A SYSTEMATIC REVIEW OF CORTISOL LEVELS IN WILD AND CAPTIVE ATLANTIC BOTTLENOSE DOLPHIN (*Tursiops truncatus*), KILLER WHALE, (*Orcinus orca*), AND BELUGA WHALE (*Delphinapterus leucas*).

by

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ABSTRACT

Systematic Review of Mean Cortisol Levels in Wild and Captive Atlantic Bottlenose Dolphins (*Tursiops truncatus)*, Killer Whales (*Orcinus orca)*, and Beluga Whales *(Delphinapterus Leucas).*

Shelby Proie

Cortisol levels can be used as a tool to measure stress in wild and captive cetaceans. Cortisol is the primary glucocorticoid found in most mammals including humans and cetaceans. This systematic review compiles all published studies conducted on cortisol levels in wild and captive Atlantic bottlenose dolphin (*Tursiops truncatus*), killer whales (*Orcinus orca*), and beluga whales (*Delphinapterus leucas*) and compares the reported mean cortisol levels between 1) wild and captive members of the same species, 2) references to sampling time, 3) wild and captive members of different species, and 4) studies on captive Atlantic bottlenose dolphins throughout time. The results show that sampling methodology affects mean cortisol levels in all three species. Cortisol levels obtained in wild cetaceans reflect similar levels to samples collected in captive animals that were sampled utilizing non-husbandry methodology. Samples obtained from both wild and non-husbandry practices reflected significantly higher cortisol levels than those sampled utilizing captive husbandry methodology. Additionally, cortisol samples of wild Atlantic bottlenose dolphins may display elevated levels within an hour of chase initiation, which challenges the claim that chase and capture sampling techniques produce accurate baseline cortisol levels of this species in the wild. Finally, mean cortisol levels of captive Atlantic bottlenose dolphins have not significantly decreased as captive care has evolved over time. Future studies need be conducted to corroborate these results, specifically studies that analyze fecal glucocorticoid concentrations so invasive sampling methodology that may skew results is minimized.

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This topic was the entire reason I decided to attend graduate studies. Through my interaction with wild and captive cetaceans, I wondered if anthropogenic stress was a significant component of their existence. I was not able to answer that question, but I now know the importance of establishing accurate methodology to analyze this question in the future. The ability to gauge and monitor stress in wild and captive cetaceans not only allow us to create a better environment for them to live in, but us as well.

For all of those whom inspired me, especially: Lolita, Kibby, Delphi, JJ, the Key West resident pod, Ric O'Barry, Howard Garrett, Sheri Sullinger, Naomi Rose, Ingrid Visser, Lori Marino, Dr. Miwa Suzuki for sharing data and answering all of my questions, and Kevin Francis for his advise and guidance. Lastly I wish to thank my family, boyfriend Mikey, and close friends, especially Katie, for their encouragement, which gave me the motivation to complete this achievement.

CHAPTER 1

INTRODUCTION

A Systematic Review of Cortisol Levels in Common Bottlenose Dolphin, Killer Whale, and Beluga Whale[1]

In the past decade the first species of cetacean to be driven to extinction, due to anthropogenic activity was the Yangtze River dolphin (*Lipotes exillifer*) (Morisaka et al. 2010). Currently the Vaquita (*Phocoena sinus)* and Maui's dolphin (*Cephalorhynchus hectori maui)*, a subspecies of Hector's dolphin, are among the most endangered animals on the planet with a population of around 150 for the former and 50 for the latter (Jefferson, 2012). A likely factor in the decrease of these species of cetacean along with many others is stress related ailments caused by anthropogenic stressors (Morisaka et al. 2010). According to Morisaka et al. (2010) the preservation of these animals can only be attempted with a clear understanding of "physiology, psychology, and behavior." Analyzing stress hormones in cetaceans living in both wild and captive environments can lead to better strategies to conserve their environment and establish better individual care. Studies of stress levels in common and endangered cetaceans may lead to better conservation strategies for all at-risk cetacean species in environments facing increasing anthropogenic stressors.

[1] Definitions: In this study, wild will be synonymous with natural environment, captive will be synonymous with artificial

This thesis is related to stress levels of common bottlenose dolphins (*Tursiops truncatus)*, killer whales (*Orcinus orca)*, and beluga whales (*Delphinapterus leucas)* in wild and captive environments. The aim is to assess stress levels between animals of the same species living in different environments, cortisol levels in reference to sampling time, stress levels between species living in different environments, and cortisol levels in response to evolved captive care. These are extremely difficult questions to answer due to numerous factors including convoluted sampling methodology and the large expense of sample collection and analysis which results in a relatively small number of studies that have been conducted on cortisol levels in wild and captive cetaceans. Studies that have been conducted encounter confounding factors and methodological errors. Because cetaceans live most of their lives below the surface of water bodies, accurate cortisol levels in wild cetaceans may be biased by having to capture or restrain the animals in order to obtain a sample, thus exciting the stress response in these animals (St. Aubin et al. 1996, Thomson & Geraci 1986). The premise of this thesis is that a better understanding of cetacean stress can be accomplished by systematic review of all the data presently available in one conclusive review.

Hypotheses

I have four specific research questions, which are followed by three null and research hypotheses. The first of each category is the research question followed by the research hypothesis (H_1) then null hypothesis (H_0). 1: Do captive

Atlantic bottlenose dolphin, killer whale, and beluga whale of the same species exhibit higher mean levels of cortisol than their wild counterparts? H_1: Captive members of these species will exhibit higher mean levels of cortisol than their wild counterparts due to constant exposure to anthropogenic stressors. H_0: Mean cortisol levels will not vary between wild and captive members of these species.

2: When do cortisol levels increase in wild cetaceans (using *Tursiops truncatus* as a model) during sampling methodology? H_1: When cortisol samples are taken from wild Atlantic bottlenose dolphins within an hour of chase initiation the samples will not display significantly elevated cortisol levels (see St. Aubin et al. 1996 and Ortiz and Worthy, 2000). H_0: There will be no difference in cortisol levels sampled within one hour of chase initiation and after one hour of chase initiation.

3: Do Atlantic bottlenose dolphin, killer whale, and beluga whale species collectively differ between species in their circulating mean cortisol levels in wild and captive environments? H_1: Mean cortisol levels will vary between species and environments. H_0: Mean cortisol levels will not vary between species and environment.

4: Have mean cortisol levels in captive Atlantic bottlenose dolphins trended over time? H_1: Mean cortisol levels will show a decreasing trend over time in captive Atlantic bottlenose dolphins. H_0: No trend will be detectable in captive Atlantic bottlenose dolphins over time.

To answer these questions I assembled a compilation of published journal articles along with unpublished data. With the data gathered from previous studies I completed a systematic review of mean cortisol levels recorded in three species of wild and captive cetacean: Atlantic bottlenose dolphin, killer whale, and beluga whale. I anticipate that this research project will result in significant understanding of how these three common species of cetacean cope with stress in two different environments: natural (wild) and artificial (captive).

Captive studies of cortisol levels have been used to determine baseline, resting, diurnal, and circulating levels for each species in a captive environment; mean cortisol levels can also be used to observe if stress levels have decreased as new husbandry practices have been implemented to improve the health and living conditions of the cetaceans. The completed research on cortisol levels in wild cetaceans is subject to confounding variables of stressful conditions due to the collection method. Accurate studies of cortisol levels in wild cetaceans can provide a baseline for captive animals, but collection methods in the wild may produce skewed data. Collection methods that involve retrieving serum samples, such as biopsy or chase, capture, restraint, are invasive and may excite the stress response prior to or during sample collection. Data collected in these studies could serve as a display of high-moderate stress in these cetaceans. Non-invasive methods such as fecal collection can produce a more accurate analysis of normal circulating cortisol levels.

How Analyzing Cortisol Levels can Impact Wild and Captive Cetaceans

Humans have always had a connection with cetaceans. As research has advanced our fascination and respect for these species has increased. These three cetacean species are currently viewed as some of the most intelligent and socially bonded animals on Earth. Delphinids, like bottlenose dolphin and killer whales display self-awareness (Reiss & Marino, 2001), a quality only expressed by humans and a few other species of primate. Anthropogenic stressors are increasingly present in both natural and artificial environments. With current research able to completely assess lifespans of cetaceans in captivity the ethics of keeping these species in captivity has been questioned (Rose, 2011). Stress is an important factor that impacts every facet of cetacean lives, much like humans. Prolonged exposure to stressors, know as chronic stress, can cause immunosuppression and reproductive problems (Curry, 1999), which are commonly found in animals living in artificial, captive environments (Rose, 2011). The ability to analyze stress in cetaceans can help target what is inducing in the stress response and help alleviate it with better best management and husbandry practices (Morisaka et al., 2010).

Anthropogenic stressors are becoming more prevalent due to overpopulation, overconsumption, and global climate change. Stress can cause psychological and physiological problems. Highly intelligent and social animals like cetaceans may be more vulnerable to the effects of stress than other species due to their longevity and wide ranges (Curry, 1999). Prolonged exposure to stressors, chronic stress, can cause fatal complications (Curry, 1999), which are

commonly found in animals living in artificial, captive environments (Marine Mammal Inventory Reports, obtained from National Marine Fisheries Service via Freedom Of Information Act). This study will serve as the first systematic review of its kind to assess the stress levels through mean cortisol concentrations of wild and captive common bottlenose dolphin, killer whales, and beluga whales by combining and comparing all published data on record. The ability to analyze stress in cetaceans can help target what is inducing the stress response and help alleviate it with a better understanding for these organisms and their environment, and in time may be applied to conserve endangered wild cetacean species and their habitat.

My contribution to the field of cetacean endocrinology takes on the issues associated with serum collection as an indicator of baseline stress levels in the wild and compares it to non-invasive fecal cortisol collection. By comparing the cortisol levels of animals that were assessed in an invasive manner to others that were either collected voluntarily or with non-invasive fecal collection more accurate baseline cortisol levels may be produced and less invasive methodology may become the norm. It will demonstrate the importance of analyzing trends in glucocorticoids between fecal and serum samples and show how that can be used a good indicator of stress level (Wasser 2013, personal communication). It also displays the importance of realizing and reducing confounding factors when at all possible, by comparing mean cortisol levels, instead of individual samples between studies, and the importance of conducting longer studies where circadian

rhythm and seasonality can be taken info effect to produce the most accurate analysis.

Delimitations, Limitations, and Assumptions

Studies comparing cortisol levels in wild and captive terrestrial animals have displayed problems with reproduction and consistently higher levels of cortisol in captive animals (Terio et al., 2004, Rangel-Negrín et al., 2009). A study on captive African elephants discovered that cortisol levels increased as enclosure size decreased (Stead et al., 2000). Captive cheetahs have displayed significantly higher levels of cortisol than their wild counterparts (Terio et al., 2004). A study on Yucatan spider monkeys concluded that captive animals display the higher levels of cortisol, while animals living in fragmented forest display lower levels of cortisol than the captive animals, but higher levels than animals living in un-fragmented forests (Rangel- Negrín, 2009). Recent studies related to stress hormone analysis in wild and captive terrestrial animals have employed fecal glucocorticoid (FGC) monitoring. This is a non-invasive way to collect feces from wild and captive animals for FGC metabolite analysis.

Conversely, studies on marine mammals have displayed the opposite. In studies conducted on harbor seals (*Phoca vitulina*) and harbor porpoises (*Phocina phocina*) concentrations of cortisol were higher and occupied a more varying range than captive animals (Gardiner & Hall, 1997, Buholzer et al., 2004). Other studies have noted the invasive methodology (chase, capture, restraint) associated

with blood sample collection cannot be employed without eliciting a stress response and influencing the results (Stead et al., 2000, Liar et al., 2009). To date no studies have been completed utilizing FGC monitoring in a population of wild and captive cetaceans of the same species for analysis.

Only one study conducted on stress hormone analysis of wild killer whales was available for this systematic review, Ayres et al., (2012). The study utilized fecal glucocorticoid (FGC) metabolites to assess glucocorticoid levels in wild Southern Resident Killer Whales. FCG analysis is not comparable to serum, plasma, or salivary cortisol measurements where the parent hormone cortisol is present. Captive killer whale studies are present in this analysis, but the wild study is only being used as a guide to encourage migration to non-invasive stress hormone monitoring in wild and captive cetaceans.

One study (Spoon & Romano, 2012) uses estimated cortisol levels in the captive beluga whales sampled obtained from a figure published in that study. Estimations were made based on the samples shown in that graph. In total the mean cortisol value estimated could be ±1 μg/dl. When those levels are applied to the analysis statistical significance or lack thereof is not altered. Contact with the living author was made via phone message on 3/15/2013. If the exact levels are possible to obtain they will be implemented into the analysis.

CHAPTER 2

LITERATURE REVIEW

Stress

Stress can cause psychological and physiological problems in all animals. Highly intelligent and social animals like cetaceans, may be more vulnerable to the effects of stress than other species due to their longevity and wide ranges (Curry, 1999). The concept of stress was recognized as far back as 450 BC Hippocrates's concept viewed health as a state of harmonious balance and disease a disruption to that balance (Chrousos and Gold 1992). Harmonious balance is a homeostasis, viewed as an ideal state of equilibrium within the body where all systems are working and interacting in the correct way to fulfill the needs of the body and the mind. Something that disrupts the homeostasis such as a stressor can cause imbalance in the mind and body (Curry, 1999). It wasn't until 1936 that the word stress was accepted in a biological context (St. Aubin & Dierauf, 2001). A current biological definition comes from Dr. Vahdettin Bayazit and defines stress as "a physiologic response to events perceived as potentially or actually threatening the integrity of the body" (Bayazit, 2009). Physical and behavioral effects of stress in cetaceans are relatively limited, although changes in adrenal and thyroid hormone levels have been documented since the 1970's (Curry, 1999). Cetaceans are exposed to a large number of environmental (natural) and anthropogenic (unnatural) stressors throughout their lives, in the

wild and in captivity. Monitoring stress hormones in cetaceans may lead to better conservation strategies and positive stewardship towards their habitat (Noren et al. 2011).

Stress hormone monitoring in cetaceans is used to observe the animal's adaptation to environmental changes and physical stimuli (Schmitt et al., 2010). It is valuable to monitor stress and its effects on an animal's state of wellbeing (Schmitt et al., 2010). Free-ranging, wild cetaceans can experience stress in numerous ways such as noise, predation, fisheries, ecotourism, climate change, declining food sources, diseases, social issues, pollution, and habitat degradation, among others (Schmitt et al., 2010). Captive cetaceans can also face a variety of stressors such as isolation, social instability, depression, lack of stimulation, food quality, confined spaces, artificial water, unnatural noises, and human interaction (Rose, 2011). These factors can cause acute and chronic physiological stress responses. The acute stress response is short in duration and anxiety invoking, making behavioral assessment an effective tool used to recognize it in animals (St. Aubin & Dierauf, 2001). Capture myopathy and problems with thermoregulation can be attributed to acute stress as well (Curry, 1999).

Frequent, intermittent, and/or repetitive stressors may induce the chronic stress response (St. Aubin & Dierauf, 2001). Constant exposure to chronic stressors can cause habituation, sensitization, and desensitization (Dantzer & Mormede, 1995). Chronic stress can cause stress-induced pathologies along with changes in immune system and impaired growth and reproductive functions (Curry, 1999). Immunosuppression is often the greatest threat of exposure to

chronic stress in cetaceans (St. Aubin & Dierauf, 2001). Physiological conditions have the ability to change concentrations of metabolites, minerals and enzymes in the body (Tryland et al., 2009). The close relationship between the nervous and immune system dictates the ability of a perceived physiological stressor to impact an animal's immune system (Spoon & Romano, 2012). Glucocorticoids (cortisol) which increase in the presence of a stressor, impact many aspects of the immune system including white blood cell production. Thomson & Geraci (1986) discovered that lymphoctytes, the white blood cells that contain T, B, and NK cells can decrease by 50% when dolphins are under a moderate stress response. They also found that eosinophil granulytes, white blood cells that combat parasites and infections, decreased immediately as a stressor was perceived and continued to decline until the stressor was gone, not recovering to normal numbers until the next day (Thomson & Geraci, 1986). Decreased levels of lymphocytes and eosinophils, along with hyperglycemia are classic characteristic of a classic stress leucogram, and have the ability to lower immunity especially when facing chronic stress (St. Aubin & Dierauf, 2001).

Changes in levels of stress hormones and other hematologic parameters are not the only effects cetacean experience when living with constant acute and chronic stressors. Clark et al. (2006) found that adrenal mass increased in beached bottlenose dolphins that were under acute and chronic stress. The adrenal gland was on average 2 times larger in chronically stressed animals than acutely stressed animals. It is apparent that exposure to chronic stressors impact cetaceans in many different ways, and has the possibility of causing an

exaggerated stress response that can cause deterioration and lead to death (Cowan, 2000). The culmination of acute and chronic stressors can occur and intensify over time (Curry, 1999). Because cetaceans are exposed to a large variety of stressors, stress level monitoring is an important tool that may help humans create better environments with lower levels of anthropogenic stressors.

Cetaceans are exposed to a large number of anthropogenic and environmental stressors throughout their lives, in wild and captive environments, which for this paper, will be interchanged with natural and artificial environments. Assessing stress hormones in captive cetaceans may help caretakers eliminate or manage potential stressors presented to the animal's to facilitate better health (St. Aubin & Dierauf, 2001). In the wild cetaceans are regularly exposed to natural and anthropogenic stressors. Collecting data on stress hormones may lead to important management decisions that could contribute to lessening the effects of stress on cetaceans (St. Aubin & Dierauf, 2001). Although not all aspects of stress are negative and some studies even suggest that the acute stress response could even be healthy in small quantities (St. Aubin and Dierauf, 2001), continuous exposure to chronic stressors often results in distress and can be fatal (Thomson & Geraci, 1986).

Cetacean Stress Physiology

The physiology of stress in cetaceans is similar to most other mammals (Schmitt et al. 2010). Although it is composed of a series of related events, there

is variation between species and from individual to individual within species (St. Aubin & Dierauf, 2001). The stress response is composed of a quick mode driven by the medulla's releases of catecholamines, which enacts the fight-or-flight response and increases alertness known as the acute stress response. Catecholamine release is followed by a delayed sustained response that is initiated by the release of glucocorticoids, which coordinate brain and body function (Wells, 2012). When exposed to a stressor the autonomic nervous system (ANS) is triggered. The ANS is composed of two branches the sympathetic nervous system, which is responsible for the fight-or-flight response and the parasympathetic nervous system which, is designed to mitigate the stressor. The sympathetic nervous system triggers the adrenal medulla to release catecholamines, epinephrine and norepinephrine, into the blood (Martineau, 2007). Concurrently with the catecholamine release, the chronic stress response is controlled by the hypothalamic-pituitary-adrenal axis that when faced with a stressor will releases corticotrophin-releasing hormone (CRH). CRH then targets the anterior pituitary to release adrenocorticotropin (ACTH), which triggers the release of mineral- and glucocorticoids from the adrenal gland (Groschl, 2008). These adrenal hormones create energy production, regulate the immune system, increase metabolic processes and assist in osmoregulation, which can allow adaptation to the stressor (Schmitt et al. 2010). Although activation of the HPA axis is necessary to successfully respond and adapt to stress prolonged stimulation of the HPA axis in cases of exposure to chronic stressors can be life threatening (Wells, 2012). To analyze stress hormones in cetaceans the steroid hormones are

collected and analyzed using a variety of methods. Steroid hormones include chemical compounds that are secreted by the adrenal cortex, which include mineral- and glucocorticoids.

Stress Hormones

Stress hormones are released in the presence of an acute stressor and can continue to be exhausted during chronic stress. A stressor increases physical and/or psychological demands and to cope with it the body releases several adaptive hormones: adrenocorticotropin by the anterior pituitary, gluco- and mineralocorticoids by the adrenal cortex, epinephrine from the adrenal medulla, and norepinephrine from the sympathetic nerves. Secondary endocrine components include prolactin, growth hormone, thyroid hormones, and vasopressin among other pituitary hormones (St. Aubin & Dierauf, 2001). These hormones are involved in complex interactions, regulate each other and help the body adapt to stressors (Axelrod & Resine, 1984).

Catecholamines

Catecholamines are the first line of defense and first responders to a stressor. These agents are fast acting and necessary to increase vigilance, alertness, arousal and attention when presented with a stressor. They involve activation of the cardiovascular system and metabolism. Catecholamines are typically produced during the acute stress response, but have also been reported to

increase during dives in marine mammals. The hypothalamic-pituitary-adrenal axis is activated by a stressor, which triggers the release of neurotransmitters (Axelrod & Resine, 1984). The well-known neurotransmitters dopamine, norepinephrine, and epinephrine are all catecholamines. They activate the amygdala, which triggers an emotional response. Catecholamines excite the HPA axis that triggers the secretion of glucocorticoids and can suppress short-term memory, concentration, and inhibition (Wells, 2012). Catecholamines are induced rapidly and can subside quickly (St. Aubin & Dierauf, 2001). Numerous studies on wild and captive cetaceans support the hypothesis that the same stress response pathways that exist in humans are present in marine mammals.

Mineralocorticoids

Aldosterone is the principle mineralocorticoid in mammals. Zona glomerulosa cells, which are prominent in marine mammals, produce aldosterone (St. Aubin, 2001). In most animals aldosterone is not viewed as a stress hormone, but it is active in the stress response of cetaceans (St. Aubin & Dierauf, 2001). Aldosterone primarily functions to provide water conservation and electrolyte balance including sodium transport and potassium excretion (Schmitt et al. 2010). Ion regulation and changes in blood pressure activate aldosterone production and releases it via the adrenal gland (Schmitt et al. 2010). ACHT production, which occurs during the stress response, elicits an unusually high elevation of aldosterone in bottlenose dolphins (Thomson & Geraci, 1986) and belugas (St. Aubin & Geraci, 1989), when compared with other mammals (St. Aubin et al.

1996). Although levels of aldosterone regularly increase 4-5 times that of base level readings during human induced stressors no significant changes in sodium, potassium, or chloride have been documented (Schmitt et al. 2010). Similar elevations in aldosterone correlated with capture and handling stress during experiments in bottlenose dolphins (Thomson and Geraci, 1986; St Aubin et al., 1996) and belugas (St. Aubin and Geraci, 1989). This discovery has lead to the hypothesis of aldosterone functioning to maintain ion homeostasis during stress and its function as a primary indicator of stress in cetaceans (Schmitt et al. 2010). Aldosterone has increased during known stressful events such as capture and handling in bottlenose dolphins (Thomson & Geraci, 1986; St. Aubin et al., 1996) and belugas (St. Aubin & Geraci, 1989). In other species of odontocetes, however, aldosterone has not been observed to rise in the presence of an acute stressor (St. Aubin et al. 2013). This could be due to a state know as "aldosterone escape" which can occur when aldosterone levels are elevated for a prolonged amount of time (Turban et al. 2003).

Glucocorticoids

Glucocorticoids are comprised of corticosteroids including corticosterone and cortisol. They are known in endocrinology for the role they play in the stress response. Cortisol is the most prominent glucocorticoid in cetaceans according to studies to date (St. Aubin & Dierauf, 2001). Cortisol is one of the initial adrenal hormones to increase when introduced to acute and chronic stress (Möstl & Palme 2002). Cortisol can stay elevated for up to five hours after presented with a

stressor (St. Aubin & Geraci, 1989), and usually peaks between 1-2 hours (St. Aubin et al., 2001). This increase makes cortisol one of the most reliable and common stress hormones collected to analyze the stress response in cetaceans (Schmitt et al. 2010). Cortisol is commonly used in stress analysis because it is known to be indicative of the stress response in cetaceans. Glucocorticoids are important to monitor because they have three functions in stress. According to the Handbook of Marine Mammal Medicine, they alter metabolism to increase energy; they permit catecholamines to act on metabolic pathways and blood vasculature; and they provide adaptations to distress by minimizing immunological reaction to prevent tissue damage (St. Aubin & Dierauf, 2001). They can inhibit gonadotropin, growth hormone and thyroid stimulating hormone secretion, which contribute to reproductive, growth, and thyroid function (Tsigos & Chrousos, 2002). Cortisol is able to inhibit thyroid hormones like as thyroxine and triidothyronine (T_4 and T_3). The inhibition of these hormones along with elongated lag time of 6-8 hours for T_3 and more than 20 hours for T_4 explain why these hormones aren't used as primary indicators of stress on their own. Cortisol functions under stress by providing the body with energy through gluconeogenesis and can act as an anti-inflammatory agent and decrease white blood cells, which may cause immunosuppression during times of prolonged psychological and physical stress (Kravitz et al, 2005).

Although basal cortisol levels are usually lower in cetaceans than terrestrial mammals (Martineau, 2007), cortisol levels are usually significantly raised when the animal is in distress or highly stressed (St. Aubin et al. 2001). A

possible explanation to the low level of circulating cortisol in cetaceans has to do with the hormone-binding capacity of the plasma. Studies on belugas and bottlenose dolphins show that the bound fraction of cortisol represents 50% or less of the total circulating hormone (St. Aubin, 2001). The high level of circulating hormone may account for small increases of cortisol to translate into more unbound hormone and greater availability to affect the animals. Although this is debated, it is thought that when presented with a stressor cortisol levels are elevated within thirty minutes (St. Aubin & Geraci, 1986). Prolonged exposure to raised cortisol levels can have deleterious effects on reproductive function along with the neurological and immune systems (Martineau, 2007).

Glucocorticoids influence immunological factors by causing an anti-inflammatory reaction and suppressing the immune system to keep cells and cell mediators in check. Some of the cell mediators stimulate CRF secretion, which increases ACTH and cortisol and weaken the immune response (St. Aubin & Dierauf, 2001). Leukocyte counts can be used to recognize stress in cetaceans. The classic stress leukogram depicts characteristics of cellular blood changes that are commonly present during the stress response (ie an increase in cortisol). A classic stress leukogram consists of lower counts of eosinophil and lymphocytes and increased circulating neutrophils, which causes an increase in white blood cells (St. Aubin et al., 2013). This trend has been found in bottlenose dolphins and belugas exposed to various stressors including transport (Medway et al., 1970; Reidarson & McBain, 1999), capture (St. Aubin & Geraci, 1989;1992) or injection with ACTH (Thomson & Geraci, 1986; St. Aubin & Geraci, 1989).

These hormonal changes in reaction to elevated glucocorticoid levels due to exposure to stress may disrupt the ability to fight infection (St. Aubin & Dierauf, 2001).

Many aspects of the stress response can cause alterations in reproductive functions by inhibiting the reproductive process. Elevations of glucocorticoids may cause reproductive complications by inhibiting secretion of gonadotropin-releasing hormone, blocking the release of luteinizing hormone along with follicle-stimulating hormone, and altering the gonadal response to LH and FSH secretion (St. Aubin & Dierauf, 2001). Although studies have not been conducted in most cetaceans to evaluate these claims it is know that TH levels can be drastically altered by stress in belugas (St. Aubin & Geraci, 1989; 1992).

Cortisol is the primary glucocorticoid produced in cetaceans. Although it varies between species roughly 95% of cortisol in the body binds to corticoid-binding globulin (90%) and albumin (5%). The remaining 5% are free for target cells (Curry, 1999). Cortisol concentration is reported to be a good indicator of a stress response in cetaceans because it is rapidly secreted during both acute and chronic stress response (Naka et al., 2006; Suzuki et al., 1998). Cortisol levels are significantly lower in cetaceans than humans. According to the National Institutes of Health, humans who display a diurnal circadian rhythm in cortisol secretion exhibit higher levels in the morning than evening. Normal cortisol levels in humans range from 165.64-634.57 nmol/L depending on sampling time. In captive bottlenose dolphins who are also thought to exhibit a diurnal circadian cortisol secretion pattern, Suzuki et al. (1998) found the mean baseline cortisol

level in captive Atlantic bottlenose dolphins sampled in the AM to be around 11 nmol/L with a range of 5.5-19.3 nmol/L. This comparison displays that normal AM circulating cortisol levels in humans are on average 33-126 times higher than what is thought to be normal in bottlenose dolphins.

The cortisol:corticosterone ratio is 5:1 in whales and dolphins that have been sampled (Thomson & Geraci, 1986; Ortiz & Worthy, 2000) in comparison to 10:1 in humans (Raubenheimer et al., 2006). Cortisol levels have been known to increase within the first five minutes of exposure to a stressor in humans; in bottlenose dolphin's increases have been observed in the first ten minutes (Orlov et al., 1988). Cortisol levels have been used in the past to gauge how cetaceans experience, cope, and adapt to stressors in their environments, whether they be captive or wild (Thompson & Geraci, 1986, St. Aubin et al., 1996, Ortiz & Worthy, 2010, Spoon & Romano, 2012).

Cortisol levels have been studied in accordance with the stress response during known stressful activities including capture and handling. Cortisol levels increased in bottlenose dolphins (Thomson & Geraci, 1986; St. Aubin et al., 1996) and belugas (St. Aubin & Geraci, 1989, 1992) during capture and handling processes in most studies. One study conducted on bottlenose dolphins showed no change in cortisol during capture and handling procedures (Ortiz & Worthy, 2000). Factors such as habituation and desensitization may have contributed to the static cortisol levels in Ortiz & Worthy, 2000.

Stressors

Numerous cetaceans, like humans, are self-aware sentient beings (Reiss & Marino, 2000). Stressors are not interpreted the same between individuals and species (St. Aubin & Dierauf, 2001). Cetaceans are often living with various stressors present in their daily lives originating from natural and anthropogenic sources. Cetaceans' life history traits include long life spans, late maturity, low reproductive potential, and feeding near the top of the food chain which makes them particularly susceptible to anthropogenic stressors (Fair & Becker, 2000). In the wild, natural stressors like predation, prolonged fasts, extended dives, and altered weather patterns, coupled with anthropogenic environmental stressors like increased boat traffic, habitat degradation, pollution, and harassment can induce stress by increased concentrations of mineral- and glucocorticoids like aldosterone and cortisol (St. Aubin & Dierauf, 2001). In captivity, most stressors are of anthropogenic origin because the animals is kept in human care, these stressors include condensed living spaces, human interaction, chemically treated water, food quality, man-made social structure, and removal from family bonds. The stressors faced in captivity likely increase levels of mineral- and glucocorticoids in individual animals, although for conclusive data more research needs to be completed.

Like humans, cetaceans individual and group responses to stressors vary. In some situations experience and acclimation can dull the stress response in some individuals for possible stressful events. Different individuals may exhibit a

stress response when new stimuli are placed in an enclosure, but others may not (St. Aubin & Dierauf, 2001). In some situations where stressors are introduced it is plausible that heightened response levels in some individuals and/or populations could predispose them to the chronic effects of prolonged hypothalamus-pituitary-adrenal axis stimulation including immunosuppression and reproductive problems (St. Aubin, 2002a).

Acoustic Stressors

Anthropogenic acoustic disturbances are the most studied cause of cetacean stress (Rolland, et al. 2012). Acoustic disturbances can range from ship traffic to navy sonar to whale watching boats (Fair & Becker, 2000). Cetaceans are incredible auditory creatures routinely using acoustic measures to communicate, navigate, and find prey. Cetaceans are primarily very social animals with some species such as southern resident killer whales living in maternal family groups for their entire lives (Ford et al., 2000). This displays the importance of communication and social interaction within cetaceans. Odontocetes (toothed whales, dolphins, and porpoises) use echolocation to detect prey sources and navigate in deep, dark waters, using a complex system of sending out a pulse of sound from the melon that bounces off of an object and gets absorbed by the lower jaw allowing the animal to detect in what direction, what size, and how far away that object is (Berta et al., 2006). It has been hypothesized that mysticetes (baleen whales) also use auditory cues to detect density of their small prey sources and for navigation purposes (Berta et al.,

2006). Increased frequency of anthropogenic auditory prevalence in the marine environment can disturb every normal behavior of cetaceans including sleeping, foraging, and mating. Its effects can range from short to long-term displacement (Fair & Becker, 2000). Exposure to high or cumulative noise levels in cetaceans can cause elevated stress levels, which have the ability to lead to a compromised immune system and depressed reproductive functioning (Fair & Becker, 2000).

A recent study conducted by Rosalind Rolland, of the New England Aquarium proved that heavy shipping noise in the Bay of Fundy causes increased levels of stress in the endangered population of North Atlantic right whales (*Eubalaena glacialis*) (Rolland et al. 2012). This study had obtained fecal samples from North Atlantic Rights Whales in the Bay of Fundy from 2001-2005 (Rolland et al., 2012). These samples were analyzed in conjunction with noise recordings taken from the bay during the time of sampling, paying special attention to the noise recordings from September 2001, the year "9/11" terrorist events practically halted all commerce (Rolland et al., 2012). The observations showed that on September 9, 2001, nine ships were observed navigating through the Bay of Fundy while on September 13, 2001 only three ships were observed (Rolland et al., 2012). The decrease in ships contributed to a decrease in low frequency shipping noise, which is around the same frequency North Atlantic right whales communicate in (Rolland et al., 2012). The results of comparing the stress hormones in the fecal samples collected in September of 2001 showed a significant decrease in stress hormones analyzed in the days immediately following September 11th than before September 11th when shipping was

unaffected (Rolland et al., 2012). These results were only consistent with September of 2001, which indicates the North Atlantic Right Whale population inhabiting the Bay of Fundy have an increased stress response to an anthropogenic acoustic presence (Rolland et al., 2012). These endangered whales may be susceptible to immunosuppression and/or repressed reproduction due to chronic elevated stress levels (Rolland et al., 2012). This study can aide in the conservation strategies to help increase the population of North Atlantic right whales by acting as scientific evidence to prove that shipping noise is directly related to the physical well-being of these highly endangered animals.

Environmental Contaminants

Cetaceans also experience chronic stress effects from exposure to environmental contaminants (Fair & Becker, 2000). Since many cetaceans have long life spans and many odontocetes occupy the top of the food chain lipophilic contaminants have the ability to bio-accumulate in the animals stored lipids (blubber) (Martineau, 2007). In Southern Resident Killer Whales among other odontocetes a trend of higher contaminant levels in males and juveniles has been recorded because females often pass accumulated contaminants to their offspring through maternal transfer and lactation (Krahn et al, 2007). Lipophilic contaminants including persistent organochlorine pollutants (POPs), which consist of polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and dichlorodiphenyltrich-lorethane (DDT) among others, are well known immunosuppressors and can target the adrenal glands and alter stress

responses (Martineau, 2007). Immunosuppression can cause increased susceptibility to diseases and pathogens, which could lead to premature mortality (Martineau, 2007).

Southern resident killer whales (SRKW) reside in the Salish Sea off the coast of Washington State for around six months every year (Ford et al., 2000). They often enter the waters of Puget Sound. They have been observed as far south as Monterey Bay, California and as far north as Southeastern Alaska (Ford et al., 2000). Because of the animals extended close proximity to civilization they are constantly exposed to environmental contaminants (Balcomb, 2012). Persistent organic pollutants (POPs) in combination with other stressors such as an increase in anthropogenic noise, and a decline in prey have the ability to cause immune and endocrine system disruption (Krahn et al., 2007).

Detecting immune functions in free-ranging cetaceans is difficult due to their constant mobility and difficulty in obtaining samples. An approach devised to analyze immune functions requires comparing a population that is hypothesized as being contaminated (i.e. inhabiting waters close to shore in urban areas) to another group of the same species that is less exposed to pollutants (farther off shore or close to shore in a less unpopulated area) (Martineau, 2007). This approach helped evaluate the conclusion that beluga whales (*Delphinapterus leucas*) living in the highly polluted St. Lawrence Seaway display a more compromised immune system and were more susceptible to disease and parasites than belugas inhabiting the waters of coastal Alaska (Martineau, 2007). With respect to the Southern Resident Killer Whales, a study being conducted by the

University of Washington's School of Conservation Biology has reported that Transient killer whales who feed on marine mammals have the highest levels of PCB's in comparison when Northern and Southern Resident Killer Whales which corroborate past studies conducted on free ranging Northern, Southern residents and transient killer whales (Ross et al., 2000). At the "Ways of Whales" workshop on January 28, 2012 at Camp Casey Conference Center (Coupeville, Whidbey Island) Jessica Lundin's preliminary research displayed Southern Resident Killer Whales having substantially higher levels of PCB's that than of Northern Resident Killer Whales, who have a larger population and frequently inhabit less urbanized areas. Ross et al. (2000) found that age, sex, and dietary preference had direct effects on PCB concentrations in free ranging pacific killer whales. Lundin went on to show that Southeastern Alaskan resident killer whales show the smallest levels of accumulated PCB's because of their more remote location and low exposure to the toxins. All Southern Killer Whales that were sampled in a 2007 study have PCB levels that have exceeded the thresholds for health effects per established in a study done in harbor seals (Krahn et al. 2007). Increased exposure to toxins and their ability to target adrenal gland function to alter stress response function, including over and under production of mineral- and glucocorticoids (cortisol and aldosterone), can increase impacts on the animal's immune system and reproduction when amalgamated (Martineau, 2007). The ability to link increased levels of toxins to increased levels of stress in cetaceans can be used to impose improved environmental protection with the

discharge of the few mentioned toxins still being produced and lead to healthier chemical regimes.

Food/Prey Scarcity

Food scarcity has long been hypothesized to play a role in stress levels of cetaceans. In a study conducted by the University of Washington's School of Conservation Biology (UWSCB), Samuel Wasser and Katherine Ayres have been monitoring fecal hormone samples of Southern Resident Killer Whales since 2006 and continue to do so. The southern resident killer whales have undergone extreme population declines over the past fifteen years, an over 20% decline in the years between 1995-2005, with little knowledge of why (Krahn et al., 2002). According to the UWSCB primary hypothesis of this population drop is the increased stress levels that correlate with a decrease of primary prey supply.

Southern resident killer whales are genetically different from others of their same species (Morin et al., 2006). One of the reasons these orcas are categorized differently is because of their primary prey choice, Pacific salmon. Orcas around the world eat different food sources ranging from other marine mammals, herring, squid, to even sharks. The Southern Residents obtain 96% of the nutritional needs from Pacific salmon (*Oncorhynchus spp*.), 63% are obtained from exclusively Chinook salmon (Ford et al, 2000). Even in the presence of other potential food sources, these orcas choose to forage for Chinook salmon (*Oncorhynchus tshawytscha*), which are the largest of the salmonoid species in

the Pacific Northwest and potentially the most threatened (Ford et al, 2000). Pacific salmon require a clean, disease and parasite-free marine habitat to thrive. Current salmon runs are declining along the Washington Coast runs are 1.8% of historic run size; Puget Sound runs are 8%, the Columbia Basin 1.7%, the Oregon Coast 7%, and British Columbia 36.2% (Lackey, 2000). The combination of Washington, Oregon, Idaho, and California salmon runs are 5.2% of historic salmon runs (Lackey, 2000).

In the hundreds of fecal samples collected by University of Washington's School of Conservation Biology glucocorticoid and triiodethyonine levels were measured. Glucocorticoid levels increase in response to acute psychological and nutritional stress and triiodethyonine, a hormone produced by the thyroid gland is responsible for the regulation of metabolism, decrease in response to longer term nutritional stress, but are unaffected by psychological stress lowering metabolism (Ayres et al., 2012). This study supports the trend of higher glucocorticoid (i.e. cortisol) levels associated with times of highest nutritional stress (Ayres et al., 2012). Analysis has rendered the highest glucocorticoid levels in 2007 where salmon runs were at their recorded lowest, and the lowest levels of glucocorticoids were recorded in 2009 when salmon runs were more abundant (Ayers et al., 2012). The analysis has also indicated the lowest stress (lowest glucocorticoid levels) in southern resident killer whales are recorded in July and August each year which coincide with peak salmon runs in the Salish Sea (Ayers et al., 2012). The seasonal trends of glucocorticoid levels reflect stress levels increasing and decreasing with nutritional stress imposed by primary prey

abundance (Ayres et al., 2012). This research supports the importance of salmon protection for the survival of these endangered whales.

Fisheries Practices

Stress in cetaceans is not just limited to acoustic and environmental origination. Fishery induced stress has been studied in several species, especially in the waters of the eastern tropical Pacific Ocean (Curry, 1999). In the Eastern tropical Pacific Ocean tuna purse-seine fisheries are prevalent, using dolphins as scouts to catch yellowfin tuna (Curry, 1999). Three particular dolphin species are commonly involved with this practice, pantropical spotted (*Stenella attenuate)*, spinner (*S. longirostris*), and common (*Delphinus spp.)* because of their unknown involvement with tuna (Hammond, 1983). Three of these dolphin stocks are currently depleted: the northeastern offshore spotted dolphin, the eastern spinner dolphins, and the coastal spinner dolphin (Curry, 1999). The methods employed by purse-seine fisheries have the ability to cause significant stress to the dolphins involved (Curry, 1999).

Purse-seine tuna fisheries in the Eastern tropical Pacific Ocean often use the technique of encircling a pod of dolphins that have comingled with a substantial amount of tuna with a purse-seine net, which can be 1.6 km long and 200 m deep (Curry, 1999). This process can take anywhere from 40 minutes to over three hours, causing the dolphins caught in the net to huddle at the bottom or near the surface to seemingly gain the farthest distance from the vessel/s involved

(Norris et al, 1980). Even though some escapement strategies are employed by the fishermen to remove the dolphins from the net many get entangled in the net and drown (Curry, 1999). Although dolphin mortality estimates exceeded 4.5 million from 1959-1972 (Wade, 2007), estimates throughout the late 1990's speculate that over 3,000 dolphins die each year from the effects of tuna purse-seine fisheries in the Eastern tropical Pacific Ocean (Curry, 1999).

The effects of stress from purse-seine fishing may affect many more dolphins than it kills. More than half of the dolphins observed when encircled by purse-seine nets display signs that have generally been accepted as signs of stress and agitation in cetaceans including headslaps, tailslaps, thrashing, and bunching (Norris et al, 1980). Other behaviors associated with stress in dolphins that have been observed are hyperactivity, mostly in spinner dolphins trying to escape from the net, which can lead to physical exertion, and passive behaviors such as floating and sinking (Norris et al, 1980). These nets may capture the same pods of dolphins found in the Eastern tropical Pacific Ocean as frequently as once per week (Edwards & Perkins, 1998). Using four subsets of current research to identify responses to stress including laboratory/captive animals, domestic animals, clinical research, and studies of free ranging animals; the conclusion is that prolonged exposure to stressors can cause compromised immune and reproductive function, and is hypothesized to be a cause of the declining numbers of the northeastern offshore spotted dolphin, eastern spinner dolphin, and the coastal spinner dolphin stocks (Curry, 1999). These dolphin populations have shown evidence of acute stress by displaying elevated circulating glucocorticoid

levels (Curry, 2002). These prolonged, increased glucocorticoid levels can be a direct reflection of fisheries induced stress in cetaceans. Although other fisheries such as long line and gillnetting expose cetaceans to stressors, the knowledge gained from these studies on purse seine netting have lead to the direct implementation of dolphin escape apparatuses from nets and dolphin by-catch, but more effective conservation strategies need be employed to protect the three endangered species chronically harassed by this fishery.

Stress Hormone Sample Collection

The collection method for obtaining stress hormone samples in cetaceans varies depending upon the captive or wild status of the animal being targeted. Five employed sample collection methods for stress hormone analysis in cetaceans are: saliva, blow, fecal, blood, and blubber. All methods could be employed in each setting but not all are practical. The most common stress hormone collection method in a captive setting is blood serum samples via venipuncture. Due to establishing positive reinforcement behavioral protocols many captive cetaceans exhibit trained husbandry behaviors such as fluke rising for medical procedures (Schmitt et al. 2010). Previous wild studies often elicited a stress response in the process by chasing and restraining the animals to obtain a serum sample (St Aubin et al. 1989). Currently, biopsy sampling of blubber is the most common in large species of cetaceans, including most mysticetes (Noren & Mocklin, 2011). Methods such as fecal collection are now being regularly employed (Hunt et al., 2006). Each method has a different level of invasiveness

ranging from very invasive to relatively non-invasive depending upon the animal and setting. The level of invasiveness has the ability to alter the results of the analysis of stress hormones depending on how the animals reacted to the collection method. Stress levels and habituation to stressors vary within different species and individuals of the same species (Fair & Becker, 2000).

The collection of stress hormones in cetaceans varies depending on the species of cetacean being targeted and if it inhabits a captive or wild setting. Five sample collection methods for stress hormone analysis that are currently being utilized in cetaceans are saliva, blow, fecal, blood, and blubber. All methods could be employed in a captive setting but not all are practical in a wild setting, such as collection of a blood sample. The most common stress hormone collection in a captive setting is blood samples because most captive cetaceans are taught husbandry behaviors such as raising its fluke above water for blood sample collection, by using positive reinforcement (Schmitt et al., 2010). In the wild setting biopsy sampling of blubber is still the most common (Noren & Mocklin, 2011) yet more recently researched methods such as fecal collection are now being regularly employed (Hunt et al., 2006). Each method has a different level of invasiveness ranging from very invasive (biopsy) to relatively non-invasive (fecal collection). The level of invasiveness has the ability to alter the results of the analysis of stress hormones depending on how the animals reacted to the collection method. Stress levels and habituation to stressors vary within different species and individuals of the same species (Fair & Becker, 2000).

Since cetaceans are exposed to a wide variety of stressors by physical, chemical, and biological factors, these methods of monitoring stress hormones in cetaceans are an important tool to gauge the physical and emotional health of the animal. Physical stressors include fishery-interaction, pollution, acoustic influences, and climate change. Chemical stressors include exposure to chemicals, harmful algal blooms, metals, pesticides, and oil pollution. Biological stressors include disease, parasites, decline in prey, and habitat degradation (Fair & Becker., 2000). A cetacean's reaction to a stressor can be documented in a variety of ways including visual surface observation while the animal is in the vicinity of a stressor or collecting a sample for analysis of stress hormones (Curry, 1999). A thorough conclusion would ideally be based on both a visual observation of the animal and an analysis of stress hormone production.

Saliva

Saliva can be used as a less invasive source to analyze steroid hormone levels (Groschl, 2008). It is refuted as a technique that reduces the natural stress response of the body when collecting plasma and serum. Saliva can be analyzed to measure stress hormones because it is a carrier of signal molecules, which can be transported into the salivary glands from blood vessels or produced by the glands independently (Groschl, 2008). The rate at which these hormones can be transferred from the blood to saliva is determined by the passage through lipophilic layers of the capillaries and glandular epithelial cells. Steroids tend to be transferred through these barriers rather quickly because they are lipophilic

(Groschl, 2008). Cortisol is a stress hormone produced via the pituitary-adrenal cortex axis and is responsible for the chronic stress response (Groschl, 2008). Salivary gluco- and mineralocorticoid samples have been used to measure stress levels in captive Delphinids (Hogg et al., 2009).

The process of collecting saliva is usually performed with commercial collection devices that are composed of a nonabsorbent collection pad that must be kept in the mouth for at least one-minute, and a glass or plastic vesicle used to house the sample on the pad (Groschl, 2008). Centrifugation is used to remove the saliva from samples for testing and use. Centrifugations use centrifugal forces to separate liquid from solid components by rotating at high speeds and require the recording of force, time, and temperature (Rice University, 2005). Stress hormones can then be analyzed in a number of ways utilizing immunological and chromatographic methods (Groschl, 2008). Immunoassays are biochemical tests that can measure the amount of a substance in a solution. Immunological analysis involves nonradioactive enzyme-linked immunosorbant assay (ELISA) methods, which determines if a particular protein is present and renders results with colored products (Groschl, 2008). Some companies offer FDA-approved assays for measurement of salivary steroids as an alternative to ELISA (Groschl, 2008). Chromatographic assays separate a mixture of compounds and chromatographic methods include combining liquid chromatography with mass spectrometric (LC-MS) detection for quantification of salivary steroid hormones, which have been more reliable than immunological methods (Groschl, 2008). Liquid chromatography uses a pump to separate compounds and their components, and

mass spectrometry determines the mass of molecules using a mass spectrometer (Niessen, 2006). LC-MS is an instrument that includes a high performance liquid chromatograph connected to a mass spectrometer, which allows for analysis of a much wider range of compounds (University of Bristol 2005). Testosterone and progesterone levels were viably measured from a moderate sample size of baleen whales in this study via the above-mentioned methods (Groschl, 2008).

Cortisol is a key stress hormone that increases the rate of gluconeogenesis during stress and is frequently measured in salivary analysis. Because cortisol levels usually rise when an animals is approached with an invasive method such as venipuncture, saliva samples offer a more reliable standing stress rate by being less invasive, thus eliciting less of a stress response (Groschl, 2008). A consensus among the scientific community is that higher levels of salivary cortisol are collected in people and animals under chronic stress (Groschl, 2008). Some problems with using saliva as method to measure stress hormones such as cortisol, arise from a natural enzymatic conversion process. Large portions of cortisol are converted into cortisone in the salivary glands by 11β-hydroxysteroid dehydrogenase II, and then to ketoform, which is inactive. (Groschl, 2008). This process often creates discrepancies in data of cortisol collection including falsely increased measurements of both glucocorticoids, cortisol and cortisone (Groschl, 2008). Cortisol levels are also influenced by timing of collection because of the circadian rhythm of natural adrenal secretion (Groschl, 2008). Future research needs to be completed to test the accuracy of using saliva samples to measure stress hormones in cetaceans.

Blow

A relatively new method that has been developed is to use cetacean blow, the air exhaled out of a cetacean's blowhole, to measure steroid hormones. This may also serve as a method to collect mineral- and glucocorticoids such as cortisol and aldosterone in a less invasive way (Hogg et al., 2005). Currently this method has been employed to collect and analyze testosterone and progesterone for reproductive studies in free ranging cetaceans including northern right whales and humpback whales. Because steroid hormones are defined by the Encyclopedia Britannica as "any of a group of hormones that belong to the class of chemical compounds known as steroids; secreted by three steroid glands—the adrenal cortex (mineral- and glucocorticoids), testes, and ovaries" and can be grouped by the receptors to which they bind, mineral- and glucocorticoids bind with androgens (which includes testosterone), estrogens, and progestogens (which includes progesterone) are all within the five groups of steroid hormones. Analyses of stress hormones could be completed with similar methods to testosterone and progesterone analysis (Hogg et al., 2005).

Cetacean blow contains lung mucosa, which is collected by using cotton gauze, or nylon stocking fastened to a bamboo ring at the end of a pole ranging in length from 10-15 meters (Hogg et al., 2005). The pole would be extended off the bow of a small vessel and held above the blowhole of the animal when they exhaled. Wind, weather, and sea conditions affected the quality of the samples by interfering with the proximity to the whale and concentration of the sample (Hogg

et al., 2005). Only a small sample is needed for correct processing usually less than (50 μL) allowing the validated method of liquid chromatography-mass spectrometry (LC-MS) that has been used on captive dolphins to be employed (Hogg et al., 2005). Two forms were evaluated using LC-MS including a gradient scan for mass-to-change rations and selected ion monitoring (SIM) mode analysis for testosterone and progesterone (Hogg et al., 2005). This method was the first documented use of lung mucosa to gather steroid hormones, especially reproductive hormones.

This collection technique allows for a quick, less invasive method for gathering steroid hormone samples from wild and captive cetaceans that may spend little time at the surface (Hogg et al., 2005). The exchange of steroid hormones in blow samples are hypothesized to retain more data the longer that animal can stay submerged and the larger the lung volume. Vascularization of their lungs allows more compounds to diffuse from the blood stream into the lung mucosa (Dellman & Eurell, 1998). This theory allows blow samples to be viewed as a mix of organic material instead of just air and water (Hogg et al., 2005). In theory this process could be used to evaluate other steroid hormones such as mineral- and glucocorticoids to analyze stress levels in wild and captive cetaceans.

Fecal

Fecal samples have been collected and used to determine age and reproductive rates of free-swimming cetaceans since 2005 (Rolland et al., 2005). More recently this same fecal collection technique has been applied to measure fecal glucocorticoids levels to analyze adrenal activity and physiologic stress in free-swimming cetaceans, including North Atlantic right whales and Southern resident killer whales (Hunt et al., 2006). This technique has also been employed on a variety of terrestrial mammals due to the lack of confrontation or interaction with the animal to obtain the sample. Since glucocorticoids are released by the adrenal gland in response to a variety of stressors, they end up in the feces by being released from the adrenal gland into the blood then excreted through the bile into the gut that renders them as metabolites in feces (Wasser et al., 2000).

Although increased levels of glucocorticoids can be caused by predictable events such as pregnancy, elevated levels of glucocorticoids generally indicate an adrenal stressor is present (Balm, 1999). Elevated levels of glucocorticoids have been reported in a North Atlantic right whale that was fatally entangled in a net (Hunt et al., 2006). Glucocorticoid levels should be relatively low in juvenile, pre-adolescent, and non-reproductive animals (Hunt et al., 2006).

Fecal hormone analysis provides some problems including the comingling of metabolized fecal hormones with many unidentified metabolites that have unstable antibody affinities (Wasser et al., 2000). Cross-reactively from one

hormone elevating levels of another may also affect glucocorticoid levels in cetaceans (Hunt et al., 2006).

Fecal collection can be completed by trailing the animal from as far as a quarter of a mile or by using a detection K-9 to locate the floating cetacean feces (Ayres et al., 2012). Detections K-9's are dogs that are trained to alert their handler to floating fecal sample locations (Ayres et al., 2012). Samples then are removed from the water by use of a nylon mesh dip-net attached to a common boat hook (Hunt et al., 2006) or a specialized apparatus (UWSCB, 2012). As much of the sample is retrieved as soon as possible and drained of remaining salt water, then in many cases 50-250g are froze within an hour of collection (-20 degrees Celsius) and stored for later analysis (Hunt et al., 2006). Variation is then removed from the samples by a process of freeze drying, mixing, pulverizing, and sifting, this process assists in equalizing hormone content (Wasser et al., 1996). The samples are then weighed and boiled for 20 minutes in 5 ml 90% ethanol water or 100% ethanol (Wasser et al., 2000). The sample is then centrifuged for ten minutes, suspended in 5 ml 90-100% ethanol, vortexed for a minute, and finally re-centrifuged (Wasser et al., 2000). The sample is then dried, and re-dissolved in 1.0 ml methanol, and then the supernatant diluted in phosphate-buffered saline (PBS) a technique designed by Wasser et al., in 1994. This method allows for steroid extraction recoveries (Wasser et al., 1994).

Another assay to analyze glucocorticoid content in fecal samples is by using a non-boiling, vortexing extraction method (Schwarzenberger et al., 1991). This method involves the sample being confined in a tube with 2.0 ml 90%

methanol, vortexed for 30 minutes, and then centrifuged for 20 minutes at 500g and then the supernatant diluted in PBS. Both extraction methods mentioned have about a 90-100% hormone accuracy rate and were first tested by recovering injected glucocorticoid levels in a variety of species (Wasser et al., 2000).

The samples are then cleaned by being passed through a 0.2 micro meter filter, spin cartridge, and diluted with 5 ml of 80% methanol then analyzed via High Performance Liquid Chromatography (HPLC) (Wasser et al., 2000). Using a Radioimmunoassay various cortisol antibodies are then used to examine fecal GC metabolites including Pantex 031, Incstar CA-1529, CUS R1222, along with a corticosterone antibody ICN 07-120102 (Wasser et al., 2000). The corticosterone ICN antibody has been proven to reliably detect adrenal activity in a wild range of mammalian species, due to its higher cross-reactivity with the dominant fecal GC metabolites (Wasser et al., 2000). The corticosterone ICN antibody was raised in rabbits and shows a low cross-reactivity to cortisol along with binding well to fecal metabolites of cortisol and aldosterone (Hunt et al., 2006).

In North Atlantic right whales, fecal samples reliably measured immunoreactive fecal glucocorticoids showcasing pregnant/lactating females along with one animal caught in a net, and a yearling female exhibiting the highest levels of glucocorticoids, hypothesized from the added stressors of being pregnant, entangled, and weaning (Hunt et al., 2006). The lowest fecal GC levels were expressed in immature animals (Hunt et al., 2006). Fecal samples from North Atlantic right whales also showed an increased concentration of glucocorticoids with increased ship noise in the Bay of Fundy observed before,

during, and after the September 11th terrorist events (Roland et al., 2005). Fecal GC analysis has successfully shown elevated levels at times of hypothesized physiological or social stress, thus rendering it useful in a vast array of conservation and management applications, while still remaining the most non-invasive method to conduct stress hormone analysis (Wasser et al., 2000).

Blood

Historically the most commonly practiced method to obtain mineral- and glucocorticoid levels from animals were from a plasma or serum blood sample (Sheriff et al., 2011). Stress hormone concentrations can be obtained via venipuncture, although this method is most commonly practiced in the captive setting and usually not practical for use on free ranging wild cetaceans. A blood sample alludes to a clear depiction of the state of the animal at that moment which is composed of the endogenous cycles, immediate prior experience, and longer-term experiences (Sheriff et al., 2011). Cetaceans like most mammals, have cortisol as the primary measureable glucocorticoid (Wasser at el., 2000).

In a captive setting blood for stress hormone (ACTH, cortisol, aldosterone) analysis is usually collected via phlebotomy. Captive cetaceans are well versed in a variety of husbandry techniques including tail fluke presentation, which allows relatively easy access to fluke veins (Schmitt et al., 2010). A sample can then be collected using a three-quarter inch 19-gauge butterfly-catheter and 20mL syringe, among other methods (Schmitt et al., 2010). In the

review of a particular study preformed on beluga whales (*Delphinapterus leucas*) compelling results were obtained by incorporating natural diurnal rhythm, collecting blood three times daily for 5 days at predetermined times which accounted for natural cycling of hormones; blood collected 20 minutes before and after a 30 minute wading-contact session for 5 days; and sampled during out of water examinations where the animals is removed from the water by a stretcher where blood samples were collected for 20 minutes, 45 minutes, and one hour after being removed from the water (Schmitt et al., 2010).

Plasma or serum blood samples were collected, plasma is blood collected via an anti-coagulant and then spun on a centrifuge to remove red blood cells. Serum is blood obtained without an anti-coagulant and coagulates on its own then is spun (Sheriff et al., 2011). The main difference between plasma and serum blood samples is that plasma samples contain fibrinogen and serum samples do not (Sheriff et al., 2011). Fibrinogen is a protein produced by the liver than acts as a fibrous coagulant (Medicine Plus, 2012). Plasma samples were placed in plastic Ethylenediaminetetraacetic acid (EDTA) tubes and serum into serum-separator tubes and then chilled on ice for at least 30 minutes before processing (Schmitt et al., 2010). The chilling and centrifuge processes minimize steroid metabolism (Sheriff et al., 2011). The EDTA and serum tubes were centrifuged at 1500 rpm for 10 minutes and the separated plasma and serum were then placed in plastic vials at -70 degrees Celsius (Schmitt et al., 2010). Storing blood at temperatures below -20 degrees Celsius in a non-frost-free freezer will maintain stable glucocorticoid levels (Sheriff et al, 2011). The serum and plasma were

measured for Adrenocorticotropic hormone (increases production and release of corticosteroids), cortisol, and aldosterone by automated chemiluminescent enzyme immunoassays, which are a light-emitting reaction used to monitor an enzyme label or its products (Kricka, et al., 1987). Each analysis was duplicated to assess accuracy where cortisol, aldosterone, and ACTH had a less than 5% variation for intra-assay coefficients (Schmitt et al., 2010). Glucocorticoid concentrations vary in wildlife from a few nanograms/milliliter to thousands of nanograms/milliliter so it is important to create a species-specific baseline for the animals being analyzed (Sheriff et al., 2011). Data then needs to be further analyzed to create likely baseline stress hormone concentrations for the cetacean species being analyzed (Schmitt et al., 2010).

The use of stress hormone analysis from blood samples is unique because unlike other matrices used for stress hormone analysis plasma glucocorticoids measure total glucocorticoid concentrations (Sheriff et al., 2011). Because free glucocorticoid concentrations are the critical measurement the equilibrium dissociation constant of corticosteroid-binding globulin (CBG, the main plasma protein transport for cortisol) and the maximum corticosteroid binding capacity of the blood sample is needed (Sheriff et al., 2011). The equilibrium dissociation constant of CBG is calculated by using saturation binding data and is calculated for a species as a whole and the maximum corticosteroid binding capacity of the blood sample is calculated for each individual plasma sample (Sheriff et al., 2011). The above-mentioned values along with the total glucocorticoid count will render a free hormone concentration (Sheriff et al., 2011).

Blood plasma and serum stress hormone analysis is an invasive technique whether it is employed in captivity or the wild. The animal being sampled is ultimately being put through a physical stressor for the sample extraction process, which may lead to altered cortisol and aldosterone levels (Schmitt et al., 2010). Less invasive techniques such as fecal glucocorticoid monitoring, which render similarly accurate results, are gaining increased popularity for their ability to be taken from a distance with less of a physical or emotional stress response from the animal being tested. This is especially true of cetaceans, which are usually hyperaware of their situation (Sheriff et al., 2011).

Tissue

Biopsy techniques were developed to obtain skin and blubber samples from cetaceans in a non-lethal manner (Noren & Mocklin, 2011). Blubber samples contain steroid hormones, which include mineral- and glucocorticoids along with other well studied hormones such as progesterone and androgens (Hogg et al., 2005). To obtain the biopsy sample the use of manual or remote biopsy methods are chosen based on size and behavior the species being targeted (Noren & Mocklin, 2011). Smaller cetaceans inhibiting shallow waters can sometimes be biopsied by both manual and remote methods, while larger cetaceans are usually biopsied by remote methods (Noren & Mocklin, 2011). Manual biopsy methods are done by hand and remote biopsy methods by use of an aluminum pole-mounted biopsy tips or darts being expelled from a crossbow, compound bow, or gun. Choice of the manual or remote method for obtaining a

cetacean biopsy sample depend on body size, skin and blubber thickness, and swimming speed of the targeted animal (Noren & Mocklin, 2011). The size of the biopsy dart depends upon the depth and structure of the blubber layer being targeted (Noren & Mocklin, 2011).

A common way to obtain a biopsy on free ranging cetaceans is to use pneumatic darts fired through a dart gun (Noren & Mocklin, 2011). The use of a dart gun allows the vessel to be at a greater distance from the animal. Although accuracy increases as distance decreases, small vessels usually fire the biopsy dart when the animal is 5-30 meters from the bow for small to mid sized odontocetes (SWFSC, 2005) and 20-50 meters from the bow for large mysticetes (Noren et al., 2005). Although techniques and darts can vary a common protocol for gaining biopsy surveys of free-ranging cetaceans involves blubber samples equating to less than one gram being collected by using a biopsy dart with a stainless steel tip between .7cm and 4.0 cm (Fossi et al, 2000). The dart is fitted with an appropriate sized stop to prevent intrusive penetration and ensured recoil, an average size stop for targeting killer whales is 2.5 cm according to NOAA's Southwest Fisheries Science Center (SWFSC). NOAA'S SWFSC also states that after samples are collected the darts are thoroughly disinfected between usages. Biopsies are usually taken between the dorsal fin and the top part of the caudal peduncle, pending upon aim and species (Noren & Mocklin, 2011). Once the sample is collected it is immediately put into liquid nitrogen or stored in a cell medium (Noren & Mocklin, 2011). Glucocorticoids can be assessed by enzyme

immunoassays, which use enzyme-bound antibodies to detect antigens (Porstmann et al., 1992).

Because of the invasive nature of employing biopsy-sampling techniques the behavioral effects of employing these techniques vary among cetaceans, and many observations may be considered inconclusive because of the large amount of time spent submerged where observation is usually obstructed (Noren & Mocklin, 2011). Another invasive aspect of biopsy sampling is the physical impact on the animal. A biopsy dart it leaves a small wound (Norton et al., 2011), usually equaling the size of the sample, ~1 gram (SWFSC, 2005). From the limited amount of data collected via biopsy protocol of free-ranging cetaceans no adverse heath effects from the wound have been observed and the wound often heals quickly with relatively little scarring (Noren & Mocklin, 2011). However, one report of a biopsy dart stopper malfunctioning possibly killed a short-beaked common dolphin (Bearzi, 2000). Positive physiological response to the biopsy, which included healing and healed wounds were reported in killer whales (Barrett-Lennard et al., 1996), indo-pacific humpback dolphins (Jefferson, et al., 2008), bottlenose dolphins (Weller et al., 1997, Berrow et al., 2002, Parsons et al., 2003, Gorgone et al., 2008, Bruce-Allen et al., 1985) and southern right whales (Best et al., 2005). In most species sampled a behavioral response to contact with the biopsy dart was exhibited ranging from a shaking motion to fleeing the area, with the stronger responses exhibited by smaller odontocetes (Noren & Mocklin, 2011). Some of the information gained from biopsy sampling of cetaceans includes genetic information, prey preferences, foraging ecology, contaminant

loads, and physiological processes such as fertility and stress levels (Noren & Mocklin, 2011). Because of the massive gain of information that can be used in conservation of these species and the relatively minor observed disturbance to the animals being sampled, biopsy sampling is viewed as having more positive than negative impacts on the species being sampled (Noren & Mocklin, 2011).

Environments

Natural and artificial environments differ in many ways for cetaceans. In the wild many species are able to travel hundreds of miles a day and come in contact with stressors that would not be present in an artificial environment such as predators, food scarcity, and pollution. They are able to live in a natural social structure, rest, hunt, socialize, mate, and travel at will. Artificial environments often include a forced social structure or no social structure, higher or lower water temperatures, altered diet, chemically treated water, forced human interaction, lack of stimuli, and lack of space (Rose, 2011).

Natural (Wild)

In the wild Atlantic bottlenose dolphin, beluga whales, and killer whales occupy different habitats and are often socially bonded with their pods (O'corry-crowe, 2008, Ford, 2008, Wells & Scott, 2008). Killer whales do not have any known predators, other than humans, but belugas and Atlantic bottlenose dolphins are subject to predation. Each species maintains a unique diet based on the region

they occupy. They may be migratory, transient, or resident populations. Complications with measuring stress hormones in a natural environment include collection methods and ability to locate and sample the animals without eliciting a stress response (Noren & Mockland, 2011).

Artificial (Captive)

Artificial environments vary across the world and can range from multimillion-dollar aquaria to small traveling carnivals with plastic pools. Cetaceans can be kept outdoors or indoors and in a natural or artificial environment. The water can be chlorinated, chemically treated, or pumped in from a natural marine source. Their diet consists of dead fish of varying degrees of quality depending on the facility they are in, and are often supplement with excess vitamins and fresh water for hydration. Social structures are constructed by humans and may consist of the same or different species inhabiting the same enclosure or solitary confinement. Some are placed in facilities with climates similar to their natural location others are exposed to climates that differ greatly from where they would naturally inhabit. Some facilities have regularly scheduled performances others just have viewing. Predation is usually not an issue unless aggression within the social structure is exhibited.

Importance of Cortisol Monitoring

In cetaceans monitoring stress hormones is used to observe the animals adaptation to environmental changes and physical stimuli (Schmitt et al. 2010). It is valuable to monitor stress and its effects on an animal's state of wellbeing (Schmitt et al. 2010). Free-ranging, wild cetaceans can experience stress in numerous ways such as noise, predation, fisheries, ecotourism, climate change, declining food sources, diseases, social issues, pollution, and habitat degradation, among others (Schmitt et al. 2010). Captive cetaceans can also face a variety of stressors such as isolation, social instability, depression, lack of stimulation, poor food quality, special confinement, artificial water, loud, unnatural noises, and forced human interaction (Rose, 2011). These factors can cause acute physiological stress responses, which can cause capture myopathy and problems with thermoregulation. Chronic effects include stress-induced pathologies along with changes in immune system and reproductive functions (Curry, 1999). The culmination of acute and chronic stressors can occur and intensify over time (Curry, 1999). Because of the known stressors to cetaceans, stress level monitoring in both natural and artificial environments are an important aspect that can help humans create better environments with lower levels of anthropogenic stressors for the animals living within.

Comparing Cortisol Levels in Wild and Captive Animals

Captive studies have developed better methodology for obtaining cortisol samples. Animals have been taught husbandry commands that are performed on a daily basis allowing for adaptation for sample collection exposing their superficial fluke veins, thus negating the stimulus that would elicit a stress response (Schmitt et al. 2010). A direct comparison between basal cortisol levels of wild and captive cetaceans cannot be obtained from these methods, a comparison of stress vs. resting cortisol levels between the two areas and different species can be assessed, and be a helpful contribution to marine mammal science. Recently, the use of fecal glucocorticoids has been used for obtaining cortisol levels in wild cetaceans (Wasser et al. 2000). This methodology is non-invasive and allows for a proper comparison of basal cortisol levels between wild and captive cetaceans. Fecal glucocorticoid analysis also produces more reliable basal cortisol estimates than blood serum concentrations because they reflect the total amount excreted, whereas blood serum levels have been known to change quickly in reference to a stressor (Möstl & Palme, 2002). A fecal glucocorticoid sample will reflect values expressed between 12-24 hours prior to collection depending upon species, while blood serum samples will reflect in time concentrations, hence the variability (Möstl & Palme 2002) Based on this thesis research, as detailed below, I propose that in the future fecal glucocorticoids from both captive and wild animals will be the most accurate measurement of cortisol levels in wild and captive cetaceans, and in turn be the best way to assess stress in these animals. To

date no published study has ever attempted to collect data from fecal matter of wild and captive cetaceans of the same species for comparison.

Comparisons of Stress Hormones Across Taxa

In a study conducted on West Indian Manatees (*Trichechus manatus)*, a species of Sirenian, Larkin et al. (2010) concluded when fecal matter is analyzed and compared in wild and captive West Indian manatees the cortisol levels depend on what region the animals are being sampled in and the state of captive care (captive manatees in the USA had higher levels of cortisol than their wild counterparts and vice versa for manatees tested in Mexico). Confounding factors that come into play are size, temperature, and number of animals, season, sex, age, reproductive status, and access to food sources in the wild and captive environments. In another study conducted by Rangel-Negrin et al. (2009) in the Yucatan Peninsula this method has been preformed and expressed conclusive results in spider monkeys (*Ateles geoffroyi yucatanensis).* Spider monkeys living in preserved forests exhibit statistically significant lower levels of cortisol than captive spider monkeys held at zoos and as pets.

In the literature examined a basal cortisol level has been estimated and accepted for captive cetacean, pinniped, and some terrestrial mammal species (Thomson & Geraci 1983, Gardiner & Hall 1997, Rangel-Negrin 2009). Wild estimates are more variable due to gaining access to the sample, as mentioned above. Confounding factors are very prevalent and can be included or avoided

depending upon specific study and methodology. Some common confounding factors include age, sex, size of tank, human presence, seasonality, indoor/outdoor location, animals housed with, time in captivity, wild or captive born, among others for captive animals (Spoon & Romano 2012). Wild animals have their own set of confounding factors including age, sex, reproductive status, seasonality, anthropogenic impacts (sound, toxins, presence), prey availability, sample methodology (Ayres et al. 2012). The best way to avoid many confounding factors is to obtain as much data as possible about the animals being sampled and their environment. Serum to fecal to salivary cortisol can't be directly compared, but trends can be analyzed between them (Wasser, personal communication). Most published literature to date uses serum cortisol samples.

Like humans, different species will display different basal or resting cortisol levels to reflect natural circulation rhythm and/or stressors in their lives (Morisaka et al. 2010). The main animals I have assessed in my literature review include beluga whales, killer whales, and bottlenose dolphin, each of these species have different resting circulating levels of cortisol (Schmitt et al. 2010, Suzuki et al. 1998, St. Aubin et al, 1996). These species of cetacean were chosen because of the amount of literature published on them both in captive and wild environments. The literature provides as accurate estimate of baseline circulating levels of cortisol for captive species of these animals (Schmitt et al. 2010, Suzuki et al. 1998, St. Aubin et al. 1996), along with attempts to gather wild estimates (Thomson & Geraci, 1986, Ayres et al. 2012, St. Aubin et al., 1996). The wild estimates more likely represent a highly to moderately stressed animal than non-

stressed animals (St. Aubin et al., 1996). The animals in captivity may represent a mildly to moderately stressed animal due to constructions of living in an artificial environment, especially if that animal was wild caught (Rose, 2011).

Most comparisons of cortisol levels in wild and captive cetaceans conclude that wild animals have higher circulating levels of cortisol, which is in contrast to many terrestrial species (St. Aubin et al. 1996, Ortiz & Worthy, 2000, Thomson & Geraci, 1986). This is hypothesized to be true because of the stress the animals are put through to obtain the samples (Thomson & Geraci, 1986). In studies conducted on other species with methodology reflecting non-invasive sample collection, such as fecal, captive animals tend to have higher levels of resting cortisol than their wild counterparts (Rangel-Negrin et al. 2009). This is hypothesized to be due to the generic structure and lack of stimuli when living in a captive environment, among other factors including correlating cortisol levels with the display of stereotypic behaviors (Liu et al. 2006).

Serum cortisol samples obtained from wild cetaceans cannot be concluded as normal resting cortisol levels even if the animals are confined in their natural environment for weeks with nets. It still does not represent normal patterns and structure, and could, and probably does express a medium to mild stress response elevating cortisol levels (St. Aubin et al., 2001). As cetacean endocrinology becomes more recognized as an important tool to analyze cetacean health, more non-invasive measures are being implemented to gain insight into estimates of stress levels in free ranging cetaceans by analyzing fecal glucocorticoids. Non-invasive fecal sampling first gained acceptance in the early 2000s

(Schwarzenberger, 2007, Wasser, 2000) is gaining popularity and providing an accurate assessment of fecal glucocorticoid levels in free ranging cetaceans without eliciting a stress response during collection. Once this is the accepted methodology, and studies end and are published the literature review will greatly differ. Use of non-invasive sample collection needs to be, and to an extent has been the excepted method for cetacean endocrinology, especially when referring to stress hormones. The ability to have little to no influence on the animal's behavior when collecting the sample is crucial to obtaining a resting cortisol level. Although this methodology is rather difficult to conduct in a marine environment, it is not impossible, in fact it is very plausible and has been deemed effective by government agencies and cutting edge research institutions such as NOAA and The University of Washington's School of Conservation Biology.

In previous literature a common assay and unit for analysis of cortisol levels would be helpful, radioimmunoassays (RA) are the most widely excepted forms of analysis and I^{125} kits seem to be the most widely used (Ayres et al. 2012, St. Aubin & Geraci, 1989), yet other radioimmuno- kits are still utilized along with electrochemiluminescence immunoassay (ECL-IA) kits (Noda et al. 2006, Naka et al. 2007). Differentiations between the two do not seemingly obstruct the analysis. Published fecal glucocorticoid research on cetaceans is limited, but is becoming increasingly popular (Rolland et al. 2012, Ayres et al. 2012). Most cortisol comparisons between wild and captive marine mammals are deemed inconclusive due to invasive attempts to obtain samples for the wild animals, although through different methodology (i.e. obtaining samples directly after

capture, after weeks, and after months) offer a unique insight on the stress response of wild animals (Thomson & Geraci, 1986). This field of research itself is rather limited. Some articles that were reviewed include published and unpublished research on stress hormones in beluga whales (Tryland et al. 2006, Schmitt et al. 2010, St. Aubin et al. 2001, Spoon & Romano 2012, Lair et al. 1997, St. Aubin & Geraci 1989), killer whales (Ayres et al. 2012, Suzuki et al. 2003, Suzuki et al. 1998, Lyamin et al. 2005), and bottlenose dolphins (Suzuki et al. 1998, Noda et al. 2006, Naka et al. 2007, Houser et al. 2011, Blasio et al. 2012, Pedernera-Romano et al. 2006, Thomson and Geraci 1986, Ortiz & Worthy, 2000, St. Aubin et al. 1996). Each species has unique features and provides confounding factors for the analysis, but the elimination of all confounding factors would be impossible. The most reliable studies provided many samples and were preformed over a long period of time to eliminate many of those factors (Suzuki et al. 1998, 2003) and they allow insights to circadian rhythms and seasonality that were not taken into account during shorter studies.

Present studies have given the first glimpse of stress hormone levels in wild and captive cetaceans. It allows a base to draw upon when establishing better sample collection and analysis. Each study has similar methodology that has been the standard in marine mammal practice, but isn't always the most conclusive. Although, without the previous studies on cetacean endocrinology, cortisol levels would not be known in many species of wild and captive cetacean (Schmitt et al. 2010). The captive studies have given a baseline of circulating cortisol levels for captive cetaceans (Pedernra-Romano et al., 2006, St. Aubin & Geraci 1989;1992 ,

Blasio et al. 2011, Suzuki et al. 1998, Thomson & Geraci 1986). They have found seasonality differences (Spoon & Romano 2012), diurnal differences (Morisaka et al. 2010, Schmitt et al. 2010, Suzuki et al. 2003), and reproductive differences among and within species (St. Aubin et al., 1996). Without these studies that information would not be available due to constraints in wild animals without a bias, although with implementation of non-invasive collection methods all of that will change and wild animals will be able to be conclusively and continuously assessed (Ayres et al. 2012).

Prolonged exposure to stressors, chronic stress, can cause fatal complications (Curry, 1999), which are commonly found in animals living in artificial, captive environments (Marine Mammal Inventory Reports, obtained from NMFS via FOIA). This study will serve as the first analysis of its kind to assess the stress levels of wild and captive common bottlenose dolphin, killer whales, and beluga whales by combining and comparing all published data on record. The ability the analyze stress in cetaceans can help target what is inducing the stress response and help alleviate it with a better understanding for these organisms and their environment, and in time may be applied to conserve endangered wild cetacean species and their habitat.

Stress Hormone Analysis

A variety of immunoassays have been used to determine free cortisol levels in serum and plasma samples obtained in the studies that were used for this

systematic review (See Appendix A). The three main categories of assays utilized in these studies were Radioimmunoassays (RIA) (74% of studies), Chemiluminscence immunoassays (CLIA) (17% of studies), and Enzyme-limed immunosorvent (ELISA) which are synonymous with Enzymeimmunoassys (EIA) (~3% of studies) (See Appendices B, C, D). CLIA's include chemiluminescentnyzme immunoassays (CLEIA), electrochemiluminescent immunoassays (ECLIA), and immunochemiluminesence assays (ICLIA). All three of these types of immunoassays are based on binding competition. Cortisol from the animal and labeled cortisol compete for binding sites on the anti-cortisol antibody that has a high affinity and specificity for cortisol. The main difference between these assays is the detection methods. RIA's use radioactive detection; ELISA/EIA's use photometric detection; and CLIA's use luminescence detection (IBL International, Hamburg, Germany). Other cortisol detection methods utilized in the studies were time-resolved fluoroimmunoassay (TR-FIA) used ~3% and an altered method of the Porter-Sibler chromogens test (~3% of studies). TR-FIA's are also based on binding competition, but detected via fluorescence. The Porter-Sibler chromogens test measures the side chain of cortisol metabolites using spectrophotometry. RIA's, ELSIA/EIA's, CLIA's are all similar methods for measuring serum cortisol and can produce comparable results in animals and humans (Singh et al., 1997; Suzuki, personal communication). TR-FIA's also produce comparable levels of free cortisol (Suzuki, personal communication).

Problems

Captive animals or wild caught animals often died during these studies due to complications from capture, refusal to eat, infections, or administration of hormones to mimic the stress response (Thomson & Geraci, 1986, St. Aubin & Geraci, 1989). In many studies wild animals were chased for hours and held in nets for several more hours and cortisol levels never exceeded a peak ~3 hours into the stressful activity even if the perceived stressor continued (Thomson & Geraci, 1986). The reason for that conclusion is not known but it is postulated that acute and chronic stress may produce different effects and although finding a measurable physiological stress response may be productive in certain situations (Dierauf, 1990), it may prove difficult to research the effects of long-term low level stress, which could be studied in an artificial captive environment.

Cetacean researchers are beginning to question the value of chase/capture/release techniques for establishing baseline cortisol levels (St. Aubin et al., 2013). Despite several decades of research that includes collection of cortisol levels in captive and wild cetaceans, no researchers to date have attempted to conduct a systematic review to compare cortisol levels across environments and species. These elevated cortisol levels often collected as a result of chase and restraint serve as a display of high-moderate stress in wild cetaceans; some that have been collected via non-invasive methods, serve as normal circulating cortisol levels. Although studies in the past, present, and future have and are being funded to analyze stress hormones in wild cetaceans, few if any, are

using fecal cortisol levels to establish wild baseline cortisol levels. To date studies on Southern Resident Killer Whales conducted by the University of Washington and NOAA are using fecal cortisol levels to assess stress in wild killer whales (conservationbiology.net). Another project funded by the Office of Naval Research in conjunction with NOAA and the University of Washington is collecting stress hormone data from a managed dolphin population to analyze season variation across multiple matrices, diurnal variation in hormone production, adrenocortical sensitivity, and thyroid challenges but not collecting any data from wild cetaceans (Houser et al., 2012). Analyzing individual animals cortisol levels can lead to many confounding variables such as age, sex, diurnal rhythm, seasonality, and reproductive status. It is important to reduce confounding factors when at all possible, by either only comparing mean cortisol levels or extrapolating data from longer studies where circadian rhythm and seasonality is taken info effect to produce the most accurate analysis.

CHAPTER 3

METHODOLOGY

Data Collection

Systematic reviews are beginning to emerge in ecology-based fields, including conservation management plans, due to their ability to provide a quantitative and qualitative assessment of published results (Roberts et al, 2006). Systematic reviews play an integral role by assembling data sets from different studies researching a static element and reporting if any trends are apparent. For this thesis, 27 methodological and reporting aspects have been derived to structure a successful systematic review. In this review 20 of those steps[2] are completed which follow the format for a functional systematic review of cortisol levels in three species of wild and captive cetaceans.

[2] Produce an explicit protocol, explain the background of the review, clearly state a question that the review was the address, clearly define the question elements, define search terms used to identify sources of evidence, document a detailed systematic literature search of sources, search for unpublished literature which is held by non-governmental organizations, governmental departments and/or charities, define inclusion/exclusion criteria for identification of relevant evidence, document reasons for inclusion/exclusion for each study, undertake an assessment of each studies quality/validity, provide standardized, documented and repeatable methods for how data was extracted from each of the studies accepted within the review synthesis, provide a formal assessment and estimation of the possible risk of publication bias of those studies accepted within the final synthesis of the review, provide a qualitative synthesis of the evidence in the text of the review and/or tabulated each accepted studies' findings, undertake a quantitative synthesis of the evidence according to the methods section, undertake a quantitative analysis of the evidence, investigate any sources of heterogeneity within the dataset, report key findings, identify an evidence gap/lack of data to properly answer the main question of the review, identify other evidence gaps or areas lacking knowledge in light of the review's findings, provide recommendations for future topics/questions that still require investigation, and advise on the methodology of future experiments.

This systematic review consists of data that exists entirely in publications and unpublished data from published studies. I conducted a text search using the terms: “cortisol cetacean”, “glucocorticoids cetacean”, “stress cetacean”, “cortisol Atlantic bottlenose dolphin”, “cortisol bottlenose dolphin”, “cortisol *Tursiops truncatus”*, “glucocorticoids Atlantic bottlenose dolphin”, “glucocorticoids bottlenose dolphin”, “glucocorticoids Tursiops truncatus”, “stress Atlantic bottlenose dolphin”, “stress bottlenose dolphin”, “stress *Tursiops truncatus”*, “cortisol killer whale”, “cortisol orca”, “cortisol *Orcinus orca”*, “glucocorticoids killer whale”, “glucocorticoids orca”, “glucocorticoids Orcinus orca”, “stress killer whale”, “stress orca”, “stress *Orcinus orca”*, “cortisol beluga whale”, “cortisol white whale”, “cortisol beluga”, “cortisol *Delphinapterus leucas”*, “glucocorticoids beluga whale”, “glucocorticoids white whale”, “glucocorticoids beluga”, “glucocorticoids *Delphinapterus leucas”*, “stress beluga whale”, “stress white whale”, “stress beluga”, “stress *Delphinapterus leucas”*. When an applicable article was of interest the data was collected in a separate spreadsheet, methods were noted, and authors were credited. I choose studies that exhibited similar methodology and validated assays were used for analysis. I researched out to all living authors in each study and received some unpublished data from them. Data that showed individual cortisol levels were analyzed to show mean cortisol levels to lower the impact of confounding variables. I attempted to obtain all information of possible confounding variables from all authors that were still living for possible analysis (See Appendices E, F, G, H).

I have not personally collect any new data. I am in contact with all known researchers in the field of cetacean stress hormone endocrinology and am constantly alerted to new results and publications as this progressed. If a viable amount of was left before the data had to be analyzed the new data was added into the systematic review. The data being analyzed are from peer-reviewed journals such as *Marine Mammal Science, Journal of Comparative Psychology, Brain Behavior and Immunity, Journal of Wildlife Diseases, Aquatic Mammals, Comparative Biophysiology and Chemistry, Canadian Journal of Fisheries and Aquatic Science, General and Comparative Endocrinology*, and *Nature*.

Cortisol levels were measured by validated radioimmunoassay (RIA), chemiluminescent immunoassay (ECLIA, ICLIA, CLEIA), and enzyme immunoassay methods (EIA/ELISA). Cortisol antibodies used in all three of these method exhibited comparable hormone-bind in human and animal samples (Singh et al., 1997). Mean cortisol levels and standard deviations for each group: captive *Tursiops truncatus*; wild *Tursiops truncatus*; captive *Orcinus orca*; wild *Orcinus orca*; captive *Delphinapterus leucas*; wild *Delphinapterus leucas* along with individual whole cortisol numbers from as many of the studies as possible. An animal/group of animals were considered to be "captive" if they were held in human care, whether it be in an open or closed containment, for ≥ 1 year. The unpublished data came from renowned researchers in the field of cetacean endocrinology that have either chosen to not publish cortisol levels in the studies they conducted or haven't published any results yet. Sampling methodology is

broken down into three categories: wild, non-husbandry, and husbandry (See Table 1).

Table 1: Sample Methods Abbreviation key. Assistance in understanding the sample methods column in "Basic Information" tables on Atlantic bottlenose dolphins, killer whales, and beluga whales.

Sample Methods	Definition
Chase, capture, restraint (C/C/R)	Usually a wild practice of chasing the animal with a vessel, netting them, hauling onto a deck or driving into shallow water to obtain a sample.
Involuntary non-husbandry (NH)	A captive practice that consists of driving the animal to shallow water, draining the pool, or hauling the animal out of water in to obtain a sample.
Voluntary husbandry (H)	A captive practice where the animal is taught to respond to a visual command by presenting a body part (usually caudal fin) to be sampled then is rewarded for the behavior.

Cetacean endocrinology is an important but relatively small field that began in the late 1970's. Specialization in stress hormone analysis has become recognized as an accurate way to assess stress levels in cetaceans and is increasingly seen in current research. Most studies that have been completed and/or published were from a core group of authors such as: Dr. David St. Aubin, Dr. Sammuel Wasser, Dr. Miwa Suzuki, Todd Schmitt DVM, Dr. Rudy Ortiz, and Dr. Tracy Romano. I have used their data with much appreciation and respect.

Conversion

To more accurately display the data obtained from past studies on cortisol levels in wild and captive Atlantic bottlenose dolphins, killer whales, and beluga whales I converted mean cortisol levels to the two most utilized units (one in Système International (SI) and the other in traditional unites), which were micrograms/deciliter (µg/dl and ug/dl) and nanomoles/liter (nmol/l). Out of the 25 studies, nine gave mean cortisol levels in nmol/l, ten in µg/dl, five in ng/ml, and one that measured FGC in ng/g. The only unit that could not be converted to µg/dl and nmol/l was the FGC study done on wild killer whales because FGC studies measure dried fecal matter for glucocorticoid metabolites not the parent hormone of cortisol. The conversions were done in Microsoft Excel for Mac 2011, version 14.3.1, last update-installed 14.3.1 (µg/dl to nmol/l and vice versa) or by an online medical calculator MedCalc 3000 (http://medcalc3000.com/Basic.htm) (ng/ml to µg/dl). To convert from nmol/l to µg/dl you need to obtain the molar mass of the hormone which is 362.46 g/mol to find the conversion factor which is 27.59 according to the Endocrinology Conversion Factors, Michigan State University, Diagnostic Center for Population & Animal Health www.dcpah.msu.edu/sections/endocrinology/WEBCD.ENDO.REF.002.pdf).

To convert mean cortisol levels from µg/dl to nmol/l you multiply the level (µg/dl) by 27.59 and get the level in nmol/l. To convert the mean cortisol level from nmol/l to µg/dl you divide the level (nmol/l) by 27.59 and get the level in µg/dl. For example if your mean cortisol level was 0.2 µg/dl and you wanted to

convert it to nmol/l you would multiply 0.2 x 27.59=5.51 nmol/l. Then to convert it back you take 5.51/27.59=0.2 µg/dl. I preformed these conversions in Microsoft excel and with a calculator to double-check for accuracy. To convert the mean cortisol levels with a unit of ng/ml to µg/dl I used the online medical calculator with the drop down box and selected ng/ml for the first box and µg/dl for the second box then typed in the level in the first box and the conversion for µg/dl appeared in the second box. The math behind this conversion was double checked by calculator with the knowledge of 1 µg=1000 ng and 1 dl=100 ml. Once the conversion from ng/ml to µg/dl was complete then the mean cortisol level was multiplied by 27.59 to obtain the level in nmol/l. These two unit will be displayed throughout this analysis and in all charts and tables to allow for fluid viewing, understanding, and access to data.

Statistical Analysis

Statistical analysis software programs were utilized on my personal MacBook Pro for analysis of the data collected from all studies mentioned below to assemble accurate mean cortisol levels for each species in a wild and captive environment along with comparisons between environments, between species, and in Atlantic bottlenose dolphin an analysis of trends in mean cortisol levels over time in captivity**.**

Microsoft Excel

Microsoft Excel for Mac 2011, version 14.2.5 was utilized for spreadsheets and descriptive statistics. Descriptive statistics including mean cortisol levels and standard deviation of the mean[3] were preformed by using the average, standard deviation. All functions were preformed in triple to account for human error.

Odontocete Physiology

Atlantic bottlenose dolphins, killer whales, and beluga whales are part of the suborder odontoceti, which means toothed whales. One of the most important features that members of odontoceti possess is a satellite dish shaped skull, which is thought to give it the ability to echolocate (Berta et al., 2006, p). Echolocation is a projection of sound that is directed by the melon to locate objects such as prey and impasses (Berta et al., 2006, p.). When it reaches an object it then bounces back and is collected through the lower jaw of the animal to produce a sonar image of the surroundings and distance to objects. They also possess a thick spinal cord to help with quick movements to capture prey (Berta et al., 2006).

Odontocetes display other important systems that have evolved to suite an aquatic lifestyle. These include respiratory, thermal regulation, diving

[3] Standard deviation of the mean was used in place of standard deviation in this analysis. It was derived from the standard deviation between the means and does not include all numbers obtained to compose the original mean. It is used to show variance between mean cortisol levels obtained in different studies.

adaptations, and advance renal systems. The main adaptation of the respiratory systems is the regression of the single blowhole to the forward ventral region (Berta et al., 2006). This allows breathing air at the surface without having to exert excess energy. Thermoregulation is important because bodies loose heat faster in water than in air. Blubber and countercurrent heat exchange are the most important ways that these animals thermoregulate (Berta et al., 2006). Cetaceans possess veins that run along arteries so blood from cooler areas such as flukes and fins warm up from traveling close to the warmed arterial blood so minimum heat is loss. The process can also be reversed by expanding arteries by increasing blood flow which causes the capillaries and veins along the surface of the skin to expand which allows some of the arterial blood to return along the peripheral veins which are close to the surface of the skin and the heat is lost through the skin (Berta et al., 2006).

Diving adaptations are essential for capturing prey and evading predators. Key components for diving include adaptations to pressure such as a flexible ribcage and lack of air sinuses in skull; adaptations to anoxia such as a greater capacity for oxygen storage and elevated hemoglobin in the blood and myoglobin in muscle, bradycardia, and peripheral vasoconstriction (Berta, et al., 2006).

Another important adaptation for living in a marine environment is the ability to osmoregulate. Cetaceans accomplish this by limiting salt intake and limiting water loss. Limiting salt intake is done by gaining fresh water from prey tissues along with metabolic water that is released when fats or carbohydrates are broken down from digestion. Limiting water loss is done by a highly efficient

reniculate kidney, which produces hyperosmotic urine, and an inability to perspire (Berta et al., 2006).

The combination of metabolic and anatomic adaptations cetaceans possess for living in an aquatic salt-water environment and similarities between stress physiology with terrestrial animals may give cetaceans a unique advantage (Schmitt et al., 2010). These adaptations allow enhanced escape strategies along with sensory advantages that come with the imposed stress of breathing air while residing in a marine environment. The culmination of these marine adaptations may allow the animal to better adapt or cope with the perception and physiologic response to a perceived stressor (Fair & Becker, 2000).

Stress in the Wild

Monitoring stress hormones in wild cetaceans allows scientists to gather reference points from healthy and unhealthy populations. Anthropogenic and environmental stressors including pollutants, disease, and habitat degradation due to anthropogenic activities and global climate change are increasingly impacting cetacean populations (St. Aubin et al., 2001). The frequency of mass mortality events including strandings has aided in need to search for answers. Current parameters such as capture and release sampling may not accurately depict baseline levels of stress hormones due to invasive methodology (St. Aubin et al., 1996). Baseline stress hormone levels are needed for wild cetaceans so as anthropogenic and environmental threats continue to increase impacts on stress

levels can be documented. This is especially important in animals like beluga whales that inhabit a narrow habitat range that is more drastically impacted by environmental impacts such as global climate change (Schmitt et al., 2010). Although adaptations such as strong social networks, that are often observed in cetaceans, may lessen the impacts of stressors, the increasing appearance of noise, fisheries, diseases, oil spills, harmful algal blooms, chemical contaminants, and habitat change will continue to increase the burden (Fair & Becker, 2000). Reference points of stress hormones are needed to assess the health of the animals and may assist in the development of better conservation techniques aimed to lowering anthropogenic and environmental stressors (Tryland et al., 2006).

Some studies such as St. Aubin (1996) claim that wild sample collection methodology that included surround, capture, and release tactics did not elicit signs of distress in the animals being sampled. Cortisol levels in these studies may be subjected to inaccuracy of baseline numbers due to the invasive nature of sample collection and the onset of the stress response and cortisol increases within the first ten minutes of exposure to a stressor (Orlov et al., 1991). Even though cortisol levels are not known to peak until 1-2 hours after confronted by a stressor (Thomson & Geraci, 1986) and some studies have enacted sample collection in less than one hour from chase (Ortiz & Worthy, 2010) the impacts that these chase, capture, release studies may have on wild populations when cortisol levels do rise is unknown.

Stress in Captivity

Stress plays a role in the overall health of animals living in both a wild and captive environment. Cetaceans are often kept in a captive environment due to their popularity to the general public (Noda et al, 2006). It is important for the staff at marine mammal venues to monitor the animal's health, including exposure to stressors, and continually improves captive conditions to minimize stress (Noda et al., 2006). This stress management is an essential element of captive care (Waples & Gales, 2006). Captive animals have increased demands put on them, because they are compelled to adapt to the new environment (Orlov et al., 1988). Captive cetaceans face a different set of stressors than their wild counterparts, which has lead to recognizable health problems (St. Aubin & Dierauf, 2001). A captive environment usually includes environmental changes such as transportation and introduction of novel stimuli, which has been reported to increase cortisol levels in bottlenose dolphins and beluga whales (Spoon & Romano, 2012, Copland & Needham, 1992, Noda et al., 2006). According to Waples & Gales (2002) stressors in a captive environmental can include social factors such as changes in group dynamics, competition over resources, unstable dominance hierarchies; and physical factors such as changes in food quality, reduced stamina, confinement, and forced human interaction (Fair & Becker, 2000). Due to the logistical issues with obtaining blood samples from free ranging

cetaceans, much of what we know about stress hormones in cetaceans come from captive studies (Medway et al., 1970, Orlov et al., 1988;1991).

It has been proposed that a combination of both behavioral observation and stress hormone analysis be imposed to more accurately detect stress in captive cetaceans, although no statistically significant relationships have emerged (Ortiz & Worthy, 2000; St. Aubin & Dierauf, 2001; St. Aubin et al., 1996; Thomson & Geraci, 1986; Waples & Gales, 2002; Curry, 1999). There is good reason to postulate that behavioral observations can be implemented to assess cetacean well-being (Esch et al., 2009). Behavioral signs of acute stress in dolphins and belugas have been identified as loss of appetite, social instability, and vocalizations along with changes in respiration and dive times (Wapes & Gales, 2002, Castellote & Fossa, 2006). In wild cetaceans bursts of energy and lethargy have been noted in acutely stressed dolphins (Curry, 1999). It is often easier to detect signs of acute stress in captive animals due to their smaller range and dependence on caretakers. Problems associated with continuous stress hormone monitoring of captive cetaceans include the invasive act of blood sample collection that can lead to infection at collection site and depletion of blood supply.

Chronic stress can lead to decreased fitness, physiological problems, along with reproductive and immune suppression (St. Aubin & Dierauf, 2001). Being able to reconginize the signs and symptoms of chronic stress is of importance to captive facilities. Behavioral cues of dolphins exhibiting chronic stress are thought to be associated with changes in behavior such as lethargy and withdrawn

tendencies, along with a decrease in appetite (Thomson & Geraci, 1986; St. Aubin & Dierauf, 2001). Internal signs of chronic stress include gastric ulcers, compromised immune system, and reproductive problems (St. Aubin & Dierauf, 2001; Waples & Gates, 2006). Different species may adapt to stressors in captivity in different ways. Orlov et al. (1991) proposed that animals that have a larger range of habitat and diet, such as beluga whales, might have increased tolerance to stressful conditions when compared to other cetaceans. Behavioral observation along with adaptation is important to include when evaluating stress in cetaceans. Combining those parameters with hematology which can show signs of stress including decreased lymphocytes and eosinophil's, increases in neutrophils, and elevated glucocorticoids (Thomson and Geraci, 1986, St. Aubin & Dierauf, 2001) will allow for an accurate analysis of stress in captive cetaceans. The importance of monitoring both hematological and behavior parameters to evaluate stress in both captive and wild cetaceans is of the upmost importance.

Social parameters are being recognized as playing a growing role in stressors faced by captive cetaceans. The often manipulated and artificial social structures presented to captive cetaceans have the ability to cause several forms of social stressors in these highly social animals. Social stressors include threats, changes in relationship, dominance, and competition. In captive killer whales, a smaller male, has displayed higher cortisol levels that don't reflect seasonality that has been displayed in the other animals in his enclosure (Suzuki et al., 1998; 2003). The inability to escape a dominant individual, which, may not exist in a wild environment, can lead to chronic stress and even death. Currently, a young

killer whale captured in the Netherlands was moved to a captive facility in Spain. Due to the artificial and unstable social hierarchy at this facility the young animal is exposed to near constant harassment and raking from the other animals (Visser, personal communication). This type of social problem has not been reported as observed in wild killer whales (Visser, personal communication). Sweeney 1990, notes that captive dolphins may experience social stress due to artificial groupings, social changes, and subordination; if true these behaviors should be constantly monitored in a captive environment to minimize the possibility of immunosuppression or even death.

Due to the numerous social problems being reported in captive cetaceans behavioral monitoring is an important component to evaluate stress in captive cetaceans but continuous monitoring of stress hormones may play a large role in increasing quality and quantity of life in these animals. Many cetaceans are thought to display a defense mechanism, where they masks signs of illness until it is in relatively advanced stages when symptoms such as loss of appetite and lethargy may be detected (Waples & Gates, 2006). Because of this aforementioned defense mechanism it may be important to monitor behavioral cues in a captive environment to assess stress in animals along with routine cortisol analysis that could be completed by noninvasive fecal gathering.

Atlantic bottlenose dolphin (Tursiops truncatus)

The Atlantic bottlenose dolphin, otherwise known as coastal bottlenose dolphin, or Atlantic bottlenose dolphin (*Tursiops truncatus*) is most likely the most well-known and most studied cetacean. They are found in warm-temperate to tropical seas and are widely distributed where surface temperatures range from 10°C-32°C (Wells & Scott, 2008). Their small-medium size and ability to adapt to human presence has made them a popular subject in wild and captive research studies. In the wild they live in pods of 2 to 15 individuals, but can congregate with up to 1000 depending upon location (Wells & Scott, 2008). Females often stay in loose family groups, nursery groups, or mixed sex juvenile groups and adult males often wonder on their own or as strongly bonded pairs. They prefer coastal environments putting them in constant contact with human civilization and numerous anthropogenic stressors, although an offshore ecotype has recently been identified. Anthropogenic activities that lead to pollution of their environment lead to bioaccumulation of toxins in the animals. In some countries such as Japan, Peru, Sri Lanka, and the Faroe Islands Atlantic bottlenose dolphins are caught for aquaria and hunted for food (Wells & Scott, 2008).

Atlantic bottlenose dolphins were first publicly displayed at the Brighton Aquarium in 1883, then at the New York Aquarium in 1914. They are the most prevalent cetacean in captivity with over 3000 living in aquaria worldwide (Corkeron, 2008). In the United States about 70% of Atlantic bottlenose dolphins are used for public display where the rest are used for military or research

opportunities. They have more success breeding in captivity than the other two species being studied in this analysis. Their size and intelligence makes them competent performers in many forms of entertainment such as shows at aquaria, movies, TV series, and interactive programs such as "swim-with" experiences and touch pools. Atlantic bottlenose dolphins are also often participants in "Dolphin Assisted Therapy" (DAT) where they interact with people who have disabilities. Although no research has been completed that conclusively proves the effectiveness of such programs. Anthropogenic stressors are prevalent in captivity due to confinement and dependence (Corkeron, 2008).

Biology

Atlantic bottlenose dolphins are in the family Delphinidae, which also includes killer whales (*Orcinus orca*). They range in size from 2.5-3.8 meters and are distributed throughout the world's oceans (Wells & Scott, 2008). They have a generalized appearance of a medium sized robust body, falcate dorsal fin, and gray coloration with a rounded, elongated rostrum. They exhibit a highly social behavior and have signature whistles and clicks that identify individuals and pods. They can hunt individually or cooperatively and methods vary depending upon location and prey source. They are generalists or specialists by region that commonly prey on squid and fish, demonstrating a preference for sciaenids, scombrids, and mugilids (Wells & Scott, 2008). They are typically brief divers usually surfacing twice a minute but can hold their breath around 8 minutes on average. Juveniles may remain with their mother for up to six years, which

exhibits a high maternal investment. Their main predators are sharks, killer whales, and humans. Maximum lifespans are ~60 for females and ~50 for males (Wells & Scott, 2008).

Status

The International Union for Conservation of Nature lists Atlantic bottlenose dolphins as "least concern" with around 600,000 thought to be living in the wild.

Wild Studies Examining Cortisol levels

Obtaining cortisol samples from wild Atlantic bottlenose dolphins often includes a chase, capture, and restraint to collect a blood/serum sample (St. Aubin, 2001). This methodology makes it challenging for the researcher to interpret if the act of obtaining a cortisol samples may produce misleading information due to the invasive act of collection (St. Aubin, 2001). The animals are often restrained in nets and/or hauled up on boats to obtain a blood/serum sample, thus stimulating the stress response prior to collection. Many studies use wild serum cortisol samples to simulate a medium-high stress response rather than a baseline (St. Aubin et al, 2001). (See Table 2)

Table 2: Atlantic Bottlenose Dolphin Cortisol Studies, Basic information. Species, Author (s) of study mean cortisol levels were obtained from, whether the study was conducted in a wild or captive environment (black wild/red captive), sample type, mean cortisol in nmol/L and µg/dl, number of animals sampled in each study, number of samples analyzed to determine mean cortisol levels in each study, range of individual cortisol levels in nmol/L and µg/dl, sample method, and assay used for measurement of cortisol levels.

Atlantic Bottlenose Dolphin	**Wild**: Captive	**Mean F**	**Mean F**	**# Animal**	**# Sample**	**Range**	**Range.**	**Sample**	**Analysis**
Author (s)	Sample type	nmol/L	µg/dl	N	N	nmol/l	µg/dl	Method	Assay
Thomson & Geraci, 1986	serum	100	3.6	38	38			C/C/R	RIA
St. Aubin et al., 1996	serum	71.7	2.6	36	36	33.1-113.1	1.2-4.1	C/C/R	RIA
Ortiz & Worthy, 2000	plasma	77.3	2.8	31	31	27.6-154.5	1.0-5.6	C/C/R	RIA
Medway et al., 1970	plasma	66.2	2.4	8	8	46.9-82.77	1.7-3.0	NH	Silber-Porter
Thomson & Geraci, 1986	serum	35	1.3	2	2	30-120	1.09-4.35	NH	RIA
Orlov et al., 1988	serum	90.34	3.3	40	40			NH	RIA
Orlov et al., 1991	serum	90.3	3.3	18	18			NH	RIA
Copland & Needham, 1992	plasma	90	3.3	6	6			NH	RIA
St. Aubin et al., 1996	serum	52.4	1.9	36	36	13.8-110.36	0.5-4.0	H	RIA
Suzuki et al., 1998	serum	10.5	0.38	2	10	5.5-22.1	0.2-0.8	H	RIA
Reidarson & McBain, 1999	serum	63.5	2.3	6	6	35.87-91	1.3-3.3	H	ICMA
Reidarson & McBain, 1999	serum	69	2.5	2	6	49.7-85.5	1.8-3.1	H	ICMA
Noda et al., 2006	plasma	160	5.8	5	5			NH	ECLIA
Ridgeway et al., 2006	serum	14.3	0.52	1	4	9.9-18.2	0.36-0.66	H	RIA
Pedernera-Romano et al., 2006	serum	19.7	0.7	7	7	6.59-64.46	0.24-2.34	H	RIA
Naka et al., 2007	plasma	10.2	0.37	5	5			H	EIA
Ridgeway et al., 2009	serum	44.1	1.6	1	6			H	RIA
Ortiz et al., 2010	plasma	u.d.	u.d.	2	2			H	RIA
Houser et al., 2011	serum	15.49	0.56	2	9	u.d.-39.4	u.d.-1.43	H	RIA

Blasio et al., 2012	serum	15.5	0.56	6	14	11.9-20.4	0.43-0.74	H	RIA
Blasio et al., 2012	serum	16.3	0.59	4	6	13.2-20.4	0.48-0.74	H	RIA
Suzuki & Komaba,, 2012 (UP)	serum	35.87	1.3	3	77	8.27-110.36	0.3-4.0	H	TR-FIA

Ortiz & Worthy, 2000

31 wild Tursiops truncatus (17 M, 14 F) were caught off the coast of Beaufort, North Carolina and monitored for plasma adrenal steroids (cortisol, aldosterone, adrenocorticotropin) and arginine vasopressin (AVP). AVP is neurohypophysial hormone that is derived from a preprohormone that is synthesized in the hypothalamus. Its primary function in mammals is to retain water and constrict blood vessels. It plays an active role in homeostasis because of its regulation of water, glucose, and salts in the blood. It was analyzed in this study to see if correlations existed between AVP and any of the adrenal steroids. Samples were taken within 40 minutes from the fluke vein and were analyzed via a commercially available RIA kit, DPC, L.A, CA. Cortisol had significant positive correlations with corticosterone; and showed a correlation that was not statistically significant with aldosterone. A significant correlation between cortisol and AVP was not observed.

Cortisol levels ranged form 1.0-5.6 µg/dl, and showed no differences in samples taken within or above 20 and 36 minutes. This may be due to the

observation of cortisol levels peaking between 1-3 hours in cetaceans (St. Aubin et al., 2001). The majority of the animals in this study were said to have not experienced a physiological stress response within the 40 minutes of capture and sampling. It is important to keep in mind that each animal differs in their stress response so it may be illogical to conclude that all free-ranging bottlenose dolphins do not experience an acute stress response within 40 minutes of sampling and cortisol levels are not biased when invasive sampling methods are present. Time of year was not indicated so seasonality may also play a part in the cortisol levels, typically spring/summer months contribute to lower cortisol levels than fall/winter months (Orlov et al., 1988).

Wild/Captive Studies Examining Cortisol Levels

A compilation of studies that researched cortisol levels in both wild and captive populations of Atlantic bottlenose dolphins.

St. Aubin et al., 1996

Adrenal hormone analysis was completed on 36 wild and 36 "semi-domesticated" captive Atlantic bottlenose dolphins. The captive animals were part of a Naval Program from two states and included 18 males and 18 females between the ages of 4-33. The percentage of wild caught/captive born animals was not clear. Blood samples were collected via voluntary behavior from their tail flukes irregularly between February 1988 and March 1992. Assaying was

performed using a commercially available RIA kit, NCS Diagnostics, Willowdale, Ontario. Serum cortisol levels ranged from 0.5-4.0 mg/dl. These levels were deemed close to a baseline cortisol sample for captive bottlenose dolphins due to the process of voluntary collection and amount of time kept in captivity.

For comparison, 36 (18M;18F) wild caught Atlantic bottlenose dolphins were captured, sampled, then released in Sarasota Bay, FL as part of the Sarasota Dolphin Research Program between June 1988 and July 1990. 14 of the samples were taken in winter months; 22 in summer months to account for seasonal variability. The capture process was conducted by encircling the animals with nets then bringing them onboard a vessel for examination and sample collection. Times between the capture process and blood collection ranged between 23-260 minutes, with a mean of 80 minutes. Assaying was performed using a commercially available RIA kit, NCS Diagnostics, Willowdale, Ontario. Serum cortisol levels ranged from 1.2-4.1 mg/dl and were slightly elevated in females. These cortisol levels were similar to ranges observed in other wild bottlenose dolphins captured from sampling (Thomson & Geraci, 1986).

Age or season did not seem to have an effect on cortisol levels. Adrenal hormones showed the greatest variance among wild and "semi-domesticated" captive animals, with the "semi-domesticated" animals displaying lower levels of cortisol, although distress was not behaviorally observed. The increase of adrenal hormones in the wild dolphins suggests a mild stress response. Wild animals sampled within an hour of capture showed similar levels of cortisol as the captive animals. The similar levels of cortisol between both groups of dolphins may be

have occurred because cortisol levels may not have reached their peak thought to occur between 1-2 hours or because the "semi-domesticated" animals were also exhibiting a mild stress response.

Thomson & Geraci, 1986

Ten captive bottlenose dolphins between the ages of 4-15 were sampled in this study to test for stress associated with capture and handling. In June 1983 the first 3 captive bottlenose dolphins kept in a outdoor sea pen, and all females around 4 years old. Each animal was captured and sampled two different ways, samples were collected from the flukes or dorsal fin during both capture methods. Calm-capture, where the animal was corralled in its sea pen and hoisted onto a stretcher within 10 minutes and then moved to a carrier and kept wet for up to 6 hours; and chase-capture where the animal was chased for 3 hours prior to being handled the same as mentioned above. Each of these techniques occurred on alternating days. Blood samples were taken before the animal was removed from the water (10 minutes after capture), after it was in a carrier (15 minutes after removal from the water), along with at 30 minutes, 1, 3, and 6 hours after being kept in the carrier, and once before the animal was released back into the water. Cortisol levels for each experiment were analyzed by RIA, New England Nuclear, Boston, MA. Serum cortisol levels were 30 and 40 nmol/L in the first samples collected after calm-capture, but were elevated to 80-120 nmol/L after the first hour they were removed from water where the stayed for the remainder of the 6

hours. This suggests that even calm-capture evokes the stress response in bottlenose dolphins. After chase-capture initial cortisol levels were 60 and 80 nmol/L and evened out in the range of 60-100 nmol/L for the next six hours.

4 days after the conclusion of the capture study 1 of the dolphins refused to eat for 3 days. The other two animals were used in another study to test the effects of synthetic ACTH on adrenal hormone production. For this study the animals were captured within 20 minutes and given 50 IU of Cortrosyn with blood samples collected at 1, 1.5, 3, 3.5, 4 and 5 h after administration of the drug. Cortisol levels were 60-100 nmol/L after Cortrosyn injection, which is simulated ACTH although it is unclear whether cortisol levels rose because of arousal or Cortrosyn injection. One of the animals in this study died within 48 hours of injection and the necropsy showed congested adrenal glands.

In October of that same year seven other captive dolphins between the ages of 8 and 15 were used to compare the effects of synthetic ACTH to the first three animals. Using a similar protocol except for elongated capture and restraint times (~4 hours total) 3 of the animals were given 50 IU of Cortrosyn, the other 4 were given the same amount of ACTHar. The animals showed similar patterns of mildly elevated cortisol levels after injections but it is also unknown whether capture and handling or injection played a role in cortisol concentrations. A dolphin in this study died within 5 days of injection with ACTHar and showed signs of adrenal congestion.

Spanning the entire time of the experiments blood samples from 38 wild bottlenose dolphins were also collected. The animals were chased then held in a net for up to 5 hours while samples could be collected before the were lifted onto a platform for blood collection. Mean serum cortisol level of the wild dolphins was 100 nmol/L, which was similar to captive dolphins after chase-capture and synthetic ACTH injection, which suggests a mild stress response due to sample collection methods. Although additional synesthetic ACTH injection after capture and handling did not rise cortisol above peak levels displayed after capture and handling, due to the deaths of 2/9 dolphins given the synthetic hormone it may be dangerous to elicit a stress response by stimulating the adrenal cortex in already stressed bottlenose dolphins.

Captive Studies Examining Cortisol Levels

Captive Atlantic bottlenose dolphin serum cortisol levels have been estimated to vary between 0.6-3.6 μg/dl among different ages and sexes (Thompson & Geraci, 1986; Orlov et al., 1988; St. Aubin et al., 1996; Suzuki et al., 1998; Ortiz & Worthy, 2000). When the animals are adjusted to a captive environment and are taught common husbandry practices, such as displaying the fluke vein, blood/serum collection can be completed within minutes, and the results most likely produce the most accurate baseline for cortisol levels (St. Aubin et al., 2013) within this species. Captive studies have show that age and season does not play a role in cortisol production in semi-domesticated Atlantic bottlenose dolphins (St. Aubin et al. 1996). Research has shown that Atlantic

bottlenose dolphin (*Tursiops truncatus*) typically exhibit lower cortisol levels than Indo-Pacific bottlenose dolphins (*Tursiops aduncas*) (Suzuki, personal communication). (See Table 2)

Orlov et al., 1988

In a study conducted by Orlov et. al., in 1988 40 wild bottlenose dolphins were sampled during "normative" conditions. A standard RIA kit was used for analysis. A serum cortisol range of 4-9 nM were displayed after capture when exposed to experimental three and six hour stressors to discover how well they adapt to stressors. The animals were given a tranquilizer prior to capture to decrease the acute stress response. This may skew control cortisol levels by affecting normal free ranging cortisol levels. The results concluded that due to shifts of adaptive hormones that are active in the acute and chronic stress response (cortisol, insulin, triiodothyroine, and thyroxin) the hormonal changes of the stress response in these animals reacted in a similar way as terrestrial mammals.

Animals were captured and moved to a marine base in the former USSR. The experimental stress occurred through a process of lifting the animals out of a pen and placing them in a seawater-filed bath for 3 and 6-hour periods. During the three-hour experimental stress, blood was taken from the "tail vein" at 15, 30, 45, and 60 minute intervals; during the six hour experimental stressor samples were obtained by the same methodology at 1, 3, and 6 hour intervals. During the study on days 1,3-4 razing the animals out of the pen and into the bath exhibited higher cortisol levels than the actual immobilization in the bath did. Increases in

cortisol occurred within one hour of raising the animals into the bath, then lead to relatively stable levels during immobilization (3 and 6 hours) until transport back to the pens. These stable levels of mean cortisol may reflect a "recovery period". Mean cortisol levels peaked at 24 hours, then decreased from day 3-4 to day 7, although the levels never reached the established "norm" during those periods. Possible issues with data include the knowledge of bottlenose dolphins exhibiting seasonality in cortisol levels displaying high levels in fall/winter than spring/summer months. This may be due to an evolutionary process, the increase of unfavorable environmental factors occurring in fall/winter months. An interesting note is that the author describes one of the animals sampled during the experimental stress as "aggressive" which is rare, and two as docile.

Orlov et al., 1991

Eighteen captivity adapted bottlenose dolphins that were kept at the Institute of Evolutionary Morphology and Animal Ecology, USSR, were sampled for this study. Blood was taken from the fluke vein and analyzed by a RIA kit produced by Sorin, France. Seasonal variation trends were recorded similar to Orlov 1988, displaying higher serum cortisol levels in winter/spring and lower levels in summer/fall. Ion concentrations differed more dramatically in the bottlenose dolphins than in beluga whales that were also being sampled. Detailed methodology was absent on the bottlenose dolphin sampling times and other parameters because this study was mostly focused on beluga whales and

translated. It is probable that methodology was similar to the protocol in Orlov et al., 1988. Mean cortisol levels for the animals were identical to the results for Orlov et al., 1988 but were similar to other studies on wild bottlenose dolphins, not captivity adapted animals.

Blasio et al., 2012

Ten (6 F, 4 M, 3 born in captivity, 7 wild caught) captive Atlantic bottlenose dolphins were sampled for serum cortisol levels in open (n=6) and closed (n=4) facilities. 20 samples (14, open; 6 closed) were taken from the caudal vein during routine voluntary husbandry practices. Assessment was preformed using a solid-phase RIA kit, Cort CT2 Bio International, France. Cortisol levels ranged from 0.43-0.74 μg/dl in open faculties and 0.48-0.74 μg/dl in closed facilities, these levels fall in the range of cortisol levels from other captive studies, which is the relatively large range of 0.4-3.6 μg/dl (Ortiz & Worthy, 2000, St. Aubin et al., 1996, Suzuki et al., 1998). Confounding factors include health, age, gender, and season. No statistically significant differences were found in in cortisol levels between animals living in open and closed captive environments. Although behavior differences such as more time spent resting in closed captive environments were observed.

Copland & Needham, 1992

Six captive dolphins (4M;2F) were moved by air from one facility to another in Australia. One dolphin was 6 months another was 4 years, the remaining 4 were ≥10 years. The animals were last fed over 24 hours before transport. At 5 AM on the date of transport the adult animals were given 3 mg/kg of diazepam to reduce travel anxiety. Serum samples were taken ~4 hours after the diazepam was administered, ~1.5 hours after capture, and ~6.5 hours before the second samples. Plasma cortisol was measured by Ameriex Cortisol RIA kit, code IM. 2021, Amersham Pty. Ltd., North Ryde, New South Wales 2113, Australia. Serum cortisol rose during transport and other classic stress indicators of the leukogram such as lymphopaenia (low lymphocytes) and eosinopaenia (low eosinophil granulocyte) observed. The results show that even when given an anti-anxiety drug these dolphins still exhibited a stress response during transport.

Houser et al., 2011

Four (3M;1F) adult Atlantic bottlenose dolphins (2 control; 2 experimental) were housed individually in above groups pools for a 10 day period and sampled for cortisol and aldosterone. The control animals were housed in ambient water temperature, where the experimental animals were exposed to decreased water temperatures in the range of 4.2-16.6°C. Blood serum samples were collected every 2-3 days between 0745-1000 hours by voluntary husbandry behavior, tail fluke presentation. Samples were analyzed via RIA kit TKCO1, Siemens

Healthcare Diagnostics, Deerfield, Illinois 60015-0778, USA. Cortisol levels were elevated in the experimental animals that were exposed to declining temperatures, although cortisol levels were not as high as observed in capture and handling studies. These results may demonstrate that bottlenose dolphins exhibit a more intense stress response to capture and handing than to lowering water temperatures. It is interesting to note that one control animal had cortisol levels below detection in some samples, and the other control animal displayed a higher cortisol level on day 4 in the pool than both of the experimental animals. This demonstrates the variability in recognition of a stressor between different animals of the same species. The study was preformed to see how thermal stress affected cortisol and aldosterone production.

Naka et al., 2007

Blood samples were obtained voluntarily from the fluke vein of 5 captive bottlenose dolphins (age 4-22) kept at Kamogawa Sea World, Chiba, Japan. The animals were healthy and on no medications at the time of sampling. Samples were collected when the animals were in a natural "floating" position, and then 10 minutes after the pool was drained in a "landed" position. Plasma cortisol was measured using a Cortisol EIA kit, Cayman Chemicals, Ann Arbor, USA. Mean cortisol concentrations rose from 3.7 ng/ml in the floating position to 10.6 ng/ml in the landed position, although the increase was not statistically significant. Heart rate was recorded in three of the animals in floating and landed position and displayed a higher but not statistically significant value in landed animals.

Plasma cortisol levels of the captive animals in this study fall within the range of known levels assessed in wild members of the same species (St. Aubin et al., 1996). Mean cortisol levels expressed in wild Atlantic bottlenose dolphins could be exhibiting a low-moderate stress response due to the chase and capture methodology for sample collection (Ortiz & Worthy, 2000).

Noda et al., 2006

Ten captive female bottlenose dolphins weighing between 200-250 kg were utilized in this study. All were wild caught in waters surrounding Japan and kept at a captive facility for at least five years. Before being transported to another facility 6 hours away, 6 of the animals treated with a total of 40 mg/kg of bovine lactoferrin/day for seven days. Lactoferrin a glycoprotein secreted in bodily fluids. It is known for its ability to contribute positively to the immune system and protect animals from pathogens by increasing the activity of neutrophils. The five control animals were not treated. All animals were deemed healthy and sexually mature. Blood serum samples were taken before transport after the pool was drained and the animals were loaded into transport units. After 6 hours of transport another sample was taken upon arrival at the receiving facility. Samples were taken from superficial vessel on the ventral side of the fluke. Analyses of cortisol levels were determined by electrochemiluminescence immunoassay using rabbit antibody.

Before transport mean serum cortisol levels were higher than referenced cortisol values, but showed no significant variation between groups. After transport the treated group showed decreased levels of mean serum cortisol while the untreated groups levels remained the static. The animals in this study displayed higher levels of cortisol than members of the same species sampled in a captive environment (St. Aubin et al., 2001) before transport. This could be due to the amount of time taken to drain the pools (~3 hours) before the first blood sample was taken. These same animals exhibited lower levels of mean serum cortisol before the experiment, making it difficult to separate the stress of draining the pool vs. the stress of the actual transport. After transport the group treated with lacoferrin displayed a reduced levels of mean serum cortisol, which may indicate that bovine lactoferrin, may reduce cortisol levels in bottlenose dolphins during transport.

Ortiz et al., 2010

Two captive male bottlenose dolphins we sampled voluntarily from their caudal flukes for blood serum. Two samples were obtained over 2 days while the animals were eating regularly (5 kg/d). The next samples were taken during a fasting period of 38 hours at 14, 24, and 38 hours. After the 38 hour fast dolphins were fed their normal diet for 24 hours where samples were collected at 5, 12, and 24 hours. Cortisol was measured by a commercial radioimmunoassay, DPC, LA, CA, USA. Plasma cortisol and aldosterone levels were undetectable or below sensitivity for the assay used in this experiment. This would make the levels lower than commonly seen in other captive and wild members of the same species

(Thomson & Geraci 1986; St. Aubin et al., 1996; Ortiz and Worthy 2000, Noda et al. 2006, Naka et al., 2007, Suzuki et al., 1998).

The seemingly lack of circulating cortisol in these two animals could be due to reduced activity and age or adrenal atrophy, which occurs when the adrenal hormones are chronically used to the point of atrophy. Fasting has been known to cause an increase of circulating cortisol in seals (Ortiz et al., 2001;2003), although documentation has never been published on cetaceans. The results of this study may be used to indicate the possibility of adrenal atrophy in captive bottlenose dolphins exposed to chronic stressors; display that the small levels of glucocorticoids present in many samples from bottlenose dolphins may indicate that a low stress response is occurring due to detection; or arise from a technical mistake during the sample collection or analysis protocol.

Reidarson & McBain, 1999

Two male captive dolphins, ages 10 and 13, were given a single oral dose of dexamethasone at .11 mg/kg. Dexamethasone is a corticosteroid that is clinically used when adrenal hormone production is low. It is used to treat inflammation and allergies in humans (http://www.nlm.nih.gov). Blood samples were taken voluntarily from the fluke vein immediately post-dose, then 1, 1.5, 2, 7, and 17 days thereafter. Analysis was conducted by immunochemiluminescence assay, Chiron Diagnostics, East Walpole, Massachusetts 02032, USA. Within 24

hours they animals expressed lympohopenia and neutrophilia, usually characteristic of a stress leukogram. Cortisol levels decreased from between 2-3 μg/dl at administration to undetectable levels after 24 hours of administration and returned to detectable levels within 48 hours. The base cortisol levels collected at administration of the medication reflect levels collected from a control group of six bottlenose dolphins sampled similarly without the administration of a medication (1.3-3.3 mg/dl). Cortisol levels may have decreased with administration of dexamethasone due to its ability to suppress the release of ACTH. It is important to note that dexamethasone is a known anti-inflammatory agent and immunosuppressant.

Ridgeway et al., 2006

Five 120-h (5 day) dolphin vigilance session were conducted between July and February. Blood samples were taken immediately prior to and after 120-h vigilance sessions from voluntary fluke presentation, a common husbandry behavior. Analysis conducted with a commercially available RIA kit, Diagnostic Products Corporation, L.A., CA) validated by Romano et al. (Romano et al., 2004). Cortisol levels were within normal ranges (6.6-3.6 $\mu g 1^{-1}$) (St. Aubin et al., 1996) and no changes were observed before and after the experimental sessions. These results may display that cortisol along with other hormones associated with the stress response such as epinephrine and norepinephrine do not fluctuate during sleep deprivation, which was supposed to be simulated by continuous vigilance

over 5 day periods. The detection rate for these two animals was 87-99%. These tests were conducted between July and February, a seasonal variation in cortisol levels have been shown in bottlenose dolphins displaying lower cortisol levels in summer/fall months and higher levels in spring and summer months (Orlov et al., 1988). Since cortisol levels did not rise pre and post study it could be hypothesized that uni-hemispherical sleep patterns occur in bottlenose dolphins, which allows auditory vigilance without showing adverse reactions of sleep deprivation. It should also be noted in this study that the fish "rewards" for correctly pressing the paddle to identify the 1.5-s sound were part of the daily diet, which may have been motivation for the animals to stay vigilant during nocturnal hours where respiration rates and reaction rates were reportedly slower.

Ridgeway et al., 2009

Two captive bottlenose dolphins (1M;1F) were tested for auditory vigilance in three 72 and four 120 h experiments. In the experiments the animals were trained to press a paddle when a 1.5 s goal tone at 70kHz was played throughout at varying times when a 0.5 s tone was more constant. Upon pressing the paddle for the correct tone the animal received a fish "reward" which was actually part of the standard 24-hour diet. Serum cortisol levels were collected from the 26-year-old female dolphin immediately prior to and post one 120h vigilance session. Blood/serum samples were taken from the fluke vein during voluntary presentation and analysis was conducted with a commercially available

RIA kit, Diagnostic Products Corporation, L.A., CA) validated by Romano et al. (Romano et al., 2004).

Cortisol levels were normal at 1.2 μg/dl^{-1} pre and 2.0 μg/dl^{-1} post 120h session (St. Aubin et al., 1996). The results corroborate a similar study conducted by the same primary author conducted in 2006. Displaying results that captive bottlenose dolphins may not show classic signs of sleep deprivation, including increased cortisol levels, after 120h near constant vigilance sessions due to uni-hemispherical sleep. As noted in the pervious study seasonality, as well as the fact that the food "reward" that was part of the normal diet of the animal may have played a part in the vigilance due to hunger. In non-experimental conditions captive dolphins are rarely fed a portion of their diet in the nocturnal hours, due to displaying characteristics of a diurnal species.

Suzuki et al., 1998

Two bottlenose dolphins (1M;1F) that were wild caught but held in captivity for at least 13 years were sampled twice a month from September to December via voluntary tail fluke presentation. Samples were collected in the AM hours. The total range of mean serum cortisol levels between both animals was 1.6-6.5 ng/ml with the female showing slightly higher levels. These two animals displayed high levels of cortisol upon arrival at the facility, but has since decreased and stabilized. During this study an RIA system for serum cortisol measurement specifically in bottlenose dolphins and killer whales was developed.

Two antibodies were tested: FKA404 reacted only with a cortisol fraction; FKA402 completely cross-reacted with 21-DOC which is a precursor to cortisol. This study not only observes cortisol levels of two captive bottlenose dolphins, but also warns in cross reactivity of certain antiserums when analyzing cortisol levels in cetaceans. The mean cortisol levels for 2 animals in this study were lower than in previous studies (Medway et al., 1970;St. Aubin et at., 1996) which could be due to cross reactivity issues in the other assays or due to acclimation and/or individuality.

Medway et al., 1970

Eight bottlenose dolphins that were housed at the Montréal Aquarium were sampled in this study. Blood samples were collected via venipuncture from the dorsal or ventral flukes. After initial blood samples were taken 2 of the animals were injected with 10 mg of dexamethasone and sampled again in 8 hours. Dexamethasone is known to reduce cortisol levels in humans within 24 hours. Analysis was preformed by a modification of the Silber-Porter method (Peterson et al., 1957). The range of plasma cortisol levels from the initial samples were 1.7-3.0 μg/100 ml, 8 hours later the two animals injected with dexamethasone had a plasma cortisol level <1 μg/100 ml. It is important to note that control samples were not taken after 8 hours in the 6 other animals that were not injected with dexamethasone so a comparison in not applicable. It is thought that cortisol levels peak within 1-2 hours of confrontation with a stressor

(Thomson & Geraci, 1986), so it is plausible to hypothesize that either all the animals sampled at the start of this study were experiencing a mild stress response, dexamethasone may have depressed plasma cortisol levels, or normal circulating levels of plasma cortisol in these 8 animals are <1 μg/100 ml.

Pedernera-Romano et al., 2006

Four bottlenose dolphins (2F;2M) housed in two different aquaria in Mexico City were trained to give saliva samples before each first meal. Both aquaria had similar space and water conditions, along with entertainment shows and therapy sessions. The dolphins were all wild caught and between 8-20 years old and have spent at least 2.5 years in captivity. Salivary cortisol measurement is applicable because of its less invasive nature and ability to be used in long-term cortisol studies without the possibility of bacterial infection from a puncture mark created during venipuncture. Cortisol is found in saliva because of its known solubility of in lipids of cell membranes. The saliva samples were collected via trained voluntary behavior between 9-930h.

A control group of 7 dolphins from a different aquarium in Puerto Vallarta, Mexico were used to obtain time-matched samples of saliva and blood/serum cortisol levels. In these animals 4 were in captivity for 5 years between the ages of 9-11 and trained to voluntarily allow serum and saliva samples. The remaining 3 dolphins in the control group have been in captivity for

1 years between the ages of 4-5 had to be restrained for sampling. Samples from these animals were taken between 1000-1100 hours with saliva obtained 3-4 after serum collection. Cortisol from both types of measurements was measured in RIA kits (Cort CT2, CIS Bio International, France).

The time-matched study displayed that saliva cortisol values represented about 27% of blood values with a correlation value of .73 between time-matched values. That percentage is similar to humans and some other primate species. Serum cortisol ranged from 6.59-64.46 nmol l^{-1} while saliva cortisol levels ranged from 1.43-15.72 nmol l^{-1}. Saliva cortisol levels were elevated in the animals that had been kept in captivity for a year when compared to the animals that had been in captivity for five years. The samples obtained from the four animals for sole saliva analysis displayed the individuality of stress reactions between members of the same species in similar conditions with some animals displaying detectable levels of cortisol 3/31 days and others showing detectable cortisol levels only 11/31 days. The adopting of less invasive techniques such as saliva sampling in captive cetaceans may allow health assessments relating to stress to occur more often without the adverse effects of possible infection and loss of blood which can occur from constant blood hormone monitoring techniques.

Suzuki & Komaba, 2012

I received unpublished data from Miwa Suzuki and her colleague Masayuki Komaba who collected the serum cortisol samples in three Atlantic bottlenose dolphins kept at Kujukushima Aquarium - Umikirara (Nagasaki, Japan). The animals were sampled between May 26, 2009 and September 13, 2011 during all seasons. Samples were obtained from the animals during husbandry procedures from the fluke vein, from the animals when lifted from the enclosure in a stretcher, and when some of the animals were on antibiotics. Antibiotics did not significantly affect the cortisol levels ($p>0.05$) in the animals so they were included in the mean cortisol levels for this analysis. Animals that had samples taken after being lifted from the pool did display a significant increase ($p=0.05$) in cortisol levels and were excluded from the mean cortisol levels for this analysis. 77 samples were collected. The samples were analyzed by TR-FIA, DELFIA system, verified for cortisol in bottlenose dolphins by Suzuki et al (1998), PerkinElmer, Walthom, Massachutes.

Killer Whale (Orcinus orca)

The Killer Whale or orca is easily recognized and widely distributed. It is second only to humans as the most widely distributed mammal on Earth (Ford,

2008). It is found in all oceans and most seas, from the Arctic to the Antarctic, but prefers coastal temperate waters. Three subspecies and ten ecotypes have been identified. The subspecies have differing mitochondrial DNA that suggest some groups have not interbred for centuries (Morin et al., 2006). The three subspecies include fish eating residents, mammal eating transients and fish eating offshores. Depending upon the subspecies pods can vary from tight knit family groups that never leave their mothers in certain resident populations, to smaller hunting groups in transient populations, to large groups of up to 100 in the seldom studied offshore populations (Ford, 2008). Their wide coastal distribution makes them a prime target for research both in the wild and captivity, and also puts them in close proximity to many anthropogenic stressors. They are still caught for aquaria in countries such as Russia and Japan (Ford, 2008). They are not usually hunted for consumption due to the high accumulation of toxins found in their blubber.

The first killer whale to be put on display was captured in 1964 with the purpose of being used as a model for a sculpture at the Vancouver Aquarium. The Seattle aquarium quickly followed by capturing the first killer whale for exhibition purposes in 1965. These animals were caught from local resident populations and between 1965-1973. 45 members of the Southern Resident orca community were captured and another 13 died in the process. This was about half of the population at that time. Only one animal out of the 45 captured is still alive and on display at the Miami Seaquarium. In 1976 wild killer whale captured were outlawed in Washington State due to the negative impacts on the local population.

Orca captures ceased in the US in 1976 as well since no other state had a local population. According to a constantly updated web resource called "orcahome.de" that tracks captures, births and deaths of killer whales in captivity worldwide maintained by a collaboration of killer whale enthusiasts, based on publications of all types. 131 orcas have been caught for aquaria worldwide, 12 are alive at the time of publication. Currently 46 orcas are in captivity worldwide (http://www.orcahome.de/orcastat.htm). The mortality rate of infants, defined as ≤6 months, in captivity is 50.2%, the wild figure is unknown due to difficulty of observation (Rose, 2011). In captivity orcas can be grouped in artificial pods or kept alone. Anthropogenic stressors are prevalent in captivity due to confinement and dependence (Rose, 2011).

Biology

Killer whales range in size from 6-9 meters and are the largest member of the Delphinidae (Ford, 2008). They have striking black and white coloration with an eye patch that varies in size depending upon ecotype. They are sexually dimorphic with males being substantially larger and possessing a much larger appendages including dorsal and pectoral fins. They can be individually identified by their unique saddle patch and dorsal fins (Ford, 2008). They are thought to be the most highly socialized animal other than humans and pods have developed their own distinct dialect (Ford et al., 2000). They usually hunt cooperatively and have developed specialization for different ecotypes and regions. They are typically brief divers usually surfacing every 10-20 seconds

but can hold their breath for over 15 minutes if necessary. Juveniles will remain with their mothers until the next calf is born, usually around 6 years, and may stay for their entire lives. Killer whales have no natural predators other than man. Maximum lifespans in the wild around ~90 for females and ~60 for males, average lifespans are in the 50's for females and 30's for males (Ford et al., 2000). In captivity the average lifespan is around 25 years for both males and females (Rose, 2011).

Status

The International Union for Conservation of Nature lists killer whales as "Data Deficient" with an estimated 50,000 thought to be living in the wild. Certain subspecies such as the Southern and Northern Resident communities are listed as endangered in the United States and Canada.

Wild Studies Examining Cortisol Levels

Collecting cortisol samples from wild orcas have recently evolved. Due to their large size and inhabitation of harsh climates chase, capture, and release is not a logical strategy to obtain a blood/serum sample. In the past, tissues samples were obtained by use of a biopsy gun, which shoots a pneumatic dark at the animal during a focal follow (Noren & Mocklin, 2011). This method is relatively invasive and has the potential to cause an infection at the sample site and cause the animals to avoid research boats in the future. Currently in Washington state

the University of Washington's School of Conservation Biology along with NOAA are collecting fecal samples from killer whales to analyze cortisol concentrations. These samples can be collected from up to ¾ of a mile away and are relatively non-invasive. Fecal cortisol takes around 12-24 hours to metabolize so the sample collected reflects the conditions of the previous day (Ayres, personal communication). In a recent study fecal cortisol samples were used to determine that Southern Resident Killer Whales exhibit higher levels of cortisol when their primary prey source is low, and vise versa (Ayres et al., 2012). The same study also demonstrated that SRKW cortisol levels were highest when a smaller number of recreational, commercial, and research vessels were around, suggesting that prey availability plays a larger role in stress levels than human vessel presence (Ayres et al., 2012). This methodology allows access to baseline cortisol levels of wild killer whales, something that has yet to be established in Atlantic bottlenose dolphins and beluga whales.

Ayres et al., 2012

The endangered Southern Resident Killer Whales are composed of three pods (J, K, and L) and often inhibit the inland waters of the Salish Sea from May-October where they forage for their primary food source Chinook salmon (*Oncorhynchus tshawytscha*). Declines in Chinook salmon runs in the western US may create stress for these animals; this is deemed the inadequate prey hypothesis. While inhabiting the Salish Sea commercial, recreation, and research

vessels often observe the animals, sometimes in large quantities that may also act as a stressor; this is called the vessel impact hypothesis. While in the inland waters SRKWs often feed on Frasier River Chinook salmon run which are usually at their peak in August-September, the same months vessel numbers peak. The inverse is often true as well, with the Chinook run lower in spring/early summer, along with fewer vessels.

This study obtained 154 fecal samples from the 88 members of the three pods between 2007-2009. Most samples were obtained from J pod (n=113). Fecal samples allow noninvasive sample collection by trailing the animals from up to ¾ mile away and scooping floating fecal matter for stress hormone analysis. RIA kit ^{125}I corticosterone, #07-120103; MP Biomedicals, Costa Mesa, CA was used for fecal hormone metabolite analysis. Results of analysis concluded that glucocorticoid levels were lowest when Fraser River Chinook salmon were peaking, although vessel abundance was also peaking at that time, and vice versa. The range of fecal glucocorticoids in these animals was 500-3,500 ng/g. The comparison between fecal GCs of male SRKWs (n=34) for a stranded male killer whale that later had to be euthanized varied greatly (1000 ng/g for SRKW males; 28,000 for stranded male). The results of this study demonstrate that prey abundance plays a larger role in elevating glucocorticoids than vessel presences. It should be noted that during years of low prey abundance vessel impacts might evoke more of a physiological reaction in the stress response.

Captive Studies Examining Cortisol Levels

Orca serum cortisol levels have been estimated to be around 0.4 μg/dl in a stable captive environment (Suzuki et al., 1998). Captive orcas are often taught husbandry practices that allow routine medical procedures to take place without exciting the stress response. These practices include presentation of the fluke for routine blood sampling. These blood serum samples most likely represent a baseline cortisol level for captive orcas. It is unknown if a cortisol baseline for captive orcas could be applied to wild orcas because of differences in environment and lifestyle. A study has shown that factors such as time of day and season can play a role in cortisol production of captive orcas. Killer whales have exhibited decreasing cortisol until 18:00 hours then fluctuations until increasing the following morning (Suzuki et al., 1998). Seasonality played a factor in the males and female orca being studied. The pregnant female showed cyclic changes in cortisol concentrations at 4-month intervals (Suzuki et al., 1998) while the males exhibited lower cortisol concentrations in the summer than in the winter. Elevated mean cortisol levels in captive killer whales have been documented in the autumn, over spring (Suzuki et al., 2003). Another study showed that cortisol levels in two females killer whales during pregnancy and 3-5 weeks post partum found no significant increases in cortisol levels (Lyamin et al., 2005), although the time of sample collection during the pregnancy was not listed so it could be within the similar 4-month intervals that Suzuki et al. revealed. (See Table 3)

Table 3: Killer Whale Cortisol Studies, Basic information. Species, Author (s) of study mean cortisol levels were obtained from, whether the study was conducted in a wild or captive environment (black wild/red captive), sample type, mean cortisol in nmol/L and µg/dl, number of animals sampled in each study, number of samples analyzed to determine mean cortisol levels in each study, range of individual cortisol levels in nmol/L and µg/dl, sample method, and assay used for measurement of cortisol levels.

Killer Whale	wild: captive	mean F	mean F	# Animal	# Sample	Range	Range	Sampling	Analysis
Author	Sample type	nmol/L	µg/dl	N	n	nmol/L	µg/dl	Method	Assay
Suzuki et al., 1998	serum	5.52	0.2	3	18	n.d.-11	n.d.-0.4	husbandry	RIA
Suzuki et al., 2003*	serum	8.28	0.3	7	319	n.d.-52.42	n.d.-1.9	husbandry	RIA
Lyamin et al., 2005	plasma	7.17	0.26	3	3	2.8-22	0.1-0.8	husbandry	RIA
Lyamin et al., 2005	plasma	9.66	0.35	3	3	8.3-13.8	0.3-0.5	husbandry	RIA

*Supplemental data from study utilized.

Lyamin et al., 2005

Three female killer whales were sampled by voluntary fluke presentation when pregnant and then again 3-5 weeks post partum. The samples were taken to attempt to discover what was responsible for the decrease in sleep behavior after the calves were born. Although no statically significant increases were found between cortisol levels when the animals were pregnant vs. 3-5 weeks after birth of their calf, there was greater variation in the range of cortisol in the pregnant animals (0.9-7.4 ng ml^{-1}) than 3-5 post partum (2.8-4.1 ng ml^{-1}). This study depicts that killer whale mothers and neonates displaying very little resting behavior during the first 3 weeks of giving birth and birth, and that resting behavior slowing increases from 4-8 weeks while returning to pre-calf resting patterns at around 8 weeks throughout adulthood.

Suzuki et al., 1998

Three killer whales (2M;1F) that were wild caught but held in captivity for at least 10 years were sampled twice a month from September to December via voluntary tail fluke presentation. Samples were collected in the AM (900-1000h) and PM (1600-1700h) hours to account for the circadian rhythm of cortisol production often observed in terrestrial diurnal mammals. The total range of mean serum cortisol levels between all animals in the AM was 1.4-4.0 ng/ml and 0.2-2.8 nm/ml in the PM. Although the mean cortisol levels in all of the animals tended to decrease in the PM only the female's cortisol levels were significantly different between the AM and PM. The difference in cortisol levels between the female and two males could differ due to social rank. Naturally killer whales are matriarchal and in captivity females often harass males. One male that showed the lowest diurnal mean cortisol variation has been observed getting harassed by the female in this study. Those observations may depict that social rank plays a part in cortisol production.

During this study an RIA system for serum cortisol measurement specifically in bottlenose dolphins and killer whales was developed. Two antibodies were tested: FKA404 reacted only with a cortisol fraction; FKA402 completely cross-reacted with 21-DOC which is a precursor to cortisol. This study not only observes cortisol levels of three captive killer whales, but also warns in cross reactivity of certain antiserums when analyzing cortisol levels in cetaceans. Seasonality could have also played a part in the mean cortisol levels of the

animals being studied other Delphinids have shown seasonal patterns in cortisol production.

Suzuki et al., 2003

Three wild caught captive killer whales (2M; 1F), being kept at Kamogawa Sea World, Japan were utilized in the study to determine diurnal and seasonal patters in killer whales. For the diurnal change study 2, 11-year-old captive killer whales (1M; 1F) wild caught in 1988 were sampled via venipuncture through voluntary tail fluke presentation at 0900, 1200, 1500, 1800, 2100, 2400, 0300, and 0600 h. Serum cortisol levels were measured by RIA developed specifically for bottlenose dolphins and killer whales by the main author of this study in a previous study. Minimum levels of cortisol were observed at 2400; maximum levels at 0600, but a pattern of fluctuations developed between 1800 and following morning. The female tended to display higher cortisol levels and greater fluctuations throughout the day than the male in this study, sometimes peaking at 2100.

The annual change study obtained samples from 3 killer whales (2M; 1F) wild caught but maintained at the same facility for at least 10 years and sampled in the same methodology twice a day between 0900-1000 and 1600-1700. All the animals were adults, but the female in the annual change study was pregnant. Serum cortisol levels were analyzed by the same RIA mentioned above. Males displayed seasonal variation with higher cortisol levels recorded in fall and winter

months, similar findings have been reported in bottlenose dolphins (*Turisops truncatus*) in winter months (Orlov et al., 1988). The pregnant female exhibited cyclic cortisol concentrations every four months, but this may be due to her pregnancy during the time of the study. Cortisol concentrations were significantly lower in the female and one of the males in the AM than the PM. The other male sampled showed a trend of this diurnal patter, but results were not significant for him. This could be due to his low social rank, which may burden him with a more constant stressor. It is important to note that Progesterone showed a negative correlation with cortisol, increasing when cortisol decreases. Stress hormones like cortisol are known reproductive hormone suppressants.

Beluga Whale (Delphinapterus leucas)

Beluga whales are also known as white whales. They inhabit the Arctic and subarctic. They are medium sized and live in fluid pods of 2 -10 individuals, although they can form herds composed of 1000s (O'corry-Crowe, 2008). Females are known to form large nursery groups while adult males may be seen singly or form a separate pod of 6-20 individuals. Due to the harsh climate belugas take part in a predictable yearly migration usually returning to their natal sites in the spring (O'corry-Crowe, 2008). They tend to inhabit coastal areas, which made them a target for research and commercial harvesting. A distinctive population of belugas inhabiting the St. Lawrence Seaway in Canada has unusually high cancer rates due to smelting operations in the twentieth century. At the time of death some of the animals were so polluted with heavy metals and

organohalogens they had to be labeled as hazardous waste. Increasing anthropogenic threats causing habitat degradation such as oil and gas development in the Arctic and global warming continue to add obstacles to beluga survival (O'corry-Crowe, 2008).

Beluga whales were one of the first cetaceans to be held in captivity (cetabase.com). In 1861 a beluga from the St. Lawrence Seaway was put on display at Barnum's Museum in New York. Currently about 200 belugas are captive throughout North America, Europe, and Japan. Although some facilities have had success with breeding captive belugas the gene pool in low and new wild caught specimens are still regularly used to stock aquaria. Some aquaria offer "swim-with" programs with belugas. Most belugas in captivity are used for public display but a small fraction is used in military exercises. Anthropogenic stressors are prevalent in captivity due to confinement and dependence.

Biology

Belugas are in the family Monodontidae. They range in size from 3.5-5.5 meters and are easily identifiable by their white coloration. The exhibit many differences from Atlantic bottlenose dolphins and killer whales including lacking a dorsal fin, un-fused neck vertebrae, rounded melon, thick blubber, yearly molting, and small appendages (O'corry-Crowe, 2008). These adaptations allow for better maneuvering and thermoregulation in the Arctic ice packs. They are highly social within their groups and can make up to 50 different vocalizations

(O'corry-Crowe, 2008). They have developed signature calls for individuals and in one case have even mimicked human speech. They hunt in groups and individually to feed on fish, crustaceans, sandworms, and cephalopods. They have the ability to dive up to 1000 meters and remain submerged for 25 minutes when hunting or navigating dense ice packs. Juveniles stay with their mother for around 3-4 years until another calf is born (O'corry-Crowe, 2008). Their main predators are killer whales, polar bears, and humans. Males and Females live on average 35-50 years in the wild, but maximum lifespan is around 80 years old (O'corry-Crowe, 2008).

Status

The International Union for Conservation of Nature lists beluga whales as "near threatened" with an estimated 150,000 alive in the wild. Certain populations including the Cook Inlet belugas near Anchorage, Alaska along with the Hudson and Ungava Bay pods in Canada are listed as endangered. Two other populations in Canada, the Cumberland Bay and St. Lawrence River estuary belugas are listed as threatened.

Wild Studies Examining Cortisol Levels

Obtaining cortisol samples from wild beluga whales has numerous obstacles resulting from location and habitat preferences, but their habituation to

specific estuaries in summer make them a target for researchers (St. Aubin & Geraci, 1989; 1992). Previous studies conducted on wild beluga whales have obtained blood/serum samples from chase, capture, and restraint (St. Aubin & Geraci, 1989; St. Aubin et al., 2001). Since many cetaceans show an increase of cortisol during onset of the acute stress response with cortisol levels peaking within 1-2 hours, the invasive methodology preformed to gather blood/serum samples may not depict an accurate cortisol baseline (St. Aubin et al., 2001). Chase, capture, and transport of the belugas all caused elevations in serum cortisol levels (St. Aubin & Geraci, 1989). Further studies determined that serum cortisol did not show age or sex related differences in wild beluga whales that were sampled during tagging or capture, although an increase in cortisol did occur in both sexes and all ages of animals tested (St. Aubin et al., 2001). (See Table 4)

Table 4: Beluga Whale Cortisol Studies, Basic information. Species, Author (s) of study mean cortisol levels were obtained from, whether the study was conducted in a wild or captive environment (black wild/red captive), sample type, mean cortisol in nmol/L and µg/dl, number of animals sampled in each study, number of samples analyzed to determine mean cortisol levels in each study, range of individual cortisol levels in nmol/L and µg/dl, sample method, and assay used for measurement of cortisol levels.

Beluga	**Wild**: Captive	**Mean F**	**Mean F**	**# Animal**	**# Sample**	**Range**	**Range**	**Sample**	**Analysis**
Author	Sample	nmol/L	µg/dl	N	N	nmol/L	µg/dl	Method	Assay
St. Aubin & Geraci, 1989	Plasma	90	3.3	42	41	18-196	0.7-7.1	C/C/R	RIA
St. Aubin & Geraci, 1992	plasma	110	4	10	158	80-204	2.9-7.4	C/C/R	RIA
St. Aubin et al., 2001	plasma	89.1	3.2	183	115	15.5-204.4	0.6-7.4	C/C/R	RIA
Tryland et al., 2006	serum	125.1	4.5	21	21	53-219	1.9-7.9	C/C/R	CLEIA
Orlov et al., 1991	serum	82.6	2.9	10	16			NH	RIA
Schmitt et al., 2010	serum/plasma	49.7	1.8	3	104	18.2-115	0.66-4.17	H	CLEIA
Spoon & Romano,	serum	27.6	~1	7	27			H	CLEIA

St. Aubin & Geraci, 1989

Forty-two sub-adult beluga whales were captured in the Seal and Churchill River estuaries in western Hudson Bay during July 1985 and 1970. Large adults and females with calves were purposely avoided to reduce confounding factors such as age and reproductive hormones. Blood/serum samples were taken during restraint within one hour. Analysis was conducted using a commercial I^{125}RIA kit, New England Nuclear, Boston, MA . Cortisol levels ranged from 18-196 nmol$\square L^{-1}$. All but six of the animals were released immediately after the samples were collected. It has been documented that cortisol levels often peak within 1-2 hours of capture and restraint (Thomson & Geraci, 1986). That knowledge may contribute to the mean cortisol level calculated from the animals sampled within an hour of capture representing a low-medium stress response and not an accurate assessment of a basal or baseline level. The exact time that cortisol levels begin to rise during the cetacean acute stress response is unknown, but it is plausible to hypothesize that changes in cortisol levels being occur immediately after presented with a stressor.

Six belugas that were captured for serum samples were taken into captivity for 10 weeks. Blood samples were taken 30 minutes to one hour after capture, when they arrived at the holding facility after 2-3 hours of transport, and irregularly during the next 70 days. Cortisol levels were highest after the 2-3 hour

transport to the holding facility, which could be viewed as a medium-high stress response, where most showed a declining trend during the first five days in a captive environment. Spikes were documented in most animals between capture, transfer (2-3 hours), and at irregular intervals during the first five days in the holding facility. One animal's cortisol levels peaked prior to its first day in captivity and slighting rose for the preceding five days, perhaps indicating that different individuals may adapt to stress in different ways.

An interesting note in this study is that out of 21 samples taken within an hour of capture the mean cortisol level is only slightly higher than the mean cortisol level determined from 77 samples of the six animals taken captive in the recovery phase, days 7-70 (both numbers between 80-120 nmol□L^{-1}). This could be due to the mean cortisol levels at both capture and after 70 days in captivity indicating a baseline for the animals in both environments or indicate that mean cortisol levels collected during acute stress within one hour of capture are similar due to acute stress still being exhibited in a captive environment up to 70 days later.

St. Aubin & Geraci, 1992

Ten juvenile beluga whales (8M; 2F) were captured in the Churchill River in Manitoba, Canada in July 1988 (4) and July 1989 (6). Blood samples were collected from the caudal peduncle 30-40 minutes after capture (10-20 minute approach followed by up to 20 minutes to obtain sample). Samples were analyzed

by commercially available RIA kits, NCS Diagnostics, Willowdale, Ontario. The mean cortisol level for the 10 animals ranged from 80-205 nmol/L.

The animals were then transported to holding facilities and held for up to five days and then released. Cortisol levels remained steady or increased from capture throughout the first 24 hours at the hold facility. Samples were collected at the holding facility at low and high tides. During low tides the pools were drained to 0.8 meters, which caused an increase in cortisol levels by at least 10 nmol/L in over 77% of the animals. The inverse occurred with 70% of the animals exhibiting a cortisol level of at least 10 nmol/L 5-6 hours after being kept in shallow water, about 30 minutes before the pool was refilled to 1.65 m.

These results illustrate that cortisol levels were altered in these animals by perceiving a stressful event (ie pool draining) and on some occasions spiked to mean levels at capture during these events. It is important to note that 2 animals were given an injection of TSH (10 IU of bovine TSH) after initial blood sampling at capture and two more after being taken into the holding facility. Although adrenal hormones like cortisol play a role in thyroid hormone production, the reverse is not thought to occur. Application of TSH could play a role in the analysis of mean cortisol levels during the time these animals were analyzed in captivity.

St. Aubin et al., 2001

Over fifteen years 183 beluga whales were sampled in the Canadian Arctic. The animals were sampled during attempts to apply tracking instruments (55), obtained during capture for aquaria, research, or carcass from Inuit hunters. The animals were mostly sampled during the summer, all other variables such as age, season, sex, stock, and year were randomly selected. 151 animals were live captured. The approach lasted from 5-15 minutes. 32 of the samples were from animals shot for food by Inuit hunters where chase time was similar. Samples were analyzed by commercially available RIA kits, NCS Diagnostics, Willowdale, Ontario. Cortisol showed no age or sex related differences. Cortisol samples were analyzed for 115/183 animals displayed a range of 15.5-204.4 nmol/L.

Tryland et al., 2006

Between 1996 and 2001, 221 blood/serum samples were collected from 21 wild beluga whales in three areas off Svalbard, Norway. The whales were were live captured and restrained while blood was collected. Amount of time from start of chase to sample collection was not noted in article, but personal communication with Dr. Tryland has rendered the time of 10-20 minutes. Samples were analyzed via a commercially available RIA kit, Immunite One

system, DPC, L.A., CA. Cortisol concentrations were comparable but higher than previously reported for beluga whales sampled in the Canadian Arctic waters, although other blood chemistry levels were similar. Cortisol concentrations ranged from 53-219 nmol/L. These samples may display elevated cortisol levels in comparison to samples collected during studies on the Canadian belugas due to the relatively extended time from chase, capture, and sample collection (St. Aubin et al., 2001). Cortisol levels are thought to peak in cetaceans about 1-2 hours after exposure to a stressor (Thomson & Geraci, 1986).

Captive Studies Examining Cortisol Levels

Captive studies conducted on stress hormones of beluga whales are more prevalent than wild studies. The captive beluga whale serum cortisol levels have been estimated to vary between 0.7-3.2 μg/dl among different ages and sexes (St. Aubin et al., 2001). Blood/serum is collected via a husbandry practice in a captive environment (Schmitt et al., 2010, Spoon & Romano, 2012, St. Aubin et al., 2012). The act of displaying the fluke for unhindered access to the fluke vein can alleviate stress during collection. Captive beluga whale cortisol levels have shown a circadian rhythm similar to those in terrestrial mammals with higher concentrations in the morning than in the evenings (Schmitt et al., 2010). Cortisol levels have differed in belugas that had been translocated to a new captive environment with a peak during arrival at the new enclosure and decent to baseline levels once acclimated (Spoon & Romano, 2012). (See Table 4)

Schmitt et al., 2010

Three long-term captive beluga whales estimated to be around the same age (19) at time of study, residing in an outdoor exhibit were sampled to determine baseline, diurnal, and stress induced changes of stress hormones in the autumn (September and October). The animals were all wild caught from the Hudson Bay, non-reproductively active, and deemed healthy at time of study. Blood serum samples were obtained from the ventral fluke veins by signaling the animals to display their fluke via common husbandry behavior. Over 6-8 weeks Blood was collected under these conditions three times daily to reflect a baseline reading. Blood was also collected 20 minutes before and 20 minutes after a wading-contact session, where 6-8 public participants entered the pool and were tactility active with the animals, and during an out of water examination. For an out of water examination to occur the animal must be separated from its group and directed into a medical pool which is quickly drained so the animal can be placed on a stretcher, lifted up, and placed on the pool deck (Stage 1). During the time on the pool deck medical procedures including an endoscopy are preformed lasting 45 minutes to 1 hour (Stage 2). Blood samples were taken 20 minutes after initial separation for the exam, following the 45-minute to 1-hour exam, along with 1, 12, 16, and 24 hours post exam. Samples were measured by automated chemiluminescent enzyme immunoassay, IMMULITE Coat-A Count, DPC, L.A., CA.

Baseline mean serum cortisol levels ranged from .66-4.17 μg/dL with a mean level of 1.8 μg/dL. Baseline endogenous cortisol concentrations from these animals were higher in the morning than afternoon and evening, displaying a cyclic trend that has also been documented in captive killer whales (Suzuki et al., 1998). Mean cortisol levels along with others stress hormones including ACTH and aldosterone were all significantly elevated during the out of water exam. Mean cortisol concentrations rose 4X baseline levels (3.8 μg/dL) in stage one of the out of water examination peaking at 7.9 μg/dL in stage two, then returned to 3.8 μg/dL one hour following the exam, and then returned to the baseline level of 1.8 μg/dL, 12 hours post out of water exam. The changes in mean cortisol levels during pre-post and during the out of water examination provides a timeline of an acute stress response on belugas. Past studies on beluga whales have indicated that seasonality effects cortisol levels, with samples taken in spring more elevated than samples taken in fall (Orlov et al., 1991). No statistically significant results were found in samples taken pre and post wading contact session, but time of day was not indicated so it may play a part in the analysis.

Spoon & Romano, 2012[4]

This study observed changes in cortisol levels in beluga whales associated with a predicted stressor, which was the introduction of seven beluga whales to their enclosure. 3 resident beluga whales at the Mystic Aquarium (Mystic, CT,

[4] The mean cortisol levels obtained from seven animals in Spoon and Romano (2012) was estimated based on individual cortisol levels posted in a graph in the publication. It is possible that the mean cortisol level may be slightly skewed.

USA) were introduced to 4 belugas being transported from the John G. Shedd Aquarium in Chicago. Baseline samples were obtained from the animals being transported along with the three belugas that reside at the Mystic Aquarium. "Arrival" samples were taken from the transported belugas immediately after transport and before introduced in the new environment; and from the resident belugas within 5 days of the transported animals arrival. "Acclimation" samples were taken from the transported belugas 5-6 months after they arrived and from the resident belugas 4, and 8 weeks after the arrival of the transported animals. All blood serum samples were taken via venipuncture from voluntary fluke presentation except the samples obtained when the transported animals were still restrained for transport. Cortisol levels were analyzed using chemiluminescent enzyme immunoassay, Immulite, Siemens, L.A., CA, USA.

The three resident belugas displayed elevated levels of E and NE but did not display any statistically significant differences in mean serum cortisol concentrations between baseline, arrival, and acclimation periods. All 4 transported belugas showed statistically significant differences in cortisol levels from baseline, arrival, and acclimation, rising from baseline to peak during the arrival phase and decreasing during the acclimation phase. This observation may indicate that transport is an acute stressor to beluga whales. All whales displayed an increase in E and NE, while just the transported whales had increased cortisol levels, indicating that the process of relocating and introducing these animals causes an environmental change that may be perceived by these animals as a

stressor. It also may indicate that the transport was a greater stressor than the environmental changes that the resident belugas were exposed to.

Orlov et al., 1991

Ten beluga whales that were captured off of Russia were studied during adaptation to a captive lifestyle in this study. Blood samples were taken 1, 3, 8, and 11 days after the animals were captured and transported to a Research Institute in Vladivostok, Russia. After the initial study on adaptation the animals were held for a year to study seasonality of hormones along with other blood constituents. The animals were kept in 6 feet of water to allow for blood sample collection from the fluke vein. Serum cortisol was analyzed by a RIA assay produced by IBOKh, Belorussian SSR or Sorin, France. Seasonality was found in the animals, displaying lower levels of cortisol in spring/fall and higher levels in winter/spring. This may be due to the harsh climatic impacts they face living in the arctic. Mean cortisol levels for the were similar to other studies conducted on wild captured animals and only held for a short amount of time, not an entire year.

Acute stress was noted by increase in cortisol levels within days 1-4 with a rapid adaptation process that stabilized levels on days 8-11. Adaptability differed among animals in this study, with cortisol levels varying among individuals. Stressor experiments were preformed on 3 of the animals after the initial testing phase and the results concluded that the animal going through the greatest stressor exhibits the highest cortisol levels. Two of the animals were subjected to immobilization on a porolon bed; the larger of the two animals displayed the

highest levels of cortisol. This is thought to be due to the development of internal organ compression syndrome what was expressed in a more drastic manner in the animal weighing the most. The third animal was immobilized by being put in tank and displayed lower cortisol levels than the two animals that were presented with more severe immobilization experiments. A reference was not made between the cortisol levels of the animal kept in the tank to the animals kept in sea pens that were not exposed to any additional stressor after capture.

CHAPTER 4

RESULTS

Wild vs. Captive Mean Cortisol Levels Within Species

Mean cortisol levels[5] within each species living in both a wild and captive environment are impacted by sample collection methodology[6] (C/C/R, H, NH). Producing descriptive statistics on mean cortisol level and standard deviation of the means by combining all findings of cortisol levels in available published data allows for the most accurate interpretation of cortisol levels found in wild and captive members of these three species of cetacean.

Atlantic Bottlenose Dolphin

Mean cortisol levels differ greatly between studies completed on wild and captive Atlantic bottlenose dolphins. Sampling methodology may play a large part

[5] All cortisol samples that were analyzed in this analysis come from studies that employed sample collection via venipuncture unless otherwise noted in the text. The samples were either blood serum or plasma and cortisol levels are similar and comparable within each medium. The decision to use either serum or plasma in each analysis is based on the original author and their preferred assay used for cortisol detection. For more detailed information on each of these topics within each species please refer to the various tables supplied throughout this analysis.

[6] Husbandry (H) and non-husbandry (NH) practices will be referenced frequently in this analysis. For the purpose of this analysis husbandry practices, sampling, or methodology refers to instances where the animal is trained to present its tail fluke for voluntary sample collection. Non-husbandry practices, sampling, or methodology refers to instances when the animal is sampled using forceful techniques such as lifting or removing from the tank and/or being forced into shallow water for involuntary sample collection. All wild sample collection techniques employ chase, capture, restraint (C/C/R) techniques.

in the results of these types of studies. Results from all studies on mean cortisol levels in wild and captive studies Atlantic bottlenose dolphin display higher mean cortisol levels in captive animals sampled using non-husbandry practices (range: 35-160 nmol/L), followed closely by wild studies that employ chase, capture, restraint sampling techniques (range: 71.7-100 nmol/L). The lowest mean cortisol levels were found in captive animals that were sampled using husbandry practices (range: 10.5-69 nmol/L).

All wild studies that assessed mean cortisol levels in wild Atlantic bottlenose dolphins display higher mean cortisol levels than any captive study that employed husbandry sampling methodology. All the wild studies display mean cortisol levels exceeding 70 nmol/L (range 71.7-100 nmol/L) while over half the studies (7/12) on the captive animals sampled via husbandry practices display mean cortisol levels that were under 20 nmol/L (range 10.5-69 nmol/L).

Mean cortisol levels of wild and captive Atlantic bottlenose dolphins that were sampled utilizing non-husbandry methods are similar. Only one captive study exceeds all of the wild studies and only 1/3 of the captive studies display mean cortisol levels lower than the levels obtained in all of the wild studies. The range of mean cortisol among wild studies is 71.7-100 nmol/L and 35-90.3 nmo/L in captive animals sampled via non-husbandry methodology.

Mean cortisol levels derived from 3 studies completed on wild Atlantic bottlenose dolphins are similar to mean cortisol levels from six studies on captive Atlantic bottlenose dolphins that utilize non-husbandry sampling, but are on

average much higher than the cortisol levels obtained from ten studies that employ husbandry sampling (Figure 1).

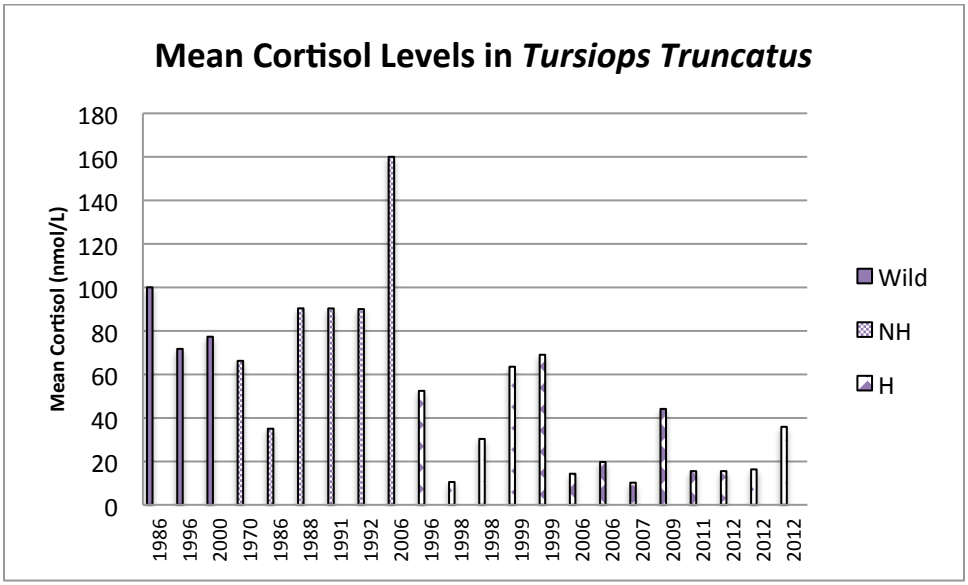

Figure 1: Mean cortisol levels from three studies on wild animals sampled (N=105, n=105)[7] and 16 captive studies including 10 captive studies that employ H sampling[8] (N=93, n=408) and six captive studies that employ NH sampling (N=79, n=79).

Mean cortisol levels were significantly higher in wild Atlantic bottlenose dolphins than in captive ones. The mean cortisol levels are more greatly differentiated when comparing the results of wild animals to the ten studies on animals that were sampled utilizing captive husbandry sampling. When comparing mean cortisol levels from three wild studies to six captive studies that employed non-husbandry sampling techniques the mean cortisol levels were very similar. Great differences existed in mean cortisol levels of captive animals that were sampled via husbandry and non-husbandry practices.[9] Cortisol samples

[7] N=number of animals sampled, n=number of samples analyzed

[8] Ortiz et al. (2010) which, produced undetectable cortisol levels were included.

[9] Ortiz & Worthy (2010) was not included in the comparisons because cortisol levels were undetectable. The study sites adrenal atrophy as a possible explanation. The RIA used for analysis in their study is a common commercially available kit (Immulite, DPC) used in 5 other studies on cortisol levels in Atlantic bottlenose dolphins for this analysis and has an

collected by practicing non-husbandry sampling methodology are much higher on average than samples collected by husbandry practices.

Based on studies, average mean cortisol levels[10] are higher in Atlantic bottlenose dolphins that have been sampled utilizing both non-husbandry methodology in captivity and chase, capture, restraint methodology in the wild. Large differences are present when comparing mean cortisol samples from wild and non-husbandry collection methods to mean cortisol levels collected via husbandry practices (Figure 2). The mean cortisol level derived from three studies on wild Atlantic bottlenose dolphins is 83.0±15.0[11] nmol/L (3.0±0.54 µg/dl). The mean cortisol level obtained from 10 studies on captive animals that voluntarily allowed sample collection to occur though trained a husbandry practice is 30.6±20.6 nmol/L (1.1±0.7 µg/dl). The mean cortisol levels from six studies on captive animals that employed involuntary non-husbandry sampling methodology is 88.3±41.2 nmol/L (3.2±1.5 µg/dl).

analytical sensitivity of 5.5 nmol/L (0.2 µg/dl). Contamination of samples during collection or analysis could also explain the undetectable cortisol levels (Siemens Healthcare, personal communication).

[10] Unconventional descriptive statistics were used in this analysis due to the heterogeneous nature of the data of this project. Since individual cortisol values for each animal from each study were not available when compiling mean cortisol levels an average mean cortisol levels is displayed. This value is a "mean of means" or synthesized means.

[11] Unconventional descriptive statistics were used in this analysis due to the heterogeneous nature of the data. Since individual cortisol values for each animal from each study were not available a standard deviation of the means was used. The standard deviation of the means is a standard deviation of mean cortisol levels. It is important to note in this study that the standard deviations given within each group are not normal standard deviations and are only being used to depict variance among the mean cortisol levels being examined.

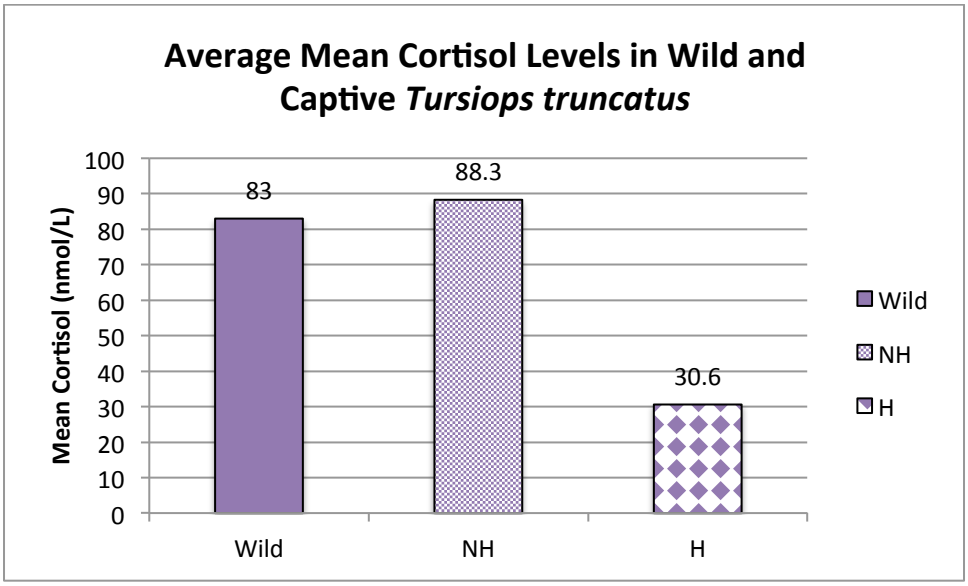

Figure 2: Mean cortisol levels in Atlantic bottlenose dolphins from three wild studies (N=105, n=105), ten captive H studies (N=93, n=408), and six captive NH studies (N=79, n=79).

Cortisol Collection Time in Wild Atlantic Bottlenose Dolphins

Conflicting estimations of when cortisol levels begin to increase in response to an acute stress response have been published (Ortiz & Worthy, 2000, St. Aubin et al., 1996, Ortiz et al., 1991, Thomson & Geraci, 1986). Two studies claim that cortisol levels do not rise in significant levels until an hour after chase ensues. To evaluate these claims I compared specific results of those two studies with all data from captive NH and H studies. These two studies conducted on wild Atlantic bottlenose dolphins contest the claims that sample collection time of <1 hour does not reflect elevated cortisol levels (Ortiz & Worthy, 2000, Aubin et al., 1996). Ortiz and Worthy (2000) sampled wild Atlantic bottlenose dolphins for cortisol levels within or below 36 minutes of chase and greater than 36 minutes

after chase ensues. The mean cortisol levels between those two groups did not differ by much. In St. Aubin et al., 1996, wild Atlantic bottlenose dolphins were sampled within an hour of chase ensuing and displayed similar results to both groups sampled in Ortiz and Worthy (2000). Both of these studies concluded that cortisol levels are not greatly impacted and little acute stress is shown when animals are sampled within an hour of the chase (St. Aubin et al., 1996, Ortiz and Worthy, 2000). When these results are compared to captive studies on Atlantic bottlenose dolphins they reflect similar values to cortisol samples that were obtained from six studies using non-husbandry sampling and display greatly elevated values when compared to cortisol samples of animals from ten studies that utilized captive husbandry collection methods (Figure 3).

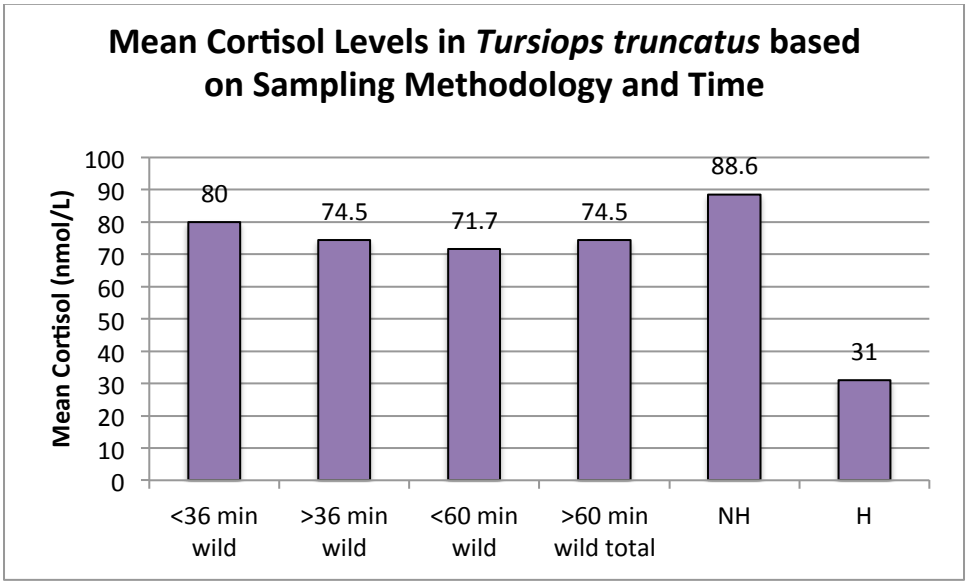

Figure 3: The columns referencing 36 minutes is data from Ortiz & Worthy (2000), the columns referencing 60 minutes is data from St. Aubin et al. (1996), the NH columns is based on six studies, and the H column represents ten studies.

Killer Whale[12]

Mean fecal glucocorticoid (FGC) levels derived from one study (Ayers et al., 2012) that collected 154 samples from Southern Resident Killer Whales displayed a mean FGC level of 1008.27 ng/g. Mean cortisol levels collected from nine captive killer whales sampled via voluntary husbandry practices from three studies is 7.66±1.75 nmol/L (0.28±0.06 μg/dl). FGC's are measurements of fecal metabolites rather than measurements of the parent hormone cortisol, thus, direct comparisons cannot be made between mean levels of FGC's and mean cortisol levels, which is the reason these levels are not depicted in this study. It is possible for trends in FGC's to be compared to trends in cortisol levels to depict stress.

Mean cortisol levels derived from four studies that employed husbandry sampling depict relatively similar results (Figure 4). In three of the four studies in this analysis, one or all of the animals being sampled were pregnant although that variable does not seem to effect the small variation in mean cortisol levels analyzed.

[12] Analysis was only performed on captive killer whale mean cortisol levels because the wild study measures FGC metabolites not cortisol so the numbers cannot be directly compared.

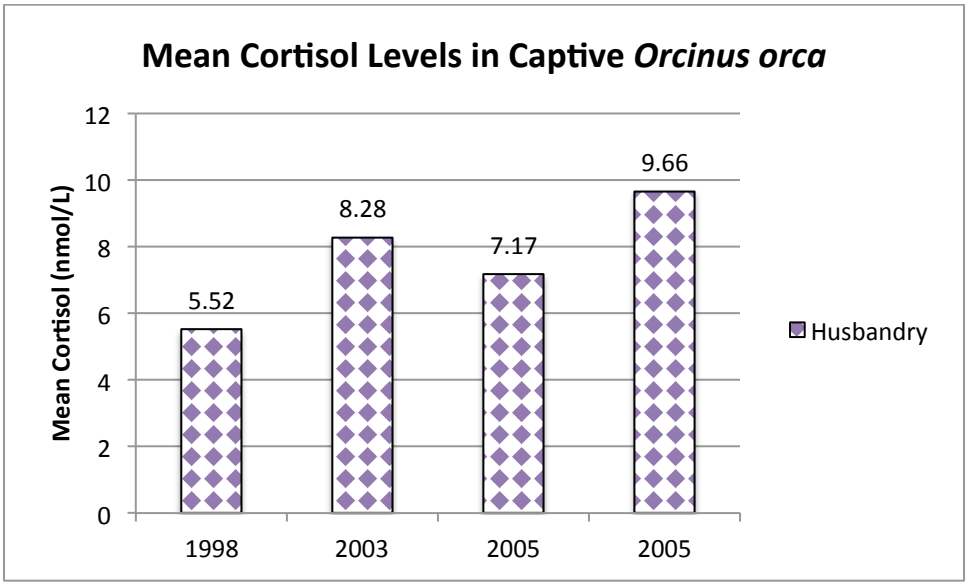

Figure 4: All three cortisol levels of captive killer whales were including (N=10, n=325), Lyamin et al. (2005) conducted two separate analyses.

Beluga Whale

Mean cortisol levels are slightly higher in beluga whales that were sampled in the wild (range: 90-125.1 nmol/L) when compared to captive belugas that were sampled utilizing non-husbandry methodology (range: 82.6-117.5 nmol/L). Mean cortisol levels from captive belugas as a whole (husbandry and non-husbandry sampling) was nearly half that, and mean cortisol levels obtained from animals sampled via husbandry techniques were the lowest (range: 27.6-49.7 nmol/L).

Mean cortisol levels from studies on wild and captive beluga whales that were sampled utilizing non-husbandry methodology were strikingly similar

(range: 82.6-125.1 nmol/L). Captive animals sampled using husbandry methodology were much lower (range 27.6-49.8).

Mean cortisol levels in wild beluga whales and captive NH were consistently higher than cortisol samples from captive H animals (Figure 5). Mean cortisol levels derived from four studies on wild beluga whales is 103.55±17.3 nmol/L (3.75±0.63µg/dl). Mean cortisol levels from two captive studies that employ non-husbandry sampling is 100.1±24.7[13] (3.6±0.9). Mean cortisol levels derived from two studies on captive beluga whales sampled through husbandry methodology is 38.65±15.63 nmol/L (1.4±0.57 µg/dl).

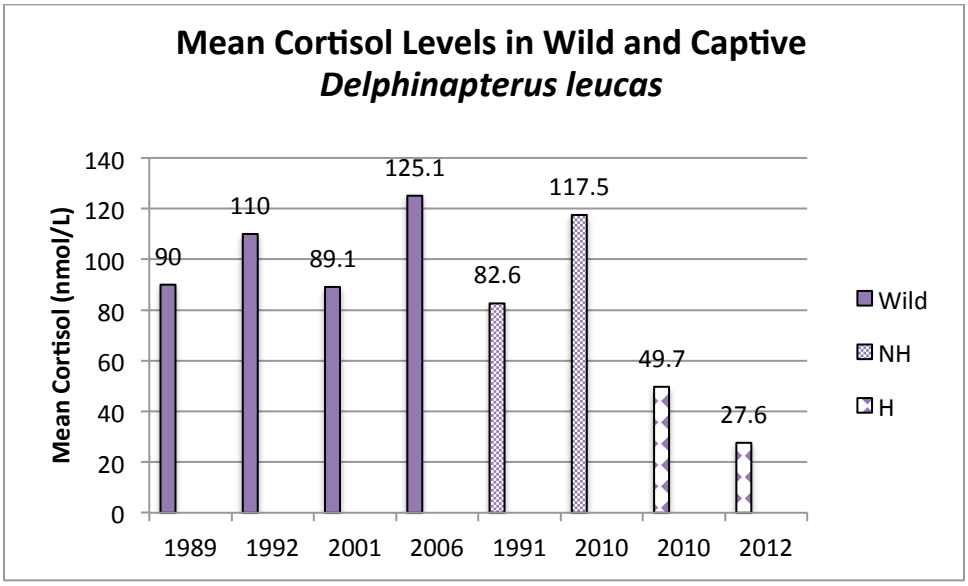

Figure 5: Mean cortisol levels from 4 studies on wild beluga whales (N=256, n=335), from two captive H studies (N=10, n=131), two captive NH studies[14] (N=13, n=33)

[13] One study, Spoon & Romano, 2010, the mean cortisol levels was estimated by averaging cortisol levels from a figure in the publication.

[14] Another aspect of Schmitt et al.'s (2010) study was included in this graph, to display an attempt to provoke stress in a captive beluga by taken the sample during an out of water examination.

Mean cortisol levels are significantly higher in the wild beluga whales that were sampled when compared to the captive studies that employ husbandry sample collection methods. The mean cortisol levels are extremely similar when comparing the studies on wild belugas to the studies on captive belugas that were sampled via non-husbandry practices. Mean cortisol levels were also much higher in captive animals that were sampled via non-husbandry methodology when compared to animals sampled utilizing husbandry methodology (Figure 6).

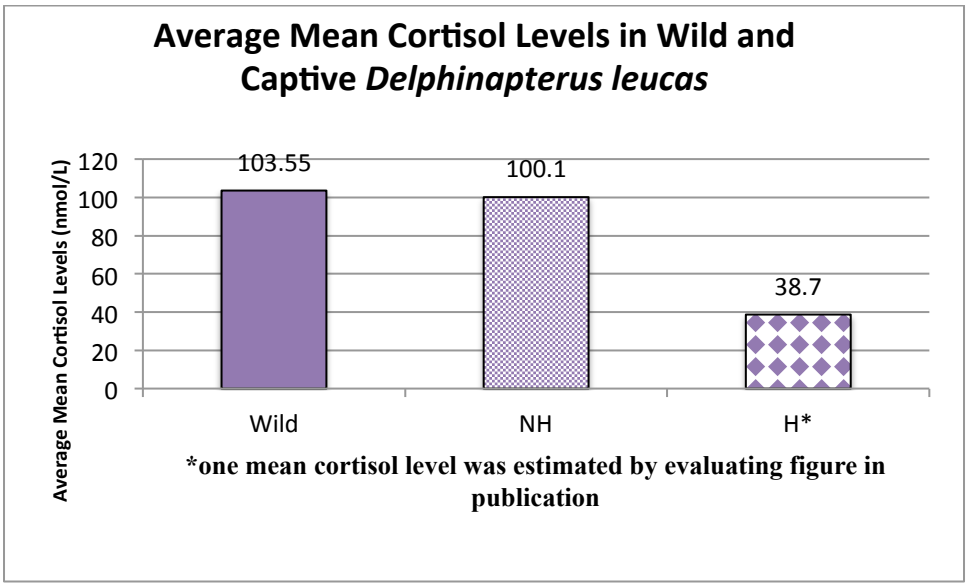

Figure 6: Mean cortisol levels from four studies on wild and three studies on captive beluga whales were included (N=276, n=482).

Wild vs. Captive Mean Cortisol Levels Among Species

In all three species, mean cortisol levels obtained from wild studies are higher than cortisol levels obtained in captive studies as whole (Figure 7[15]). Wild

[15] Killer whales are data deficient in wild studies

sampled beluga whales display the highest mean cortisol levels among wild studies (range: 90-125 nmol/L) and captive beluga whales display the highest mean cortisol levels among the captives species studied (range: 27.6-82.6 nmol/L). Captive killer whales sampled for cortisol concentrations displayed the lowest mean cortisol levels of all wild and captive species sampled (5.5-9.7 nmol/L).

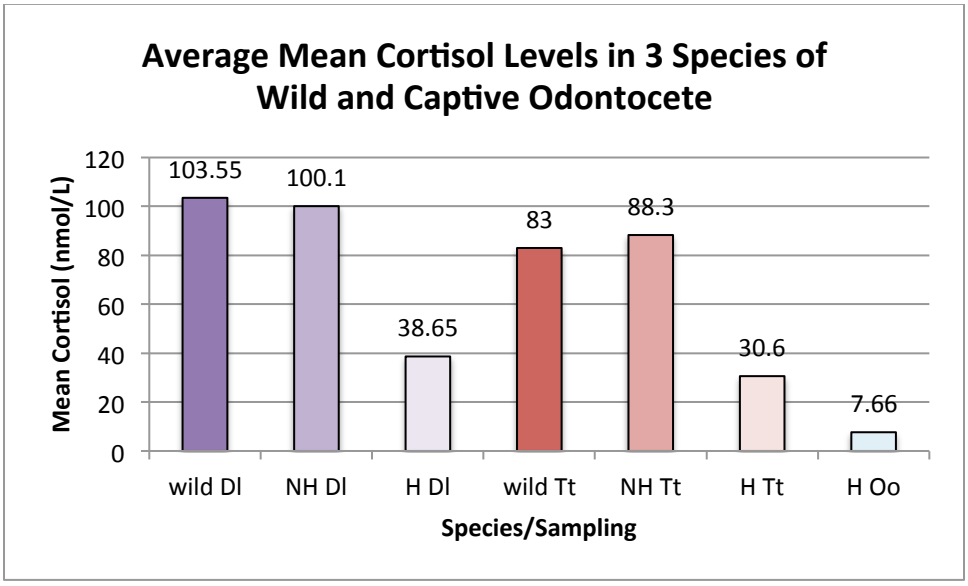

Figure 7: All 30 studies were included (N=563, n=1399)

Among captive beluga whales, Atlantic bottlenose dolphins, and killer whales sampled in previous studies that included both husbandry and non-husbandry sampling methodology beluga whales displayed the highest mean cortisol levels (range: 27.6-82.6 nmol/L), followed closely by Atlantic bottlenose dolphins (range: 10.5-160 nmol/L). Mean cortisol levels from captive killer whales were much lower than both other species, but this may be due to all studies that collected cortisol levels in captive killer whales only practiced husbandry sampling methodology (range: 5.5-9.7 nmol/L).

Wild and Captive Atlantic Bottlenose Dolphin and Beluga Whale

Mean cortisol levels derived from four studies conducted on 187 wild beluga whales is 103.55±17.3 nmol/L (3.75±0.63µg/dl). The mean cortisol level derived from three studies on 105 wild Atlantic bottlenose dolphins is 83±15.0 nmol/L (3.0±0.54 µg/dl). Results from comparing mean cortisol levels from studies on wild beluga whales to studies on wild Atlantic bottlenose dolphins are similar. Similar mean cortisol levels were also observed when comparing all captive studies on each species, including those utilizing husbandry practices, and those utilizing non-husbandry practices.

Captive Killer Whale and Atlantic Bottlenose Dolphin

Mean cortisol levels in three studies on 10 captive killer whales that were sampled utilizing husbandry methodology were compared to ten studies on 93 captive Atlantic bottlenose dolphins where husbandry practices were also utilized for sample collection. The results displayed higher cortisol levels in captive Atlantic bottlenose dolphins than in captive killer whales when husbandry sampling was employed.

Captive Beluga Whale and Killer Whale

Three studies that assessed mean cortisol level in 10 captive killer whales via voluntary husbandry sampling methodology were compared to two studies that assessed mean cortisol levels in 10 captive beluga whales that were sampled

utilizing voluntary husbandry practices. Mean cortisol levels were significantly higher in the captive belugas.

Trends in mean Cortisol Levels Throughout Time in Captivity

Assessing trends in mean cortisol levels throughout time in captive Atlantic bottlenose dolphins may help determine if evolved captive care contributes to decreased exposure to stress in these animals. Studies have analyzed cortisol levels in captive Atlantic bottlenose dolphins since 1970 and are ongoing. Captive care has evolved immensely in the past 42 years due to a better understanding of cetacean needs. Analyzing mean cortisol levels throughout time in captivity will display if the captive animals are currently experiencing statistically significant decreases in stress and may assist in developing more effective animal husbandry and care.

Atlantic Bottlenose Dolphins

Many of the early studies on cortisol levels of this species utilized non-husbandry sampling techniques. All published studies prior to 1996 utilized NH sampling, whereas only one study after 1996 employed NH sampling. Visually, a slight declining trend may look apparent, but due to the possible impacts of sampling methodology a definitive trend cannot be conclusively assessed due to less stress in the environment over time (Figure 8).

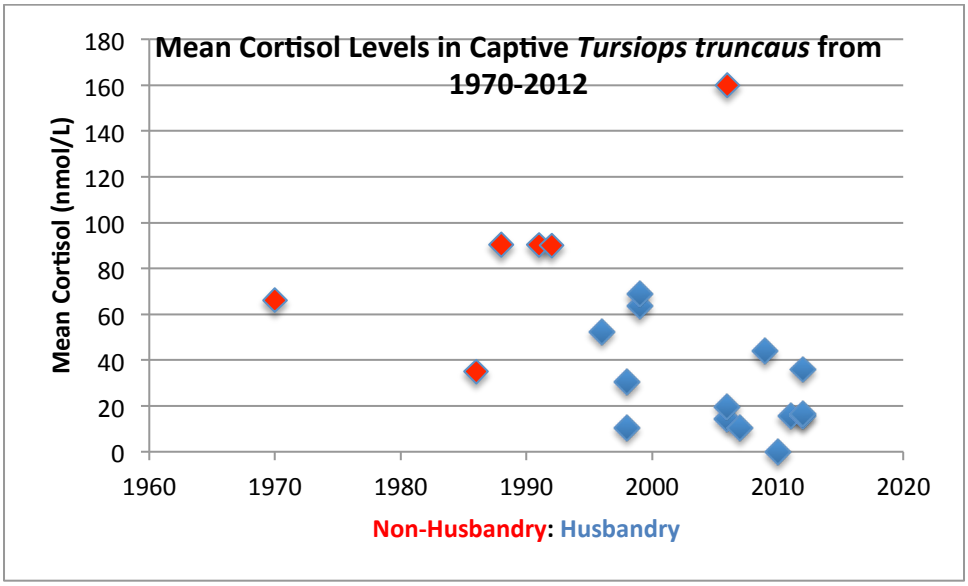

Figure 8: Mean cortisol levels in captive Atlantic bottlenose dolphins from 1970-2012. All 17 studies on captive animals were included (N=172, n=487). Some studies conducted multiple analyses or supplied supplemental data[16] that account for the extra points.

In this comparison between mean cortisol levels obtained from studies conducted from 1996-2012 a trend line was undetectable. Studies that employed non-husbandry sampling methodology were excluded from the trend map (Figure 9).

[16] Blasio et al., 2012, Reirdson & McBain et al., 1999, Suzuki et al., 1998

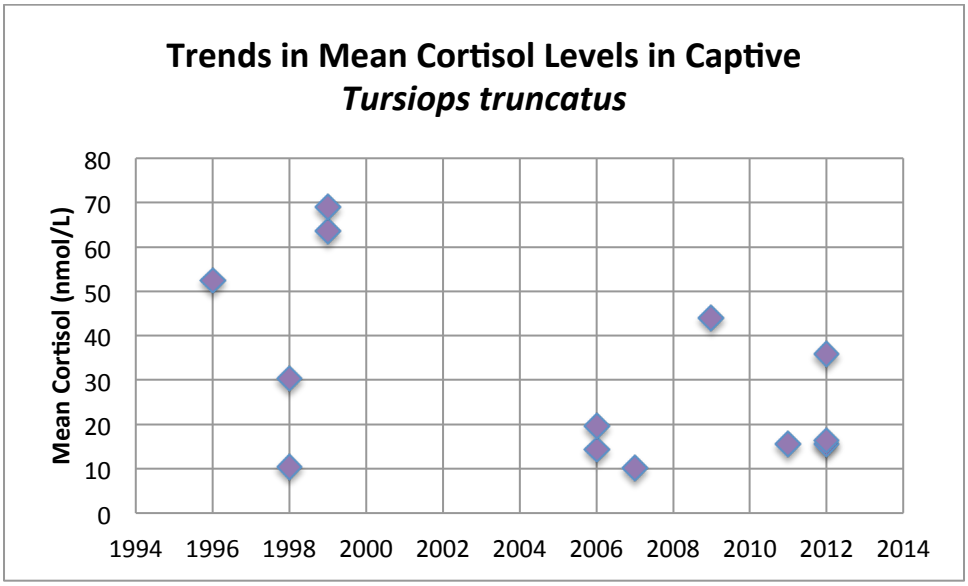

Figure 9: Mean cortisol levels collected in 10 studies on captive Atlantic bottlenose dolphins that utilized H sampling[17] (N=91, n=406). Some studies conducted multiple analyses or supplied supplemental data that accounts for the extra points[18]. The two figures in 1999 seem like outliers in the data set[19].

[17] Ortiz et al. (2010) was not included because the cortisol levels were undetectable

[18] Blasio et al., 2012, Reirdson & McBain et al., 1999, Suzuki et al., 1998

[19] The authors, Reirdson & McBain were contacted via email and assured me they followed the published procedures but those two points seem anomalous enough to take into consideration when assessing this figure.

CHAPTER 5

DISCUSSION

Analysis

This systematic review displays that sample methodology is a consistent confounding variable in every research questions that was asked. Because of the unanticipated impact that sampling methodology had on results it would be inappropriate to portray any definitive results. Nevertheless, some interesting trends were observed within each research question. The lack of definitive answers underscores the need to design and implement a protocol that would decrease the impact of sampling methodology when collecting samples for cortisol analysis.

Despite the problems with sampling methodology, I can make some preliminary conclusions about my four hypotheses based on this systematic review. First, comparison of mean cortisol levels between wild and captive members of the same species provided insight on whether sampling methodology affects the results and demonstrated the importance of implementing new methodology for future studies. Second, comparison of mean cortisol levels from studies on wild Atlantic bottlenose dolphins that were sampled in under one hour to captive studies, assisted in displaying that due to invasive sampling methodology the acute stress response may be activated upon chase initiation and

display elevated levels of cortisol when sampled in <60 minutes. Third, comparison of mean cortisol levels between species assessed if a species may respond to a similar stressor (capture or captivity) in varying degrees. Fourth, analyzing the trends in mean cortisol levels of captive Atlantic bottlenose dolphins assisted in determining if the animals stress levels have declined in a captive environment as captive care has improved due to heightened knowledge of cetacean needs.

Wild vs. Captive Mean Cortisol Levels Within Species

My first research hypothesis was that captive members of the same species would exhibit higher levels of cortisol than their wild counterparts due to the constant presence of anthropogenic stressors. The results actually displayed elevated cortisol in wild species. These results are significant because invasive sampling methodology conducted on the wild cetaceans utilized for this study may be the cause of raised cortisol levels. The invasive nature employed to obtain the sample for cortisol analysis may have increased cortisol before and during the collection process. These results suggest the important of implementing noninvasive sampling methodology to accurately assess resting cortisol levels in wild cetaceans during future studies. Because sampling methodology is such a pronounced confounding variable it would be inaccurate to conclude that captive cetaceans have a lower resting cortisol level than wild cetaceans or to assume that captive cetaceans are “less stressed” than their wild counterparts.

Atlantic Bottlenose Dolphin

The mean cortisol levels were similar in wild studies conducted on Atlantic bottlenose dolphin and in samples collected in captivity via non-husbandry methods. The similar mean cortisol levels throughout these studies may indicate that the stress evoked from wild sample collection methods involving chase, capture, restraint may be similar to the stress that arises from involuntary captive sampling methodology, which includes moving the animals to shallow water (Medway et al., 1970), out of water examinations (Thomson & Geraci, 1986, Orlov et al., 1988;1991), and out of water examinations before or during transport (Copland & Needham, 1999, Noda et al., 2006) even if the animals have been exposed to this activity in the past. The mean cortisol levels expressed during chase, capture, restraint of wild Atlantic bottlenose dolphin and involuntary, non-husbandry practices in captive Atlantic bottlenose dolphins may be exhibiting a mild-moderate stress response depending upon the individual animal (St. Aubin et al., 1990).

Mean cortisol levels obtained from the studies conducted on wild Atlantic bottlenose dolphins were on average higher than mean cortisol levels obtained from the studies conducted on captive Atlantic bottlenose dolphins using both husbandry and non-husbandry sampling methods. These results display the activation of the stress response during chase, capture, and restraint and the difficulty associated with trying to obtain baseline cortisol levels in wild Atlantic

bottlenose dolphins. An even greater difference in mean cortisol levels was found between the mean cortisol levels collected in the studies on wild animals and the mean cortisol levels collected from the animals in captive studies that only employed husbandry sampling. This number may be the greatest display of variation between mean cortisol levels that are collected when the HPA axis is stimulated and when it may not be in Atlantic bottlenose dolphins. This aspect of the study is focusing mainly on comparing mean cortisol levels derived from an acute stressor, so that is not to say that the animals in captivity are not experiencing a chronic stressor in some way. It is just depicting the difference in mean cortisol levels in these animals when different sample collection methods are employed.

The average of mean cortisol obtained from studies on captive Atlantic bottlenose dolphins sampled by husbandry practices was significantly lower than samples obtained from non-husbandry methodology. These results may display that animals of the same species that are often involuntarily exposed to invasive medical procedures may not get conditioned to those actions and still exhibit a stress response when compared to members of the same species who voluntary participate in sample collection. Thomson & Geraci (1986) noted that captive Atlantic bottlenose dolphins that were often exposed to out of water examinations still exhibited higher cortisol levels which are associated with a stress response during these examinations.

Cortisol Collection Time in Wild Atlantic Bottlenose Dolphins

My second research hypothesis stated that based upon findings in previous studies cortisol levels would be lower in cetaceans that were sampled within an hour of chase initiation. The two studies, Ortiz & Worthy (2000) and St. Aubin et al. (1996), that collected cortisol samples in wild Atlantic bottlenose dolphin populations infer that because the samples were collected in under one hour from chase initiation, the acute stress response was not activated to the point where cortisol levels would noticeably increase. Because of that inference the studies hypothesized that their samples may reflect near baseline cortisol levels in wild Atlantic bottlenose dolphins. Ortiz and Worthy (2000) find no significant differences in cortisol levels collected <36 minutes from chase initiation and cortisol levels collected in samples >36-60 minutes from chase initiation (Figure 10).

	Restraint time[a] (min)		Total capture time (min)	
	<20	>20	<36	>36
n	17	11	19	12
AVP (pg/ml)	3.2 ± 0.1	3.6 ± 0.2	3.2 ± 0.1	3.5 ± 0.2
Aldosterone (pg/ml)	228.3 ± 37.8	252.7 ± 37.1	253.5 ± 32.7	199.8 ± 35.3
Cortisol (μg/dl)	2.8 ± 0.2	2.9 ± 0.3	2.9 ± 0.2	2.7 ± 0.3

Figure 10: From Ortiz & Worthy (2000), mean cortisol levels in wild Atlantic bottlenose dolphins sampled within 36 or after 36 minutes from chase initiation.

St. Aubin et al. (1996) stated that when wild Atlantic bottlenose dolphins were sampled within one hour of encirclement their cortisol levels were not statistically significantly different to levels collected from "semi-domesticated" dolphins that were sampled using husbandry practices, even though the only variable that displayed statistical significance in variation of cortisol levels was status (wild or semi-domesticated) (Figure 11).

		Wild (at capture)			Semidomesticated			
Hormone	Sex	*n*	$\bar{x} \pm SD$	Range	*n*	$\bar{x} \pm SD$	Range	Significance
Cortisol (μg/dL)	female	18	2.7 ± 0.6	1.7–4.1	18	1.8 ± 0.7	0.5–3.1	<0.01
	both	36	2.6 ± 0.8	1.2–4.1	36	1.9 ± 0.8	0.5–4.0	<0.01

Figure 11: From St. Aubin et al. (1996), mean cortisol levels in wild Atlantic bottlenose dolphins sampled within an hour of capture.

Although both of these studies collected their cortisol samples within on hour of chase initiation, the average mean cortisol levels were much higher than they were in captive studies were husbandry methodology was employed. The mean cortisol levels from these two wild studies were very similar to mean cortisol levels obtained from captive animals sampled via non-husbandry practices. These results suggest that wild chase, capture, restraint sample collection methodology that occurs in under one hour and involuntary captive sampling methodology elicit similar stress responses in Atlantic bottlenose dolphins and the wild collection methods employed even when sample are collected in under one hour do not reflect baseline cortisol levels in wild Atlantic bottlenose dolphins.

Their hypothesis, which states that if sample collection occurs within an hour after chase initiation the acute stress response is not active enough to display significant increase in cortisol levels, is not supported by the data collected for this analysis. When compared to all data available on mean cortisol levels of wild and captive Atlantic bottlenose dolphins, the compiled data is in fact leaning towards supporting the alternate hypothesis: that the acute stress response is activated to a point where cortisol levels rise within the first 36 minutes of chase initiation until sample collection. The preliminary results from analysis of the data echoes the need for non-invasive wild sample collection methods if a cortisol baseline in wild cetaceans is ever to be established.

Killer Whale

Data on mean serum/plasma cortisol levels in wild killer whales was unavailable and to the best of my knowledge non-existent. One study on fecal glucocorticoids assessed in free ranging killer whales (Ayers et al., 2012) is referenced but cannot be compared to captive killer whale mean cortisol levels due to differences in the hormones assessed. Fecal glucocorticoids analyze the binding rate of cortisol metabolites and serum/plasma measures the binding rate of the parent hormone, cortisol. Trends can be analyzed between the two methodologies but for this analysis that protocol is not applicable. Killer whales were still included in this analysis because of the data present in captive animals that can be used to analyze comparisons between other species of odontocetes kept in captivity.

Beluga Whale

The similar mean cortisol levels between studies of wild beluga whales and the studies of captive beluga whales using non-husbandry practices to collect samples suggests that wild sample collection methods involving chase, capture, and restraint evoke HPA stimulation and cortisol production similar to the stress that arises from involuntary captive sampling methodology, which includes moving the animals to shallow water (Orlov et al., 1991) and out of water examinations (Schmitt et al., 2010). The small variation between samples obtained in captivity via husbandry and non-husbandry sampling methodology could be due to the small sample sizes or a difficulty for these animals to adapt to a captive environment. If a difficulty of adapting to an artificial environment contributes to the higher levels of cortisol in husbandry sampled captive beluga whales is in complete dissonance with Orlov et al.'s (1991) prediction that because beluga whales are exposed to a greater numbers of climatic and nutritional stressors they would display greater adaptation to stressful situations than other cetaceans. In contrast, beluga whales display the highest mean cortisol levels recorded in this review when sampled via husbandry protocol in captivity.

Averages of mean cortisol levels were much higher in the wild studies when compared to captive studies that employed husbandry sampling. These results display the difficulties in attempting to obtain baseline data in wild and captive beluga whale populations due to invasive sampling methodology.

Baseline cortisol levels in wild beluga whales will not be established through currently employed invasive serum/plasma collection methods. This information also aides in challenging the few studies (St. Aubin et al., 1996, Ortiz & Worthy, 2010) that suggest that baseline cortisol samples are able to be obtained from wild cetaceans if sampling is completed in under one hour, before cortisol levels are thought to peak in cetaceans exposed to acute stressors (Thomson & Geraci, 1986, St. Aubin et al., 1996)

Wild vs. Captive Mean Cortisol Levels Among Species

My third research hypothesis predicted that mean cortisol levels would vary among the different species being studied. A prediction that larger cetaceans that are seemingly less likely to adapt well to captivity would display lower levels of mean cortisol was not observed in this review. No significant difference was found between mean cortisol levels of any of the three species living in captivity and wild when compared to each other[20]. My study suggests these three species of odontocetes may have similar levels of circulating cortisol and adapt in a similar manner to sample collection in the wild and captivity. Some studies have suggested a negative correlation between size of animal and cortisol production, but that was not apparent in this analysis.

Utilizing descriptive statistics such as mean cortisol levels and standard deviation of the means to detect differences and similarities of mean cortisol

[20] Killer whales were not included because of lack of data.

levels between species of odonotocete may assist in assessing adaptation to stressors in wild and captive members of these species. It is important in both wild and captive environments to be aware of how sensitive to stressors cetaceans are and by comparing mean cortisol levels between three species of odontocetes it may be possible to assess if one species is more reactive to stressors than others, or if one species may naturally produce higher of lower levels of cortisol than another.

Mean cortisol levels obtained from wild studies are higher than cortisol levels obtained in captive studies as a whole, in both beluga whales and Atlantic bottlenose dolphins. (Killer whales are data deficient in wild studies). Wild sampled beluga whales display the highest mean cortisol levels among wild studies (range: 90-125 nmol/L) and captive beluga whales display the highest mean cortisol levels among the captives species studied (range: 27.6-82.6 nmol/L). Captive killer whales sampled for cortisol concentrations displayed the lowest mean cortisol levels of all wild and captive species sampled (5.5-9.7 nmol/L).

Wild and Captive Atlantic Bottlenose Dolphin and Beluga Whale

Although mean cortisol levels obtained from studies on wild beluga whales were on average greater than levels in wild Atlantic bottlenose dolphins, a

difference is not detectable. These results suggest that chase, capture, restraint methodology employed for blood serum/plasma collection activates the HPA axis and increases cortisol levels in both beluga whales and Atlantic bottlenose dolphins, two species of cetacean that are often researched in the wild and captured for use in captive displays. Even though beluga whales seem to display higher cortisol levels in almost all wild and captive studies a larger variation in average mean cortisol levels is found in wild beluga whales and wild Atlantic bottlenose dolphins. When captive members of these species are sampled the variation decreases. The similarities in mean cortisol levels between captive belugas that were sampled via husbandry practices and captive Atlantic bottlenose dolphins that were sampled via husbandry practices, and captive belugas that sampled by non-husbandry practices and captive Atlantic bottlenose dolphins that were sampled by non-husbandry practices help display that both of these species of captive cetacean produce similar levels of cortisol when kept in a captive environment. Variation in cortisol production due to sampling methodology reflects similar trends in changes among these species.

Captive Killer Whale and Atlantic Bottlenose Dolphin

Captive killer whales display significantly lower mean cortisol levels when compared to captive Atlantic bottlenose dolphins that were sampled through voluntary husbandry practices. One of the original hypotheses stated that larger

odontocetes may not adapt to captivity as well as smaller members of that family, but mean cortisol levels obtained from all the animals included in captive husbandry sampling were 7.66 nmol/L in killer whales and 30.6 nmol/L in Atlantic bottlenose dolphins. This was the only aspect of species to species comparisons that yielded significant results. The original hypothesis was still rejected even though these results were yielded because the smaller species, Atlantic bottlenose dolphin displayed higher mean cortisol levels than the larger, killer whale species. It is important to note that both killer whales and Atlantic bottlenose dolphins are in the same family, Delphinidae, and these results may assist in conveying that cetacean species within the same family may either produce different amounts of cortisol, react to stressful situations in different manners, or display different abilities of adaptation to captivity. It is also important to note that in 2/3 studies (Lyamin et al., 2005, Suzuki et al., 2003) 4/6 animals were pregnant which could influence mean cortisol levels.

Captive Beluga Whale and Killer Whale

Average mean cortisol levels were greater in captive beluga whales when compared to captive killer whales when animals were sampled using husbandry methodology, although the numbers were not significantly different. These results may assist in corroborating Orlov et al.'s (1991) hypothesis which states that cetaceans that are exposed to frequent climatic and dietary stressors may possess an ability to adapt to other stressors, such as introduction to a captive

environment, better than other species. Killer whales and beluga whales are in different families of odontocetes, killer whales a member of Delphinidae and beluga whales a member of Monodontidae. Both species are larger than Atlantic bottlenose dolphin and can inhabit polar seas, whereas Atlantic bottlenose dolphins reside is tropic and subtropical climates (Perrin et al., 2002). The data displays that the mean cortisol levels from nine killer whales were the lowest (7.66 nmol/L) and mean cortisol levels from the ten beluga whales were the highest (38.65 nmol/L). It is also important to note that in 2/3 studies on killer whales (Lyamin et al., 2005; Suzuki et al., 1998) 4/6 animals were pregnant which could influence mean cortisol levels.

Captive Killer Whales, Beluga Whales, and Atlantic Bottlenose Dolphins

Captive killer whales tend to display lower mean cortisol levels when sample collection involved voluntary husbandry practices when compared to captive Atlantic bottlenose dolphins and captive beluga whales. A negative correlation between body size and cortisol production has been proposed (Suzuki, personal communication), but not conclusively confirmed. This correlation was not observed in this review. If greater body mass does correlate with lower cortisol levels, lower cortisol levels would be expected to be found in captive beluga whales and higher cortisol levels in captive Atlantic bottlenose dolphins. The opposite was observed in this review, although small sample sizes could have

skewed results. More research should be completed to analyze this possibility of a correlation between cortisol production and body mass in cetaceans.

Orlov et al. (1991) proposed that animals that are exposed to frequent climatic and dietary extremes might possess heightened adaptation ability to a captive environment. This hypothesis may explain why killer whales show lower levels of mean cortisol when sampled through husbandry methodology in a captive environment, although one would assume that beluga whale would also possess this trend, which they do not. Beluga whales displayed the highest levels of mean cortisol when husbandry sampling methodology was employed (38.65 nmol/L), followed by Atlantic bottlenose dolphins (30.55 nmol/L), and killer whales (7.66 nmol/L). Wild beluga whales also displayed the largest mean cortisol levels (103.6 nmol/L) followed by Atlantic bottlenose dolphins (83 nmol/L). Studies on cortisol levels in wild killer whales are data deficient. Studies that minimize confounding variables, especially stimulation of the HPA axis during sample collection, must be implemented to more accurate assess cortisol along with other stress hormone levels in wild and captive cetaceans before accurate conclusions can be drawn.

Trends in Mean Cortisol Levels Throughout Time in Captivity

My fourth research hypothesis stated that a declining trend in cortisol levels should be observed in captive Atlantic bottlenose dolphins through time. All studies that obtained mean cortisol levels in captive Atlantic bottlenose

dolphins that were conducted before 1996 employed non-husbandry sampling methodology. Most of these studies yield mean cortisol levels that are higher than studies post-1996 that employed husbandry-sampling techniques. The range of mean cortisol levels between 1970 and 1992 is 35-90.3 nmol/L. The range of mean cortisol levels between 1996-2012 is 0-160 nmol/L. A positive or negative trend in studies conducted on captive Atlantic bottlenose dolphins that analyzed mean cortisol levels between 1996 and 2012 could not be detected. All studies included in the analysis employed husbandry sampling methodology and displayed a range of 15.5-69 nmol/L.

The lack of significant differences found in mean cortisol levels of captive Atlantic bottlenose dolphins over a study time of 1970-2012 may help combat the claim that captive marine mammal facilities have minimized stressors throughout years by learning more about captive care and animal enrichment over time. All of the studies up until 1996 employed non-husbandry sampling, which most likely contributes to the elevated cortisol levels in those studies. When the studies that utilized non-husbandry methodology were eliminated from the figure, a definitive decreasing trend is not observed. Two points from the same study in 1999 (Reirdson & McBain) may be viewed as outliers, even though the author's have been contacted to verify sampling protocol. Their results were extremely high when compared to all other studies on captive Atlatnic bottlenose dolphins sampled via husbandry practices. The levels from their study actually visually appear more similar to cortisol levels that were obtained via non-husbandry sampling in other studies utilized in this review. If those two points are eliminated

from the figure the analysis looks even less persuasive. These results, or lack thereof, should be used to display the need for non-invasive stress hormone sampling methodology such as FGC analysis in captive situations used to develop a system to minimize stress for captive cetaceans kept in a restricted and dependent artificial situation.

Findings in Comparison with Other Studies

Thompson & Geraci (1986) found that cortisol concentration in dolphins increased within 10 minutes of capture and peaked at about 90 minutes. Orlov et al. (1988; 1991) noted that an increase of cortisol in bottlenose dolphins occurred within 5 minutes of a stressor and a process of partial stabilization then recurrent intensification occurred 6-12 hours later, with a peak around 30-45 minutes. St. Aubin & Geraci (1989) noted that cortisol levels may increase, but not until after 30 minutes in their study on beluga whales. St. Aubin et al. (1996) and Thompson and Geraci (1986) both noted that cortisol levels don't elevate significantly until 1-2 hours after exposure to a stressor. Differences in mean cortisol levels in bottlenose dolphins typically range from 70-150 nmol/L in wild animals sampled by capture/forced restraint, which are thought to be experiencing a mild stress response (Thomson & Geraci, 1986; St. Aubin et al., 1989), 210-490 nmol/L in stranded animals, thought to be experience a high stress response, and 50 nmol/L in captive animals that are accustomed to handling, thought to be close

to baseline levels (Houser et al., 2011). Thompson & Geraci (1986) proposed that 30-40 nmol/L are baseline cortisol concentrations in bottlenose dolphins and those numbers reflect baseline cortisol levels in dogs and rabbits (Thompson & Geraci, 1986).

Complications in using Cortisol as a Primary Indicator of Stress

Confounding variables and natural variation that are thought to effect cortisol levels in cetaceans are age, sex, reproductive status, wild or captive status, season, and time of day. The sample collection method and analysis procedure can also cause variability in results. Although there are arguments for and against the influence of these variables I have tried to minimize them in this study by using only mean cortisol levels and studies that have used similar methodology and analysis. Studies and aspects of studies that were attempting to artificially activate acute stress in cetaceans were excluded from this analysis.

Orlov et al. (1991) found that bottlenose dolphins exhibited higher levels of cortisol in the winter and spring than in summer and fall. The reason for variability in cortisol levels within seasons may come from evolutionary adaptations for feeding, with high levels of glucocorticoids having the ability to inhibit feeding in animals anticipating energy declines (Houser et al., 2011). Beluga whales that often face harsh climatic conditions in the Arctic have displayed a seasonal trend as well. Orlov et al. (1991) discovered higher levels of

cortisol in 10 beluga whales captured off Russia in the spring and winter months. Suzuki et al. (2003) found similar seasonality trends in captive male killer whales exhibiting higher cortisol levels in winter than summer months. Concurrently, St. Aubin et al. (1996) found that season did not have an effect on cortisol levels in 18 wild and 18 semi-domesticated bottlenose dolphins.

Age is thought to play a part in cortisol production in cetaceans. Suzuki (unpublished data) has observed trends of lower cortisol levels in older animals. In a study conducted by Ortiz et al. (2010) two captive male bottlenose dolphins ages17 and 23, displayed undetectable levels of cortisol in each of their 16 samples. The hypothesis for these results is adrenal atrophy though it is unlikely that age played a large role because the mean lifespan of bottlenose dolphins is around 45 years (Perrin et al., 2002). St. Aubin et al. (2001) found no age related differences in cortisol levels in 115 wild caught belugas.

Cortisol production in cetaceans has been found exhibit diurnal circadian trends similar to humans. Studies have shown evidence of higher cortisol levels in the morning with lowest levels in the evening in killer whales (Suzuki et al., 1998; 2003). Beluga whales were found to display consistently higher cortisol levels in morning hours than evening hours (St. Aubin et al., 2001;Schmitt et al., 2010). The lowest levels of cortisol how been displayed between noon and midnight in beluga whales (St. Aubin & Ridgeway, unpublished data) and killer whales (Suzuki et al., 1998).

Although cortisol levels are thought to differ by sex no statistically significant results have been produced. St. Aubin et al. (1996) found no difference in cortisol levels obtained from 36 samples of 18 wild and 18 semi-domesticated dolphins. It may be plausible to attribute differences in cortisol levels between males and females to social ranking. Killer whales for example, exhibit a matriarchal society, Suzuki et al. (1998;2003) found that of the three animals sampled (2M:1F) the smaller, less dominant male showed less variability in cortisol production throughout the diurnal and seasonal study. More research will have to be done to establish conclusive results.

Wild or captive status can play a large part in fluctuating cortisol levels in cetaceans. As noted in the methodology section wild animals are often sampled via chase, capture, and restrain methods that is likely to elicit a stress response (Tryland et al, 2006, St. Aubin et al., 1996). Ortiz & Worthy (2000) found no difference in mean cortisol concentration of wild bottlenose dolphins sampled below 20 minutes to 40 minutes of capture. This could be due to the fact that cortisol levels are thought to peak in cetaceans between 1-2 hours of introduction to a stressor (Thomson & Geraci, 1986). St. Aubin et al. (1996) found no differences in cortisol levels relating to age or sex in his study conducted on 18 wild and 18 semi-domesticated dolphins, but did find variance between the wild and captive animals. The wild animals displayed higher cortisol levels, which may be due to the methodology used to obtain the sample.

Thompson & Geraci (1986) found that even captive animals that were constantly exposed to handling displayed higher levels of cortisol when being

handled. Noda et al. (2006) and Reirdson & McBain (1999) found that transporting captive bottlenose dolphins causes elevated cortisol levels. Naka et al. (2007) found that bottlenose dolphins increased their cortisol level when their pools were being drained before sample collection. Spoon and Romano (2012) found that in four beluga whales that were transported from one facility to another displayed elevated levels of glucocorticoids that were statistically significant from baseline samples taken before transport; arrival samples taken upon arrival to the new facility; and acclimation samples taken 5-6 months after arrival at the new facility. Orlov et al. (1991) did find that 10 wild caught beluga whales that showed elevated cortisol levels in the first four days of being captive, stabilized on the 11th day. In the same study he found that wild caught bottlenose dolphins that were held in captivity for 7 days showed a peak in cortisol levels at 24 hours that began to decrease from day 3-4, never reached the established "normal" levels for the animals being held.

Due to the low values found in cetaceans along with the narrow range and small fluctuations among stressors the ability for cortisol to be used as an absolute value of stress in cetaceans may be unreliable (St. Aubin & Dierauf, 2001). Studies on dolphins and beluga whales have displayed that the bound fraction of cortisol that is normally measured in standard immunoassays is ≤50% of the total hormone (St. Aubin & Geraci, unpublished data). The small amounts of bound hormone may mean that greater quantities of free cortisol are circulating through the body and able to cause greater effects on the animal (St. Aubin & Dierauf, 2001). The measurement of cortisol levels along with other stress indicative

blood parameters such as aldosterone levels, and lymphocyte and eosinophil counts, and behavioral observations may be the best method to clearly evaluate stress in cetaceans (St. Aubin & Dierauf, 2001).

Although cortisol is still used to gauge the effects of acute stressors on cetaceans it may be a more reliable gauge of chronic stress. A hypothesis exists that questions the ability of the cetacean adrenal gland to only store and produce small amounts of cortisol within a limited timeframe (Schmitt et al., 2010). If that hypothesis is correct it may not be possible to use cortisol levels to reliably measure acute stress. Fecal glucocorticoid measurements may be the most accurate way to monitor chronic stress in cetaceans due to the noninvasive sampling methodology and less variable fluctuations associated with them (Washburn, 2004). The importance of shifting to noninvasive sampling methods for stress hormone analysis in cetaceans should not be overlooked. Stress can be induced by human presence and increase with invasive methodology (Ortiz & Worthy, 2000; Tryland et al., 2006; St. Aubin et al., 1989). Since cortisol levels in cetaceans may begin to rise within 10 minutes of perceived stressor (Orlov et al., 1988; Thompson & Geraci 1986), results for baseline cortisol levels may become skewed. Baseline cortisol levels are important to be able to analyze environmental and anthropogenic impacts on the stress of wild and captive cetaceans. The shift towards FGC measurement and standard immunoassay analysis would allow for the creation of a collaborative database where comparisons could accurately be made and a better understanding of how

numerous stressors impact the health and prosperity of numerous cetaceans species throughout the onset of global climate change.

Complications in Methodology

When analyzing data on stress hormones in cetaceans it is important to note many confounding variables and try to reduce them as much as possible. The individual stress reaction of each animal will vary (Orlov et al., 1988; Esch et al., 2009; Houser et al., 2011), seasonal hormone fluctuations have been noted in several studies (Orlov et al, 1991; 1988; Suzuki et al., 1998; 2003), and the methodology of sample collection has the ability to alter results (Tryland et al., 2006; Schmitt et al., 2010; St. Aubin & Geraci, 1989). An ongoing disagreement about when cortisol levels begin to increase in response to an acute stress response in cetaceans is prevalent in the literature. Cortisol levels in cetaceans are thought to increase from within 5 minutes of a perceived stressor according to some researchers (Orlov at al., 1988), not until 35 minutes after a stressor is encountered (Thomson & Geraci, 1986, St. Aubin & Geraci, 1989), up to an hour after chase initiation (St. Aubin et al. 1996). This implication has the ability to affect all serum and plasma cortisol samples obtained from wild cetaceans whenever beginning of chase to sampling time is greater than five minutes. All wild studies utilized in this systematic review list chase to sample collection time as >10 minutes (St Aubin & Geraci 1989;1992: St. Aubin et al., 2001; Tryland et

al., 2006; Ortiz & Worthy, 2000; St. Aubin et al., 1996; Thomson & Geraci, 1986). It has been noted that mean cortisol concentrations in wild captured beluga whales correspond to mean cortisol concentrations of captive beluga whales placed in a stretcher for blood sampling, an act that is categorized as a "moderate" stressor (Schmitt et al., 2010). Thomson & Geraci (1986) found that mean serum cortisol levels in wild dolphins were similar to levels obtained in captive dolphins following a simulated calm- and chase capture.

It is important to monitor stress hormones of cetaceans in the wild. The knowledge gained from such studies could lead to better management practices and conservation efforts (Ortiz & Worthy, 2000). Although, it is thought that some of the current methodology employed could skew results due to activation of the HPA axis prior to and during sample collection, noninvasive stress hormone methodology exists in the form of fecal glucocorticoid monitoring. Although several populations of wild dolphins are continuously monitored via chase, capture, release methodology, including the Sarasota resident population monitored by the Sarasota Dolphin Research Project out of MOTE Marine Laboratories, studies have shown that even captive dolphins that are consistently exposed to the medical practice of being taken out of their tank for evaluation still display signs of HPA axis activation in the form of elevated cortisol (Thomson & Geraci, 1986). If the HPA axis is producing glucocorticoids in response chase, capture, release methodology it may be possible that baseline cortisol levels are not plausibly obtained from this practice (Mancia et al., 2008). Several studies advise that the sample collection methodology may cause an increase in cortisol

levels prior to sample collection (Tryland et al., 2009; Orlov et al., 1988; St. Aubin & Geraci, 1989; Schmitt et al., 2010).

Some studies indicate that if sample collection occurs within 40 minutes of chase the sample does not indicate a physiological stress response (Ortiz & Worthy, 2000; St. Aubin et al., 1996). In my analysis of 12 captive studies on cortisol levels using similar plasma/serum collection obtained during routine husbandry behavior and analyzed by comparable immunoassays on Atlantic bottlenose dolphin (*Tursiops truncatus*), mean cortisol levels are significantly higher in the two wild studies. The mean cortisol level and standard deviation in 83 individual animals obtained from 186 samples was 75.5±21.54 nmol/L (5.9±6.5 μg/dl) (St. Abuin et al., 1996; Suzuki et al., 1998; Reidarson & McBain, 1999; Reidgeway et al., 2006; Pedernera-Romano et al., 2012; Naka et al., 2007, Ridgeway et al., 2009; Houser et al., 2011, Balsio et al., 2012; Suzuki & Komaba, 2012), while the mean plasma/serum cortisol levels obtained from a total of 67 wild bottlenose dolphins from Ortiz & Worthy (2000) and St. Aubin et al. (1996) was 74.5±4.0 (2.7±0.1 μg/dl). These wild sampling methods may have the ability to physiologically impact the animals utilized for these studies (Mancia et al., 2008). We are unsure of the degree to which these animals could be impacted in their daily routines post-sampling. In captive studies where animals were exposed to an acute stressor cortisol levels have retuned to baseline levels within 24 hours; in studies where wild animals were brought into a captive environment it has taken up to 11 days (Orlov et al., 1991). Field studies conducted on wild cetaceans most likely do not reflect resting cortisol levels (Schmitt et al., 2010),

which makes comparisons of these results difficult. Although sampling stress hormone sampling of wild cetaceans is important to monitor, especially in threatened or endangered populations, it is important to continue to attempt to determine the effects of capture on the wild animals after sampling occurs (Ortiz & Worthy, 2000).

Captive animals are exposed to numerous stressors that differ from wild animals but have the same ability to lower immune system function (Noda et al., 2006). Stress levels in captive animals can originate during the chase and capture process, which has been shown by wild chase, capture, and release studies (St. Aubin & Geraci, 1989;1992, St. Aubin et al., 2001). It may continue through the transportation process, where cortisol levels have been shown to elevate (Noda et al, 2006; Copland & Needham, 1992), and into the introduction to new environmental and social parameters, where belugas that were transported from one facility to another showed increased cortisol levels until six months after introduction to the new facility (Spoon & Romano, 2012). The decreased immune function resulting from increased cortisol levels has the ability to lower several types of white blood cells and increase the risk of infection (Noda et al., 2006; Thomson & Geraci, 1986; St. Aubin & Dierauf, 2001). Stress hormone monitoring is important because elevations in cortisol levels are a precursor to decreased levels of lymphocytes and esponophils which can lead to infection (St. Aubin & Dierauf, 2001), which is one of the primary causes of death in captive dolphins kept in aquariums (Medway, 1980).

In captivity sampling methodology differs greatly from wild sample collection. Often animals are trained to perform a husbandry technique such as displaying their fluke for blood sample collection. Some animals that are newly captive, young, or disobedient may have to be lifted from their enclosure for blood sampling to occur. The act of lifting the animal from their tank for sample collection often excites the HPA axis and increases glucocorticoid production (Schmitt et al., 2010; Thomson & Geraci, 1986). The increased cortisol levels associated with HPA axis activation when animals are not conditioned to husbandry sampling practices may represent a mild to moderate stress response (Schmitt et al., 2010). The data that most likely represents true baseline cortisol levels in captive animals is sampled from long-term captive residents that have been trained to present the sample collection site during husbandry practices with seasonal and diurnal trends noted (Suzuki et al., 2003).

In captivity serum constituents including cortisol levels may be influenced by several environmental and social factors such as diet, forced human interaction, social conflict, human dictated movements, or illness (Waples & Gales, 2002). Adaptation is an important factor to evaluate in captive cetaceans (Orlov et al., 1988). If the animals cannot adapt to restricted living space, changes in character of nutrition, along with constant human interaction and dependence they may be subject to chronic stress (Orlov et al., 1988; Blasio et al., 2012). Captive studies also are limited by their low sample sizes especially for captive killer whales and beluga whales (Schmitt et al., 2012). Even in Atlantic bottlenose dolphins, which are relatively prevalent in captive facilities with

numbers estimated as over 3,000 worldwide, it is often difficult to coordinate studies between different facilities (Schmitt et al., 2010). It is known that stress in animals causes behavioral changes, which could lead to mental and physical health issues (Waples & Gales, 2002). To implement a continuous stress hormone monitoring program in captive environments where animals are susceptible to infection and disease, it is crucial to have alternative, non invasive procedures to establish baselines and detect fluctuations in cortisol levels which could be used to monitor immune system health (Pedernera-Romano et al., 2006). Fecal glucocorticoid monitoring has the ability to be implemented in both wild and captive environments so baseline cortisol levels could be logged in a database and made available to all scientists and animal caretakers.

Complications in Immunoassay Analysis

Although all of the RIA's, CLIA's, EIA's, and TR-FIA's aside from the Porter-Sibler chromogens tests have be confirmed to display comparable levels of cortisol in cetaceans, it is important to be aware of the differences in detection of the machine, antibody used, and possible contamination during processing. RIA's have been the most commonly used assay to measure free cortisol levels in cetaceans a shift to the other assays is taking place because of the risk and hassle of working with and disposing radioactive isotopes. CLIA's and EIA's are being employed more frequently than RIA's currently due to their high sensitivity and the ease of operation. Liquid chromatography-tandem mass spectrometry (LC-

MS/MS) is starting to be used in cortisol analysis because of its highly sensitive and specific tests developed for cortisol measurement, but the high cost and labor-intensive process is not widely utilized at the moment (Babic et al, 2011).

When choosing and comparing the appropriate assay to measure cortisol levels it is important to perform parallelism tests between standard and serum samples along with tests on intra- and inter-assay coefficients of variation and spike recovery tests. It is also important to note the assay sensitivity to cortisol. Many studies lack this important detail in their publications, which can be an important factor when some samples are listed as an amount of cortisol that is not detectable. You will also have to determine if the cortisol levels in the matrices you are examining are comparable. Serum and plasma samples are directly comparable to each other, saliva cortisol levels are 27% of serum/plasma levels in bottlenose dolphins (Pedernera-Romano, 2006), which is similar to the saliva:serum/plasma cortisol ratio in humans. Doing matched tests on the same animals allows comparisons to be made.

Fecal cortisol levels are not directly comparable to serum/plasma and saliva samples because cortisol metabolites are metabolized in the liver before they can be measured in the samples (Wasser et al., 2000). The most accurate way to compare fecal cortisol levels to cortisol levels collected in other matrices is by comparing trends (Wasser, personal communication). Fecal glucocorticoid metabolites (FGM) may be a more accurate way to assess effects of stress that are accumulated over time and can be analyzed by current commercially available RIA kits. The unique ability to assess the effects of stress over time is unable to

be assessed in single blood samples that along with being invasive also are not able to be used in a constant manner to assess the cumulative effects of stress in animals (Wasser et al., 2000). When measuring FGM one must take into account the lag time for the species being sampled. In cetaceans the lag time is ~24 hours (Ayres, personal communication). The nature of collecting FGM in both wild and captive environments would be less likely to elicit a stress response than chase capture and even husbandry methods. In the wild animals can be trailed by up to ¾ of a mile and samples can be collected from the surface (Ayres et al., 2012) or in tropical and subtropical climates inversion collection can occur below the waters surface (Parsons et al., 2003) A transition to non-invasive FGM method for analyzing cortisol levels in cetaceans living both in captivity and the wild may provide the best baseline for normal resting cortisol levels in these animals living in both a wild and captive environment. A long-term daily monitoring program of captive cetaceans could be implemented due to non-invasive methodology (collecting feces from the bottom of the tank or from the filter) and could lead to better care and health monitoring.

CHAPTER 6

CONCLUSION

Conclusions Drawn from Results

Although it wasn't an anticipated conclusion of this analysis, sampling methodology played a large role in elevation of mean cortisol level in wild and captive Atlantic bottlenose dolphins, killer whales, and beluga whales. Mean cortisol levels obtained from blood samples during wild studies that employed a chase, capture, restraint protocol were strikingly similar to mean cortisol levels obtained from involuntary non-husbandry blood sample collection in captive animals. Significant differences were found between mean cortisol levels obtained via husbandry and non-husbandry practices for captive Atlantic bottlenose dolphins and beluga whales. Studies conducted on captive killer whales only employed husbandry practices to obtain blood samples for cortisol analysis so no comparison could be made between husbandry and non-husbandry practices. Mean cortisol levels in the captive killer whales seem to be significantly lower than mean cortisol levels in captive beluga whales and Atlantic bottlenose dolphins that were sampled using similar methodology. These results display the importance of taking sample collection methodology into account when interpreting cortisol levels of wild and captive cetaceans when sampled during chase, capture, restraint and involuntary non-husbandry practices, such as raising the animal out of the water, placing the animal on a stretcher, or moving the animal to shallow water to obtain a sample.

The implications of sampling methodology on cortisol levels in wild sampled and non-husbandry sampled cetaceans may more clearly reflect a mild-moderate stress response than a baseline reading even if samples are collected within an hour of the perceived stressors appearance. Ortiz & Worthy (2000) and St. Aubin et al. (1996) concluded that if sample collection were secured within 30 minutes of the chase in wild Atlantic bottlenose dolphins would not display elevated levels of cortisol indicating HPA axis activity and activation of the acute stress response. If HPA activation was not displayed in wild studies where chase-sample collection occurred within 30 minutes, it is thought the cortisol levels obtained from those studies could reflect baseline cortisol levels of wild animals. It is believed that the most accurate baseline cortisol levels in Atlantic bottlenose dolphins have been obtained during voluntary husbandry sample collection in a captive environment (Suzuki et al., 1998;2003). The results of this analysis found that mean cortisol levels obtained from samples in Ortiz and Worthy (2000) and St. Aubin et al. (1996) that were collected in within 60 minutes of chase were significantly higher when compared to mean cortisol levels from studies that employed husbandry sampling on captive Atlantic bottlenose dolphins. In fact, mean cortisol levels were extremely similar when the levels obtained from Ortiz and Worthy (2000) an St. Aubin et al. (1996) were compared to mean cortisol levels obtained from Atlantic bottlenose dolphins that were sampled following involuntary non-husbandry practices, such as removing the animal from its enclosure or cornering the animal in shallow water.

The results of this analysis suggest that baseline cortisol levels in cetaceans may not reflect accurate levels when obtained by current standard sampling methodology, such as chase, capture, restraint methods in wild animals (Lair et al., 2006, St. Aubin et al., 1996, Thomson & Geraci, 1986). It is also

unknown if cortisol samples obtained from voluntary husbandry sampling methodology in captive cetaceans accurately reflect baseline cortisol levels because many environmental and social factors may conflict or alter results when naturally free-ranging animals are confined (Orlov et al., 1988). The acute stress response in cetaceans has been documented in captive Atlantic bottlenose dolphins and beluga whales in various studies (Orlov et al., 1991, Schmitt et al., 2010, Thompson & Geraci, 1986), but less in known about the chronic stress response, which has the greater likelihood of causing physical and mental impairments (St. Aubin & Dierauf, 2001).

Recommendations for Further Research

When the Marine Mammal Protection act was amended in 1997 a priority was given to establish methodology that would be able to evaluate stress in dolphins (Esch et al., 2009) via The International Dolphin Conservation Program Act (IDCP Act, U.S. Pubic Law 105-42). The priority status of stress evaluation in cetaceans is due to the increasing amount of anthropogenic and environmental stressors introduced into the marine environment. Increased cetacean mortality recently observed coupled with mass stranding events causes concern over the general health of cetaceans, and the impact that other factors such as pollution and disease have on overall stress levels (Esch et al., 2009). Wild collection of animals for captivity has caused entire populations of some cetacean species to become endangered. Keeping cetaceans in captivity may also impact the stress response due to their social nature, intelligence, diverse diets, and large natural

ranges (Southern Resident Killer Whale Recovery Plan, 2006). In many animal species exposure to chronic stress can lead to reproductive problems, immunosuppression and even death (St. Aubin & Dierauf, 2001). In the past, stress hormone analysis has been used to evaluate changes in individual or specific populations of animals to different stimuli (Schmitt et al., 2010) To accurately measure stress in cetaceans non-invasive sample collections methods may need to be implemented (Wasser et al., 2000). Scientists and researchers will only be able to analysis the impacts of environmental and anthropogenic stressors once resting values for healthy animals are established in both wild and captive environments (Esch et al., 2009).

Studies documenting the stress response in cetaceans have been conducted by measuring the presence of cortisol in response to stressors and synthetic stimulants (Thomson and Geraci, 1986, Orlov et al., 1988;1991, St. Aubin & Geraci 1989;1992, St. Aubin et al., 1996;2001;2013, Suzuki et al., 1998;2003, Ortiz and Worthy, 2000, Spoon & Romano, 2010). Stress can be measured in cetaceans by monitoring a variety of hormones and blood parameters other than cortisol. Thyroid hormones (T3 and T4) can be measured to record nutritional stress (Ayres et al., 2012, St. Aubin & Geraci, 1989). St. Aubin & Geraci (1989) discovered that T3 levels were suppressed for a 10-week captive period and that stimulation of ACTH further decreased the levels in beluga whales. Noda et al. (2006) found that when bottlenose dolphins were exposed to an elongated preparatory process (>3 hours) before being transported that the animals exhibited a stress response that was more clearly indicated on the leucogram than

monitoring of cortisol levels. A typical stress leucogram in cetaceans displays a decrease in lymphocytes and eosinophil's. Non-invasive ways to measure stress in cetaceans may be completed by behavioral analysis in both captivity and the wild, although no past studies have been able to correlate cortisol with behavioral state (St. Aubin and Dierauf, 2001). Inconsistencies in what is deemed a "stressed" behavior state may impact proper analysis and cause ambiguity. Castellote and Fossa (2006) discovered that acoustically monitoring captive belugas could be an effective method of monitoring stress and adaptation in captive animals.

Stress hormone analysis, hematology, and behavioral observations have the greatest ability to assess stress in cetaceans when used in tandem. The amalgamation of interdisciplinary practices to create a more accurate core of cetacean stress levels is extremely important. These methods can create a better ability to assess environmental and anthropogenic impacts on wild and captive cetaceans (Spoon and Romano, 2012) and in turn lead to conservation and captive care techniques. Combining different methodologies of stress monitoring in cetaceans and establishing a standard non-invasive method of sample collection and cortisol analysis such as FGM, along with an accepted standard for analysis, may allow for studies to be more accurate compared and the most accurate results yielded. The construction of a FGC database on wild and captive cetacean species would allow for accurate comparisons between species inhabiting different environments and become the first tool to compile true baseline levels of cortisol in these animals.

Appendices

Appendix A: Immunoassay Information Table. Will assist in understanding "Assay Information" tables utilized to detect cortisol levels in Atlantic bottlenose dolphins, killer whales, and beluga whales.

Immunoassay	Abbreviation	Detection Method
Radioimmunoassay	RIA	Radio-active
Enzyme immunoassay	EIA	Photometric
Enzyme-linked immune sorbent assay	ELISA	Photometric
Chemiluminscence immunoassay	CLIA	Luminescence
Electrochemiluminescent immunoassay	ECLIA	Luminescence
Immunochemiluminescence assay	ICMA	Luminescence
Chemiluminescentenzyme immunoassay	CLEIA	Luminescence
Time resolved flurometric immunoassay	TR-FIA	Flurometric

Appendix B: Atlantic Bottlenose Dolphin Cortisol Studies, Assay information. Author (s) of original study on cortisol level, sample type, assay method, assay producer, location of where the assay was produced, analytical assay sensitivity in µg/dl and nmol/L.

Author	Sample type	Assay Method	Assay System/Kit	Producer	Location	Sensitivity µg/dl	Sensitivity nmol/L
Thomson & Geraci, 1986	**serum**	**RIA**	**I ^125**	**New England Nuclear**	**Boston, Mass**	**0.36**	**10**
St. Aubin et al., 1996	**serum**	**RIA**	**Immulite**	**Intermedico***	**Willowdale, Ontario**	**0.2**	**5.5**
Ortiz & Worthy, 2000	**plasma**	**RIA**	**Immulite**	**DPC***	**L.A., Cali**	**0.2**	**5.5**
Medway et al., 1970	plasma	Silber-Porter	Chromogens Test				
Thomson & Geraci, 1986	serum	RIA	1 ^125	New England Nuclear	Boston, Mass	0.36	10
Orlov et al., 1988	serum	RIA	Kort-I-H^3		USSR or France		
Orlov et al., 1991	serum	RIA	Kort, I ^125	IBOKH	Sorin, France	0.36	10

Copland & Needham, 1992	plasma	RIA	Amerlex	Amersham Pty. Ltd	New South Wales, AUS	0.4	11
St. Aubin et al., 1996	serum	RIA	Immulite	Intermedico*	Willowdale, Ontario	0.2	5.5
Suzuki et al., 1998	serum	RIA	FKA404 antibody	Cosmo Bio	Tokyo, Japan	0.01	0.28
Reidarson & McBain, 1999	serum	ICLIA		Chiron Diagnostics	East Wapole, Mass		
Reidarson & McBain, 1999	serum	ICLIA		Chiron Diagnostics	East Wapole, Mass		
Noda et al., 2006	plasma	ECLIA	rabbit antibody	Roche Diagnostics	Indianapolis, Indiana	0.04	1
Ridgeway et al., 2006	serum	RIA	Immulite	DPC*	L.A., Cali	0.2	5.5
Pedernera-Romano et al., 2006	serum	RIA	I ^125,Cort CT2	CIS Biointernational	France	0.24	6.6
Naka et al., 2007	plasma	EIA		Caymen Chemicals	Ann Arbor, USA	0.0035	0.1
Ridgeway et al., 2009	serum	RIA	Immulite	DPC*	L.A., Cali	0.2	5.5
Ortiz et al., 2010	plasma	RIA	Immulite	DPC*	L.A., Cali	0.2	5.5
Houser et al., 2011	serum	RIA	TKCO1 (Coat-a-count)	Seimens Healthcare Diagnostics	Deerfield, Ill	0.15	4.2
Blasio et al., 2012	serum	RIA	I^125,Cort CT2	Bio International	France	0.24	6.6
Blasio et al., 2012	serum	RIA	I^125,Cort CT2	Bio International	France	0.24	6.6
Suzuki & Komaba,, 2012 (UP)	serum	TR-FIA	DELFIA system	PerkinElmer	Waltham, Mass.	0.02	0.55

Appendix C: Killer Whale Cortisol Studies, Assay information. Author (s) of original study on cortisol level, sample type, assay method, assay producer, location of where the assay was produced, analytical assay sensitivity in µg/dl and nmol/L.

Author	Sample type	Assay Method	Assay System/Kit	Producer	Location	Sensitivity µg/dl	Sensitivity nmol/L
Ayres et al., 2012	fecal GC metabolites	RIA	I 125 corticosterone	Biomedicals	Costa Mesa, CA	0.8	22.1
Suzuki et al., 1998	Serum	RIA	FKA404 antibody	Cosmo Bio	Tokyo, Japan	0.01	0.3
Suzuki et al., 2003	Serum	RIA	FKA404 antibody	Cosmo Bio	Tokyo, Japan	0.01	0.3
Lyamin et al., 2005	Plasma	RIA	I 125 cortisol	Biomedicals	Costa Mesa, CA	0.17	4.7
Lyamin et al., 2005	Plasma	RIA	I 125 cortisol	Biomedicals	Costa Mesa, CA	0.17	4.7

Appendix D: Beluga Whale Cortisol Studies, Assay information. Author (s) of original study on cortisol level, sample type, assay method, assay producer, location of where the assay was produced, analytical assay sensitivity in µg/dl and nmol/L.

Author	Sample type	Assay Method	Assay System/Kit	Producer	Location	Sensitivity µg/dl	Sensitivity nmol/L

St. Aubin & Geraci, 1989	Plasma	RIA	I^125	New England Nuclear	Boston, MA	0.36	10
St. Aubin & Geraci, 1992	Plasma	RIA	Immulite	Intermedic o*	Willowdale, Ontario	0.2	5.5
St. Aubin et al., 2001	Plasma	RIA	Immulite	Intermedic o*	Willowdale, Ontario	0.2	5.5
Tryland et al., 2006	Serum	CLEIA	Immulite 1000 System	DPC*	L.A., CA	0.2	5.5
Orlov et al., 1991	serum*	RIA	Kort, I^125,	IBOKH	Sorin, France		
Schmitt et al., 2010	serum/plasma	CLEIA	IMMULITE Coat-A-Count	DPC*	L.A., CA	0.2	5.5
Spoon & Romano, 2012*	Serum	CLEIA	Immulite	Siemens	L.A., CA	0.2	5.5

Appendix E: Confounding Variables Information Table, Abbreviation Key. Assistance in understanding the "Confounding Variables" tables for Atlantic bottlenose dolphins, killer whales, and beluga whales.

Captive Environment	Abbreviation
Indoor enclosure	ID
Outdoor enclosure	OD
Origin	Abbreviation
Wild caught	WC
Captive born	CB

Appendix F: Atlantic Bottlenose Dolphin Cortisol Studies, Confounding Variables. Author (s) of original study on cortisol levels, mean cortisol levels in nmol/L and µg/dl, season or month the study was conducted in (months in numeric form), age of the animal (s) sampled, sex of the animal (s) sampled, if the captive animals were kept in an indoor (ID) or outdoor (OD) enclosure, time it took for sample to be collected (in wild studies from initiation of chase until sample collection), time spend in captivity, origin of captive animals (wild caught or captive born), time of day when samples were collected, any significant notes listed in each study.

Author	Mean F nmol/L	Mean F µg/dl	Season	Age	Sex	ID/OD	Time to Sample	Time in Captivity	Origin	AM: PM	Notes
Thomson & Geraci, 1986	100	3.6	10				up to 5 hours				
St. Aubin et al., 1996	71.7	2.6	year round		18 M:18F		23-260 mins				
Ortiz & Worthy, 2000	77.3	2.8			M17:F14		<20-40 mins				
Medway et al., 1970	66.2	2.4			3M:5F	ID					NH
Thomson & Geraci, 1986	35	1.3	June	4	F	OD	w/in 10 mins	3	WC	both	lifted

Orlov et al., 1988	90.34	3.3	year round			OD					lifted
Orlov et al., 1991	90.3	3.3	year round			OD					lifted
Copland & Needham, 1992	90	3.3		6 mos->10 yrs	4M:2F		before transport				lifted and diazapam
St. Aubin et al., 1996	52.4	1.9	year round	4 -33	18M:18F	OD		varied	WC and CB	both	
Suzuki et al., 1998	10.5	0.38	9-12	Mature	M:F	OD		15 & 11	WC	AM	
Reidarson & McBain, 1999	63.5	2.3		12-18		OD		life	CB	both	clinically normal
Reidarson & McBain, 1999	69	2.5		10, 13	M	OD		life	CB	both	
Noda et al., 2006	160	5.8		mature			2-3 hours after drained	>5 years	WC		raised to get sample
Ridgeway et al., 2006	14.3	0.52		21	M	OD				both	
Pedernera-Romano et al., 2006	19.7	0.7		4-11				1-5 years		both	3 not husbandry
Naka et al., 2007	10.2	0.37		4-22		OD					
Ridgeway et al., 2009	44.1	1.6	3	26	F	OD				both	
Ortiz et al., 2010	u.d.	u.d.	summer	17 , 23	M				WC		
Houser et al., 2011	15.49	0.56	4-6	39, 35	1M:1F	OD		>18 years	WC	750-10 A	
Blasio et al., 2012	15.5	0.56		5-~20	2M:4F	OD		5-~20	5 WC	9-10 AM	
Blasio et al., 2012	16.3	0.59		5-~20	2M:2F	ID		5-~20	2 WC	9-10 AM	
Suzuki & Komaba, 2012 (UP)	35.87	1.3			F	OD		>1	WC		

Appendix G: Killer Whale Cortisol Studies, Confounding Variables. . Author (s) of original study on cortisol levels, mean cortisol levels in nmol/L and µg/dl, season or month the study was conducted in (months in numeric form), age of the animal (s) sampled, sex of the animal (s) sampled, if the captive animals were kept in an indoor (ID) or outdoor (OD) enclosure, time it took for sample to be collected (in wild studies from initiation of chase until sample collection), time spend in captivity, origin of captive animals (wild caught or captive born), time of day when samples were collected, any significant notes listed in each study.

Author	Mean F nmol/L	Mean F µg/dl	Season	Age	Sex	ID/OD	Time to sample	Time in captivity	Origin	AM:PM	Notes
Ayres et al., 2012			5-10		M72:F66		n/a	n/a			
Suzuki et al., 1998	5.52	0.2	9-12	2 mat:1 unmat	2M:1F	OD		≥10 years	WC	both	
Suzuki et al., 2003	5.52	0.2	year round	11, 12, & 14	2M:1F	OD		≥10 years	WC	both	pregnant
Lyamin et	7.17	0.26		11 ,23	3F	OD		many	1WC:		pregnan

al., 2005								years	1CB		t
Lyamin et al., 2005	9.66	0.35		11, 23	3F	OD		many years	1WC: 1CB		post-partum

Appendix H: Beluga Whale Cortisol Studies, Confounding Variables. Author (s) of original study on cortisol levels, mean cortisol levels in nmol/L and µg/dl, season or month the study was conducted in (months in numeric form), age of the animal (s) sampled, sex of the animal (s) sampled, if the captive animals were kept in an indoor (ID) or outdoor (OD) enclosure, time it took for sample to be collected (in wild studies from initiation of chase until sample collection), time spend in captivity, origin of captive animals (wild caught or captive born), time of day when samples were collected, any significant notes listed in each study.

Author	Mean F nmol/L	Mean F µg/dl	Season	Age	Sex	ID /OD	Time to sample	Time in captivity	Origin	AM :PM	Notes
St. Aubin & Geraci, 1989	90	3.3	July	Sub-adult	34M: 8F		w/in 1 hour	n/a	n/a		
St. Aubin & Geraci, 1992	110	4	July	juvenile	8M:2 F		w/in 1 hour	n/a	n/a		
St. Aubin et al., 2001	89.1	3.2	Summer/fall	>2	118 M:60 F		<1 hour	n/a	n/a		
Tryland et al., 2006	125.1	4.5	October	calves-adults	14M: 7F		<1 hour	n/a	n/a		
Orlov et al., 1991	82.6	2.9	Spring and Fall			OD		1 year	yes		shallow sample
Schmitt et al., 2010	49.7	1.8	Fall	19	2M:1 F	OD		~18	yes	both	not reproactive
Spoon & Romano, 2012*	27.6	~1	Fall	9-28	2M:5 F	ID/ OD					

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