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Effects of health status on pressure-induced changes in phocid immune function and implications for dive ability

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Abstract

The ability of marine mammals to cope with environmental challenges is a key determining factor in strandings and successful release of rehabilitated animals. Dive behavior is related to foraging and thus survival. While dive adaptations have been well studied, it is unknown how the immune system responds to diving and whether health status impacts immune function during diving. This study investigated the functional response of ex situ immune cells from stranded phocids to in vitro increased pressure, over the course of rehabilitation. Blood samples were drawn from stranded harbor seals (*Phoca vitulina*), gray seals (*Halichoerus grypus*) and harp seals (*Phoca groenlandica*) at the time of admit to the Mystic Aquarium, Mystic, CT and again after rehabilitation (pre-release). Phagocytosis, lymphocyte proliferation and immune cell activation were measured in vitro, with and without exposure to 2000 psi (simulated dive depth of 1360 m). Plasma epinephrine and norepinephrine, and serum cortisol were measured in vivo. All hormone values decreased between admit and release conditions. Under admit or release conditions, pressure exposures resulted in significant changes in granulocyte and monocyte phagocytosis, granulocyte expression of CD11b and lymphocyte expression of the IL2 receptor (IL2R). Overall, pressure exposures resulted in decreased phagocytosis for admit conditions, but increased phagocytosis in release samples. Expression of leukocyte activation markers, CD11b and IL2R, increased and the response did not differ between admit and release samples. Specific hematological and serum chemistry values also changed significantly between admit and release and were significantly correlated with pressure-induced changes in immune function. Results suggest (1) dive duration affects the response of immune cells, (2) different white blood 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 health status in phocids.

Keywords Seals · Dive physiology · Granulocytes · Lymphocytes · Marine mammal · Immune system

Abbreviations

IL2R	Interleukin 2 receptor
PI Staph	Propidium iodide labeled *Staphylococcus aureus*
MFI	Mean fluorescence intensity
HBSS	Hank's balanced salt solution
PBS	Phosphate-buffered saline
FITC	Fluorescein isothiocyanate
BrdU	Bromodeoxyuridine
Con A	Concanavalin A
HCT	Hematocrit
EosAbs	Absolute eosinophil count
Eos%	Percent of eosinophils
AlkPh	Alkaline phosphatase
Calc	Calcium
WBC	White blood cell count
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
Chol	Cholesterol
TP	Total protein

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, 2002), whether from initial injury, disease, the stranding event itself or human interaction (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 of 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 related to diving 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 necessary oxygen stores (Lander et al. 2003). These differences may be related to the stranding event itself and/or the necessary human handling and restraint associated with rehabilitation. Additionally, these differences may reflect an important environmental role in the development of diving ability; for example accessibility of pools, especially for young animals, during rehabilitation. If the development of blood oxygen stores can be altered during rehabilitation, it is possible that other adaptations may also be interrupted in stranded and rescued animals. Rehabilitated Steller sea lion (*Eumetopias jubatus*) pups, however, were observed to make dives of similar depth and duration to wild animals (Lander and Gulland 2003); perhaps indicating that any discrepancies in development can be overcome once animals are healthy and released. More work is clearly needed to better understand the effects of stranding and rehabilitation on dive ability in successfully released animals. The effect of dive experience during maturation of immune responses in developing young animals is of important consideration, as it determines vulnerability to dive related injury and disease once released.

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 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 in 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 (HPA) 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). Bidirectional communication allows the immune system to also regulate neuroendocrine activity (Besedovsky et al. 1985). Activity of the HPA axis, for example, can be regulated through cytokines produced during an immune response (Gaillard 1994; Sternberg 1997). Thus, an animal fighting infection or sustaining a wound may have elevated hormone levels prior to diving, which may subsequently result in an abnormal dive response. Conversely, an animal with elevated levels of catecholamines or cortisol due to natural diving adjustments may display an unusual or extreme response to an additional stressor (Talpalar and Grossman 2005). 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, 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. The response of pinnipeds in water to human disturbance has been minimally studied (Richardson et al. 1995), with more effort focused on disturbance at haul-out sites. However, 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 (Constantine et al. 2004) and changes in ascent or descent rates in elephant seals (Costa et al. 2003). Changes in dive duration, ascent rates and surface intervals in response to sonar have also been reported for several species of beaked whale (Tyack et al. 2011; Falcone et al. 2017). A sudden and/or 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 may also lead to stranding.

Because of the importance of diving behavior for foraging, traveling, as well as avoidance and escape of potential threats, marine mammals are constantly challenged not only by the changes in hormones, but also by specific characteristics of the dive, such as duration or depth. In humans, changes in immune function, facilitated by conditions of a dive, can lead to development of dive-related injury and dive related disease, such as decompression sickness (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 provide a preliminary investigation of the relationship between pinniped health, immune function and potentially demanding behaviors such as diving. Specifically, the aim of this work was to measure and compare the response of phocid immune cells to changes in pressure (i.e. simulated dives) at the time of stranding, an event associated with compromised health, as compared with healthy animals prior to release following rehabilitation. To evaluate effects on the non-specific innate

immune responses, phagocytosis, an important process by which bacteria is engulfed and removed from tissues, or cell debris is cleared following injury, was targeted. Changes in phagocytosis may lead to decreased ability to resist infection, or reduced clearance of apoptotic cells, which could perpetuate inflammation (Maderna and Godson 2003). Additionally, activation of granulocytes and lymphocytes, which primes cells for a stronger response was assessed through expression of the cell surface proteins CD11b and IL2R (interleukin 2 receptor), respectively, in order to evaluate the potential for altered immune activity post dives. We hypothesized that overall, as indicators of the neuro-endocrine response, plasma catecholamines and cortisol would decrease between admit and release conditions, reflecting a resolution of the cause of stranding. We further hypothesized that the immune response to pressure exposures would reflect the health status of the individuals, resulting in greater changes in immune function following pressure exposures in "admit" samples compared to "release".

Methods

Animal subjects and samples

Blood samples were obtained from harp seals (*Phoca groenlandica; Total n* =8), gray seals (*Halichoerus grypus; Total n* =7) and harbor seals (*Phoca vitulina; Total n* =5), 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, and cause of stranding varied between species and individuals with several animals presenting with more than one initial diagnosis. Major causes of stranding included abandoned pups, dehydration, underweight weanlings, infection, injury and rock ingestion. In general, the harbor seals were classified as abandoned pups or weanlings which were underweight and dehydrated; harp seals tended to strand with dehydration and rock ingestion; and gray seals also tended to strand as weanling pups and were diagnosed with respiratory infections, wounds and dehydration. A single harp seal was diagnosed with morbillivirus along with bacterial infections, parasites and dehydration. Admit samples were drawn prior to clinical analysis and the administration of medications; however, several animals did receive fluids.

Blood samples were drawn from the extradural intervertebral vein under manual restraint, during which animals displayed minor resistance and occasional slowing of respiration rates, but no breath hold. In cases where handling was problematic, prolonged or breathing became labored, sampling was ended as this may have impacted blood measures of interest for this study, and thus not all animals were

used for all experimental exposures. Sampling protocols were approved by Mystic Aquarium IACUC (No. 04006) and granted exemption by UConn IACUC (Exemption No. E11-011).

Where feasible, samples were also obtained pre-release, i.e. after rehabilitation and when animals were considered "healthy" for release back into the wild. These release samples were drawn at least 1 week after medications were ceased. In cases where samples were not obtained pre-release animals either did not survive or blood draws were unsuccessful due to handling/restraint difficulties as mentioned above. Despite confounding factors associated with sampling under manual restraint, it was expected that stress hormone levels would be lower in pre-release samples than admit samples due to the resolution of health challenges. 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 h of collection. Approximately 4 ml of whole blood was set aside for phagocytosis and CD11b assays for each pressure exposure. For hormone and lymphocyte assays, the remaining blood tubes were centrifuged for 10 min at 10 °C and 2000×*g* to separate blood components. One-ml aliquots of plasma were stored in Sarstedt tubes™ at − 80 °C for hormone assays. White blood cell buffy coats were removed for lymphocyte function assays, mixed with an equal volume of freezing media (10% DMSO and 90% FBS), stored at − 80 °C for 24 h and then transferred to liquid nitrogen.

Body condition

A body condition index was calculated as weight/length for both admit and release conditions. Length was measured as the straight distance between the tip of the nose and the tip of the tail.

Hematology and serum chemistries

Approximately 1 ml of EDTA whole blood was aliquoted for complete blood counts which were run in house at the Mystic Aquarium using a VetScan® HM2 Hematology Analyzer (Abaxis, Inc). A minimum of 200 µl of serum was sent to Pfizer Inc., (Groton, CT) for analysis of serum chemistries.

Hormone analysis

Cortisol concentrations were determined by Immulite® chemiluminescent assay at the Animal Health Diagnostic Center, Endocrinology Lab at Cornell University (Ithaca, NY, USA), which has previously been used for processing endocrine hormone levels in marine mammal samples (Schmitt et al. 2010; St. Aubin et al. 2013; Schwake et al. 2013). A Waters (Milford, MA, USA) 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, USA) and in St. Aubin et al. (2013).

Simulated dive exposures

Depending on the assay being run (see below) fresh blood samples or mononuclear cell suspensions (from freshly 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, provided for use (Somero et al. 1977; Field and Tablin 2012). This approach of using a pressure chamber to simulate dives allowed us to control the rate of pressure change, the target "depth" of each exposure, and the duration of each exposure. Additionally this approach allowed us to expose cells to pressures which represent the extreme end of dive capabilities. Such parameters could not be controlled or achieved using trained animals within aquaria or tagging free ranging individuals.

The chamber was brought to a temperature of 37 °C with circulating water, 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. While previous work with this pressure chamber system indicated chilling was necessary in order to avoid mixing of oil with the sample, a preliminary experiment indicated chilling itself impacted the immune responses of interest for this study. Control studies were run with mineral oil to ensure no biological effect (data not shown), and so this study was less concerned with mixing of oil with the sample. Samples were exposed to a pressure of 2000 psi (1360 m) with 2-min periods for compression and decompression. Exposures lasted for 30 min, 5 min or two repeated sessions of 5-min duration with a 1-min resting period. Temperature was monitored and kept at 37 °C throughout each pressure exposure. 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 was aliquoted into BD Falcon™ 5 ml polystyrene round-bottom tubes (BD Biosciences, San Jose, CA, USA) for control measurements. Four ml of whole blood was set aside for simulated pressure exposures. Cell counts were obtained using Trypan blue exclusion. Cell viability was assessed at > 95%. Heat killed propidium iodide labeled *Staphylococcus aureus* (PI Staph) was prepared at a ratio of 25:1, and 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 4.8×10^8 bacterial units ml^{-1}. The pressure chamber was brought to a temperature of 37 °C and bacteria were added to pressure samples immediately before compression. Control samples were placed in a 37 °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; each sample was run in triplicate. In order to stop cell activity, 10 μl of 10 mM N-ethylmaleimide was added to tubes immediately after decompression (dive) or after a further 20-min recovery period (recovery). Tubes were then placed on ice until red blood cell lysis.

CD11b expression

Two ml of fresh blood was set aside from phagocytosis assays for CD11b expression controls. Remaining blood from pressure exposures for phagocytosis was aliquoted for determination of CD11b expression. Because the pressurized cells were exposed to PI Staph for the phagocytosis assay, PI Staph was also added to controls for comparison.

One hundred μl aliquots of pressure-exposed blood, as well as non-pressure exposed control blood was incubated with 50 μl of a 1:5 dilution of mouse anti-canine CD11b IgG1 (AbD Serotec, Raleigh, NC, USA) for 30 min at 37 °C. Fifty μl of HBSS and 50 μl mouse IgG (Sigma, St Louis, MO) were added to blank and negative controls, respectively. All samples and controls were run in duplicate.

Cells were washed twice with HBSS (centrifuged for 5 min at 220×g), resuspended in 50 μl of 1:10,000 dilution of FITC-labeled goat anti-mouse IgG (Beckman Coulter, Miami, FL, USA), and incubated in the dark for an additional 30 min at 4 °C. 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 min with 1 ml of lysis buffer (0.01 M Tris; 0.001 M EDTA; 0.17 M NH$_4$ CL solution; pH 7.4). Tubes were washed twice with $1 \times$ PBS (pH 7.2) and fixed with 250 μl of 1% paraformaldehyde (pH 7.4). Tubes were stored in the dark at 4 °C until analyzed 24 h later by flow cytometry.

Interleukin 2 receptor (IL2R) expression

Buffy coat samples were removed from liquid nitrogen, thawed quickly at 37 °C and washed twice with RPMI 1640 (centrifuged for 5 min at 220×g, 20 °C). Cell pellets were re-suspended in 3 ml of RPMI 1640 and layered over 3 ml of room temperature Histopaque®-1077 (Sigma Aldrich,

St Louis, MO, USA). Tubes were centrifuged for 30 min at 400×g, 10 °C. The resulting mononuclear cell layer was removed and washed twice in $1 \times$ PBS (pH 7.2). Cell counts were obtained using Trypan blue exclusion and cell viability was assessed to be > 95%. Based on cell counts, the volume of PBS buffer in which cells were resuspended was adjusted to reach a cell concentration of 4×10^6 cells ml^{-1}. One ml of cell suspension was set aside for controls, and the remaining cell suspension was brought to a volume of 4 ml and introduced to the pressure chamber. Following decompression, the volume of pressure-exposed samples was adjusted back to reach 4×10^6 cells ml^{-1}. IL2R expression was measured using a Human IL2 biotinylated fluorokine kit from R & D Systems® (Minneapolis, MN, USA). 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) was added. All samples and controls were run in duplicate. Tubes were incubated for 1 h at 4 °C, after which 10 μl of avidin FITC (R & D Systems®, Minneapolis, MN, USA) was added followed by a 30-min 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 °C until analyzed 24 h later by flow cytometry.

Flow cytometry

Samples for phagocytosis, CD11b expression and IL2R expression were read using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and Cell Quest software. Tubes containing cells only 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 phagocytosis, propidium iodide was read at 617 nm using the FL2 channel, while the FITC signal for both the CD11b and IL2R expression was read at 518 nm in the FL1 channel. 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 5-bromo-2′-deoxyuridine (BrdU) incorporation assay (ROCHE Diagnostics, Indianapolis, IN, USA). Archived white cell buffy coats were removed from liquid nitrogen and thawed rapidly in a 37 °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 (Histopaque®-1077) as described above for the IL2R expression assay. Mononuclear cells were washed twice with media (RPMI 1640 with 0.1 μM non-essential amino acids, 100 units ml^{-1} penicillin, 100 μg ml^{-1} strepto-mycin, 0.292 mg ml^{-1} 1-glutamine, 1 mM sodium pyruvate, 0.01 M HEPES, 10% FBS, 0.1 mM 2-mercaptoethanol). Cell counts were obtained using Trypan blue exclusion and cell viability was assessed at > 95%. The volume of cell suspension was adjusted to reach a density of 1×10^6 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 1×10^6 cells ml^{-1}. One hundred μl of pressure-exposed, as well as non-exposed, cell suspensions were pipetted into Micro-test™ 96-well tissue culture plates in triplicate (BD Bio-sciences, San Jose, CA, USA) for assay.

One hundred μl of concanavalin A (Con A) (Sigma Aldrich, St Louis, MO, USA) was added to experimental wells at a final concentration of 2.5 μg ml^{-1}. Plates were incubated at 37 °C for 72 h. Ten μl of BrdU, a thymidine analog, was added to each well except for no-BrdU controls, and plates were incubated for an additional 12 h followed by centrifugation for 10 min at 300×g and the media removed. Plates were dried and stored in clean Ziploc® bags at 4 °C until fixing and developing. The plates were fixed and developed within 1 week of drying in accordance with kit protocol. Briefly, plates were incubated for 30 min at room temperature with two hundred μl of FixDenat™. One hundred μl of anti- BrdU-POD (peroxidase conjugated mon-oclonal Fab fragments from a mouse–mouse hybrid) was then added and plates were incubated for a further 90 min at room temperature. Wells were washed three times with wash solution before addition of 100 μl of substrate solution (tetramethyl-benzidine). Plates were developed for 30 min before the addition of 25 μl 1 M H_2SO_4 to stop the reaction and read at 450 nm using an EL800 microplate reader (BioTek, Winooski, VT, USA). Proliferation indices were calculated as the ratio of the optical density (OD) of stimulated cells to the OD of the unstimulated cells.

Statistical analysis

Due to small sample sizes used in this study, significance was determined at $\alpha = 0.1$ for all tests in order to increase the power for each test. Statistical power was determine to be 0.4 or higher for all tests.

Control values for all measures of immune function, as well as hormone, CBC and serum chemistry data, were compared between admit and release conditions using non parametric Wilcoxon signed rank tests for animals with data from both conditions ($\alpha = 0.1$).

Mixed effects generalized linear models were set up for each simulated dive to examine the effect of pressure on immune function within arrival and release conditions ($\alpha = 0.1$). Individuals with only admit data, due to unsuc-cessful rehabilitation or difficulty sampling in healthy ani-mals, or only release data, due to animals being transferred and already on meds before they could be sampled, were included in these analyses. Individuals were added to the model as a random effect, and fixed effects added were treatment (control, dive) and condition (admit, release).

The change in immune function measures between con-trols and pressure-exposed cells was then calculated as a percent of the control values. This standardization of val-ues was also intended to control for individual variations in baseline values based on species or cause of stranding.

For comparing the effects of pressure between admit and release conditions, only animals with paired sam-ples were analyzed. Due to the much reduced sample size ($n = 6$), species-specific differences could not be deter-mined and so all individuals were grouped together.

Non parametric Spearman Rank correlations were used to investigate the relationship between hormone values, CBC and serum chemistries, and both immune function control values and % change in immune function ($\alpha = 0.1$) for all animals (i.e. both admit and release samples, paired and unpaired). Spearman correlations were also used to compare the changes in immune function with a body con-dition index calculated from animal lengths and weights ($\alpha = 0.1$).

Results

Health parameters and control measures of immune function

Body condition

Body Condition was not significantly different between admit and release conditions (Table 1) though less vari-ability was seen in animals at release. Body Condition Indices only showed significant negative correlation with control measures of phagocytosis for the recovery period of the repeated 5-min dives ($r = -0.470$; $p = 0.042$), a pos-itive correlation with CD11b expression per cell for the 30-min control ($r = 508$; $p = 0.026$). No significant rela-tionships were found between Body Condition and IL2R expression controls.

Table 1 Plasma concentrations of catecholamines, cortisol and body condition (Mean ± STDEV) in phocids at the time of stranding (admit) and release (after rehabilitation)

	Epinephrine pg ml^{-1}		NorEpinephrine pg ml^{-1}		Cortisol µg dl^{-1}		Body condition
Paired							
Admit	318.48 ± 272.07	n = 6 (Hg = 3, Pv = 1, Pg = 2)	831.08 ± 449.35	n = 6 (Hg = 3, Pv = 1, Pg = 2)	13.72 ± 9.09	n = 6 (Hg = 4, Pv = 1, Pg = 1)	22.4 ± 5.5
Release	183.34 ± 85.6	n = 6 (Hg = 3, Pv = 1, Pg = 2)	304.49 ± 141.80	n = 6 (Hg = 3, Pv = 1, Pg = 2)	5.00 ± 2.79	n = 6 (Hg = 4, Pv = 1, Pg = 1)	24.3 ± 1.9
Non paired							
Admit	211.37 ± 137.57	n = 6 (Hg = 3, Pg = 3)	2126.42 ± 2020.65	n = 5 (Hg = 3, Pg = 2)	21.66 ± 14.15	n = 8 (Hg = 3, Pg = 4, Pv = 1)	17.9 ± 2.6

Non paired animals are those individuals which were sampled at admit but died during rehabilitation

Hematology and serum chemistries

Few clinical blood parameters showed a significant difference between admit and release conditions. Hematocrit (HCT; $Z = -1.859$; $p = 0.063$), Absolute Eosinophil Counts (EosAbs; $Z = -1.753$; $p = 0.08$), the percentage of Eosinophils (Eos%; $Z = -1.782$; $p = 0.075$), alkaline phosphatase (AlkPh; $Z = -2.366$; $p = 0.018$) and Calcium (Calc; $Z = -2.032$; $p = 0.042$) were all significantly higher in release samples as compared with admit.

Correlations were run between these parameters and control measures of immune function. Additional clinical values were also used, including white blood cell counts (WBC), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) as markers of developing oxygen stores and thus dive ability; cholesterol (Chol) and total Protein (TP) as indicators of nutritional health. Correlation results are summarized in Table 2. In general, WBC displayed significant positive correlations with control immune function while AlkPh, Calc, Chol, and EosAbs displayed negative relationships with control immune activity. TP, HCT, MCH, MCHC and Eos % had varied relationships depending on the immune function measured.

Hormones

Animals ($n = 6$) for which both admit and release data were available displayed decreased plasma hormone concentrations from admit to release (Table 1) and this was significant for norepinephrine ($Z = -1.826$; $p = 0.068$) and cortisol ($Z = -2.197$; $p = 0.028$). Animals which were sampled upon admit but did not survive to release were grouped together as 'non-paired' animals to see if hormones reflected or indicated rehabilitation success. 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. Despite changes in plasma hormones, no significant differences were found in control measures of phagocytosis or CD11b expression between admit and release conditions for paired data. However, epinephrine values were negatively correlated with control values of the % of monocytes undergoing phagocytosis (30 min; $r = -0.612$; $p = 0.005$) and positively correlated with the % of granulocytes expressing CD11b (30 min; $r = 0.875$; $p = 0.010$). Cortisol was also positively correlated with control measures of the % of granulocytes expressing CD11b following the 30-min 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-min incubations. No significant differences in control measures of IL2R expression or proliferation indices were detected between admit and release conditions, and no significant correlations between hormone values and control measures of either lymphocyte function were detected.

Effects of pressure on phagocytosis

Decreased phagocytic activity per cell was observed following the dive period of the 30-min exposures for granulocytes (Fig. 1; admit, $p = 0.060$) and monocytes (admit, $p = 0.016$; release, $p = 0.068$; data not shown). A significant increase in granulocyte phagocytosis was detected for admit samples following the recovery period of the repeated 5-min exposures (Fig. 1; MFI; $p = 0.030$). A pattern of increased phagocytic activity per cell was observed for release samples following the recovery period of this pressure exposure (Fig. 1). This pattern was also observed for the release samples following the recovery period of the single 5-min exposure (Fig. 1; $p = 0.056$).

The calculated change in granulocyte phagocytosis was significantly different between dive and recovery periods following the 30-min ($F = 2.964$; $p = 0.041$) and repeated

Table 2 Summary of significant correlations between hematology and serum chemistry values, and control values of immune functions

Pressure exposure	Immune Function	Cell type	Condition	Measure	WBC	TP	HCT	MCH	MCHC	Eos %	EosAbs	AlkPh	Calc	Chol
30 min	Phagocytosis	Granulocytes	Dive	X mean	r=0.328; p=0.059				r=-0.317; p=0.072					
				%Gated	r=0.514; p=0.002									
			Recovery	X mean				r=-0.303; p=0.081						
				%Gated					r=0.298; p=0.087					
		Monocytes	Dive	X mean	r=0.329; p=0.058			r=0.332; p=0.055				r=-0.498; p=0.003		
				%Gated		r=-0.361; p=0.033								
			Recovery	X mean								r=-0.409; p=0.02		r=-0.426; p=0.013
				%Gated	r=0.348; p=0.044							r=-0.379; p=0.03		
	CD11b	Granulocytes	Dive	X mean			r=-0.454; p=0.078		r=-0.513; p=0.025					
				%Gated					r=-0.558; p=0.013					
	IL2	Lymphocytes	Dive	X mean	r=0.559; p=0.059						r=-0.805; p=0.016			
				%Gated	r=0.833; p=0.01					r=-0.835; p=0.01				
5 min	Phagocytosis	Granulocytes	Dive	X mean							r=-0.577; p=0.039			
			Recovery	X mean							r=-0.499; p=0.082			

Table 2 (continued)

Pressure exposure	Immune Function	Cell type	Condition	Measure	WBC	TP	HCT	MCH	MCHC	Eos %	EosAbs	AlkPh	Calc	Chol
		Monocytes	Dive	X mean							r= −0.494; p=0.086			
	CD11b	Granulocytes	Dive	X mean	r=0.405; p=0.085									
	IL2	Lymphocytes	Dive	X mean	r=0.714; p=0.071			r= −0.821; p=0.023						
				%Gated	r=0.622; p=0.031									
2 × 5 min	Phagocytosis	Granulocytes	Dive	X mean								r= −0.477; p=0.045	r= −0.571; p=0.013	
				%Gated		r= −0.401; p=0.08							r= −0.455; p=0.057	r= −0.441; p=0.067
			Recovery	X mean			r=0.426; p=0.099							
				%Gated	r= −0.460; p=0.041									r=0.476; p=0.046
		Monocytes	Dive	X mean								r= −0.418; p=0.084	r= −0.510; p=0.031	
				%Gated							r=0.442; p=0.051			
				%Gated							r=0.389; p=0.09			
	CD11b	Granulocytes	Dive	X mean	r=0.407; p=0.084			r=0.457; p=0.049		r= −0.438; p=0.061			r= −0.449; p=0.062	r= −0.586; p=0.011
	IL2	Lymphocytes	Dive	X mean	r=0.810; p=0.015					r= −0.835; p=0.01	r= −0.805; p=0.016			
				%Gated	r=0857; p=0.014			r= −0.857; p=0.014	r= −0.679; p=0.094					

The exposure period is indicated by 'dive' or 'recovery'. The r statistic and p value is given for each significant result

Fig. 1 Calculated % change in granulocyte MFI for phagocytosis in stranded phocids for dive and recovery periods following all pressure exposures to 2000 psi during admit and release conditions. Data are presented as mean ± s.e.m. An asterisk (*) indicates significant pressure-induced changes from controls; a § indicates significantly different responses between dive and recovery periods (GLM; $\alpha = 0.05$)
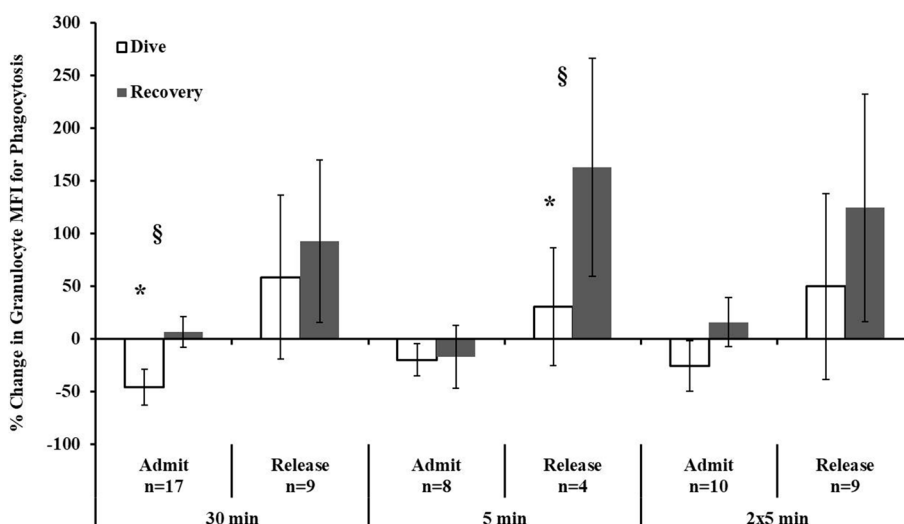
5-min ($F = 3.522$; $p = 0.025$) exposures (Fig. 1). For the 30-min exposure (admit conditions), a decrease in activity per cell was observed during the dive with values returning to control levels during recovery. Following the repeated 5-min exposures, the average granulocyte phagocytic activity per cell showed no change for the dive period, which differed significantly from the increase observed during the recovery.

Following the recovery period of the 30-min exposure, granulocytes displayed significant increases in the % of granulocytes performing phagocytosis for both admit ($p < 0.001$) and release ($p = 0.011$) conditions (Fig. 2). Significant increases in the % of positive monocytes were also detected for admit ($p = 0.001$; data not shown) and release ($p = 0.001$; data not shown) conditions following this exposure. Additionally, significant increases in the % of positive cells were detected for release samples following the

repeated 5-min exposures (Fig. 2; granulocytes, $p = 0.003$; monocytes, $p < 0.001$; data not shown).

The change in the % positive granulocytes was also significantly different between dive and recovery periods following the 30-min exposure ($F = 10.229$; $p < 0.001$). For both admit and release conditions, recovery periods showed increased % of granulocytes undergoing phagocytosis and this change was larger than changes observed during the dive period (Fig. 2). This pattern can also be observed for the 5-min and repeated 5-min exposures.

Combined effects of health and pressure on phagocytosis-paired samples

For animals with paired data, significant differences between admit and release conditions were detected in phagocytic activity per cell ($F = 3.431$; $p = 0.037$), and % positive

Fig. 2 Calculated change in % granulocytes positive for phagocytosis for dive and recovery periods following all pressure exposures to 2000 psi for both admit and release conditions. Data are presented as mean ± s.e.m. An asterisk (*) indicates significant pressure-induced changes from controls; a § indicates significantly different responses between dive and recovery periods (GLM; $\alpha = 0.05$). The symbol ¥ indicates s.e.m bar too large to portray on the scale of the graph and data should be interpreted cautiously
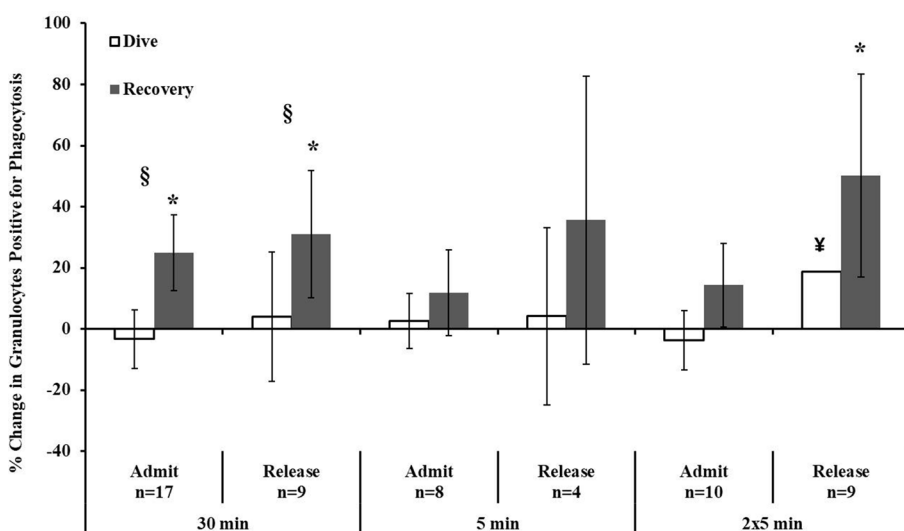
granulocytes ($F = 5.37$; $p = 0.001$), as well as phagocytic activity per cell ($F = 9.465$; $p < 0.001$) and % positive monocytes ($F = 3.21$; $p = 0.045$) for the 30 min exposure, though pairwise comparisons were unable to indicate if this significance occurred during the dive or recovery periods. In general, admit conditions displayed decreased measures of phagocytosis, while release conditions displayed increases or minimal change from controls (Fig. 3). Changes occurring for release conditions appear smaller than those measured for admit conditions. Additionally, significant changes were found in the % of granulocytes undergoing phagocytosis ($F = 4.331$; $p = 0.050$) and the phagocytic activity per monocyte ($F = 5.806$; $p = 0.021$) following the 5-min exposure. Pairwise comparisons were only significant for the % of granulocytes undergoing phagocytosis 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 (Fig. 3). This pattern was also observed for the recovery period and for the phagocytic activity per cell for both granulocytes and monocytes (data not shown).

Body condition

Significant correlations were found between body condition and changes in monocyte phagocytosis activity per cell following the 30-min dive period ($r = 0.552$; $p = 0.004$). The change in the % of monocytes performing phagocytosis was also significantly correlated with body condition for both the dive ($r = 0.728$; $p < 0.001$) and recovery period ($r = 0.607$; $p = 0.001$) of the 30-min exposures. In all cases, the correlations were positive indicating larger changes in monocyte phagocytosis for animals with more robust body condition

(e.g. not emaciated). No significant relationships were detected between body condition and granulocyte function.

Hematology and serum chemistries

Correlation results are detailed in Table 3. The response of both granulocytes and monocytes to changes in pressure was positively correlated with WBC for the 30-min exposure and MCHC for the repeated 5-min exposure. Significant negatively correlations were detected for TP and AlkPh, EosAbs, HCT and Calc.

Hormones

There was a significant positive correlation between epinephrine and changes in the percent of monocytes positive for phagocytosis during both the dive ($r = 0.684$; $p = 0.001$) and recovery period ($r = 0.574$; $p = 0.010$) of the 30-min exposures. Pressure-induced changes in the % monocytes performing phagocytosis following the recovery period of the 30-min dive were negatively correlated with measures of cortisol. Cortisol also displayed a significant negative correlation with the percent of granulocytes undergoing phagocytosis following the 5-min exposures ($r = -0.750$; $p = 0.02$), as well as with the average phagocytic activity per monocyte following the recovery period of this dive ($r = -0.65$; $p = 0.058$). No significant relationship between norepinephrine and the response of immune cells to pressure was detected.

Effects of Pressure on CD11b Expression

Significant increases in CD11b expression per cell were detected only for admit conditions following the 30-min ($p = 0.003$) and single 5-min ($p = 0.045$) exposures (Fig. 4).

Fig. 3 Calculated change in % of granulocyte and monocyte populations positive for phagocytosis for admit and release conditions in paired animals following the dive period for all pressure exposures to 2000 psi. Data are presented as mean ± s.e.m. An asterisk (*) indicates significantly different responses between the two conditions (GLM; $\alpha = 0.05$)
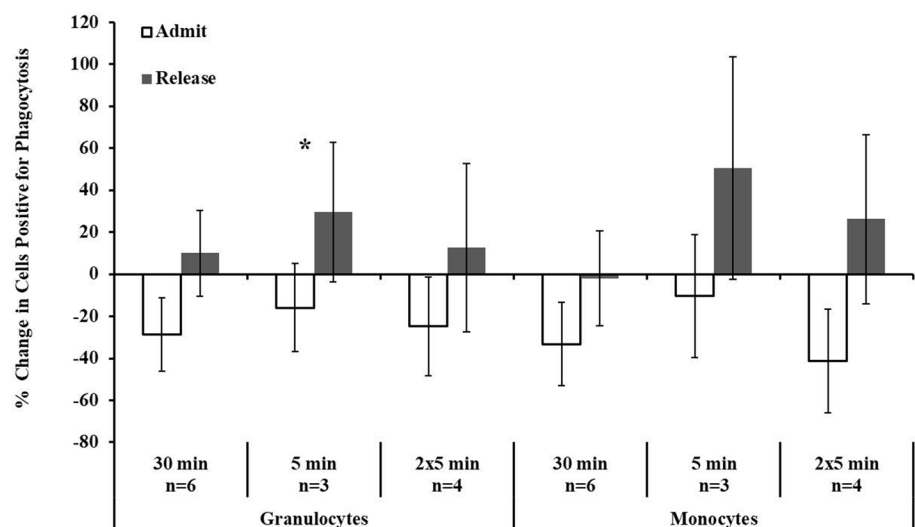
Table 3 Summary of significant correlations between hematology and serum chemistry values, and changes in immune function following each pressure exposure

Pressure Exposure	Immune Function	Cell Type	Condition	Measure	WBC	TP	HCT	MCH	MCHC	Eos %	EosAbs	AlkPh	Calc	Chol
30 min	Phagocytosis	Granulocytes	Dive	X mean	r=0.329; p=0.058			r=0.332; p=0.055				r= −0.498; p=0.003		
				%Gated		r= −0.292; p=0.089								
			Recovery	X mean								r= −0.409; p=0.02		r= −0.426; p=0.013
				%Gated		r= −0.361; p=0.033								
		Monocytes	Dive	X mean	r=0.295; p=0.090							r= −0.396; p=0.022		
				%Gated		r= −0.310; p=0.07								
			Recovery	X mean	r=0.348; p=0.044							r= −0.379; p=0.03		
	CD11b			%Gated	r=0.407; p=0.084			r=0.457; p=0.049		r= −0.438; p=0.061			r= −0.449; p=0.062	r= −0.586; p=0.011
	IL2	Lymphocytes	Dive	X mean	r=0.559; p=r=0.059									
5 min	Phagocytosis	Granulocytes	Dive	X mean							r= −0.577; p=0.039			
			Recovery	X mean							r= −0.499; p=0.082			
		Monocytes	Dive	X mean							r= −0.44; p=0.086			
	CD11b	Granulocytes	Dive	X mean				r=0.59; p=0.026				r=0.534; p=0.06		
	IL2	Lymphocytes	Dive	X mean		r=0.793; p=0.033								

Table 3 (continued)

Pressure Exposure	Immune Function	Cell Type	Condition	Measure	WBC	TP	HCT	MCH	MCHC	Eos %	EosAbs	AlkPh	Calc	Chol
2 × 5 min	Phagocytosis	Granulocytes	Dive	%Gated			$r = -0.624$; $p = 0.054$							
				X mean			$r = -0.477$; $p = 0.085$					$r = -0.452$; $p = 0.091$	$r = -0.578$; $p = 0.024$	
				%Gated		$r = -0.454$; $p = 0.077$			$r = 0.599$; $p = 0.014$					
		Monocytes	Dive	X mean			$r = -0481$; $p = 0.081$						$r = -0.501$; $p = 0.057$	
				%Gated					$r = 0.475$; $p = 0.063$		$r = 0.567$; $p = 0.022$			
				%Gated					$r = 0.431$; $p = 0.095$	$r = 0.460$; $p = 0.073$	$r = 0.642$; $p = 0.007$			
	CD11b			%Gated										$r = -0.545$; $p = 0.054$

The exposure period is indicated by 'dive' or 'recovery'. The r statistic and p value is given for each significant result

Fig. 4 Measured granulocyte MFI for CD11b expression for control and pressure-exposed cells following all dive exposures to 2000 psi, for both admit and release conditions. Data are presented as mean ± s.e.m. An asterisk (*) indicates a significant change in MFI during the dive (GLM; $\alpha = 0.05$)
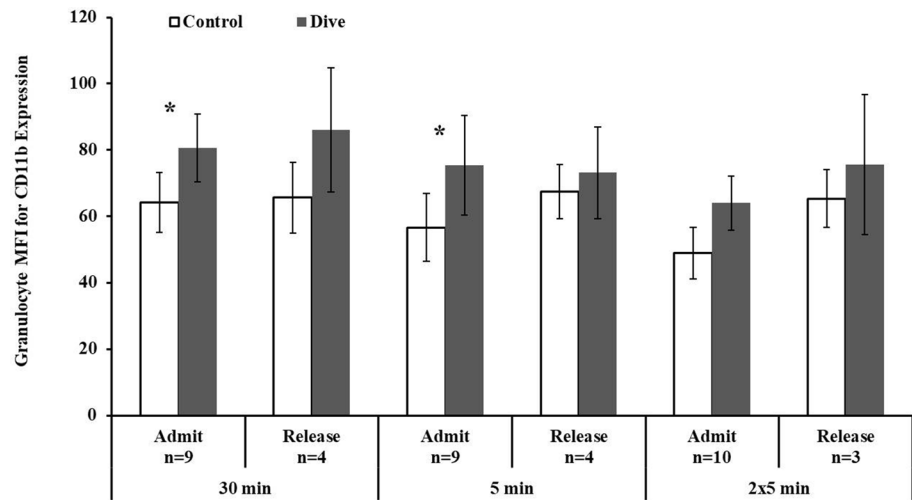
No significant effect of pressure on the % of cells positive for CD11b could be detected.

Combined effects of health and pressure on CD11b expression-paired samples

Changes in CD11b expression were not significantly different between admit and release conditions for paired samples.

Body condition

No significant correlations were found between changes in CD11b expression following pressure exposures and body condition.

Hematology and serum chemistries

Correlation results are summarized in Table 3. Changes in the expression of CD11b were positively correlated with WBC, MCH and AlkPh. Significant negative correlations were detected with Eos %, Calc and Chol.

Hormones

Changes in the expression of CD11b per cell following the single 5-min exposures were positively correlated with norepinephrine ($r = 0.718$; $p = 0.045$) and cortisol ($r = 0.884$; $p = 0.001$).

Effects of Pressure on IL2R Expression

Significant pressure-induced increases in IL2R expression were detected following all pressure exposures. Increased expression per cell (Fig. 5) was observed for admit samples following all exposures (30 min, $p = 0.021$; 5 min, $p = 0.034$; 2×5 min, $p < 0.001$). Similar patterns of increase were observed in the % of cells expressing IL2R.

Fig. 5 Measured lymphocyte MFI for IL2R expression in control and pressure-exposed cells for all pressure exposures for both admit and release conditions. Data are presented as mean ± s.e.m. An asterisk (*) indicates significant change in MFI during the pressure exposures (GLM; $\alpha = 0.05$)
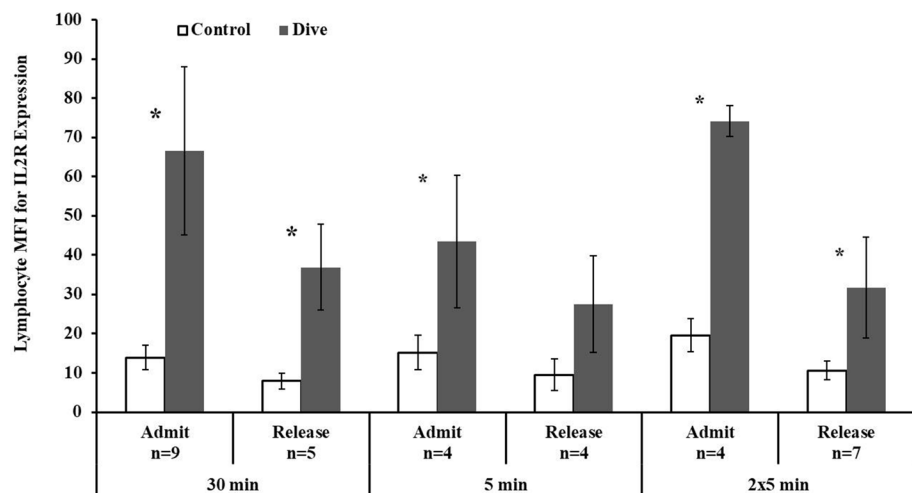
Combined effects of health and pressure on IL2R expression—paired samples

No significant differences were found in the changes in IL2R expression between conditions for paired samples.

Body condition

Body condition was not significantly correlated with changes in either measure of IL2R expression following pressure exposures.

CBC and serum chemistries

Correlation results are detailed in Table 3. Changes in IL2 displayed few significant relationships with the target clinical measures used in this study. A significant positive correlation was detected with WBC following the 30-min exposures, and with TP following the 5-min exposures. HCT displayed a negative relationship with IL2R expression for the 5-min exposures. No significant correlations were detected for the repeated 5-min exposures.

Hormones

Cortisol displayed a significant positive correlation with changes in the average expression of IL2R on lymphocytes following the 5-min dive exposure ($r = 0.771$; $p = 0.072$).

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 was observed for all pressure exposures as compared with controls. Release samples were only available for a single animal and exposure to 2000 psi for 30 min 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-min exposures ($r = -0.933$; $p = 0.021$).

Hormones

Epinephrine was positively correlated with changes in proliferation indices following a 30-min exposure to 2000 psi ($r = 0.928$; $p = 0.003$). Following a single 5-min pressure exposure, changes in IL2 receptor expression were positively correlated with both plasma epinephrine ($r = 0.872$; $p = 0.054$) and norepinephrine ($r = 0.945$; $p = 0.015$).

Hematology and serum chemistries

No significant correlations between lymphocyte proliferation and clinical blood parameters were detected for this study.

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, using stranded phocids with each individual serving as its own control (admit vs. release). Morrison et al. (2012) noted no apparent difference in dive behavior 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. This work represents a pilot investigation of this relation between health status and immune function during diving in phocids.

As expected, plasma catecholamines and cortisol decreased between admit and release conditions. 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 number or perceived intensity of these stressors on an individual. Increased stressor load is generally associated with inhibited immune responses, in part due to regulation of cell function by catecholamines and particularly glucocorticoids; 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). For this study, despite the changes in plasma hormones, no significant differences in control values of immune function measures were detected between admit and release conditions, and few significant correlations were detected between hormone levels and measures of immune function. Additionally, only a few clinical parameters were significantly different between admit and release in this study. These results may reflect small sample sizes included in this study; however, they may reflect marine mammal-specific physiological responses and robust regulation of homeostasis even in the face of physiological challenge. Circulating catecholamines, for example, also play a role in regulating the dive response during 'normal' conditions (Hochachka et al. 1995; Butler and Jones 1997; Foster and Sheel 2005), and it is likely that such changes

do not have major adverse effects of immune activity. Due to the in vitro approach of this study, the combined effects of these regulatory adjustments in circulating hormones in addition to a stress response could not be directly assessed and needs further investigation.

There were, however, significant correlations between several immune parameters (e.g. CD11b expression, monocyte phagocytosis) and hormone levels. 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, neuroendocrine activity 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).

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 (Brenner et al. 1999; Barack and Katz 2005). 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.

Few relationships between cortisol and immune function were detected in this study. High plasma cortisol levels of 29.8–86.3 $\mu g\ dl^{-1}$ have been reported for several species of Antarctic phocids (Liggins et al. 1993), while the highest cortisol values measured during this study were 38.4 and 46.8 $\mu g\ dl^{-1}$ in a gray and harp seal, respectively, which stranded with respiratory infections and did not survive in rehabilitation. The majority of cortisol values fell below 30 $\mu g\ dl^{-1}$, with release sample cortisol averaging less than 10 $\mu g\ dl^{-1}$. Binding capacity of cortisol by serum proteins was also reported to be high in Antarctic phocids. 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, a possible explanation for the lack of significant correlations with immune responses.

Few statistically significant changes were detected for CBCs and serum chemistries between admit and release conditions, and no significant differences in control measures of immune function were detected. However, the functional response of cells to pressure exposures did differ between admit and release conditions and displayed several interesting correlations with hematology and serum chemistry values.

For phagocytosis, no change or decreased function was observed in admit samples for the dive periods following pressure exposures, while increased activity was observed following further recovery periods. Samples obtained pre-release also showed an increase in phagocytic activity. Interestingly, the relationship between control measures of phagocytic activity per cell and cell activation (CD11b expression per cell) displayed opposite trends between admit and release conditions. For admit animals, decreasing measures of CD11b expression were significantly correlated with higher phagocytic activity, suggesting activity despite low activation. However, for release conditions there was a positive correlation between CD11b expression and phagocytosis; and this increase in phagocytic activity agrees with the general pattern of cell activation. Because CD11b binding is not the only pathway by which phagocytosis can be triggered in granulocytes, these results suggest that phocid granulocytes may utilize different mechanisms to carry out immune functions under different conditions.

Increases in phagocytosis have been reported in response to pressure for human cells (Madden et al. 1995). In belugas, in vitro pressure exposures resulted in a decrease in phagocytic activity during baseline conditions, while an increase in phagocytosis was observed in samples collected under stressor conditions (Thompson and Romano 2015). Differences in the response of phocid cells between admit and release may be due to stressor reduction, and/or the combined effects of stress and pressure between admit and 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; Heinemann 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.

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. Total protein measures are low in cases of malnutrition, and tended to be negatively related to changes in phagocytosis in response to pressure: an interesting result as decreased immune responses tend to be associated with malnutrition. Decreased AlkPh can also be associated with malnutrition and anemia (Kumari et al. 1993; Dover et al. 1993; Lum 1995), and levels in this study were low in admit samples and increased to release conditions. This pattern makes sense, as several animals stranded undernourished, and AlkPh significantly correlated with HCT ($r = 0.678$; $p < 0.001$). Additionally AlkPh is associated with bone growth (Rader 2017), and may also be related to development in young stranded animals. As such the slightly older release animals may also have greater investment in growth as their health issues resolve. Pressure-induced changes in both phagocytosis and CD11b showed significant negative correlations with AlkPh in this study. As AlkPh has been noted to moderate inflammatory processes by facilitating dephosphorylation of signaling proteins (Rader 2017; Koyama et al. 2002), this low AlkPh may increase responsiveness of immune cells during diving, if signaling proteins are left intact, thus potentially playing a role in increasing risk of inflammatory damage.

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 pre-release animals may have altered membrane fluidity as compared with admit. While cholesterol displayed only a few significant correlations with the % of cells undergoing phagocytosis, the relationship was always negative meaning lower cholesterol led to greater changes in the % of cell populations undergoing phagocytic activity. 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 indices were negatively correlated with changes in lymphocyte proliferation, suggesting that in underweight or emaciated animals 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 immune cells from animals in poor body condition respond abnormally to increased pressure, and thus these individuals 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.

An overall activation of lymphocytes was observed as both measures of IL2 receptor expression increased following each pressure excursion. However, though limited data were available for this study, decreased proliferation indices were observed. This is similar to results reported for belugas in Thompson and Romano (2016). 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 h 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. Redundancies within the immune system may allow a response to immunological challenge while suppressing unwanted activity.

The response of immune cells to short-duration pressure exposures (5 min, 2 × 5 min) differed in some cases from the response measured following the 30-min exposure, suggesting that duration is an important characteristic of a dive. WBC counts were positively correlated with pressure-induced changes in phagocytosis, CD11b and IL2R expression following the 30-min exposures, but not the 5-min or repeated 5-min exposures. In contrast, absolute eosinophil counts were negatively correlated with phagocytosis following the 5-min exposures, and positively correlated with phagocytosis following the repeated 5-min exposures. Increased white blood cells often occur in response to certain immunological challenges, such as bacterial infection (Peltola et al. 2006; Korppi et al. 1993; Webb and Latimer 2011). Samples with the highest values in this study were collected

at admit from individuals diagnosed with respiratory disease, bacterial or parasitic infection. These results would suggest that the immune cells of these animals are already actively responding to the infection and were more sensitive to the 30-min pressure exposures as compared to shorter exposures. Eosinophils also are associated with parasitic infection, and increasing eosinophils over the course of rehabilitation may be driven by weaning of young animals and exposure to parasites in food items as found in Greig et al. (2010). Thus, a compromised animal in the wild may still be able to perform single short dives, but increasing duration or repetitive dive bouts may increase the risk of disease development.

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 gray 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 (Serpetti et al. 2017). 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-min 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.

MCH and MCHC showed some positive relationships with the response of immune cells to pressure exposures, meaning larger changes in immune activity were measured when these clinical levels these parameters were increased.

This relationship is interesting as these measures relate to blood oxygen content, and ultimately breath hold and dive ability. Thus, it would seem that animals with the ability to dive longer (and potentially deeper) show increased immune sensitivity to pressure: a somewhat counter intuitive conclusion. In contrast, negative correlations were detected between immune responses to pressure and HCT which also increases during extended dive periods (Castellini et al. 1988). While the exact relationship between these measures and immune function responses is beyond the scope of this work, these results support the need for a better understanding of the relationship between health, development and diving in stranded phocids.

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. The opportunistic use of stranded pinnipeds in this study provided a useful repeated measures paradigm, but limited the interpretation of results due to lack of 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 gray seals or harp seals (Perrin et al. 2002), but development of deep diving ability can be age-dependent (Jorgensen et al. 2001). In this regard, measured immune responses sometimes varied greatly between individuals, resulting in very large standard errors. For example, in Fig. 2, the error bar for the dive period of the repeated 5-min exposure for release samples was too large to be displayed on the graph. This result in particular was due to a single individual with a large magnitude response having a disproportionate influence on the mean. Small sample sizes make statistical analyses of these results difficult, and data sets in this study were too small to run comparisons by species or age, or between paired and non-paired individuals. 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 monocytes 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 suggesting 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, expression of CD11b and IL2R showed significant positive correlations with plasma catecholamines and cortisol, and significant increases in expression of these proteins were found following pressure exposures, though significant differences in response were not detected between

admit and release. Lymphocyte proliferation also 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, such as dehydration, wounding or illness. 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 further investigation is needed to better understand the balance between health and physiological adaptation to demanding behaviors such as diving.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

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