnipeds is greatly variable from the generally shallow diving otariids, to the deeper diving phocids with some notably exceptional divers such as the elephant seal (*Mirounga angustirostris*) and Weddell seal (*Leptonychotes weddelli*). During diving several physiological and behavioral adaptations protect marine mammals from challenges associated with breath holding and changes in pressure. These include increased antioxidant capacities (Elsner *et al.,* 1998), decreased metabolism (Kooyman *et al.,* 1980), collapsible lungs and trachea, peripheral vasoconstriction and bradycardia (reviewed in Kooyman *et al.,* 1981).

Increased catecholamines have also been reported in diving pinnipeds. Hance *et al.,* (1982) reported increased epinephrine and norepinephrine in diving harbor seals, with changes in both hormones being greater following a second dive. While these dives were performed under forced conditions, increased catecholamines have also been reported in free diving Weddell seals (Hochachka *et al.,* 1995; Hurford *et al.,* 1996). These hormonal changes likely exert some control over certain aspects of the dive response. Peripheral vasoconstriction is controlled through sympathetic nervous activity (Foster and Sheel, 2005) and the binding of catecholamines to α receptors in peripheral tissues (Hochachka *et al.,* 1995). Increasing catecholamines in free diving Weddell seals were accompanied by a decrease in spleen size (Hurford *et al.,* 1996) likely caused by contraction due to binding of α adrenergic receptors (Foster and Sheel, 2005). This contraction leads to the release of red blood cells and thus increases blood oxygen stores (Hochachka *et al.,* 1995). Bradycardia is largely controlled by vagal activity during a dive (Foster and Sheel, 2005) though catecholamines may have modulating effects on the intensity of the response (Hochachka *et al.,* 1995). However, at the conclusion of a dive, vagal influences

decrease and the effect of catecholamines may increase, contributing to post dive tachycardia. This increased heart rate is thought to aid in recovering oxygen stores (Elliott *et al.,* 2002). In addition high plasma cortisol has been reported for phocids such as the Weddell seal and southern elephant seal during free dives (Liggins *et al.,* 1993). While the role of increased cortisol during diving is not known, it may have a protective function against dive related pathologies such as high pressure nervous syndrome (Liggins *et al.,* 1993).

Catecholamines and glucocorticoids are also released following activation of the sympathetic nervous system and hypothalamic pituitary adrenal axis in response to a stressor, and can modulate immune cell function through binding of adrenergic and adrenal steroid receptors, respectively, which are present on cell membranes (Madden *et al.,* 1995; Dabhar *et al.,* 1996). An animal with already elevated levels of catecholamines or cortisol due to natural diving adjustments may display an unusual, extreme or abnormal response to an additional stressor (Talpalar and Grossman, 2005). In addition, bidirectional communication allows the immune system to alter neuroendocrine activity (Besedovsky *et al.,* 1985), thus an animal with elevated hormone levels due to infection or injury may have an abnormal dive response. Hochachka *et al.,* (1995) reported increased plasma catecholamines in Weddell seals following diving, noting higher values (for both resting periods, as well as dives) in a single animal with obvious injury (Hochachka *et al.,* 1995) suggesting that the presence of an injury, as a stressor, in conjunction with adjustments made for diving resulted in a magnified response of the sympathetic nervous system. Where minor hormonal adjustments associated with diving may not result in altered immune function, such a magnified response may result in inhibited or augmented function.

Perception of a stressor can also result in altered behavior. While the response of pinnipeds in water to human disturbance has been minimally studied (Richardson *et al.,* 1995), decreased

diving behavior, and associated increased time at the surface have been reported in hooded seal pups (*Cystophora cystata)* following sonar exposure (Kvadsheim *et al.,* 2010). Other avoidance behaviors include increased dive durations in bottlenose dolphins (*Tursiops truncatus*) (Constantine *et al.,* 2004) and changes in ascent or descent rates in elephant seals (*Mirounga angustirostris*) (Costa *et al.,* 2003). A sudden and extreme change in dive behavior may compromise natural dive adaptation and leave an animal susceptible to dive related injury. Such changes in behavior and possibly physiological responses can also lead to stranding.

Because of the importance of diving behavior for foraging, travelling, and avoidance/escape, marine mammals are constantly challenged not only by the changes in hormones, but by specific characteristics of the dive, such as length or depth. In humans, dive related injury and dive related disease such as decompression sickness can develop following changes in immune function facilitated by dive conditions (Barack and Katz, 2005; Brenner *et al.,* 1999; Macdonald, 1982). Such pathologies have long been thought not to occur in marine mammals, and it is likely that, as with other physiological systems, the marine mammal immune system possesses a form of dive adaptation which protects them from these conditions. Despite growing interest in marine mammal immunology and the need to better understand dive adaptation of marine mammals in order to assess the effect of anthropogenic stressors on marine mammal health, the relationship between diving and immune function has not been investigated in pinnipeds to date.

The purpose of this study was to investigate the relationship between pinniped health, immune function and the ability to perform potentially demanding behaviors such as diving. Specifically, the aim of this work was to measure the response of immune cells from phocids to changes in pressure representing simulated dives, at the time of stranding as compared with release (after

rehabilitation); Plasma catecholamines and cortisol were measured as indicators of a stress response and correlated with pressure induced changes observed in immune function.

Methods

Animal Subjects and Samples

Blood samples were obtained from harp seals (*Phoca groenlandica*), grey seals (*Halichoerus grypus*), and harbor seals (*Phoca vitulina*) which were admitted to the marine mammal stranding and rehabilitation program at the Mystic Aquarium, Mystic, CT, between 2009 and 2012. Animals underwent full physical examination upon admit. Blood samples were drawn from the extradural intervertebral vein under manual restraint (Mystic Aquarium IACUC no. 04006; UConn Exemption No. E11-011). Samples were also obtained pre-release, i.e. after rehabilitation and when animals were considered "healthy" for release back into the wild. Despite confounding factors associated with sampling under manual restraint, it was expected that stress hormone levels would be lower in pre-release samples. Blood was collected in 10 ml sodium heparin vacutainers™ and placed on ice and transferred to the laboratory at Mystic Aquarium. All blood was processed within 24 hours of collection.

Hormone Analysis

Sodium heparin blood tubes were centrifuged for 10 minutes at 10^{o}C, 2000 x g to separate blood components. One ml aliquots of plasma were stored in Sarstedt tubes™ at -80^{o}C until assayed. Cortisol concentrations were determined by Immulite® chemiluminescent assay at the Animal Health Diagnostic Center, Endocrinology Lab at Cornell University (Ithaca, NY), which has previously been used for processing endocrine hormone levels in marine mammal samples (Schmitt *et al.,* 2010; Schwake *et al.,* 2013). A Waters (Milford, MA) High Performance Liquid

Chromatography machine with electrochemical detection (2465 electrochemical detector) was used to measure plasma epinephrine and norepinephrine according to methodology detailed in "Plasma Catecholamines by HPLC" (Instruction Manual, June 2001, BIO-RAD, Hercules, CA).

White blood cell buffy coats were removed for lymphocyte function assays and mixed with an equal volume of freezing media (10% DMSO and 90% FBS) before being stored at -80^{o}C for 24 hours and then transferred to liquid nitrogen.

Simulated Dive Exposures

Fresh blood samples or mononuclear cell suspensions, from thawed archived buffy coats, were exposed to simulated dives using a stainless steel pressure chamber attached to a hydraulic hand-pump filled with mineral oil. The chamber was brought to a temperature of 37^{o}C and 4 ml of sample were added through a top loading port. A thin layer of oil was pumped over the sample in order to reach the desired pressure. Samples were exposed to a pressure of 2000 psi (1360m) with 2 minute periods for compression and decompression. Exposures lasted for 30 minutes, 5 minutes or two repeated sessions of 5 minute duration with a 1 minute resting period. At the conclusion of each exposure, pressure was released by loosening valve connections, and samples were removed and aliquoted as per assay descriptions below.

Phagocytosis

One hundred µl of whole blood were aliquoted into BD Falcon™ 5 ml polystyrene round bottom tubes (BD Biosciences, San Jose, CA) for control measurements. Four ml of whole blood were set aside for simulated pressure exposures. Cell counts were obtained using Trypan blue exclusion. Heat killed propidium iodide labeled *Staphylococcus aureus* (PI Staph) was prepared at a ratio of 25:1, bacteria: leukocytes according to the protocol described in Spoon and Romano

(2012). Ten and 400 µl of PI Staph were added to control and pressure treated aliquots of blood, respectively, for a final concentration of 4.8x 10^8 bacterial units ml^{-1}. The pressure chamber was brought to a temperature of 37^{o}C and bacteria were added to pressure samples immediately before compression. Control samples were placed in a 37^{o}C water bath and all samples were allowed to incubate for the duration of the pressure exposures. Following decompression, 100 µl of pressure treated blood was aliquoted into Falcon ™ tubes. In order to stop cell activity, 10 µl of 10 mM N-ethylmaleimide were added to tubes immediately after decompression (dive) or after a further 20 minute recovery period (recovery). Tubes were then placed on ice until red blood cell lysis.

CD11b Expression

Two ml of fresh blood were set aside from phagocytosis assays for CD11b expression controls. Pressure excursions were used for both phagocytosis and CD11b expression assays concurrently, meaning pressurized cells were exposed to PI Staph. The addition of PI Staph was found to increase expression of CD11b (data not shown) and thus PI Staph was added to controls for comparability. Immediately following decompression 100 µl aliquots of pressure exposed blood, as well as control blood were incubated with 50 µl of a 1:5 dilution of mouse anti-canine CD11b antibody (AbD Serotec, Raleigh, NC) for 30 minutes at 37^{o}C. Cells were washed twice with Hank's Balanced Salt Solution (HBSS) and centrifuged for 5 minutes at 220 x g. Cells were then incubated in the dark at 4^{o}C for 30 minutes with a 1:10,000 dilution of FITC labeled goat anti-mouse IgG (Beckman Coulter, Miami, FL). Tubes were washed twice with HBSS and placed on ice until red blood cell lysis.

To lyse red blood cells, tubes from both phagocytosis and CD11b expression assays were removed from ice and incubated for 15 minutes with 1 ml of lysis buffer (0.01M Tris; 0.001 M EDTA; 0.17M NH4 CL solution; pH 7.4). Tubes were washed twice with 1x PBS (pH 7.2) and fixed with 250 µl of 1% paraformaldehyde (pH 7.4). Tubes were stored in the dark at 4^{o}C until analyzed 24 hours later with an LSR flow cytometer (BD Biosciences, San Jose, CA).

Interleukin 2 Receptor (IL2R) Expression

Buffy coat samples were removed from liquid nitrogen and thawed quickly in a 37^{o}C water bath. Samples were washed twice with RPMI 1640 and centrifuged for 5 minutes at 220 x g, 20^{o}C. Cell pellets were re-suspended in 3 ml of RPMI 1640 and layered over 3 ml of room temperature histopaque-1077 to isolate mononuclear cells (Sigma Aldrich, St Louis, MO). Tubes were centrifuged for 30 minutes at 400 x g, 10^{o}C. The mononuclear cell layer was removed and washed twice in 1x PBS (pH 7.2). Cell counts were obtained using Trypan blue exclusion. The cell suspension volume was adjusted to a concentration of 4 x 10^{6} cells ml^{-1}. Samples were separated for controls and pressure exposures. Cells for pressure exposures were brought to a volume of 4 ml and introduced to the pressure chamber. Following decompression, cells were removed from the pressure chamber, recounted and the final volume adjusted to reach 4 x 10^{6} cells ml^{-1}. IL2R expression was measured using a Human IL2 biotinylated fluorokine kit from R & D Systems® (Minneapolis, MN). Twenty five µl of both control samples and pressure exposed cells were aliquoted into Falcon™ tubes and 10 µl of biotinylated IL2 fluorokine or negative control (biotinylated soybean trypsin inhibitor) were added. Tubes were incubated for 1 hour at 4^{o}C, after which 10 µl of avidin FITC (R & D Systems®, Minneapolis, MN) were added followed by a 30 minute incubation. Finally, cells were washed twice with PBS, fixed with 250

µl of 1% paraformaldehyde (pH=7.4) and stored in the dark at 4^{o}C until analyzed 24 hours later with an LSR flow cytometer (BD Biosciences, San Jose, CA).

Flow Cytometry

Samples for phagocytosis, CD11b expression and IL2R expression were read using an LSR flow cytometer (BD Biosciences, San Jose, CA). Tubes containing only cells were used to generate forward and side scatter plots and to gate the cell populations of interest for each assay (i.e. phagocytosis- granulocyte and monocyte populations; CD11b expression -granulocytes; IL2R expression- lymphocytes). For all assays, two measures of function were collected; 1) The percent of cells within a defined population expressing a positive signal (% positive) and 2) the mean intensity of the fluorescent signal expressed by the cells (MFI) reflecting the average expression of receptors or the average uptake of PI Staph per cell.

Lymphocyte Proliferation

Lymphocyte proliferation was assessed using a colorimetric BrdU incorporation assay (ROCHE Diagnostics, Indianapolis, IN). Archived white cell buffy coats were removed from liquid nitrogen and thawed rapidly in a 37^{o}C water bath. Cells were washed twice and finally re-suspended in 3 ml RPMI 1640. The mononuclear cell layer was isolated using a histopaque density gradient as described above for the IL2R assay. Mononuclear cells were washed twice with lymphocyte proliferation media (RPMI 1640 with 0.1 µM non-essential amino acids, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.292 mg ml^{-1} 1-glutamine, 1mM sodium pyruvate, 0.01 M hepes, 10% FBS, 0.1mM 2-mercaptoehtanol). Cell counts were obtained using Trypan blue exclusion. The volume of cell suspension was adjusted to reach 1x10^{6} cells ml^{-1}. For pressure excursions, an appropriate volume of cell suspension was removed, brought to

4 ml and introduced to the pressure chamber. Following decompression, cells were recounted and reconstituted at a concentration of 1 x 10^6 cells ml^{-1}. One hundred µl of pressure exposed, as well as non-exposed, cell suspensions were pipetted into Microtest™ 96 well tissue culture plates (BD Biosciences, San Jose, CA) for assay.

Concanavalin A (Con A) (Sigma Aldrich, St Louis, MO) was added to experimental and control wells at a final concentration of 2.5 µg ml^{-1}. Plates were incubated at 37°C for 72 hours. Ten µl of BrdU, a thymidine analog, were added to each well except for BrdU controls, and plates were incubated for an additional 12 hours. Plates were centrifuged for 10 minutes at 300 x g and the media removed. Plates were dried and stored in clean Ziploc® bags at 4°C until fixing and developing. Plates were fixed and developed within 1 week of drying in accordance with kit protocol. Briefly, two hundred µl of FixDenat were added to each well for 30 minutes. One hundred µl of anti- BrdU-POD (peroxidase conjugated monoclonal Fab fragments from a mouse-mouse hybrid) were added and plates incubated for 90 minutes at room temperature. Wells were washed three times with wash solution before addition of 100 µl of substrate solution. Plates were developed for 30 minutes before the addition of 25 µl 1M H_2SO_4 to stop the reaction. Plates were read at 450 nm using an EL800 microplate reader (BioTek, Winooski, VT). Proliferation indices were calculated as the ratio of the optical density (OD) of stimulated cells to the OD of the unstimulated cells.

Statistical Analysis

Control values for all measures of immune function, as well as hormone values, were compared between admit and release conditions using Paired T-tests for animals with data from both conditions (α=0.05). Mixed effects generalized linear models were set up for each dive to

examine the effect of pressure on immune function for arrival and release conditions (α=0.05). Individuals were added to the model as a random effect, and fixed effects added were treatment (control, dive) and condition (arrival, release). The change in immune function measures between controls and pressure exposed cells was then calculated as a percent of the control values, and this change was compared between arrival and release conditions (α=0.05). For comparing the effects of pressure between admit and release conditions, only animals with paired samples were analyzed. Pearson correlations were used to investigate the relationship between hormone values and both immune function control values and % change in immune function (α=0.05). Pearson correlations were also used to compare the changes in immune function with a body condition index calculated from animal lengths and weights (α=0.05).

Results

Hormones and Control Values

Animals (n=6) for which both admit and release data were available displayed decreased plasma hormone concentrations from admit to release (Table 5-1) though this was only significant for norepinephrine (t_5=3.505; p=0.017). Non paired animals displayed higher norepinephrine than paired animals at the time of admit, though this was likely due to very high values in two individuals. While not significant, admit cortisol was also higher in non-paired animals as compared with paired animals. Since cortisol levels have been linked with fitness and overall animal health (Romero, 2004; Blas *et al.*, 2007) this may be significant in determining a successful outcome from rehabilitation efforts, but is beyond the scope of the present study.

Table 5-1: Plasma concentrations of catecholamines and cortisol (± STDEV) in phocids at the time of stranding (admit) and release (after rehabilitation). Significant differences between admit and release conditions in animals for which data from both conditions are available (paired) are indicated with an asterisk (*). α=0.05. Non-paired animals represent individuals for which only admit data were available.

		Epinephrine pg ml^{-1}		Norepinephrine pg ml^{-1}		Cortisol µg dl^{-1}	
Paired	**admit**	318.48 ± 111.07	n=6	831.08 ± 183.45	n=6	13.72 ± 3.71	n=6
	release	183.34 ± 34.95	n=6	304.49 ±57.89*	n=6	5.00 ± 1.14	n=6
Non paired	**admit**	211.37 ± 56.16	n=6	2126.42 ± 903.66	n=5	21.66 ± 5.00	n=8

No significant differences were found in measures of phagocytosis or CD11b expression between admit and release conditions for paired data. Epinephrine was negatively correlated with % of monocytes undergoing phagocytosis (r=-0.612; p=0.005) and positively correlated with the % of granulocytes expressing CD11b (r=0.875; p=0.01) following a 30 minute incubation without pressure. Cortisol was also positively correlated with control measures of the % of granulocytes expressing CD11b following the 30 minute incubation (r=0.734; p=0.024). No significant relationships were found between any hormones and control measures of either phagocytosis or CD11b expression following single or repeated 5 minute incubations. No significant differences in IL2R expression or proliferation indices were detected between admit and release conditions, and no significant correlations between hormone values and either measure of lymphocyte function were detected.

Effects of Pressure on Phagocytosis- Granulocytes

Results of mixed generalized linear models are summarized in Appendix AB. No significant changes in granulocyte phagocytosis were detected for the dive periods of any duration exposures. However a pattern suggesting decreased activity per cell (MFI) was observed for admit samples following the dive period of the 30 minute exposure (Figure 5-1).

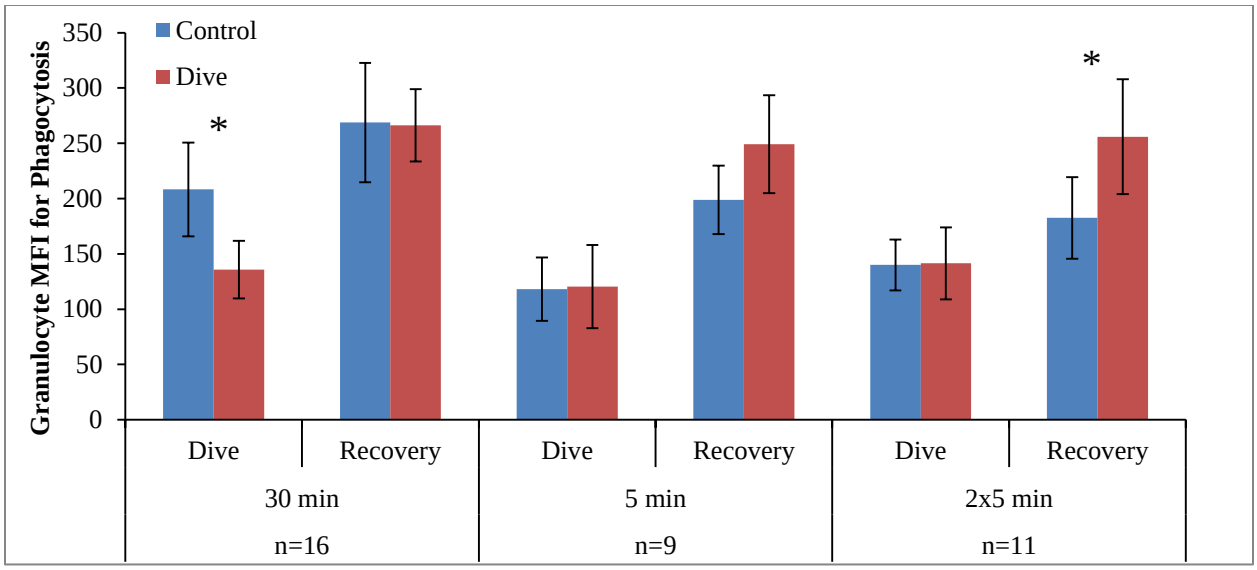

Figure 5-1: Granulocyte MFI for phagocytosis in admit samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * ($p<0.05$).

Significant increases in the % of granulocytes performing phagocytosis were detected for both admit ($p<0.001$) and release ($p=0.011$) conditions following the recovery period of the 30 minute exposure (Appendix AC; Figures AC-1, AC-2). This pattern of increased activity was also observed in the MFI for release samples for this pressure exposure (Figure 5-2). For the recovery period of the repeated 5 minute exposures, a significant increase in MFI (Figure 5-1) was detected for admit samples ($p=0.030$), while a significant increase in % of positive granulocytes (Appendix AC; Figure AC-2) was detected for release samples ($p=0.003$). A pattern of increased MFI was also observed for the release samples following the recovery period of the single 5 minute exposure (Figure 5-1; $p=0.056$).

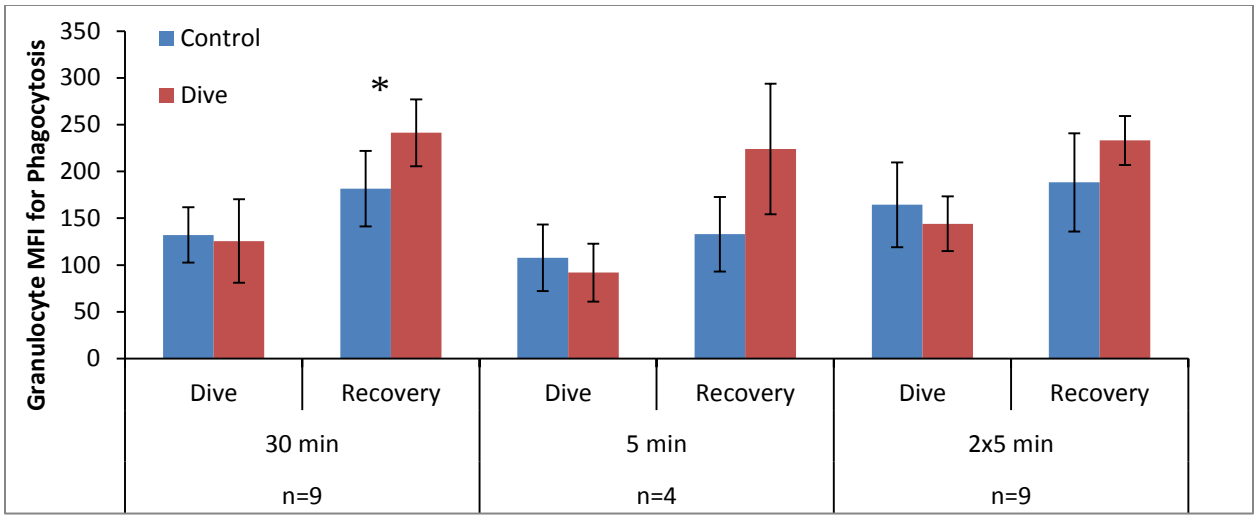

Figure 5-2: Granulocyte MFI for phagocytosis in release samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * ($p<0.05$).

Effects of Pressure on Phagocytosis-Monocytes

Monocytes showed decreased phagocytic activity per cell (MFI) for the dive period of the 30 minute exposures for both admit ($p=0.016$) and release ($p=0.068$) conditions (Appendix AC; Figures AC-3, AC-4). During the recovery period of this exposure the % of monocytes performing phagocytosis increased for both admit ($p=0.001$) and release ($p=0.001$) conditions (Appendix AC; Figures AC-5, AC-6). A similar significant increase in the % of Positive cells was detected for release samples following the recovery period of the repeated 5 minute exposures ($p<0.001$).

Combined Effects of Health and Pressure on Phagocytosis

For animals with paired data, significant differences between admit and release conditions in phagocytosis were detected for granulocyte MFI ($F=3.431$; $p=0.037$), % positive granulocytes ($F=5.37$; $p=0.001$), monocyte MFI ($F=9.465$; $p<0.001$) and % positive monocytes ($F=3.21$; $p=0.045$) for the 30 minute exposure, though pairwise comparisons were unable to indicate if this

significance occurred during the dive or recovery periods. In general, admit conditions displayed decreased measures of phagocytosis, while release conditions displayed increases or minimal change from controls. Changes occurring for release conditions appear smaller than those measured for admit conditions. Additionally, significant changes were found in the % positive granulocytes ($F=4.331$; $p=0.05$) and monocyte MFI ($F=5.806$; $p=0.021$) following the 5 minute exposure. Pairwise comparisons were only significant for the % positive granulocytes which showed a significantly different response between admit and release conditions. In this case a decrease was observed during admit conditions, but an increase, and larger change from controls, was observed during release conditions (Figure 5-3). This pattern was also observed for the recovery period, and for the MFI of both granulocytes and monocytes.

Significant correlations were only found between body condition indices and monocyte MFI following the 30 minute dive period ($r=0.552$; $p=0.004$). The change in the % positive monocytes was also significantly correlated with body condition indices for both the dive period ($r=0.728$; $p<0.001$) and recovery period ($r=0.607$; $p=0.001$) of the 30 minute exposure. In all cases, the correlations were positive indicating larger changes in monocyte phagocytosis with increased body condition. No significant relationships were detected between body condition indices and granulocyte function.

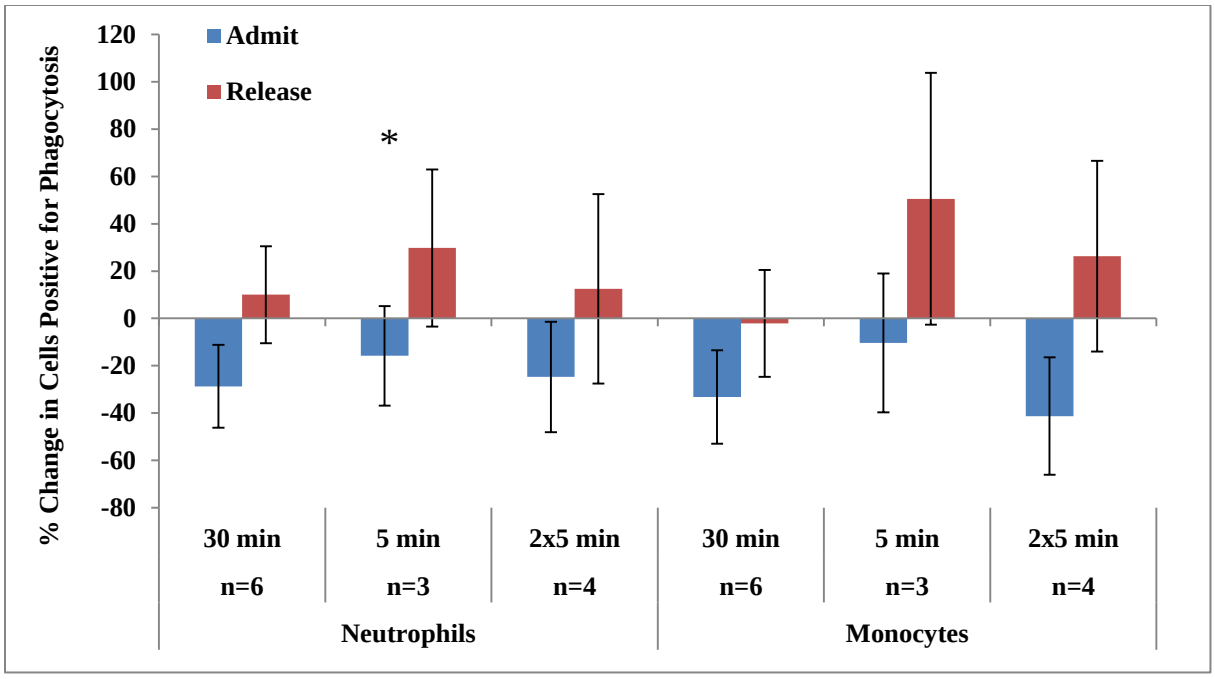

Figure 5-3: Calculated changes in the % of phocid cells positive for phagocytosis for paired admit and release samples. Data are presented as mean ± SE. Values greater than 0 indicate an increase in function, and values less than 0 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with an asterisk * ($p<0.05$).

Effects of Pressure on CD11b Expression

The results of mixed generalized linear models for CD11b expression are summarized in Appendix AD. Significant increases in expression per cell (MFI) were detected only for admit conditions following the 30 minute ($p=0.003$) and single 5 minute ($p=0.045$) exposures (Figure 5-4).

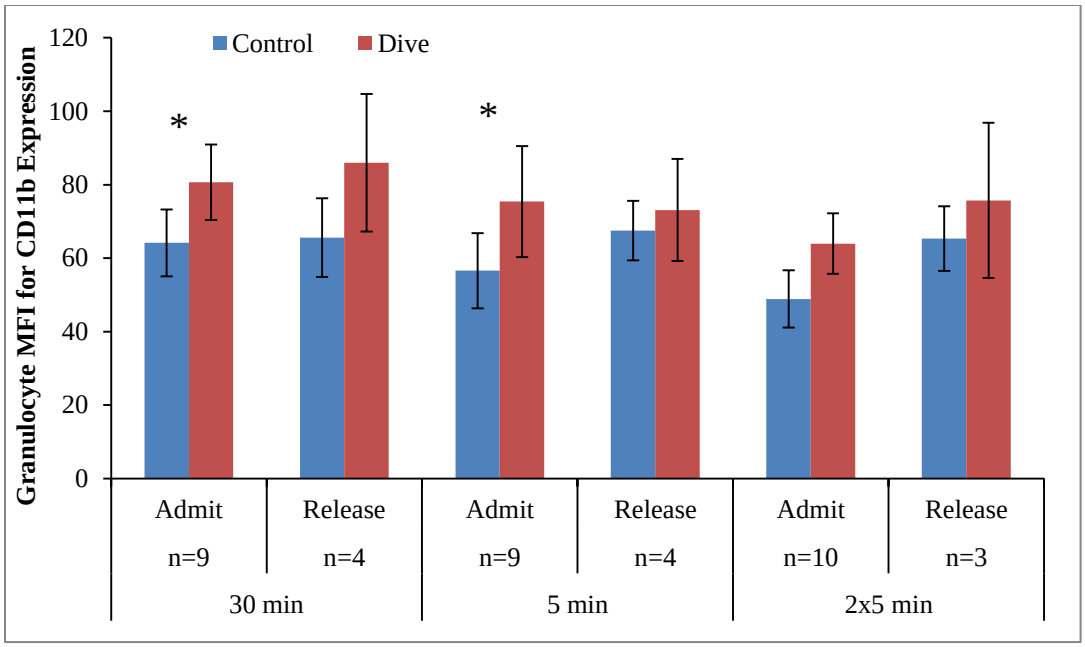

Figure 5-4: Granulocyte MFI for CD11b expression in stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * ($p<0.05$).

Combined Effects of Health and Pressure on CD11b Expression

Changes in the MFI and the % positive cells for CD11b expression were not significantly different between admit and release conditions for paired samples. No significant correlations were found between changes in CD11b expression following pressure exposures and body condition indices.

Effects of Pressure on IL2R Expression

Significant pressure-induced increases in IL2R expression were detected following all pressure exposures (Appendix AE). Increased expression per cell (MFI; Figure 5-5) was observed for admit samples following all exposures (30 minute, $p=0.021$; 5 minute, $p=0.034$; 2x5 minute, $p<0.001$), and for release samples for the 30 minute ($p=0.005$) and repeated 5 minute ($p=0.063$).

Similar patterns of increase were observed in the % of cells expression IL2R (Appendix AF; Figure AF-1).

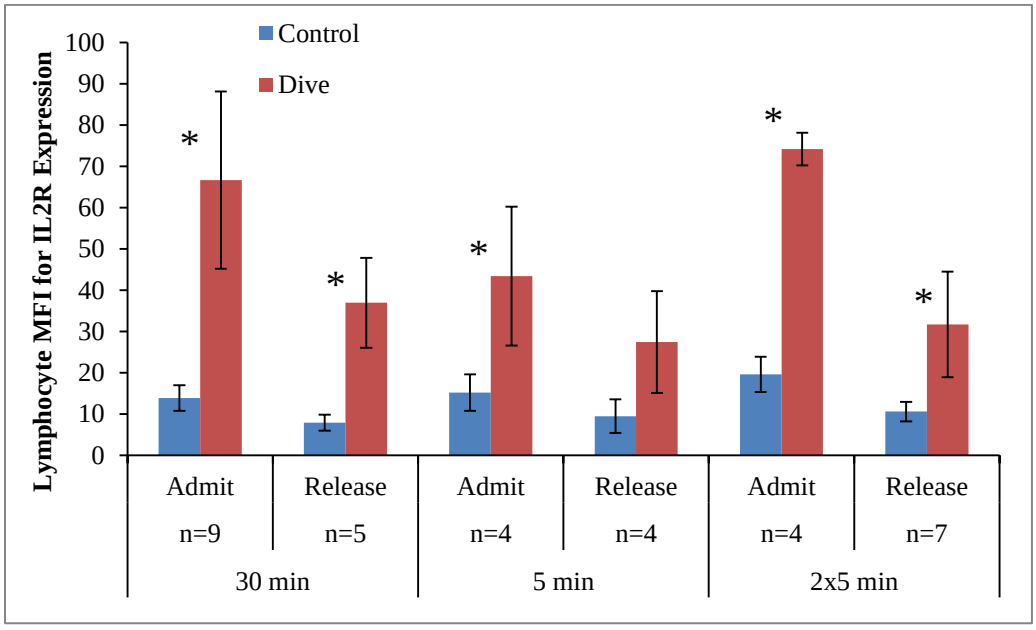

Figure 5-5: Lymphocyte MFI for IL2R expression in stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * ($p<0.05$).

Combined Effects of Health and Pressure on IL2R Expression

No significant differences were found in the changes in IL2R expression between conditions for paired samples. Body condition indices were not significantly correlated with changes in either measure of IL2R expression following pressure exposures.

Effects of Pressure on Lymphocyte Proliferation

Few samples were available for studies investigating the effect of pressure on lymphocyte proliferation and statistical significance was not detected. Overall, a decrease in proliferation indices were observed for all pressure exposures as compared with controls (Figure 5-6). Release

samples were only available for a single animal and exposure to 2000 psi for 30 minutes also resulted in a decrease in proliferation. Due to limitations in sample availability, no comparisons of the change in proliferation indices between admit and release conditions could be run. A significant negative correlation however was detected between body condition indices and the % change in lymphocyte proliferation between control and pressure exposed cells for the 30 minute exposures (r=-0.933; p=0.021).

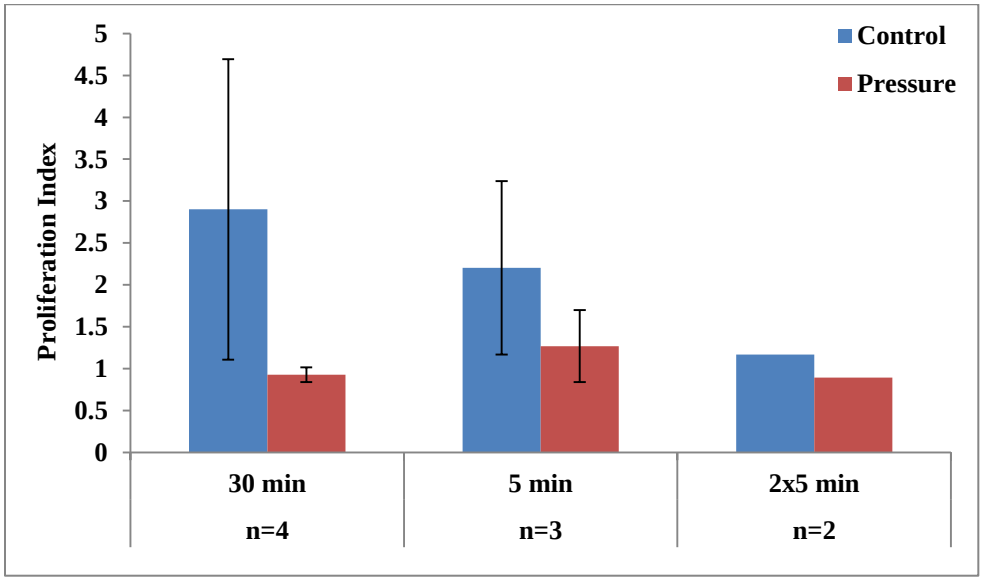

Figure 5-6: Lymphocyte proliferation in admit samples for stranded phocids following exposures to 2000G. Data are presented as the mean ± SE. Due to small sample size no error is shown for repeated 5 minute exposures.

Influence of Hormones on the Effect of Pressure

Significant correlations between plasma hormones and changes in immune function are summarized in Table 5-2. There was a significant positive relationship between epinephrine and changes in monocyte MFI for phagocytosis during both the dive (r=0.565; p=0.012) and recovery periods (r=0.58; p=0.009) as well as the % positive monocytes for phagocytosis for both the dive (r=0.865; p<0.001) and recovery periods (r=0.839; p<0.001). No significant

relationship was found between hormone concentrations and changes in the % positive granulocytes following pressure exposures. However changes in the MFI of granulocytes (i.e. the expression of CD11b per cell) was positively correlated with norepinephrine ($r=0.718$; $p=0.045$) and cortisol ($r=0.884$; $p=0.001$) following a single 5 minute exposure to 2000 psi. Epinephrine was positively correlated with changes in proliferation indices following a 30 minute exposure to 2000 psi ($r=0.928$; $p=0.003$). Following a single 5 minute pressure exposure, changes in IL2 receptor expression were positively correlated with both plasma epinephrine ($r=0.872$; $p=0.054$) and norepinephrine ($r=0.945$; $p=0.015$).

Table 5-2: Summary of significant correlations between catecholamines or cortisol and pressure induced changes measured in immune function for phocids. The exposure period is indicated by 'dive' or 'recovery'. Positive correlations are indicated by (+). No significant relationship is indicated by a dash (-).

Assay	Cell Type	Measure of Function	Epinephrine	Norepinephrine	Cortisol
Phagocytosis	**Granulocytes**	**MFI**	-	-	-
		% Positive	-	-	-
	Monocytes	**MFI**	dive (+), recovery (+)	-	-
		% Positive	dive (+), recovery (+)	-	-
CD11b expression	**Granulocytes**	**MFI**	-	(+)	(+)
		% Positive	-	-	-
IL2R expression	**lymphocytes**	**MFI**	(+)	(+)	-
		% Positive	-	-	-
Proliferation	**lymphocytes**	**index**	(+)	-	-

Discussion

The focus of this study was to evaluate whether the ability of seals to mount an immune response during and following diving was altered depending on health status. Stranded seals with each serving as its own control (admit vs. release) were the subjects of the study. In an investigation of dive behavior, Morrison *et al.,* (2012) noted no apparent difference between rehabilitated harbor seals and wild animals, suggesting that recovery from stranding and time spent in rehabilitation did not adversely affect the dive ability of these animals. Differences in immune function and the response of immune cells to pressure however, may be more subtle with cumulative long term effects.

For admit conditions, general trends suggested decreased or no change in phagocytic activity for the dive periods following pressure exposures even though general patterns of increased expression of CD11b (suggesting granulocyte activation) were observed. In contrast increased function was observed following further recovery periods. Patterns of change suggesting increased phagocytosis were noted for release conditions.

The effects of pressure *per se* on cell function may be facilitated by changes in membrane structure and associated membrane processes (Macdonald, 1982; Heineman *et al.,* 1987; Somero, 1992; Kato and Hayashi, 1999). The process of phagocytosis involves aggregation of membrane receptors (Greenberg, 1999; Aderem and Underhill, 1999), development of pseudopodia, as well as membrane invaginations and membrane fusion (Murphy *et al.,* 2008). Thus, phagocytosis can be inhibited by the direct effect of pressure on the membrane, resulting in decreased membrane fluidity and volume due to ordering effects, regardless of a change in activation state of the cell.

In addition, actin has been reported to undergo pressure induced de-polymerization (Haskin and Cameron, 1993) which could inhibit pseudopod formation and extension of membranes to surround particles for engulfment.

Release samples, however, showed opposite patterns, with an increase in phagocytic activity that agrees with the general pattern of cell activation. Increases in phagocytosis have been reported in response to pressure for human cells (Madden *et al.,* 1995; Chapter 2). Differences in this response may be due to stressor reduction, and/or the combined effects of stress and pressure between admit and release conditions. Other factors, such as nutritional status or age can also play a role. Many young animals which strand may be malnourished upon admit and gain weight and improve body condition over the course of rehabilitation. Membrane characteristics such as fluidity are in part determined by cholesterol and fatty acid composition. Dietary sources of polyunsaturated fatty acids (PUFA) have been shown to affect cell membrane composition of PUFAs (Fan *et al.,* 2003; Switzer *et al.*, 2004) and thus at release animals may have altered membrane fluidity as compared with admit. Body condition indices were calculated from weight and length measurements, and thus are related to nutrition, but may also be affected by illness (i.e. a sick animal may not be eating and thus have poor body condition). Body condition was negatively correlated with changes in lymphocyte proliferation, suggesting that in animals with poor body condition, lymphocytes are more sensitive to increased pressure. In contrast, there was a positive correlation between body condition and monocyte MFI for phagocytosis. These results suggest that animals in poor body condition respond abnormally to increased pressure, and thus may be less capable of coping with the challenge of diving. It is interesting to note that the response measured during release conditions (healthy, acceptable body condition) resembles that observed for humans, a non-dive adapted species.

Moreover, it is interesting to note that the relationship between control measures of MFI for phagocytosis and control measures of CD11b displayed opposite trends between admit and release conditions (data not shown). For admit animals, decreasing measures of CD11b expression were significantly correlated with higher phagocytic activity. However, for release conditions there was a positive correlation between CD11b expression and phagocytosis. While CD11b binding is not the only pathway by which phagocytosis can be triggered in granulocytes, these results suggest the phocid granulocytes may utilize different mechanisms to carry out immune functions under different conditions.

An overall activation of lymphocytes was observed as both measures of IL2 receptor expression increased following each pressure excursion. However, though limited data was available for this study, decreased proliferation indices were observed. Macdonald (1982) reports that procession of the cell cycle and cell division are inhibited by increased pressures, and decreased lymphocyte proliferation occurs in response to phytoheamagglutinin stimulation following pressure exposure. Again, this could be an effect of mechanical properties of membrane, and altered fluidity or binding. No relationship was observed however, between control measures of proliferation and IL2R expression, which may reflect the timing of these specific lymphocyte responses. IL2R up regulation occurs early in the response of lymphocytes, whereas lymphocyte proliferation occurs over a period of 96 hours for this study. Thus, pressure induced changes in early stages of the lymphocyte response, may not lead to changes in later stages of the lymphocyte response. Pressure effects may thus be enhancing in the short term, but inhibiting in the long term. The animals in this study were also faced with the additional stress of stranding, injury, mal-nutrition or disease, as well as handling, all of which likely impacted the response of cells to pressure exposures. These results thus support the idea that stranded phocids are less

capable of dealing with the challenges associated with diving. For healthy animals, particularly those which spend months at a time at sea diving repeatedly there must be a balance between the effects of a single dive and continuous diving in order to avoid injury or increased susceptibility to disease. Additionally, different cellular processes may be responding in different ways to changes in pressure. Redundancy within the immune system may allow a response to immunological challenge while suppressing unwanted activity.

Plasma concentrations of hormones such as catecholamines and glucocorticoids, associated with stressor conditions, can also impact immune function. As expected, plasma catecholamines and cortisol decreased between admit and release. Over the course of rehabilitation, animals may become habituated to human presence or the novelty of their environment (Lander *et al.,* 2003) and the issue that caused them to strand becomes resolved, thus decreasing the stressor load on an individual. Increased stressor load is generally associated with inhibited immune responses, however this is not always the case and many factors can regulate the response of cells to stress hormones (Dabhar, 2009; Martin, 2009; Madden, 2003; Madden *et al.*, 1995).

Despite the changes in plasma hormones, control values of immune function were not significantly different between admit and release conditions for animals with paired data. However, differences in the functional response of cells were observed between admit and release conditions. Additionally, results of this study suggest positive relationships between stress hormones and several measures of immune function including control values of CD11b expression and changes in lymphocyte proliferation and IL2R expression under pressure, with only a single negative relationship between increased epinephrine and a decrease in the % positive monocytes in control samples.

Minor increases in epinephrine have been reported to correlate with increased lymphocyte proliferation and IL2R expression in rats (Harris *et al.*,1995), and phagocytic activity of both monocytes and neutrophils has been reported to peak in conjunction with the peak in epinephrine over the course of an athletic training regime (Ortega Rincon *et al.*, 2001). There are many similarities between dive physiology and exercise physiology, and thus the relationships observed in this study between hormones and immune function are not entirely unexpected. However, there is likely a complicated relationship between dive behavior, stressor effects and immunity. This relationship may be complicated further as increased catecholamines play an important role in regulation of several aspects of the mammalian dive response, including bradycardia, peripheral vasoconstriction and splenic contraction (Foster and Sheel, 2005; Hochachka *et al.*, 1995; Hurford *et al.*, 1996).

The fact that no significant differences in control values were observed, may suggest reduced sensitivity of phocid immune cells to minor changes in plasma hormone concentrations. Because differences were observed in the response of cells to pressure exposures, it may be suggested that pressure *per se* may alter the sensitivity of these cells. The pressure used for this study represents an extreme, and these results may be particular to extreme diving conditions and may not be applicable to shallower diving situations.

Few relationships between cortisol and immune function were detected in this study. High plasma cortisol has been reported for several species of Antarctic phocids (Liggins *et al.*, 1993). Binding capacity of cortisol by serum proteins was also reported to be high in these animals. Because the effects of cortisol on physiological processes reflect the free fraction (Mendel, 1989) this ability to bind cortisol may reduce the effects on immune cells. Catecholamines are the first hormones released during a stress response and are short lived within the blood (Borysenko and

Borysenko, 1982). They are commonly associated with acute stressors and regulate the initial fight or flight response, preparing the body to deal with an immediate threat such as a predator. Immunosuppression is generally associated with the flight or fight response, yet it may be beneficial to have heightened immune responses in the face of short term stressors in case wounding, and thus the possibility of infection, should occur (Dabhar, 2002). During diving however, increased responsiveness of immune cells can lead to endothelial damage and development of dive related injury. Injury may not be extreme or apparent following a single instance however, and chronic exposures to such conditions may lead to decreased health in individuals and populations.

The response of immune cells to short duration pressure exposures (5 min, 2x5 min) differed in some cases from the response measured following the 30 minute exposure, suggesting that duration is an important characteristic of a dive. Whereas the response of swimming seals to human disturbances in the water column appears not to have been a great focus of research or observation, altered dive patterns have been reported in response to boat presence for dolphins (Constantine *et al.,* 2004) and to noise for hooded seals (Kvadsheim *et al.,* 2010) and northern elephant seals (Costa *et al.,* 2003). Changes in marine mammal dive behavior may be indicative of the degree of human activity impact on a marine community, or larger scale environmental changes. Southern elephant seals (*Mirounga leonina*) for example have recently been reported to dive deeper in warmer waters, likely because of shift in the vertical distribution of prey species (Boersch-Supan *et al.,* 2012). Dive effort, including time spent diving, is also adjusted to increase energetic gain of foraging bouts in grey seals (Austin *et al.,* 2006). Thus changes in marine mammal diving behavior reflect environmental changes and shifts in ecosystem resources, which have not only ecological implications but can also be important from the

perspective of human fisheries. Additionally, the results of this study demonstrate the potential for undesirable activity or in-activity of immune cells to result from such changes in dive behavior caused by either foraging needs or stressors such as human disturbance or anthropogenic noise.

The smallest change in proliferation was observed following repeated 5 minute exposures, and could be suggestive of a priming effect, where "memory" calls for cells to respond less to repeated pressure exposures. In humans, a habituation response has been reported for repeat dives with decreased inflammatory responses suggestive of decreased risk of decompression sickness (Errson *et al.,* 2002). Due to the repetitive nature of diving in seals, this effect may be important in modulating the effects of pressure on animal health during development of dive behavior. While more work needs to be done, this may suggest the importance of providing appropriate space for swimming/diving in rehabilitation settings.

A great deal of research has been conducted on dive abilities and adaptations in pinnipeds, yet this study was the first to investigate the relationship between immune function and diving, via simulated pressure exposures, in pinnipeds over the course of rehabilitation. There were many limitations with this study and data are difficult to interpret for several reasons. Sampling stranded animals is an opportunistic event and there is no control over age, species or reason for stranding; all of which could be important factors in modulating immune function or when considering dive capabilities. Harbor seals for example have been reported to dive deeper than either grey seals or harp seals, but development of deep diving ability can be age dependent (Jorgensen *et al.,* 2001). In this regard, variability in immune responses measured sometime varied greatly between individuals, resulting in very large standard errors. For example, in Figure 5-2 the error bar for the dive period of the repeated 5 minute exposure for release samples was

too large to be displayed on the graph. This result was due to small sample size and a single individual with a large magnitude response having a disproportionate influence on the mean. Unfortunately, data sets in this study were too small to run comparisons by species or age, or between paired and non-paired individuals. Additionally, small sample sizes make statistical analysis difficult. Nonetheless, there does appear to be an effect of pressure on both innate and adaptive immune function in phocids. Pressure exposures resulted in significant changes in the % of granulocytes and monocyte undergoing phagocytosis and in the amount of bacteria being engulfed per monocyte. These changes varied based on dive profile and time post-decompression, with overall patterns suggested larger changes following recovery periods as compared with dive periods. General patterns of decreased phagocytic function were observed for animals at the time of admit, whereas no change or increased function was detected at the time of release. In addition, significant increases in both the expression of CD11b and IL2R were found following pressure exposures. Though significant differences between admit and release conditions were not detected, cellular expression of these proteins showed significant positive correlations with plasma catecholamines and cortisol. Lymphocyte proliferation appeared to decrease following pressure exposures, and this change was negatively correlated with body condition.

The relationship between plasma hormones, body condition and immune function, as well as differences in immune responses between admit and release conditions suggest that the effect of pressure can be modulated by the presence of stressors. Thus, the results of this study suggest there is likely a complicated balance between dive behavior and health status. It is difficult however to speculate about the adaptive or deleterious effects of these changes, and more work is

needed to better understand the balance between health and physiological adaptation to demanding behaviors such as diving.

Chapter 6 BLOW COLLECTION AS A NON-INVASIVE METHOD FOR MEASURING CORTISOL IN THE BELUGA (*Delphinapterus leucas*)

Abstract

Non-invasive sampling techniques are increasingly being used to monitor glucocorticoids, such as cortisol, as indicators of stressor load and fitness in zoo and wildlife conservation, research and medicine. For cetaceans, exhaled breath condensate (blow) provides a unique sampling matrix for such purposes. The purpose of this work was to develop an appropriate collection methodology and validate the use of a commercially available EIA for measuring cortisol in blow samples collected from belugas (*Delphinapterus leucas*). Nitex membrane stretched over a petri dish provided the optimal method for collecting blow. A commercially available cortisol EIA for measuring human cortisol (detection limit 35 pg ml^{-1}) was adapted and validated for beluga cortisol using tests of parallelism, accuracy and recovery. Blow samples were collected from aquarium belugas during monthly health checks and during out of water examination, as well as from wild belugas. Two aquarium belugas showed increased blow cortisol between baseline samples and 30 minutes out of water (Baseline, 0.21 and 0.04 µg dl^{-1}; 30 minutes, 0.95 and 0.14 µg dl^{-1}). Six wild belugas also showed increases in blow cortisol between pre and post 1.5 hour examination (Pre 0.03, 0.23, 0.13, 0.19, 0.13, 0.04 µg dl^{-1}, Post 0.60, 0.31, 0.36, 0.24, 0.14, 0.16 µg dl^{-1}). Though more work needs to be done, this study suggests that blow sampling is a good candidate for non-invasive monitoring of cortisol in belugas. It can be collected from both wild and aquarium animals efficiently for the purposes of health monitoring and research, and may ultimately be useful in obtaining data on populations which are difficult to handle directly.

Introduction

Glucocorticoids, such as cortisol, provide important information for wildlife health and conservation efforts; serving as indices of fitness and stressor load as well as for monitoring the response of individuals or populations to stressors (Bonier *et al.,* 2009; Sheriff *et al.,* 2011). Physiological consequences of increased cortisol, which include reallocation of energy resources (Bonier *et al.,* 2009; Breuner *et al.,* 2008), inhibition of growth, reproduction and immune function (Bonier *et al.,* 2009; Breuner *et al.,* 2008; Romero and Butler, 2007), may be beneficial or pathological and ultimately affect the fitness of an individual.

Typical methodology for measuring changes in stress hormones involves blood sampling. Animals in zoos or aquaria, such as marine mammals, can be trained to participate in behavioral blood draws; however, for free ranging animals the process of chase, capture and restraint are stressors in themselves and the data need to be interpreted with consideration of confounding effects of sampling procedures. For some free-ranging species, such as large baleen whales which cannot be restrained or handled, blood sampling is simply not feasible. Alternative matrices for steroid hormone measurements have been successfully collected for both terrestrial and marine animals, including feces, urine, hair, (Sheriff *et al.*, 2011; Hogg *et al.,* 2009; Amaral, 2010), feathers (Bortolotti *et al.,* 2008) and blubber in marine mammals (Mansour *et al.,* 2002; Kellar *et al.,* 2006; Kellar *et al.,* 2009). While these sampling procedures may be less invasive than blood sampling, they pose their own challenges. For example, there is a risk of environmental contamination when collecting feces or urine from cetaceans. Additionally, the hormone content in these matrices may accumulate over different time periods (e.g. days to months) and thus may be difficult to associate with any particular event or stressor.

Over the past few years, exhaled respiratory condensate or "blow" has been a focus for non-invasive biological sampling in order to monitor steroid hormones, such as testosterone and progesterone in bottlenose dolphins (*Tursiops truncatus*) (Hogg *et al.,* 2005), humpback whales (*Megaptera novaeangliae)* and north Atlantic right whales (*Eubalaena glacialis*) (Hogg *et al.,* 2009), as well as for monitoring bacterial communities (Acevedo-Whitehouse *et al.,* 2010) and genetics (Frere *et al.,* 2010). Exhalations are composed not only of gas and water vapor, but also molecular aerosols, lung mucosa and associated proteins (Mutlu *et al.,* 2001; Horvath *et al.,* 2005). Cortisol has been measured in exhaled breath condensate from healthy bottlenose dolphins, including a pregnant female (Amadei *et al.,* 2008) and humpback whales (*Megaptera novaeangliae*) (Dunstan *et al.,* 2012). Hunt *et al.,* (2013b) measured several steroid hormones, including cortisol, in blow collected from north Atlantic right whales.

Methodologies for measuring cortisol in blow samples include radio-immunoassay (RIA) and enzyme-immunoassay (EIA) (Hunt *et al.,* 2013a), as well as high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) (Hogg *et al.,* 2009; Kellar *et al.,* 2009). HPLC-MS requires expensive specialized equipment and training, while RIA's require the use of radioisotopes (Sheriff *et al.,* 2011). Commercially available EIA's are more portable for field work, and do not require expensive equipment or radioisotopes and therefore provide a more convenient method for monitoring hormones in blow of marine mammals at zoos and aquaria.

The purpose of this work was to evaluate the use of blow as an alternative matrix for monitoring cortisol levels in belugas (*Delphinapterus leucas*). To do this we 1) developed an appropriate sampling protocol for collecting blow in beluga whales 2) validated a commercially available enzyme immunoassay for measuring cortisol in whale blow and 3) measured cortisol content of

blow and blood in aquarium and wild belugas before, during and following events known to stimulate activity of the hypothalamic pituitary adrenal (HPA) axis.

Methods

Subjects

Paired blow and blood samples were obtained utilizing positive behavioral reinforcement from four beluga whales, two females (~30 years old) and two males (~9 and 26 years old) housed at the Mystic Aquarium, Mystic, CT (IACUC protocol # 11001). Paired samples were also collected from wild belugas during live capture-release health assessments in Bristol Bay, AK (n=15) and Point Lay, AK (n=1) (NMFS Marine Mammal Research Permit No. 14245, Alaska Department of Fish and Game Permit No. 14610).

Preliminary Experiments

Determination of Collection Device and Material for Blow

Preliminary experiments were carried out using samples collected only from aquarium animals. The volume of blow collected was measured for 3 different collection devices; 50 ml conical tubes, 250 ml Nalgene™ bottles, and Petri dishes (100mm x 15mm). Petri dishes consistently returned the largest volumes of blow and were chosen as the collection device for all further work.

Petri dishes were covered with one of four different collection materials; cotton gauze, nylon stocking cleaned by sonication for 20 minutes (Hogg *et al.*, 2009), nitex membrane (110 µm pore size) (Elko Filtering Co., Miami, FL) and tulle netting (Michaels Stores, Waterford, CT), both cleaned with 70% ethanol dehydrant. Each collection material was held in place on the petri dish with an elastic band.

On average ≤30 µl of sample were recovered from cotton gauze and tulle, while ≥ 50 µl were recovered from nylon stocking and nitex membrane (Figure 6-1).

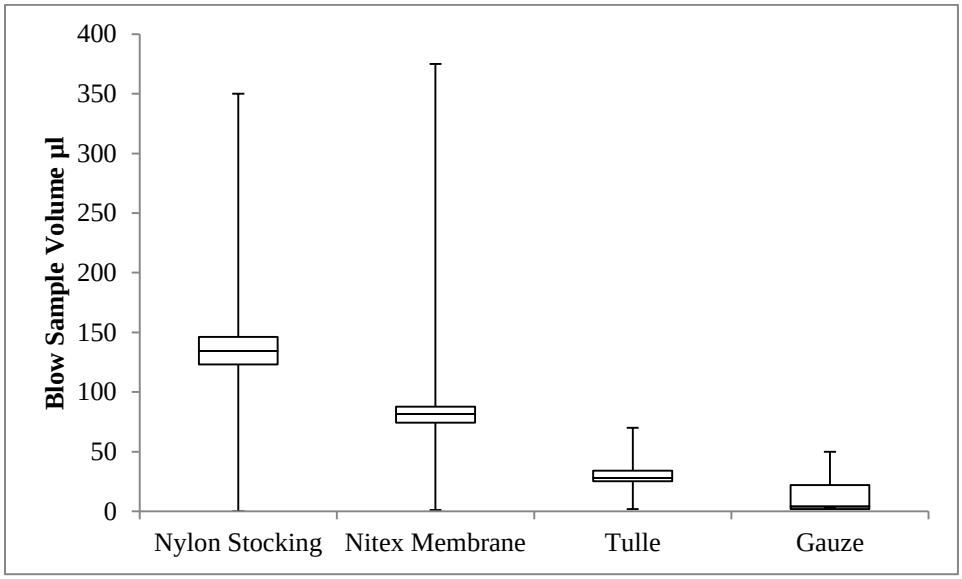

Figure 6-1: Volumes of blow samples recovered from nylon stocking, nitex (nylon) membrane, tulle netting and gauze following 4 exhales. For each collection material sample collection data are pooled for three animals. For all collection materials there was at least one sample from which no blow could be recovered. Largest volumes were collected from nylon stocking and nitex membrane.

Preliminary experiments to determine the best collection material were run using an EIA from Cayman Chemical (Ann Arbor, MI; cat#500360) which has previously been utilized and validated for marine mammal samples at the Mystic Aquarium. Linearity testing for nylon stocking and nitex membrane revealed best fit lines with slopes of 1.112 ± 0.068 and 1.002 ± 0.032, respectively (Figure 6-2). However, subjective comparison of dilution lines suggests that nylon stocking may interfere with the assay more than the nitex membrane, as higher dilutions varied more from 1:1 ratio of expected: observed. Nitex membrane covered petri dishes were then chosen as the collection device and material for the remainder of the study.

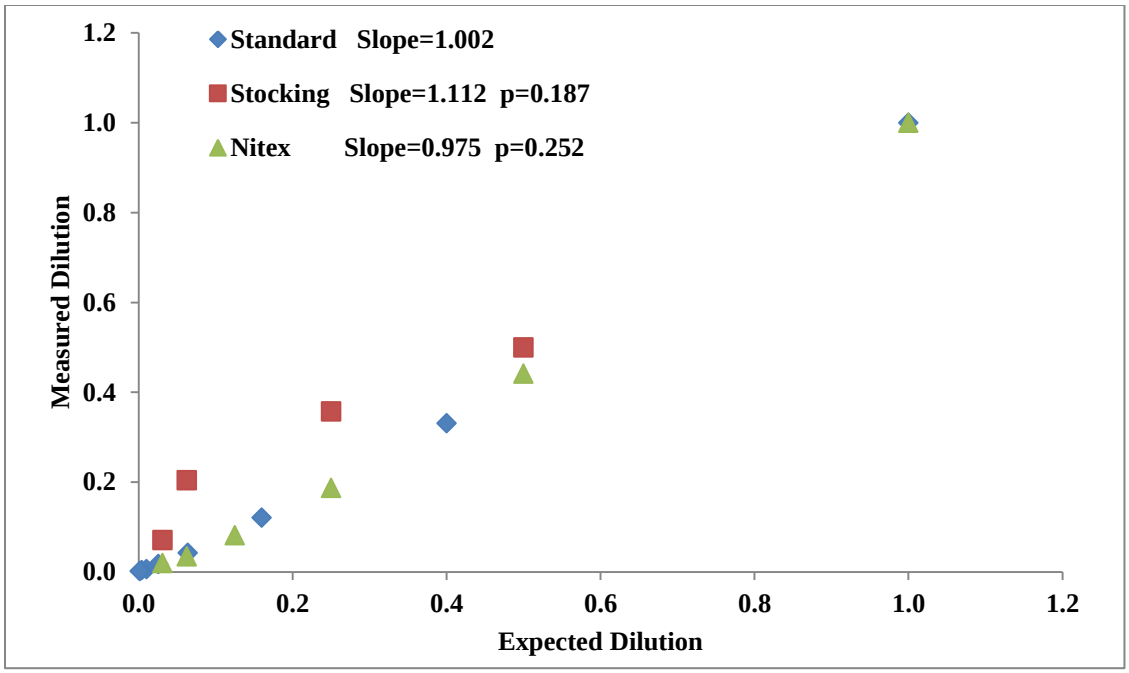

Figure 6-2: A representative dilution series comparing cortisol standard with blow samples recovered from nylon stocking and nitex membrane. The nylon stocking line shows deviation from linearity at higher dilutions. Nitex membrane shows good linearity.

Determination of Enzyme Immunoassay

Immunoassay kits tested for suitability of use with blow, were purchased from Salimetrics (State College, PA; cat#1-3002), MP Biomedical (Solon, OH; cat #07M-21602), Enzo Life Sciences (Miami, FL; cat #ADI-900-071), Cayman Chemical and Arbor Assays (Ann Arbor, MI; cat # K003-HI). Blow samples from three animals (1 male, 2 females) were pooled for kit testing, with a pooled plasma sample run simultaneously as a control. Parallelism results for all kits are shown in figure 6-3. In addition, the dilution for achieving 50 % B/Bo (ratio of sample cortisol bound compared with maximum binding) and the required volume of sample to reach this value was also calculated for each kit (Table 6-2). Based on a combination of these results, as well as simplicity of methodology and analysis, the Cayman Kit was chosen for all further validation.

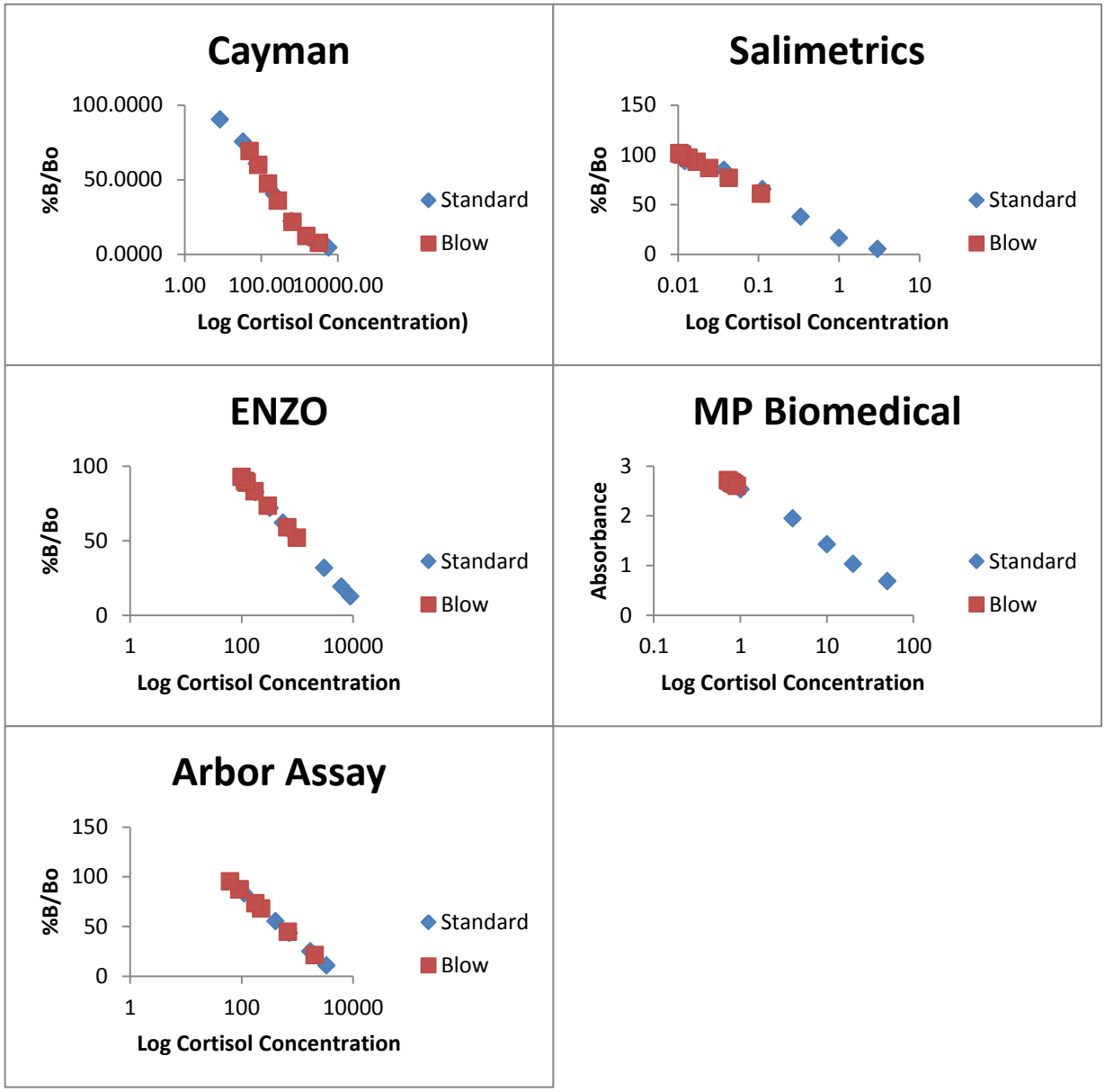

Figure 6-3: Parallelism results for pooled blow (n=3) for 5 commercial enzyme-immunoassay's. The % of kit vs. sample bound cortisol (%B/Bo) could not be calculated for MP Biomedical due to differences in kit protocols and absorbance values were used in this case.

Table 6-1: Calculated volumes of blow required per well to reach 50%B/Bo (the middle of the standard curve) for each cortisol EIA tested. MP biomedical did not provide measurements necessary to calculate the %B/Bo and so absorbance values were used during assessment (indicated by ND=no data)

Assay Kit	Vol of unknown required per well (µl)	Dilution for 50% B/Bo	Actual Vol. of sample needed per well (µl)
Cayman	50	1:4 to 1:16	3.125-12.5
Arbor Assays	50	1:2	25
MP Biomedical	25	ND	ND
ENZO	100	1 to 1:2	50-100
Salimetrics	25	1	25

Sample Collection

Blow Collection and Processing

Based on preliminary experiments petri dishes with nitex membrane were used for all sample collection. Collection dishes were kept refrigerated or on ice for at least 30 minutes immediately prior to sample collection to aid in the condensation of sample, based on the development of collection devices for human breath condensate studies (Mutlu *et al.,* 2001;Horvath *et al.,* 2005).

The whales at Mystic Aquarium were trained through positive behavioral reinforcement to exhale on signal. Baseline samples (n=4) were collected from aquarium belugas stationed with their head resting on the exhibit beach. A single exhale was performed first to clear water from the blow hole. The collection device was held inverted approximately 2-4 inches from the blow hole for the duration of 4 or 8 exhales. While 4 deep exhales were found to provide a decent volume of sample during preliminary experiments, the exhales in two animals were short and shallow chuffs, thus 8 exhales were targeted in these animals to increase the volume of sample

collection. “Stressor” samples were obtained during out of water examinations (OWE) by attaching petri dishes to one end of a pole extended over the blow hole (n=3). For the OWE, animals were lifted out of the water on a stretcher and placed on padding upon the exhibit beach for examination including gross full body exam, blood sampling, and veterinary assessment. Sampling occurred 1 week and 24 hours prior to the OWE, and for 10 minute intervals throughout the 30 minute examination. At the conclusion of the examination, animals were returned to the water and samples were collected at a further 24 hours, 48 hours, 72 hours and 96 hours post OWE. At the end of sample collection, petri dishes were covered and secured with the membrane inside and placed on ice.

Wild belugas were captured and handled according to Norman *et al.,* (2012). Animals were guided into shallow water using several small boats and captured in netting suspended between two boats. For handling, animals were removed from the net and each whale was supported so that the blowhole remained out of the water between breaths. One exhale was allowed to occur before collection in order to clear water from the blowhole. For animals in deeper water, a plastic gasket was placed around the blowhole to aid in keeping water out (Figure 6-4). On average, 2 exhales were collected per plate. Following sample collection, membranes were secured inside petri dishes. Plates were stored inside plastic bags and placed in a cooler with an ice pack until they could be transferred to a -20^{o}C freezer (<10 hours). Plates were then shipped back to the Mystic Aquarium on dry ice.

Figure 6-4: Collection of blow from a wild beluga in Bristol Bay, AK during live-capture and release health assessment studies. A plastic gasket was placed around the blowhole in order to minimize water contamination of blow samples. The blow plate was then held inverted in the center of the gasket for two repeated exhales.

In the lab, the collection material was used to wipe the inside of the dish in order to collect any sample which passed through it, and placed inside a 50 ml conical tube with a plastic stopper, made from the sterile plunger of a 20cc syringe. Conical tubes were centrifuged at 2800 x g for 30 minutes at 10^{o}C. The centrifuged condensate was then pipetted into 1.25 ml Sarstedt tubes and stored at -80^{o}C to avoid degradation (Amaral, 2010) until assay.

Blood Collection and Processing

Blood samples were obtained from belugas at the same sessions during which blow samples were collected. Blood was drawn from the ventral or dorsal flukes and collected in 10 ml sodium heparinized vacutainer™ tubes. Blood tubes were kept on ice until centrifugation at 2000 x g for 10 minutes at 10^{o}C. Plasma was aliquoted into 1.25 ml tubes and stored at -80^{o}C. Subsequently, one ml aliquots were sent to the AHDC Endocrinology Laboratory at Cornell University, Ithaca, NY, for cortisol analysis (Schmitt *et al.*, 2010; Schwake *et al.*, 2013) via Immulite ® chemiluminescent enzyme immunoassay (Siemens Medical Solutions, Malvern, PA, cat # LKC01).

Cortisol Enzyme Immunoassay

Validation of Cayman Chemical EIA

Blow samples were thawed at room temperature and centrifuged at 2060 x g for 15 minutes. This centrifugation step is recommended when working with saliva samples in order to remove mucins which precipitate during freezing (Salimetrics Salivary Cortisol EIA user Manual, 2011) and so was tested with blow samples. Pellets were observed in many of the blow samples, particularly those of larger volume. Following centrifugation the supernatant was removed for use in the assay. Blow samples were pooled for males (n=2) and females (n=2) separately. All samples were run in duplicate. Parallelism was tested by serially diluting pooled samples 1:2, 1:4, 1:8, 1:16 and 1:32, and comparing the cortisol concentrations with the percent bound (B/Bo) for blow pools and the standard curve. Intra-assay variability was calculated from eight replications of triplicate wells of a pooled blow sample. Inter-assay variability was calculated

using 6 cortisol standards, provided with the kit, between 40 and 4000 pg ml^{-1} and averaging the % CV over three separate assays.

Cortisol standards were also used to test accuracy of cortisol in blow samples, and recovery of cortisol from the collection membrane. For accuracy, four standards (41, 102.4, 256, and 640 pg ml^{-1}) were spiked into male and female pools of blow and the measured cortisol (minus cortisol measured in un-spiked samples) was compared with the known values of the standard. Three standards (102.4, 256, and 640 pg ml^{-1}) were spiked directly onto collection membranes and retrieved following the same centrifugation protocol as blow samples to test for recovery.

Monthly, OWE and wild samples were run in duplicate at a dilution of 1:4. Where cortisol concentrations were outside the 20-80% B/Bo of the kit, samples were rerun at an adjusted dilution, volume permitting.

Statistics

A goodness of fitness ANCOVA association test was run in order to assess parallelism for the Cayman Chemical EIA. A Shapiro-Wilk test for normality revealed a significant deviation from normal in data from monthly samples, OWE as well as wild belugas ($p< 0.001$). Following a log transformation, data from monthly samples were normal and repeated measures ANOVA was used to compare monthly cortisol values. However, data collected during the OWE and from Bristol Bay whales required a non-parametric Kruskal Wallis ANOVA to compare cortisol values in blow over several time points during the course of the OWE.

Because stressed populations show greater variance in physiological responses (Anderson *et al.*, 2011) heterogeneity of variance was also investigated by calculating the absolute differences

between each data point and the mean, and comparing baseline, 30 minutes out of water and 24 hours post out of water examination using a non-parametric repeated measures ANOVA.

Correlations were conducted in order to compare changes in blow and plasma cortisol for the OWE's, as well as compare blood and blow cortisol for 'pre' samples from Bristol Bay animals. Non-parametric paired Wilcoxon tests were used to compare pre and post cortisol values in blow for Bristol Bay belugas. For all tests, significance was determined at α=0.05.

Results

Cortisol Kit Validation

Following determination of nitex membrane as the collection material and a collection protocol during preliminary experiments, several commercial cortisol EIA's were tested and the Cayman Chemical cortisol EIA was chosen for further validation based on preliminary parallelism results, and kit requirements. Kit sensitivity is 35 pg ml^{-1}. Intra and inter assay CV's were calculated to be 8.26 and 11.79 respectively, both of which fall within the acceptable range for recommended use of the kit.

Parallelism results were repeated for this kit using separate pools of male and female blow. Both pools displayed curves parallel to the standard curve ($F_{2,12}$ =0.813;p=0.467) (Figures6-5). Pooled blow samples were spiked with four standards (41, 102.4, 256, and 640 pg ml^{-1}). Cortisol measured in un-spiked samples was subtracted from spiked samples, and the resulting cortisol value was compared with standards (Figure 6-6). Males displayed a slope of 0.9999 ±0.102 while females displayed a slope of 1.086±0.038. Regression analysis revealed significant relationships for both males and females (α=0.05) suggesting good accuracy of the kit. Three

standards were used to spike nitex membrane (102.4, 256, and 640 pg ml^{-1}). Measured cortisol concentrations were between 80 and 110% recovery.

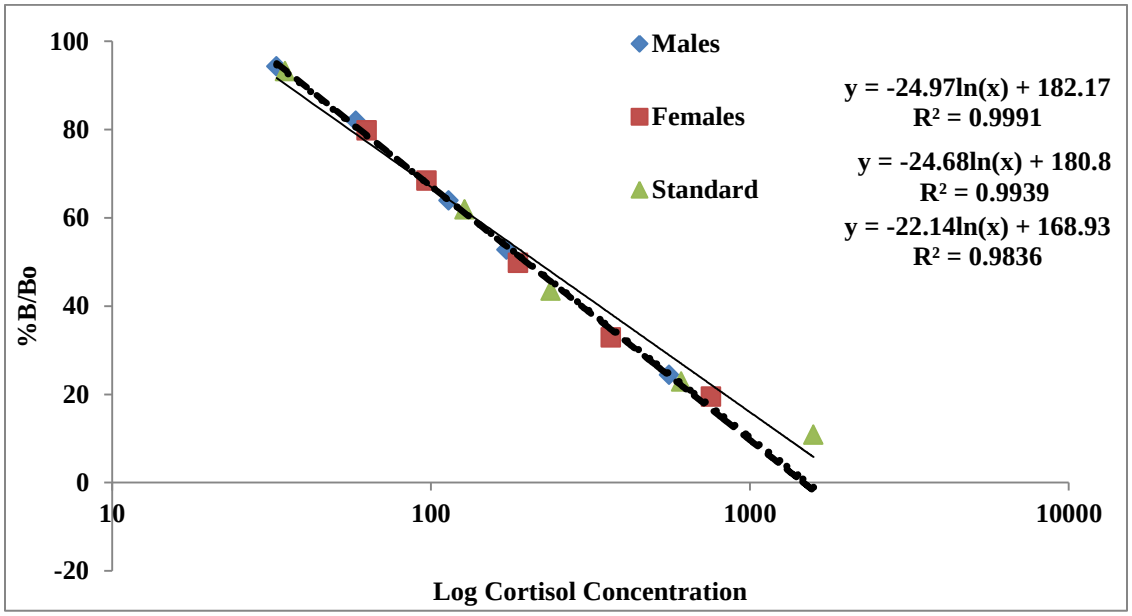

Figure 6-5: Relationship between (Log) cortisol concentration and the %B/Bo for the Cayman kit standards and for blow pooled between males and females showing parallelism.

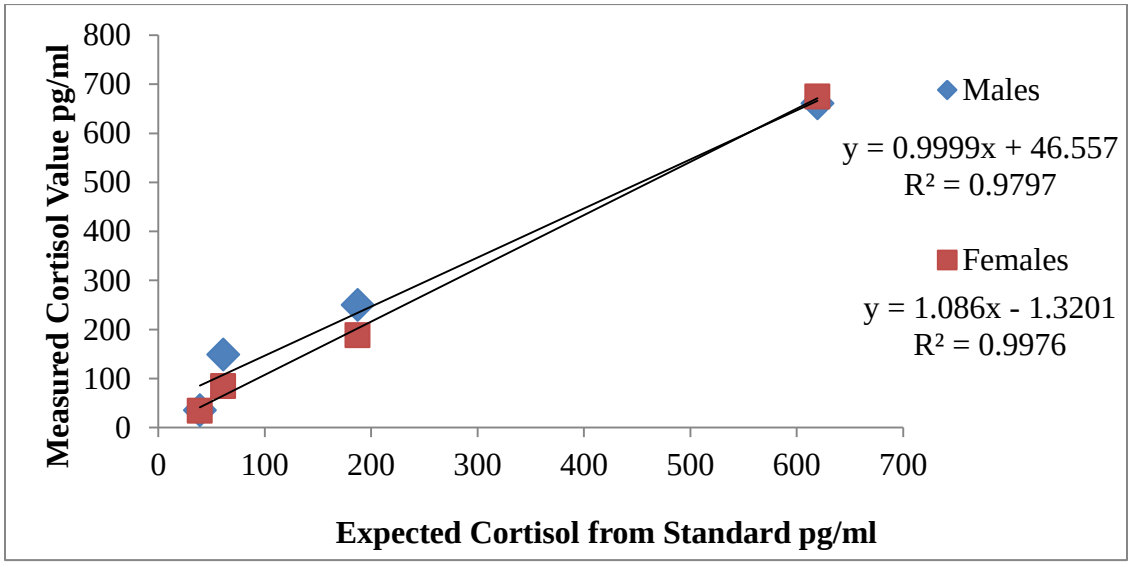

Figure 6-6: Results of accuracy testing for the Cayman kit using pooled male and female blow samples. Measured cortisol values are calculated with the actual sample cortisol subtracted and compared against the known standard concentrations.

Cortisol Values

All samples were run in duplicate. Non-specific binding, blank, maximum binding wells, and standards were run on all plates. Plates were accepted if the maximum binding wells displayed absorbance values between 0.3 and 1.0 with the blanks subtracted. Sample results were accepted if the percent bound fell within the 20-80% range and CV's for duplicates were no larger than 30%.

Monthly Values

Cortisol concentration was measured in blow samples collected from aquarium belugas during monthly health checks between 2011 and 2013 (Table 6-3). Missing values reflect samples which ran outside the 20-80% B/Bo range of the cortisol kit or occur where samples were not obtained due to either extreme low volumes (<5 µl) or whale behavior. The majority of blow samples contained less than 1 µg dl^{-1} cortisol (average 0.22 µg dl^{-1}), though some variation between months is apparent. No significant differences in monthly cortisol values measured in blow were detected ($p>0.05$) although increased blow cortisol concentrations were observed in three animals during late summer and fall of 2011, with a peak occurring in Dec 2011. Data were also binned in season by year (winter= Jan, Feb, Mar; spring= April, May, June; summer= July, Aug, Sept; fall= Oct, Nov, Dec). Significant differences in cortisol were detected ($p=0.042$) (Figure 6-7).

Table 6-2: Monthly cortisol values of blow for individual animals between 2011 and 2013. ND= not detectable or outside the 20-80 % B/Bo range of the Cayman kit. I = insufficient volume recovered to run sample. Ø = no sample collected.

		Animal1	Animal2	Animal3	Animal4
2011	April	0.7548	1.0470	0.3004	Ø
	May	0.2094	ND	ND	Ø
	June	ND	0.0374	0.1271	Ø
	July	0.1221	I	0.4549	Ø
	August	0.0963	0.0431	0.0474	Ø
	Sept	0.1827	0.4465	0.1276	Ø
	Oct	0.4559	0.5089	I	Ø
	Nov	0.6080	0.5409	0.4819	Ø
	Dec	0.8421	4.6000	1.5080	Ø
2012	Jan	0.7309	0.4999	0.2205	Ø
	Feb	0.1500	0.1109	0.0660	0.8648
	Mar	0.2107	ND	0.1733	0.2862
	April	ND?	Ø	ND	Ø
	May	ND?	Ø	ND	ND
	June	0.4807	0.1468	Ø	0.0623
	July	0.0970	0.0970	2.5250	0.3979
	August	I	0.0941	0.3343	0.0889
	Sept	I	0.3024	ND	0.0614
	Oct	0.2119	0.0359	ND	0.2650
	Nov	ND	ND	ND	0.0527
	Dec	0.0291	0.0197	0.0313	0.0557
2013	Jan	0.2417	Ø	0.0629	0.0412
	Feb	0.1070	Ø	0.0881	Ø
	March	Ø	Ø	0.0563	Ø
	April	ND	0.0296	0.0216	ND
	May	ND	0.1494	0.0880	Ø
	June	Ø	Ø	Ø	Ø
	July	0.0717	0.1905	Ø	Ø
	August	Ø	Ø	Ø	Ø
	Sept	Ø	0.3054	0.7554	Ø
	Oct	Ø	0.2501	Ø	0.0910

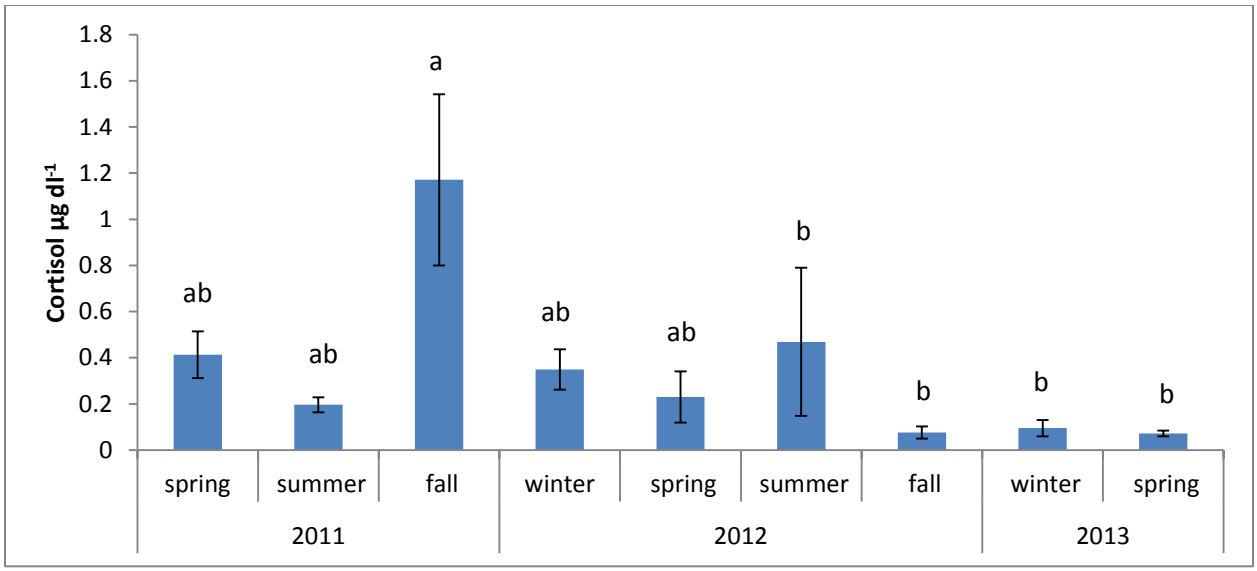

Figure 6-7: Average cortisol concentrations in blow (± SE) binned by season for 2011, 2012 and 2013. Letters indicate similarities and differences between seasons (i.e. all seasons marked with an 'a' are similar, but differ from those marked with a 'b'). Cortisol values were higher during the fall of 2011.

Out of Water Examination

Breaths for each of the animals involved in the OWEs were noted to be shorter and shallower than during most monthly collections. All animals showed significantly ($p=0.039$) elevated plasma cortisol at 30 minutes on the beach as compared with baseline samples (Figure 6-8a), with levels returning to baseline by the 24 hours post examination. Animals 1 and 2 also displayed increased cortisol in blow at the 30 minute time point though statistical significance was not detected ($p>0.05$) (Figure 6-8b). Heterogeneity of variance for each data point was calculated as the absolute value of the difference between the point and the mean for baseline, 30 min and 24 hour post time points. Heterogeneity of variance was significantly greater for the 30 minute time point as compared to baseline or 24 hour post samples ($F=11.991;p=0.020$). A significant positive correlation was found between blow and plasma cortisol ($r=0.561$; $p=0.005$). Animals 1 and 2 displayed similar patterns in blow and blood cortisol over the course of OWE 1

while animal 3 did not display any apparent relationship between cortisol in blow and blood (Figure 6-9).

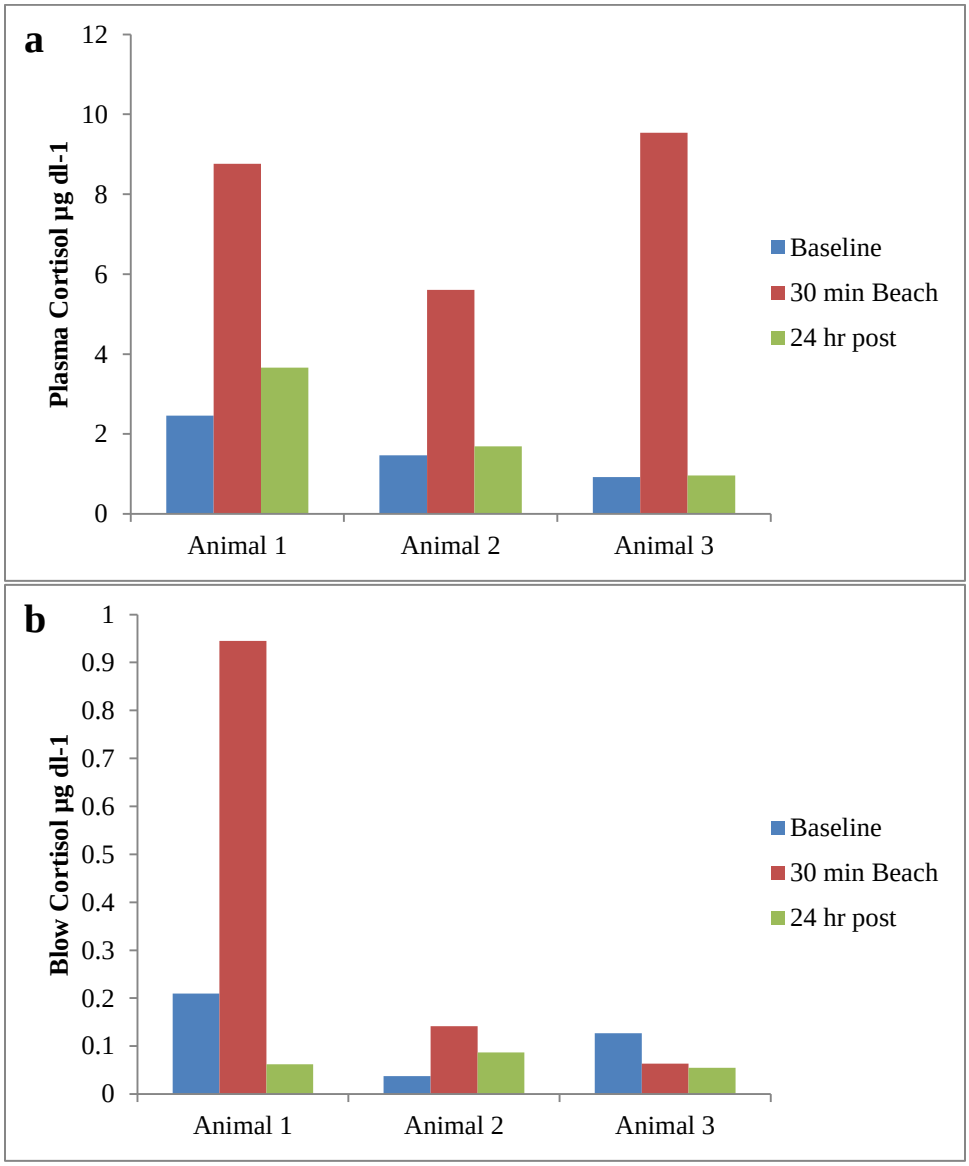

Figure 6-8: a) plasma and b) blow cortisol concentrations for baseline samples, 30-minutes out-of-water, and 24 hours post out of water examination for three aquarium belugas involved in OWE 1. Plasma cortisol was significantly elevated at the 30 minute time point ($p<0.039$).

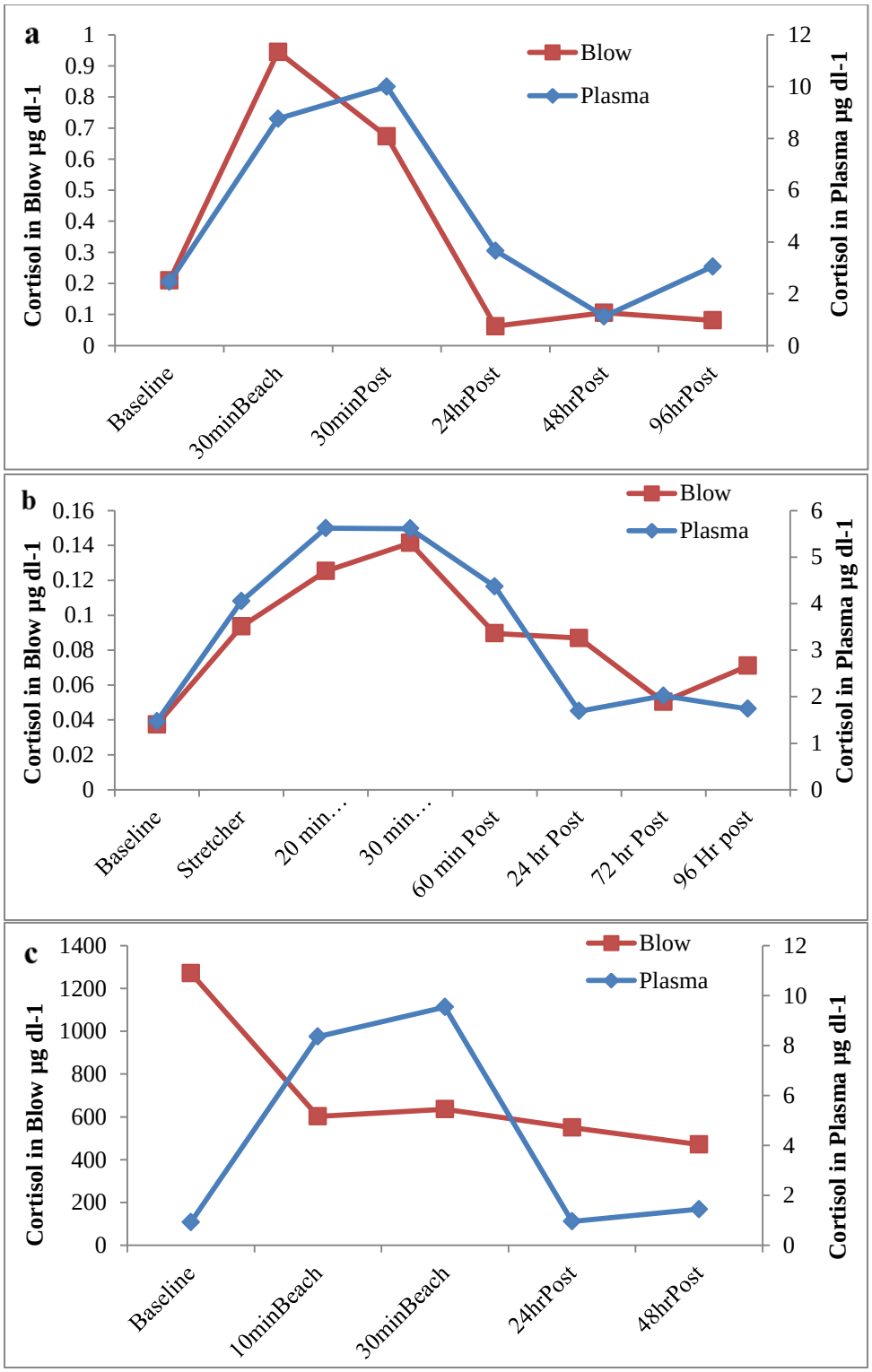

Figure 6-9: Blow and Plasma cortisol concentrations measured in a) animal 1, b) animal 2, and c) animal 3, over the time course of an OWE. Missing time points reflect samples with volumes too low to run with the assay or where results fell outside the 20-80 % B/Bo range.

Wild Animals

The volume of blow condensate recovered from one to two exhales per wild beluga ranged from 0 to 800 µl (Table 6-4). In 2012, two out of nine 'pre' and two out of nine 'post' plates were found to be dry once returned to the lab. For one animal, the same plate was used for both 'pre' and 'post' sampling and so was removed from analyses for this experiment. In 2013, four out of ten 'pre' and four out of ten 'post' plates were found to be dry. Dry plates were excluded from this analysis as no volume of sample could be recovered.

The single whale sampled from Pt. Lay, AK had a blow cortisol concentration of 0.0853 µg dl^{-1}. For belugas from Bristol Bay, cortisol concentrations measured in blow were below 1 µg dl^{-1} and several were lower than the detection limits of the kit (<0.0035 µg dl^{-1}) . Plasma cortisol values were only available for the initial 'pre' sample and there was no significant relationship between plasma and blow cortisol ($p>0.05$). A significant increase in cortisol values in blow was detected between pre and post samples for Bristol Bay belugas ($p=0.028$) (Figure 6-10). The magnitude of increase between pre and post blow samples ranged from an 8.8 % increase to a nearly 2000% increase (median= 109.2%; mean=399.2%). One animal however displayed a large decrease (65%) in cortisol between pre and post samples in 2013. This animal was actively splashing during restraint and sample collection, and it is suspected that the collection plate became contaminated with water leading to dilution of the cortisol content measured.

Table 6-3: Volumes and cortisol concentrations of blow samples recovered from live captured belugas in Bristol Bay, AK during 2012 and 2013, and a single animal from Pt. Lay, AK. Ø indicates no sample recovered from membranes.

Animal ID	Volume µl	Cortisol µg dl^{-1}	Volume µl	Cortisol µg dl-1	Change (Pre to Post)
DLBB12-01	Ø	---	10	0.3079	---
DLBB12-02	Ø	---	Ø	---	---
DLBB12-03	15	0.0311	25	0.5948	Increase
DLBB12-04	200	0.2303	90	0.3079	Increase
DLBB12-05	25	0.1277	10	0.3636	Increase
DLBB12-06	75	0.1947	400	0.2379	Increase
DLBB12-07	800	Outside kit range	Ø	---	---
DLBB12-08	450	0.0165	800	Outside kit range	---
DLBB12-09	300	Outside kit range	Ø	---	---
DLBB13-03	Ø	---	50	0.0994	---
DLBB13-05	200	0.1651	Ø	---	---
DLBB13-06	15	0.1325	50	0.1441	Increase
DLBB13-07	50	0.1636	Ø	---	---
DLBB13-08	50	0.038	100	0.1646	Increase
DLBB13-09	250	0.0163	500	0.0057	Decrease

Pt Lay		
Animal ID	**Volume µl**	**Cortisol µg dl^{-1}**
108771	200	0.0853

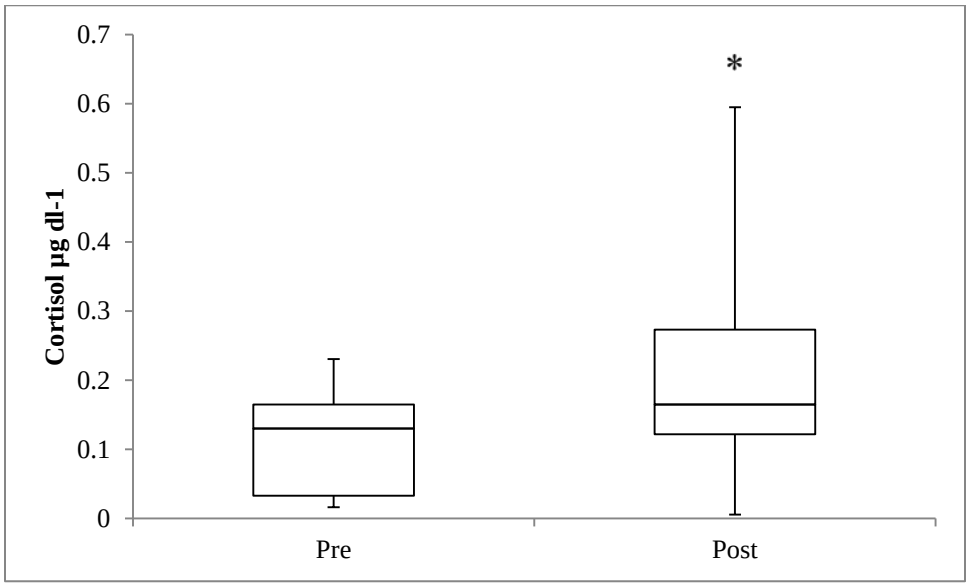

Figure 6-10: Cortisol concentrations in blow samples collected 'pre' and 'post' full examination for Bristol Bay belugas. A significant increase in blow cortisol was detected, and is indicated by an asterisk (*)(p<0.05).

Discussion

The results of this work show that cortisol can be successfully detected in exhaled breath condensate, or blow, from belugas using an EIA, and that blow sampling is a good candidate for monitoring changes in cortisol associated with stressor events in belugas. Such methodology has application for both aquarium and wild animals as it will allow for non-invasive, repeated collection of biological information.

It is important to note that the depth of the breath (full exhale vs. shallow chuff) seemed to play a role in the volume of sample collected. This is particularly true for aquarium belugas, where 4-8 repeated exhales were required to collect an appreciable volume of sample. When animals were removed from the water and placed upon the exhibit beach during the OWE, their breathing also became shallower accounting for no sample being recovered from several time points. In

contrast, wild animals sampled in Bristol Bay, AK performed deep and extended exhales and >50 µl of sample were collected with only 1 or 2 exhales. An explanation for this difference may be that wild animals likely exhale at or near full capacity in order to clear CO_2 built up from diving or to replenish oxygen stores, and the effect on cortisol content should be investigated in future studies. Variation is also seen in recovered condensate volumes from the Bristol Bay animals and this may be due to differences between sample collectors and animal behavior (i.e. remaining still vs. moving head from side to side). Additionally samples collected from animals earlier in the course of a day remained in a cooler for longer periods than later animals and this may have led to greater evaporation before plates were transferred to a freezer.

Monthly samples showed increased cortisol content in blow during the fall of 2011, returning to baseline in January and February of 2012. It is interesting to note that a fourth whale was introduced to the beluga habitat at the aquarium in the fall of 2011. Changing social dynamics could have acted as a stressor, resulting in activation of the HPA axis and a rise in circulating cortisol. Increased cortisol in blow was also found for 2 out of 3 animals after 30 minutes out of the water for examination. The differences observed in the magnitude of changes may reflect individual variability in perception of the stressor. Heterogeneity of the variance was also found to be significantly greater at the 30 minute time point as compared with baseline or 24 hours post out of water time points. The increase in the variation of cortisol values suggests increased variation in the neuroendocrine response of individual animals. Such increased variation has been linked with exposure to environmental stressors such as contaminants, or novel environments (Orlando and Guillette, 2001). Plasma cortisol however, did not display the same significant increase in variation at the 30 minute time point. This disconnect between blow and blood may be due to timing of sampling in these two matrices and the temporal relationship

between changes in blow vs. changes in plasma. More work thus is needed to investigate this relationship and understand both the timing and rate of hormone changes in blood and blow. Variation seen in aquarium animals during the OWE may also reflect other physiological differences related to animal age (~30 years old vs 9 years old) or sex (2 females vs. 1 male). Indeed, the male, and also youngest animal had a markedly different behaviorally response to the OWE, and was the only animal which did not show an increase in blow cortisol. Additionally, individual perception of the stressor, shaped by experience, can affect the neuroendocrine response.

For wild animals, an increase in blow cortisol was found between 'pre' and 'post' exam samples. Plasma cortisol was only available for 'pre' samples and so could not be compared over the course of the restraint period. The magnitude of change in blow cortisol concentration between 'pre' and 'post' samples however showed some variability, with one animal in particular showing a much larger increase than the rest. The variability in this response may be explained by individual experience and modified stressor responses of individual animals due to past exposure to pursuit, boat presence, entanglement or stranding. This animal was also the only juvenile female sampled for this study, and thus age and sex may also have influenced the glucocorticoid response. Additionally, no relationship was observed between blood and blow for 'pre' samples collected from Bristol Bay animals. We attribute this to the variability in the time of sampling blow in relation to blood during the examination.

Due to gaps in monthly blood collection no conclusions could be reported as to the relationship between blow and blood cortisol in monthly samples from aquarium animals. However, a significant positive correlation between cortisol values in blow and blood was detected over the course of the OWE, suggesting that blow is a useful alternative matrix for monitoring

glucocorticoid responses to stressors. We were able to measure cortisol monthly (baseline conditions) in aquarium belugas, suggesting that cortisol is present in blow even without an obvious stressor event. Establishing normal patterns of variation in these samples could lead to determining a threshold for detecting large deviations in cortisol content indicative of a stress response.

Cortisol measured in blow, however, may reflect only a portion of the total cortisol in plasma. In blood, cortisol can be bound by corticosteroid binding globulin (CBG) or can be free and available to bind with cells and thus exert influence on biological function. Salivary cortisol is thought only to consist of free cortisol (Kirschbaum and Hellhammer, 1994; Gozansky *et al.,* 2005) which passes to saliva via passive diffusion (Hogg *et al.,* 2009; Vining *et al.,* 1983). If passive diffusion is also the mechanism by which cortisol enters the lungs, blow samples may also contain only the free fraction of cortisol. Both free cortisol and CBG can be measured in blood samples (Lewis *et al.,* 2005; Clerico *et al.,* 1982; Pretorius *et al.,* 2011; Coolens *et al.,* 1987) though these analyses were beyond the scope of this study. Variability in the CBG binding should be considered as a possible explanation for lack of a relationship between blow and plasma, and could account for some of the variability observed between individuals in the cortisol values measured in blow during the OWE. Further work should attempt to correlate blow cortisol with the unbound fraction of blood cortisol.

Results of this study demonstrate the ease and efficiency with which blow samples can be obtained. Behaviorally, the ability to sample blood from the whales was less consistent than the ability to sample blow during multiple months for all animals, in part due to behavioral challenges. Knowing that blow samples could still be collected, for monitoring hormones or other health parameters when blood cannot be obtained, has important potential within aquaria

for both clinical and research applications. For example, the response of cetaceans to changes in their environment or social structure can be monitored in the blow since blood sampling behaviors may be disrupted.

There is no universal standard for a dilution effect in exhaled condensate studies, though the need for one is widely accepted (Katial, 2012). Urea has been used in human studies, however lack of complete validation of this method and great variability even with subjects has led to some criticism (Horvath *et al.,* 2005; Katial, 2012; Effros *et al.,* 2004; Borrill *et al.,* 2008). Attempts to validate urea as a marker of dilution for blow in our laboratory were inconclusive and not reported for this study, though the authors recognize the importance for such work. This study aimed to minimize dilution by environmental water, however dilution by water vapor through the respiratory tract, condensation on the plate during collection and differences in lung condensate production between deep breaths and shallow chuffs could be responsible for several outlying events noted in this study; 1) the apparent decrease in blow cortisol for animal 3 during the OWE, 2) lower blow cortisol values in wild animals than in aquarium animals and 3) the single animal from Bristol Bay which showed a large decrease in blow cortisol between pre and post samples.

Use of blow is a relatively new approach to research and health monitoring, and this study is the first to show that blow can be collected and used to measure cortisol in belugas. Information concerning the health status of populations can be gained by comparing not only cortisol values, but also the variance in cortisol measurements, in blow samples between healthy populations such as Bristol Bay and endangered populations such as the Cook Inlet animals. Differences in cortisol (e.g. higher baseline levels) can indicate decreased ability of a population to cope with new disturbances (Wingfield *et al.,* 1997). Long term monitoring of cortisol in blow samples

can be useful in assessing the impact of anthropogenic or environmental stressors such as underwater drilling or climate change.

Collection of blow carries potential for providing a suite of information on the health status of individuals, as it likely also contains proteins, such as cytokines and other markers of health, stress or disease. Having this non-invasive approach will be an important tool for research and wildlife conservation and medicine since blow can be sampled more frequently than blood. More work however is needed to better describe the relationship between blood and blow cortisol. Constraints on this study prevented repetition of the OWE's or use of ACTH challenge, however additional time course studies should be conducted as feasible.

In summary, an appropriate methodology for collecting blow samples from belugas was developed and a commercial EIA validated and used to measure cortisol in beluga blow. This technique provides a means of assessing individual health status without invasive handling and sampling, and is an important tool for monitoring animals in aquaria. Additionally, the accessibility of this methodology (e.g. ease of obtaining collecting materials) and non-invasive nature of sampling could provide access to free ranging populations, or different species, that are otherwise difficult to handle directly; increasing research opportunities and providing information about threatened or endangered populations. This technique also holds promise for monitoring the neuroendocrine response of cetaceans to anthropogenic activities or changing environmental conditions.

Chapter 7 CONCLUSION AND SIGNIFICANCE

The ocean environment is changing; habitats are being destroyed (Agardy, 1997; Turner *et al.*, 1999; Lotze *et al.,* 2006), noise pollution is increasing (Richardson, 1995), the distribution and availability of food species are changing (Garrison and Link, 2000; Richardson and Schoeman, 2004; Perry *et al.,* 2005), temperatures are rising and ice is melting (Comiso *et al.,* 2008; Hoegh-Guldberg and Bruno, 2010). A major contributor in driving these changes is human activity. The ultimate result is that human exploitation of the oceans is impacting the health of marine life, including marine mammals. An example of this is facilitation of disease epidemics through changes in environmental parameters and species distributions (Harvell *et al.,* 1999). Biology (presence of blubber), life histories (long-lived) and charisma have made marine mammals good indicators of changing environmental conditions which are also of concern to human health, leisure and economics. Emerging and spreading disease information can be gained by monitoring infections in coastal populations or stranded animals. In addition, the bio-accumulation of pollutants, including persistent organic contaminants, in the blubber reflect environmental loads and health risks. Belugas are one species which have been used as sentinels for ecological risk in the Great Lakes-St Lawrence Basin (Fox, 2001). Pinniped species that strand or haul out near heavily populated or visited beaches increase the potential for interactions with humans or domestic pets, causing concern for disease transmission. Understanding the relationship between marine mammal physiology and the ocean environment could prove useful in understanding and monitoring outbreaks of potentially zoonotic diseases as changes in immune function caused by anthropogenic activities can leave individuals, or populations, less able to fight infection (DeSwart *et al.,* 1996; Burek *et al.,* 2008).

The first aim of this work was to further our knowledge of marine mammal immunology by evaluating the functional response of immune cells to simulated dive excursions. Dive behavior

represents physiological challenges that can result in serious injury, disease and even animal death. The response of human divers to the challenges associated with diving are highly varied, and exposures to increased pressure can result in no apparent injury, or in barotraumas, neurological dysfunction, and inflammatory damage associated with DCS (reviewed in Macdonald, 1982) . The effects of increased pressures on cellular functions have been discussed previously and occur by altering membrane characteristics, reaction volumes or protein denaturation (reviewed in Macdonald, 1982; Heineman *et al.,* 1987; Siebenaller and Garret, 2002). Although a few studies have investigated cellular adaptations and the effects of pressure on marine mammal erythrocytes and platelets (Ahmed *et al.,* 1989*;* Field, 2000; Williams *et al.,* 2001; Castellini *et al.,* 2001), none have considered how cells of the immune system may function during, or in response to, a dive. Proper regulation of immune function is critical for maintaining health, and changes in immune function play important roles in leading the development of dive related infections and injury associated with decompression sickness; conditions from which marine mammals have long been thought not to suffer. Diving seals have been suggested as models for research into ischemia and reperfusion injuries due their hypoxic tolerance and anti-oxidant capabilities (Zenteno-Savin *et al.,* 2002), and understanding how marine mammals avoid dive related pathologies has potential application in human medical research to develop treatments or therapies for decompression, hypoxia and hypothermic related pathologies in dive communities as well as clinical settings.

The measures of immune cell function targeted for this study are hardly representative of the vast and complicated mammalian immune response. The results of this work, however, do suggest that certain immune functions, including phagocytosis and lymphocyte proliferation, in marine mammals can be altered in response to diving. These findings may be surprising considering

that marine mammals are highly adapted to diving and rarely present any symptoms of dive related injury or disease. However, it is possible that the changes observed in immune function serve an adaptive role in these animals. For example, lack of an activation response or decreased activity of granulocytes could mean these cells are less reactive to the presence of gas nuclei or bubbles. Indeed, a less reactive immune system has been suggested to be one mechanism by which marine mammals avoid development of decompression sickness (Fahlman *et al.,* 2006) even though extremely high tissue super saturation can occur and the presence of non-symptomatic gas bubbles could be more common than previously thought (Hooker *et al.,* 2012). Further evidence of gas saturation are the reports of bubbles, which likely arose from rapid decompression and off gassing of saturated tissues, in stranded dolphins, harbor seals harbor seals and harbor porpoises (Moore *et al.,* 2009; Dennison *et al.,* 2012).

Altering the target pressure, as well as the rate of compression and decompression resulted in some variation in the response of beluga cells. The change in response, however, was not consistent (e.g., deeper dives always leading to larger increases), and small sample sizes make it difficult to interpret these results. Additionally, responses varied between marine mammal species and between marine mammals and humans. For example, belugas tended to show decreased phagocytic activity in response to exposures to 2000 psi. Stranded pinnipeds showed some decreases under the same exposures, but not for all conditions and not to the same extent as seen in the belugas. In complete contrast, humans tended to display increased phagocytic activity. These differences may reflect species-specific dive capabilities. Although the majority of beluga dives tend to be shallow, they are capable of dives to over 1000m. The deepest dives for each of the pinniped species targeted for this work are several hundred meters shallower, and humans are by far the shallowest free divers. Associating the largest decreases in granulocyte

activity with the deepest divers supports the idea that this reduction in activity could serve some adaptive advantage. These results also suggest that the risk for developing dive related pathologies may be species specific.

The second purpose of this work was to aid in investigating the potential for anthropogenic stressors to alter the response of immune cells to diving and this was accomplished by comparing the response of marine mammal immune cells to pressure excursions between different health conditions representative of stressor exposures. The effect of pressure on the function of immune cells for both belugas and pinnipeds showed some differences between baseline ('healthy') and stressor conditions, including a period of chronic inflammation in belugas. Thus, altered physiological states can affect the ability of marine mammals to respond appropriately to challenges of diving. Even though a definitive relationship could not be described from these results, in some cases, the response of marine mammal cells during stressor conditions resembled that observed in humans. The biological significance of these changes in immune function is difficult to ascertain as either increased or decreased immune function can be both beneficial or deleterious depending on other situational variables. For example, increased activity is good for fighting infection but an abnormal immune response could indicate auto-immune processes. Nonetheless, results of this study are the first evidence that cellular responses to diving in marine mammals can be altered under stressor conditions (results are summarized in Appendix AG) and it is possible that these changes result in increased susceptibility of marine mammals to dive related injury, or long term consequences such as decreased fitness.

Emboli and lesions, similar to signs of DCS in humans, have been reported in animals which stranded in close proximity to naval sonar activities and have been suggested to have arisen through behavioral responses (i.e., changes in diving behavior; Jepson *et al.,* 2003; Fernandez *et*

al., 2005). This change in behavior could have been brought about by a startle response during diving which: 1) could have been of unusual intensity due to physiological adjustment made during diving (Talpalar and Grossman 2005), and 2) could have led to damage due to inappropriate immune activity resulting from the combination of changes in stress hormones and the presence of gas bubbles. In many cases it is likely that the effects of anthropogenic activities or stressors will not be as apparent as mass strandings, and subtle changes in immune function may manifest in long term changes in individual and population health.

The effects of a perceived stressor may be situational (Breuner *et al.,* 2008); dependent on the stressor, individual experience and life history stage, and environmental factors. Monitoring the relationship between the behavior, physiology and stressors is important in order to assess individual and population conditions as these have complex interactions with, and effects on, fitness. For example, Constantine *et al.,* (2004) report that the presence of dolphin-watching tour boats alters the time spent resting in bottlenose dolphins. The authors noted a previous study which found that mother and calf pairs dive for increasing durations in the presence of boats. In addition, increased usage of deep water channels in the presence of boats has also been reported for bottlenose dolphins (Constantine *et al.,* 2004). With the presence of boats serving as a stressor, an increase in stress hormones result in these avoidance behaviors (diving) which in and of itself presents challenges to homeostasis, or may interrupt foraging or rest periods. Even though avoidance of an immediate threat may be beneficial, the interaction between the increased hormones and the regulation of immune function during the dive may have undesirable effects.

Chronic stressor exposure is linked with decreased fitness and measures of glucocorticoids, such as cortisol, are increasingly being used as indicators of stress, animal health and fitness. High

baseline cortisol is usually associated with reduced population fitness reflective of greater challenges (Bonier *et al.,* 2009) with a negative correlation between body condition and baseline cortisol (Breuner *et al.,* 2008). High fecal glucocorticoids in right whales also have been associated with shipping traffic and noise (Rolland *et al.,* 2011) suggesting chronic stress responses. In this study, increased plasma cortisol was found in belugas exposed to a 30 minute out of water physical examination, wild belugas under restraint and in stranded pinnipeds admitted to rehabilitation. Though changes in stress hormones were observed between conditions, there were few significant relationships between immune responses and hormones. Thus more work is needed to characterize the effects of stress on immune cells in marine mammals. Changes in cortisol observed during stressor conditions have been reported to be more limited than observed in other mammals (St Aubin and Dierauf, 2001). It is possible that, due to the important roles neuroendocrine hormones play in regulating aspects of the dive response (Hance *et al.,* 1982; Hochachka *et al.,* 1995; Hurford *et al.,* 1996) immune cells may be less reactive to their effects during a stress response. Changes in the sensitivity of immune cells to increased glucocorticoids have been reported in other species with repeated exposures (Martin, 2009). In addition, aldosterone has been suggested to be more important in the stress response of marine mammals as compared with terrestrial animals (St Aubin and Dierauf, 2001) and should be investigated in future studies.

Like all physiological functions, mounting an immune response costs energy (Demas, 2004). If immune activity is prolonged, the energetic demand increases and can ultimately affect fitness by depleting energy available for other functions (Lochmiller and Deerenbay, 2000). Hanssen *et al.,* (2004) suggest that the cost of mounting immune responses to disease may be dependent on other conditions (e.g. breeding) and that consequences may not be apparent but manifest in long

term changes, such as failure to breed in following years. Thus certain species (with shorter reproductive cycles) may be better candidates for investigating impacts of anthropogenic stressors than others.

In marine mammals, diving and foraging behaviors are linked. Diving for benthic foraging has been reported for belugas (Martin *et al.,* 1999), harbor seals (Lesage *et al.,* 1999), as well as adult grey seals (Tucker *et al.,* 2007). Factors such as vertical prey distribution and depth of foraging habitats place some constraints on diving, and there is likely a balance between the costs of diving and the benefits of foraging at particularly depths (Tollit *et al.,* 1998). If human activity results in changes in prey distribution this balance can be altered; e.g. increased costs of diving and decreased gains from foraging. Whereas changes in prey distribution and abundance may better indicate environmental impacts of anthropogenic activity, it is important to evaluate the potential impact of such changes on higher trophic levels. Concern over the decline in Steller sea lion populations for example, highlighted changes occurring in fish stocks, including economically important species (Reddy *et al.,* 2001).

Jaquet *et al.,* (2000) comments that the repetitive and extreme dive behavior associated with foraging in male sperm whales is necessary to meet their energetic demands and decreased fitness may be the result of altering this behavior. During lactation, female Weddell seals lose energy stores and thinner animals have been noted to dive deeper in pursuit of higher return prey patches than females in more robust body condition (Sato *et al.,* 2002). Additionally, a study of energy budget models in killer whales (*Orcinus orca*) suggests that avoidance behaviors associated with human disturbance may not be costly per se, but can result in decreased ability to meet the energetic demands of other behaviors if it alters foraging or habitat use (Williams *et al.,* 2006). If energetic demands are not met, it is possible that cells of the immune system will

not respond appropriately to challenges associated with diving. If dive behavior is altered and an abnormal immune response is triggered, as the results of this study suggest, essential energy stores may be affected. Conversely, decreased responsiveness of immune cells can have short term implications on health, but may positively affect fitness in the long term by freeing energy for more immediately vital functions (Viney *et al.,* 2005).

Furthermore, diet can play an important role in cellular adaptation to pressure. Membrane composition is important in determining rigidity and sensitivity to changes in pressure. High cholesterol content for examples has an ordering effect on membranes, and leads to increased sensitivity to pressure changes (Hazel and Williams, 1990). Differences in membrane cholesterol have been noted between elephant seal and human platelets (Field, 2000) as well as between elephant seal erythrocytes and terrestrial mammals such cows, horses and dogs (Williams *et al.,* 2001). In addition, polyunsaturated fatty acids (PUFA's) are also important components of the membrane, playing a role in pressure adaptation. European eels (*Anguilla anguilla L.)* that migrate to depth regulate the PUFA content of cell membranes prior to vertical migration (Vettier *et al.,* 2006). Ahmed (1989) found no difference in the amount of fatty acids between seal and human platelets, but notes that seals have increased eicosapentaenoic acid content which, when consumed in the diet of humans results in altered sensitivity of platelets.

Thus, this dissertation has demonstrated a link between diving and cellular immune function in marine mammals. Understanding this link is important for determining the consequences of human activities on marine mammal populations. However, complex physiology and the relationships between neuroendocrine activity, immune function, foraging success and energetics and fitness, suggest there is a larger intricate relationship between diving and health which was beyond the scope of this study. The consequences of anthropogenic activities on this

relationship, particularly chronic sub lethal consequences, are mostly unknown and cannot be derived from this work alone.

Several limitations of this study are recognized. Many physiological adjustments occur during diving including changes in neuroendocrine hormones and, for some pinniped species, changes in temperature. While the *in vitro* approach utilized in this study allowed the effects of pressure *per se* to be investigated, the influence of these other factors cannot be ignored when extrapolating to free diving marine mammals. In addition, the pressures used for this study represent extreme dives and responses presented here may differ from what occurs during shallow dives to <500m. Due to limitations with the pressure chamber experimental setup, exposures to corresponding pressures could not be performed reliably. Because dives up to the depth of lung collapse pose an increased risk of tissue super-saturation and thus bubble formation, the effects of shallow dives should be considered for future studies.

Furthermore, human blood samples used for pressure exposure experiments in chapters 2, 3 and 4 were shipped on cold packs overnight to the Mystic Aquarium. Thus, these samples were approximately 24 hours old before immune function experiments as compared with marine mammal blood samples which were run fresh. This difference is sample handling could not be avoided, and may have had an impact on the results.

This research was intended as an initial descriptive study, with the hopes of initiating interest in future immune studies in marine mammals. Eventually such information may aid in evaluating the impact of noise and other human activities on free ranging cetacean populations. For species or populations that cannot be worked with directly and blood samples cannot be drawn, alternative mechanisms of sampling are needed. I have shown that blow (exhaled breath

condensate) can be collected from belugas, and used to monitor changes in cortisol associated with a specific stressor event. This methodology has huge application as it is possible to collect blow samples from large whales in the wild (Hogg *et al.,* 2009; Acevedo-Whitehouse *et al.,* 2010), and also to collect samples from endangered populations which cannot be handled. Though there may be some sampling bias, with populations under long term surveillance for which there are photo identification catalogs it may be possible to serially sample the same individuals over time and monitor changes in hormones that could be associated with anthropogenic events (e.g. drilling, sonar). In addition, blow sampling is an excellent alternative to blood sampling because it can be collected serially within a short period of time with no effect on the animal. This ability to repeatedly sample is important when looking at cortisol, and other hormones which follow diurnal patterns of secretion, however by using blow the risks associated with multiple blood draws, such as infection, are eliminated.

If a relationship between blow hormones and immune function can be established, it may be possible to extrapolate information from these *in vitro* studies to make informed hypotheses concerning the immune status of animals and the impact of anthropogenic activities. My study suggested a relationship between increased blow cortisol and decreased granulocyte expression of CD11b and increased lymphocyte proliferation. In addition, the decrease in lymphocyte proliferation observed in pressure exposed cells was larger in samples corresponding with higher blow cortisol. Interestingly, no relationships were found between plasma cortisol and lymphocyte proliferation in belugas. This difference in result, could be due to differences in the free/bound cortisol content of blow and plasma. The Immulite® system used to measure plasma cortisol measures the total cortisol, and so includes both bound and free fractions of cortisol. It is possible that the cortisol in blow reflects only free cortisol, similar to what has been reported

for saliva (Kirshbaum and Hellhammer, 1994; Gozansky *et al.*, 2005). Thus blow may provide a relatively accurate measure of biologically active cortisol.

Information gathered from blow sampling can also be useful for monitoring glucocorticoids as an indicator of stressor load in a population over time, and in comparative studies monitoring the condition of wild populations inhabiting geographically distinct areas. For example, populations of beluga can be found in northern Alaska, southern Alaska as well as in eastern Canada (Nunavut, Hudson Bay), each of which is exposed to different environmental challenges and anthropogenically driven stressors. The ability to collect biological samples (e.g. noninvasive blow) and interpret analyses (e.g. cortisol concentrations) will be useful in estimating overall health in these populations. In conservation research, cortisol has been used as an indicator of environmental and life history challenges which are faced by individuals or populations and to estimate fitness (Blas *et al.*, 2007; Bonier *et al.*, 2009; Shallin Busch and Hayward, 2009). High loads could lead to exhaustion of energy resources and the inability of animals to handle additional challenges, leading to reduced fitness (Romero *et al.,* 2009). Thus, by comparing cortisol between populations the ability for animals to cope with human activities may be inferred and populations with increased vulnerability to disturbances may be identified (Wingfield *et al.*, 1997).

Ultimately, this research has shown that there is a relationship between diving behavior and immune function in marine mammals and it is possible for anthropogenic activities to alter this relationship and thus impact marine mammal health. This study only considered the challenges of changing pressure associated with diving however, and marine mammals must also contend with hypoxia and reperfusion, as well as the hormonal adjustments which regulate the mammalian dive response. Future work should thus include *in vivo* studies utilizing free ranging

animals in order to observe the combined effects of these physiological changes on immune function. More work is needed before such information can become applicable to monitoring and conservation efforts, however, this study does support the need for further investigation of the relationships between diving, health and the potential impacts of stressors.

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Appendices

Appendix A

Mixed generalized linear model results for beluga baselines (n=4) and humans (n=4) for phagocytosis following all pressure exposures.

Phagocytosis			Beluga Model				Human			
			F	p	Dive	Recovery	F	p	Dive	Recovery
2000G	Granulocytes	MFI	38.82	<0.001	<0.001	0.775	1.385	0.276	0.999	0.113
			10.507	0.001	<0.001	0.402	2.452	0.114	0.04	0.955
			2.226	0.137	0.296	0.086	0.134	0.875	0.616	0.93
			16.147	<0.001	<0.001	0.092	5.188	0.017	0.133	0.012
		% Positive	21.318	<0.001	<0.001	0.138	0.358	0.704	0.97	0.409
			4.694	0.023	0.295	0.01	9.694	0.001	0.32	<0.001
			1.409	0.27	0.114	0.809	1.196	0.325	0.779	0.344
	Monocytes	MFI	5.987	0.01	0.003	0.652	1.022	0.38	0.17	0.962
			1.095	0.356	0.16	0.833	0.033	0.967	0.803	0.964
			1.83	0.191	0.099	0.443	3.208	0.66	0.245	0.037
		% Positive	6.389	0.009	0.002	0.861	0.329	0.724	0.905	0.429
			1.421	0.267	0.115	0.75	0.509	0.61	0.452	0.522
1000G	Granulocytes	MFI	3.982	0.039	0.074	0.055	2.335	0.129	0.799	0.052
			4.159	0.033	0.204	0.019	0.872	0.435	0.844	0.208
			4.694	0.023	0.007	0.815	19.071	<0.001	<0.001	0.949
		% Positive	25.187	<0.001	0.371	<0.001	4.561	0.025	0.156	0.017
			71.887	<0.001	0.17	<0.001	5.031	0.018	0.064	0.023
			2.917	0.08	0.028	0.767	7.789	0.004	0.001	0.24
	Monocytes	MFI	3.162	0.067	0.7	0.023	9.33	0.002	0.064	0.001
			3.285	0.061	0.11	0.069	4.5	0.026	0.013	0.246
			1.604	0.229	0.093	0.795	0.206	0.816	0.579	0.765
		% Positive	3.085	0.07	0.7	0.024	30.57	0.072	0.156	0.063
			3.174	0.066	0.935	0.021	12.545	<0.001	0.001	0.012
			0.777	0.475	0.753	0.244	0.11	0.896	0.644	0.998
2000R	Granulocytes	MFI	3.494	0.052	0.023	0.395	1.98	0.167	0.062	0.998
			34.268	<0.001	<0.001	<0.001	1.277	0.304	0.141	0.686
			0.877	0.433	0.519	0.265	0.012	0.988	0.97	0.883
		% Positive	5.006	0.019	0.008	0.299	1.628	0.224	0.436	0.123
			5.751	0.012	0.059	0.014	7.818	0.004	0.67	0.001
			1.048	0.371	0.958	0.165	3.845	0.041	0.358	0.018
	Monocytes	MFI	0.949	0.406	0.97	0.185	8.382	0.003	0.001	0.813
			15.934	<0.001	0.002	<0.001	0.207	0.815	0.545	0.857
			2.158	0.146	0.306	0.056	0.078	0.925	0.717	0.889
		% Positive	3.122	0.069	0.178	0.053	1.701	0.211	0.119	0.4909
			4.618	0.026	0.954	0.008	0.944	0.41	0.478	0.261
			1.435	0.264	0.14	0.495	0.391	0.682	0.475	0.623
1000R	Granulocytes	MFI	1.657	0.218	0.435	0.119	7.675	0.004	0.001	0.917
			4.579	0.025	0.325	0.011	1.187	0.328	0.338	0.252
			1.536	0.242	0.224	0.238	1.202	0.324	0.609	0.162
		% Positive	4.572	0.025	0.155	0.014	3.14	0.068	0.029	0.43
			10.452	0.001	0.326	<0.001	8.718	0.002	0.002	0.043
			1.262	0.307	0.384	0.205	1.022	0.38	0.327	0.324
	Monocytes	MFI	27.152	<0.001	<0.001	<0.001	2.761	0.09	0.03	0.973
			7.726	0.004	0.087	0.003	2.755	0.09	0.077	0.176
			4.275	0.03	0.392	0.012	1.176	0.331	0.327	0.263
		% Positive	27.69	<0.001	<0.001	<0.001	2.756	0.09	0.047	0.291
			36.473	<0.001	<0.001	<0.001	8.33	0.003	0.002	0.068
			7.182	0.005	0.017	0.012	1.005	0.386	0.33	0.329

Appendix B

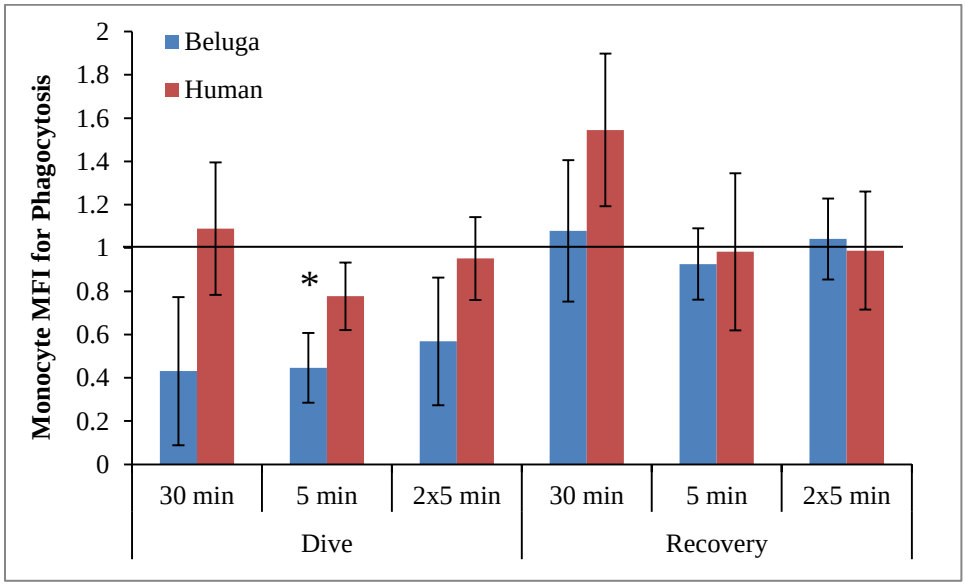


Figure B 1: Monocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

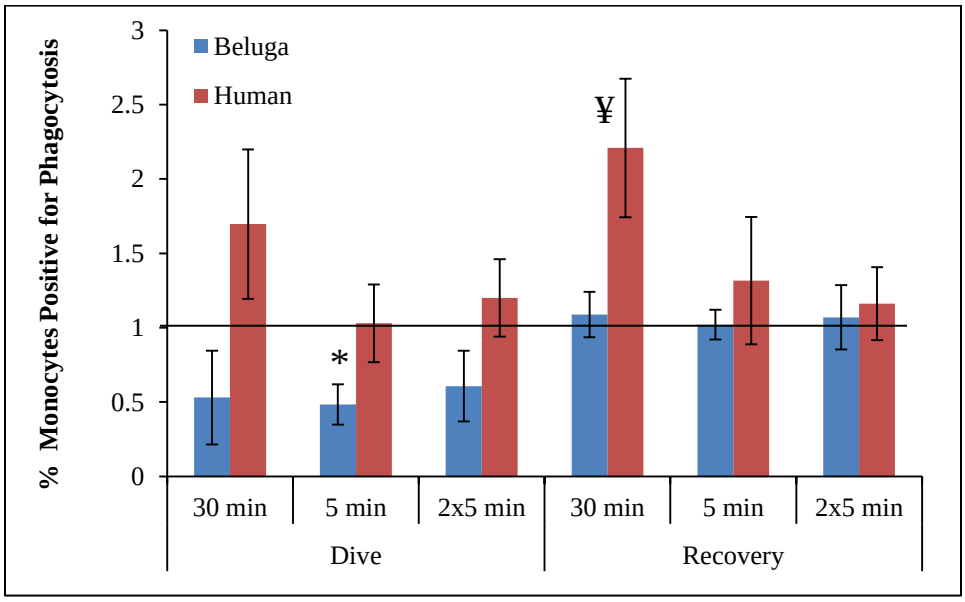


Figure B 2: % Monocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

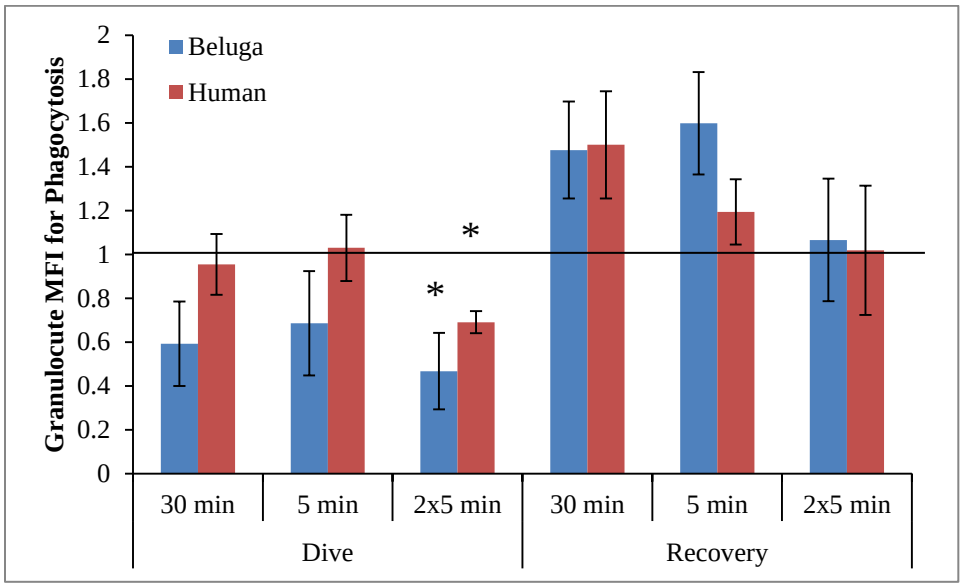


Figure B 3: Granulocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

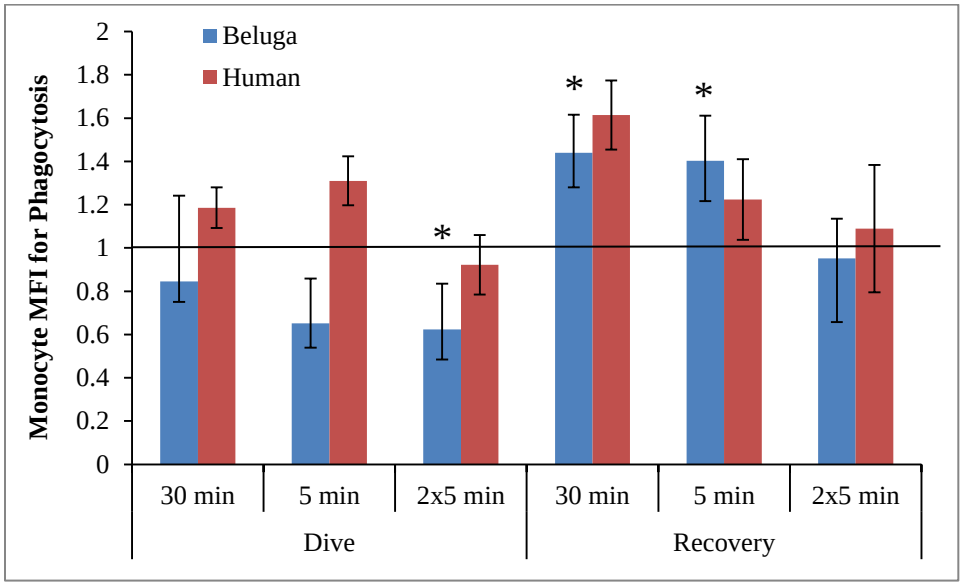


Figure B 4: Monocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

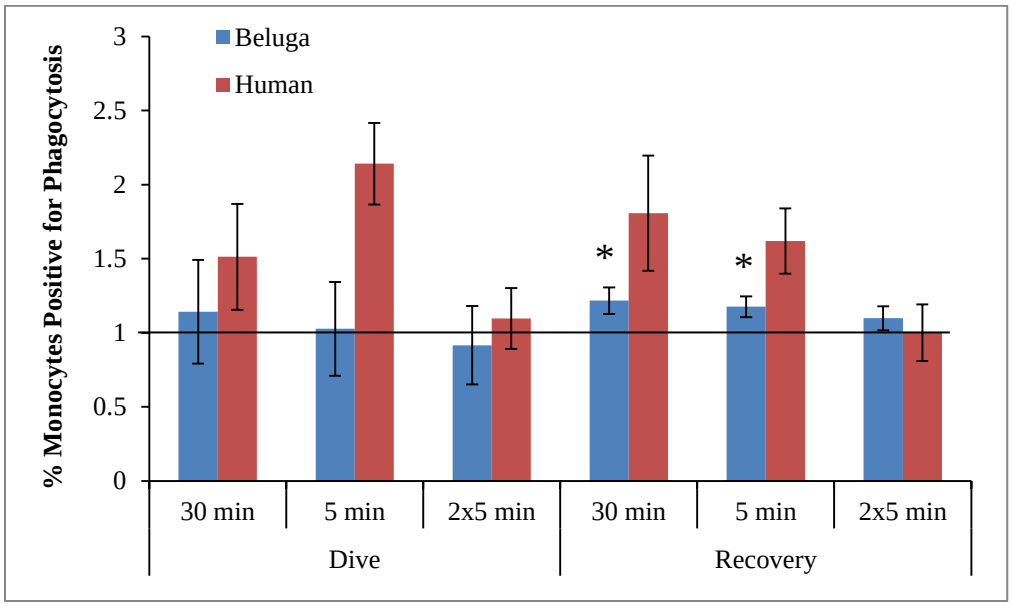


Figure B 5: % Monocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

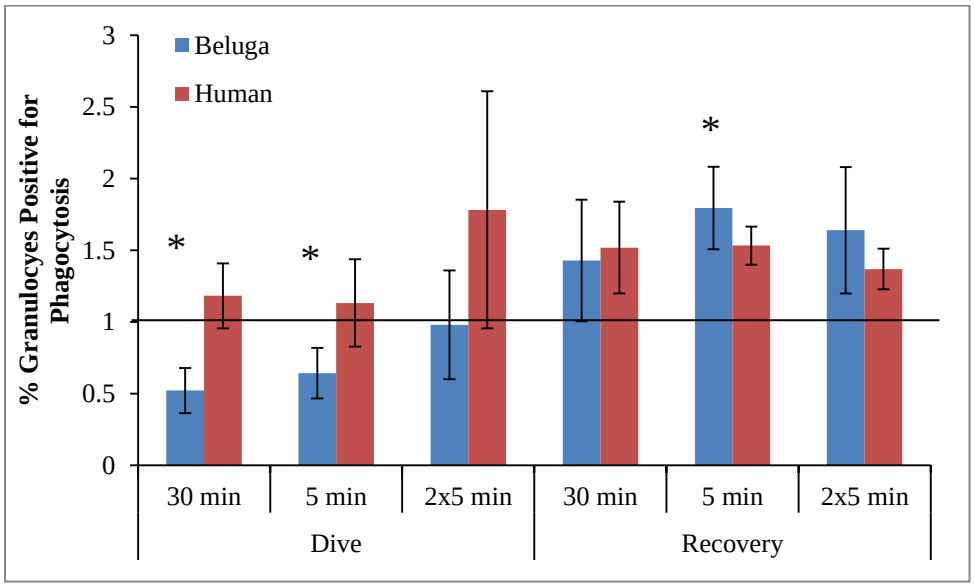


Figure B 6: % Granulocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

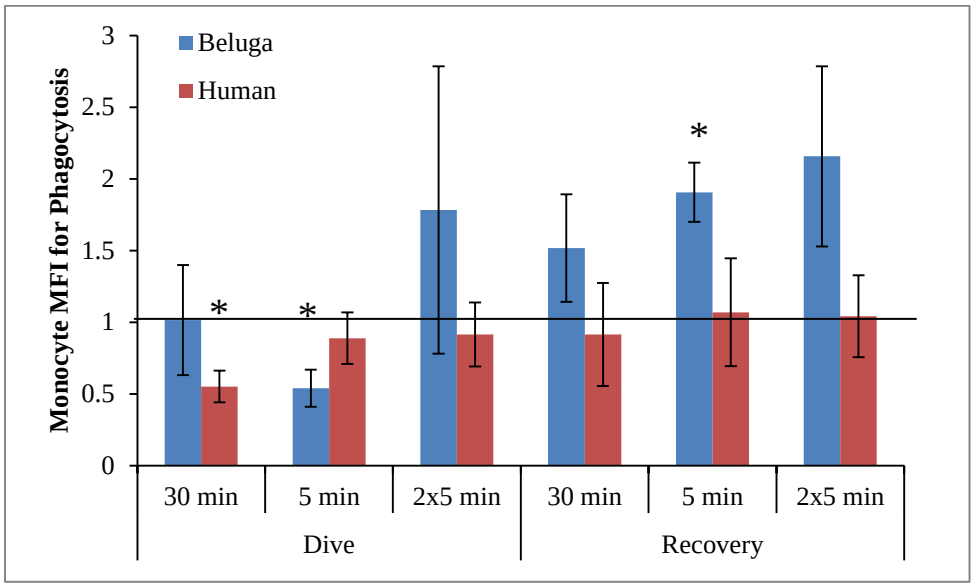


Figure B 7: Monocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

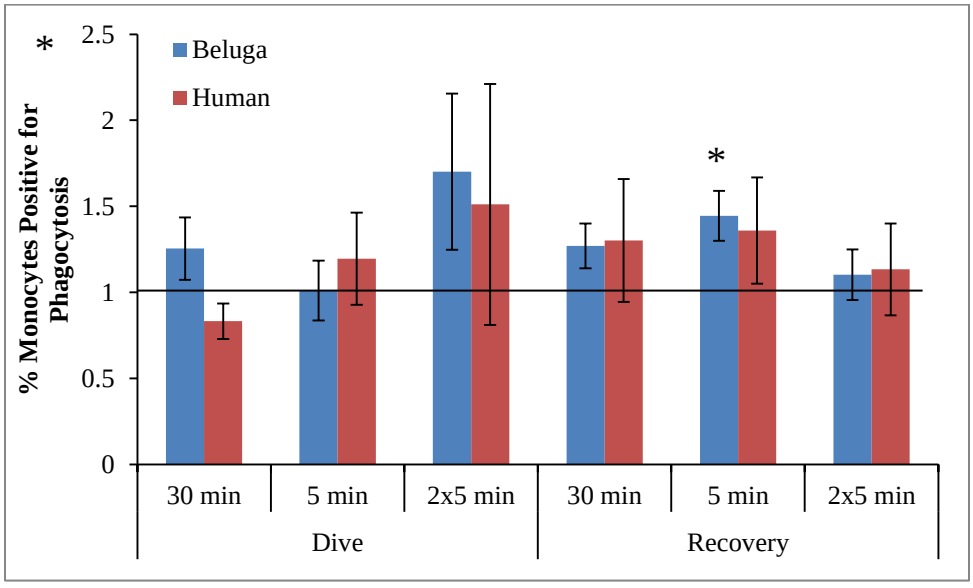


Figure B 8: % Monocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

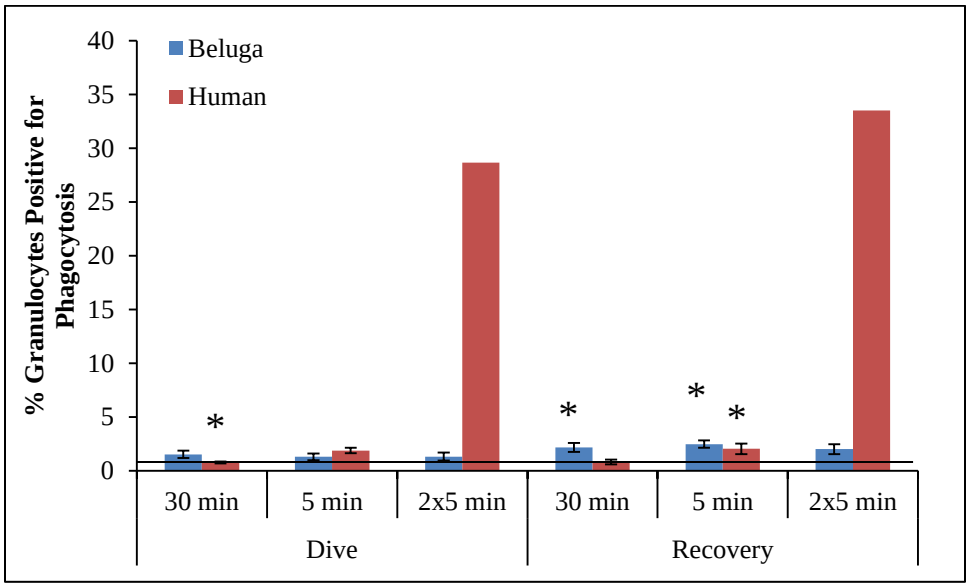


Figure B 9: % Granulocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

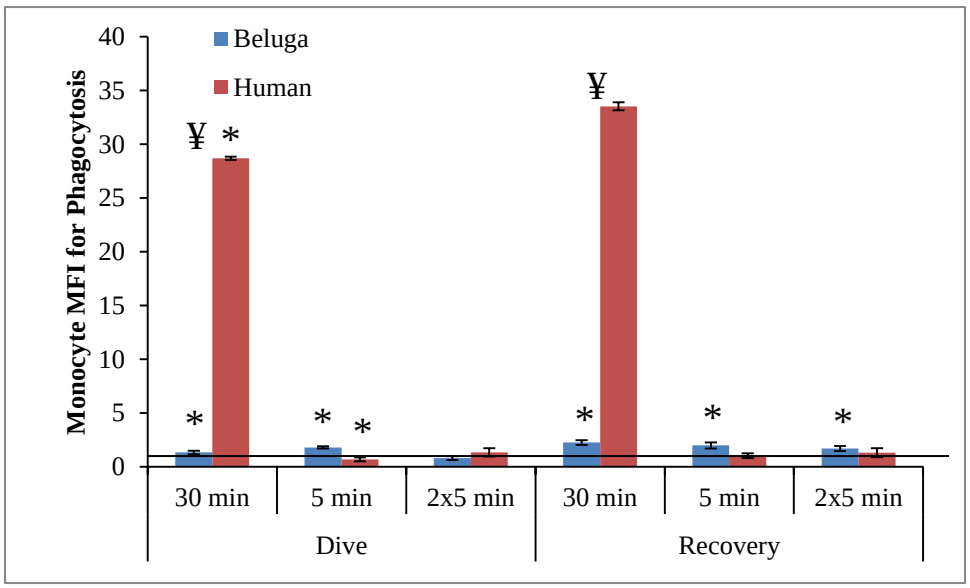


Figure B 10: Monocyte MFI for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

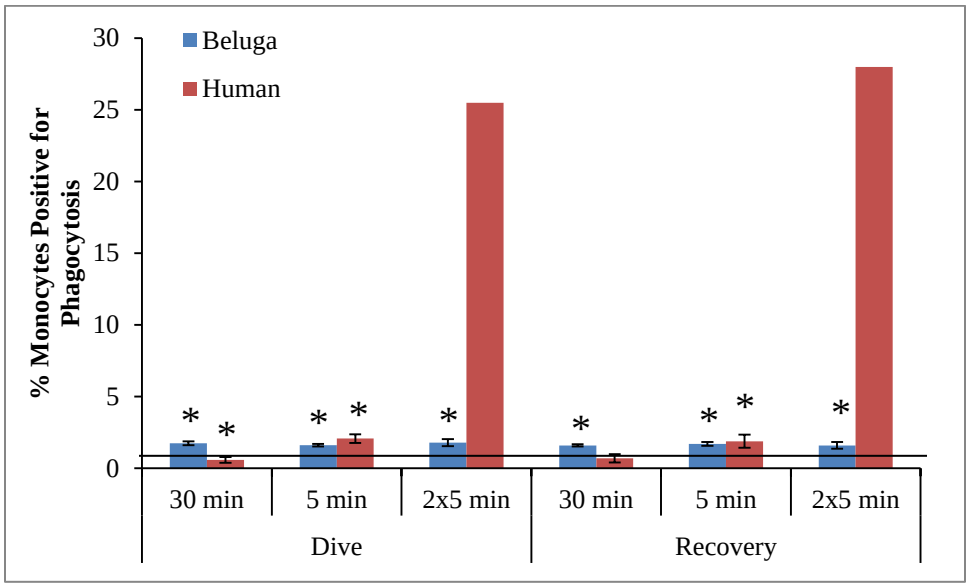


Figure B 11: % Monocytes positive for phagocytosis in beluga (n=4) and human (n=4) samples measured following exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Significant differences between belugas and humans are indicated with ¥ ($p<0.05$).

Appendix C

Mixed generalized linear model results comparing phagocytosis data between dive profiles for beluga baselines.

Phagocytosis between dive profiles			Dive		Recovery	
			F	p	F	p
Granulocytes	MFI	30 minute	5.77	0.002	3.669	0.021
		5 minute	2.249	0.099	2.59	0.068
		2x5 minute	5.699	0.003	0.751	0.529
	% Positive	30 minute	3.098	0.039	13.867	<0.001
		5 minute	7.587	<0.001	4.634	0.008
		2x5 minute	2.714	0.059	1.66	0.193
Monocytes	MFI	30 minute	15.236	<0.001	5.246	0.004
		5 minute	3.765	0.019	17.918	<0.001
		2x5 minute	1.261	0.302	4.609	0.008
	% Positive	30 minute	7.072	0.001	12.104	<0.001
		5 minute	33.531	<0.001	9.314	<0.001
		2x5 minute	8.189	<0.001	2.757	0.056

Appendix D

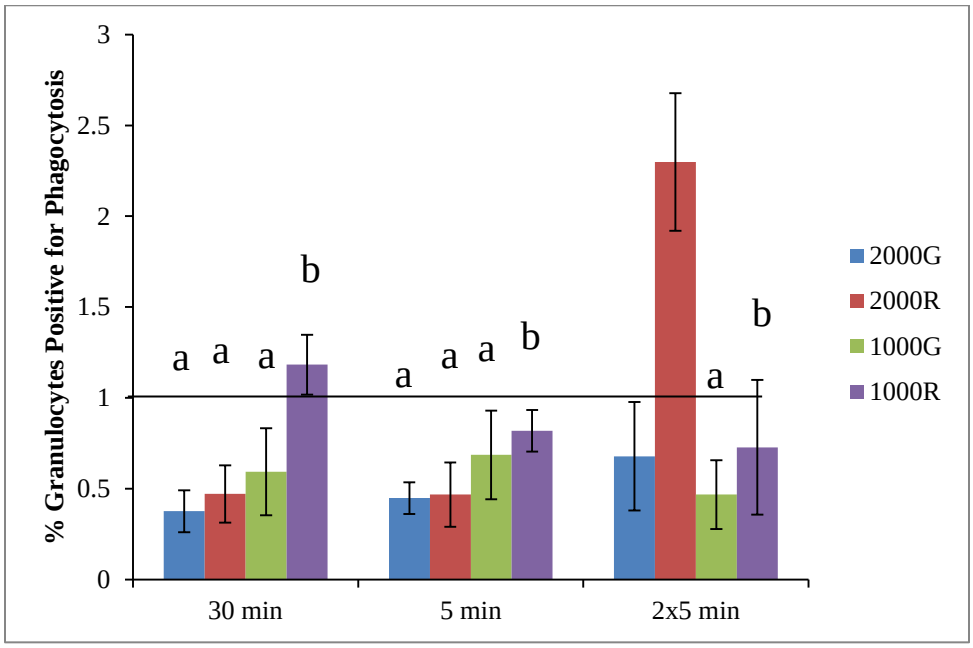


Figure D 1: % Granulocytes positive for phagocytosis in belugas (n=4) for the dive periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

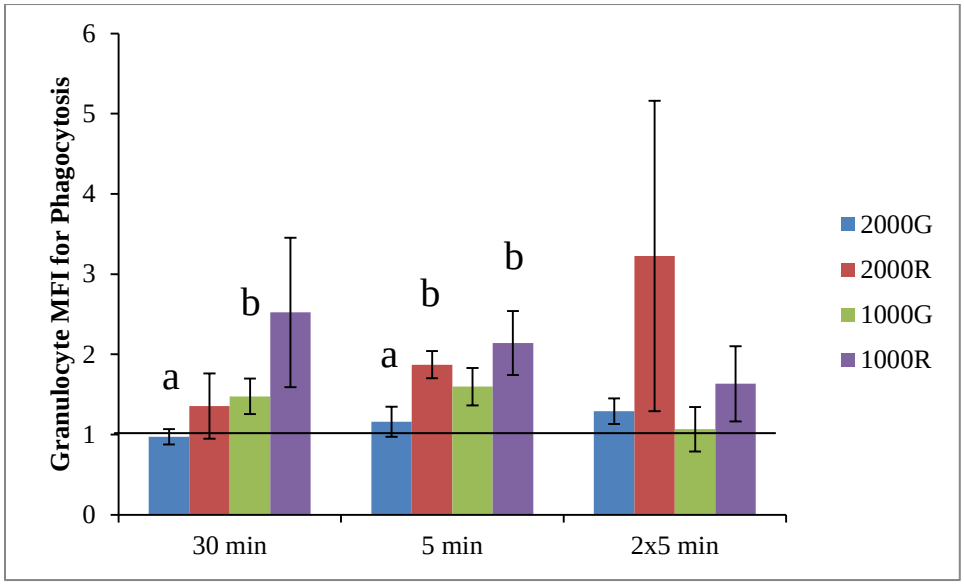


Figure D 2: Granulocyte MFI for phagocytosis in belugas (n=4) for the recovery periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

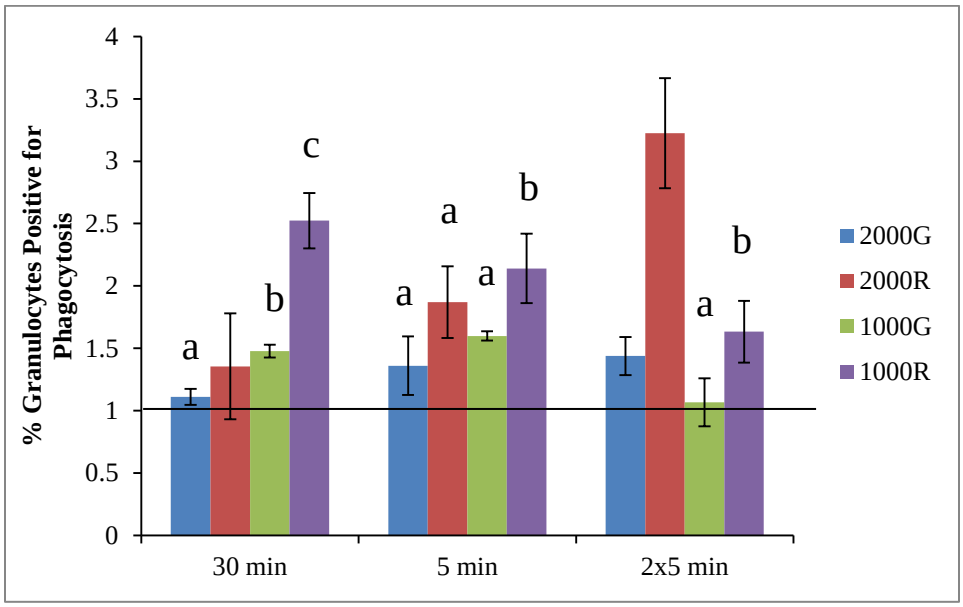


Figure D 3: % Granulocytes positive for phagocytosis in belugas (n=4) for the recovery periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters ($p<0.05$).

Appendix E

Mixed generalized linear model results comparing phagocytosis data between exposure durations.

Phagocytosis between durations			Dive		Recovery	
			F	p	F	p
Granulocytes	MFI	2000G	0.781	0.466	5.176	0.001
		1000G	3.297	0.048	1.345	0.274
		2000R	0.486	0.619	0.915	0.41
		1000R	4.632	0.016	0.521	0.599
	% Positive	2000G	0.284	0.754	2.18	0.128
		1000G	0.546	0.584	2.214	0.124
		2000R	1.149	0.328	0.301	0.742
		1000R	0.146	0.864	0.459	0.636
Monocytes	MFI	2000G	0.217	0.806	0.386	0.683
		1000G	0.215	0.807	1.09	0.347
		2000R	1.749	0.188	0.898	0.416
		1000R	19.708	<0.001	3.253	0.05
	% Positive	2000G	0.201	0.819	0.099	0.906
		1000G	0.157	0.855	0.091	0.913
		2000R	1.014	0.373	2.976	0.064
		1000R	0.536	0.59	0.819	0.449

Appendix F

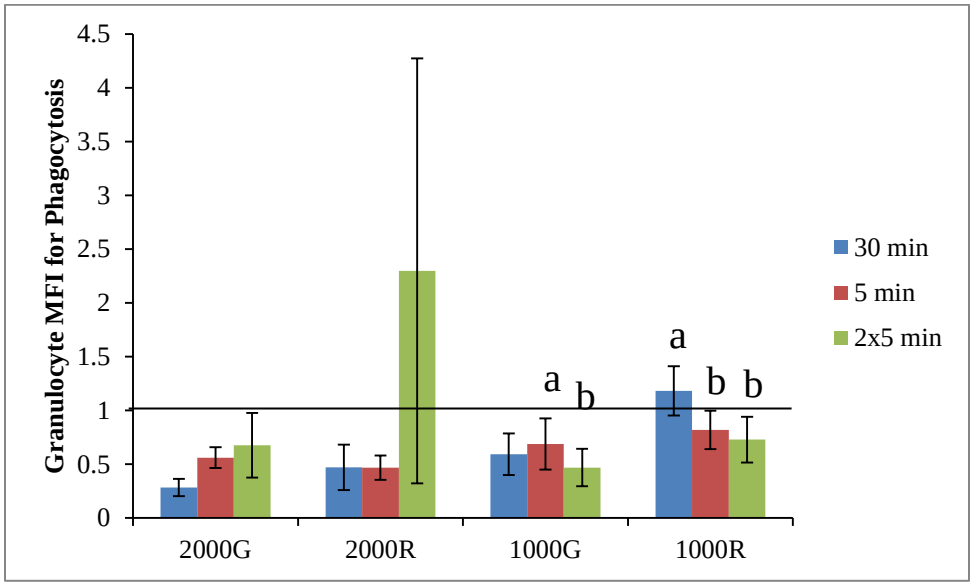


Figure F 1: Granulocyte MFI for phagocytosis in belugas (n=4) for the dive periods of all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

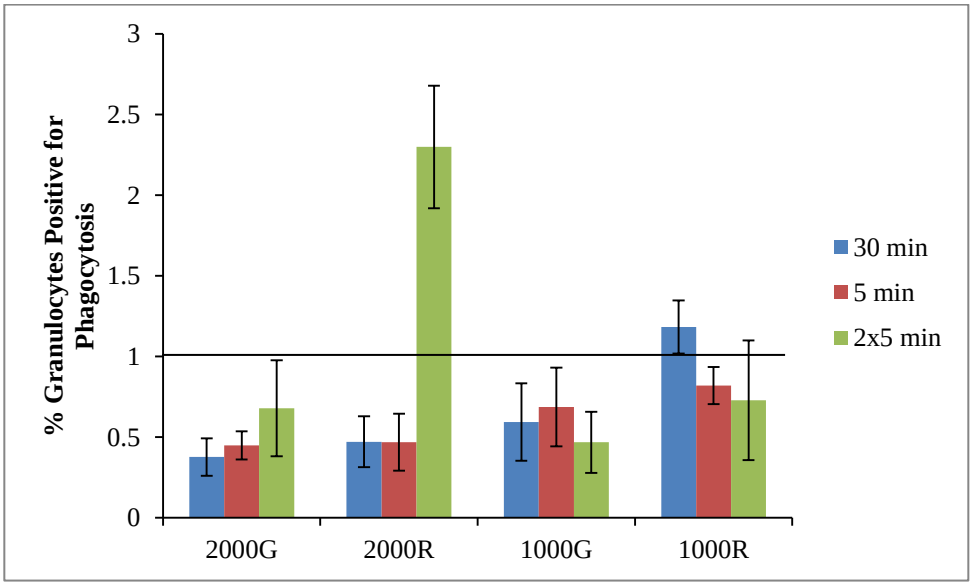


Figure F 2: % Granulocytes positive for phagocytosis in belugas (n=4) for the dive periods of all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

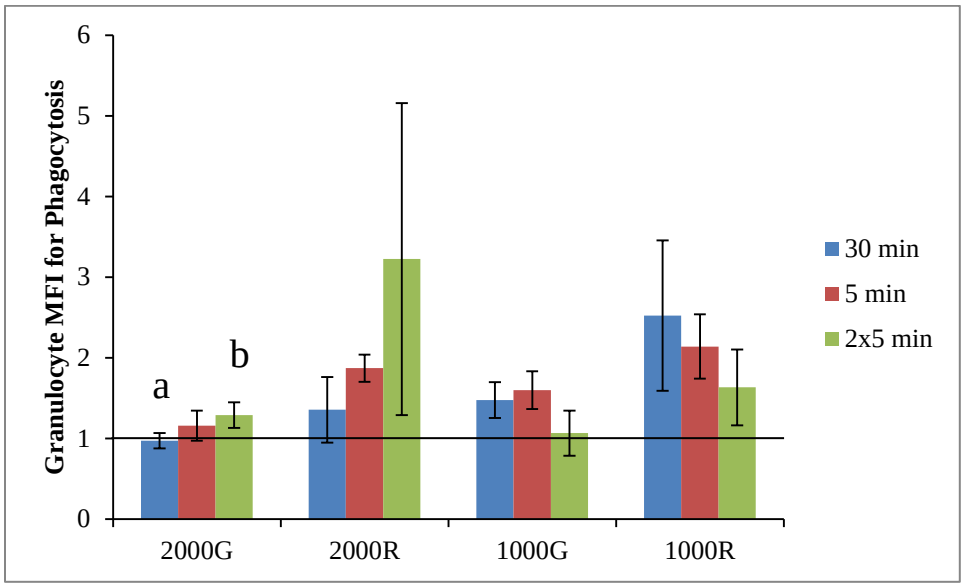


Figure F 3: Granulocyte MFI for phagocytosis in belugas (n=4) for the recovery periods of all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

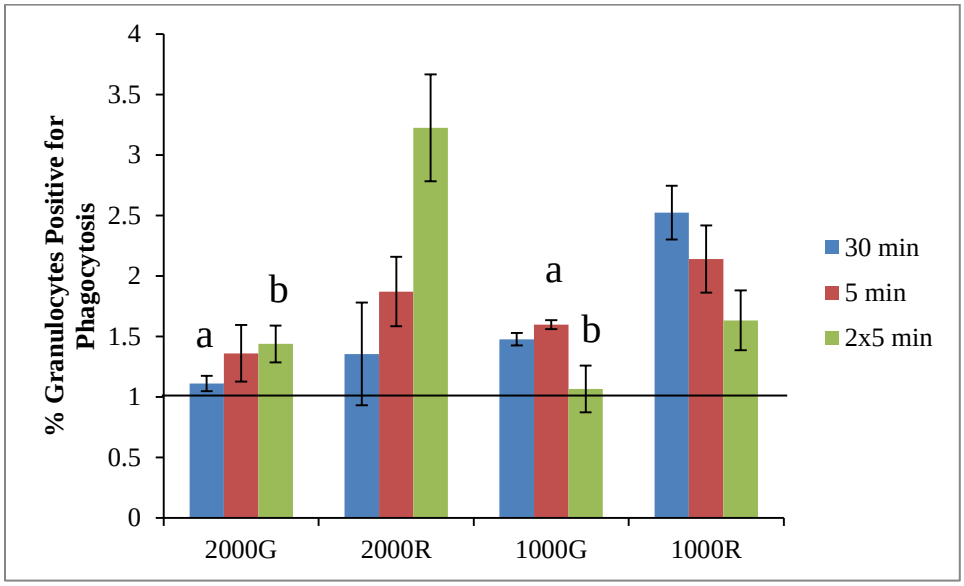


Figure F 4: % Granulocytes positive for phagocytosis in belugas (n=4) for the recovery periods of all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

Appendix G

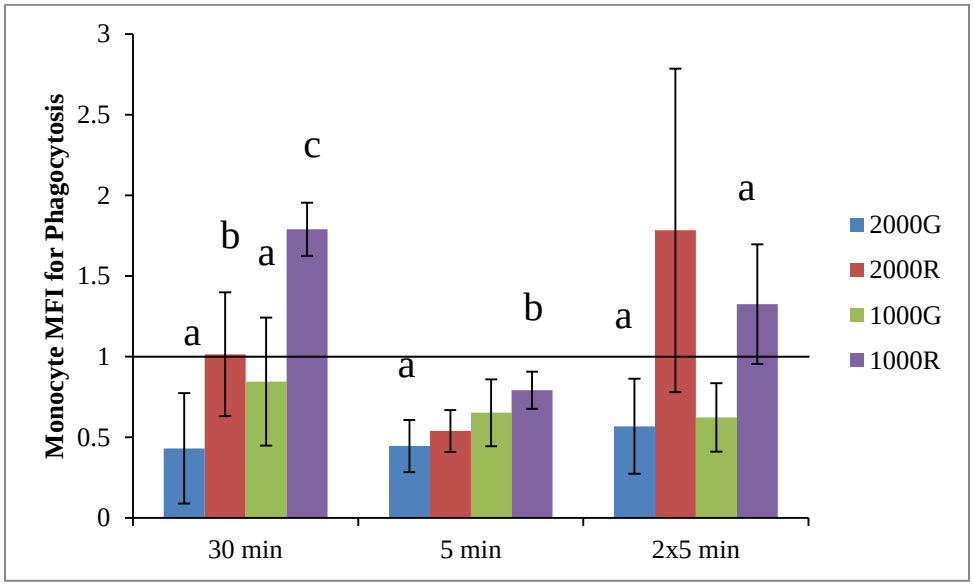


Figure G 1: Monocyte MFI for phagocytosis in belugas (n=4) for the dive periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

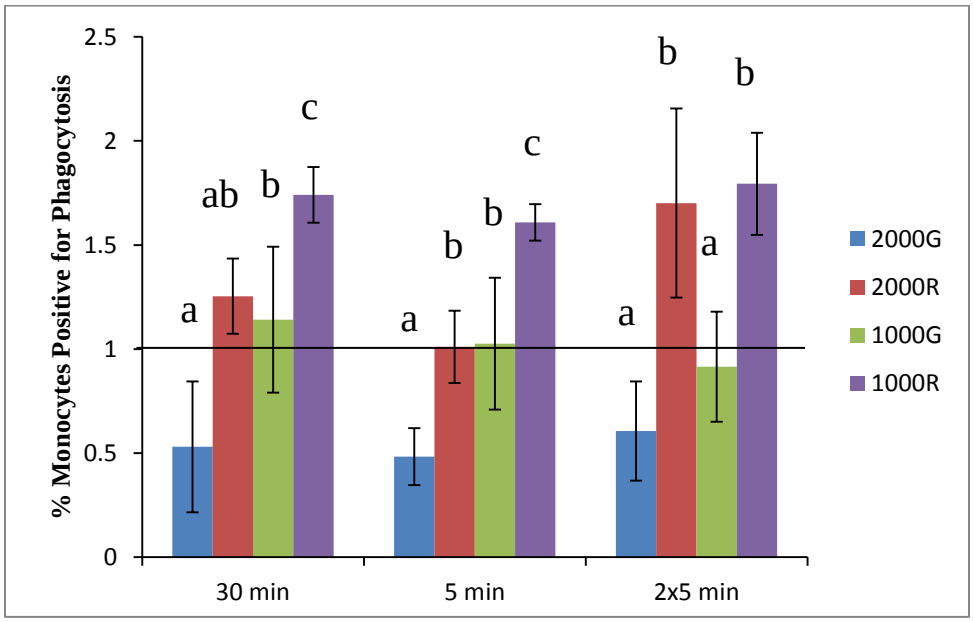


Figure G 2: % Monocytes positive for phagocytosis in belugas (n=4) for the dive periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

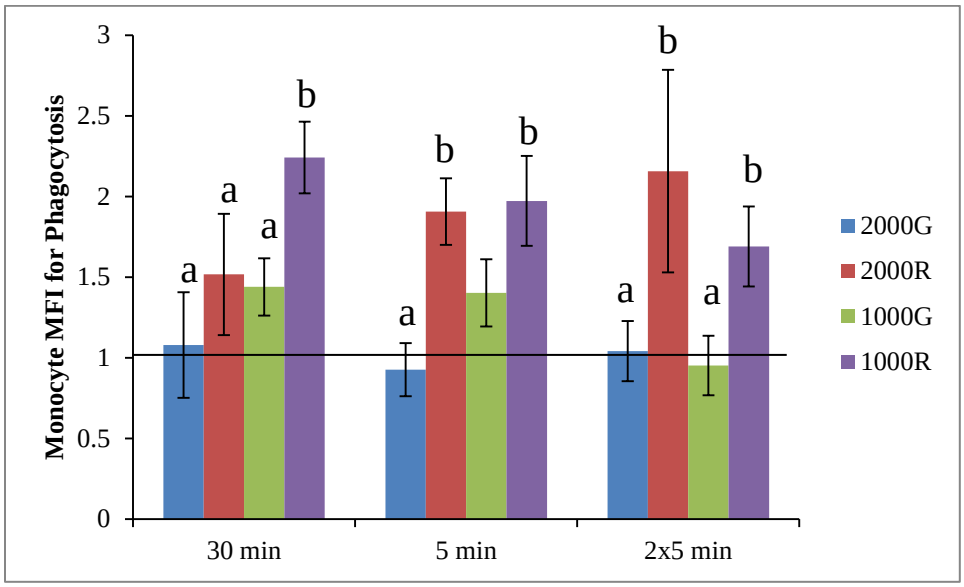


Figure G 3: Monocyte MFI for phagocytosis in belugas (n=4) for the recovery periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

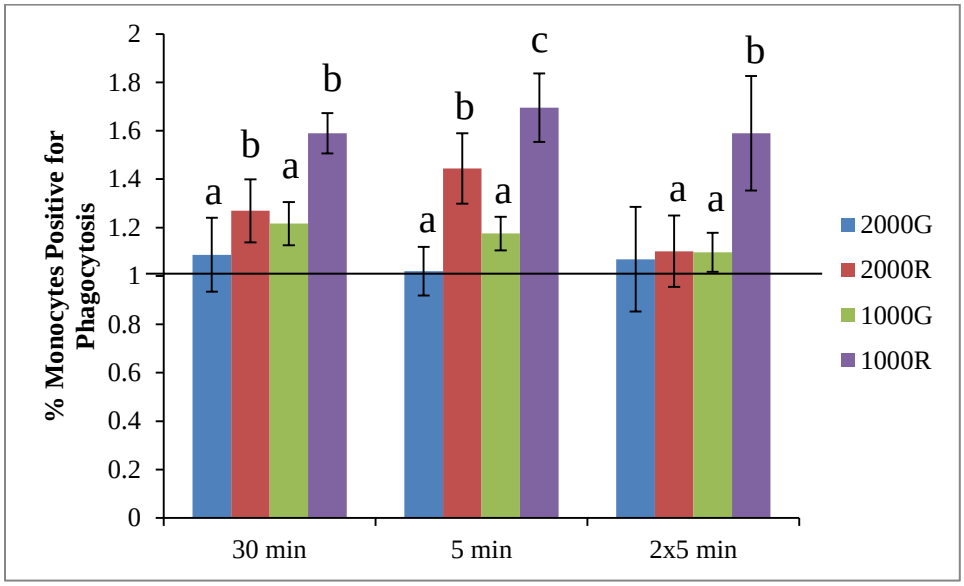


Figure G 4: % Monocytes positive for phagocytosis in belugas (n=4) for the recovery periods of all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

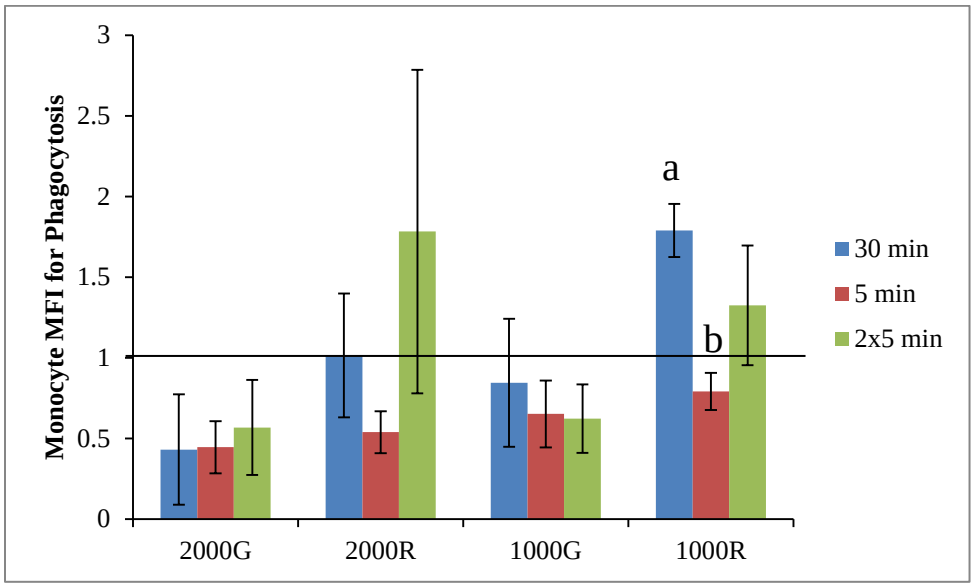


Figure G 5: Monocyte MFI for phagocytosis in belugas (n=4) for the dive periods of all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

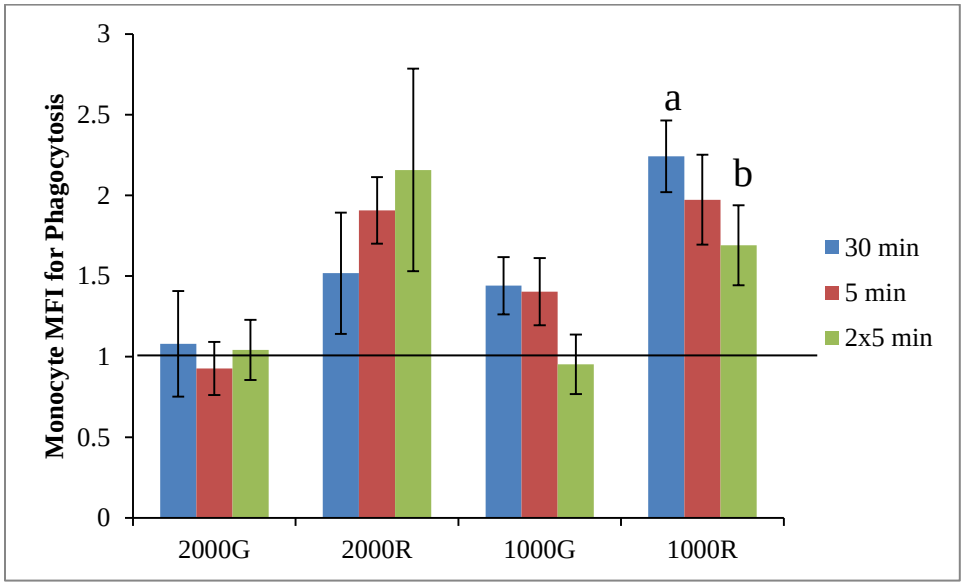


Figure G 6: Monocyte MFI for phagocytosis in belugas (n=4) for the recovery periods of all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. For each duration significant differences between pressures are indicated with letters (p<0.05).

Appendix H

Mixed generalized linear model results for CD11b expression.

CD11b Expression		30 minute		5 minute		2x5 minute	
		F	p	F	p	F	p
2000G	MFI	0.78	0.46	1.954	0.152	62.331	<0.001
	% Positive	2.39	0.101	0.651	0.526	34.458	<0.001
1000G	MFI	1.474	0.238	0.894	0.41	0.101	0.904
	% Positive	1.254	0.294	0.447	0.642	0.613	0.546
2000R	MFI	0.25	0.78	1.151	0.324	0.204	0.816
	% Positive	0.124	0.883	1.151	0.324	0.066	0.936
1000R	MFI	3.104	0.053	0.51	0.603	0.46	0.634
	% Positive	4.793	0.012	4.379	0.017	5.577	0.006

Appendix I

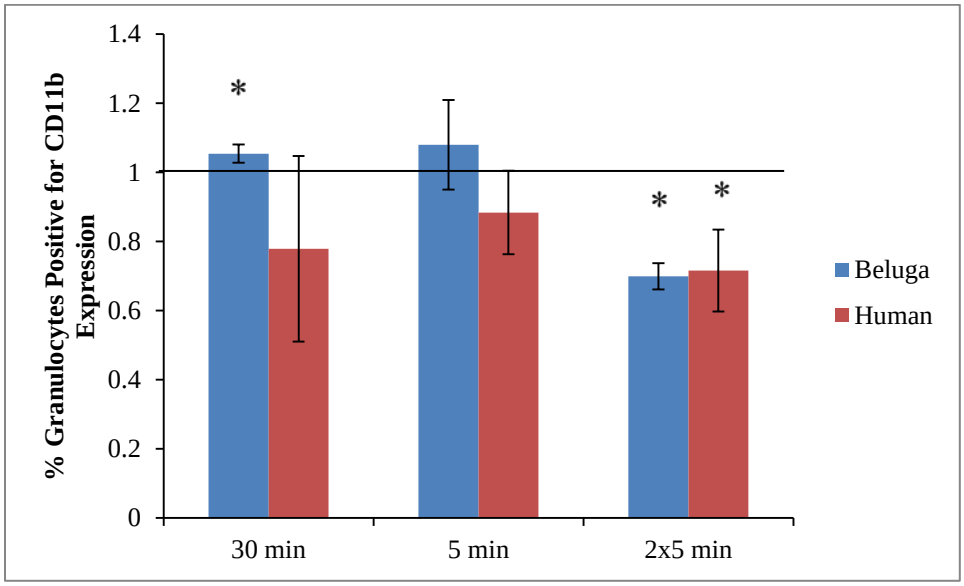


Figure I 1: % Granulocytes positive for CD11b expression in belugas (n=4) and humans (n=4) following exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures.

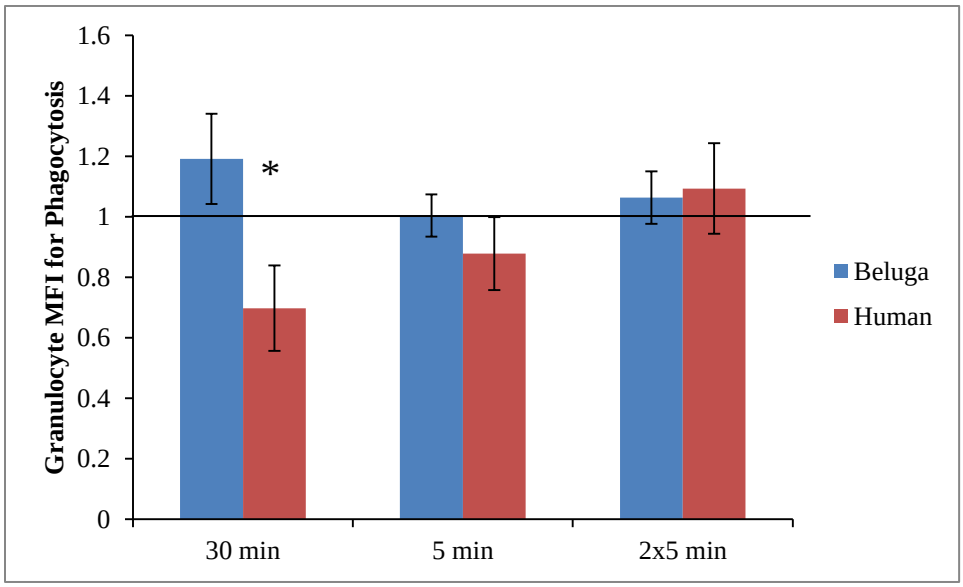


Figure I 2: Granulocyte MFI for CD11b expression in belugas (n=4) and humans (n=4) following exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$).

Appendix J

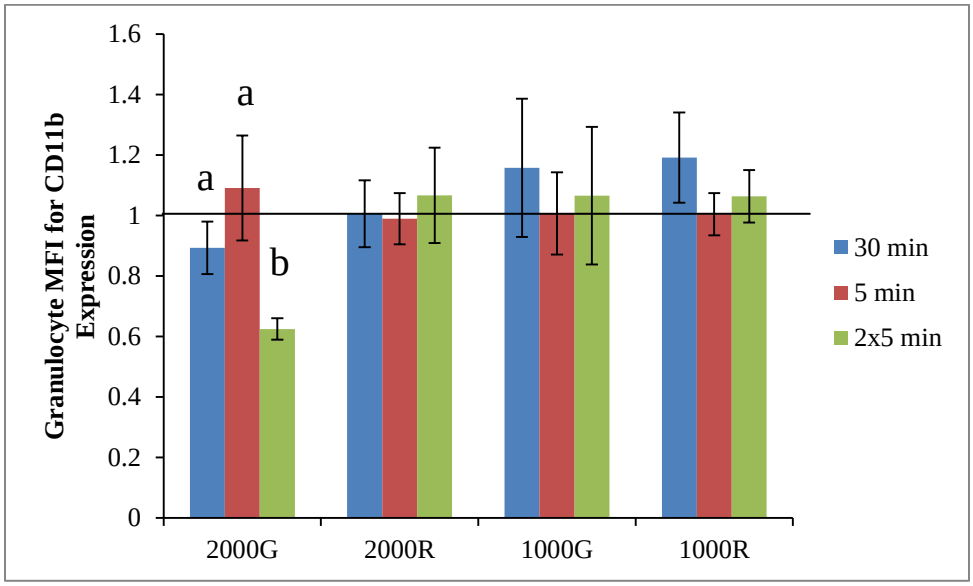


Figure J 1: Granulocyte MFI for CD11b expression in belugas (n=4) for all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between durations are indicated with letters (p<0.05).

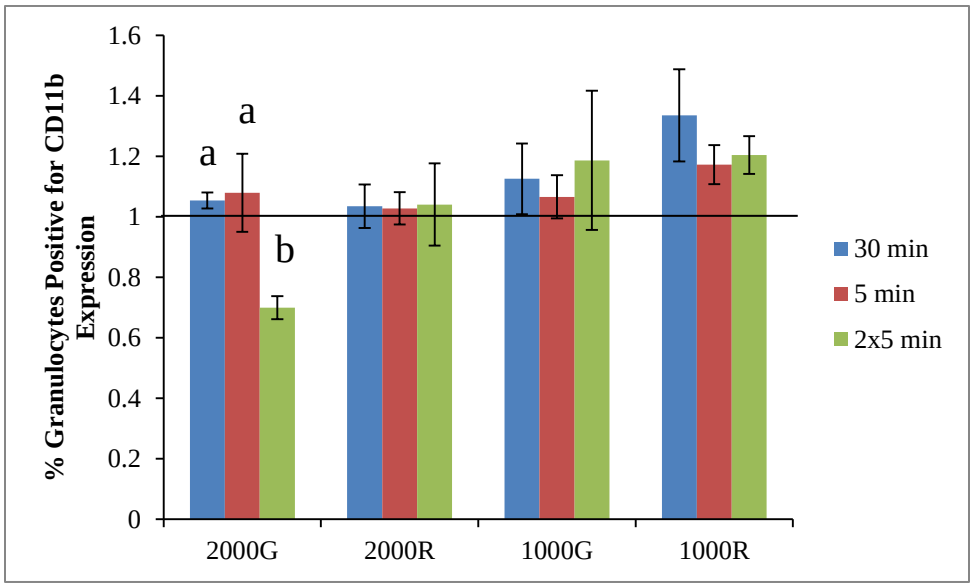


Figure J 2: % Granulocytes positive for CD11b expression in belugas (n=4) for all duration exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between durations are indicated with letters (p<0.05).

Appendix K

Mixed generalized linear model results for phagocytosis data from OWE conditions in belugas.

OWE				Phagocytosis			
				F	p	Dive	Recovery
2000psi	Granulocytes	MFI	30 minute	41.41	<0.001	<0.001	0.011
			5 minute	37.124	<0.001	<0.001	0.165
			2x5 minute	1.999	0.216	0.656	0.1
		% Positive	30 minute	8.538	0.018	0.009	0.134
			5 minute	17.197	0.003	0.001	0.107
			2x5 minute	13.066	0.007	0.003	0.133
	Monocytes	MFI	30 minute	396.28	<0.001	<0.001	0.051
			5 minute	112.281	<0.001	<0.001	0.135
			2x5 minute	443.946	<0.001	<0.001	0.791
		% Positive	30 minute	82.824	<0.001	<0.001	0.072
			5 minute	84.876	<0.001	<0.001	0.053
			2x5 minute	53.303	<0.001	<0.001	0.37

Appendix L

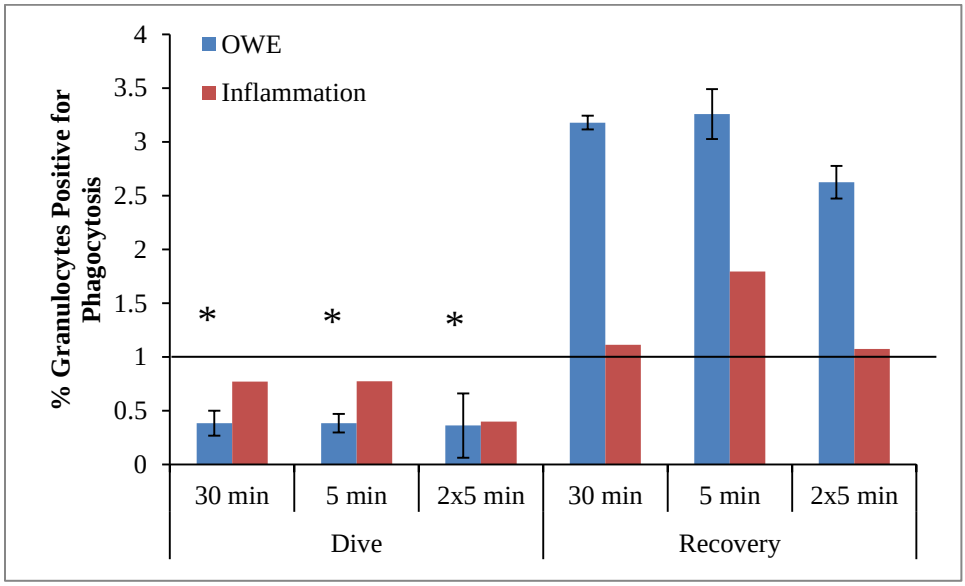


Figure L 1: % Granulocytes positive for phagocytosis during OWE (n=3) and inflammation (n=2) conditions in belugas for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with as asterisk * ($p<0.05$). Due to small samples size no statistics could be run for inflammation data.

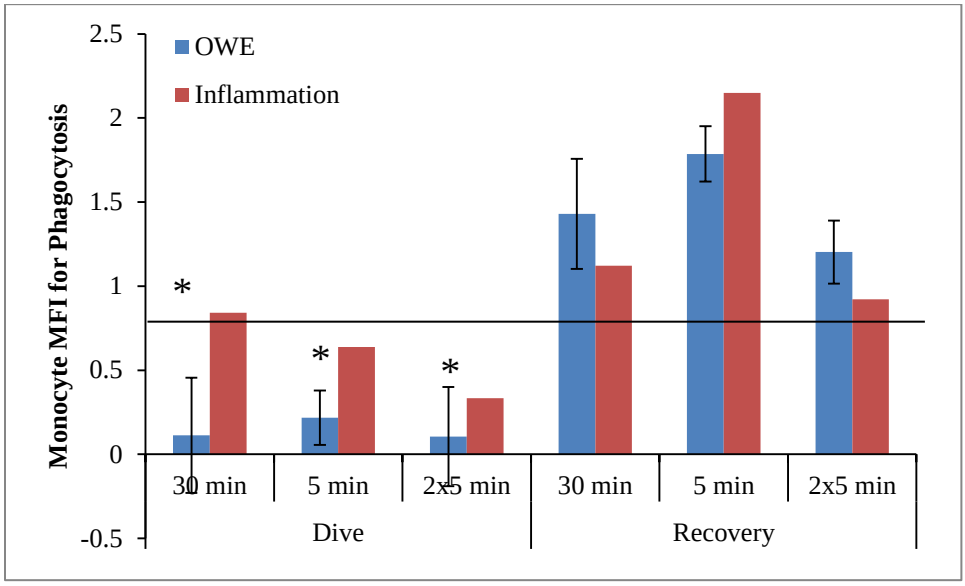


Figure L 2: Monocyte MFI for phagocytosis during OWE (n=3) and inflammation (n=2) conditions in belugas for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with as asterisk * ($p<0.05$). Due to small samples size no statistics could be run for inflammation data.

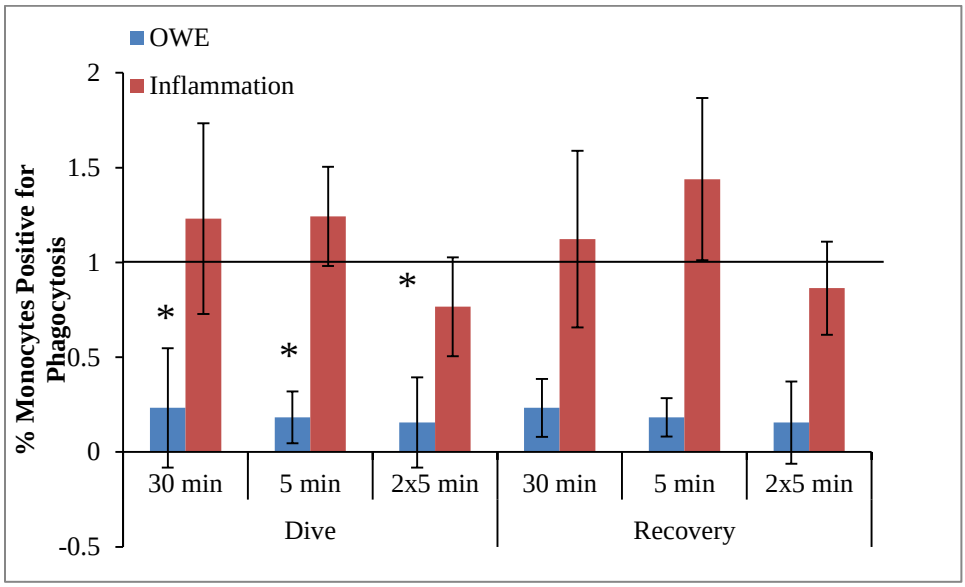


Figure L 3: % Monocytes positive for phagocytosis during OWE (n=3) and inflammation (n=2) conditions in belugas for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with as asterisk * ($p<0.05$). Due to small samples size no statistics could be run for inflammation data.

Appendix M

Mixed generalized linear models for CD11b expression during OWE conditions in belugas.

OWE		CD11b	
		F	p
2000G	MFI	3.393	0.083
	% Positive	2.503	0.133

Appendix N

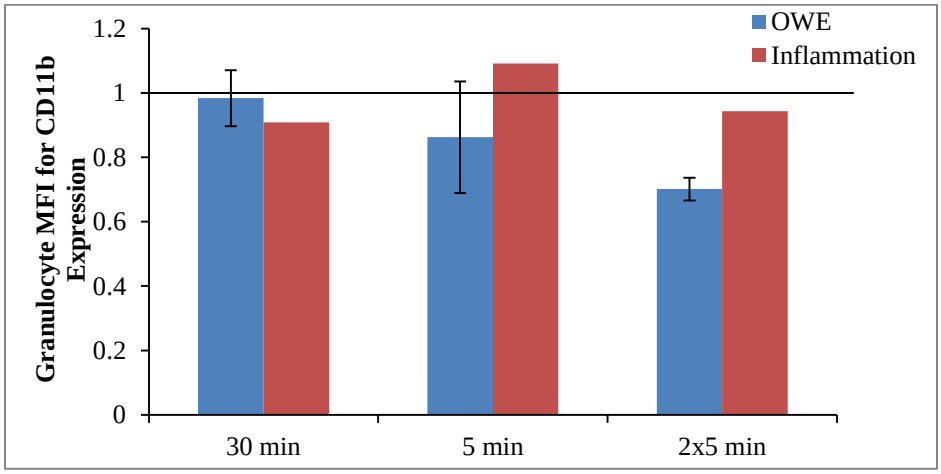


Figure N 1: Granulocyte MFI for CD11b expression during OWE (n=3) and inflammation (n=2) conditions in belugas for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small samples size no statistics could be run for inflammation data.

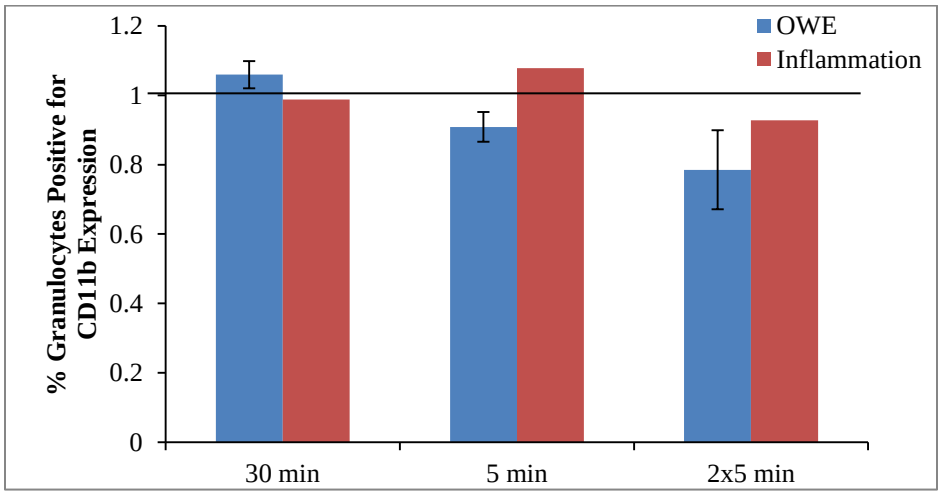


Figure N 2: % Granulocytes positive for CD11b expression during OWE (n=3) and inflammation (n=2) conditions in belugas for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures.). Due to small samples size no statistics could be run for inflammation data.

Appendix O

Generalized mixed linear model results comparing phagocytosis results between conditions in belugas and humans.

Phagocytosis between conditions			Dive		Recovery	
			F	p	F	p
Granulocytes	MFI	30 minute	3.108	0.072	11.229	0.001
		5 minute	1.03	0.379	1.068	0.37
		2x5 minute	0.176	0.84	2.267	0.136
	% Positive	30 minute	4.189	0.036	5.237	0.019
		5 minute	1.283	0.304	1.231	0.318
		2x5 minute	3.729	0.047	0.849	0.446
Monocytes	MFI	30 minute	3.139	0.071	2.542	0.11
		5 minute	1.828	0.193	2.462	0.117
		2x5 minute	0.535	0.596	0.42	0.959
	% Positive	30 minute	2.506	0.113	4.638	0.026
		5 minute	7.191	0.007	2.896	0.089
		2x5 minute	9.011	0.002	0.213	0.811

Appendix P

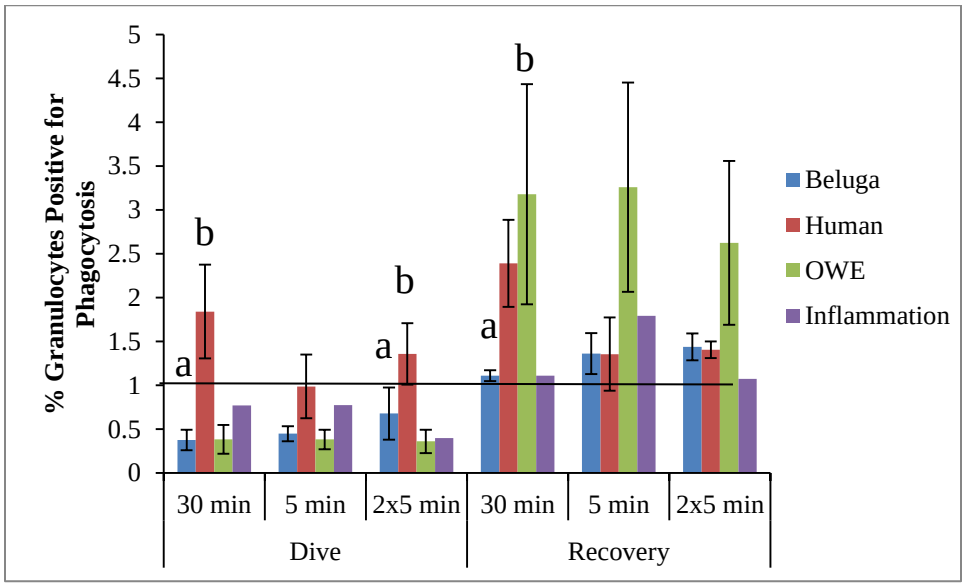


Figure P 1: % Granulocytes positive for phagocytosis during baseline (n=4), OWE (n=3) and inflammation (n=2) conditions in belugas, and humans for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with letters (p<0.05). Due to small samples size no statistics could be run for inflammation data.

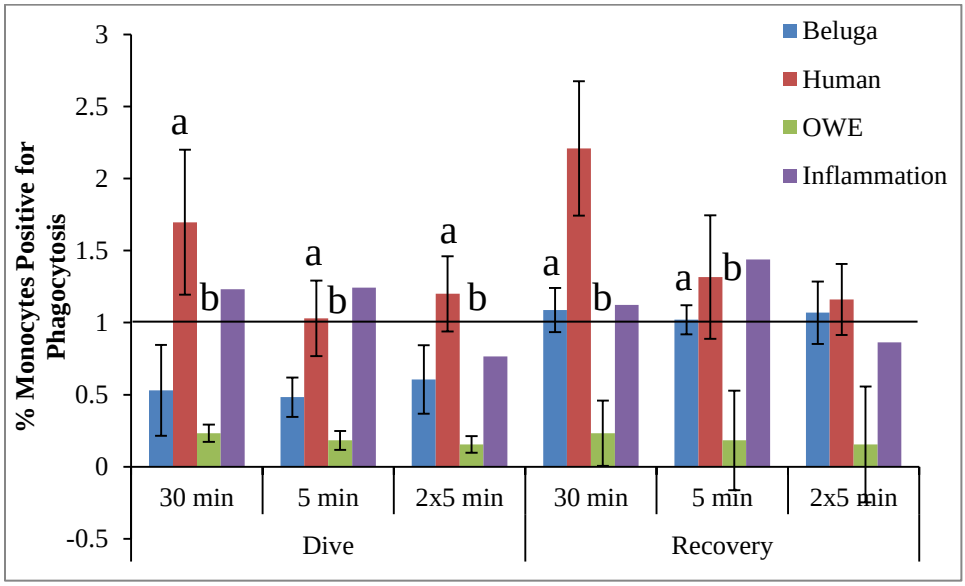


Figure P 2: % Monocytes positive for phagocytosis during baseline (n=4), OWE (n=3) and inflammation (n=2) conditions in belugas, and humans for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with letters (p<0.05). Due to small samples size no statistics could be run for inflammation data.

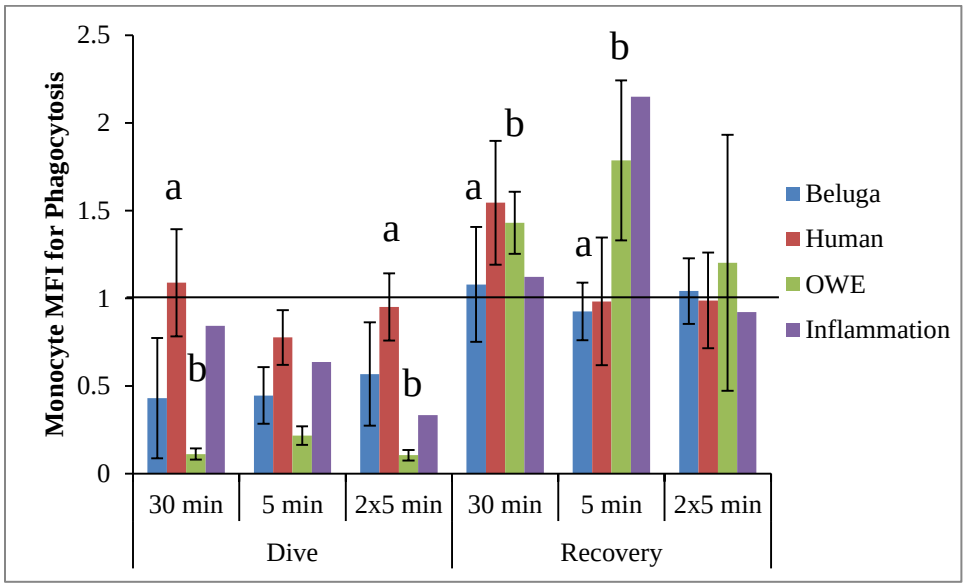


Figure P 3: Monocyte MFI for phagocytosis during baseline (n=4), OWE (n=3) and inflammation (n=2) conditions in belugas, and humans for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between conditions are indicated with letters ($p<0.05$). Due to small samples size no statistics could be run for inflammation data.

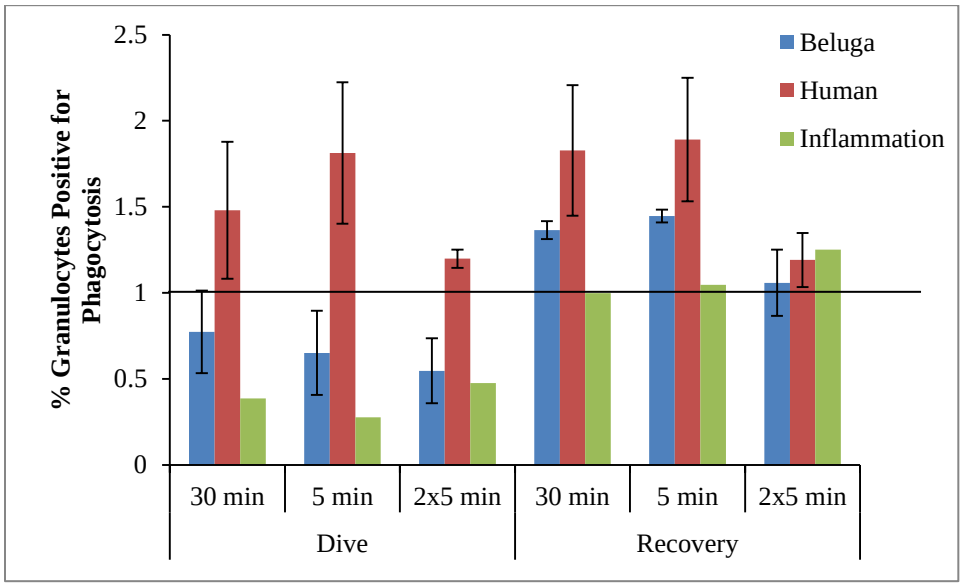


Figure P 4: % Granulocytes positive for phagocytosis during baseline (n=4) and inflammation (n=2) conditions in belugas, and humans for all duration exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small samples size no statistics could be run for inflammation data.

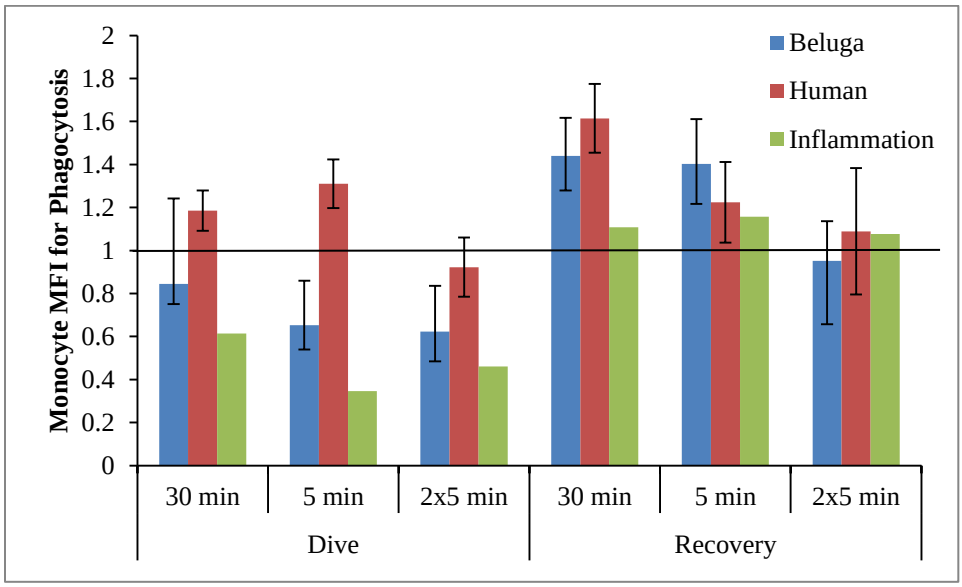


Figure P 5: Monocyte MFI for phagocytosis during baseline (n=4) and inflammation (n=2) conditions in belugas, and humans for all duration exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small samples size no statistics could be run for inflammation data.

Appendix Q

Mixed generalized linear model results comparing CD11b expression between conditions in belugas and humans.

CD11b between conditions		F	p
MFI	30 minute	0.28	0.759
	5 minute	1.07	0.359
	2x5 minute	0.807	0.458
% Positive	30 minute	1.4	0.267
	5 minute	0.634	0.54
	2x5 minute	0.524	0.599

Appendix R

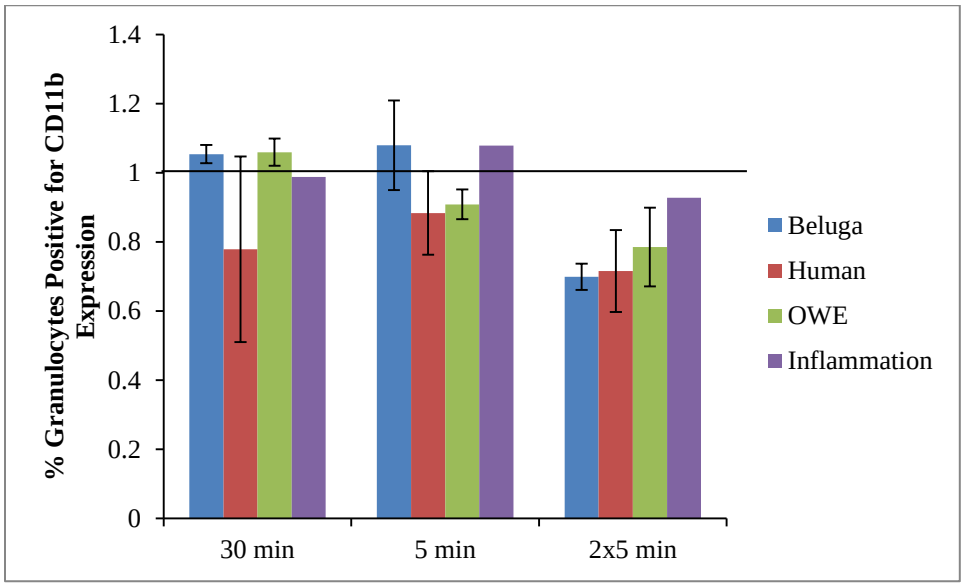


Figure R 1: % granulocytes positive for CD11b expression during baseline (n=4), OWE (n=3) and inflammation (n=2) conditions in belugas, and humans for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Due to small samples size no statistics could be run for inflammation data.

Appendix S

Mixed generalized linear model results for Lymphocyte Proliferation for beluga baseline and stressor conditions as well as humans.

Proliferation		Beluga			Human
		Baseline	OWE	Wild	
30 minutes	F	27.78	19.27	2.44	1.525
	p	<0.001	<0.001	0.125	0.212
5 minutes	F	3.5	18.475	2.014	0.952
	p	0.042	<0.001	0.157	0.396
2x5 minutes	F	3.568	5.588	4.348	1.325
	p	0.04	0.024	0.045	0.279

Appendix T

Mixed generalized linear model results comparing proliferation results between conditions in belugas and humans.

Between Conditions	F	p
30 minute	10.251	<0.001
5 minute	5.073	0.004
2x5 minute	3.904	0.014

Appendix U

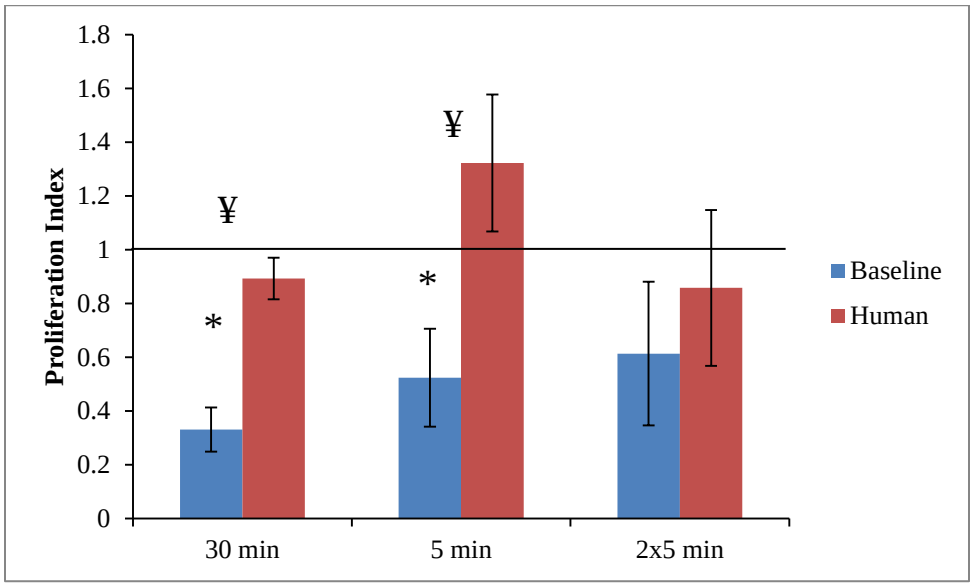


Figure U 1: Proliferation indices for beluga baselines (n=4) and humans for all duration exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

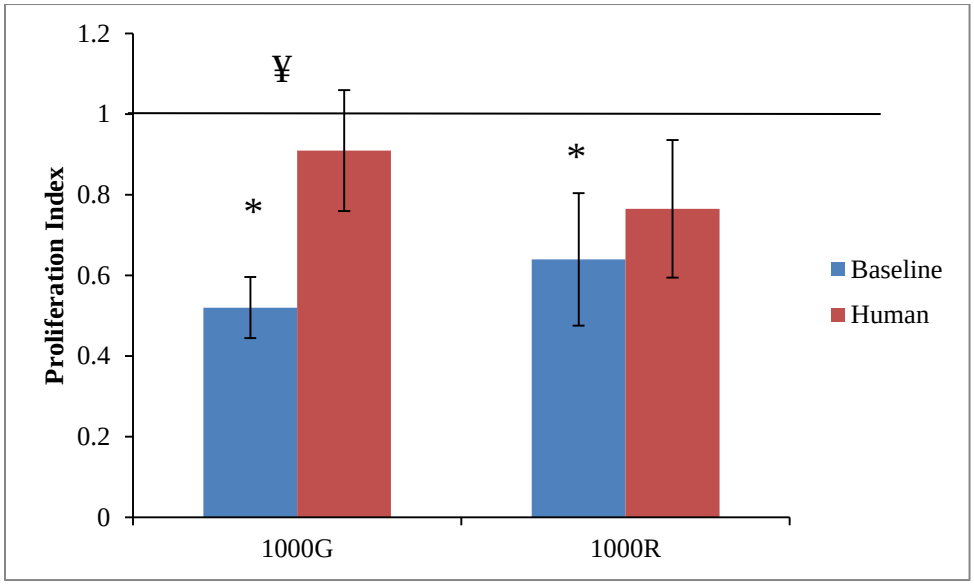


Figure U 2: Proliferation indices for beluga baselines (n=4) and humans for all duration exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

Appendix V

Mixed generalized linear model results comparing proliferation results between dive profiles and durations for beluga baselines.

Between dives	F	p
30 minute	1.472	0.258
5 minute	2.31	0.147
2x5 minute	0.033	0.857

Durations	F	p
2000G	0.872	0.436
2000R	0.872	0.126

Appendix W

Mixed generalized linear model results for PBMC IL2R expression in belugas and humans following pressure exposures.

IL2R Expression		30 minute		5 minute		2x5 minute	
		F	p	F	p	F	p
2000G	MFI	5.97	<0.001	3.547	0.011	4.276	0.004
	% Positive	4.889	0.002	0.785	0.539	9.733	<0.001
1000G	MFI	6.749	0.002	58.271	<0.001	46.053	<0.001
	% Positive	2.649	0.078	1.599	0.209	1.588	0.202
2000R	MFI	6.352	0.003	5.383	0.007	3.946	0.024
	% Positive	29.423	<0.001	1.396	0.254	1.413	0.25
1000R	MFI	6.273	0.003	1.137	0.327	0.856	0.429
	% Positive	2.323	0.105	1.436	0.245	2.187	0.12

Appendix X

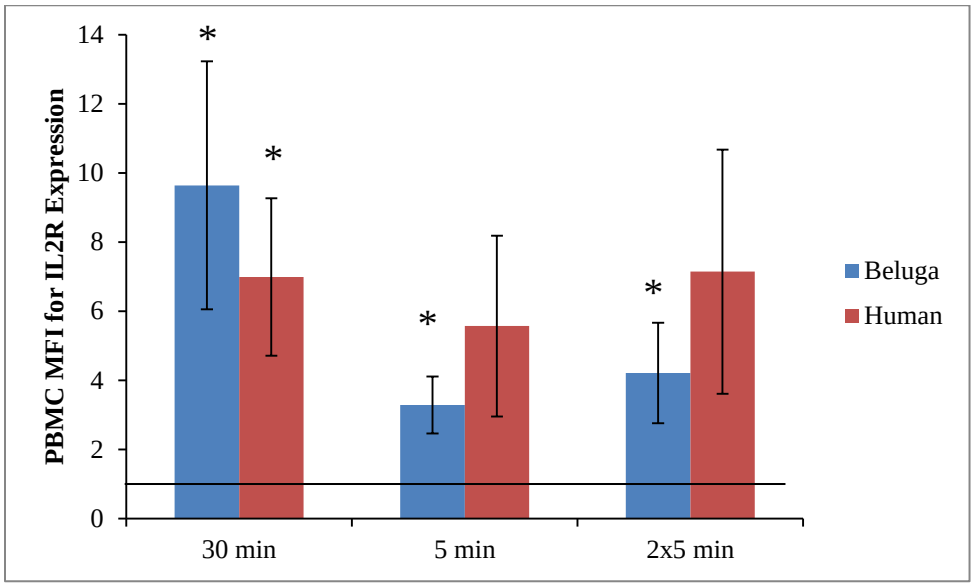


Figure X 1: PBMC MFI for Il2R expression in beluga baselines (n=4) and humans for all duration exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

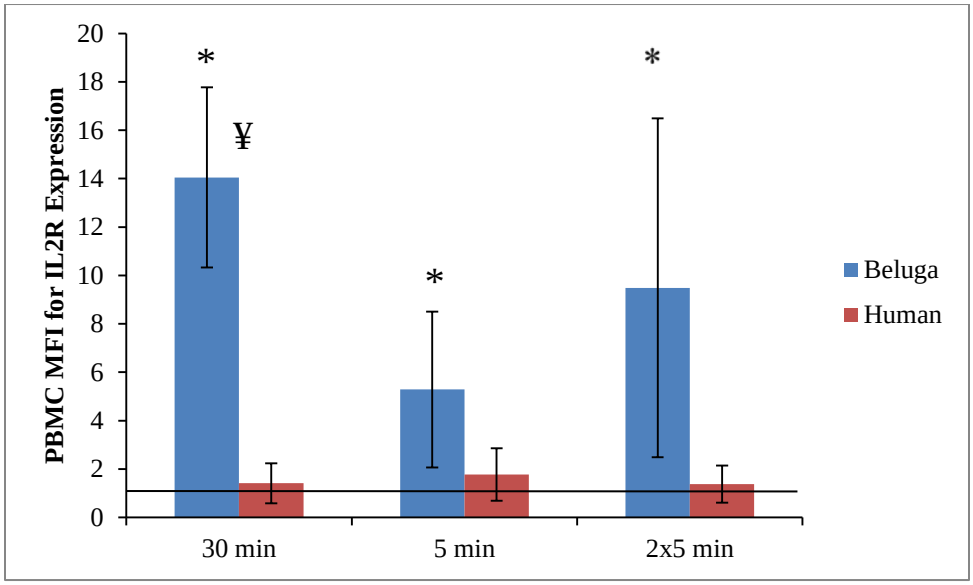


Figure X 2: PBMC MFI for IL2R expression for baseline (n=4) and inflammation conditions (n=2) in aquarium belugas and humans for all duration exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05). Significant differences between belugas and humans are indicated with ¥ (p<0.05).

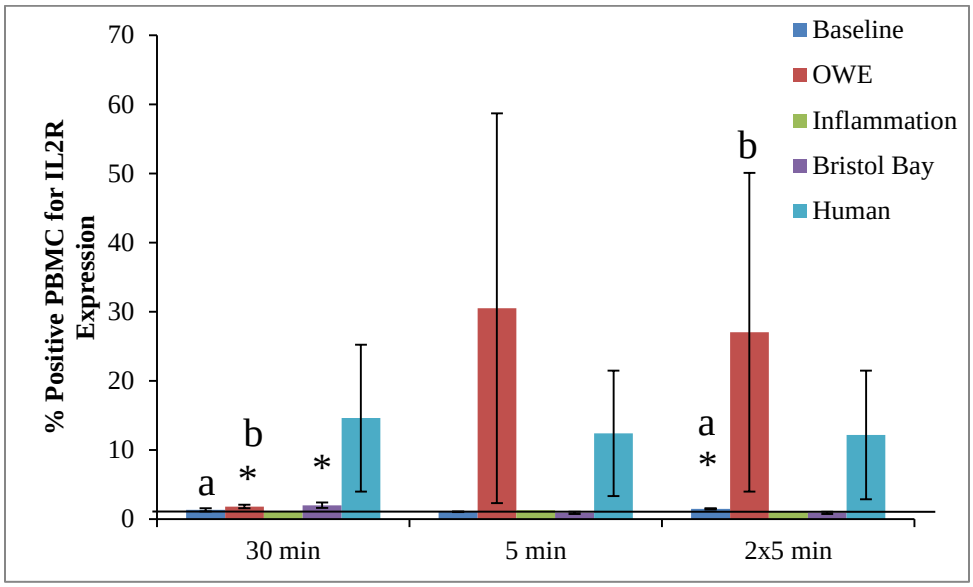


Figure X 3: % PBMC positive for baselines(n=4), OWE (n=3), Inflammation (n=2) conditions in aquarium belugas, wild belugas from Bristol Bay (n=9) and humans (n=4) for all duration exposures to 2000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$). Significant differences between conditions are indicated with letters ($p<0.05$).

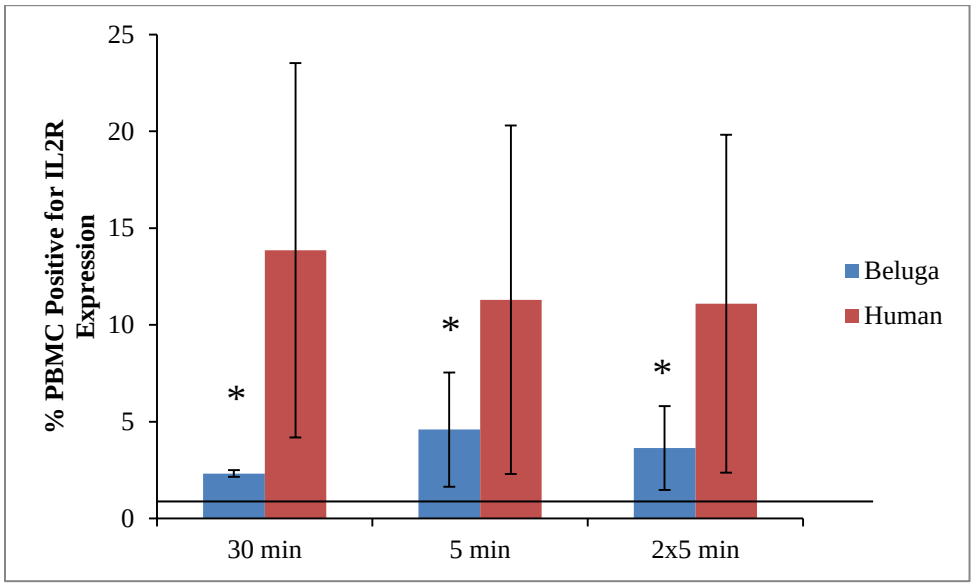


Figure X 4: % PBMC positive for IL2R expression for beluga baselines (n=4) and humans for all duration exposures to 2000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * ($p<0.05$).

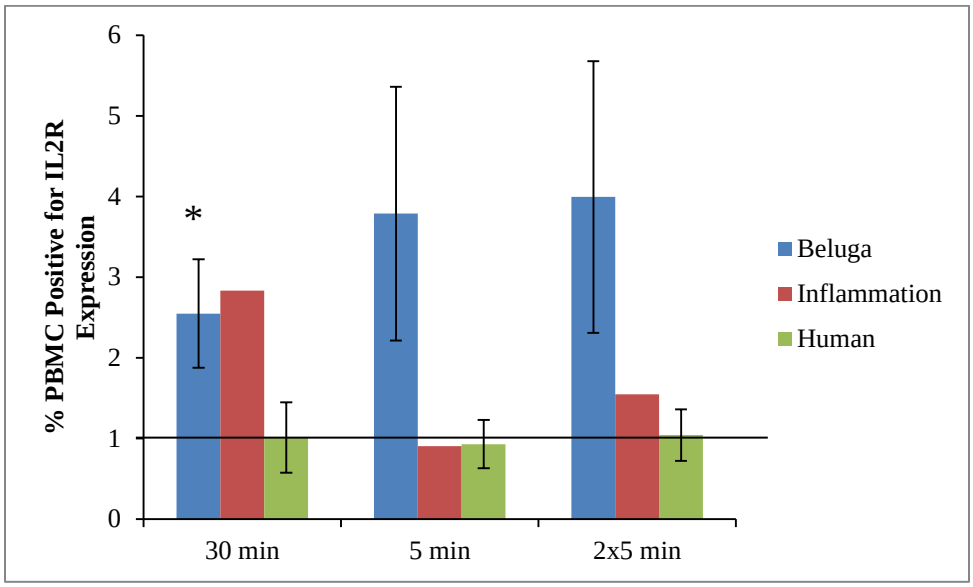


Figure X 5: % PBMC positive for IL2R expression for baseline (n=4) and inflammation conditions (n=2) in aquarium belugas and humans for all duration exposures to 1000G. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

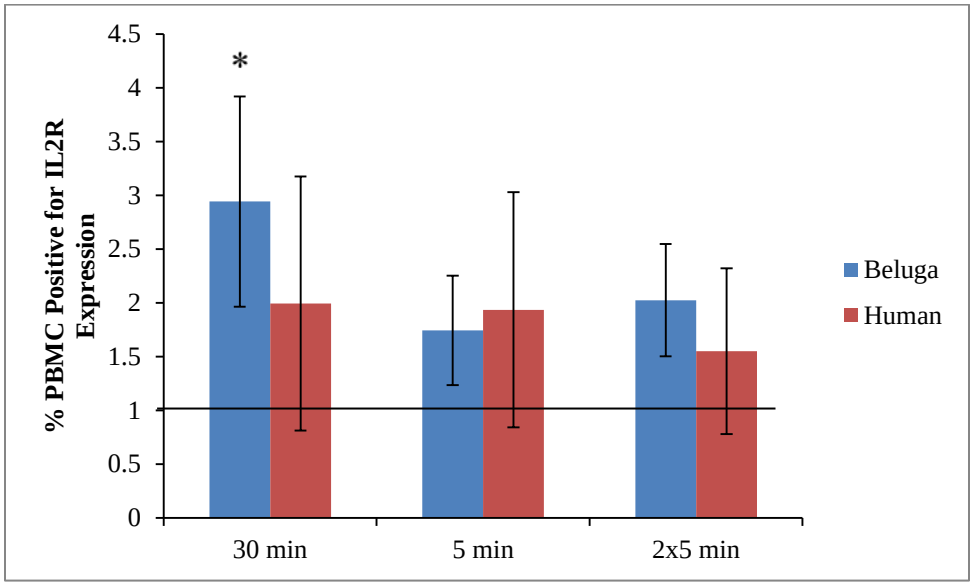


Figure X 6: % PBMC positive for IL2R expression for baseline (n=4) and inflammation conditions (n=2) in aquarium belugas and humans for all duration exposures to 1000R. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences from controls are indicated with an asterisk * (p<0.05).

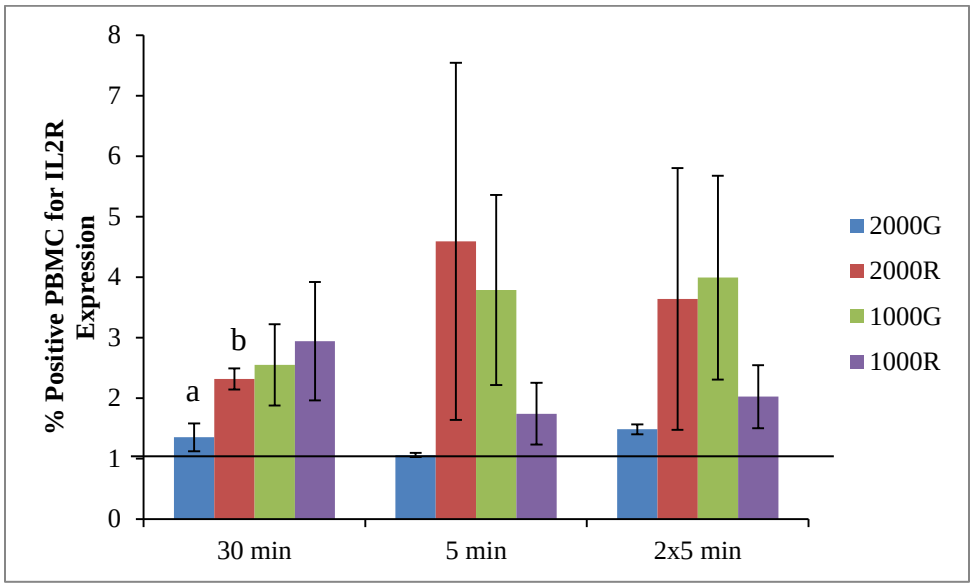


Figure X 7: % PBMC positive for IL2R expression for beluga baselines (n=4) following all pressure exposures. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures. Significant differences between dive profiles are indicated with letters ($p<0.05$).

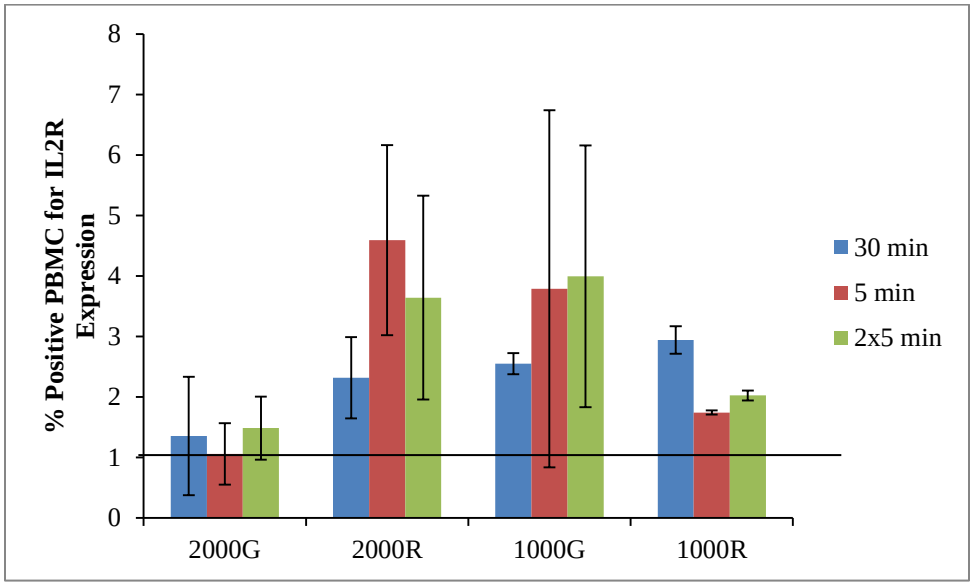


Figure X 8: % PBMC positive for IL2R expression for beluga baselines (n=4) compared between exposure durations. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures.

Appendix Y

Mixed generalized linear model results comparing IL2R expression between dive profiles for beluga baselines.

Between Profiles		F	p
MFI	30 minute	1.369	0.268
	5 minute	82.563	<0.001
	2x5 minute	6.368	0.001
% Positive	30 minute	4.297	0.001
	5 minute	2.076	0.121
	2x5 minute	1.403	0.258

Appendix Z

Mixed generalized linear model results comparing IL2R expression between exposure durations for beluga baselines.

Between Durations		F	p
MFI	2000G	2.468	0.099
	1000G	2.265	0.118
	2000R	1.939	0.159
	1000R	1.447	0.249
% Positive	2000G	12.232	<0.001
	1000G	0.506	0.607
	2000R	0.479	0.623
	1000R	0.592	0.559

Appendix AA

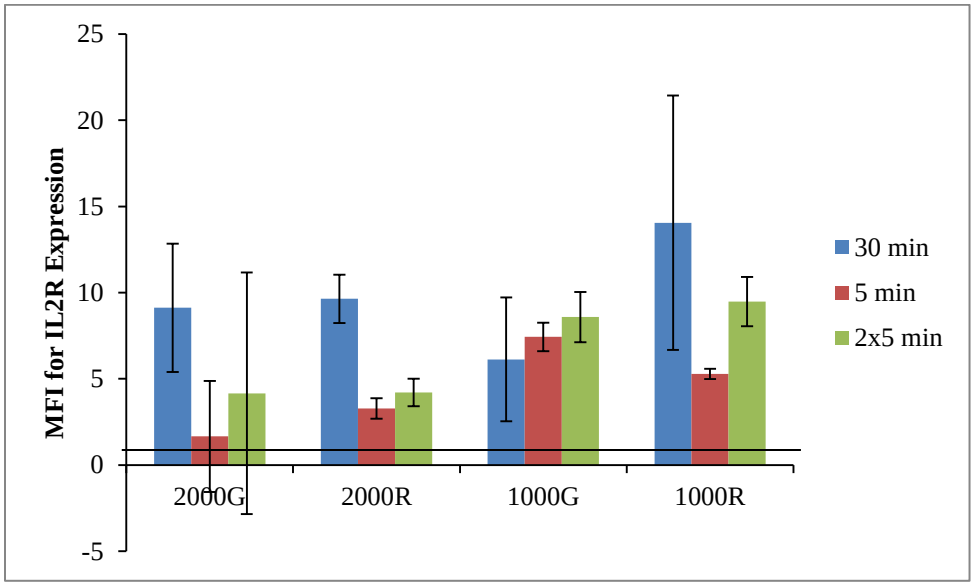


Figure AA 1: PBMC MFI for IL2R expression for beluga baselines (n=4) compared between exposure durations. Data have been normalized to controls (represented by the solid line at 1) and presented as the mean ± SE. Values greater than 1 indicate increased function, and values less than 1 indicate decreased function following pressure exposures.

Appendix AB

Mixed generalized linear model results for phagocytosis in stranded phocids.

Phagocytosis				Admit Dive		Recovery		Release Dive		Recovery	
				F	p	F	p	F	p	F	p
2000G	Granulocytes	MFI	30 minute	3.497	0.066	0.272	0.604	0.001	0.97	3.212	0.082
			5 minute	0.022	0.884	2.023	0.165	0	0.997	2.712	0.128
			2x5 minute	0.247	0.622	5.055	0.03	0.001	0.98	1.506	0.229
		% Positive	30 minute	6.131	0.016	0.076	0.783	0.102	0.751	7.265	0.011
			5 minute	0.117	0.734	0.931	0.342	0.042	0.842	4.48	0.056
			2x5 minute	0.448	0.507	0.1	0.753	0.949	0.337	10.725	0.003
	Monocytes	MFI	30 minute	0.001	0.976	13.737	<0.001	3.54	0.068	2.548	0.119
			5 minute	0.028	0.867	0.23	0.635	0.542	0.476	0	0.998
			2x5 minute	0.843	0.364	0	0.987	0.508	0.481	1.931	0.175
		% Positive	30 minute	2.198	0.143	11.451	0.001	1.106	0.3	12.512	0.001
			5 minute	0.113	0.739	0.701	0.409	0.216	0.651	1.389	0.261
			2x5 minute	0.004	0.952	0.578	0.452	1.483	0.232	26.593	<0.001

Appendix AC

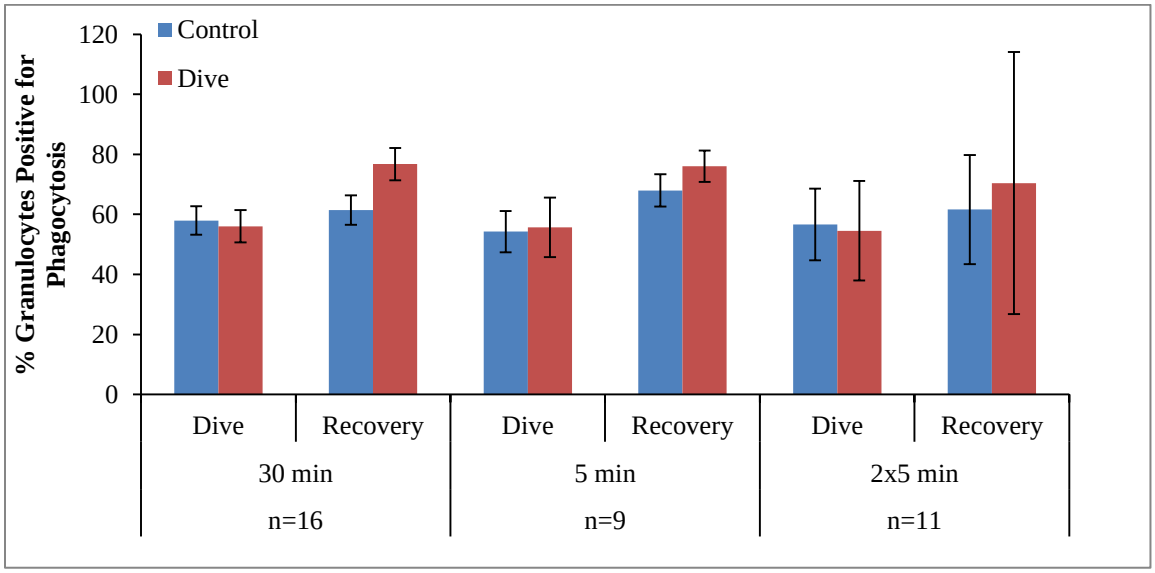


Figure AC 1: % Granulocytes positive for phagocytosis in admit samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * (p<0.05).

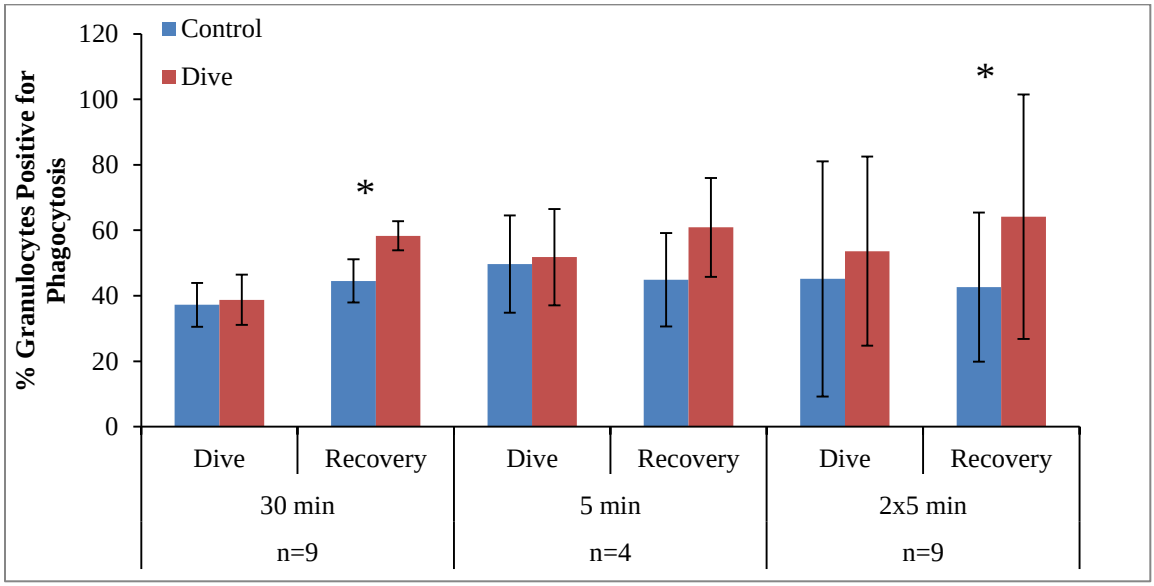


Figure AC 2: % Granulocytes positive for phagocytosis in release samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * (p<0.05).

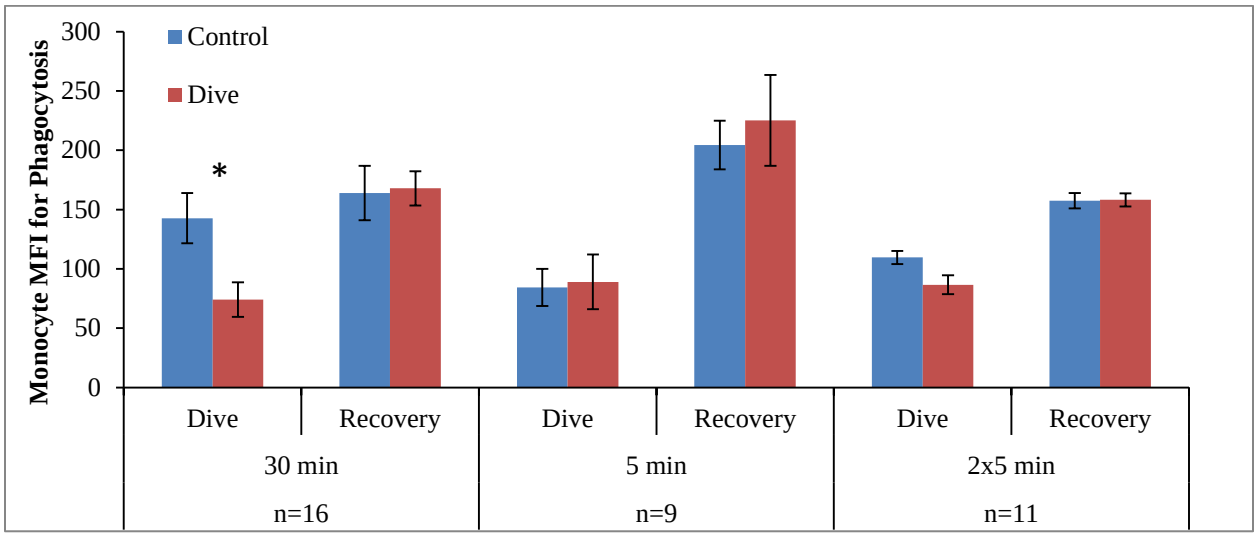


Figure AC 3: Monocyte MFI for phagocytosis in admit samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * (p<0.05).

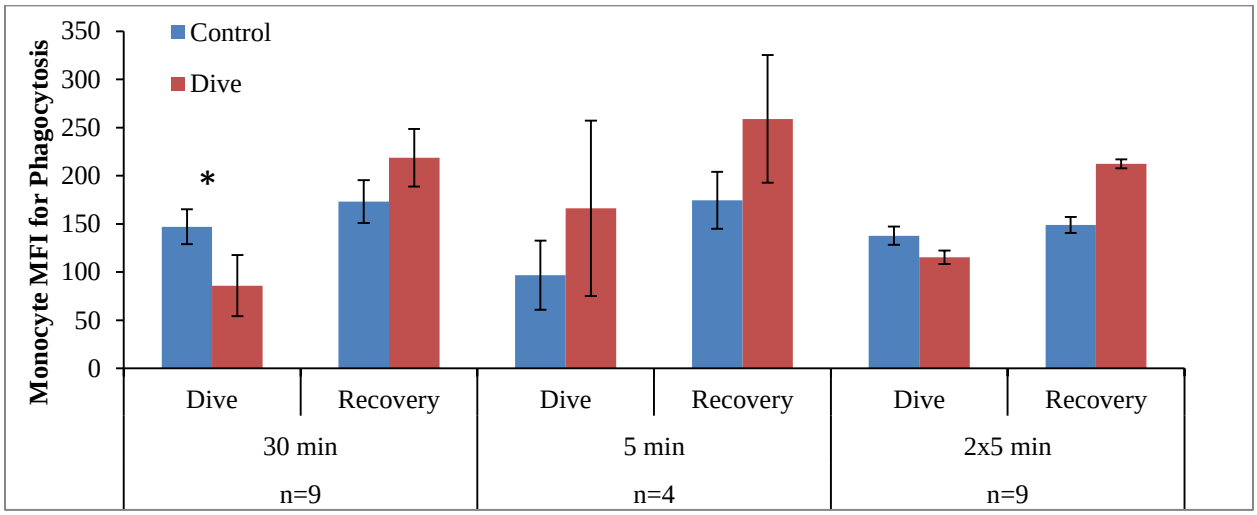


Figure AC 4: Monocyte MFI for phagocytosis in release samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * (p<0.05).

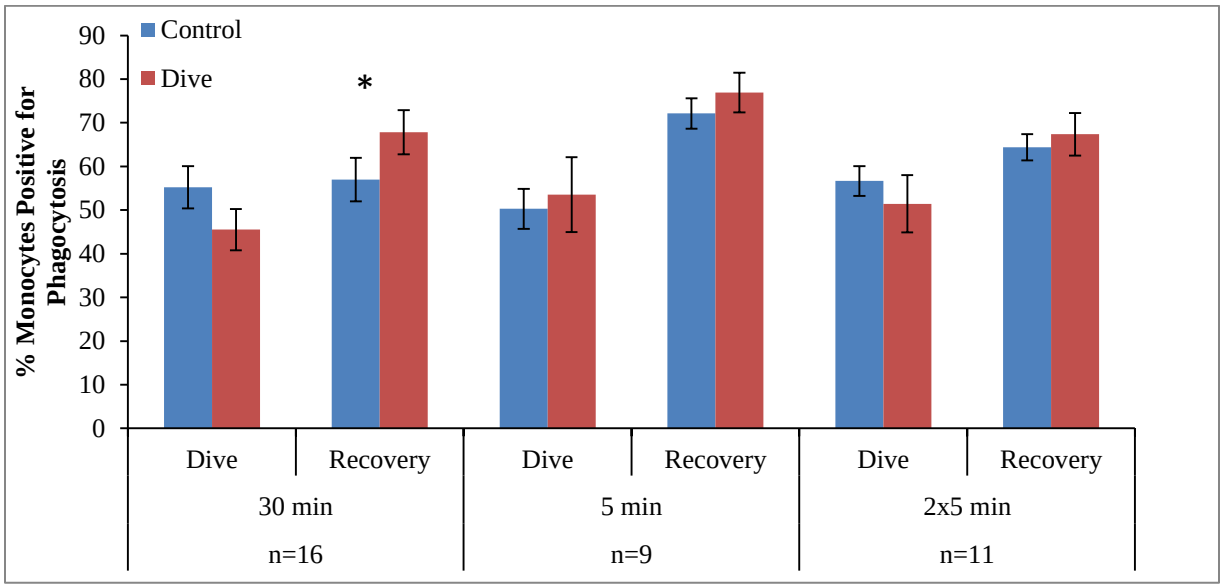


Figure AC 5: % Monocytes positive for phagocytosis in admit samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * (p<0.05).

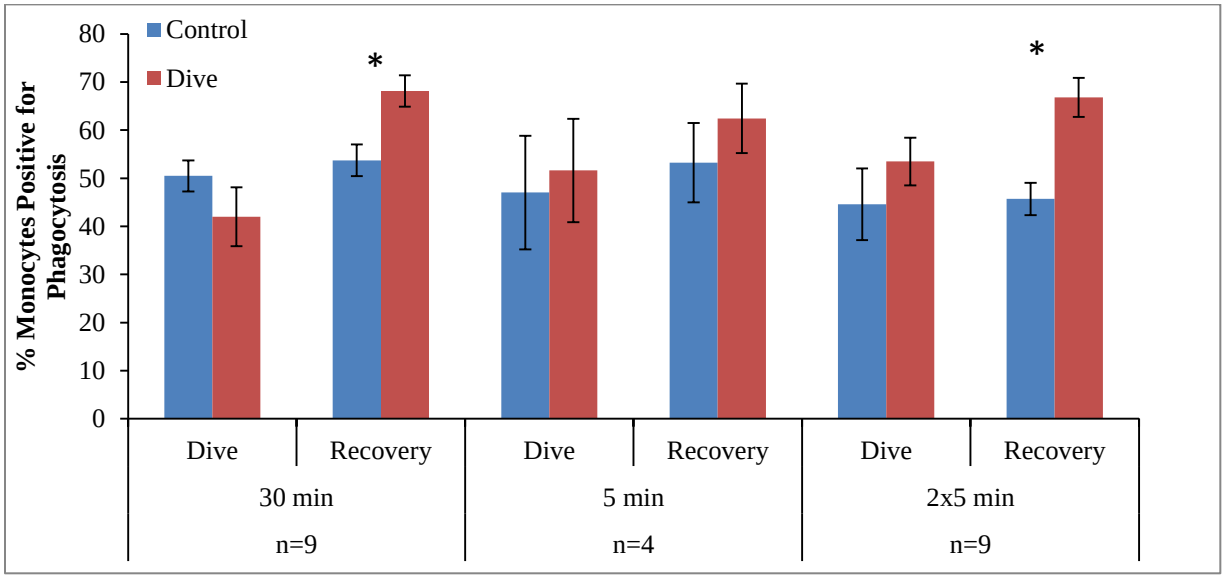


Figure AC 6: % Monocytes positive for phagocytosis in release samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * (p<0.05).

Appendix AD

Mixed generalized linear model results for CD11b expression in stranded phocids.

CD11b Expression			Admit		Release	
			F	p	F	p
2000G	MFI	30 minute	11.73	0.003	0.346	0.563
		5 minute	2.17	0.157	2.093	0.164
		2x5 minute	4.558	0.045	0	0.999
	% Positive	30 minute	0.014	0.906	0.592	0.45
		5 minute	1.869	0.185	0.202	0.657
		2x5 minute	0.308	0.585	0	0.997

Appendix AE

Mixed generalized linear model results for IL2R expression in stranded phocids.

IL2R Expression			Admit		Release	
			F	p	F	p
2000G	MFI	30 minute	6.023	0.021	9.331	0.005
		5 minute	5.868	0.034	0	0.995
		2x5 minute	321.915	<0.001	3.912	0.063
	% Positive	30 minute	7.687	0.01	10.493	0.003
		5 minute	2.41	0.152	5.846	0.036
		2x5 minute	63.783	<0.001	3.263	0.09

Appendix AF

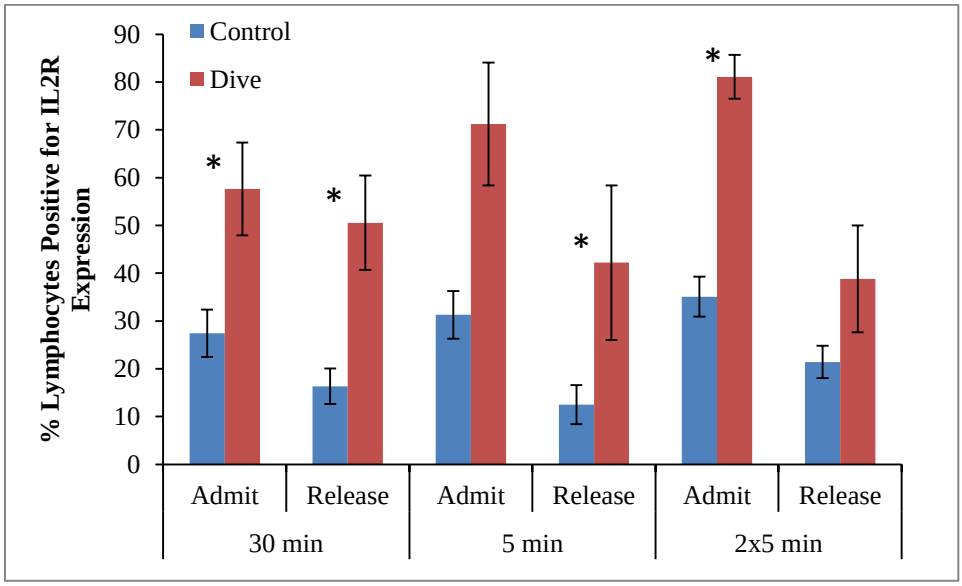
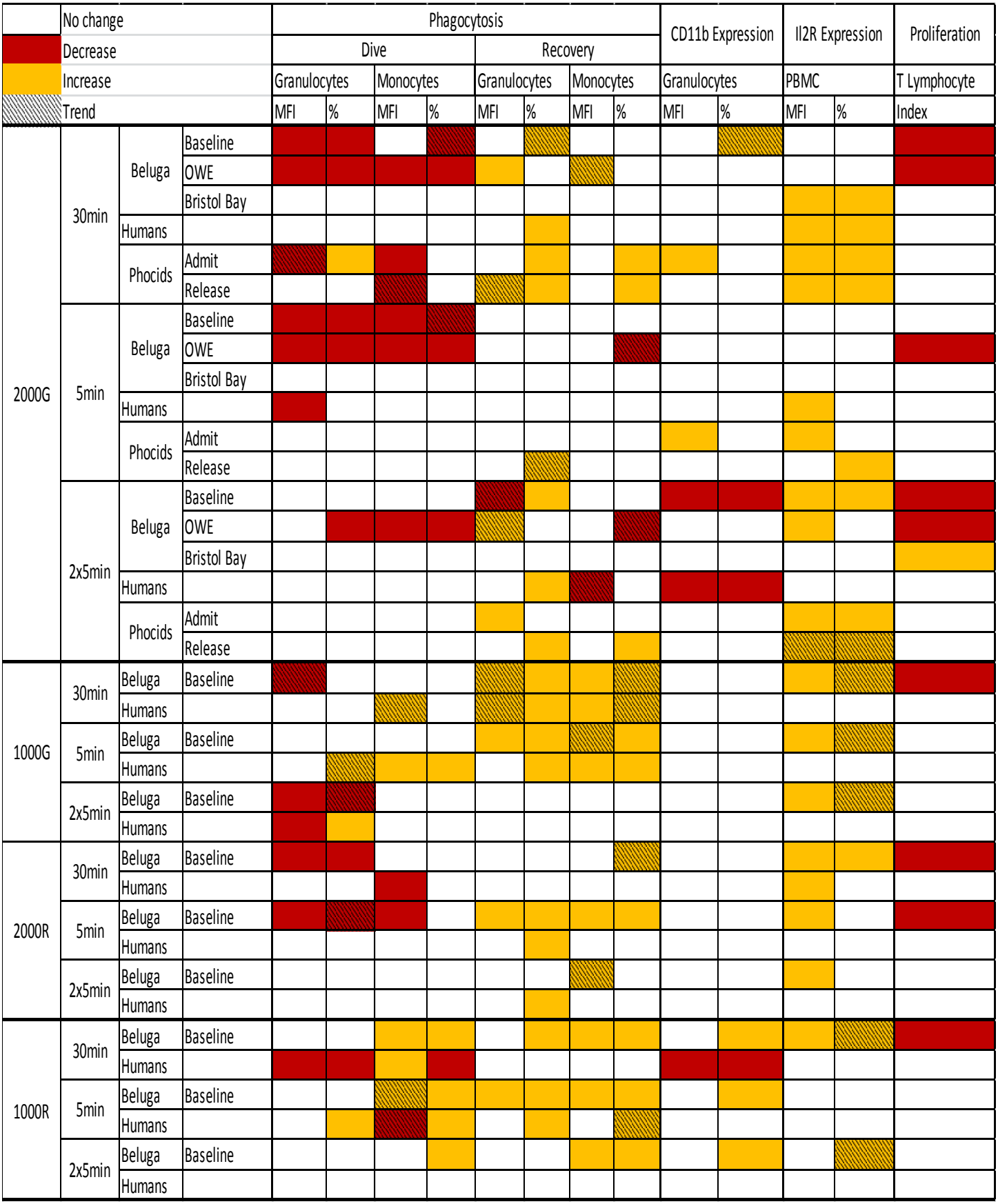


Figure AF 1: % Lymphocytes positive for IL2R expression in admit and release samples for stranded phocids measured following exposures to 2000G. Data are presented as the mean ± SE. Significant differences between controls and pressure exposures are indicated with an asterisk * ($p<0.05$).

Appendix AG


No change
Decrease
Increase
Trend
Phagocytosis
Dive
Recovery
Granulocytes
Monocytes
Granulocytes
Monocytes
MFI
%
MFI
%
MFI
%
MFI
%
CD11b Expression
Granulocytes
MFI
%
Il2R Expression
PBMC
MFI
%
Proliferation
T Lymphocyte
Index
2000G
30min
Beluga
Baseline
OWE
Bristol Bay
Humans
Phocids
Admit
Release
5min
Beluga
Baseline
OWE
Bristol Bay
Humans
Phocids
Admit
Release
2x5min
Beluga
Baseline
OWE
Bristol Bay
Humans
Phocids
Admit
Release
1000G
30min
Beluga
Baseline
Humans
5min
Beluga
Baseline
Humans
2x5min
Beluga
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Humans
2000R
30min
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Humans