FINAL REPORT
Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (*Tursiops truncatus)*

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LONG-TERM GOALS

Concern for the conservation of marine mammals continues to rise as they are exposed to multiple anthropogenic and natural environmental stressors (Fair and Becker, 2000; Bossart, 2006; 2011). Stress responses play a critical role in allowing animals to cope with environmental perturbations. As such, there remains a large gap in our knowledge about the pathophysiological effects of both acute and chronic stress to guide management decisions on these important species. A paucity of research exists that characterizes basic stress responses required to better understand multiple stressors and biologically significant effects. As top predators, marine mammals can serve as an integrator and intensifier of the diverse stressors and hazards present in the environment. The growing concerns about anthropogenic stress include multiple stressors faced by marine mammals from increased environmental exposures to pathogens, pollution, noise and other acoustic influences. Studies that evaluate these effects in marine mammals are lacking since much of the baseline data related to physiology and health are not available. Given the limited amount of data specific to free-ranging marine mammal populations, our study used managed-care animals to bridge this gap and generate baseline data for stress parameters to ground hypotheses regarding wild populations. The bottlenose dolphin is a particularly relevant species to study stress, as it is the most common cetacean in near coastal waters, with a cosmopolitan distribution, widely represented along the U.S. coast and an important resource for the U.S. Navy.

Although the basic neurophysiology of the mammalian stress response is well understood, there remains much to be discovered about how the components of this system interact with one another, especially in marine mammals for which much less is known. In captive marine mammals, the stress response has been shown to adhere to the classic stress response model with activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in release of adrenocorticotropic hormone (ACTH) from the pituitary into the bloodstream, and secretion of cortisol and other glucocorticoids from the adrenal cortex (St. Aubin and Geraci, 1989a; 1990; St..Aubin et al., 1996). The involvement of the sympathetic nervous system (SNS) in the stress response is responsible for release of the catecholamines (e.g., epinephrine and norepinephrine) (Romano et al., 2004). Our recent study examined these stress parameters and found that wild dolphins exhibited a typical mammalian response to the acute stress of capture and restraint (Fair et al., 2014). However, limited information exists on a comprehensive evaluation of stress in marine mammals and the relationship between the associated stress hormones and related hormones such as the thyroid which play a critical role in numerous metabolic and physiological processes including regulation of basal metabolic rate, oxygen consumption, carbohydrate and lipid metabolism (Larsen, et al. 1992; Yen 2001; Zoeller, et al. 2007). Altered thyroid hormone status has been observed with capture and handling of beluga whales as part of the stress-mediated changes in hormone secretion and metabolism (St Aubin and Geraci, 1988). Stress responses in the immune system are also mediated by the endocrine system via the HPA axis. Evidence predominately from animal studies also indicate thyroid hormone involvement in immune responses to environmental and stress-mediated suppression (Davis 1998; Dorshkind and Horseman 2001).

Studies have shown differences in traditional markers of the stress response between captive and free-ranging marine mammals. For example, lower levels of thyroid hormones have been observed in captive pinnipeds, manatees and cetaceans compared with their free-ranging counterparts (St. Aubin et al., 1996; Ortiz et al., 2000; Myers et al., 2006). In order to consider inherent differences between managed-care and free-ranging animals it is critical to develop baseline measurements of stress biomarkers in both types of populations. Thus, our study design incorporates free-ranging as well as managed-care dolphins subject to different environmental conditions. Assessing baseline stress biomarkers in populations of Atlantic bottlenose dolphins (*Tursiops truncatus*) that live under a range of different environmental conditions and accompanying levels of stress will provide a means of evaluating basic physiological responses to stress using established and novel endpoints.

The overall goal of this research is to characterize the pathophysiology of stress in wild and managed-care bottlenose dolphins and to establish relationships between markers of the stress response applying a broad array of assessments (i.e., immunological evaluations, dependent hormonal endpoints, hematology, serum chemistry parameters, metabolism and protein expression). In order for scientists within and beyond the US Navy to understand and assess the physiological condition of animals in the wild, particularly in regions where animals are exposed to acoustic and other anthropogenic stressors, it is important to determine baseline levels of stress markers under varying environmental conditions and the relationship between these markers. This research addresses this critical need and incorporates new technologies to provide an integrative measure of stress as applied to the bottlenose dolphin.

OBJECTIVES

Our objectives were to assess baseline stress biomarkers in four populations of Atlantic bottlenose dolphins (*Tursiops truncatus*): 1) managed-care Georgia Aquarium (GA) dolphins, 2) managed-care U.S Navy Marine Mammal Program (MMP) dolphins in open-ocean enclosures; 3) wild dolphins from the Indian River Lagoon, (IRL) FL, and 4) wild dolphins from Charleston, (CHS) SC.

APPROACH

A suite of classical stress markers (ACTH, aldosterone, cortisol) and thyroid hormones were measured in collaboration with Dr. Houser and colleagues of the National Marine Mammal Foundation. Chatecholamines, and immunological markers (immunophenotyping (B+T cell lymphocyte subsets, MHCII expression) were measured by Dr. Romano of Mystic Institute for Exploration.

Natural killer cell activity, and lysozyme activity was measured by Dr. Margie Peden-Adams; IgG, and pathogen antibody titers were measured by Dr. Charles Rice. We also incorporated a suite of cytokines (IL4, IL10, IL17, CD69, TNFα, IFNg, IFNa, MX1, IL2-Ra, and FADD) measured by Dr. Jeffrey Stott of the University of California, Davis. Well-characterized baseline stress evaluation using classic stress hormones were paired with biomarker expression using proteomics and metabolomics to provide additional stress profile information and inter-relationships in hormones/biomarkers among different matrices and across populations.

We assessed this suite of stress-related parameters in the four populations of bottlenose dolphins as described below. A total of 49 single collections were obtained from wild dolphins (27 from the Indian River Lagoon, FL and 22 from Charleston, SC). Additionally, 10 GA and 10 MMP dolphins were sampled multiple times during a 12-month period. This approach was used to provide a comparative study among dolphins that live under varying conditions.

Sample Collection

Group 1-managed-care Georgia Aquarium bottlenose dolphins

The most controlled population consisted of managed-care indoor dolphins housed at the Georgia Aquarium (controlled water and air temperatures 76 ºF, limited/minimal pathogens, etc.). Samples were obtained from 10 Atlantic bottlenose dolphins (*Tursiops truncatus*) managed by the Georgia Aquarium (GA) at the new main aquarium facility in Atlanta, GA and at Marineland, FL. GA is a USDA APHIS approved and inspected facility and a member of Association of Zoos and Aquariums (AZA) and the Alliance of Marine Mammal Parks and Aquariums. The dolphins interacted with GA guests starting in April 2011 in a themed performance schedule and encounter sessions. GA's facility in Atlanta is a recently completed state-of-the-art indoor naturally and artificially lighted dolphin facility consisting of 5 pools with a total volume of over 2 million gallons and pool depths ranging from 12 feet to 30 feet. A medical pool with a false bottom that can be elevated for medical procedures is available. The entire volume of seawater in the facility is turned over once every 60 minutes and life support systems include sand filtration, ozonation and protein skimmers. Dolphins are trained for voluntary husbandry behaviors including the collection of blood samples through the fluke arteriovenous plexus. A 12-month sample collection period of dolphins from Georgia Aquarium was completed in August 2012 under the direction of Dr. Gregory Bossart. Samples were obtained from 10 individual dolphins as part of a preventative medical program with multiple collections during the 12-month period for a total of 37 samples (Table 1).

Group 2-managed-care U.S. Navy bottlenose dolphins

This group involved dolphins managed by the United States Navy Marine Mammal Program (MMP). The MMP maintains approximately 70 bottlenose dolphins for use by the Navy in tasks involving underwater object detection and swimmer interdiction. Most of the MMP dolphin population resides at SSC Pacific in San Diego, California, within open-water, netted enclosures (water temp 50-60º F). Access to the population, under permission of the MMP, allowed investigators to collect repeated samples throughout the year from animals of both genders, of multiple age groups, and differing reproductive states. All procedures were approved by the Institutional Animal Care and Use Committee of the Biosciences Division, Space and Naval Warfare Systems Center Pacific and followed U.S. Department of Defense guidelines for the care of laboratory animals. The dolphins were housed in floating net pens within San Diego Bay with ambient water temperatures throughout the year of the sample collection. The dolphins were trained for voluntary blood samples through the arteriovenous plexus of the fluke, and basic husbandry behaviors that permit the collection of other biological samples. A 12-month sample collection period was completed in August 2012 with collection of 65 samples from 10 dolphins in partnership the Marine Mammal Program (MMP) under the direction of Dr. Dorian Houser (Table 1).

Groups 3&4- wild bottlenose dolphins

This group was comprised of populations of free-living estuarine dolphins from two coastal areas of the southeast United States (Figure 1) as part of the Dolphin Health and Risk Assessment (HERA) Project. These dolphin populations live under a greater range of environmental conditions, water teperatures 45-86^{o}F, nutritional/forage stress, pathogen/toxin/contaminant exposures, predators and other stressors. The HERA project is a comprehensive, integrated, multi-disciplinary research project that was initiated as a collaborative effort between the National Ocean Service's Center for Coastal Environmental Health and Biomolecular Research, South Carolina and Harbor Branch Oceanographic Institution, Florida to assess the health status of Atlantic bottlenose dolphins (*Tursiops truncatus*) in two southeast U.S. locations: Charleston, SC (CHS) and the Indian River Lagoon, FL (IRL). CHS (32°46'35"N, 79°55'51"W) included the Charleston Harbor, portions of the main channels and creeks of the Ashley, Cooper, and Wando Rivers, and the Stono River Estuary. IRL assessments were conducted between Titusville, FL (28°36'43"N, 80°48'27"W) and Stuart, FL (27°11'51"N, 80°15'10"W) and included portions of the Mosquito Lagoon, Indian River, Banana River, north and south forks of the St. Lucie River, and Sebastian Inlet. The Dolphin HERA Project incorporates standardized asssessment techniques (Fair et al., 2006b) allowing comparison of health-related data. The large dataset allows insight into effects of variables on various measurements such as thyroid hormones in which marked variation were found to be associated with age, reproductive status and geographic location (Fair et al. 2013). Except as indicated, all markers of stress, endocrine assays, immune function tests and clinical pathology parameters have been measured consistently in contract laboratories since the inception of HERA in 2003. Similar tests and/or validation of methods were used by Dr. Houser on those analyses being conducted under his direction (adrenocorticotropic hormone (ACTH), aldosterone, cortisol, thyroid hormones). In July 2011, samples were collected from 26 dolphins during capture–release health assessments conducted in the Indian River Lagoon, FL (IRL) under National Marine Fisheries Permit No. 14352 (permit dates from 2009-2014) issued to Dr. Gregory Bossart and approved by the Harbor Branch Oceanographic Institutional Animal Care and Use Committee (IACUC). Collections of 19 samples from wild dolphins in Charleston, SC occurred in August 2013 as part of the HERA Project under NMFS Permit No. 14352-02 (Table 1).

Collection Summary

Table 1 provides a summary of demographic data on dolphins from the above 4 groups of dolphins. A total of 147 samples were collected during the study period. Samples collected include multiple samples from each of the Navy dolphins (n=65) as well as from Georgia Aquarium dolphins (n=37) while single samples were obtained from both wild dolphin populations (IRL = 26 and CHS =19). The majority of the samples were obtained from males for all groups except for the Georgia Aquarium in which females constituted the largest group (60%). Mean ages of the dolphins ranged from 14.0 yrs for IRL dolphins, 16.9 yrs for Georgia aquarium dolphins, 17.8 yrs for CHS dolphins and 26.8 yrs for Navy dolphins.

Hematology/Serum Chemistry/Protein Electrophoresis/Hormone Analyses

Rationale: Standardized methods for hematology, serum chemistry and serum protein electrophoresis have been incorporated in our studies under the Dolphin HERA Project since 2003 and have been described previously (Fair et al., 2006b). A single laboratory (Cornell

University, Veterinary Diagnostic Laboratory) has evaluated all samples assuring consistency and attention to QA/QC standards. The results of these investigations (Fair et al., 2006; Goldstein et al., 2006) have been published previously and combined with data from other wild dolphin populations to establish reference ranges and assess variability across populations (Schwacke et al., 2008). Additionally, thyroid reference values were established for both IRL and CHS dolphins (Fair et al., 2011). Hematology, serum chemistry, serum protein electrophoresis (SPEP) and reproductive hormones (progesterone, estradiol, testosterone) were determined by the Animal Health Diagnostic Laboratory (AHDL) at Cornell University (Ithaca, New York).

Methods: Blood and other required biologic samples were collected and analyzed by methods previously described (Bossart et al, 2001) as part of routine dolphin preventative health maintenance examinations for managed dolphin groups. Techniques used for the capture, sampling and release of wild dolphins are described elsewhere (Fair et al., 2006b). Once restrained, blood samples were drawn, generally within the first 10 min after capture, from the periarterial rete in the flukes using a 19-gauge butterfly catheter. Whole blood was collected in EDTA vacutainer tubes for complete blood count (CBC) and serum collected in serum separator tubes (Beckon, Dickinson, and Co., Franklin Lakes, NJ). The tubes were carefully inverted after venipuncture, allowed to clot for approximately 30 minutes and centrifuged at 1,500 rpm for 10 minutes. Serum was collected in cryovials and samples for the various analyses stored at -80ºC until analyzed. Dolphins were then placed on a processing vessel for veterinary health examinations and tissue sample collection. The techniques for hematology, protein electrophoresis, and serum chemistry have been described previously (Goldstein et al.,2005; Fair et al., 2006a) Briefly, white and red blood cell counts, hemoglobin, total platelets and other red blood cell parameters were determined using an automated analyzer (Bayer ADVIA 120, Bayer Diagnostics, Tarrytown, NY, USA). Differential leukocyte counts were performed by microscopic examination of modified Wright-Giemsa stained blood smears (Bayer Healthcare, Tarrytown, NY, USA). A microhematocrit tube was centrifuged for 5 min at 11,700 rpm, and the manual hematocrit was interpreted by visual inspection against a standard calibration. Automated hematocrit (HCT), hemoglobin (Hb), red blood cells (RBC), mean corpuscular platelet volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cells (WBC), platelets, and mean platelet volumes (MPV) were determined by using an automated analyzer (Bayer ADVIA 120, Bayer Diagnostics, Tarrytown, NY). Serum protein electrophoresis was performed on an automated analyzer (Rapid Electrophoresis, Helena Laboratories, Beaumont, TX, USA). Automated hemoglobin, red blood cell count, mean corpuscular platelet volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), white blood cell count (WBC), and total platelets were determined by an automated analyzer (Bayer ADVIA 120, Bayer Diagnostics, Tarrytown, NY, USA). The concentrations of serum chemistry analytes were determined with an automated analyzer (Hitachi 917, Roche, Indianapolis, IN, USA) and included analyses for sodium, potassium, chloride, bicarbonate, anion gap, blood urea nitrogen (BUN), creatinine, uric acid, calcium, phosphorus, magnesium, glucose, total direct and indirect bilirubin, cholesterol, triglycerides, iron, and fibrinogen. Enzyme activity was determined for alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and amylase. Fibrinogen concentration was determined by the method of Schalm using heat precipitation.

ACTH, Cortisol, Aldosterone and Thyroid Hormone Analyses:

Rationale: The adrenocorticotropic hormones, ACTH, cortisol, and aldosterone play a critical role in responding to stress. Circulating levels of these constituents increase in response to stress while prolonged stress may have detrimental effects. Assessing the physiological response to stress in free-ranging dolphins is difficult as the capture and blood collection induces stress. Capture-release studies have investigated hormonal responses in wild dolphins and studies in managed-care dolphins have demonstrated activation of the HPA axis during capture, handling and transport. Thryoid hormones play important roles in regulating many metabolic functions maintaining homeostasis and developmental processes.

Methods: Drs. Dorian Houser and Cory Champagne analyzed blood samples for ACTH, aldosterone, cortisol and thyroid hormones.

Aldosterone: Aldosterone concentrations were generally low in dolphin serum samples. We therefore performed a steroid extraction to increase the detectable quantity of aldosterone prior to radioimmunoassay. Sample aldosterone was extracted into an organic solvent by adding 4 mL dichloromethane (Sigma-Aldrich Inc, St Loius MO: cat# 65463) to 1 mL of serum in 15 mL glass test tubes and vortexed. The aqueous serum and dichloromethane layers were allowed to separate for several minutes and the dichloromethane layer was collected into a second test tube. The extraction was repeated three times, collecting the dichloromethane extract into a single 50 mL test tube and the remaining serum was discarded. Samples were then evaporated to dryness on a vacuum centrifuge. Dried samples were reconstituted in 500 µL of steroid free serum (Siemens, Inc). Extracted aldosterone was then assayed according using a commercially available RIA kit (Siemens Inc, catalog #TKAL1; see Houser et al 2011); we corrected the assay value by the dilution factor of the extraction to calculate the aldosterone concentration from the original serum sample. We tested the extraction efficiency using tritiated aldosterone standards and found greater than 90% recovery; therefore, no correction for extraction efficiency was conducted. The detection limit of the kit is 30.5 pM; the extraction method used increased the realized sensitivity of the assay by at least one-half. Aldosterone analysis was done on all groups of dolphins except for the IRL dolphins as there was insufficient sample.

Cortisol: Cortisol concentrations were generally low in dolphin serum samples. We modified the protocol from a commercially available cortisol RIA kit (Siemens Inc; catalog #TKCO1; see Houser et al 2011) by increasing the serum sample volume from 25 µL to 100 µL. We tested the validity of this modification by comparing cortisol standards assayed using various volumes up to 150 uL and found 100% linearity (i.e. cortisol concentration using 100 µL was four times that at 25 µL). The serum concentrations were corrected for the increased volume simply by dividing by four. The detection limit of this kit is 5.5 nM.

ACTH: ACTH was assayed using a commercially available Enzyme Immunoassay (EIA) kit (Alpco, Salem NH; cat # 21-ACTHU-E01). The detection limit of the kit is 0.05 pM.

Thyroid Hormones: Serum thyroid hormone concentrations, triiodothyronine and thyroxine (T_3 & T_4, respectively) were assessed using commercially available kits to detect each of free and total (both bound with carrier protein and unbound hormone) T_3 and T_4. All thyroid kits were acquired from Siemens Inc and conducted according to the kit protocols. The limits of detection were as follows: total T_3, 0.1 nM; free T_3, 0.3 pM; total T_4, 3.2 nM; free T_4 0.13 pM.

Catecholamine (epinephrine, norepinephrine and dopamine) analysis:

Rationale: Catecholamines are "flight or fight hormones" released by the adrenal glands in response to stress and can cause general physiological changes that prepare the body for physical activity (e.g., increased heart rate, blood pressure, glucose). Catecholamines are part of the

sympathetic nervous system (SNS) and those most abundant are epinephrine (adrenaline), norephinephrine, (noradrenaline) and dopamine. High catecholamine levels in blood are associated with stress, which can be induced from psychological reactions or environmental stressors such as elevated sound levels, intense light, or low blood sugar.

Method: Catecholamines were measured by Dr Tracy Romano of Mystic Aquarium. High performance liquid chromatography (HPLC) with electrochemical detection was used to measure and quantify dolphin catecholamines (NOR, EPI, DA) following the methodology detailed in the manufacturers (BioRad, Hercules, CA) manual on Plasma Catecholamines by HPLC. Briefly, catecholamines were extracted from 1-2 mL of heparinized plasma using 50 mg alumina (BioRad, Hercules, CA) in a conical centrifuge tube containing 200 µl working internal standard (BioRad urinary and plasma catecholamines internal standard, 195-6035[e]) and 1.0 mL 1M Tris buffer (with 2% EDTA, pH 8.6). Samples were mixed and then centrifuged at 900 relative centrifugal force (rcf) for 5 min at 2-5ºC. After discarding the supernatant, the alumina was washed twice with 1.0 mL of HPLC-grade water before adding 200 µl of 0.1M phosphoric acid. To extract the catecholamines from the alumina, the alumina-acid mixture was vortexed for 30 sec then centrifuged at 1200 rcf for 5 min at 2-5ºC. The supernatant was transferred to an autosampler vial (BioRad, Hercules, CA). Samples were analyzed on a HPLC system (1515 isocratic pump, 717 autosampler) with Electrochemical Detection (2465 electrochemical detector[f]. Quantitation of EPI, NOR, and DA was based on comparing peak height ratios relative to an internal standard in both the unknown sample and a plasma calibrator specimen (BioRad plasma catecholamines by HPLC Calibrator, 195-6066; Siemens Medical Diagnostics, Los Angeles, CA). Data were analyzed using Breeze software (Waters, Milford, MA).

Immunology Analysis:

Rationale: *Stress produces profound effects on the immune system.*

In this study on stress markers, we have included assessments for immunophenotyping *(B+T cell lymphocyte subsets, MHCII expression)*, natural killer cell activity, IgG, and pathogen antibody titers. Immunophenotyping quantifies the numbers of cells in each of the different cell types and subsets. In marine mammals, phenotypic changes have been observed with disease and can be indicative of host insult by a number of pathogens (DeGuise *et al*., 1997; Schwartz *et al.,* 2005). Natural killer (NK) cells play an important role in innate immunity by lysing and killing nonself/foreign cells, tumor cells and viral infected cells (Kane *et al*., 1996). Their activity is another commonly measured functional component of the innate immune response (Descotes, 2004; Kane *et al*., 1996). Functional assays of the adaptive immune response are important when measuring immunocompetence. Crucial to this arm of immunity is the ability of lymphocytes to proliferate when activated. We also evaluated a suite of cytokines in collaboration with Dr. Jeffrey Stott. Cytokines are a group of soluble proteins or glycoproteins involved in intercellular communication. . The study of cytokines and immunocellular function are important components for assessing the overall health and stress of animals and humans. In marine mammals, the relevance of biological properties and molecular analysis of cytokines has been only recently addressed (Bradley and Reynolds, 2002; King and Stott, 2002). Recently an increased number of cytokine molecules have been identified in *T. truncatus*, which will help in furthering health assessment in this species and evaluating the effects of stressors (reviewed by Vechionne et al., 2008). For a more comprehensive evaluation of the health status and condition of immunological function in *T. truncatus*, it is important to obtain additional information on this species' hematopoietic system and cytokine function. Thus, we incorporated a suite of cytokines (IL4,

IL10, IL17, CD69, TNFα, IFNg, IFNa, MX1, IL2-Ra, and FADD) in the immunological analysis in samples collected from the dolphin subject groups.

Methods: *Immunophenotyping (B+T cell lymphocyte subsets, MHCII expression) by Dr Tracy Romano, Mystic Aquarium.*

Peripheral blood leukocytes (PBLs) were isolated by a slow spin technique within 2 h of blood collection. Whole blood was centrifuged at 59 rcf in a swinging bucket rotor for 25 min at 10ºC. PBLs were collected by gently swirling the buffy coat into the plasma and transferring the cells into a new tube. Following centrifugation at 377 rcf for 5 min, the plasma was removed and the cell pellet gently resuspended in 1 ml of complete media (RPMI-1640, 10% fetal bovine serum, 50 IU penicillin and 50 mg streptomycin). Numbers of nucleated PBLs were determined using a hemacytometer. Briefly, $1x10^6$ cells/ml were labeled with 50 µl of monoclonal supernatant for 30 min at 4ºC. Antibodies to be used include: Q5/13, a human monoclonal antibody that cross-reacts with dolphin class II molecules; CD4, a cetacean-specific monoclonal antibody that recognizes T helper lymphocytes; IgG, dolphin-specific anti-IgG antibodies that bind to B lymphocytes; and CD8, a cetacean-specific monoclonal antibody that reacts with T cytotoxic lymphocytes. Monoclonal supernatant of the myeloma cell line P2X63-AG8.653 (The Scripps Research Institute, La Jolla, CA, USA) was used as a negative control. The cells were washed three times with HBSS before incubation with fluorescein isothiocyanate conjugated affinity purified goat anti-mouse or goat anti-rabbit F(ab)'2 IgG for 30 min at 4ºC in the dark (Beckman Coulter, Miami, FL, USA). Cells were then washed twice in phosphate-buffered saline and re-suspended in 250 µl of 1% paraformaldehyde for analysis by flow cytometry. Samples were analyzed on an LSR flow cytometer (BD Biosciences, San Jose, CA, USA). Forward/side scatterplots were used to gate lymphocytes based on their size and low degree of granularity. Ten thousand gated events were analyzed by histogram statistics.

Immunological assessments for natural killer cell and lysozyme activity were conducted by *Drs. Peden-Adams*

Peripheral blood leukocytes (PBLs) were isolated from whole blood samples by a slow spin technique (700 rpm in a swinging bucket rotor for 25 min) within 36 h of blood collection. The PBLs were resuspended in 1 ml of complete media (RPMI-1640, 10% fetal bovine serum, 50 IU penicillin and 50 mg streptomycin). Numbers of nucleated PBLs were determined using a hemacytometer following RBC lysis with zap-o-globin or 0.17 M ammonium chloride. Cells were then be diluted as required for each measurement (Bossart et al.,2008; Reif et al., 2009). Natural killer (NK) cell activity was assessed via an *in vitro* cytotoxicity assay using 51Cr-labeled Yac-1 cells as detailed in Bossart et al., (2008) and Reif et al., (2009). Superoxide production, a measure of respiratory burst, was determined using PBLs by assessing nitroblue tetrazolium (NBT) conversion. Plates were assessed for absorbance at 620 nm with a spectrophotometer (SpectraCount; Packard, Meridian, CT, USA). Results shall be reported as the stimulation index (AU stimulated/AU unstimulated) and specific details.

Immunological analysis of IgG, and pathogen ELISAs was conducted by Dr. Charles Rice, Clemson University.

The concentration of IgG was determined in a sandwich capture ELISA using two monoclonal antibodies as previously described (Beck and Rice 2003). Specific antibody activities against select marine bacteria were determined in an ELISA system also described previously (Beck and Rice 2003). Cultures of the following bacteria will be obtained from ATCC and subcultured in

their recommended broths: *Escherichia coli, Erysipelothrix rhusiopathiae, Mycobacterium marinum, Vibrio cholerae and Vibrio parahemolyticu*s. Bacteria were formalin-killed, diluted to an O.D. of 200 nm, and coated onto ELISA plates. Using an indirect ELISA system with mAb BB10-2 (Beck and Rice 2003), serum antibody titers were determined at a 1:200 dilution.

Cytokine expression was determined (IL4, IL10, IL17, CD69, TNFα, IFNg, IFNa, MX1, IL2-Ra, FADD) by Dr. Jeff Stott, University of California, Davis. Three cytokines were related to lymphocyte activation (IL-2Rα, IFNγ, IL-4), two are pro-inflammatory (TNFα, IL-17), two antiviral (MX-1, IFNα), IL-10 is anti-inflammatory and CD69 is involved in leukocyte activation. .

Method: Blood for quantitation of select leukocyte gene transcripts was obtained from PaxGene vacutainer tubes. Total RNA was extracted using a commercial kit and cDNA synthesized with using a commercial reverse transcription kit and stored at −20 °C until analysis. Real-time PCR analysis was conducted as follows. Briefly, five µl of diluted (1:5) cDNA is added to a master mix containing 12.5µl of QuantiTect SYBR Green® Master Mix [5mM Mg^{2+}] (Qiagen, Valencia, CA), 1µl each of forward and reverse sequence specific primers, 0.5µl of Uracil-N-Glycosylase (Invitrogen, Carlsbad, CA), and 5.0µl of RNase-free water; total reaction mixture is 25µl. Samples were loaded into 96 well plates in duplicate and sealed with optical sealing tape (Applied Biosystems, Foster City, CA). Reaction mixtures containing water, but no cDNA, were used as negative controls. Amplifications were conducted on a 7300 Real-time Thermal Cycler (Applied Biosystems, Foster City, CA). Reaction conditions are as follows: 50°C for 2 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 31 seconds, an extended elongation phase at 72°C for 10 minutes, followed by a dissociation step. Product specificity was monitored by analysis of melting curves and periodically confirmed by sequencing the amplified products. Threshold crossing (CT) values for cytokine genes of interest were normalized to the S-9 ribosomal gene.

Proteomic analysis:

Rationale: An antibody-based protein microarray was used to determine expression levels of a suite of stress-associated proteins in skin biopsy samples collected from the wild dolphins. The custom array was originally developed to detect stress protein expression in bears (Carlson, 2011; Carlson et al., in preparation), and is broadly applicable to other wildlife including cetaceans. The array determines expression of 31 proteins associated with four key aspects of the stress response: the hypothalamic-pituitary-adrenal axis, immune function, oxidative stress, and cellular proteotoxicity. Using a statistical approach originally used to assess allostatic load in elderly humans (Seeman et al., 2001), the array produces a quantitative measure of cumulative long-term stress in small (50-100 mg) skin biopsies (Carlson, 2011; Carlson et al., in preparation). Since sufficient quantities of skin were only available from biopsies of wild dolphins, we explored the potential use of blood (buffy coat and plasma) to assess application of the stress protein microarray in these matrices. The use of blood samples would then provide a more direct comparison between managed-care and wild dolphins, and in future work may allow this technology to be applied in situations where more invasive skin biopsy sampling is not possible. Nonetheless, skin samples from wild dolphins will be useful in relating stress proteins to classic stress markers and also to metabolomic markers in blood. Two CITES permits were obtained by Dr. Fair in order to ship samples to Dr. Janz in Canada for proteomic analysis.

Method: Expression profiles of 31 stress-associated proteins were determined in skin biopsy samples from wild dolphins using a custom antibody-based protein microarray recently developed in Dr. Janz's laboratory (Carlson 2011; Carlson et al. in preparation). Sample processing, protein labeling, microarray hybridization and quantitation of protein expression were based on established methods (Haab, 2001; Haab and Zhou, 2004). Briefly, skin samples were ground under liquid nitrogen in a mortar and pestle. Proteins were isolated from powdered skin, buffy coat and plasma using lysis buffer, concentrated using centrifugal ultrafiltration, and labeled with the fluorescent cyanine dyes Cy3 and Cy5 (GE Healthcare, Piscataway, NJ, USA). Protein isolates from individual dolphins were labeled with Cy5. Relative protein expression were determined by comparing individual Cy5-labeled samples to a pooled protein standard labeled with Cy3, which consists of equal quantities of protein isolated from all dolphins within the group. Equal amounts (80 µg) of Cy3- and Cy5-labeled protein, run in triplicate, were hybridized for 1 hr on the microarray, washed with phosphate-buffered saline-Tween (pH 7.4), and dried under nitrogen gas. Microarray fluorescence detection were performed using an Axon Instruments GenePix 4000B scanner (Molecular Devives, Sunnyvale, CA, USA). Mean scanned fluorescence values of each of the 31 stress-associated proteins from each individual dolphin were standardized by dividing by the mean fluorescence obtained from the pooled sample run on the same array to obtain a single relative protein expression value for each protein, which can then be used for statistical analyses. To simplify statistical analysis, we have adopted an approach used previously in humans (Seeman et al., 2001) to identify a "stress protein index" (Carlson, 2011; Carlson et al., in preparation), as follows. For each of the 31 stress proteins within an experimental group, the top quartile of expression among all dolphins was determined. Each dolphin with a stress protein expression value within the top quartile received a point (1), and these values were summed for all proteins. Thus, an individual dolphin can hypothetically be assigned a stress protein index score between 0 to 31. Depending on the statistical relationships being examined, all 31 proteins can be used in this manner, or subsets of proteins falling into one of the four stress protein categories (HPA axis, oxidative stress/inflammation, apoptosis/cell cycle, and cellular proteotoxicity) can be used to investigate relationships among other stress markers, health indices and environmental variables being assessed in this research.

Metabolomic analysis:

Rationale: Metabolomics is the study of small molecule metabolite profiles in a systematic manner of the unique chemical fingerprints that specific cellular processes leave behind (Daviss, 2005). Metabolites are the intermediates and products of metabolism and in the context of metabolomics, this usually represents molecules less than 1 kDa in size. Since the metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes mRNA gene expression data and proteomic analyses may not tell the whole story of what might be happening in a cell. Thus, metabolic profiling can give an instantaneous snapshot of the physiology of that cell and complement information obtained from genomic or proteomic methods. Therefore, we included both metabolomic and proteomic tools to give a more complete picture of the dolphin stress profile.

Method: All serum samples for metabolomic experiments were conducted in the lab of Dr. Mark Styczynski using coupled gas chromatography/mass spectrometry (GC-GC-MS). Pre-processing of include deproteinizing and lyophilization. Principal Components Analysis (PCA) was used to summarize the multi-dimensional data output from the GC-GC-MS analysis into two-dimensional scatter plots that capture the majority of variance in the data set. These plots

were then be subject to a battery of exploratory analyses wherein metabolomic snapshots compared against independent variables (e.g. sex, location, health status, infection, mortality, etc.) to determine if metabolomes cluster according to tested variables (Viant et al., 2003; Viant 2007). Loading plots will be used to identify which groups of metabolites are affected in similar functional fashion by the independent variables. These plots contain much of the metabolic information necessary to help generate subsequent homeostatic models for dolphin metabolism. Having identified key metabolomic shifts associated with independent variables, these were then be extracted from GC-GC-MS output tables, score plots and loading plots for information relevant to specific areas of interest. In addition to the multivariate analyses of quantitative spectral data, linguistic analyses of lists of candidate compounds were also undertaken to extract patterns regarding the frequency of specific metabolites in different groups, which can then be tested by cross tabulation or other, non-parametric methods. This approach can also identify key bioindicator metabolites that are closely allied with health status or specific disease states. One sample from each animal was analyzed on a Leco Pegasus IV two dimensional gas chromatography-mass spectrometry system. Data acquisition was performed per standard protocols, including sample preparation and work-up, randomization of samples across batches within the experiment, and frequent injection of pooled quality control samples to allow for correction of instrument drift. The resulting data was processed through a combination of instrument manufacturer software, in-house data processing software, and publically available data processing and interpretation software. Additionally, longitudinal samples for the Navy dolphins were run separately on the same instrument. Six to seven samples were available for each of the ten Navy animals. Samples and data were processed identically to the approaches described above for the multi-group analyses.

Data Management, Quality Assurance and Analysis (John Reif and Adam Schaefer, Harbor Branch Oceanographic Institute, Ft. Piece, FL)

A Microsoft Access database framework was completed to include all variables for the data collected in this study. Thus far, this database contains all data for completed parameters submitted from the various researchers and laboratories using Quality Assurance/Quality Control processes. Descriptive statistical analysis for each population was conducted to calculate the mean, standard deviation, range and quartile values for all test variables (excluding proteomics and metabolomics which were analyzed separately). Normality was assessed using the Sharpiro-Wilks test and variables were log transformed to meet test assumptions. Inter-population comparisons were conducted initially in ANOVA, followed by ANCOVA to control for potential confounding by age and gender. The final models incorporated a repeated measures design since Navy and GAI dolphins were sampled on multiple occasions. In order to assess potential temporal variation among Navy and GAI samples, a repeated-measures ANOVA with population and time as main effects and an interaction term (population*time) was used. To compare mean values across study populations a nested repeated measure ANOVA using study location as a fixed effect and individuals as a random effect was used. Post-hoc comparisons of location means was conducted by least significant differences. The potential effects of serum cortisol concentration on multiple downstream markers were evaluated in a multivariable linear mixed regression model. Statistical significance was established at $p < 0.05$. All analyses were conducted using SPSS version 22 (IBM Corp. 2011, Armonk, NY).

WORK COMPLETED

Comparison of stress and immune markers in managed-care and wild dolphins

Stress Markers - Descriptive statistical analysis for each population is presented in Tables 2-5 containing means and standard deviations for all test variables. Tables 6-9 provide inter-population comparisons incorporating a repeated measures design to compare mean values across study populations. The ANOVA p values indicate the significance of overall comparisons across the four population groups. The post-hoc p values presented for each of the inter-population comparisons can be used to assess differences between individual populations of dolphins for each parameter. The mean values in tables 6-9 are unadjusted. Comparison of mean values following repeated measures analysis of variance between the managed groups and the wild dolphins indicated significant differences in several hematological (Table 6) and serum chemistry parameters (Table 7). Generally, differences were found between managed care and wild populations. Significant differences across populations were found for several red blood cell parameters including total RBCs, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. Wild dolphins had slightly higher levels of RBCs than managed care dolphins. All hematology values appeared to be within normal limits. Wild dolphins had lower MCH than managed care dolphins. Significant differences were also found for total white blood cells and white blood cell types across population groups. Total white blood cells and absolute numbers of lymphocytes, eosinophils and platelets were significantly higher in wild dolphins.

There have been limited studies comparing hematologic parameters between managed-care dolphins to wild dolphins. Wild dolphins were reported by Asper et al., (1990) to have significantly higher WBC, lower neutrophils and a higher percentage of eosinophils than captive dolphins. The present study found similar results for WBC and eosinophils while differences in neutrophil levels were only found between the two managed-care groups. The lower WBC and eosinophil levels in captive animals are thought to be associated with reduced exposure to infectious agents and parasitism (Asper et al 1990).

Serum chemistry analyses were performed on both wild populations and only the GA managed-care dolphins. These comparisons showed higher creatinine, glucose, AST, AP and iron in GA dolphins versus either of the wild dolphin groups. Differences in creatinine, a product of muscle metabolism cleared by renal excretion, may reflect dissimilar levels of protein intake and/or exercise or larger individuals, which we will consider during the next phase of analysis. Higher blood glucose values in GA dolphins could be due to a higher plane of nutrition. Lower levels of alkaline phosphatase and serum iron are associated with various disease states in dolphins. Both AST and AP were higher in GA dolphins. Generally, AST is a nonspecific serum analyte that when elevated may indicate –hepatic, cardiac and/or skeletal muscle injury with the concurrent elevation of other serum analytes such as ALT, GGT, and others (Bossart et al. 2001). The latter analytes were not elevated in GA dolphins thus the higher AST levels are likely not clinically significant. AP levels in bottlenose dolphins vary with age, nutritional status and the presence of infection or inflammation (Bossart et al. 2001; 2010). Low AP levels are used as a nonspecific but important prognostic indicator of general state of health (Fothergill et al. 1991). Rapid decreases in AP in dolphins are frequently indicative of a disease state and can be observed with various infectious diseases including lobomycosis and subclinical morbillivirus infection

(Bossart et al. 2003, 2010; Reif et al. 2006). The pathologic mechanism for decreased AP levels in dolphins with disease is unknown but it postulated to be associated with AP's ability to act in endotoxin detoxification as observed in rats and humans (Bossart et al. 2001, Poelstra et al., 1997). Acute disease with associated endotoxemia may consume and deplete available serum AP, and so, with resolution of disease, circulating levels of AP return to normal. Another possible cause for low AP is related to a negative nutritional status and since inanition may be a clinical component of many dolphin diseases, this alternate mechanism may account for decreases of SAP in this species (Bossart et al, 2001). Thus, the higher AP in GA dolphins may reflect a more positive nutritional status and/or the absence of infection or inflammation compared to the wild dolphin groups. It is also known that variables such as age and gender can influence iron, and AST (Fair et al., 2006; Goldstein et al., 2006). Interestingly, cholesterol was significantly higher in CHS dolphins (160.7 mg/dL) compared to IRL dolphins (143.0 mg/dL) and GA dolphins (143 mg/dL) which may be associated with their higher perfluorinated chemical burdens (Fair et al., 2013). The higher iron levels in managed-care dolphins are consistent with other studies that have reported higher iron levels (Mazzaro et al., 2012).

Protein electrophoresis showed GA dolphins with significantly different levels of most serum fractions measured compared to wild dolphins. GA dolphins had lower levels of total protein due to lower total alpha globulins, total beta globulins and total gamma globulin protein fractions than wild dolphins. These results suggest that dolphins in an aquarium environment have lower exposure to foreign antigens and lower levels of several classes of immune globulins in response. GA dolphins had a relative hyperalbuminemia which is likely not clinically significant or is the result of a higher nutritional plane.

A suite of hormones (cortisol, ACTH, thyroid, aldosterone and catecholamines) was measured to assess baseline levels stress markers. There were significant differences between groups. Wild dolphins had higher levels of ACTH, (IRL149 pg/mL followed by CHS with 59.19 pg/mL, GA dolphins, 24.56 pg/mL and Navy dolphins 6.82 pg/mL). Sampling procedures for blood collections from managed dolphins occurred via voluntary husbandry behaviors as indicated in the methods section. These dolphins should theoretically be under less physiologic stress than those living under free-ranging or managed conditions where samples must be acquired under restraint. Sampling procedures can influence stress responses and time-dependent hormone release as recently demonstrated (Fair et al., 2014) The differences in ACTH concentrations between the two the wild dolphin populations is consistent with recent results reported in our paper on acute stress during capture-release studies in which CHS dolphins had lower ACTH values than IRL dolphins (Fair et al., 2014). Similarly, cortisol levels were significantly lower in managed-care dolphins (Navy 0.98 ug/dL; GA dolphins 0.46 ug/dL) than IRL dolphins (1.25 ug/dL). However, cortisol levels in Navy dolphins were not significantly different than CHS (0.84 ug/dL); only GA dolphin cortisol levels were lower and these cortisol levels when combined with the GA ACTH data suggest a comparative state of less physiologic stress. The cortisol value for both wild dolphin populations during previous capture-release studies were higher than those obtained in the 2013 study as part of this current work (CHS 2.36 ug/dL; IRL 2.59 ug/dL) (Fair et al., 2014). However, the earlier analyses for cortisol and ACTH were done in another laboratory and it is likely that methodological differences between laboratories explain the differences. Aldosterone was just recently analyzed and preliminary results indicate that the

CHS wild dolphins had mean levels (101.5 ±SD 125.4 pmol/L) five to seven times higher than the navy dolphins (14.6 17.1 pmol/L) and GA dolphins (18.1 ± SD 28.5 pmol/L), respectively.

Navy dolphins had significantly higher dopamine levels than IRL dolphins but were not different from either the CHS or GA dolphins. Epinephrine was significantly higher in both managed-care dolphins compared to their wild counterparts, which would seem counterintuitive as blood samples were collected by voluntary husbandry techniques. Norepinephrine was significantly lower in both managed-care groups compared to the CHS dolphins. Wild dolphins differed in both epinephrine and norepinephrine concentrations (CHS epinephrine 634.4 pg/mL; norepinephrine 219.4 pg/mL; IRL epinephrine 142.2 pg/mL; norepinephrine 80.8 pg/mL). Our previous results indicate that concentrations of catecholamines change rapidly over time following chase, net encirclement and capture (Fair et al., 2014). This fact and the high variances in the mean values suggest that the catecholamines may be a less reliable biomarker for studies of stress in bottlenose dolphins.

Numerous differences were observed in concentrations of thyroid hormones among the four groups of dolphins. The highest levels of total T_4 were found in CHS dolphins (12.0 ug/mL) followed by GA (11.0 ug/mL); IRL (10.6 ug/mL) and Navy dolphins (9.9 ug/mL). Navy dolphins had significantly lower T_4 than GA and CHS dolphins, while IRL levels of T_4 were significantly lower than GA; IRL and CHS also differed significantly. Conversely, the highest levels of T_3 levels were observed in Navy dolphins followed by CHS, IRL, and GA dolphins (Navy dolphins were significantly higher than all other groups.) Free T_4, the active form of thyroid hormone, was highest in CHS dolphins followed by GA, Navy and IRL dolphins (note: Navy dolphins were significantly lower than GA and CHS, while GA dolphins were significantly higher than IRL dolphins; CHS and IRL dolphins also exhibited significant differences. No clear pattern in thyroid hormones was found.

Water temperature, and thus seasonal effects, are likely variables affecting specific hormones such as thyroid. Other biological and environmental variables including contaminants such as polybrominated diphenyl ethers known to affect thyroid metabolism (McDonald 2002), may also contribute to differences. As previously, described, higher levels of thyroid hormones in CHS compared to IRL dolphins may constitute an adaptive phenomenon to their colder environment (Fair et al. 2011). Differences in levels of thyroid hormones between groups of wild dolphins and inconsistent differences between wild and managed care dolphins suggest that thyroid hormones may be of limited use in studying stress responses in the wild. However, free T_4 related overall to cortisol (Table 10) which may be useful in further exploring as a possible stress marker.

Immune Markers - Comparison of mean values between the managed dolphin groups and the wild dolphins indicated significant differences in several immune parameters (Table 9). Managed-care dolphins had significantly lower absolute numbers of MHCII+ cells, CD2+ T cells, CD4+ helper T cells and mature CD21 B cells compared to wild dolphins. These parameters reflect differences in cell-mediated immunity between the groups. The relatively large differences in these parameters between wild and managed care dolphins suggest that these differences are important. Further, serum IgG as well as NK cell activity was significantly lower in managed-care dolphins compared to the wild dolphin groups. IgG binds pathogens and toxins and has an important role in antibody-dependent cell-mediated cytotoxicity. NK cells are cytotoxic lymphocytes that constitute a major component of the innate immune system with roles

in rejection of tumors and virus-infected cells. There were no differences observed between the two managed-care dolphin groups (Navy, GA) regarding absolute numbers of MHCII+ cells, CD4+ helper T cells, CD21 B cells-mature and NK cell activity. The GA dolphins had the lowest CD21 B cells-mature, serum IgG, and lysozyme concentration among the dolphin groups, probably reflecting lower exposures to foreign antigens as discussed below.

A suite of nine cytokines was measured and compared among the four groups of dolphins. GA and navy dolphins had significantly higher expression of the two pro-inflammatory cytokines IL-17 and TNF as well as anti-viral MX1 and INFα, anti-inflammatory IL-10, and two cytokines involved with lymphocyte activation, IL-4 and IL-2Rα, compared to one or both wild groups. Navy dolphins generally had the highest levels of expression for all the cytokines although the magnitude of these differences was small and of uncertain biological significance. Between the two wild dolphin groups, only INFα and IL-10 were significantly different, with higher levels in the CHS dolphins.

Specific antibody titers against several common marine bacteria (*E. coli, E. rhusiopathiae, V. cholera, V. parahaemolyticus, M. marinarum*) were measured as an indication of the general immune response. Antibody titers were significantly higher in wild dolphins compared to one or more of the managed dolphin groups (Table 9). No differences were observed between the two managed-care dolphin groups with their antibody titers to *V. cholera* and *E.coli* but differences occurred in the other three organisms (*E. rhusiopathiae, V. parahaemolyticus, M. marinarum).*

Overall, the differences found in the dolphins' immune parameters appear to reflect differences in the environmental conditions under which these four dolphin populations live. The differences in antibody concentrations against marine microorganisms suggest that managed-care dolphins have reduced antigenic exposure to infectious agents including parasites. GA dolphins live under the most controlled environmental conditions (i.e., constant water and air temperature 76 ºF, salinity, pH, lighting, noise and diet with limited/minimal exposures to pathogens, toxins and contaminants). Therefore, one would expect their exposures to pathogens and challenges to their immune system to be low compared to the other groups. For certain responses such as IgG, NK, lysozyme activity and some antibody titers, this indeed was the case. Important differences observed between managed-care and wild dolphins in a wide range of immune parameters appear to reflect differences in their environmental exposures. Managed-care dolphins typically do not carry a parasite burden as the parasite life cycle is terminated by freezing of food fish or some may be treated with anthelmintics. In contrast, parasites are ubiquitously found in wild dolphins.

Since cortisol is often considered the key marker of a long-term stress response, the relationships between serum cortisol and multiple downstream markers were evaluated in a multivariable linear mixed regression model (Table 10). Significant associations were found between cortisol and the following six parameters: free T_4, ACTH, MHCII+ cells, CD4+ helper T cells and antibody titers to *M. marinarum* (note*: p = 0.06 for E.coli antibody)* Cortisol, as the main circulating glucocorticoid in marine mammals prepares an animal to respond to a threat although prolonged release(s) can be detrimental (Butler and Romano, 2007). As cortisol is difficult to measure in marine mammals without incurring a stress response, downstream markers found to be associated with cortisol response in this study may be useful prognostic indicators of stress in

dolphin populations. The most promising marker in this regard may be free T_4, since this hormone is significantly correlated with serum cortisol and is also regulated through the hypothalamic-pituitary axis. However, further work is needed to resolve some of the inconsistencies in the data described above.

Further Analyses:
The complex suite of stress-related parameters assessed in this study require further statistical and modeling to integrate the stress variables and their inter-relationships in managed-care and wild dolphins. Initial efforts were directed at conducting cross-population comparisons using an analysis of variance to identify potential differences in the levels of each marker; e.g., between GAI and Navy dolphins, between GAI and HERA dolphins etc. in order to develop an understanding of the magnitude of potential differences between groups of animals of the same species living under disparate environmental conditions. Additional analysis will be performed on markers that are available from two or more matrices (blood, urine, saliva etc.). Correlation coefficients will be calculated initially to evaluate the strength of these relationships and the variability across substrates. Future analyses may examine the effects of anthropogenic contaminants on the stress markers in each population as data made available from other sources permit.

Proteomics – Our work with Dr. David Janz involved using a novel antibody-based protein microarray to determine expression levels of 33 stress-associated proteins (Table 11) in small skin biopsy samples collected from bottlenose dolphins. The microarray, originally developed for our research in free-ranging grizzly/brown bears, measures expression of proteins associated with four key aspects of the stress response: hypothalamic-pituitary-adrenal (HPA) axis, apoptosis/cell cycle, proteotoxicity/cellular stress, and oxidative stress/inflammation. As the largest organ in the body of mammals, skin responds to multiple endogenous and exogenous stressors and is thus well-suited as a tissue for monitoring stress in wildlife. Since skin has recently been discovered to possess an independent, functional HPA axis, our research thus applies exciting advances in cutaneous neuroendocrinology to the emerging field of conservation physiology.

Our objectives in year 1 were methodological, and focused on (1) evaluating the applicability of the microarray to dolphin skin biopsy specimens, and (2) determining whether the microarray could reliably measure stress proteins in white blood cells (WBCs; consisting of neutrophils, eosinophils, basophils, lymphocytes and monocytes) and plasma collected during routine blood sampling of dolphins. We predicted that many of the 33 antibodies on the microarray would specifically recognize dolphin proteins since they were initially selected from a large panel (>250) of broadly reactive commercial antibodies that specifically cross-reacted with bear proteins. We also predicted that we could successfully isolate and concentrate proteins in blood matrices (WBCs and plasma) so that such samples could also be utilized for the microarray. Since collection of skin samples is a more invasive procedure compared to blood collection, particularly in managed-care dolphins, we attempted to modify our technique to expand the applicability of the microarray to blood matrices. We were successful in achieving both of these initial methodological objectives. The microarray recognized all 33 stress-associated proteins in dolphin skin samples. In addition, we were able to sufficiently concentrate proteins isolated from WBCs and plasma so that the microarray also recognized all 33 stress-associated proteins in

these blood matrices. To our knowledge, this is the first time such a proteomics approach has been achieved in any wildlife species.

In years 2-3 we determined stress-associated protein expression in selected skin, WBC and plasma samples using the microarray, which were collected from a total of n=45 individual wild and managed-care bottlenose dolphins. Skin samples were not collected from managed-care dolphins (note: restricted in GA dolphins as part of a USDA defined preventative medicine program). We initially analyzed these data for normality, homoscedasticity and outliers, and then applied natural log or square-root transformations to achieve normality. Protein expression was evaluated as (1) individual proteins, (2) cumulative protein expression within each of the four functional groups (e.g., HPA proteins), and (3) total cumulative protein expression. This enabled us to conduct parametric statistical comparisons (using repeated measures ANOVA or linear mixed modeling) of protein expression in skin, WBC and/or plasma among the following groups of dolphins:

1. Skin vs. WBC vs. plasma in Indian River Lagoon (wild) dolphins (n=8);
2. Skin vs. WBC in wild male dolphins from Indian River Lagoon (n=7) and Charleston Harbor (n=11);
3. Skin in male and female dolphins from Indian River Lagoon (n=16) vs. Charleston Harbor (n=12); and
4. WBC in wild dolphins (Indian River Lagoon [n=9] and Charleston Harbor [n=12]) vs. managed-care dolphins (Georgia Aquarium [n=10] and San Diego Naval facility [n=7]).

A variety of potential predictor variables obtained from the dolphin dataset was used to test for associations with stress-associated protein expression. This was achieved using linear mixed model analysis, with a stepwise backward selection approach starting with a global model containing all potential predictor variables. Akaike's Information Criterion (AIC) was used to sequentially eliminate least important variables based on *F*- and *P*-values (<0.05). Potential predictor variables included:

1. Age, sex, population (IRL, CHS, Georgia Aquarium, and San Diego Naval facility), and confinement status (wild vs. managed-care);
2. mRNA transcript abundance of selected proteins (AIF, HSP90, HO2, SOD1);
3. Plasma hormones (ACTH, cortisol, estradiol, progesterone, testosterone, total and free T4, and total T3);
4. Hematology (PCV, Hb, RBC counts, neutrophils (N), lymphocytes (L), N:L ratio, monocytes, and eosinophils); and
5. Routine serum biochemistry (e.g., electrolytes, glucose, serum proteins, and functional enzymes; 31 variables).

With such a large number of variables, the results of these statistical analyses are complex, and the following general conclusions can be made.

1. There were many statistically significant differences in protein expression among skin, WBCs and plasma, although these differences were protein-specific with no clear trends

for differences among matrices. This is not surprising since the relative importance and roles of specific proteins will differ among matrices.

2. Wild male dolphins had significantly greater expression levels for 10/33 proteins in skin compared to females. Overall, there were several sex differences in protein expression, although this was limited by the relatively small number of females sampled.
3. There were many differences in skin and WBC protein expression when comparing wild populations (IRL and CHS). In skin, 13/33 proteins exhibited greater expression in IRL dolphins compared to CHS dolphins (Figure 2).
4. There were few age differences in protein expression.
5. When comparing protein expression in WBCs (where we could compare wild vs. managed-care dolphins), 24/33 proteins were significantly different between wild and managed-care dolphins (Figure 3). These differences were protein-specific with 15 proteins greater in wild dolphins and 9 proteins greater in managed-care dolphins.
6. Protein expression in skin and WBCs showed many consistent associations with measures of plasma endocrinology, hematology, and serum biochemistry; this was particularly true for plasma endocrinology.
7. There were no consistent associations between protein expression and mRNA transcript abundance in skin and WBCs, although this was limited by the number of comparisons (4) that could be made. Apoptosis-inducing factor (AIF) gene expression was positively associated with AIF protein expression in WBCs, which is likely of biological significance due to the rapid turnover (high rate of apoptotic cell death) of WBCs.
8. Although individual protein expression values were significantly associated with multiple predictor variables as described above, cumulative protein expression within functional categories and total cumulative protein expression were not as consistently associated with predictor variables.

In combination with other research being conducted in these dolphins, this proteomics research should provide unique insight into stress responses in multiple tissues and matrices. Based on the results described above, a manuscript (in preparation) will be submitted to a peer-reviewed journal in early 2015 (e.g., *Conservation Physiology*). It is possible that a second manuscript will be written depending on findings from the other studies conducted by collaborators and integration with the proteomics work.

Metabolomics - Metabolite profiles have been measured by Dr. Mark Styczynski for one animal from each of the four groups using untargeted GCxGC-MS analysis with subsequent annotation of measured analytes using mass spectral databases. Using all measured analytes and using principal components analysis to reduce the dimensionality of the dataset in an unsupervised fashion, there is clear separation between the four groups in the first two to three reduced dimensions. This suggests significant metabolic differences between the animals. Using only the annotated metabolites, separation is still present but is weaker, indicating that poorly annotated portions of the dolphin metabolome may be critical in distinguishing populations from each other. These annotated metabolites have been the basis for initial biological interpretation of the results, which indicates that there is likely some difference between the groups due to sample acquisition (i.e., fasted vs. nonfasted), but that much of this separation is not explained by sample acquisition differences. Continuing work is focused on biological analysis and interpretation of the analytes that most strongly distinguish the animal groups from each other, as well as

"temporal" metabolite profiling for samples for which there are multiple samples in order to assess intra-animal variability.

Overall analysis - In the four-group analysis, the resulting data indicated substantial differences between all of the groups (Figure 4). Using all of the analytes measured and tracked during our analyses, hierarchical clustering easily separated the four groups quite easily, suggesting substantial metabolic differences between the groups of animals. Principal components analysis, a dimensional reduction technique, similarly identified clustering and separation of the different groups of animals within the first two to three principal components. However, the clustering was not as strong when only analytes that were annotated to known metabolites (based on mass spectral similarity) were considered in the data analysis. In this case, the Indian River Lagoon animals still separated out fairly strongly from the rest of the animals, and the Navy animals still separated out fairly strongly from the rest of the animals, but the clustering for both of these cases was not perfect (meaning incorrect assignment of a member of that group to another cluster or assignment of a member of a different group to one of those clusters). Principal components analysis suggested similar misclustering, but with a more evident interpretation: in the first two to three principal components, the animals' metabolite profiles tended to cluster together by group, but those clusters showed overlap that was not evident when considering all of the analytes originally measured.

We conclude from this that some aspect of metabolism that is not well-characterized in existing mass spectral/biochemical databases is actually a strong driver of the differences in metabolism between certain groups of animals (in particular, the Indian River Lagoon animals from the Charleston animals, and to some extent from the Georgia Aquarium animals). This was fairly surprising; when unknown analytes are removed from consideration, we typically only see a small change in separation between groups in principal components space. To see such a substantial change in the first two principal components based on the exclusion of unknown analytes suggests a disproportionate importance of poorly characterized sections of metabolism.

In the longitudinal analysis, there was little evidence of highly individual-specific metabolite profiles (Figure 5). Using hierarchical analysis and all measured analytes, only two animals had more than half of their samples cluster together; the best separated animal had five of its six samples cluster together, with the sixth in a distant cluster, while the next-best separated animal had four samples cluster together and another three samples cluster in the next most related cluster (though with a number of other samples in that cluster). A different animal had two sets of three samples each cluster together, but fairly distantly from each other. Principal components analysis yielded similar interpretations, highlighting that a few animals seemed to have metabolite profiles more dissimilar from the rest, but that the profiles generally exhibited substantial overlap. However, while any arbitrary pair of animals may have significant overlap, there may be some separation between subgroups of animals; further analysis of clinical and other metadata parameters, including gender, may yield further insights. Also of note is that neither method of interpretation provided substantial evidence for temporal clustering of samples; that is, there did not seem to be any factors that affected all animals' metabolomes similarly and simultaneously.

Biochemical interpretation -Analysis at the single-analyte level, restricted to only those analytes that have been assigned a tentative identification based on mass spectral similarity to known spectral libraries, has provided a foundation for biochemical interpretation and analysis that is currently being expanded. A common mode of interpretation is to look at the analytes most heavily weighted in principal components analysis (with principal component one responsible for the separation of the Navy animals and principal component three responsible for the separation of the other three groups). This approach identified a number of fatty acids (including eicosapentaenoic acid, heptadecaenoic acid, and docosahexaenoic acid) as playing a dominant role in separating the three non-Navy groups. Some of these fatty acid levels may very well be diet-associated, and dietary confounders are difficult to remove from this analysis.

Using ANOVA to analyze the data reveals over forty different tentatively annotated metabolites that exhibit differential profiles across the groups. Probing more deeply into some of these analytes reveals a number of different metabolite-specific patterns. For example, some analytes like guanine and hypoxanthine seem to have "graded" behavior, with three or more apparent average "levels" in the four groups. Other analytes, like eicosapentaenoic acid, are typically below the detection limit in one group but detectable at fairly similar levels across other groups. Other analytes, like benzoic acid, appear to be elevated in only one group. Extensive biochemical interpretation of the potential meaning of these differences, including enrichment analysis based on known metabolite pathways, is ongoing. (However, we note that while pathway-level interpretation can be useful for identification of overall trends, mechanistic inferences from these analyses based on circulating serum levels are typically not possible.)

Expected or possible future manuscripts: We expect at least one, possibly two, manuscripts to emerge from this work in the next two to six months. Immediate work will focus on a comprehensive description of all metabolomics data generated to date, including the four populations with longitudinal data for two of the populations. Based on this content, the working title of this manuscript is "Serum metabolomics of four dolphin populations." We hope to generate this manuscript in the next two months. An additional manuscript may come from an integrative analysis of the metabolomics data with the data collected by other participants in this project. In particular, we hope to focus on building simple statistical models (partial least squares, etc.) that use metabolomics data to fit or predict other clinical chemistry parameters. This is a longer-term priority, which we would aim to complete in the next six months. The working title of this manuscript will be "Metabolomics-based prediction of stress indicators in bottlenose dolphins."

RESULTS

This study provides a well-characterized baseline evaluation of classic stress hormones paired with biomarker expression using new technologies that describe the natural variation and inter-relationships in hormones/biomarkers across dolphin populations maintained under differing environmental conditions. This study is the most detailed study to date considering the scope and comprehensive suite of measurements, sample size among four different groups including repeated measures over a year for managed-care dolphins. We found multiple significant differences among the four dolphin populations. As expected, the greatest number of differences were found between managed-care groups and wild dolphins reflective of the different

environments in which they live. Many of the differences found between managed-care dolphins and wild dolphins are consistent with their cleaner environment and reduced antigenic stress. On the other hand, the biological significance of these differences cannot be determined with the existing data. It is likely that many of the statistically significant differences may not be accompanied by a biologically significant response or health effect. An additional, important caveat is that some of the statistically significant differences described may be due to chance in view of the relatively large number of comparisons conducted.

The assessment of stress variables and response in managed-care animals has important implications for the assessment and interpretation of stress in wild bottlenose dolphins. Considerable individual variation exists in hematology and blood chemistry measurements and individual baselines can be obtained in managed-care dolphins. However, only a single sample is obtained from wild dolphins. Stress can have a dramatic effect on some serum chemical constituents and as such, can make interpretations difficult in both wild and captive animals that may be stressed during sampling. The results of this study highlight differences between managed-care and wild dolphins. In order for the US Navy to understand and assess the physiological condition of animals in the wild, particularly in regions where animals are exposed to acoustic and other anthropogenic stressors, it is important to determine baseline levels of stress markers for comparisons with potentially exposed populations. Differences between managed care and wild dolphins show that these parameters may be affected by local environmental conditions, irrespective of anthropogenic stressors. This research assists in addressing this critical need and furthermore incorporates the use of new technologies with classic parameters to provide an integrative measure of stress biomarkers extending our knowledge and application of such measures. Presentations and publications from this project are listed below. Additional manuscripts intended for publication are planned.

PUBLICATIONS

Presentations:

Houser, D.S., Champagne, C., Bossart, G.D., Fair, P.A. 2011. Estimating the impact of specific stressors requires comparisons to minimal stress conditions. Presentation at the Stress Workshop, Marine Mammal Biennial Conference, Tampa, FL., December 2011.

Fair, P.A., Schaefer, A.M., Reif, J.S., Houser, D.S., Romano, T.A., Champagne C.D., Bossart, G.D. Stress Markers in Managed-Care and Wild Dolphins, 2013 Bienniel Conference on Marine Mammals, New Zealand, December 2013.

Reif, J.S., Schaefer, A.M., Romano, T.A., Stott, J.L., Rice, C.D., Bossart, G.D., Houser, D.S., Champagne, C.D., Fair, P.A. Immune Markers in Managed-Care and Wild Dolphins. 2013 Bienniel Conference on Marine Mammals, New Zealand, December 2013.

Publications:

Lewis, L., Lamb, S.V., Schaefer, A.M., Reif, J.S., Bossart, G.D., Fair, P.A. 2013. Influence of collection and storage conditions on ACTH measurements in dolphins (*Tursiops truncatus*). 39(4) 324-329.

Fair, P.A., Schaefer, A.M., Houser, D.S., Bossart, G.D., Romano, T.A., Champagne C.D., Stott, J.L., Rice, C.D., Reif, J.S. Stress and immune markers in managed-care and wild Dolphins (in preparation).

Janz, D. and co-authors. Application of an antibody-based protein microarray to assess stress in managed-care and wild dolphins (in preparation).

Styczynski, M. and co-authors. Metabolomic differences in serum from managed-care and wild dolphins (in preparation).

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Table 1. Demographic data for Bottlenose Dolphins from Managed Care and Wild Populations.

	Navy	GAI	IRL	CHS
Individuals	10	10	26	19
Blood Samples	65	37	26	19
Gender				
Male	6 (60%)	4 (40%)	15 (58%)	12 (63%)
Female	4 (40%)	6 (60%)	11 (42%)	7 (37%)
Mean Age (SD)	26.8 (9.2)	16.9 (8.9)	14.0 (7.0)	17.8 (7.5)

Table 2. Unadjusted means (± SD) for hematological parameters for managed-care and wild bottlenose dolphins, by site.

	Navy		GAI		IRL		CHS	
	mean	std	mean	std	mean	Std	mean	Std
Manual Hematocrit (%)	42.41	2.67	40.12	3.21	42.52	2.79	41.74	3.31
Hemoglobin (g/dl)	14.62	0.87	13.59	1.09	14.46	0.92	13.76	0.99
RBC (10x3cells/ul)	3.32	0.29	3.24	0.26	3.66	0.30	3.55	0.26
Mean Corpuscular Volume (MCV) (fl)	128.40	6.86	124.14	4.53	153.70	192.04	118.05	7.48
Mean Corpuscular Hemoglobin (MCH) (pg)	44.37	2.85	42.19	1.87	39.78	2.50	38.95	2.48
Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dl)	34.58	0.65	33.93	0.39	34.07	0.62	33.05	0.78
WBCs (10x3cells/ul)	5.89	1.62	9.18	1.73	10.39	3.15	12.49	3.10
Segmented Neutrophils (10x3cells/ul)	3.78	1.04	6.23	1.61	4.99	2.02	4.85	1.62
Band Neutrophils (10x3cells/ul)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lymphocytes (10x3cells/ul)	1.00	0.48	1.07	0.48	1.95	0.87	2.17	1.01
Monocytes (10x3cells/ul)	0.31	0.11	0.45	0.12	0.28	0.16	0.31	0.21
Eosinophils (10x3cells/ul)	0.79	0.60	1.38	0.62	3.20	1.56	5.02	1.66
Basophils (10x3cells/ul)	0.02	0.02	0.01	0.01	0.06	0.07	0.12	0.15
Platelets (10x3/ul)	101.08	30.54	121.49	33.50	164.56	39.38	190.74	45.65

Table 3. Unadjusted means (± SD) for serum chemistry and protein electrophoresis parameters for managed-care and wild bottlenose dolphins, by site.

	GAI		IRL		CHS	
	mean	std	mean	std	mean	std
Sodium	156.17	1.27	155.73	2.79	154.95	2.82
Potassium	4.29	0.19	4.03	0.27	4.09	0.38
Chloride	119.53	2.46	115.73	3.31	114.05	3.15
Bicarbonate	26.36	2.81	24.73	3.45	24.26	4.05
AnionGap	14.56	2.21	38.88	2.66	20.89	5.88
BUN	52.28	5.29	.	.	57.16	7.08
Creatinine	1.63	0.40	1.13	0.24	1.16	0.13
Calcium	9.04	0.33	8.82	0.33	8.90	0.38
Phosphorous	4.42	0.54	4.28	0.57	4.88	0.60
Magnesium	1.60	0.10	1.56	0.13	1.48	0.12
Total Protein	6.33	0.31	7.25	0.39	7.22	0.49
Albumin	4.80	0.22	4.46	0.30	4.23	0.34
Globulin	1.54	0.28	2.78	0.50	2.99	0.65
Albumin/Globulin ratio	3.25	0.69	1.65	0.32	1.49	0.42
Glucose	98.25	10.95	86.77	7.77	85.84	13.27
Alanine Aminotransferase	63.82	34.25	39.15	16.78	60.58	45.21
Aspartate Aminotransferase	458.16	290.64	263.69	119.35	283.58	159.71
Alkaline Phosphatase	379.96	177.71	158.96	79.08	150.68	92.33
Gamma Glutamyltransferase	31.42	27.56	26.69	6.80	26.21	17.65
Total bilirubin	0.09	0.03	0.07	0.05	0.10	0.00
Direct bilirubin	0.05	0.04	0.01	0.03	0.10	0.00
Indirect bilirubin	0.04	0.03	0.06	0.05	0.10	0.00
Amylase	2.87	0.28	3.00	0.00	3.00	0.00
Cholesterol	142.95	28.21	121.50	20.18	160.74	29.32
Iron	116.38	20.40	95.27	33.89	86.47	28.86
PROTEIN ELECTROPHORESIS						
Total Protein	6.33	0.31	7.25	0.39	7.22	0.49
Albumin	4.80	0.22	4.46	0.30	4.23	0.34

Globulin	1.54	0.28	2.78	0.50	2.99	0.65
Albumin/Globulin ratio	3.25	0.69	1.65	0.32	1.49	0.42
Alpha1Globulins	0.23	0.03	0.30	0.05	0.28	0.06
Alpha2 Globulins	0.94	0.09	1.01	0.08	1.21	0.13
Total Alpha Globulins	1.17	0.08	1.31	0.11	1.48	0.13
Beta1 Globulin	0.17	0.03	0.22	0.03	0.21	0.05
Beta2 Globulin	0.21	0.03	0.24	0.09	0.27	0.08
Total Beta Globulin	0.38	0.02	0.45	0.11	0.48	0.11
Gamma Globulins	1.08	0.28	2.19	0.36	1.98	0.58

Table 4. Unadjusted means (± SD) for endocrine parameters for managed-care and wild bottlenose dolphins, by site.

	Navy		GAI		IRL		CHS	
	mean	std	mean	std	mean	Std	mean	std
ACTH (pg/mL)	6.82	2.36	24.56	12.47	149.04	81.29	59.19	40.58
Cortisol (ug/dL)	0.98	0.62	0.46	0.32	1.25	0.48	0.84	0.31
Estradiol (pg/mL)	.	.	18.64	9.11	22.83	3.12	20.47	4.71
Progesterone (ng/mL)	.	.	0.82	1.49	0.45	0.79	3.64	7.77
Testosterone (ng/mL)	.	.	10.58	.	4.42	5.11	2.41	3.73
Total T4 (ug/g)	9.85	1.67	11.99	1.45	10.56	2.76	12.02	4.18
Total T3 (ng/g)	88.90	9.87	62.92	10.43	74.21	20.44	67.65	31.00
Free T4 (ng/dL)	1.22	0.20	1.48	0.17	1.13	0.32	1.67	0.47

Table 5. Unadjusted means (±SD) for immunological parameters for managed-care and wild bottlenose dolphins, by site.

	Navy		GAI		IRL		CHS	
	mean	std	mean	std	mean	std	mean	std
MHCII+ (Absolute Nos.)	874.88	567.38	924.38	434.69	1810.30	849.48	1618.63	1139.40
CD2 T Cells (Absolute Nos.)	415.67	248.27	586.08	296.78	1114.07	401.31	995.26	691.96
CD4 Helper T Cells (Absolute Nos.)	207.54	104.60	281.23	135.03	711.26	316.23	578.89	441.41
CD21 B Cells-mmature (Absolute Nos.)	361.71	274.87	308.79	222.62	530.59	465.36	530.11	461.71
CD19 B Cells - immature (Absolute Nos.)								
Granulocytic phagocytosis%	.	.	.	.	18.17	21.52	.	.
Monocytic phagocytosis%	.	.	26.68	12.75	8.24	9.85	.	.
Lysozyme Concentration (ug/ul)	9.62	4.40	3.83	5.38	.	.	8.94	10.65
IgG1 (mg/ml)	9.59	3.36	8.21	2.77	13.80	1.65	15.19	16.02
T Cell 1.25	91.13	225.16	38.97	128.99	10.67	17.66	524.93	430.14
T Cell 2.5	88.02	236.63	34.81	129.03	11.57	19.02	675.21	577.25
T Cell 5	43.17	166.74	13.20	55.05	5.23	9.81	478.93	438.59
B Cell LPS 60	1.86	3.53	1.42	0.66	7.19	22.88	111.67	97.02
B Cell LPS 120	8.72	40.48	12.20	47.98	10.13	25.06	156.16	125.91
NK	3.52	8.65	3.76	10.15	19.24	18.38	11.04	2.60
ANTIBODY TITERS								
Mycobacterium marinarum	142.36	59.89	148.14	84.35	192.45	32.39	130.16	123.00
Erysipelothrix rhusiopathiae	133.94	51.15	86.86	54.04	194.76	20.23	228.33	230.50
Vibrio cholera	294.10	100.69	233.91	74.36	385.03	46.83	286.17	272.00
Escherichia coli	140.15	51.73	89.35	45.60	204.36	46.10	270.33	250.00
Vibrio parahaemolyticus	121.88	43.55	107.14	34.80	181.90	24.10	197.94	173.00
CYTOKINE EXPRESSION								
TNF	13.06	0.86	11.61	0.89	10.87	1.45	10.90	1.10
IL-17	17.29	2.12	16.26	2.60	14.11	1.76	14.81	1.18

MX1	14.94	2.71	13.83	1.50	13.46	2.78	13.92	1.44
INFα	9.79	1.30	9.03	1.48	7.70	1.49	8.81	0.88
IL-4	16.75	1.06	16.67	2.37	12.47	1.38	13.11	1.07
IL-10	10.79	0.92	9.69	1.01	8.90	1.31	9.88	0.88
CD69	10.68	0.96	9.27	1.02	8.87	0.81	.	.
IL2Rα	12.75	0.93	11.68	0.78	11.28	0.94	11.09	0.78
INFγ	15.66	0.88	14.61	1.17	13.03	2.54	13.95	1.24

Table 6. Repeated Measures Analysis of Variance for Hematological Parameters for Managed Care and Wild

	Navy		GAI		IRL		CHS		ANOVA	Navy vs GAI	Navy vs IRL	Navy vs CHS	GAI vs IRL	GAI vs CHS	IRL vs CHS
	mean	std	mean	std	mean	std	mean	std	p-value						
Manual Hematocrit (%)	42.41	2.67	40.12	3.21	42.52	2.79	41.74	3.31	0.18	.328	.145	.515	.328	1.000	.820
Hemoglobin (g/dl)	14.62	0.87	13.59	1.09	14.46	0.92	13.76	0.99	0.01	.086	.079	.278	.255	.107	.000
RBC (10x3cells/ul)	3.32	0.29	3.24	0.26	3.66	0.30	3.55	0.26	0.00	.908	.001	.032	.010	.179	.551
Mean Corpuscular Volume (MCV) (fl)	128.40	6.86	124.14	4.53	153.70	192.04	118.05	7.48	0.04	1.000	.918	.000	.001	.997	.001
Mean Corpuscular Hemoglobin (MCH) (pg)	44.37	2.85	42.19	1.87	39.78	2.50	38.95	2.48	0.00	.211	.050	.007	.000	.000	.677
Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dl)	34.58	0.65	33.93	0.39	34.07	0.62	33.05	0.78	0.00	.122	.935	.005	.157	.000	.000
WBCs (10x3cells/ul)	5.89	1.62	9.18	1.73	10.39	3.15	12.49	3.10	0.00	.050	.005	.018	.000	.000	.067
Segmented Neutrophils (10x3cells/ul)	3.78	1.04	6.23	1.61	4.99	2.02	4.85	1.62	0.02	.012	.225	.185	.240	.392	.993
Lymphocytes(10x3cells/ul)	1.00	0.48	1.07	0.48	1.95	0.87	2.17	1.01	0.00	.997	.027	.006	.014	.003	.817
Monocytes (10x3cells/ul)	0.31	0.11	0.45	0.12	0.28	0.16	0.31	0.21	0.06	.215	.037	.143	.979	1.000	.936
Eosinophils (10x3cells/ul)	0.79	0.60	1.38	0.62	3.20	1.56	5.02	1.66	0.00	.777	.004	.000	.000	.000	.000
Basophils (10x3cells/ ul)	0.02	0.02	0.01	0.01	0.06	0.07	0.12	0.15	0.51	.988	.509	.013	.752	.036	.092
Platelets (10x3/ul)	101.08	30.54	121.49	33.50	164.56	39.38	190.74	45.65	0.00	.656	.023	.000	.000	.000	.130

Table 7. Repeated measures analysis of variance for serum chemistry parameters for managed-care and wild bottlenose dolphins, adjusted for age and sex, by site.

	GAI		IRL		CHS		ANOVA	GAI vs IRL	GAI vs CHS	IRL vs CHS
	mean	std	mean	std	mean	Std	p-value			
Sodium	156.17	1.27	155.73	2.79	154.95	2.82	0.43	.389	.045	.223
Potassium	4.29	0.19	4.03	0.27	4.09	0.38	0.03	.000	.006	.369
Chloride	119.53	2.46	115.73	3.31	114.05	3.15	0.00	.000	.000	.003
Bicarbonate	26.36	2.81	24.73	3.45	24.26	4.05	0.32	.444	.299	.902
AnionGap	14.56	2.21	38.88	2.66	20.89	5.88	0.00	.000	.001	.000
BUN	52.28	5.29	.	.	57.16	7.08	0.07	.	.074	.
Creatinine	1.63	0.40	1.13	0.24	1.16	0.13	0.00	.000	.000	.929
Calcium	9.04	0.33	8.82	0.33	8.90	0.38	0.24	.212	.543	.746
Phosphorous	4.42	0.54	4.28	0.57	4.88	0.60	0.00	.810	.105	.003
Magnesium	1.60	0.10	1.56	0.13	1.48	0.12	0.02	.647	.035	.077
Glucose	98.25	10.95	86.77	7.77	85.84	13.27	0.01	.014	.011	.954
Alanine Aminotransferase	63.82	34.25	39.15	16.78	60.58	45.21	0.04	.111	.964	.081
Aspartate Aminotransferase	458.16	290.64	263.69	119.35	283.58	159.71	0.01	.011	.035	.924
Alkaline Phosphatase	379.96	177.71	158.96	79.08	150.68	92.33	0.00	.000	.000	.964
Gamma Glutamyltransferase	31.42	27.56	26.69	6.80	26.21	17.65	0.68	.714	.690	.995
Total bilirubin	0.09	0.03	0.07	0.05	0.10	0.00	0.05	.176	.913	.077
Direct bilirubin	0.05	0.04	0.01	0.03	0.10	0.00	0.00	.002	.010	.000
Indirect bilirubin	0.04	0.03	0.06	0.05	0.10	0.00	0.11	.688	.093	.185
Amylase	2.87	0.28	3.00	0.00	3.00	0.00	0.01	.016	.020	1.00
Cholesterol	142.95	28.21	121.50	20.18	160.74	29.32	0.00	.065	.017	.000
Iron	116.38	20.40	95.27	33.89	86.47	28.86	0.00	.000	.000	.716

PROTEIN

ELECTROPHORESIS

Total Protein	6.33	0.31	7.25	0.39	7.22	0.49	0.00	.000	.000	.968
Albumin	4.80	0.22	4.46	0.30	4.23	0.34	0.00	.013	.000	.036
Globulin	1.54	0.28	2.78	0.50	2.99	0.65	0.00	.000	.000	.408
Albumin/Globulin ratio	3.25	0.69	1.65	0.32	1.49	0.42	0.00	.000	.000	.449
Alpha1 Globulin	0.23	0.03	0.30	0.05	0.28	0.06	0.00	.001	.051	.247
Alpha2 Globulin	0.94	0.09	1.01	0.08	1.21	0.13	0.00	.168	.000	.000
Total Alpha Globulins	1.17	0.08	1.31	0.11	1.48	0.13	0.00	.004	.000	.000
Beta1 Globulin*	0.17	0.03	0.22	0.03	0.21	0.05	0.01	.005	.019	.911
Beta2 Globulin	0.21	0.03	0.24	0.09	0.27	0.08	0.15	.545	.136	.447
Total Beta Globulins*	0.38	0.02	0.45	0.11	0.48	0.11	0.03	.090	.026	.692
Gamma Globulins	1.08	0.28	2.19	0.36	1.98	0.58	0.00	.000	.000	.282

* Log10 used

Table 8. Repeated Measures Analysis of Variance Endocrine Parameters for Managed Care and Wild Bottlenose dolphins, adjusted for age and sex, by site.

	Navy		GAI		IRL		CHS		ANOVA	Navy vs GAI	Navy vs IRL	Navy vs CHS	GAI vs IRL	GAI vs CHS	IRL vs CHS
	mean	std	mean	std	mean	std	Mean	std	p-value						
ACTH (pg/mL)	6.82	2.36	24.56	12.47	149.04	81.29	59.19	40.58	0.00	.043	.000	.000	.000	.001	.000
Cortisol (ug/dL)	0.98	0.62	0.46	0.32	1.25	0.48	0.84	0.31	0.00	.000	.040	.418	.000	.004	.021
Estradiol (pg/mL)	.	.	18.64	9.11	22.83	3.12	20.47	4.71	0.08	.	.	.	.	.	.
Progesterone (ng/mL)	.	.	0.82	1.49	0.45	0.79	3.64	7.77	0.09	.	.	.	.	.	.
Dopamine (pg/mL)	45.17	22.43	60.62	62.77	30.00	0.00	41.88	8.47	0.00	.265	.117	.810	.030	.448	.640
Epinephrine (pg/mL)	1221.86	211.62	1476.67	721.17	142.16	128.29	634.40	199.23	0.00	.033	.000	.020	.000	.000	.020
Norepinephrine (pg/mL)	72.67	39.04	89.52	109.91	80.76	77.48	219.43	110.93	0.00	.973	.993	.000	.994	.000	.000
Testosterone (ng/mL)	.	.	10.58		4.42	5.11	2.41	3.73	0.23	.	.	.	.	.	.002
Total T4 (ug/g)	9.85	1.67	11.99	1.45	10.56	2.76	12.02	4.18	0.00	.000	.278	.001	.027	.853	.036
Total T3 (ng/g)	88.90	9.87	62.92	10.43	74.21	20.44	67.65	31.00	0.05	.000	.001	.000	.012	.282	.234
Free T4 (ng/dL)	1.22	0.20	1.48	0.17	1.13	0.32	1.67	0.47	0.00	.002	.243	.008	.000	.051	.000

Table 9. Repeated measures analysis of variance for immunological and parameters for managed-care and wild bottlenose dolphins, adjusted for age and sex, by site.

	Navy		GAI		IRL		CHS		ANOVA	Navy vs GAI	Navy vs IRL	Navy vs CHS	GAI vs IRL	GAI vs CHS	IRL vs CHS
	mean	std	mean	std	mean	std	mean	std	p-value						
MHCII+ (Absolute Nos.)	874.88	567.38	924.38	434.69	1810.30	849.48	1618.63	1139.40	0.01	.985	.000	.000	.000	.000	.007
CD2 T Cells (Absolute Nos.)	415.67	248.27	586.08	296.78	1114.07	401.31	995.26	691.96	0.00	.044	.000	.000	.000	.000	.014
CD4 Helper T Cells (Absolute Nos.)	207.54	104.60	281.23	135.03	711.26	316.23	578.89	441.41	0.00	.169	.000	.000	.000	.000	.590
CD21 B Cells-mature (Absolute Nos.)	361.71	274.87	308.79	222.62	530.59	465.36	530.11	461.71	0.00	.139	.000	.000	.000	.000	.000
Lysozyme Concentration (ug/ul)	9.62	4.40	3.83	5.38	.	.	8.94	10.65	0.00	.001	.	.589	.	.030	.
IgG1 (mg/ml)	9.59	3.36	8.21	2.77	13.80	1.65	15.19	16.02	0.00	.024	.000	.000	.000	.000	.000
NK Cells	5.371	3.885	3.517	4.214	18.706	3.981	10.455	3.579	0.04	.761	.033	.368	.010	.205	.119
ANTIBODY TITERS															
Mycobacterium marinarum	142.36	59.89	148.14	84.35	192.45	32.39	130.16	123.00	0.01	.000	.000	.040	.021	.000	.000
Erysipelothrix rhusiopathiae	133.94	51.15	86.86	54.04	194.76	20.23	228.33	230.50	0.00	.023	.000	.000	.000	.000	.004
Vibrio cholera	294.10	100.69	233.91	74.36	385.03	46.83	286.17	272.00	0.00	.058	.000	.001	.000	.000	.000
Escherichia coli	140.15	51.73	89.35	45.60	204.36	46.10	270.33	250.00	0.00	.079	.000	.000	.000	.000	.000
Vibrio parahaemolyticus	121.88	43.55	107.14	34.80	181.90	24.10	197.94	173.00	0.00	.000	.000	.000	.000	.000	.007
CYTOKINE EXPRESSION															
TNF	13.06	0.86	11.61	0.89	10.87	1.45	10.90	1.10	0.00	.049	.000	.000	.351	.449	1.00
IL-17	17.29	2.12	16.26	2.60	14.11	1.76	14.81	1.18	0.00	.586	.000	.005	.010	.188	.554
MX1	14.94	2.71	13.83	1.50	13.46	2.78	13.92	1.44	0.41	.714	.306	.678	.970	1.00	.904
INFα	9.79	1.30	9.03	1.48	7.70	1.49	8.81	0.88	0.00	.570	.000	.238	.035	.975	.025
IL-4	16.75	1.06	16.67	2.37	12.47	1.38	13.11	1.07	0.00	.999	.000	.000	.000	.000	.429
IL-10	10.79	0.92	9.69	1.01	8.90	1.31	9.88	0.88	0.00	.130	.000	.163	.211	.972	.018
CD69	10.68	0.96	9.27	1.02	8.87	0.81	.	.	0.00	.	.	.	.	.	.

IRL2Rα	12.75	0.93	11.68	0.78	11.28	0.94	11.09	0.78	0.00	.041	.000	.000	.607	.331	.881
INFγ	15.66	0.88	14.61	1.17	13.03	2.54	13.95	1.24	0.01	.611	.002	.108	.113	.808	.359

Table 10. Relationship between serum cortisol concentrations and selected parameters.

Dependent Variable	Type III Sum of Squares	Mean Square	F	Sig.	Adjusted R Squared
Total T4 (ug/g)	18.12	18.12	2.48	0.25	.27
Total T3 (ng/g)	210.64	210.64	.41	0.52	.08
Free T4 (ng/dL)	.30	.30	4.91	0.06	.56
ACTH (pg/mL)	12784.12	12784.12	4.53	0.03	.62
Dopamine (pg/mL)	3.01	3.01	.00	0.76	.07
Norepinephrine (pg/mL)	175678.31	175678.31	1.77	0.19	.69
Epinephrine (pg/mL)	5975.14	5975.14	.61	0.44	.27
MHCII+ (Absolute Nos.)	31856.98	31856.98	.07	0.01	.32
MHCII+ (%)	104.06	104.06	.49	0.33	.19
CD2 T Cells (Absolute Nos.)	.31	.31	.00	0.23	.43
CD4 Helper T Cells (Absolute Nos.)	31274.11	31274.11	.44	0.01	.44
CD21 B Cells-CD 21 B Cells-Mature (Absolute Nos.)	155.80	155.80	.002	0.11	.11
pctCD2	19.64	19.64	.235	0.31	.67
pctCD4	120.13	120.13	1.06	0.01	.74
pctCD21	18.12	18.12	.193	0.18	.22
Lysozyme Concentration (ug/ul	1.50	1.50	.09	0.28	.20
IgG1 (mg/ml)	5.61	5.61	1.58	0.64	.73
Mycobacterium marinarum	328.77	328.77	.14	0.05	.21
Erysipelothrix rhusiopathiae	176.00	176.00	.12	0.70	.67
Vibrio cholera	426.40	426.40	.13	0.19	.55
Escherichia coli	3649.130	3649.130	.759	0.06	.46
Vibrio parahaemolyticus	302.599	302.599	.121	0.95	.37

Table 11 . Physiological function categories of protein microarray antibodies on the custom microarray.

Category	**Protein**
Hypothalamic-pituitary-adrenal axis	adrenocorticotropic hormone (ACTH) (Biodesign BDE54057M), arginine vasopressin receptor (AVP) Receptor V1a (Santa Cruz sc30025), corticotrophin-releasing hormone receptor (CRH)-Receptor 1/2 (CRHR-1/2) (Santa Cruz sc5543), glucocorticoid receptor (GR) (Santa Cruz sc1002), C-terminal proopiomelanocortin (POMC) precursor (Abcam ab32893), prolactin (Santa Cruz sc7805)
Apoptosis and cell cycle	apoptosis inducing factor (AIF) (Santa Cruz sc13116), annexin II (Santa Cruz sc1924), annexin IV (Santa Cruz sc1930), caspase 1 (Santa Cruz sc514), caspase 2 (Lab Vision rb1699), caspase 6 (Sigma c7599), epithelial (E)-cadherin (Santa Cruz sc31020), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay Designs 905-734-100)
Cellular stress	cytokeratin (Abcam ab9377), glucose regulated protein (grp78 / BIP) (Sigma G9043), heat shock protein (hsp)110 (Sigma H7412), hsp27 (Stressgen SPA524), hsp40 (Sigma H4038), hsp60 (Sigma H3524), hsp70 (Santa Cruz sc24), hsp70 inducible (i) (Stressgen SPA810), hsp90 (Stressgen SPS771)
Oxidative stress and inflammation	chemokine (CC-motif) receptor (CCR) 5 (Sigma C8604), cyclooxygenase (COX)2 (Santa Cruz sc7951), heme oxygenase (HO)-2 (Santa Cruz sc11361), endothelial nitric oxide synthase (eNOS) (Abcam ab5589), inducible (iNOS) (Sigma N7782), peroxiredoxin 3 (PRDX3) (Sigma P1247), superoxide dismutase (SOD)1 (Santa Cruz sc8637), SOD2 (Abcam ab13533)

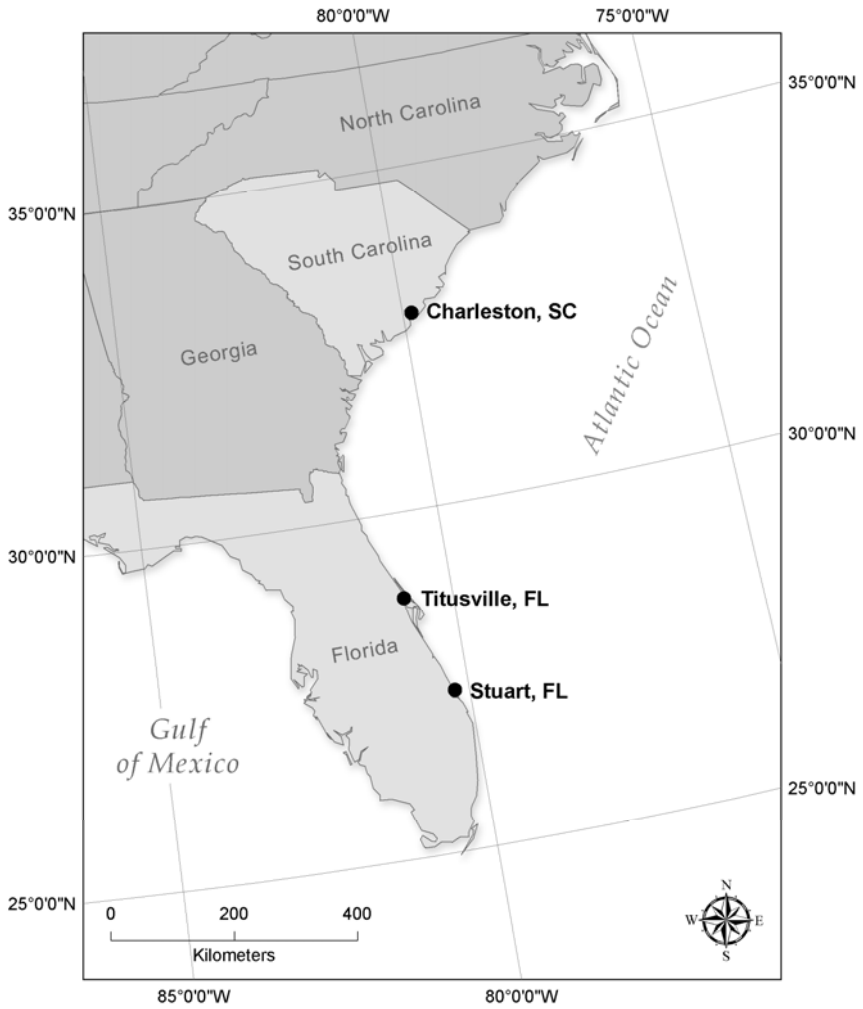

Figure 1: Health assessment studies of populations of wild Atlantic bottlenose dolphins conducted along the eastern coast of the United States at two estuarine sites - Charleston, SC and the Indian River Lagoon, FL, between Titusville and Stuart, FL.

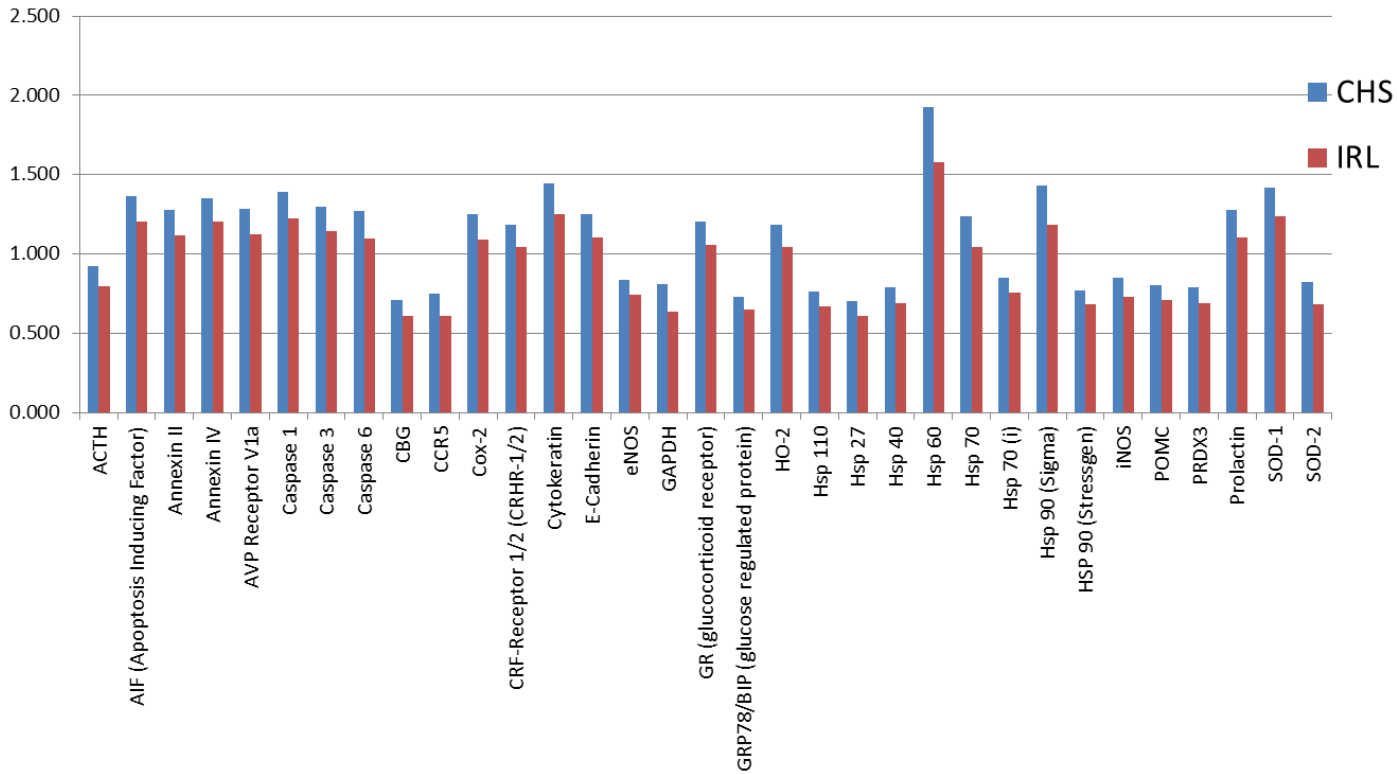

Figure 2. Stress proteins (n=33) detected in dolphin skin samples of wild dolphins from Charleston (CHS) SC and the Indian River Lagoon (IRL), FL

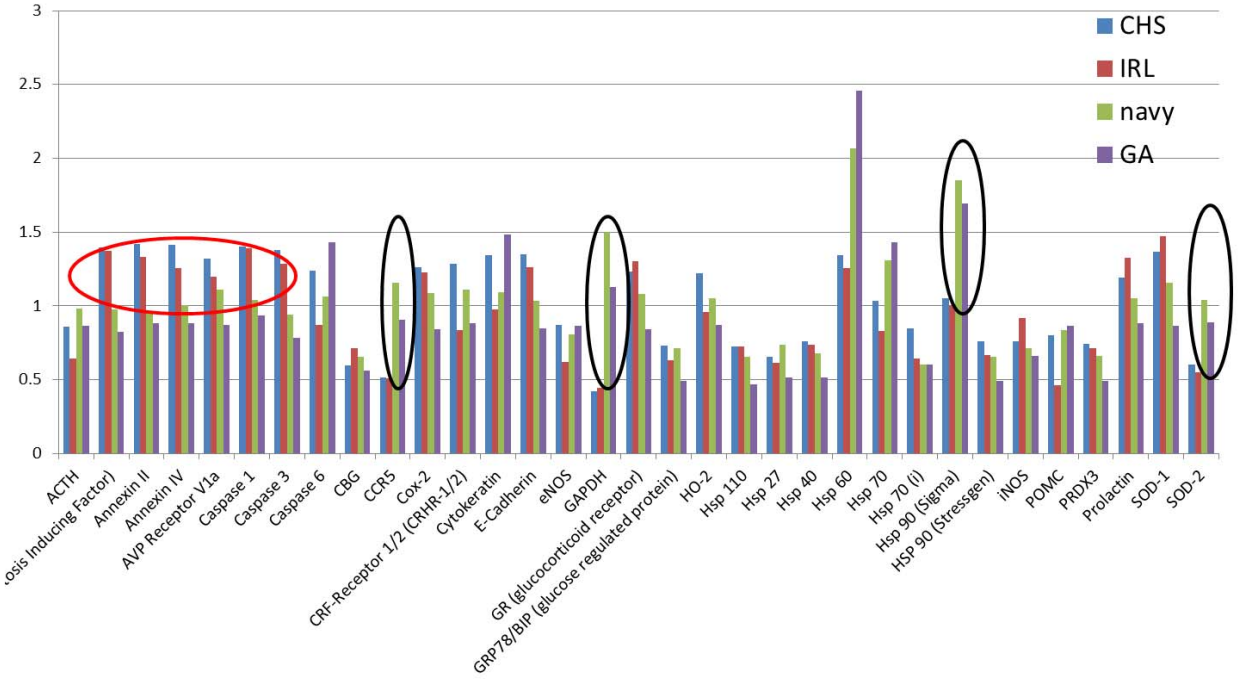

Figure 3. Stress proteins (n=33) detected in white blood cells of wild dolphins from Charleston (CHS) SC and the Indian River Lagoon (IRL), FL and managed-care dolphins from the Navy and Georgia Aquarium (GA). The wild dolphins (red circles) denote closer association with expression of several proteins; the managed-care dolphins (black circle) also indicate closer association with several proteins.

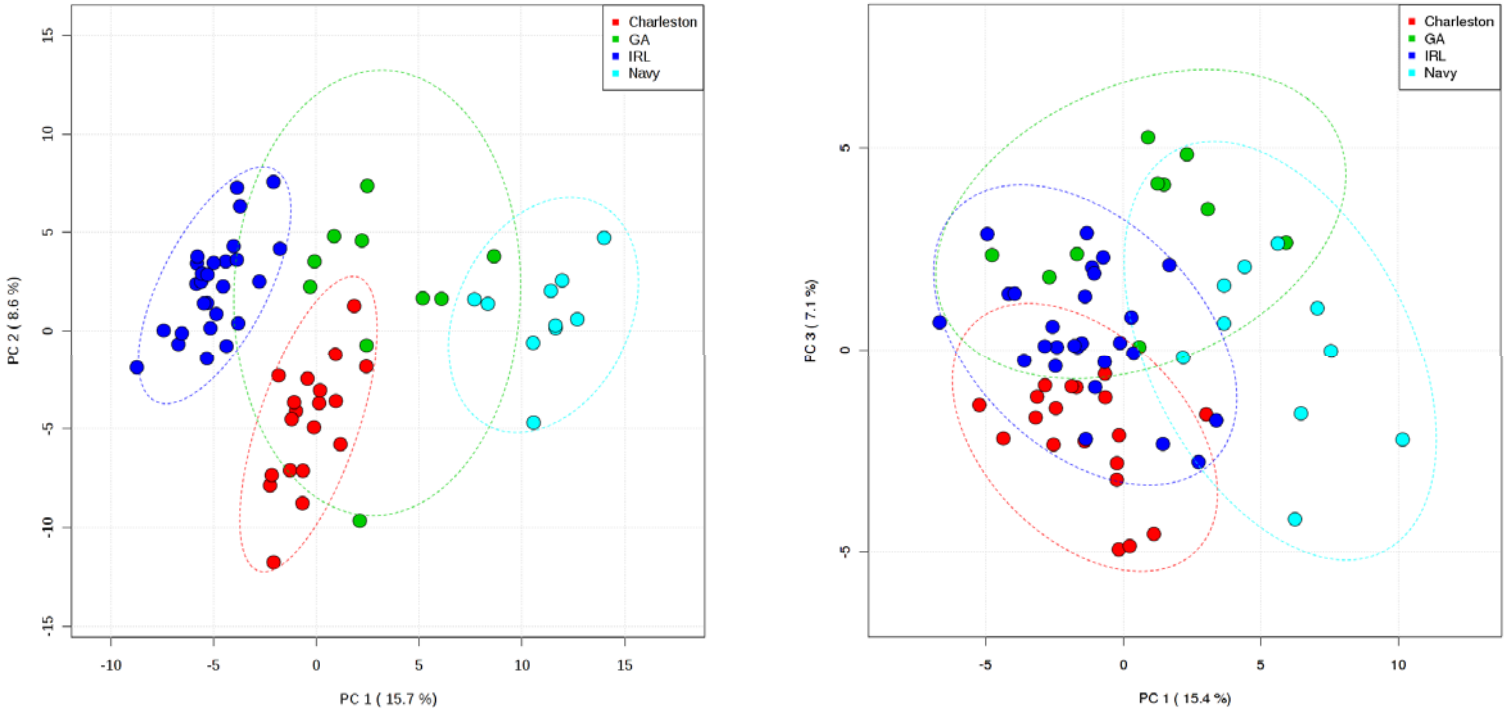

Figure 4: Principal components analysis plots demonstrating separation of the groups (a) with and (b) without the inclusion of analytes that have not been annotated to analytes. Separation decreases (and is strongest using PCs 1 and 3, compared to PCs 1 and 2), when unknown analytes are removed. This suggests that poorly characterized parts of metabolism contribute substantially to metabolic differences between the animals.

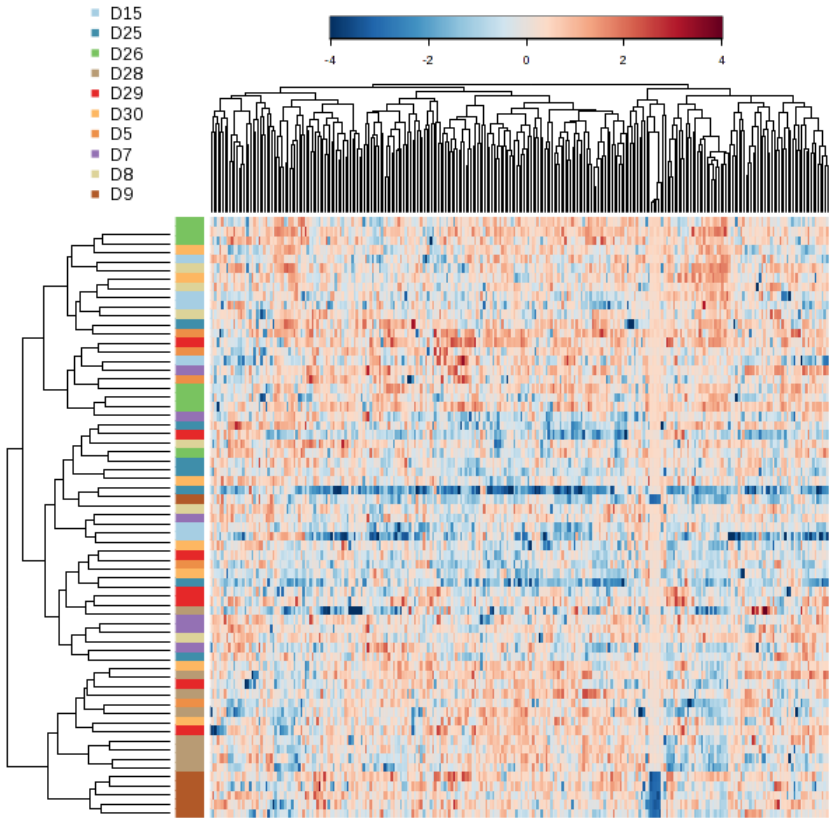

Figure 5: Heatmap and clustergram of the longitudinal Navy samples, with samples clustered in rows (where different colors represent different animals), and analytes clustered in columns. With few exceptions, there does not seem to be a major animal-specific effect in the metabolism of these animals; that is, metabolite profiles are more likely to reflect either inherent noisiness or response to external factors rather than the unique metabolism of any individual animal.