Stress Hormones and Their Regulation in a Captive Dolphin Population

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LONG-TERM GOALS

This research aids our understanding of how the stress response operates in marine mammals by evaluating markers of stress in a captive dolphin population. This research effort will determine baseline levels of putative stress hormones and evaluate the functional consequences of increased stress in the bottlenose dolphin (*Tursiops truncatus*) through the assessment of non-traditional biochemical markers.

OBJECTIVES

Marine mammals are potentially affected by multiple environmental stressors, many of which are anthropogenic. The resulting stress response provides for immediate physiological needs and manages recovery from acute stressors. When environmental stressors become chronic, however, stress-response mechanisms can become detrimental, reducing survival and both reproductive effort and success. Assessing levels of stress and their functional consequences are therefore critical to evaluating the effects of anthropogenic disturbance on species of concern, including the influence of U.S. Naval activities on marine mammals.

This research project leverages another ONR-funded effort investigating stress markers in bottlenose dolphins at the U.S. Navy Marine Mammal Program (MMP) and is composed of two broad components: 1) assessing baseline variability in stress hormones and 2) evaluating physiological and metabolic alterations that occur during stress.

The specific research objectives of this effort are to (1) establish protocols for improved sensitivity of low-level corticosteroids (cortisol and aldosterone) frequenctly observed in cetaceans; (2) determine the regulatory role of corticosteroid binding globulin (CBG) in corticosteroid action; (3) assess the role of reverse triiodothyronine (rT_3) in the counter-regulation of thyroid hormone action; and (4) determine the impact of hormone variation associated with the stress-response on the function of metabolic pathways using metabolomic analyses.

APPROACH

Key Individuals and Collaborations

This research study is being conducted in close association with another ONR-funded effort: grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*; PI: Dorian Houser, PhD; National Marine Mammal Foundation; hereafter referred to as the *Parent Project*. Hormone assays are being conducted in collaboration with Dr Daniel Crocker (crocker@sonoma.edu) at Sonoma State University; Department of Biology, Rohnert Park, CA; 94928. Metabolomic sample processing is being conducted by *Metabolon, Inc*. and in consultation with the Science Development Director, Jeff Buckthal (JBuckthal@metabolon.com).

Study Approach

This study capitalizes on three experimental components of the Parent Project. (1) Normal variation in stress and metabolic hormones is being evaluated by collecting samples from dolphins throughout the year (*temporal variation*). Thirty dolphins were sampled to assess temporal variation in hormone levels. To evaluate the sensitivity of hormone axes, hormone stimulation experiments were conducted on the (2) HPA axis, and will be performed on the (3) HPT axis (*HPA* and *HPT stimulation* studies, respectively). During these stimulation experiments, an animal's hormonal and physiological response to a simulated stressor can be evaluated. The HPA axis was stimulated in bottlenose dolphins using an out-of-water stress protocol. The observed response was similar to that of ACTH administrations (see Parent Project for further details). The HPT axis will be activated using thyrotropin-releasing hormone (TRH) in a separate set of experiments. The project described here extends the suite of biomarkers assayed in the Parent Project and attempts to improve on processing methods in order to improve quantification of certain stress biomarkers. Four project tasks are being conducted.

Task 1—Improved quantification of circulating corticosteroids

Bottlenose dolphins have low circulating levels of corticosteroids (cortisol and aldosterone). Accurate quantification requires highly sensitive assays to detect variation at or near the typical detection limit of most commercially available immunoassay kits. By modifying existing protocols, this project is evaluating assay techniques to determine the most efficient, reliable, and cost-effective means of measuring circulating corticosteroids in bottlenose dolphin.

Task 2—Assessment of corticosteroid binding globulin

Most corticosteroids in circulation are bound with a carrier protein, primarily corticosteroid binding globulin (CBG). Only unbound hormones, however, are thought to interact with receptors and elicit a response at target tissues. Consequently, variation in carrier proteins like CBG can mediate the metabolic influence of hormones. CBG may in fact be an accurate marker of long-term stress as it does not seem to vary with acute stress in some species (Chow et al, 2010). This project will therefore assess temporal variation in CBG concentration in the bottlenose dolphin.

Task 3—The influence of the HPT axis on rT_3

Variability in, and sensitivity of, the HPT axis is being investigated in the Parent Project. Under stress conditions, rT_3 production can be increased, leading to reductions in energy use by blocking T_3 receptors (Weissman, 1990). This resultant reduction in energy use may be an important energy conserving mechanism necessary to endure stressful periods. We are therefore quantifying rT_3 concentrations for normal variation and during stimulation of the HPA and HPT axes.

Task 4—Functional analysis using metabolomics
The principal role of hormones is to influence metabolic pathways. There are numerous metabolic pathways and thousands of resultant compounds that are likely influenced by hormones associated with the stress response. Many of these compounds can be simultaneously identfied using a broad-based metabolomics technique and the identified compounds can be associated with up- and down-regulation of associated metabolic pathways (Goodacre, et al, 2004) thereby establishing some of the metabolic consequences of stress. We are therefore conducting metabolomic analyses of HPA and HPT axes stimulation to evaluate the functional consequences of increased stress in the bottlenose dolphin.

WORK COMPLETED

Task 1—Sampling for the temporal variability component of the Parent Project is complete; 1,092 samples were collected during the first two years of sampling. We assessed and established reliable methods to measure cortisol at low concentrations and used this to measure cortisol concentrations in samples from the Parent Project. The studies of seasonal and and daily variability (years 1 & 2, respectively) are completed as well as HPA stimulation studies. Cortisol has been measured in all associated samples. We assessed and established reliable methods to measure aldosterone at low concentrations in dolphin serum and used this to measure aldosterone concentrations from samples from the Parent Project. Samples from the study of seasonal variability (year 1) and HPA axis stimulation are completed and aldosterone has been measured in all relevant samples. Aldosterone assays from the study of daily variability (year 2) remain to be completed. The commercial provider of the aldosterone radioimmunoassay (RIA; Siemens Inc.) has, however, recently stopped production of the assay. We therefore must test and validate a new assay. We have selected a commercially available RIA with a high degree of sensitivity (MP Biomedicals; Santa Ana, CA) and with some modification this assay should perform satisfactorily.

Task 2—At the last ONR program revew we consulted with Dr R. Boonstra regarding CBG assays. He has developed reliable and accurate techniques for measuring circulating CBG concentrations from various species and generously agreed to assist with this work. We are therefore collaborating with Dr Boonstra's laboratory to conduct these measurements rather than develop new methods. The assays of CBG will be performed Jan - May 2015.

Task 3—Assessments of rT3 have not yet been performed.

Task 4—HPA axis stimulations have been completed in the Parent Project; cortisol and aldosterone have been measured from these samples. This hormone response was used to select appropriate samples for metabolomics assessment. A study protocol is in place and these samples are currently being analyzed by Metabolon; preliminary results should be available in the fall of 2014.

RESULTS

We have established a reliable method of measuring corticosteroids from dolphin serum at low circulating concentrations. We modified a protocol from a simple, affordable, and commercially available RIA kit (Siemens coat-a-count TKCO1). We conducted a validation of the RIA using serially diluted serum samples and found excellent parallelism with the standard curve. We determined that there were no interferring substances in dolphin samples by comparing steroid-extracted (purified) and non-extracted serum samples and found excellent agreement between the two

measurements (± 9%) across a four-fold dilution. There was no detectable cortisol present in steroid-stripped serum, indicating there is no cross-reactivity with the assay antibody and other matrix compounds. We established a method of measuring aldosterone by first extracting steroids from a larger volume of dolphin serum (~1 mL) into an organic phase (dichloromethane) before performing RIA. Extracted hormones are dried and reconstituted in assay matrix so this procedure should be applicable to a range of assays used across laboratories. With the initial peer-reviewed publication from this project we will promulgate the cortisol and aldosterone assay protocols in *supplementary material* to promote consistency in hormone assays within the marine mammal field.

Corticosteroid concentrations were low in this managed dolphin population (mean cortisol concentrations were 18.1 (sd 11.0) and 16.8 (sd 10.2) nM in years 1 and 2, respectively). Although consistently low compared to other studies (e.g. Ortiz & Worthy, 2001), cortisol concentrations varied daily, seasonally, and with sex (see Figure 1).

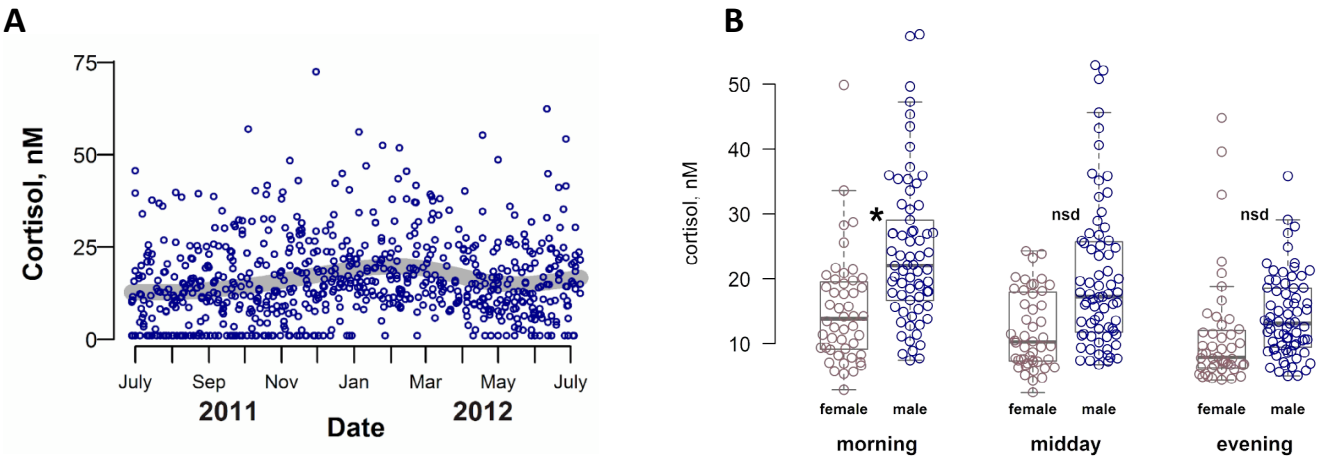

Figure 1. (A) Circulating cortisol concentrations were consistently low among 30 captive dolphins managed by the U.S. Navy MMP (mean 18.1, sd 11.0, nM; data from 731 samples collected between July 2011 - 2012). (B) Cortisol concentrations varied daily and by sex—concentrations were highest in the morning in both sexes and varied by sex in the morning (asterisk* indicates significant difference while "nsd" indicates no significant difference between sexes within sample time).

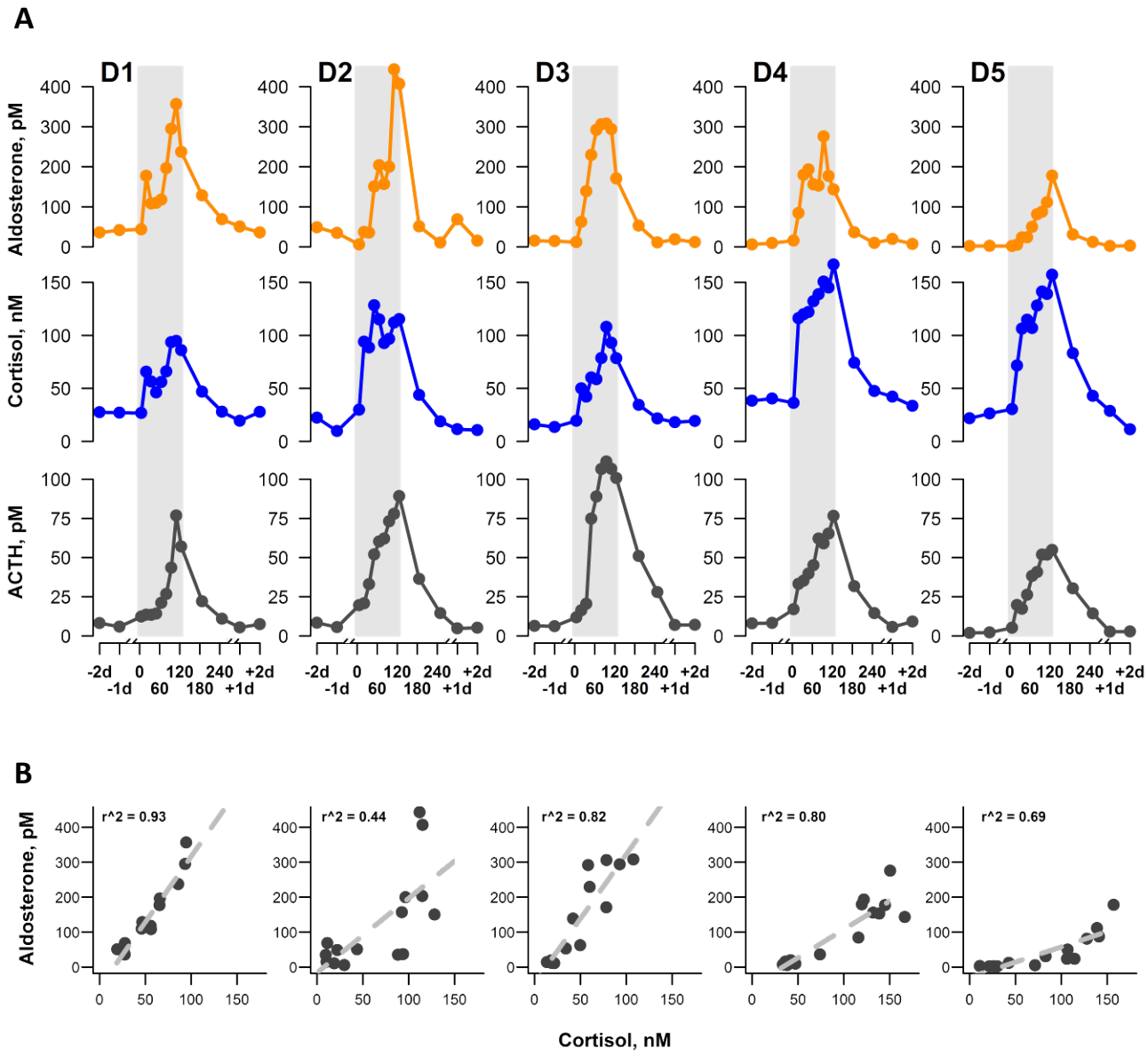

Figure 2. (A) Dolphins exhibited a characteristic stress response during out-of-water restraint. The two-hour restraint period is shown within the gray box and baseline samples were collected two days before (-2d, -1d), 1 and 2 hours after the conclusion (+180 and +240 minutes), and 1 and 2 days following (+1d, +2d) the stress tests (note a break in the time-scale axis before and after the stress tests). Adrenocorticotropic hormone (ACTH), cortisol, and aldosterone responses are shown for each of the five study animals, D1 - D5. (B) The release of aldosterone and cortisol were associated within each dolphin; linear fits of aldosterone vs. cortisol concentrations, and corresponding r^2 values, are shown for each subject, D1 - D5 (linear regressions, $p < 0.01$ for all cases).

IMPACT/APPLICATIONS

Marine mammals negatively influenced by acoustic disturbances or other U.S. Navy activities potentially experience a "stress response." The stress response can be detected by changes in stress markers, including select hormone concentrations, alterations in metabolic pathways, and potentially certain metabolite levels. The stress response can influence survival and reproduction and, therefore, may have population-level effects (Wikelski & Cooke, 2006). The additional characterization of hormones, hormone regulators, and metabolites during baseline and simulated stress conditions, as described in the current proposal, provides a mechanism by which to better detect the presence and magnitude of the physiological responses of marine mammals exposed to anthropogenic sound. In accordance with National Research Council recommendations (2005), the work described in this proposal, in concert with the Parent Project, will establish baseline and activated levels for putative stress markers in marine mammals.

RELATED PROJECTS

Grant #N000141110436, *Variability of hormonal stress markers collected from a managed dolphin population*; PI: Dorian Houser, PhD; National Marine Mammal Foundation. This project is the parent project from which samples have been and continue to be obtained.

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PUBLICATIONS

Houser, DS, Champagne, CD, Crocker, DE, Kellar, NM, Cockrem, J, Romano, T, Booth, RK and Wasser, SK. Natural variation in stress hormones, comparisons across matrices, and impacts resulting from induced stress in the bottlenose dolphin. In: *Effects of Noise on Aquatic Life II*, Popper, A and Hawkins, A, eds. [*in press*].