

## **The Bottlenose Dolphin (*Tursiops truncatus*) as a Model to Understand Variation in Stress and Reproductive Hormone Measures in Relation to Sampling Matrix, Demographics, and Environmental Factors**

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### **LONG-TERM GOALS**

Our overarching goal is to develop indicators and methods to quantify chronic stress in bottlenose dolphins. Much research has focused on the stimuli which induce stress in marine mammals, as well as the hormonal mediators of the stress response. Stress may be induced by a variety of factors, including noise, pollutant or toxin exposure, presence of predators, loss of prey, and/or habitat changes. The stress response is complex and difficult to study experimentally in marine mammals due to ethical and logistical considerations, but has been well characterized in other laboratory mammal species. In mammals as well as other vertebrates, the stress response has two modes of operation: The fast mode involves the rapid release of fast-acting agents, such as catecholamines, by the medulla which drive the fight-or-flight response, enhancing vigilance, alertness, arousal, and attention. The catecholamines in turn play a major role in excitation of the hypothalamic-pituitary-adrenal (HPA) axis, initiating a hormonal cascade which culminates in stimulation of the adrenal cortex to secrete glucocorticoids (GCs). The delayed but more sustained response driven by GCs coordinates brain and body functions to cope with stress and facilitate recovery, adaptation, and re-establishment of homeostasis. These functions include mobilization of substrates for energy metabolism, suppression of immune and inflammatory reactions, and inhibition of bone and muscle growth. Studies of both captive and free-ranging individuals support the existence of these same stress response pathways in marine mammals.

While the HPA axis and physiological processes driven by the GCs are essential for an individual's ability to respond and adapt to stress, prolonged stimulation can overly burden the body's regulatory systems and induce deleterious effects. Prolonged elevation of GC hormones can lead to chronic immune suppression and inhibition of other energy-expending hormonal systems, including disruption of reproductive function along the hypothalamo-pituitary-gonadal axis, all of which may cumulatively lead to decreased survival and/or inability to reproduce. For this reason, developing indicators and methods to quantify chronic stress in marine mammals is essential for understanding risks and long-term consequences for populations.

## OBJECTIVES

Using the bottlenose dolphin as a model species, specific objectives for this project are:

- Determine correlation of hormone measures (cortisol, T3, T4, FT4, reproductive hormones) between blood and blubber
- Develop a comprehensive understanding of factors that influence stress hormone levels and establish reference intervals for blood and blubber measurements, determining necessary stratifications by sex, age and/or sampling season.
- Examine relationships among the various hormone measures, and conduct preliminary screening analysis to examine potential relationships between the stress hormones and other health measures including immune function.

## APPROACH

The challenge of dealing with free-ranging marine mammals can be reduced through selection of species for which a broad base of biological information is already available and situations where appropriate samples can be readily obtained. Long-term studies of resident populations of well-known species such as common bottlenose dolphins (*Tursiops truncatus*) can help to promote an understanding of the natural variation in hormones and/or biomarkers of the stress response in free-ranging marine mammals as it relates to life history or natural cycles.

The Chicago Zoological Society's (CZS) "natural laboratory" situation in Sarasota Bay, Florida, where a resident population of bottlenose dolphins has been studied for more than 40 years, including demographic studies, capture-release health assessments, remote biopsy sampling, behavioral studies, and characterization of stress and stressors, provides unique opportunities to address questions related to stress. Serum hormones (cortisol, aldosterone, thyroid and reproductive hormones) have been routinely measured in blood as part of the health assessment which also includes a complete physical examination, morphometric measurements, hearing test, and sampling of blood, skin, blubber, urine, feces and blowhole swab. The collected tissue samples are analyzed for a broad suite of diagnostics.

Using the Sarasota model, we have more recently initiated studies targeting populations in heavily impacted coastal sites to gain an understanding of the effects of biological and chemical stressors on dolphin population health. Capture-release studies have been conducted in the Florida Panhandle where we are investigating the effects of chronic algal toxin exposure, and along the Georgia coast where we are examining the impacts of high exposure to legacy chemical contaminants. In all of these capture-release projects, we have collected data on reproductive, and thyroid hormones, as well as indicators of functional immunity (*e.g.*, lymphocyte proliferation, neutrophil and monocyte phagocytosis), all measured simultaneously from the same individuals and processed by the same laboratories to ensure inter-study comparability.

We are meeting our proposed objectives by leveraging this existing collection of data and expanding sampling to include additional reference populations. One of the additional reference populations in a northern latitude site will be sampled across seasons to elucidate potential seasonal variation in blubber hormone measures.

Recent work by our collaborative team has supported the use of blubber as a sampling matrix for measuring hormone concentrations. Blubber as a sampling matrix provides an advantage in that samples can be readily obtained using remote biopsy, which is relatively inexpensive and entails less harassment compared to capture-release sampling. In addition, while the logistics of capture-release sampling generally limit its utility to investigations of coastal cetaceans, remote biopsy has the potential to be a powerful tool for investigations across a range of habitats, from estuarine to nearshore and pelagic populations.

Remote biopsy samples will be collected to obtain sufficient sample sizes, to acquire samples across 4 seasons and to ensure derived reference intervals are applicable across geographic sampling sites. Additional remote biopsy sampling will be conducted across all 4 seasons in estuarine and coastal waters near Charleston, South Carolina, and across 2 seasons in the Ashepoo, Combahee and Edisto (ACE) Basin, also in South Carolina.

The Charleston site is home to a resident stock of bottlenose dolphins that has been studied since 1994; these dolphins are one of the northernmost year-round resident stocks on the U.S. coast. Additionally, since 1997, over 200 remote biopsy samples have been collected from Charleston dolphins, with no adverse impacts. Selection of this northerly stock should enable identification of seasonal variation in hormone concentrations, if such variation exists. The ACE Basin site was selected as an additional site for estimation of reference intervals because it is a relatively undeveloped area, home to a National Estuarine Research Reserve, and due to its proximity to the NOAA Charleston Laboratory.

The project is a collaborative effort led by Dr. Lori Schwacke (NOAA/National Ocean Service (NOS)/National Centers for Coastal Ocean Science (NCCOS)) and Dr. Randall Wells (Chicago Zoological Society). Other collaborators and co-PIs are Eric Zolman, NOAA/NOS/NCCOS, Dr. Nicholas Kellar, NOAA/National Marine Fisheries Service (NMFS), Southwest Fisheries Science Center, Dr. Patricia Rosel, NOAA/NMFS Southeast Fisheries Science Center, Dr. Stephanie Venn-Watson, National Marine Mammal Foundation, and Dr. Teri Rowles, NOAA/NMFS, Office of Protected Resources.

## **WORK COMPLETED**

All of the planned capture-release fieldwork has been completed. Matched blood and blubber samples were collected from dolphins in Sarasota Bay during May 2009 (n=20), May 2010 (n=10), May 2011 (n=15), and May 2012 (n=16). Additional blood samples, without blubber, were collected in July 2012 (n=10). More than 2,100 hormone measures previously obtained for Sarasota Bay dolphins are being applied to this project.

Remote biopsy sampling to collect blubber samples across seasons was conducted during Fall 2011 and Winter, Spring, Summer 2012 in waters near Charleston, SC, and conducted in the ACE Basin during Winter and Summer 2012 (Table 1). In total, 118 blubber samples were collected for hormone analysis. At a minimum, 65 of the 80 dolphins sampled near Charleston and one dolphin from ACE Basin were matched to the local photo-identification catalog. This completes the fieldwork component.

**Table 1. Number of biopsy samples collected near Charleston, SC and in the ACE Basin, SC.**

	<b>Fall 2011</b>	<b>Winter 2012</b>	<b>Spring 2012</b>	<b>Summer 2012</b>
Charleston	20	20	20	20
ACE Basin		<u>15</u>		<u>23</u>
<b>Total</b>	20	35	20	43

Laboratory analysis of remote biopsy blubber samples was scheduled to begin in September 2012 and we had originally planned to use commercially available enzyme-immuno (EIA) assay kits that are designed for measuring a single select hormone per assay kit and which provide an indirect measure of hormone concentration. However, ideally we would like to obtain a more complete understanding of hormone interactions and potential roles in stress response by measuring a broader suite of hormones, without having to collect multiple blubber samples. Therefore, we developed a collaboration with the National Institute of Technology (NIST) and Medical University of South Carolina (MUSC) partners at the Hollings Marine Laboratory. Dr. Ashley Boggs and Dr. Louis Guillette have developed a new approach using solid phase extraction (SPE) to liquid chromatography tandem mass spectrometry (LC-MSMS) to extract and directly quantify multiple classes of hormones from a single sample. Dr. Boggs has validated the method for the suite of hormones listed in Table 2 and is currently testing this method for use with dolphin plasma. Dr. Boggs will use samples provided by our project to validate the same method for blubber. Therefore, rather than limit our analyses to a few hormones which can currently be measured via EIAs, we are working with Dr. Boggs to analyze the collected biopsy samples for a much broader suite of hormones using her newly developed LC-MSMS method. The analyses of our remote biopsy samples will begin as soon as the method has been validated using blubber (~ January 2013).

**Table 2: Steroid Classes and Compounds Validated for LC-MSMS**

<b>Androgens</b>	<b>Estrogens</b>	<b>Progestogens</b>	<b>Corticosteroids</b>
Testosterone	Estradiol†	Progesterone	Corticosterone
Androstenedione	Estrone†	17-OH Progesterone	Cortisol
17-Methyltestosterone	Estriol†	Pregnenolone	11-deoxycortisol
Dehydroepiandrosterone	Ethynylestradiol†+	17-OH-Pregnenolone	21-deoxycortisol
Trenbolone+	Equilin†	11-keto-Progesterone	Cortisol-d4*
Adrenosterone	Equilenin†+	Norgestrel+	
Dihydrotestosterone	17-a-Estradiol†	Pregnandienetrione	
Testosterone-d5*	Methoxyestrone†	Dehydroprogesterone	
	Estradiol-13C6*†	Progesterone-d9*	

\* Internal standard

† Require derivatization on ESI

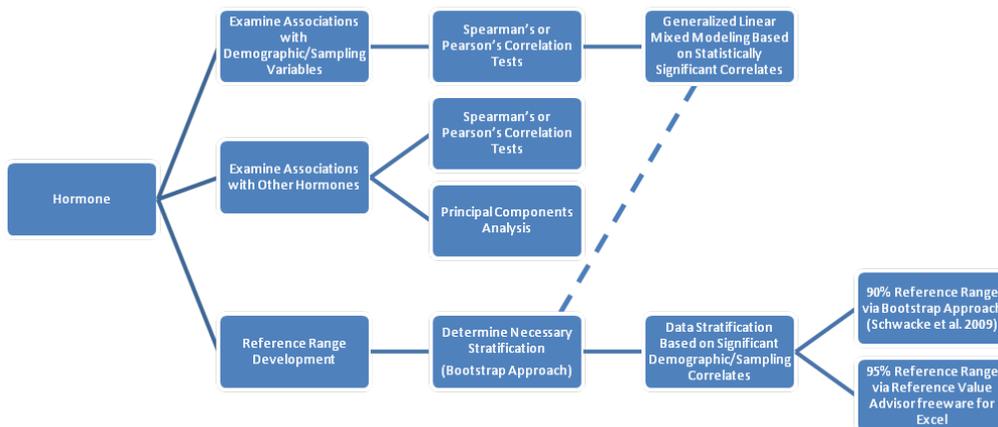
+ Synthetic or medically prescribed hormone

A workshop to begin statistical analysis and modeling of available hormone data was held August 29-31 at the Hollings Marine Laboratory in Charleston, SC. PIs Schwacke and Wells met with HML collaborators (Drs. Boggs, Guillette) to discuss the newly developed LC-MSMS methods, and also with Dr. Leslie Hart (NOAA/NCCOS), an epidemiologist assisting with the statistical analyses.

## RESULTS

Hormone analysis of blubber samples from previous capture-release health assessments in Sarasota using the EIA method were completed. Although data are still being finalized, preliminary results indicate a strong correlation between blubber cortisol level and elapsed time from initial capture to biopsy extraction. These results suggest a much faster accumulation of cortisol in the blubber than what was expected. These initial data are currently being reviewed and finalized, and will then help to guide future experimental/assessment plans to better understand the dynamics of cortisol in blubber.

Although hormone analysis for the remote biopsy blubber samples is not scheduled for completion until FY13, we have begun statistical analysis for development of reference intervals using the available blood data from dolphin capture-release work in Sarasota. An analytical plan (Figure 1) was developed during the workshop held in late-August. For all stress-related hormones (cortisol, aldosterone, T3, T4, and Free T4), correlational tests and Generalized Linear Mixed Models (GLMM) will be used to determine associations between each individual hormone and demographic/sampling parameters. The GLMM results will determine potential stratifications for development of reference intervals, but the GLMM tests for differences in means rather than differences in the tails (95<sup>th</sup> percentiles) of the distributions. While this may suggest a need for stratification, the final decision to stratify/partition data will be based on the bootstrap approach used by Schwacke *et al.* (2009) for hematologic and serum biochemical reference intervals.



**Figure 1. Planned analytical process for reference range development.**

## IMPACT/APPLICATIONS

We expect to better define the range of natural variability of stress hormones for bottlenose dolphins, as well as stress hormone responses to a variety of natural and anthropogenic stressors. By examining relationships between stress hormones in blood and blubber, we hope to enhance the utility of remote biopsy sampling as a tool for measuring stress hormones, and reduce the need for dolphin capture-release -- a stressful, expensive, and logistically complex activity -- to obtain stress hormone measures. We will also examine potential relationships between stress hormone measures and longer-term dolphin health indicators in order to identify potential impacts of stress.

## **RELATED PROJECTS**

A matching project is being conducted under the leadership of Dr. Lori Schwacke of the NOAA/National Ocean Service Hollings Marine Laboratory (Project No. N0001412IP20053). The current project provides samples from Sarasota Bay bottlenose dolphins and assists with the projects conducted primarily by Dr. Schwacke and her team, in Barataria Bay, St. Joseph Bay, and coastal Georgia. Data analyses are being performed jointly.

## **REFERENCES**

Schwacke LH, Hall AJ, Townsend FI, Wells RS, Hansen LJ, Hohn AA, Bossart GD, Fair PA, Rowles TK (2009). Hematologic and serum biochemical reference intervals for free-ranging common bottlenose dolphins (*Tursiops truncatus*) and variation in the distributions of clinicopathologic values related to geographic sampling site. *Am J Vet Res*, **70**(8):973-985.