

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

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LONG-TERM GOALS

The overall goal of the proposed 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 in cetaceans and immune function, dependent hormonal endpoints, hematology and serum chemistry parameters, biomarkers of stress, inflammation and metabolism and health status.

OBJECTIVES

Objective 1 – To characterize multiple stress markers in managed-care bottlenose dolphins.

Objective 2 – To characterize multiple stress markers in semi-domesticated bottlenose dolphins

Objective 3 – To characterize multiple stress markers in wild bottlenose dolphins

Objective 4 – To integrate the information obtained from these three populations of bottlenose dolphins in order to develop a validated model of stress and its pathophysiologic effects on the bottlenose dolphin.

APPROACH

We plan to assess baseline stress biomarkers in the following three populations of Atlantic bottlenose dolphins (*Tursiops truncatus*): 1) managed-care (Group 1), 2) semi-domesticated (Group 2), and 3) wild (Group 3). This approach will provide a truly comparative study among bottlenose dolphins that live under a range of different and varying stressors.

To develop further understanding on stress in wild and managed-care dolphins and the association between classic measures of stress and new technologies, a research team comprised of scientists from federal, academic, and managed-care marine mammal facilities are collaborating on this project to develop integrated measures of stress using a comparative study design. Additionally, we are partnering with Dr. Dorian Houser, who leads the “Behavioral Response of Dolphins to Signals Simulating Mid-Frequency Active Sonar” and collaborators in a joint effort for the purpose of integrating traditional markers of stress with novel markers of stress.

WORK COMPLETED

SUBTASK 1 - *Collection of samples from Group 1 (managed-care Georgia Aquarium bottlenose dolphins) to characterize multiple stress markers*

This subtask was completed in 2012. A 12 month sample collection period of dolphins from Georgia Aquarium (group 1) was completed in August 2012. Samples were obtained from 9 individual dolphins with multiple collections during the 12 month period for a total of 32 samples. Several analyses including hematology, immunology and endocrine, have been completed and the remaining analysis will be conducted in FY13. Additionally, 10 dolphins from the navy (group 2) were sampled on multiple dates with a total of 64 samples collected during the 12-month study as a comparative group.

SUBTASK 2 - *Collection of samples from Group 3 (wild bottlenose dolphins)*

This subtask is now completed with the collection of samples from wild dolphins in Charleston, SC in August 2013. A total of 22 dolphins were safely captured with samples collected from 19 dolphins.

Within 2 weeks, all samples collected were sent to the appropriate laboratories for analysis including those to Dr. Janz in Canada under our CITES Permit. Previous collections from dolphins (n=27) in the Indian River Lagoon, FL were completed during capture–release health assessments as part of the Dolphin Health and Risk Assessment (HERA) Project. All animal capture and sampling protocols for both of these collections were conducted 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).

SUBTASK 3 - *Catecholamine (epinephrine, norepinephrine and dopamine) analysis shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted by Dr Tracy Romano.*

Catecholamine analysis has been completed on the Group 3 IRL dolphins. Catecholamine analyses were completed on the first several months of collections from Group 1 and Group 2 dolphins. All remaining samples have been sent to Dr. Romano and are in the process of being analyzed.

SUBTASK 4 - *Immunological assessments for immunophenotyping (B+T cell lymphocyte subsets, MHCII expression) shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted by Dr Tracy Romano.*

All immunophenotyping assessments have been completed on Group 1, Group 2 and the Group 3 IRL dolphins. These assessments have been completed for the recent collections of samples from Group 3 CHS dolphins and the data is currently being synthesized.

SUBTASK 5 - *Immunological assessments for lymphocyte proliferation, natural killer cell activity shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins).*

All immunological assessments for this task (i.e., lymphocyte proliferation and natural killer cell activity) have been completed on Group 1, Group 2 and the Group 3 IRL dolphins. These assessments have been completed for the recent collections of samples from Group 3 CHS dolphins and the data is currently being synthesized.

SUBTASK 6 - *Immunological analysis of IgG, CRP, and pathogen ELISAs shall be determined on samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins). These analyses will be conducted by Dr. Charles Rice.*

All immunological assessments for this task have been completed on Group 1, Group 2 and the Group 3 IRL dolphins. These assessments have been completed for the recent collections of samples from Group 3 CHS dolphins and provided to the PIs.

SUBTASK 7 - *The following cytokines shall be determined (IL4, IL10, IL17, CD69, TNF α , IFN γ , IFN α , MX1, IL2-R α , FADD) in the collected blood samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins) as outlined in Table 2. These analyses will be conducted by Dr. Jeff Scott.*

All immunological cytokine assessments have been completed on Group 1, Group 2, and the Group 3 IRL dolphins. Samples from Group 3 CHS dolphins are currently being analyzed.

SUBTASK 8 – *Proteomic analysis of samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins) will be conducted by Dr. David Janz.*

This work involves using an antibody-based protein microarray, initially developed for grizzly and brown bears, to determine expression levels of 33 stress-associated proteins in small biopsy samples collected from bottlenose dolphins. The microarray measures expression of proteins associated with four key aspects of the stress response: hypothalamic-pituitary-adrenal (HPA) axis, apoptosis/cell cycle, proteotoxicity, and oxidative stress/inflammation. The microarray was originally developed to determine stress-associated proteins in skin biopsy samples which will be relevant and applicable to collection of dart skin biopsies from free-ranging dolphins. The collection of skin samples is a more invasive procedure compared to blood collection, particularly in managed-care dolphins. Thus, we have also focused our research on modifying our technique to expand the applicability of the microarray to blood matrices.

In year 1, we isolated and concentrated proteins from the white blood cell fraction (“buffy coat”) of centrifuged whole blood samples collected from a single Indian River Lagoon dolphin and were able to measure 25 of the 33 stress-associated proteins. In year 2, we modified the technique to further concentrate white blood cell proteins, and were successful in measuring expression levels of all 33 stress proteins. We then expanded this work further to attempt to measure stress proteins in the blood plasma fraction. We developed a technique to concentrate plasma proteins sufficiently to run them on the microarray, and were successful in detecting expression of all 33 stress-associated proteins. To our knowledge, this is the first time this has been achieved in any wildlife species. We then received further dolphin skin, white blood cell, and plasma samples from the Indian River Lagoon population, and determined stress protein expression levels in 8 individual dolphins. Depending on the results from the other concurrent studies, we will be running additional samples in October-November 2013 to compare stress protein expression in wild dolphins (Charleston Harbor) with managed care dolphins (Georgia Aquarium and Navy dolphins; in these cases only white blood cell and plasma samples are available). In combination with other research being conducted in these dolphins, this research should provide unique insight into stress responses in multiple tissues and matrices.

SUBTASK 9 - *Metabolomic analysis of samples from dolphin groups (Group 1 - managed-care bottlenose dolphins; Group 2 – semi-domesticated bottlenose dolphins; Group 3 – wild bottlenose dolphins) will be conducted by Dr. Al Dove in association with colleagues at Georgia Tech, Atlanta, GA.*

All samples for metabolomic analysis were sent to Dr. Dove from collections from all groups as indicated in Subtasks 2 and 3. The GC-MS analytical protocols have been refined and samples from 2011 wild IRL dolphins have been completed in the laboratory of Dr. Styckzynski at Georgia Tech. These analyses will continue on samples from the other groups as a single batch run in September thus, reducing batch variability. Once stress measurements from this study are completed these will be integrated with metabolic profiles to identify key metabolic markers. The discriminant analyses may indicate which analytes may be most useful as metabolic biomarker candidates in dolphins, as well as revealing underlying metabolic mechanisms of diseases.

SUBTASK 10 - *Data Management, Quality Assurance and Analysis*

The Microsoft Access database framework was completed to include all variables for the data collected in this study. Thus far, data has been entered for all parameters which have been completed and submitted from the various researchers and laboratories using Quality Assurance/Quality Control

processes. Descriptive statistical analysis of data is proceeding for each population including mean, standard deviation, range, quartile values followed by further appropriate statistical tests.

RESULTS

The project was initiated in June 2011 as funding was received by both Dr. Fair (N0001411IP20081) and Bossart (N000141110541). Below are listed several accomplishments and further information is presented under subtasks for FY13.

1. Completion of sample collection of all groups was accomplished with the final collection of samples from Group 3 Charleston dolphins in August 2013.
2. Total samples collected include: 1) Group 1 - 32 during a 12-month sample collection period from Group 1 managed-care dolphins at Georgia Aquarium completed in August 2012, 2) Group 2 - 56 during a 12-month sample collection period in partnership the Marine Mammal Program on a collaborative study with managed-care dolphins at the U.S. Navy in August, 2012; 3) Group 3 – 27 from IRL dolphins in 2011 and 19 from CHS dolphins in 2013.
3. Sample analyses have been completed for hematology and immunology tests for all samples collected thus far. Completion of the remaining tests and measurements are in process.
4. Data on hematology, serum chemistry, immune, hormone and stress biomarker data that have been completed for this project from the various researchers and laboratories have been entered into the relational database developed for this project. Based on the samples collected the number of tests being done there will be over 14,000 data points for this study.

This project is on target for meeting the outlined objectives within specified timeframe.

IMPACT/APPLICATIONS

Well-characterized baseline stress evaluation using classic stress hormones paired with biomarker expression using new technologies will provide needed information on natural variation and inter-relationships in hormones/biomarkers among different matrices and across populations maintained under differing environmental conditions. The assessment of stress variables and response in managed-care animals will have important implications for the assessment and interpretation of stress in wild bottlenose dolphins. Approaches and results developed in this proposal to assess the measurement and burden of stress may also be generalized to other marine mammal species.

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 the relationship of stress measures not only between tissue matrices but also between managed-care and wild dolphins. This proposal addresses this critical need and furthermore incorporates the use of new technologies to provide an integrative measure of stress with classic parameters extending our knowledge and application of such measures.

PRESENTATIONS/PUBLICATIONS

ABSTRACTS - Oral presentations accepted for 2013 Biennial Conference on Marine Mammals – December, New Zealand:

1. Patricia A. Fair, Adam M. Schaefer, John S. Reif, Dorian S. Houser, Tracy A. Romano, Cory D. Champagne, Gregory D. Bossart, ***Stress Markers in Managed-Care and Wild Dolphins***
2. John S. Reif, Adam M. Schaefer, Tracy A. Romano, Jeffrey L. Stott, Charles D. Rice, Gregory D. Bossart, Dorian S. Houser, Cory D. Champagne, Patricia A. Fair ***Immune Markers in Managed-Care and Wild Dolphins***

PUBLICATION

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*). Aquatic Mammals 39(4) 324-329.

RELATED PROJECTS

The Dolphin Health and Risk Assessment Project has several publications (including in press and/or submitted) that are related or applicable to studies on stress including:

- Bergfelt, D.R., Steinetz, B.G., Reif, J.S., Schaefer, A.M., Bossart, G.D., Mazzoil, M.S., Zolman, E., Fair, P.A. 2013. Evaluation of single-sample analysis of progesterone in combination with relaxin for diagnosis of pregnancy status in wild bottlenose dolphins (*Tursiops truncatus*). Aquatic Mammals, 39(2), 198-206.
- Bossart, G., Arheart, K., Leppert, L., Roberts, K., McCulloch, S., Goldstein, J., Gonzalez, C., Sweeney, J., Stone, R., Fair, P.A., Cray, C. Protein electrophoresis of serum from healthy Atlantic bottlenose dolphins (*Tursiops truncatus*). Aquatic Mammals 2012, 38(4), 412-417.
- Cray, C., Arheart K., Leppert, L., Roberts, K., McCulloch, S., Goldstein, J., Gonzalez, C., Fair, P.A., Bossart, G. Acute phase protein quantitation in serum samples from healthy Atlantic bottlenose dolphins (*Tursiops truncatus*). Journal of Veterinary Diagnostic Investigation 25(1) 107–111.
- Fair, P.A., Schaefer, A.M, Reif, J.R., Bossart, G.D., Lamb, S.V., Romano, T.A. Stress response of wild bottlenose dolphins (*Tursiops truncatus*) during capture-release health assessment studies (submitted).
- Goldstein, D., Schaefer, A.M., Reif, J.S. McCulloch, S.D., Fair, P.A., Bossart, G.D. 2012. Clinicopathologic findings from Atlantic bottlenose dolphins (*Tursiops truncatus*) exhibiting cytologic evidence of gastric inflammation. Journal of Zoo and Wildlife Medicine. Journal of Zoo and Wildlife Medicine 43(4):730-738.
- Lee, R.F., Brinkley, K., Adams, J.D., Peden-Adams, M., Bossart, G.D., King, L., Fair, P.A. DNA Strand Breaks (Comet Assay) in Blood Lymphocytes from Wild Bottlenose Dolphins. Marine Pollution Bulletin (in press).
- Mazzaro, L.M., Johnson, S.P., Fair, P.A., Bossart, G., Carlin, K.P., Jensen, E.D., Smith, C.R., Andrews, G.A., Chavey, P.S., Venn-Watson, S. 2012. Iron Indices among bottlenose dolphins (*Tursiops truncatus*): Identifying populations at risk for iron overload. Comparative Medicine 62:6 508-515.