Final Workshop Proceedings for

Effects of Stress on Marine Mammals Exposed to Sound

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Executive Summary

The Office of Naval Research sponsored a workshop entitled 'Effects of Stress on Marine Mammals Exposed to Sound' that was held in Arlington, Virginia, 4-5 November 2009. The workshop brought together 20 researchers, veterinarians and the federal permitting agency personnel to discuss the current state of stress-related research stress in marine mammals. The purpose of this workshop was to assemble a cross-section of researchers in the field of stress physiology and behavioral research to identify the state-of-the-art science in stress physiology as it may apply to marine mammals, identify research needs for marine mammal stress-related research, and evaluate available or developing technologies for measuring indicators of stress ultimately in free-ranging marine mammals.

Research Recommendations
Workshop participants developed recommendations for stress-related research on marine mammals and recommendations related to technological needs for conducting stress-related research on marine mammals. Research recommendations without prioritization, included:

1) **Stress-Related Research**

- Promote an understanding of the natural variation in hormones and/or biomarkers of the stress response in free-ranging marine mammals as it relates to the life history state (e.g. sex, reproductive state, age) and physiological state (e.g. migrating vs. not migrating, fasting vs. nonfasting) of the individual and investigate whether these hormones and/or biomarkers have predictable annual cycles or diurnal cycles.

- Better understand and characterize the relationships among hormones (e.g. cortisol, corticosterone, aldosterone, catecholamines, reproductive hormones, thyroid hormones, etc.) or other biomarkers (e.g. immune function, cortisol receptors, etc.) in different matrices (e.g. blood, saliva, blubber, feces, urine, blow etc.).

- Validate the accuracy and precision of measurements of hormones and biomarkers in various matrices in captive and free-ranging marine mammals. Determine if there are 'biologically significant' differences in hormone or biomarker levels as measured among different matrices and/or between captive and free-ranging animals.

- Define and compare the quantitative and temporal relationships of hormones across the different matrices in captive and free-ranging marine mammals (e.g. how is circulating cortisol reflected in measurements made in the blubber or feces).

- Evaluate the potential effects of sample collection methods (e.g. chase, capture, restraint, behavioral conditioning, non-invasive vs. invasive, etc.) on hormone and biomarker measurements.

- Develop a conceptual framework to discuss and consider stress-related research in marine mammals.

  Identify reference populations or defined acoustic exposure events where the stress responses to varying levels of acoustic exposure can be quantified and compared for a given marine mammal species. Identify populations where known individuals can be identified with sufficient frequency to examine stress measures in relation to known sex, age and physiological state.
- Evaluate and determine the viability of grouping marine mammal species to assess/predict stress in free-ranging marine mammals based on taxonomy, ecological niche and/or habitat, diving physiology, social response to predation, or functional hearing groups.

- Develop a conceptual model and a mathematical framework to evaluate the cumulative and/or synergistic effects of multiple stressors (a component of the mathematical framework may be to quantify the dose/response relationships of multiple stressors that may be acting cumulatively or synergistically).

- Consider experimental design when investigating the effects of multiple stressors, or parsing the stress budget, including:
  - collection of metadata and timing of sample collection (i.e. chase/handling time and proximity, diurnal variation,etc.) ,
  - measurement of an individual before and after a specific stressor, and measurements should, when possible, include parallel measurements of other biomarkers including the use of genomics, proteomics, metabolomics,
  - measurement of multiple stressors when possible, with specific means to identify cumulative or synergistic effects of stressors,
  - confounding factors in the design of experiments.

- Evaluate potential population level effects of stressors and/or multiple stressors by:
  - collecting information on reproductive rate of a population (e.g. calf/pup counts, hormone measurements)
  - collecting information on variability of responses in individuals
  - using long-term and detailed demographic datasets that are key to linking stressors to population level effects

2) **Technological Needs**

- Develop a library of expression events in genomics (e.g. microarrays, cDNA libraries are already generated from dolphin WBC and right whale skin).

- Invent devices that can closely approach marine mammals and/or directly contact them (e.g. a cyamid-like robot or other such device) for ultrasound measurements, blubber extraction, heart rate monitoring, and/or blood, breast milk, urine and feces collection.

- Develop a collection mechanism and analytic approach for whale saliva and blow to conduct quantitative saliva and breath analyses of hormone and/or biomarker levels.

- Develop sensor, sampler, strips, and/or detectors to detect presence/absence of hormones and their relative levels in an animal during normal diving activities (surface and underwater).

- Produce a heart rate monitor using penetrating tags or suction cup attachments considering the variation in anatomy among species and taxonomic groups.

- Partner with existing endocrine labs to develop diagnostic laboratory facilities to support stress-related research on marine mammals.

- Develop and validate existing stress-assessment technologies that have not been used for marine mammal species; e.g. test existing hormone assay kits that have not previously been used for marine mammals.

Overview of Workshop

The workshop was organized and sponsored by the Office of Naval Research (ONR) - Marine Mammals and Biological Oceanography Program (MMB), which is part of the U.S. Department of Navy (DON). ONR is the Science & Technology branch of DON charged with supporting basic and early-applied research. The MMB program is guided by, though not limited to, research and technology development related to a better understanding of the potential effects of sound on marine mammals. A key research topic in the MMB Program is how acoustic stressors may affect marine mammals and their populations.

Marine mammals are exposed to a variety of potentially stressful anthropogenic and natural environmental inputs in both the wild and captive environments. Potential stressors include noise, pollutants, threatening stimuli such as fishing gear, habitat disruption, ecosystem changes in free-ranging animals, and transport/restraint, novel environments, and social interactions for animals maintained under human care. For the most part, the stress response in captive marine mammals under controlled conditions has been shown to conform to the classical definition of the generalized stress response, which is defined by activation of the hypothalamic–pituitary–adrenal (HPA) axis resulting in elevated levels of glucocorticoid (GC) hormones (i.e. cortisol and corticosterone; St. Aubin and Geraci, 1989, 1990; St. Aubin et al., 1996). The involvement of the sympathetic nervous system (SNS) in the stress response is immediate and acute in terrestrial and marine mammals and is characterized by the release of the neurohormones norepinephrine and epinephrine (i.e., the catecholamines) (Romano et al., 2004; Young and Landsberg, 1998), with evidence for profound activity during stressors such as stranding or predation (Mashburn and Atkinson, 2007, Cowan and Curry, 2008; Eskesen *et al.*, 2009). Hormones involved in the stress response are required for basic metabolic processes, such as the involvement of GC in regulating metabolic pathways. This holds true for marine mammals , which demonstrate natural variations in GC and adrenal sensitivity as a function of season, time of day, reproductive state, maturity, and dietary state (Schmidt et al., 2010; St. Aubin et al., 1996; Suzuki *et al.*, 2003; Atkinson and Mashburn , 2004; Hunt *et al.*, 2006; Houser *et al.*, 2007; Lidgard *et al.*, 2008; Mashburn and Atkinson, 2008; Rosen and Kumagai, 2008), as well as variations in catecholamines during normal behaviors such as diving (Hochachka *et al.*, 1995; Hurford *et al.*, 1996). To effectively characterize the hormonal stress response, it is important to understand the natural rhythms of GC and catecholamines that support an animal's normal biological functions, i.e. natural variability must be understood so that the impact of additional stressors can be quantified (Reeder and Kramer, 2005).

Little is known about long-term effects of stress on individuals and populations in marine mammals. Prolonged exposure to stress may result in immune system suppression, reproductive failure, accelerated aging, and slowed growth. Indeed, adrenal exhaustion has been observed in marine mammals deemed 'chronically stressed' (Clark *et al.*, 2006). Allostasis, which literally means 'stability through change', is the maintenance of an animal's physiological stability in spite of perturbations or change to its internal or

external environment. It has been proposed as a way to conceptualize the integration of short-term and cumulative stress, and provides a conceptual framework to evaluate the potential additive effects of stressors to animals (Korte *et al.*, 2005; Korte *et al.*, 2007; Gersten, 2008). The concept has been embraced by the National Research Council (NRC) with respect to determining the potential impact of anthropogenic sound to marine mammals (National Research Council (NRC), 2005).

In 2004, the United States National Academy of Sciences convened a NRC panel to clarify the term 'biologically significant' in terms of the effects of ocean noise on marine mammal populations. The NRC panel produced the 2005 report entitled 'Marine Mammal Populations and Ocean Noise: Determining When Noise Causes Biologically Significant Effects'. The report presented a conceptual model that outlines how the behavior of marine mammals responds to anthropogenic sound and how population level effects could be inferred on the basis of observed behavioral changes. This model, the Population Consequences of Acoustic Disturbance (PCAD) model, is a heuristic model that defines several levels of potential effects of anthropogenic sound on marine mammals, ranging from behavioral effects to effects on life functions (e.g. feeding, breeding, migrating), to effects on vital rates (e.g. adult survival, reproduction), to population level effects. Between each of these levels are 'transfer functions', which are the relationships between levels, most of which are poorly understood.

The NRC report identified a number of research recommendations to better understand the biologically significant effects of sound on marine mammals. Recommendation 4 indicated that the use of GC and other serum hormone concentrations to assess stress should be developed, validated, and calibrated for various marine mammal species, age-classes, and life-history conditions. The report identified the need to establish dose-response curves for those indicators as a function of sound exposure characteristics. The report also recommended that research should seek to determine whether reliable long-term stress indicators exist and, if so, whether they can be used to differentiate between noise-induced stress and other sources of stress in representative marine mammal species. Glucocorticoids were specifically identified in the report as potentially being mechanistically involved in the translation of behavioral effects into altered rates of reproduction and mortality (PCAD transfer functions). If glucocorticoids are not the primary mechansim, they may well be indicators of the cascade of effects leading from behavioral changes to alterations in survival and reproduction (PCAD transfer functions).

The purpose of this workshop was to assemble a cross-section of researchers in the field of 'stress research', identify the state-of-the-art science in stress physiology as it may apply to marine mammals, and identify progress and research needs in the field since the 2005 NRC report. The goal of the workshop was to identify specific research needs and evaluate available or developing technologies for measuring hormonal, neuroendocrinological, cardiological, and biochemical indicators of stress in marine mammals.

The objectives for this workshop included:

- Review state-of-the-art stress physiology research that could be applied to a marine mammal model;
- Review and identify research needs related to recently developed means of measuring hormones, such as catecholamine and glucocorticoid or other hormones, via blood, fecal sampling, sloughed skin, or collection of exhaled blow for application in marine mammal investigations;

- Identify key hormonal, cardiological, neuroendocrinological, and/or biochemical indicators that have promise in investigating the effects of stress on free-ranging marine mammals exposed to anthropogenic sound, particularly mid-frequency active (MFA) sonar;

- Identify technological needs related to sampling hormonal, cardiological, neuroendocrinological, and/or biochemical indicators in free-ranging marine mammals;

- Identify existing research protocols in the marine mammal field that would facilitate development and testing of devices or methods (data logger and/or biomarkers) for measuring stress responses in marine mammals.

Organization of the Workshop

The workshop commenced with a brief overview of the ONR research program and how ONR interests in the effects of stress on marine mammal populations fits into ONR research topics, specifically – Physiology and Stress and Population Level Effects of Sound Exposure.

The first portion of the workshop consisted of a series of presentations by researchers that conduct stress research on marine and/or terrestrial animals and humans to identify the state-of-the-art science in stress physiology. Each researcher was asked to give a detailed overview of their research on using hormones or other biomarkers to assess the effects of stressors on animals and/or humans. See Appendix 3 for key points from each presentation.

The second portion of the workshop was a facilitated group discussion addressing the following topics:

- Define stress/stressors/stress responses

- Define and/or differentiate between acute and chronic stress responses

- Conceptual framework for interpretation (i.e. allostasis)

- Sampling (e.g. fecal, skin, blubber, mucous, blood) and sample types (e.g. hormones, metabolites)
- Baseline sampling and data
- Representative marine mammal species/groups for stress markers
- Multiple-stressors or the 'stress budget'
- Measures of stress and population level effects
- Technological needs to investigate stressors in marine mammals

The workshop was facilitated by Dr. Michael Weise (ONR). These discussions provided a forum for researchers, veterinarians, and the federal permitting agency to have an open and honest discussion about the status of stress research as it applies to the field of marine mammalogy.

Discussion - Define stress/stressors/ stress responses

The term "stress" has a diversity of meanings in common parlance. It is frequently used to describe the physiological or psychological state of an organism. It is also often used to describe any of a suite of responses by an animal exposed to anthropogenic activities when considering marine mammals this could include such things as ocean noise, fishing, and vessel interactions. Compounding issues with creating a commonly accepted definition of stress, the perception that an animal in a given situation is experiencing stress is usually subjective. A brief survey of the scientific literature on stress over the last ten years finds a number of terms seemingly used interchangeably, including, stress, stress response, and stressors. Most uses have negative connotations, yet there is a mounting body of literature that demonstrates some moderate levels of stress (e.g. dietary restriction, exercise) are beneficial to an organism (Gersten, 2008). Due to the heterogeneity of meaning to which the term stress is applied, the scientific implementation of stress as a concept has become difficult without implementation of a more precise and operational definition.

The first workshop discussion, following the presentations, was focused on defining stress and other related terms. While the term stress has been commonly used in the scientific literature it was generally agreed upon by this group that the subjective connotations of the term rendered it less than useful in a scientific context. Moberg (1987) describes stress as an abstract concept and hence describes the difficulty in defining stress. The preferred terms that offer a more scientifically defensible definition were 'stressor' and 'stress response'. A stressor was defined as an internal or external perturbation that presents a challenge for survival or reproduction or that causes an animal to perceive that a problem exists. A stressor can be anything that induces a stress response and is often classified as either physical or psychological. A stress response was

defined as a suite of physiological, behavioral, and perceptual responses that serve to re-establish or maintain homeostasis in response to stressors, which vary in magnitude, duration, and frequency of occurrence. The physiological stress response has been well studied with respect to stimulation of the SNS and the neuroendocrine system, and the HPA axis. The activation frequency, magnitude and duration of the HPA/SNS response relative to the underlying physiological condition of the animal determines if the response is beneficial or potentially harmful to the animal.

Discussion - Define acute vs chronic stress response

The term 'stress' or stress response has a negative connotation, however; as the group indicated, animals have evolved mechanisms to physiologically detect and respond to short-term, or acute, changes in their environment along with real (or perceived) threats from predators and social interactions. Stressors may elicit eustress (good stress), neutral stress or distress in animals. Eustress elicits responses that are beneficial to an animal's well being, reproduction, maintenance of homeostatic state, etc., while distress evokes responses that interfere with well-being, reproduction, etc. and are capable of inducing overt pathologic changes, and neutral stress evokes responses that are neither harmful nor helpful (Breazile, 1987). The result of physiological action is the mobilization of energy reserves and/or alteration in behavior that enables the organism to combat the source of stress or the stressor; the general term for this action is the stress response. Acute stressors were defined as stressors that can last from seconds to minutes; whereas, long-term or chronic stressors can last for days, weeks, and even months. The chronic stress response can be deleterious to the animal depending on the magnitude, frequency and duration of the stressor, and the underlying physiological state of the animal. Chronic stress response can be particularly devastating to an individual that is already compromised, such as an animal that is too young to have developed physiological mechanisms typical of older animals, an animal that is pregnant, or an animal that is under increased allostatic loads such as those experienced while undergoing puberty. The deleterious effects of the chronic stress response are known to include inhibition of growth, reproduction, and immune system function, potentially resulting in a decrease in overall fitness (Bonier *et al*. 2009; Romero and Butler 2007)

In defining acute versus chronic stress, it must first be noted that the adrenal gland is composed of two distinct tissue types: the cortex (or outer layer) and the medulla (inner core). Both the cortex and the medulla secrete very distinct hormones. The cortex secretes the glucocorticoids and mineralcorticoids commonly associated with stress response, while the medulla, under direct neural control, secretes epinephrine and norepinephrine. The classic description of a response to a chronic stressor was advanced by Hans Seyle and is known as "general adaptation syndrome (GAS) (Seyle, 1973). GAS is comprised of three stages: the alarm reaction, the stage of resistance, and finally, the stage of exhaustion. The alarm reaction is comprised of physical changes (enlargement) of the adrenal glands as well as physiological changes marked by increased secretion of glucocorticoids from the adrenal cortex. Continued exposure to the stressor results in the second stage, the stage of resistance, during which glucocorticoid secretion is both prolonged and further increased. Should exposure to the stressor continue and become

chronic, normal function becomes impaired and characterizes the stage of exhaustion during which glucocorticoid secretion decreases precipitously from its elevated state, and typically results in death. Acute stress, or the "fight or flight" response can be differentiated from the initial stage of GAS in that secretions from the adrenal medulla play a larger role in responses to a stressor, primarily due to the brevity of the response.

Discussion - Conceptual framework for interpretation

There is a need for a conceptual framework to consider previous, current, and future stress-related research. Biomedical research on human stress responses provides a theoretical framework that can assist in conceptualizing and ultimately measuring the cumulative effects of multiple stressors on individual animals (NRC 2005).

The framework, or *allostatic theory*, relies upon the concept of *allostasis*, which encompasses the processes by which an animal maintains physiological stability in spite of change (McEwen and Wingfield, 2003; Gersten, 2008). The cumulative cost of these processes is referred to as the *allostatic load*, and it has been proposed that increases in the allostatic load leads to increased risk of poor health. Allostatic theory can be conceptually integrated with energy budgets and life-history events (McEwen and Wingfield, 2003), making it a potentially useful tool for modeling impacts resulting from exposure to various stressors. It is one way to conceptualize the integration of cumulative stress and is predicated on the idea that the effect of a given stressor is contingent upon multiple factors related to the animal's condition (e.g. species, sex, nutritional, and reproductive states; McEwen and Wingfield 2003). One factor is an understanding of the underlying physiological state of the animal when presented with a stressor. This is required to effectively understand how the stress response of the animal impacts the animal. For example, the stress response of an animal that is sick when it experiences a stressor may be different from that of an animal that is healthy when it experiences the same stressor. Indeed, the physiological state of the animal may be such that the stressor produces a beneficial response (eustress). The potential beneficial or deleterious effects of the stress response will depend on the underlying physiological state of the animal; although whether this directly relates to the allostatic load of the individual is currently under debate (Gersten, 2008). In order to interpret the potential effects of a stressor on an animal there is a need to understand where on the allostatic curve an animal may be, or how close to allostatic overload the animal is at the time of exposure to the stressor. Presumably, the closer an animal is to allostatic overload the more susceptible it is for additional stressors to produce deleterious effects.

The relevance of allostatic theory as applied to stress-related research in marine mammals was discussed. Workshop participants agreed that a commonly accepted conceptual framework was needed to discuss and consider stress-related research and its findings. Participants also agreed that allostatic theory has its detractors. The *allostasis concept* has received criticism for its assumptions and variant models and methods of integrating its component ideas have been proposed (Romero *et al.*, 2009; McEwen and Wingfield, 2010). However, no alternative to the current application of allostatic theory was identified.

Discussion – Sampling matrices and sample types

The NRC (2005) report identified GC as the primary, if not, exclusive hormone identified to assess stress in marine mammals. Because of the role of GC in the general adaptation syndrome (Selye, 1973), cortisol in particular has been extensively studied as a key stress hormone. However, it was apparent from workshop presentations that there are a variety of other hormones and/or biomarkers that are indicative of the stress response in humans, terrestrial and marine mammals. These compounds can be assessed in a number of matrices including, plasma, saliva, urine, feces, hair, skin, lacrimal and vaginal secretions, blubber and whale blow. While the characteristics and dynamics of hormones in many of these matrices (i.e. plasma, saliva, urine, feces) are well characterized in humans and some terrestrial animal models, it was generally agreed upon by workshop participants that the characteristics and dynamics of hormones and biomarkers in the samples obtained from marine mammals were not well understood.

A key research need identified by workshop participants was an understanding of sample parallelism between sample types; i.e., there is a need to better understand and characterize the relationship among hormones (e.g. cortisol, aldosterone, catecholamines, thyroid hormones, etc.) and other biomarkers (e.g. immune function indicators, cortisol receptors, etc.) and in different matrices (i.e. plasma, saliva, feces, urine, etc.). The reliability of being able to measure a hormone or biomarker and the range of concentrations it exhibits within different sample types needs to be determined. Furthermore, it needs to be determined whether other substances in the various matrices sampled can interfere with reliable measurements of hormones and biomarkers (e.g. cross-reactive substances in radioimmunoassay). The physiological state of the animal (e.g. reproductive state, state of the immune system, nutritional status, etc.) needs to be taken into consideration when making comparisons among hormones and biomarkers collected from different matrices as differing physiological states may not be equally reflected across matrices. Data collected from these studies will provide information on the suitability of sampling a particular matrix for a specific hormone/biomarker or family of hormones/biomarkers.

Participants also identified the need to validate the accuracy and precision of measurements of hormones and biomarkers in different species and in various sample matrices. Because hormone metabolism and excretion routes vary between different animals, assays require careful validation for each new species. Further, each sample type reflects a unique integration of physiological processes and likely has a different temporal signature associated with the time course of hormone integration in the tissue sampled. For example, while serum GC levels change within a few minutes of adrenal activation, fecal GC measures integrate levels of the serum hormone over 24-28 hours previous to sampling (Millspaugh and Washburn, 2004). For other matrices such as blubber, the relationship to serum hormone levels has not been determined. Participants agreed that there is a need to define and compare the temporal signature of hormones/biomarkers in the different sample matrices of both captive and free-ranging marine mammals. Biological validation of hormones and biomarker measurements should take into account potential degradation (due to exposure to the elements, delays in ensuring stable storage,

or multiple bouts of freeze/thaw cycles in transferring samples, etc.), which may give false measurements and the proper methods of collection and preservation in the field. It was noted that differences in the accuracy and precision of these measurements should be evaluated as to their biological significance, and potential for species-specific differences in these measurements needs to be considered.

Participants noted that the metadata of sample collections must account for collection methodologies as the process of obtaining samples may itself be a stressor. In these cases, the physiological signal of interest being measured may be masked by a stress response to the sample collection itself. Sample collection methods might include chase, restraint, non-invasive, or invasive means, some of which have been demonstrated to affect levels of stress hormones in both wild and captive marine mammals (Koopman *et al.*, 1995; St.Aubin *et al.*, 1996; Ortiz and Worthy, 2000; St.Aubin, 2002; Lidgard *et al.*, 2008; Mancia *et al.*, 2008). In order to assess the potential effects of collection methods on the hormones/biomarkers being measured, detailed metadata on sample collection methods and timing should be recorded and ideally compared to alternative sampling methods within the same species. Further, there is a need to understand how samples are affected by environmental factors and storage protocols following collection. Under field conditions, samples may not be processed immediately after collection and may not be stored on ice during transport to the laboratory. In other instances, samples may be exposed to the environment for some time prior to collection (e.g. fecal, skin, or fur samples). For samples that are stored, it also needs to be determined how storage conditions (e.g. -20 vs. -80°C; storing feces dried vs. undried) and the duration of storage prior to processing relates to sample degradation. Collectively, there is a need to determine optimal storage temperatures and procedures, processing methods, and contamination issues in order to ensure the greatest consistency and comparability of measurements made across different studies.

Discussion - Baseline sampling and data

Marine mammals, as well as terrestrial mammals, are not static with respect to the production of stress hormones or other stress-related biomarkers. Indeed, hormones typically associated with the general adaptation syndrome are critical to normal metabolic functions. For example, cortisol, in its normal mode of action, contributes to the regulation of glucose availability by stimulating hepatic gluconeogenesis and inhibiting peripheral glucose uptake. Therefore, in marine mammals, quantifying the potential significance of a hormone/biomarker response induced by an acoustic exposure requires an understanding of the natural variation in baseline hormone/biomarker levels. Baseline measures are critical to characterizing the variation in hormones/biomarkers necessary to support normal biological functions. Understanding the natural variability of these stress indicators will permit concentrations measured in marine mammal samples to be interpreted in context, i.e. that is, relative to levels typically expected for an animal's defined physiological state.

Hormones and biomarkers vary as a function of age or age class, sex, reproductive status, molt status, fasting status, and health status – contaminant and parasite loads, disease

state, immune status, nutritional status, body composition/condition, etc. Hormones and biomarkers may also show seasonal fluctuations and predictable diurnal variations. Furthermore, the site of sampling on the body, the geographic location of the animal when sampled, and associated environmental conditions (i.e. El Niño events) may all contribute to variability in sample measurements. Additionally, hormones and biomarkers vary between species. Several studies have shown increased adrenal activity as a function of social status (and by extension, reproductive success) including high concentrations of glucocorticoids in association with dominance under free-ranging conditions in African wild dogs (Creel et al 1996, 1997), ringtailed lemurs (Cavigelli, 1999) and Japanese macaques (Barrett, et al, 2002) while elevated glucocorticoids in subordinates have been shown in other free-ranging species; among these are spotted hyena (Goymann, et al, 2001), olive baboons (Sapolsky, 1992), and sparrows (Rohwer and Wingfield, 1981; Schwabl et al., 1988). Other studies found no difference in glucocorticoid concentrations and status (van Schaik et al, 1991; Robbins and Czekala, 1997; Lynch, 2002). Further, high corticosterone concentrations associated with pregnancy, parturition and lactation have been shown to negatively affect reproduction in a broad range of species (Mohberg, 1985; Pottinger, 1999.), while producing little effects in others (Goymann et al, 2001).

Workshop participants agreed that any interpretation of hormones/biomarker measurements should take into account the baseline context of the measurement, i.e. animal's physiologic condition, relative allostatic load, and spatial, temporal and environmental factors. Captive animals were identified as a valuable resource for investigating the variability of baseline measurements under controlled conditions, as well as for designing experiments focused on responses to stressors. Long-term repeatable access to animals permit longitudinal studies to be conducted in which seasonal, diurnal, and life-history stage variations in hormone/biomarker levels can be characterized across multiple matrices. Such characterizations could also permit the temporal and magnitude relationships of a hormone/biomarker between different sampling matrices to be determined (see "Sampling and sample types," above).

Discussion - Representative marine mammal groups

Broadly characterizing the stress response in marine mammals will require representative marine mammal species or groups to be identified (NRC 2005). There are too many species of marine mammals, many of which are rare or difficult to access, to allow broad scale sampling across a large cross-section. Workshop participants discussed the types of groupings that may be suitable to permit extrapolation between species with some degree of confidence. Workshop participants discussed how to assess and evaluate these groupings, and made suggestions as to what they might be.

Several general grouping possibilities were identified and discussed. These included groupings based on taxonomy, ecological niche or habitat use, diving physiology, sociobiology, response to predation, and sensitivity to sound or functional hearing groups (Southall *et al.*, 2007) or existing allostatic load or cumulative environmental, anthropogenic or other pressures. Any proposed groupings of species or model species would necessarily require justification. General taxonomic groupings included the

phocids, otariids, odontocetes, mysticetes, and odobenids (walrus). Grouping according to sensitivity to sound included animals that seem to be tolerant or curious of novel sounds (e.g. delphinids) as well as those that have shown a propensity to strand in the presence of anthropogenic sound sources (e.g. beaked whales). Suggested groupings based on ecological niche or habitat use included coastal and offshore ecotypes, income and capital breeders, and social and solitary species. Grouping based on sociobiology may include dominant or subordinate members of a social group, or those that cluster for sociobiological reasons. Groupings based on dive behavior or physiology included surface (shallow diver) and deep diving species, inhalation and exhalation divers, and foraging specialists and generalists. Groupings based on behavioral responses to predation included animals grouped into either flight or fight responders to potential predators or those that present protective responses to the threat of predation. Grouping based on allostatic or cumulative load would include groupings of animals known to be under pressure from at least one or more stressors that may be additive to the stressor that is the subject of study. An example would be a population that is experiencing known competition for food resources (e.g. interspecific competition or that due to fisheries) that then is subject to anthropogenic underwater sounds. Within any of these groups, the ideal populations to select for study, at least initially, would be those in which samples can be reliably tied to known sex, age, and reproductive state categories (e.g. populations under long-term study with a high proportion of known individuals, or populations in which age, sex and reproductive state can be determined by other means - visual, genetic, reproductive hormones, etc.). All participants agreed that baseline data needed to be collected and that a comparative approach (across species) would be the most instructive approach given the paucity of data on stress responses in marine mammals, and that not all of the ideal or realistic study animals may reside in US waters.

Discussion - Multiple-stressors or the 'stress budget'

Marine mammals are exposed to a variety of anthropogenic and natural environmental inputs in both the wild and captivity that may induce a stress response including noise, pollutants, threatening stimuli, habitat disruption, changing water temperatures, novel environments and social interations. Any measure of a stress response in a free-ranging (or captive) marine mammal will reflect an integrated response to multiple stressors of both natural and anthropogenic origin. The summation of these natural and anthropogenic stressors produces the 'stress budget' (allostatic load) of an individual animal, a concept which was previously discussed with respect to allostatic theory. The difficulty in determining the impact of a particular stressor on an animal is in teasing apart the stress budget and the cumulative effect of the multiple stressors experienced by an animal. Conversely, determining the effects of a particular stressor on a population requires determining the variability in individual responses within the population and its cumulative effect on reproduction and survival. Assessing the potential effects of a single stressor is complicated by the fact that it may not only be cumulative with other stressors, but it may act synergistically as well, amplifying the impact to the animal beyond that which each stressor may cause individually.

During this discussion workshop participants reiterated the need for a better understanding of baseline measures of hormones/biomarkers in marine mammals because the state of the individual and the temporal and spatial context of measurements were relevant to the consideration of multiple stressors. Similar to individual stressors, multiple stressors may be more biologically relevant at certain times or life history stages (e.g. multiple stressors during reproduction vs. during molt). This discussion resulted in an agreement that baseline measurements included not only measures of natural variation in hormones/biomarkers within an individual, but within a population as well.

Workshop participants identified research needs related to conceptual and modeling approaches for assessing multiple stressors or to parsing the stress budget in marine mammals. The need to incorporate the use of a decision tree to conceptually consider the effects of multiple stressors was identified, as was a need to develop a mathematical framework to evaluate the cumulative and/or synergistic effects of multiple stressors. Such a model might incorporate a component that quantifies the dose/response relationships of multiple stressors acting cumulatively or synergistically. To investigate the effects of multiple stressors, workshop participants identified a number of factors to consider in the design of experiments and the collection of data. First, care needs to be taken to record metadata related to the sample design and the method and timing of sample collection (i.e. chase/handling time and proximity) in order to determine the potential introduction of stressors beyond that which is being investigated. Confounding factors should also be considered in the design of experiments in order to reduce the probability of inaccurate interpretations (existing datasets might be useful in identifying confounding factors). To determine the potential stress response related to a specific stressor, studies should be designed that quantify the hormone/biomarker of interest in the individual before and after application of the stressor. Measurements in these studies should include parallel measurements of multiple other biomarkers (e.g. application of genomics, proteomics, metabolomics) so that biochemical pathways associated with the stress response can be better characterized. Other study designs should attempt to measure the response to multiple stressors in a manner that permits the identification of cumulative or synergistic effects.

Discussion - Measures of stress and population level effects

The acute stress response in animals results in physiological changes (e.g. the mobilization of energy reserves) that enable an organism to respond to short-term changes in its environment. These responses are potentially critical for survival; however, repeated acute stress responses or long-term or chronic stressors can be deleterious to an animal. What is deleterious to an animal to the point of having 'biological significance' is a subject of much debate. The NRC (2005) attempted to clarify the term 'biologically significant' in terms of the effects of ocean noise on marine mammal populations, with specific recommendations to use GC and other serum hormone concentrations to assess the stress response in marine mammals. In the PCAD conceptual model, GC and other serum hormones were identified as possible indicators of behavioral effects that are translated into altered rates of reproduction and mortality (PCAD transfer functions). Since the NRC report, there is evidence that the stress response in animals and behavior

are not necessarily coupled. In other animal systems, elevated stress hormones have been found in the absence of overt behavioral changes, and in marine mammals, behavioral changes have been observed in the absence of an observable stress response (D. Houser, unpublished). A stress response could therefore be potentially translated into altered rates of reproduction and mortality in the absence of behavioral changes. These findings argue further for the need to characterize baseline hormone/biomarker levels and to perform experimental perturbations, as the reliance on behavioral responses to perturbations within wild populations may not be sufficient to adequately characterize the effects to the population or observed individuals.

As indicated by the NRC (2005), long-term and detailed demographic datasets are key to linking stressors to potential population level effects. Workshop participants recommended that investigators collect information on reproductive rate, as a population parameter, when measuring stressors and stress responses to evaluate population level effects. Reproductive rates are quite sensitive to stress physiology and can be measured in a number of ways from calf/pup counting to hormone measurements in various matrices. Also, immune competence, a critical factor for survival, can be affected by stress leading to suppression of the immune response and leaving an animal susceptible to pathogens and disease or an enhancement of the immune response which can lead to autoimmune disease (Romano et al., 2002). This, in turn, can potentially lead to population level effects depending on the extent that the stressor affects the population and the status of the population. Information on the variability of stress responses in individuals, including reproductive measures, can be used to infer population level effects and should be collected and evaluated.

In a parallel effort, ONR is sponsoring a PCAD working group (Sep 2009 – Sep 2011). This working group has been tasked with translating the conceptual PCAD model (NRC 2005) into a mathematical framework so that population-level effects of changes in marine mammal behavior can be evaluated. As recommended by the NRC (2005), there is still a need to establish dose-response curves for those indicators (hormones/biomarkers) as a function of sound exposure characteristics. Once this relationship is established, the developing PCAD framework might be used to evaluate if and to what extent the stress response coupled or independent of behavioral responses is precipitated through the PCAD model transfer functions (i.e. into population level impacts).

Discussion - Technological needs to investigate stressors in marine mammals

Compared to the extensive literature on stress-related studies in terrestrial animals, research on the stress responses of marine mammals is in the early stages of development. Studying marine mammals presents unique challenges because many species are elusive and visible for only brief periods of time while hauled-out or at the surface, home ranges can include huge areas of ocean limiting accessibility for continuous monitoring (or repeated sampling) throughout the year, and many species can not be easily captured or sampled using traditional methods. For example, blood sampling is not yet feasible in large, free-ranging whales. Therefore, conducting stress

research on marine mammals requires novel approaches to obtaining physiologic data and samples. In addition, measurement of stress responses in marine mammals should expand upon reliance on GC hormones as a single indicator, and incorporate other stress-related hormones (e.g. thyroid hormones, aldosterone) and biomarkers into the study design. Furthermore, recent technological advances for studying stress physiology and metabolism in humans and terrestrial animals (e.g. microarrays, metabolomics, cDNA libraries) should be investigated for application to marine mammals.

Several factors limit the real time measurement of existing stress hormones and biomarkers, including the invasive nature of many of the samples matrices and the laboratory nature of the analyses. Much discussion centered on technological improvements that could be made to utilize existing assays on new media, as well as new technologies on routine samples. Recommendations related to technological needs for investigating the effects of stressors and the stress response in marine mammals, included:

- Develop a library of expression events in genomics (i.e. microarrays and cDNA libraries are already generated from dolphin WBC and Right whale skin).

- Invent a cyamid-like robot (or other such device) for ultrasound measurements, blubber extraction, heart rate monitor, and/or blood, breast milk, urine and feces collection.

- Develop a collection mechanism for whale saliva and blow to conduct saliva and breath analyses of hormone and/or biomarker levels.

- Develop sensor, sampler, strips, and/or detectors to detect presence/absence, low/med/high hormone levels in an animal during normal diving activities (surface and underwater). Sensor development might require development of a catheterization technique for cetaceans. This may require more detailed anatomical studies for cetaceans.

- Produce a heart rate monitor using penetrating tags or suction cup attachments considering the variation in anatomy among species and taxonomic groups. This may require more detailed anatomical studies for cetaceans.

- Develop and enhance existing diagnostic and research laboratory facilities to support stress-related research on marine mammals.

- Several achievable short-term actions that would support research needs listed above, included:
    - develop a conduit between researchers and diagnostic labs to facilitate communication
    - mine existing datasets for information (including archived samples)
    - validate existing, commercially available assay kits that have not yet been used for marine mammals (i.e. aldosterone, thyroid hormones). Once

assays are validated for marine mammals, expand datasets for these hormones
- search medical/nutritional research for ideas beyond the scope and expertise of workshop participants.
- set up a facility to temporarily or permanently house stranded or live captured marine mammals of various sizes to conduct basic stress-related research (for example - lack of any facility for stranded baleen whales hampers basic research on baleen whale stress physiology)
- develop international collaborations to conduct research on animals where there are well-defined stressors (or lack of stressors) for comparative studies (e.g. southern sea lions, southern right whales, western gray whales).

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Appendix 2 - Workshop Agenda

Day 1 – 4 November

0730 Registration and Coffee

0830 Introductions
- Brief overview ONR program
- NRC Reports & background
- Workshop goals / structure
- Introductions

0900 Atkinson – 'Response, Adaptation, Detection and Mitigation: Stress Physiology in Marine Mammals'

0920 Houser – 'Behavioral Response of dolphins to signals simulating MFA sonar'

0940 Crocker – 'Stress responses to handling and immobilization in northern elephant seals**'**

1000 Break

1030 Janz – 'Determination of long-term stress markers in skin and hair samples collected from free-ranging mammals**'**

1050 Cockrem – 'Blubber cortisol and the measurement of stress in free-living marine mammals'

1110 Keller – 'Blubber cortisol: Assessing chronic stress response in cetaceans from projectile biopsies**'**

1130 Ponganis – 'Heart Rate: Measurement and Interpretation'

1200 Lunch

1300 Rolland – 'Assessing Stress and Stressors in North Atlantic Right Whales**'**

1320 Hunt – 'Cort alone can't tell the whole story: case studies in stress assessment of North Atlantic right whales and sea turtles'

1340 Wasser – 'Distinguishing between multiple co-occurring disturbances in killer whales'

1400 Romano – 'Stress, Environment and the Immune Response: Investigations in Wild and Captive Cetaceans'

1420 Break

1450 Romano/Gulland – 'CHESS'

1510 Gulland – 'Assessment of stress response in rehabilitating California sea lions: effects of handling, anesthesia and domoic acid poisoning**'**

1530 Fair – 'Bottlenose Dolphin Health & Risk Assessment Project: Measures of Stress**'**

1550 Hogg – 'Determination of steroid hormones in whale blow: Is it possible?**'**

1610 – Discussion:

- **Define Stress, Active vs Chronic**
- **Conceptual Framework for Interpretation (i.e. Allostasis)**

1715 Adjourn

1830 Social

Day 2 – 17 March

0800 Discussion Topics:

- **Sampling and sample type**
- **Baseline sampling and data**
- **Representative marine mammal species/groups for stress markers**
- **Multiple-stressors or parsing out the stress budget**
- **Measures of stress and population level effects**
- **Technological needs**

1000 Break

1030 Discussion

1200 Lunch

1300 Discussion

1400 Break

1430 Discussion

1715 Wrap-up / Adjourn

Appendix 3 - Presentation Summaries

Shannon Atkinson (University of Alaska Fairbanks)

Title: 'Response, Adaptation, Detection and Mitigation: Stress Physiology in Marine Mammals'

- "Stress"
  - The nonspecific response of the body to any demand made upon it (Hans Selye 1950).
  - A state produced by an environmental or other factor which extends the adaptive response of an animal beyond the normal range (J.R. Brett 1958).
- "Acute stress"
  - Normal physiological actions taken by the animal to detect and respond to short-term changes in the animal's environment
  - Detection and response to acute stressors is good!
- "Chronic stress"
  - The prolonged physiological actions taken by the animal to detect and respond to long-term changes in the animals environment
  - Response to chronic stressors can be deleterious
- There are primary (i.e.: reflexes, epinephrine, serotonin, norepinephrin, catecholamine's, etc) and secondary responses (i.e.: glucocorticoids, mineralocorticoids, thyroid, leptin, etc.)
- Physiological mitigation
  - Physiologically adapt to change (i.e.: habituation or desensitization)
  - Maintain stressors as acute
  - Use chronic stress response in order to maintain homeostasis
  - Worst case
    - Become refractory
    - Leads to adrenal exhaustion
- There can be a predisposition to negative effects of stress response
  - Poor nutritional status
  - High body burden or exposure to contaminants
  - Naïve populations facing a new disease
  - Forced overcrowding
  - Altered ambient noise or elevation in sound levels
- Studies:
  - captive Steller sea lions (SSL) – ACTH Challenges
    - Used fecal samples
    - Different seasonal signatures
    - Big differences between sexes
    - Females similar to juveniles
    - Adults have a less varied response compared to juveniles
  - Wild SSL studies
    - Used scat on haulout – know age since deposited
    - Saw an effect due to predation

        - Is predation an acute stressor?
        - Response was quick, but ended quickly as well
    - Health of captive SSL
        - Spikes in corticosterone with a salmonella infection and branding
        - There was an aldosterone response to ACTH challenge with a seasonal signature
    - Harbor Seal ACTH study
        - Response depended upon body size – highest response from smallest animal
        - Strong relationship between body mass and endocrine/immune function – except in adult females
    - Rehab animals
        - Stress high at first, but decreased pre-release
    - Noise
        - Yakutat & Cook Inlet belugas have an aversive response to acoustic exposure
- Summary
    - Need reliable baselines from which samples can be compared
    - Need access to biological tissues (includes blood, feces, and blubber)
    - Need to account for predisposing factors (i.e.: age, sex, species, season, etc)
    - Need discussions with colleagues – more workshops
    - Need to find out the relationship between feces, blubber, and serum – is there a temporal signature
    - What is the role of aldosterone?

Dorian Houser (Biomimetica)

Title: ‘Behavioral Response of dolphins to signals simulating MFA sonar’

- Data contributing to risk function form are from:
    - Fleet animals for TTS studies (Finneran and Schlundt 2004)
    - Right whale response to acoustic alarm (Nowacek et al 2004)
    - Killer whale reaction to sonar in Haro Strait (2003)
    - There are limitations to data
- Controlled exposure studies
    - Access to diverse group of animals with known life history
    - Trained for beneficial behaviors
    - Have access to a large sample size (30 dolphins and 15 sea lions)
    - Procedure is a A-B-A test with a 10 trial control session and then a 10 trial exposure session – the dolphin or sea lion receives the same exposure across all 10 exposure sessions
- Responses to sound exposure
    - Investigate “stress”
        - Some overt behaviors are traditionally coupled with signs of agitation (i.e.: tail slaps, chuffing, etc)
        - Heart rate

        - Hormones – traditional indicators of acute and chronic stress
    - Data collection is done by both audio and visual means
- Behavioral analysis
    - A priori predict the types of behavioral reactions – overt reactions, refusal to participate, response times, respiration rate, etc.
    - Enlist independent marine mammal scientists to score the severity of behavioral reactions
        - Scorers generally agreed on relative order of severity in responses, but there was variation in how people scored individual responses
    - There will be a behavioral analysis of sessions by blind observers
- Application to risk function
    - Data amenable to risk function analysis
    - Multiple trials permits investigation of acute acclimation/sensitization
- Heart Rate
    - Some animals are trained to wear a harness that a heart rate monitor and acoustic dosimeter can be attached to
    - Data collection and display – keeps a running average of heart rate and sample analysis window down to 2-seconds
    - Analysis of short-term stress response (startle response) – data collection time aligned with video/audio so heart rate can be matched to time of sound exposure
    - Potential issues: diving heart rate variability and surface tachycardia/diving bradycardia
- Hormone Analysis
    - Looking at:
        - Cortisol and aldosterone – potential relationship to prolonged cold stress in dolphins, has a potential confounding effect due to social isolation/artificial environment, and could be a potential response to an acute stressor
        - Epinephrine – not measured much on marine mammals, could be an acute stress marker
    - Take voluntary blood samples
        - Several days before testing, immediately following exposure, and one week after the test
    - There is an on-site centrifuge and liquid nitrogen flash-freeze
    - Analysis will be done via RIA
- Status and outlook
    - Testing began in August 2009
        - 20 dolphins tested
        - The rest should be completed within 5 months
        - Start testing sea lions after dolphins completed
    - Heart rates collected on 3 animals (should be collected on 1/3 of animals when completed)
    - Blood collections on all animals to date (analyses should be in soon)

Daniel Crocker (Sonoma State University)

Title – 'Stress physiology of elephant seals'

- Looking at impacts of handling and immobilization on metabolism
    - Using tracer kinetics (i.e.: glucose production, glucose cycle, PEP cycle, etc)
- Are there stress/handling artifacts
    - Chemical immobilization
        - Duration
        - Compare this to non-drugged or restrained
    - Doing work in field or transporting to lab
- Stress hormones = glucoregulatory hormones
    - Cortisol and epinephrine
    - Stress effects (i.e.: handling, natural stress, etc)
    - Diel patterns
    - Changes with fasting
- Cortisol response to fasting
    - Validated for northern elephant seals (Ortiz et al 2003, Champagne et al 2005)
    - Little change except for lactating females
    - Sex differences in yearlings
    - Diel pattern – decreases with time
- Prolonged chemical immobilization
    - 2 ½ hours for whole procedure
    - Increased cortisol and aldosterone
    - Depends on timing within fasting period/season
- Acute stress does NOT impact reproductive success
- Cortisol increases with physical restraint and transport to the lab
- Working on validating epinephrine testing
    - Getting validating kits
    - Checking on sample stability
        - Mimic field handling procedures at lab
        - Examine degradation in freezer
- Metabolomics – looks at all hormones and metabolites combined
    - Could be a good way to view stress response
- Summary:
    - Evidence for stress impacts from drugging
        - 2 ½ hours into immobilization
        - Does not perturb glucose homeostasis
        - Suppressed late in fast?
    - Transport to the lab and physical restraint more stressful than drugging
        - Response to drugging more dramatic at lab
    - Possible diel pattern to cortisol levels in elephant seals
    - Acute stress of handling/immobilization does NOT impact reproductive success

David Janz (University of Saskatchewan)

Title: 'Determination of long-term stress markers in skin and hair collected from free-ranging mammals'

- Developed/validated techniques to detect long-term stress in grizzlies
  - Created a custom protein array – "bear stress chip"
  - Looking at hair cortisol concentrations
- Why skin?
  - Skin possesses the functional equivalent of HPA axis
  - Potential for skin to be collected remotely without capturing bears (or other wildlife)
- How it works
  - Skin sample taken
    - Individual bear samples are labeled with red fluorescent dye; pooled reference sample is labeled with green fluorescent dye; nitrocellulose coated slides are printed with antibodies
  - Antibody-based protein array (similar to gene chips) was developed initially with 32 antibodies recognizing stress-associated proteins in bears
  - 4 different categories of stress-associated proteins measured
    - HPA axis
    - Oxidative stress and inflammation
    - Cellular stress and proteotoxicity
    - Apoptosis and mitosis (cell death and proliferation)
- Results
  - Exploratory analyses indicates significant correlations between certain skin stress proteins and other variables including:
    - Clinical serum markers (GGT, AP, urea:creatinine ratio, etc)
    - Serum stress markers (cortisol, hsp70)
    - Hair cortisol
    - GIS home range data (% protected, % regenerating forest, mean RSF [resource selection function] score, road density)
    - Region (population unit)
    - No differences between male and female bears for any protein, nor among capture methods (leg snare, culvert trap, helicopter dart)
- Challenges
  - Large number of variables (stress, animal health, environmental) "dilute" statistical capabilities
  - Current focus is on prioritizing/categorizing variables
  - Skin sample collection and storage
- Hair cortisol: a relevant measure of long-term stress
  - Can be collected non-invasively (barbwire snags)
  - Avoid confounding influence of acute capture stress
  - Hair shaft cortisol represents longer term (weeks to months) accumulation
  - Hair cortisol recently validated as a marker of chronic stress in non-human primates

- Lab & field validation
  - Only need 5-10 hairs
  - No age/sex effects
  - Does vary by body region (neck is highest)
  - Layer of skin measured WILL affect measure of cortisol
- Future research
  - Expand number of stress-associated proteins on microarray
  - Application to other species: microarray "works" in polar bear, ringed seal, and moose
  - Investigate relationships between stress markers and other measures of animal health and environmental stressors

John Cockrem (Massey University)

Title: 'Blubber cortisol and the measurement of stress in free-living marine mammals'

- Stress is a state when:
  - The HPA axis is activated in response to a physical or emotional stressor
  - Cortisol secretion increases
- Stress responses help animals adjust to changes in their environment
- Response to physical stressor
  - Activates the HPA axis
- Response to emotional stressor
  - Key is perception of threat
- Varies by individual
  - If an animal perceives a threat it experiences a loss of control (response)
  - If an animal doesn't perceive a threat there is no loss of control (no response)
- Measure cortisol concentrations in:
  - Plasma
  - Feces
  - Urine
  - Saliva
  - Blubber
- Cortisol concentrations provide an objective measure of the degree of stress that an animal is experiencing or has experienced
- Example: do laying hens experience more stress in cages compared with other housing systems?
  - By this definition of stress – stress is a state when the HPA axis is activated with increased secretion of corticosterone in response to a stressor
  - If there is no difference between 2 groups of hens in their corticosterone concentrations then, at the time samples were collected, one group of hens was not experiencing more stress than the other group
  - There was no consistent difference in plasma or fecal corticosterone between hens at a cage farm, two barns, and two free range farms

    - There was no evidence from this study that hens in one housing system experienced more stress than hens in other housing systems
    - Due to public perception, the science was believed to be dishonest
- Stress in marine mammals
    - Blubber cortisol: a potential "non-invasive" method to measure stress in free-living marine mammals
        - Develop laboratory extraction and assay methods
        - Conduct biological validations to determine relationships between plasma and blubber cortisol concentrations
        - Measure blubber cortisol concentrations within populations of marine mammals to quantify variation between
            - Individuals
            - Seasons
            - Locations
        - Compare blubber cortisol concentrations and hence experiences of stress between groups of marine mammals in different situations
    - Blubber cortisol: extraction and assay methods
        - Homogenize tissue
        - Solvent extractions
        - Measure cortisol by radioimmunoassay
    - Blubber cortisol: biological validation
        - Measure plasma and blubber cortisol concentrations in captive animals
            - Exposed to different stimuli
            - Given cortisol implants
    - Blubber cortisol variation
        - Measure blubber cortisol concentrations within a population of marine mammals to quantify variation between individuals, seasons, and locations
    - Blubber cortisol: stress in animals in different situations
        - Compare blubber cortisol concentrations and hence experiences of stress between groups of marine mammals in different situations (i.e.: not exposed to people, exposed to people, exposed to sonar)
- Conclusions
    - Animals have behavioral and physiological responses to stimuli from the environment
    - Stress responses involve activation of the HPA axis and increased secretion of cortisol
    - Cortisol concentrations provide an objective measure of the degree of stress that an animal is experiencing or has experienced
    - Blubber cortisol concentrations can potentially be used as a "non-invasive" method to determine whether groups of free-living marine mammals in different situations experience different levels of stress

Nick Kellar (National Marine Fisheries Service)

Title: 'Blubber cortisol: Assessing chronic stress response in cetaceans from projectile biopsies'

- Advantages of biopsy sampling
  - The community has a lot of experience with projectile biopsying
  - Samples can be obtained quickly
    - There can be a high rate of sample acquisition depending on species
    - Appears to be minimal disturbance to animals when collecting samples
  - Lipid laden blubber tissue accumulates steroid hormones in relatively high concentrations
  - Biopsy samples are routinely collected for a wide range of research objectives: genetics, diet and trophic analyses, contaminant loading, etc.
  - Photo ID images can be easily acquired simultaneously
  - Likely measures endocrine activity over the previous hours/days/weeks – not minutes
  - Lipophilic biomarkers most readily measured
- Disadvantages of biopsy sampling
  - Not easily compared to terrestrial animals
  - Does create a small wound in the animal
  - May change an individual's behavior over time
- Adrenal Steroid Hormones: glucocorticoids and aldosterone
  - Glucocorticoids are the most widely used indicators of stress
  - Ephemeral rise in glucocorticoids area a beneficial response to acute stressors
  - Prolonged activation of HPA axis leads to: reduced reproduction, immune suppression, and an overall decrease in fitness
  - Some stressors that effect glucocorticoid levels include: predator abundance, limited food supply, pollution, exposure to humans, habitat change, etc.
  - Likely have limited role in blubber, but accumulate readily
  - Aldosterone has glucocorticoid potency equivalent to corticosterone
- Challenges and potential research needs for blubber glucocorticoids
  - Dynamics of glucocorticoid levels in blubber are unknown – clearance, residency, blood/blubber relationship
  - No/limited ground truth opportunities
  - Very few comparable terrestrial studies on glucocorticoid levels in adipose tissue
- Bowhead Cortisol levels
  - Cortisol levels have been quantified in Bowhead blubber
  - Blubber vs. serum – weak relationship, levels are an order of magnitude higher in serum vs. blubber (this relationship is opposite in reproductive hormones

- Spotted Dolphin Pregnancy relative to ETP fishing
    - Determining glucocorticoid concentration or other stress markers is NOT enough
    - Assessing the effects of stress on populations is critical
    - Though estimating mortality is difficult in marine mammal populations, assessing reproduction is tenable
    - It has been demonstrated that pregnancy can be determined from blubber progesterone concentrations – the same blubber samples that can be used to measure cortisol
    - We have used this to determine the effects of potential stressors such as the chase and encirclement of dolphins in the tuna fishery on pregnancy rates
    - Found the highest pregnancy rates in areas with low fishery effort
    - $\Delta\mu$ permutation tests indicate that pregnant animals have significantly lower average fishery-exposure indices in all three ambits that were examined
    - The interpretation of these results indicate that fishery effects on reproduction are complex
- Lessons learned
    - Need to analyze data from hundreds of animals – there is so much individual variation in stress response that a small sample size won't capture the variation within populations
    - Need to have enough power to determine how a stressor will effect a population
    - Stress measurements can provide plausibility of causation or potential mechanisms
    - But need to show effect at population level: either in survival or reproduction
    - All blubber isn't created equal – need to take into account species, where blubber is taken from, etc. However, relative levels of different hormones can be captured in population level sampling of groups that are of interest and as long as sampling is not methodologically different between comparison groups – the variation due to anatomical location or species will be captured in resulting measurements, minimizing biases

Paul Ponganis (UC San Diego / Scripps Institute of Oceanograph)

Title: 'Heart Rate: Measurement and Interpretation'

- Orca ECG beached vs. in water
    - When animal is beached you can easily see signal
    - When animal is in water there is a lot of noise that masks the signal
- Diving animals
    - Heart rate lowers when animal is diving (bradycardia) and increases when at the surface (tachycardia)
    - Harbor seal tests

        - Exhibited bradycardia in both naïve and trained submersions
        - In a test where the animal was kept down after the trained period – the heart rate was lowered further to compensate
- Heart rate measurement
    - ECG or acoustic signal
    - Signal detection and verification – R wave detector not accurate enough
        - Need to develop custom peak detector software (digital ECG)
    - Heart rate profile
- Heart rate interpretation
    - Diving bradycardia – Onset, Intensity, Fluctuations, Ascent
    - Surface tachycardia – Intensity, Duration
- Lessons learned
    - To interpret data you have to take context into account
        - Animal can control heart rate depending upon the situation

Rosilind Rolland (New England Aquarium)

Title: 'Assessing Stress and Stressors in North Atlantic Right Whales'

- This work assessed various stressors in North Atlantic Right Whales by looking at fecal steroid hormone metabolites – particularly glucocorticoids
- There is a detailed catalog of NA right whales that includes
    - Photo-identification of individuals
    - > 46,000 sightings of over 500 individuals
    - Up to 30 years of associated life history data on individual whales (age, sex, calving history, genetic profile, habitat use patterns, behaviors, associations, etc)
    - Used in association with fecal hormone results to develop baseline profiles
- Whale health profiles
    - Over last decade techniques have been developed to assess health of NA right whales using:
        - Visual health assessment using photographs
        - Entanglement scars from fishing gear
        - Fecal sampling studies (endocrinology, marine biotoxins, diseases, genetics, energetics)
    - 1/3 of fecal samples (95 out of 271 samples) collected have been associated with a known right whale using photo-id coupled with fecal DNA genetic profiles
    - Able to look at relationship between fecal hormone levels and age, sex, and reproductive status of known right whales
- Fecal steroid hormone metabolites
    - Looked at 3 reproductive hormones and glucocorticoids
        - Estrogens
        - Progestin's
        - Androgens
        - Glucocorticoids – "cort" (corticosterone antibody was used)
    - Assays validated using tests for parallelism and accuracy

    - Measured using ^{3}H and ^{125}I radioimmunoassays
- Fecal endocrinology
    - The androgen:estrogen ratio accurately reflects the sex of most samples from unidentified whales – this was validated using samples from identified whales
- Fecal reproductive hormone metabolites by sex and reproductive status
    - Are able to distinguish juvenile and adult males using androgen levels alone
    - Pregnant females are easy to identify by extremely elevated progestins
    - Lactating females have higher estrogens and androgens than juvenile and resting females and lower progestins than pregnant females
    - Unable to distinguish juvenile and resting females using fecal hormones at this time
- Fecal cort levels
    - Pregnant females have very elevated cort levels and lactating females have elevated cort (not as high as pregnant females) compared with immature and resting females which are not different
    - Adult males have significantly higher median levels of fecal cort over a broader range of values compared to juvenile males
        - There is a significant positive correlation between fecal cort and androgen levels in males
- Findings
    - Fecal cort levels vary according to sex and reproductive status and can be grouped into 3 categories
        - Juveniles and resting females – cort levels <50 ng/g
        - Lactating females and some mature males – cort levels between 50-100 ng/g
        - Mature males with very elevated androgen, pregnant females, and whales in poor health – cort levels >100 ng/g
- Severe entanglement example
    - "Churchill" (EG# 1102) had a severe entanglement and was in very poor condition when a fecal sample was collected (it is believed that he died shortly thereafter)
        - His fecal cort levels were very elevated at 178 ng/g
            - This suggests that the metabolites being measured reflect a chronic stress response in this whale (i.e. biological validation)
- Mild entanglement example
    - "Piper" had a simple mild entanglement – she shed the entanglement 6 months after the sample was taken
        - Her fecal cort level was only 12 ng/g
- These two examples are anecdotal, but suggest that fecal cort levels in these whales reflected the degree to which the entanglement compromised their health
- Preliminary Results for a Boat Traffic Study in the Bay of Fundy before and after Labor Day
    - There was a sample size of 137 fecal samples over 6 years

    - There was no detectable difference in cort levels before vs. after Labor Day in all years excluding 2001
    - Excluded samples from pregnant females and testosterone levels above 5,000 ng/g in the analysis (because of normally elevated cort levels)
    - In 2001 ports were closed after 9/11 and there was no boat traffic in the Bay of Fundy – cort levels were significantly lower after 9/11
    - These results suggest that boat traffic disturbance and/or noise levels resulted in elevated fecal cort levels in right whales
- Visual health assessment
    - Whale photos are scored for 4 parameters: body condition, skin condition, orange cyamids, and rake marks
        - Each parameter has a rank scale
        - Body and skin condition are the most informative so far
    - Fecal cort levels become a powerful tool when used in combination with these other health indicators
    - Data have been analyzed through 2002 and there are some compelling preliminary relationships between these scores and reproductive success in females and also survival that require further analysis and modeling
- Linking stressors to effects
    - There are multiple stressors affecting NA right whales
    - Developed methods to assess the impacts of some stressors using various metrics - fecal cort levels are one measure
    - Working on linking these metrics to significant impacts on health, reproduction, and mortality at the individual and population levels
- Benefits of fecal sampling approach
    - Minimal disturbance to whales
    - Only available method to obtain endocrinology samples in free ranging large whales – however there is intriguing preliminary work on using blow samples
    - Fecal cort assays are robust in many species
    - Fecal cort levels are averaged over time simplifying interpretation
    - By measuring reproductive hormones you can control for normal effects of sex and reproductive status on cort levels
    - Can collect data on some other potential stressors in the same sample (disease, biotoxins, etc)
- Limitations of fecal sampling
    - Sampling is largely opportunistic – can't resample deliberately
        - However, detection dogs can increase sampling rates by 4X/hour
        - Were able to resample 12 whales (2-3X) within a field season and 10 whales in different years
    - Weather dependent
    - Inability to sample throughout the year and in all habitats
    - Applicable to some, but not all wild cetacean populations
- Challenges in interpretation
    - MUST interpret cort with knowledge of the reproductive status, sex, and age class of animal

    - Control for the influence of other stressors
    - Differentiating short-term vs. chronic stress that leads to significant impacts on health, reproduction, and survival
- Research Needs
    - Additional endocrine measures – thyroid hormone, aldosterone, etc.
    - Model relationships between reproductive and stress hormones
    - Multivariate analyses of relationships between different health indicators (stress hormones, visual health assessment)
    - Model relationship between visual health, reproductive success, and survival
    - Sampling whales in different acoustic environments to determine thresholds for a stress response to be detected

Kathleen Hunt (University of Portland)

Title: 'Cort alone is not enough: case studies from North Atlantic right whales and sea turtles'

- Cort can be high for many reasons
    - NARW – cort varies with age/sex/state and there is cross-reactivity in fecal assays
    - Sea turtles – cort responds to many different stressors and thyroid hormones are a complementary measure
- Fecal cort in NARW
    - Poop is not like blood
        - Steroid hormones can be extremely high in fecal samples
        - Unknown fecal metabolites – there are many different "fecal glucocorticoid metabolites (none of which have been identified)
        - Unknown antibody affinities
- Cross reactivity in feces can be a greater problem than in plasma
    - T cross-reactivity in the fecal cort assay is "only 2.5%"
        - 100 ng/g of T "looks like" 2.5 ng/g of cort (
        - This cross-reactivity would be considered negligible in blood, but in feces can cause substantial alterations in apparent hormone level (due to high levels of certain steroids in feces)
        - Particularly an issue when one hormone is present at much higher concentrations than another
    - Example:
        - Mature males - examples from two individuals
            - Cort = 35 ng/g (male 1) or 86 ng/g (male 2)
            - Androgens = 6880 ng/g (male 1) or 15,700 ng/g (male 2)
            - A/C ratio = 197 (male 1) or 183 (male 2)
        - If these "androgens" are actually testosterone, the apparent cort could be entirely cross-reactivity
        - Strong correlation of cort with androgens in males – is it real or cross-reactivity?
- "Screamingly high" progestins in pregnant females

- Luckily for this cort assay, progesterone has no detectable cross-reactivity in the cort assay (and there is no correlation of cort with progesterone)
- Adding HPLC to our toolkit
    - Specific goals
        - Identify the fecal cort metabolites
            - How many?
            - Do whales excrete pure cortisol or pure corticosterone?
            - Do different whales excrete different cort metabolites?
        - Identify the fecal androgens
            - Are whales excreting T?
        - Look for peaks that overlap (= possible cross-reactivity)
- HPLC 101
    - High-performance (or high pressure) liquid chromatography
        - Separates steroids gradually, by polarity
    - Our methods
        - HPLC runs on cleaned, filtered fecal extract from multiple individual whales
        - The resulting 120 fractions assayed for both androgens and cort
        - Selected representative whales from various age/sex classes + "high cort whales"
- Typical results from a healthy adult male
    - Things we have learned:
        - Almost no pure T, cortisol, or corticosterone excreted
        - A cort peak in fraction #85 lines up perfectly with an andro peak
        - These to peaks both represent nonpolar metabolites
        - There are polar metabolites too
- Results from a fatally entangled whale (Churchill)
    - Same two peaks are occurring in the nonpolar area as the healthy whale
    - Plus a third peak not seen in the healthy whales
- Results from a "mysterious high-cort" yearling (an unentangled whale that has high fecal cort for unknown reasons)
    - Cort peak at 75 occurs in this whale too
    - Another new polar peak in this whale
- Summary
    - The cort assay is "seeing" several different steroid metabolites
    - None of them are pure cortisol or pure corticosterone
    - Peak #75 is the only consistent cort peak across whales
    - The whales are not excreting much pure T at all
    - Peak #86 may be DHT, and it's probably cross-reacting in the cort assay
    - Luckily, in most whales the DHT peak has only a negligible effect on the cort assay results. In adult males, however, up to half of their apparent cort level may actually be DHT.
    - Other peaks vary with the whales reproductive/age state
        - Polar peaks highly variable
        - Highly stressed Churchill had an interesting peak at #65
- Tentative conclusions

    - Fecal cort can be high for many different reasons, not all of which indicate physiological stress
        - Cross-reactivity from high androgens
        - Pregnant females always have high cort
    - Easiest fix: always run the reproductive steroids too
        - Can ID the pregnant females and adult males this way
        - Could numerically correct for androgen cross-reactivity
    - Best (but most tedious) fix: also do HPLC
        - Separate out peak #75
        - Further investigate cort metabolite profiles
- Sea Turtles: another case of "cort can be high for many different reasons"
    - Example of adding thyroid hormones to the picture
    - Two different kinds of stressors:
        - Cold-stunning and entanglement
- First question: is cort useful as a general indicator of stress?
    - Cold-stunned Kemp's Ridley sea turtles
        - Corticosterone very high on admission in both animals that ultimately survived or died
        - In turtles that survived, corticosterone decreased dramatically, and significantly during recovery
    - Entangled Leatherback Turtles
        - Corticosterone is higher in entangled turtles (note that the turtles "not entangled" were chased for capture, therefore probably not at baseline)
- Corticosterone DOES reflect stress
    - But cort was elevated both for cold-stunning and entanglement, two very different stressors (cort can be high for many different reasons)
    - If we didn't already know the story of each individual, how could we tell what the stressor was?
- Adding thyroid hormone to the picture
    - Released from thyroid gland – primary controllers of metabolic rate
        - T4 (thyroxine) – the pro-hormone released from the thyroid gland
        - T3 (tri-iodothyronine) – the active hormone
        - T3 is generally at much lower concentraton and therefore harder to detect than T4
        - Both hormones circulate mostly bound to a carrier protein, but some circulates unbound ("free"). Assays either measure the "free" hormone only, or the total hormone (e.g., four assay types exist: free-T4 assay, total-T4 assay, free-T3 assay, or total-T3 assay)
        - Tested a total-T3 assay; undetectably low in all turtles tested
        - Tested a "free-T4" assay: successfully validated for both species, detected measurable hormone in some (but not all) individuals.
- Summary of preliminary T4 results:
    - Leatherbacks – entangled turtles had HIGH cort and HIGH T4
    - Kemp's – cold-stunned turtles had HIGH cort and LOW T4 (and T4 increased as the survivor turtles recovered).

- A tentative model:
  - HIGH cort + HIGH T4 = stressor that has caused the animal to become very active; "stressed and struggling"
    - Possible stressors in this category: entanglement ("struggling to swim"), reproductive activity, migration, etc
  - HIGH cort + LOW T4 = stressor that has caused the animal to "shut down"; "stressed and still"
    - Possible stressors in this category: cold-stunning, nutritional stress (also known to have this endocrine profile), etc.
- Summary
  - High cort alone can tell us vaguely: this animal might be stressed, for who knows what reason, or it might be pregnant or it might have high androgens or it might be migrating or it might be involved in reproduction or ... etc.
  - High cort plus complementary measures (reproductive, HPLC, thyroid) can potentially tell us is it definitely stressed (not just pregnant/high T/etc) or why it is stressed (entanglement vs. cold-stunning, etc.)

Samual Wasser (University of Washington)

Title: 'Monitoring disturbance impacts on Southern Resident Killer Whales'

- Proposed threats to killer whale health?
  - Loss of prey quantity and/or quality
  - Disturbance from vessel traffic
  - Exposure to persistent toxins
- Information from feces
  - DNA – species, sex, individual, abundance, distribution
  - Hormones – stress, reproduction, nutrition, endocrine disruption
  - Pathogens – parasites, disease
  - Immunoglobins – immunosuppression and/or activation
  - Diet – habitat requirements
  - Toxins – persistent organic pollutants (POP: PCB, DDT, DDE, PBDE)
- Analogous to a health panel, without having to see the patient
- Fecal sample collection rates for right whales using opportunistic (opp.) methods vs. detection dogs (d.d.)
  - Samples collected per hour
    - 2003 opp. = 0.15 d.d. = 1.07
    - 2004 opp. = 0.28 d.d. = 0.80
    - 2005 opp. = 0.32 d.d. = 1.43
    - Overall opp. = 0.25 d.d. = 1.10
  - 4X greater detections with dogs vs. humans (100-1000m distances)
- Context
  - Boat traffic and prey availability both peak around the same time each year, enabling us to use the opposing physiological responses expected to boats are prey to distinguish between boat traffic and loss of prey hypotheses for the killer whale decline.

    - Loss of prey hypothesis predicts:
        - Glucocorticoids (GC) in feces will be negatively correlated with prey availability,
        - Whereas thyroid hormone (T3) will be positively correlated with prey availability.
    - Boat stress hypothesis predicts the opposite:
        - GCs will increase with number of whale watch boats on the water,
        - Whereas T3 will remain unchanged in response to boat traffic.
- Results and Conclusions:
    - Use of detection dogs greatly enhanced fecal sample collections from killer whales. Fecal GCs were lowest in the months when prey were most available and were highest in months when prey was least available. Fecal T3 was highest when prey was most available and lowest when prey was least available. These results suggest that the loss of prey is having a far more negative impact on killer whale physiology than is the impacts of increased boat traffic.
    - The ability to physiologically distinguish between competing psychological stress-related versus nutritional or toxin-related pressures makes these methods well-suited to address issues surrounding the impacts of sonar on killer whales.

Carolyn Hogg – via telephone (University of New South Wales)

Title: 'Determination of steroid hormones in whale blow'

- Steroid Hormones
    - Reproductive hormones – androgens, progestins, and estrogens
    - Corticosteroids
    - Some characteristics
        - All derivatives of cholesterol via metabolic pathways
        - Lipophilic
        - Diurnal and seasonal rhythms
- Current hormonal techniques
    - Plasma, Urine, Saliva, Feces
- New hormonal technique
    - Blow exudates ('whale snot')
        - Non-lethal
        - Suitable for daily collection
        - Can be collected from wild animals
- Captive pilot study
    - Dolphins at Sea World Gold Coast
        - Saliva and blow samples
- New analytical method
    - Liquid chromatography-mass spectrometry (LC-MS)
    - Solid phase extraction to concentrate samples
    - Preferred over traditional immunoassays
        - Small sample volume <50µL

        - Re-use sample for future analysis
        - Multiple analyses at once
- Pilot study results
    - Positive results for both saliva and blow samples
        - Testosterone
        - Progesterone
        - Problems with estrogens
- Stability
    - Issues with stability of hormones in both saliva and blow samples
    - Spiked samples with know concentrations of hormones and measured over time
        - At room temperature - 21ºC
        - At -20 ºC
        - At -80 ºC
    - Testosterone concentrations increased by 65% over 3 hours at room temperature
    - Progesterone concentrations increased by 25% over 2 hours at room temperature
    - Added 5ml inhibitor mix of 100mM $MnCl_2$ and 100 μg/ml amoxicillin/potassium clavulanate
    - $MnCl_2$ was for testosterone stability
    - Antibiotic was to prevent hormone degradation due to the presence of bacteria
    - Issues with stability of hormones in both saliva and blow samples
        - Stability at 21ºC – 12 hours
        - Stability at -20ºC – 4 weeks
        - Stability at -80ºC – 8 weeks
    - Extracted samples may be greater than 8 months at -80ºC
- Does it work with whales?
    - Humpback whales off Peregian Beach, Queensland with HARC in 2003 and 2004
    - NARW in the Bay of Fundy, Canada in 2005
- Sample collection
    - 5-inch embroidery ring mounted to 13-m carbon fiber pole
    - Cotton gauze
    - Cleaned stocking using 100% Acetonitrile and Milli-Q water wash
- Testosterone
    - Run time = 10 min
    - Retention time = 5 min
    - Extraction recovery
        - 50 ng/ml = 83%
        - 1 ng/ml = 86%
    - Intra-assay variability = 6.2%
    - Inter-assay variability = 7.3%
- Progesterone
    - Run time = 12 min

    - Retention time = 6 min
    - Extraction recovery
        - 50 ng/ml = 88%
        - 0.5 ng/ml = 89%
    - Intra-assay variability = 2.8%
    - Inter-assay variability = 4.4%
- Humpback whales
    - October 2003: n = 9
    - October 2004: n = 26
- NARW
    - August 2005: n = 18
- Results
    - Humpback whale testosterone
        - 2003: 2 of 9 samples
        - 2004: 2 of 24 samples
    - Humpback whale progesterone
        - 2003: none
        - 2004: 7 of 24 samples
- Quantifying hormones
    - Dilution factor
    - Sensitivity
- Other applications
    - Blow sampling allows for multiple sample collection from the same individual over time
    - Population biology
    - Genetics
    - Disease ecology
    - Lots of other possibilities

Tracy Romano (Mystic Aquarium)

Title: 'Stress, Environment and the Immune Response: Investigations in Wild and Captive Cetaceans'

- Evidence for nervous-immune system communication in cetaceans:
    - Presence of autonomic nerve fibers in lymphoid organs
        - Light and electron microscopy – presence of nerves and nerve terminals abutting cells of the immune system in cetacean lymphoid organs
        - HPLC – quantization of neurotransmitters in lymphoid organs
    - Adrenergic receptors on cetacean lymphocytes
    - Functional changes of immune cells in-vitro in presence of neurotransmitters/stress hormones
- Cloning of important immune response genes – to use as a tool in assessing immune competence (i.e. before and after stressors) and an evolutionary comparison of structure and function of cetacean immune system with other mammals, including humans

- Tools to assess immune status:
  - T helper cells (CD4)
  - T cells (CD2)
  - B cells (CD21, CD19, sIg)
  - Lymphocytes (MHC II)
  - T suppressor cells (CD8)
- Study Group of dolphins in Navy Marine Mammal Program
  - ~70 bottlenose dolphins
  - Males and females of various ages
  - Known life history
  - Research laboratory on site
- Approach
  - Catecholamines (NOR, EP, DOP)
  - Cortisol, aldosterone
  - Immunophenotyping (flow Cytometry)
  - Lymphocyte proliferation
  - Natural killer cell/neutrophil function
- Summary of neural-immune analyses on Navy Dolphins
  - NE, E, WBC – significantly > in ill dolphins
  - ACTH – significantly > in female dolphins
  - T, B, T helper, class II > in dolphins less than 18 years
  - Positive correlations: ACTH vs. T, class II; E vs. T, B, class II, T helper, WBC; NE vs. WBC; DA vs. WBC
- Investigation of the Effects of Anthropogenic Sound on Marine Mammal Health
  - TTS Experiments
    - Purpose
      - Measure masked underwater hearing thresholds before and after exposure to single underwater impulsive sounds produced from a seismic water gun
      - Measure neural-immune parameters before and after sound exposure
  - TTS Experimental Set-up
    - 2 underwater listening stations
      - S1 – fatiguing stimulus, start signal
      - S2 – hearing test tones
    - Subjects and conditions
      - Open water experiments – water gun impulsive sound
        - MUK (beluga whale)
          - 3 groups: control (no sound); low sound (8.2, 20.2, 58.6, 87.2 kPa); and high sound (116, 118, 143, 160, 198 kPa)
        - BEN (bottlenose dolphin)
          - 2 groups: control (no sound) and sound (146, 207, 220 kPa)
          - He also participated in pool experiments using tones

- 2 groups: control (no sound) and sound (130, 180, 190, 196, 198, 200, 201 dB rms re 1 μPa)

- Measurements of the nervous and immune systems during TTS experiments:
  - Catecholamines (norepinephrine, epinephrine, dopamine)
  - Hormones (cortisol and aldosterone)
  - Lymphocyte subsets (T cells, B cells, T helper cells, class II, ratios)
  - Complete blood cell counts/hematology
  - Serum chemistries
  - Banked plasma, serum, lymphocytes, RNA
- Summary of results of NI sound study
  - First attempt to study the effects of anthropogenic noise on cetacean health
  - Increased catecholamine levels after high level sound exposure for MUK
  - Increased catecholamine levels with increasing sound levels
  - Catecholamines returned to baseline levels within 24 hr
  - Increase in aldosterone after sound exposure and decrease in monocytes after sound exposure for BEN (water gun studies)
  - Appeared to be an "adjustment period" for BEN in the pool condition
- Stress of physical exam vs. human contact program
  - Objectives
    - Establish baseline or non-stressed values of stress-related hormones
    - Do hormone levels exhibit a diurnal pattern?
    - Compare different experimental stressors
    - Is plasma ACTH a better indicator of stress than cortisol or aldosterone?
  - Study Design
    - 3 adult long-term captive belugas (19 years old)
    - Blood collected via voluntary fluke presentation
    - Approximately 40 samples per whale
    - Sampling times (0930 hrs, 1300 hrs, 2000 hrs)
    - Sampling during Physical examination – stretcher, post-endoscopy, 1hr post-endoscopy
    - Wading contact session – sampling before and 20 min post session
  - Summary of results
    - Plasma ACTH and cortisol were higher in the morning than in the evening while aldosterone was higher in the evening
    - Significant differences exist between physical exam stressor and baseline stress hormone levels; no differences for wading contact programs
    - ACTH increased 5-10 fold, whereas cortisol and aldosterone showed 2-4 fold elevations during physical examination
    - Parallel trends exist between ACTH and cortisol
- Collaboration with Shedd Aquarium – collected data for future analyses pending future funding
  - Physiology: blood, saliva, feces

    - Vocalizations - recordings
    - Behavior – quantitative sessions
- Limitations of stress studies on captive marine mammals
    - Number of subjects
    - Trained animals
    - Opportunistic sampling
    - Variability
    - Health criteria - what defines a healthy cetacean?
    - Study design
- Future direction for stress studies on captive marine mammals
    - Design studies for hearing and stress
    - Design studies for measuring stress/time course (saliva, blood, feces)
    - Integrate behavior and vocalization studies with physiological studies
    - Stranded marine mammals
    - Injection of ACTH – time course/magnitude of catecholamines, immune response

Tracy Romano (Mystic Aquarium) / Frances Gulland (Marine Mammal Center)

Title: 'Research in the field'

- Subsistence hunted belugas
    - Purpose - to obtain: blood samples from live capture-released belugas and blood and organs of the immune system from subsistence hunted belugas in order to contribute to information on health status of wild belugas as well as to enhance our health studies on belugas at Mystic Aquarium
        - Samples will serve as a baseline before impact of oil and gas exploration, oil spills, climate change; also in future they can be compared with endangered populations (i.e. Cook Inlet belugas)
    - Health Assessment in belugas
        - Immune status
        - Stress hormones
        - Disease exposure
        - Archive for gene expression
        - Clinical health parameters
    - Stranded animals have highest level of catecholamines compared to chase and captured animals; sound studies
- Chase Encirclement Stress Studies (CHESS)
    - Objective – investigate potential stress-related effects of repeated chase and encirclement of dolphins associated with the ETP tuna fishery
    - Target species: pantropical spotted dolphins and eastern spinner dolphins
    - Strategy
        - Use a chartered tuna purse seiner/capture boat using standard fishing techniques
        - Carry out complementary studies focusing on impacts to survival and reproduction

        - Feasible projects given at-sea constraints on capture, handling, sampling, blood processing and storage
    - Logistics
        - Seiner captures school using standard methods
        - Rafts and personnel deployed into net for sampling, tagging, and photos
        - Swimmers restrain dolphins and lead them to sampling rafts
        - Raft stations: do bleeding/sampling/tagging, biopsy, and roto-tag 'drive-thru'
        - All dolphins released together at backdown
        - Focal dolphin tracked for recapture along with others
- Pathophysiological Studies
    - Thermal studies
    - Blood analyses
    - Immunological studies
    - Pathology
    - Skin proteins

- Does repeated chase and encirclement bring about changes in immune function?
    - Methods
        - Immunophenotyping of lymphocytes – flow Cytometry
        - Lymphocyte proliferation – mitogen proliferation assay
        - Lymphoid organ morphology – immunocytochemistry/microscopy
    - Results
        - Were only able to resample 10 animals
        - Values comparable to those in other cetaceans
        - Decreased % B cells in recaptured dolphins
        - Lymphocyte function unchanged in recaptured dolphins, but lower for B cells in recaptured dolphins after 3 days
        - No evidence of chronic changes in lymphoid organs
    - Immunology conclusions
        - Possible effect on the arm of the immune system responsible for antibody production
        - No chronic effects noted morphologically
    - Thermal Results
        - Some increase in fin and body surface temperatures with longer chase times
        - No increase in core body temperature, with the exception of one female
        - Heat flux increased during chase and escape
    - Thermal conclusions
        - Chased dolphins may generate additional heat, but appear to be able to dissipate it before becoming hyperthermic
        - Hyperthermia may be a problem in some individuals
    - Hematological Analyses
        - Total and differential WBC counts

        - RBC counts
        - Hemoglobin
        - Hematocrit
        - MCV, MCH, MCHC
        - Red cell distribution width
    - Serum Chemical Analyses
        - Electrolytes – Na, K, Cl, Na:K, Anion gap, bicarbonate, % saturation, etc
        - Metabolites – glucose, urea, uric acid, creatinine, cholesterol, etc. (total, direct, indirect)
        - Enzymes – AP, ALT, AST, amylase, CK, GGT, LDH
        - Protein – total, albumin, globulin, A:G, fibrinogen

    - Hormonal Analyses
        - ACTH, cortisol, T4, fT4, aldosterone, T3, rT3, epinephrine, norepinephrine, dopamine, estradiols, progesterone, testosterone
    - Trends in "baseline" samples
        - Time interval from start of chase to blood collection influenced levels of at least 12 serum chemical constituents and hormones and 6 hematological determinations
        - Positive correlations for Ca, glucose, Na, K, urea, uric acid, T3, fT4, WBC, lymphocytes, eosinophils, platelets, MCHC
        - Negative correlations for bilirubin, Cl, Na:K, Mg, and MCH
    - Blood Analyses
        - Levels of catecholamines, ACTH, cortisol, and muscle enzymes appear to be elevated in dolphins sampled after 1.5-4 hours confinement in the net
        - Levels of iron and thyroid hormones were significantly lower in recaptured dolphins
        - No significant elevations in muscle enzymes in recaptured dolphins
    - Blood Analysis conclusions
        - Chase and encirclement produces a measurable stress response in dolphins
        - Elevations in catecholamines highlight the risk for benign capture myopathy
        - Elevations in ACTH appear to be large and sustained during encirclement
        - Repeated chase and capture may influence thyroid and iron status
        - No changes in blood constituents signaling distress were noted
    - Necropsy
        - Acute lesions in heart, muscle, and kidney of dolphins drowning in nets are consistent with excessive catecholamine levels in blood
        - Scars observed in heart muscle demonstrate earlier, sub lethal damage

    - Necropsy conclusions
        - A 'sympathetic storm' resulting in cardiac arrest is the likely cause of death in dolphins dying in nets
        - Smaller increases in catecholamines likely result in benign cardiomyopathy with unknown functional consequences
- CHESS conclusions
    - Physiological changes indicating stress were documented in captured and recaptured dolphins during CHESS.
    - Changes were not sufficient to lead to rapid death following chase and capture.
    - Population-level conclusions cannot be made at this time and would require larger sample sizes.
    - Connections between the various projects provided complementary information and allowed identification of effects that may warrant further study.
- Overall Findings
    - Recapture sample too small to allow statistically defensible conclusions
    - Evaluation of the acute stress response relative to other species of cetaceans
        - How severe?
        - Potential to produce lesions?
        - Consequences of these lesions?
- Inferences from data
    - Significant adverse effect might occur through selective loss of individuals
    - Examples of unusual individuals
        - D61 slightly hyperthermic (exertion?)
        - D17 hypothermic, leucocytosis (underlying illness?)
- Limitation of CHESS
    - Large variation in population values may obscure trends in individuals
    - "Baseline" data are confounded by long time interval prior to sampling
    - Small number of recaptured dolphins
    - Very small number of recaptured dolphins with first-capture data

Frances Gulland (Marine Mammal Center)

Title: 'Assessment of stress response in rehabilitating California sea lions'

- Effects of rehabilitation and restraint on fecal corticosterone
    - Variability in response
    - Restraint for blood sampling increases fecal corticosterone
- Effects of surgery and anesthesia
    - Physical restraint - increases cort response, but need to take animal condition into account
    - Inhalant gas anesthesia for non-surgical purposes
    - Minimally invasive surgery
    - Invasive surgery
- ACTH stimulation tests

    - ACTH stimulation – 2 IU/kg, I.M. long-acting gel preparation
    - Timing and magnitude varied
    - There was a diminished response in DA animals
- Fecal corticosterone – RIA kit (MP Biomedicals)
    - Peak in fecal corticosterone
        - 230-500% baseline
        - 18-48 hrs post injection
- Diminished responses in DA toxicosis
    - Domoic acid is a glutamate receptor agonist
    - NMDA, kainate receptors
        - Hippocampus
        - Cardiac myocytes
        - Adrenals?
    - Circulatory eosinophilia observed
- Biotoxin effects decreased cort levels
- Research needs
    - Methods independent of handling
        - Skin/blubber biopsies, feces, hair
    - Methods independent of adrenal exhaustion, suppression
    - Methods reflective of chronic stress rather than short term activation of HPA axis
    - Genomics: telomeres
    - Metabolomics: protein folding enzymes
- Issues
    - Confounding effects on adrenal function
        - Artifacts of handling, sedation
        - Exposure to environmental biotoxins, POP alter adrenal hormones
    - Adrenal exhaustion and suppression not differentiated with hormonal measurements

Patricia Fair (National Oceanographic & Atmospheric Administration)

Title: 'Bottlenose Dolphin Health & Risk Assessment Project: Measures of Stress'

- Comprehensive dolphin screening level health assessment study
    - 2 SE U.S. sites: Charleston, SC and Indian River Lagoon, FL
    - 2003-2005
    - Develop standardized tools and methods
    - Explore relationship between health and environmental stressors
- Site investigations of dolphin populations in CHS, SC and IRL, FL to assess health
    - Concerns in IRL ecosystem
        - Destruction of sea grass habitat
        - Alteration of water flow
        - Low flushing rate
        - Declining water quality
        - Dolphin UME 2001

        - Many have been diagnosed with a variety of skin lesions
    - Concerns in CHS ecosystem
        - Increase in development and agricultural influences
        - NOAA data found high sediment contaminants
        - Two superfund sites
            - Suggests potential for detrimental health impacts although dolphins show fewer signs of compromised health
- Assessing a dolphin's health can be a hard puzzle to solve
    - Endocrine hormones – blood
    - Urinalysis
    - Cytology
    - Body condition
    - Bacteria, viruses, fungi
    - Antibiotic resistance
    - Hematology
    - Contaminants
    - Stress hormones
    - Nutrition
    - Biomarkers
    - Oxidative stress/DNA damage
    - Immunology
- Stress related measurements
    - Bioindicators of exposure
        - Contaminants (PCBs, PBDEs, pesticides, PFCs, PAHs, trace metals)
    - Bioindicators of effects
        - Immunology tests
        - Stress hormones
        - Oxidative stress
            - Surveys and epidemiology studies in humans should measure at least 1 marker of oxidative stress – isoprotanes, comet assay, c-reactive protein
- Methods/Strategy
    - Use existing laboratory tests when available – hematology, serum chemistry, hormones
    - Optimize assays – method development for dolphins; suite of immunology tests
    - Explore methods used in human medicine – isoprostanes (most established index of oxidative stress in vivo in humans)
    - Conduct quality control studies
        - ACTH – no existing data on influence of collection methods, blood components, storage condition – conducted joint study with NOAA, Cornell Endocrinology Lab, Dolphin Discovery, and HBOI
- Biomarkers

    - Definition – xenobiotically induced variation in cellular and biochemical components, in processes, in structures, and or functions that is measurable in a biological system or sample (NAS)
    - Examples:
        - Phase I reactions – oxidative step, makes compounds more hydrophilic; induction of cytochrome P450 enzymes
        - Phase II reactions – metabolites linked to endogenous compounds (sugar derivatives, peptides, sulfated) making them very water-soluble, non-toxic, ionizable, and readily excreted; changes in GST, UDP-glucuronyltransferase
        - Oxidative stress/cellular stress biomarkers – lipid peroxidation, lysosomal stability, methemoglobinemia, DNA or protein adducts, heat shock proteins, P-glycoprotein, histopathology
        - Metal exposure/toxicity – metallothioneins, poryphyrins
        - Organophosphate/carbonate exposure/toxicity – acetyl cholinesterase
- Biomonitoring endpoints
    - Indicators of exposure – process that links sources with effects
        - Body burden – whole body or tissue concentrations of contaminants
        - Biomarkers of exposure – biochemical or physiological changes which indicate that an organism has received an internal dose of a chemical
    - Indicators of effects
        - Changes in population, community, or ecosystem characteristics
        - Overt toxic injury in individual organisms (e.g. mortality, behavioral abnormalities, lesions, etc)
        - Biomarkers of effects – biochemical or physiological indicators of a toxicological response
- Stressor – contaminants
    - Background
        - High levels of Persistent Organohalogen Contaminants (POCs) have been detected in marine mammals
        - Concern of POC exposure on immune systems of marine mammals followed outbreak of morbillivirus epizootics; hypothesis: immune suppression was contributory
        - POC exposure studies in numerous laboratory animal studies reported increased immunotoxicity and susceptibility to disease
    - Objectives
        - To explore possible contaminant-induced health and immune alterations in dolphins: PCBs, pesticides, PBDEs, and PFCs were quantified and relationships between contaminant exposure and health status and immune parameters were examined
- Methods
    - Assessments
        - Health

- Capture-release studies in summer months of 2003-2005 (94 CHS; 101 IRL captured, examined, sampled, released)
- Contaminants
  - ∑ PCBs (n=71)
  - ∑ Pesticides (n=20)
  - ∑ PBDE (polybrominated diphenyl ethers) (n= 13)
  - ∑ PFCs (n=12) (perfluorinated chemicals)

- Immunological
  - Screening set of immune parameters for bottlenose dolphin

- Analysis (animals placed in 3 groups (tertiles) based on distribution of contaminant)
  - Contaminant-Immune differences were compared using maximum likelihood least squares between 1st and 3rd tertile controlling age/sex, separately per site
  - Contaminant/Immune-disease differences compared using logistic regression with age/sex controlled per site

- Health classification – expert judgment (veterinarian panel)
  - 2003-2005: evaluated 171 dolphins using physical, ultrasound, hematology, serum chemistry, cytologic, and microbiologic evaluations
    - Classifications were normal, possible disease, definite disease
  - Lobomycosis - chronic, granuloma disease of skin & subcutaneous tissues caused by fungal pathogen *Lacazia loboi* found only in dolphins and humans --1st described in humans 1931 in the Amazon basin;
  - **Papilloma** --We reported 1st cases of orogenital papilloma with novel herpes virus & papillomavirus in free-ranging dolphins (Bossart et al 2006); Preliminary evidence suggests that these tumors may be infectious, most likely having an orogenital route of transmission. Thus, sexual and cow-calf transmission may be important components in disease pathogenesis. It is unknown what role other environmental or host factors may have played in the formation of these lesions. (Bossart et al 2006).
- Disease classification – site
  - Prevalence of disease higher (not statistically) in IRL (31%) vs. CHS (20%).
  - Lobomycosis (n=10) found only in south IRL contributed to higher disease
  - Less than half of all dolphins at both sites classified as "normal" health
- Screening suite of immune markers – bottlenose dolphins
  - Immunosuppression effects may be one of the most sensitive and environmentally relevant effects of OCs.
  - No single test can evaluate an organisms' immune system – a suite of immune markers provides a more complete depiction of immune status and function. The ability to study immune function in cetaceans has been limited due to absence of routinely available tests. Thus, one of our

priority goals was to optimize a suite of immune parameters for bottlenose dolphins and we'll be publishing this as a NOAA Technical Memorandum

- Comparison of health and immune parameters
  - Immune:
    - CHS diseased dolphins – significant increase in lysozyme, increased phagocytosis, increased B cell proliferation
    - IRL diseased dolphins – significant increase in phagocytosis
  - Disease – immune and clinical markers
    - Lobomycosis
      - Dolphins had significant immunologic impairment in adaptive immunity
    - Papilloma
      - Acute phase inflammation and up regulated innate and humoral immunity in dolphins with sexually transmitted orogenital papillomas
- Perfluoroalkyl compounds (PFAs)
  - Globally detected in water, sediment, house dust, human blood, and wildlife
  - Adverse effects on: developmental and reproductive systems, immunology systems, neuro-endocrine systems, cellular integrity, and carcinogen
  - Some PFAs bioaccumulate and biomagnify through food webs
- Concentrations of PFA chemicals in wildlife and humans
  - PFA levels for CHS dolphins are higher than anything reported in dolphins, cetaceans, or mammals.
- Predicting contaminant related disease outcomes
  - Diseased dolphins
    - CHS dolphins in highest PBDE tertile significant association with disease (odds ratio of 22.3)
    - CHS dolphins in highest PFC tertile significant association with disease (odds ratio 27.4)
    - IRL dolphins – no significant disease outcome related to contaminants
- Summary: health vs. contaminants
  - Significant associations of high levels of two emerging contaminants (PBDEs and PFCs) with disease were found only for CHS dolphins
- Summary: Health vs. immune
  - Diseased dolphins vs. healthy dolphins in both sites had alterations of innate immunity – increased phagocytosis for both sites, also increased lysozyme in CHS dolphins only
  - Additionally, diseased dolphins in CHS had suppressed adaptive/humoral immunity as indicated by decreased B cell proliferation
- Summary: immune vs. contaminants
  - Increased respirations observed with increasing PCB levels in both locations

  - Lymphocyte proliferation was not associated with PCB concentrations - contrary to other studies with increased levels ----differences may be attributed to species, dose, and chemical
  - Alterations in some parameters with total pesticides, but most studies assess single pesticides or classes. This data will be further assessed by pesticide class
  - Although PBDEs have been associated with lymphoid depletion in stranded and by-caught harbor porpoises, no striking effects were noted to any degree on functional immune parameters in bottlenose dolphins
  - PFCs were associated with increased B-cell proliferation (CHS), numbers of T-cells (CHS), phagocytosis (IRL), and respiratory burst (CHS). However, little data exists in the literature at environmentally relevant concentrations of PFCs for comparison
- Stress Response
  - Measurements
    - Blood levels of ACTH, cortisol, aldosterone, epinephrine, norepinephrine, dopamine
    - Urinary cortisol
  - Evaluate capture stress (restraint, exam, sampling)
    - Relationship between stress parameters over time?
    - Do sick/diseased animals have an impaired stress response?
    - Sex, age, site variables; time (pre and post capture)
      - 2 years pre and post data; 5 years pre
- Isoprostanes (IsoPs)
  - Series of prostaglandin-like (PG) cmpds formed via free-radical peroxidation of arachiodonic acid (AA)
  - Potent biological activity
  - Large body of evidence IsoPs are reliable markers of oxidant injury both *in vivo & in vitro*
  - *Animal models (Review Fam & Morris, 1993)*
  - *Human Diseases (Alzheimer, Atherosclerosis, Crohns, Diabetes, Huntington, Rheumatic inflammation, Chronic obstructive lung disease, etc.)*
- Urinary 8-isoprostane F_{2a} (8-iso-PGF2ά)
  - Results: CHS dolphins = mean 0.43 (±0.21 S.D.) ng/mg creatinine (range 0.15-1.26 ng/mg); IRL dolphins mean 0.45 (± 0.18 SD ng/mg; range 0.22 to 0.86 ng/mg). *Mollenhauer et al. 2005 SMM*
  - *Length and gender had no affect on urine F2-isoprostane levels (32 dolphins from CHS and 11 from IRL); similar to humans (Basu & Helmersson, 2005; Block et al. 2002)*
  - F2-isoprostane levels were detectable in urine samples collected from dolphins and the range of these values was similar to those found previously in humans
- Isoprostanes
  - First report of urinary IsoPs levels in free-ranging bottlenose dolphins

    - Measurements of isoprostanes may provide a valuable and non-invasive approach to defining oxidative stress in marine mammal and other wildlife populations
    - Need for additional standards for IsoP isomers
    - Studies targeting how levels of biomarker vary during 24 hr period and between days and diseased/stressed animals (age and gender variation)
- ACTH Results
    - EDTA draw order – no effect
    - Heparin vs. serum – no effect
    - EDTA slightly higher than samples with heparin or serum
    - No difference between fresh vs. frozen samples
- Conclusion Comments
    - Environmental stressors may be impacting both populations; immune modulation associated with contaminants and disease. Both populations may be impacted, albeit more highly contaminated CHS dolphins had disease outcomes and a greater number of immune alterations associated with contaminants
    - Epidemiological study - helps to provide insight into how contaminants may affect immune and health status of bottlenose dolphins – not cause and effect
    - Immunotoxic effects of these contaminants, especially emerging PBDEs, PFCs needs further research. *Comparison to other studies may vary due to species differences, dose and chemical differences...."*
- Things to keep in mind/Lessons learned
    - Important that comparison of results in individual dolphins and populations are based on using methods which are optimized for dolphins or other species of interest using the same methods and suite of immune assays
    - Need for standards and specific assays for marine mammal species
    - Quality control/quality assurance