



MYSTIC AQUARIUM

Project No. **13007**

RESEARCH PROPOSAL and PROTOCOL REVIEW

MYSTIC AQUARIUM
Sea Research Foundation, Inc.
55 Coogan Blvd.
Mystic, CT 06355

Introduction

Mystic Aquarium is committed to research and promotes the use of its collection for the purpose of enhancing knowledge of aquatic animals through scientific investigation.

The following proposal materials are designed to address all pertinent issues regarding the use of live animals for scientific study at Mystic Aquarium. Individuals interested in conducting research must provide this information to satisfy the requirements of the Aquarium's Staff Research Committee and Institutional Animal Care and Use Committee (IACUC). It is the function of these groups to ensure that all protocols comply with the regulations and standards of the United States Department of Agriculture, as well as standards established by Sea Research Foundation. Information should be specific and comply with the format as presented.

Submission and questions regarding the preparation of this proposal should be directed to:

Gayle Sirpenski, Secretary, IACUC
(860) 572-5955 ext. 108
gsirpenski@mysticaquarium.org

I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Tracy Romano, Vice President of Research & Chief Scientist
860-572-5955 Ext. 102; tromano@mysticaquarium.org; 55 Coogan Blvd Mystic CT 06355-1997

Laura Thompson, Research Fellow
860-572-5955 Ext. 155; lthompson@mysticaquarium.org; 55 Coogan Blvd Mystic CT 06355-1997

B. Title of proposed research

Health Assessments of Wild Belugas

C. Introduction and background

Given the current threats in the Arctic including climate change, oil and gas exploration, mineral extraction, increased shipping traffic and pollution including noise, there is increasing concern for the conservation and management of beluga whales (*Delphinapterus leucas*) within Alaskan waters and beyond. The population trends vary greatly for the different management stocks and little is known about the health or immune status of these individuals. To this end there has been live capture release health assessments occurring in Point Lay and Bristol Bay, AK. These ongoing studies will provide information on the health and immune function of the wild beluga population. Furthermore some of the data obtained may be able to be utilized for comparison in other beluga populations including endangered populations e.g. Cook Inlet, where sampling and handling, may be limited or prohibited.

D. Brief summary of proposed research. (Note if the study would be done in house or in the field.)

Sample collection for the proposed research project will occur in the field and not in-house. All samples will be collected in collaboration with other research efforts and/or subsistence hunters. Samples will be transferred to Mystic Aquarium under permit # (17298).

Samples including blood, saliva, respiratory exhalation, viral swabs, skin, feces and/or tissues will be collected on live-captured and released belugas or subsistence hunted belugas as feasible. Blood samples will be used for complete blood cell counts, serum chemistries, pathogen exposure and for the isolation of peripheral blood mononuclear cells (PBMC). Archived PBMC will be used for assessing immune function, as measured by the lymphocyte proliferation assay and immunophenotyping subpopulations of lymphocytes. Tissues samples will be primarily collected from subsistence hunted whales including but not limited to blood, lymph nodes, spleen, thymus, feces and skin. These tissues will be preserved for molecular analysis, screening for infectious agents (e.g. brucella), and/or histological studies. The collection of skin from live-captured belugas will be used to assess and validate the use of skin as an alternative matrix for assessing the health of whales. The use of an alternative matrices, such as skin, is highly desirable and would allow for future studies of free-ranging cetaceans in which logistical or other constraints or concerns prevents collection of blood or other biological samples.

E. Specific goals of the proposed research.

The goals of this proposed research are to: 1) assess the integrity of samples collected in a field setting including blood and skin from capture-released and subsistence hunted whales; 2) add to our understanding of the variation of multiple biomarkers and establishment of baseline values for these biomarkers in free-ranging belugas; and 3) support the development and validation of methods for use in marine mammals and in particular the use of alternative matrices to assess the health and immune status of belugas (e.g. respiratory exhale, feces, skin).

Please note: Live capture release studies and subsistence hunts may not happen on a yearly basis for various reasons. The frequency of these events are in the hands of our collaborators and depend on permitting and funding. We are not leading the live capture effort, but taking the opportunity of these events for sampling wild whales. Moreover, if these events do occur the chances of success are highly variable given weather conditions, timing, success at capture, success at the hunt, etc. Oftentimes if they are successful 1-2 whales per field season may be sampled. We anticipate these studies to occur long-term, and will strive to take every opportunity to sample when live capture release studies occur and subsistence hunts occur pending funding. Moreover, monitoring health long term in wild belugas given anthropogenic and environmental stressors is critical. For these reasons we anticipate this project to be a long-term project.

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

Currently, non-animal models are not available as a proxy for studying the health and immune status of belugas. Therefore, this study seeks to acquire samples from free-ranging belugas that are being handled or sampled as part of other live-capture studies or in association of subsistence hunts and subsequent sampling. In an effort to reduce the impact on the animals, samples collected will be used for *in vitro* experiments in our laboratory rather than in vivo experiments.

B. Justify the choice of species to be used.

Belugas have a circumpolar distribution and are found in estuaries in the summer and along the continental shelf, where increases in exploration, development and shipping activities are currently occurring and likely to increase. Therefore, belugas are expected to experience a changing environment and will likely face many anthropogenic challenges in the near future. Further, the presence of belugas in aquaria and marine parks provides an opportunity to study the endocrine and immune systems, physiology and general health of belugas under controlled conditions.

C. Identify and justify the number of animals to be used.

The number of animals sampled will be dependent on the constraints of our collaborators permits, the field conditions and the ability to safely handle and sample whales. It is expected that between 5-10 whales would be sampled during each live capture release study and 10-20 whales sampled from the subsistence hunt.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

Biological samples including skin and blood will be collected from live-captured belugas using standard aseptic techniques. All samples will be collected by personnel qualified and experienced in the collection of samples from belugas and other marine mammals.

Blood -will be collected from the periarterial venous rete of the flukes using butterfly catheter directly into a syringe or blood tubes.

Respiratory exhalation - will be collected by placing either a conical tube or a petri dish above the blow hole while the animal exhales.

Saliva - will be collected by utilizing a piece of gauze to swab the side and back of the whale's mouth as feasible.

Feces - will be collected by inserting a catheter into the rectum.

Skin - will be collected opportunistically and as a result from inserting a trocar directly underneath the dorsal ridge from one side to the other in order to thread nylon pins that hold a satellite transmitter in place. Smaller, superficial biopsies will also be taken from normal skin as well as cutaneous lesions considered potentially significant by the field veterinary staff. A photograph will be obtained prior to biopsying. The site will be cleaned and an antiseptic applied. Depending on the shape and size of the lesion either a scalpel will be used to obtain a wedge or a punch biopsy (no greater than 8 mm, typically 6 mm) which may include a small amount of underlying blubber. Sloughed skin may also be retained for analysis.

Blood, feces and skin in addition to other tissues will be collected from subsistence harvested whales utilizing necropsy techniques.

**B. Will a test substance be administered? If yes,
1.) What is the name of the compound?**

N/A

2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes.

N/A

C. Will manual, chemical and/or mechanical restraint of the animal be necessary? If yes, explain the method of restraint.

Manual restraint of live-captured animals will be required. A tail rope will be secured to the peduncle of the whale and will be tied off to a small vessel or manually held by personnel. Additional restraint will be used when needed by placing a hoop net over the animal. Additionally, qualified personnel will use physical restraint when needed during the process and collection of biological samples.

No restraint will be needed when sampling subsistence hunted whales.

D. Indicate the level of pain the proposed procedure is expected to cause.

a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.

b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.

c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery of procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

E. Describe the method of euthanasia to be used if this were to become necessary

No euthanasia is expected.

**F. Does the proposed research involve surgery?
If yes, go to Addendum II. Surgical Protocol**

N/A

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

The study animals will be free-ranging whales and will not be housed as part of this study.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours.

A veterinarian is present during the handling of live-captured whales in Bristol Bay but not always in Pt. Lay.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics?

The study animals are free-ranging or subsistence hunted whales.

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include 1.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

Tracy Romano, PhD

1) Formal Education:

Postdoctoral training: joint appointment at the Naval Command, Control, and Ocean Surveillance Center and The Scripps Research Institute, San Diego, CA. October 1993 - June 1996.

University of Rochester, School of Medicine and Dentistry, Rochester, NY. Doctorate of Philosophy degree in Anatomy; May, 1993.

Saint Michael's College, Winooski, Vermont. Bachelor of Science degree; May, 1986.

2) Relevant General Experience: Organized and conducted four field expeditions to Churchill, Manitoba, a field expedition to the Northwest Territories, Canada, twelve expeditions to Pt. Lay, Alaska, and one expedition to Barrow, Alaska to collect tissues from beluga and bowhead whales taken by native hunters. This is a unique opportunity to obtain fresh cetacean tissues for research and requires establishing working relations with political organizations of the U.S. and Canada as well as the Inuit people, and logistical planning. Also have participated in the live capture-release of beluga whales for tagging with satellite transmitters and to obtain blood samples for health assessment. (1997, 1998, 2007, 2010, 2011, 2012, 2013, 2014 and 2015). Also in the summers 2012 and 2013 conducted a pilot study on belugas in Cunningham Inlet focusing on photo ID, quantitative behavioral assessments, acoustic monitoring and educational outreach.

3). Training in non-routine, invasive and surgical procedures: Not applicable.

Laura Thompson, Research Fellow

1) Formal Education:

University of Connecticut, Groton, CT. Doctorate of Philosophy degree in Oceanography; May, 2014.

Queen Mary, University of London, UK. Bachelor of Science degree, May, 2006.

2) Relevant General Experience: Participated in live capture-release health assessments of belugas in Bristol Bay, Alaska in 2012, 2014 and 2016 which included animal restraint, general handling and biological sampling as well as processing of samples in the field. In the summer of 2012 participated in a pilot study of beluga whales in Cunningham Inlet focusing on photo ID, quantitative behavioral assessments, acoustic monitoring and educational outreach.

3). Training in non-routine, invasive and surgical procedures: Not applicable.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

All collaborators participating in the live capture health assessments and/or sample collection from subsistent hunted whales have had experience in animal chase, capture and restraint and/or tissue sampling.

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

Not Applicable

1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).

2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?

3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.

4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.

5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.

ADDENDUM II. SURGICAL PROTOCOL

Not Applicable

1.) Have alternatives to the surgical procedure been considered and found to be inappropriate? Explain.

2.) Describe with reasonable detail the surgical procedures to be used and where it is to be performed. Include the name of the person who will be performing the surgery and that persons level of expertise in this area. (Note: All survival surgery must be performed using aseptic technique.)

3.) Will the surgery be major or minor?

4.) Will multiple survival surgeries be performed OR is there more than one major operative procedure from which the animals are allowed to recover? (Note: More than one major operative procedure is NOT allowed without prior approval from the IACUC.)

5.) What method of anesthesia will be used; who will supervise the administration of anesthesia and how will the anesthetic depth be monitored?

6.) Describe the pre- and postoperative care that will be provided. Include names of anesthetics, analgesics, antibiotics, dose and route of administration and duration.

STATEMENT OF ASSURANCE

Based upon available information, the alternatives to the use of live animals for the purpose of this study have been considered and found to be inappropriate. It is the responsibility of the Principal Investigator to assure that the protocols outlined here for the humane care and treatment of the animal are followed. The Mystic Aquarium reserves the right to discontinue the study if the health of the animal or the safety of the staff is jeopardized. Any necessary changes in the study design will be approved by the Institutional Animal Care and Use Committee (IACUC) prior to implementation. The Principal Investigator has reviewed the qualifications of all personnel involved in the study and found them to be adequate.

To the best of my knowledge, the animal research described in this protocol review does not unnecessarily duplicate previous experiments.

Joey Romond February 5, 2019
Principal Investigator Date

The Attending Veterinarian has reviewed the proposed protocols for animal use and has found them to be acceptable.

Josephine 2/5/19
Attending Veterinarian DIP. ACVIM Date

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IACUC Comments or Conditions of Approval:

F. Fabiana Matos 2/5/19
Signature of IACUC Chair Date

Approval Period: 2/5/19 - 2/4/20 Protocol # 13007
(Must be reviewed annually)

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____



Project No. 16006

RESEARCH PROPOSAL and PROTOCOL REVIEW

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I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Tracy Romano, PhD
VP of Research & Chief Scientist
Sea Research Foundation, Inc.
Mystic Aquarium
55 Coogan Blvd.
Mystic, CT 06355
Phone: (860)-572-5955 Ext. 102
FAX: (860)-572-5969
E-mail: tromano@mysticaquarium.org

B. Title of proposed research

Determination of biomarkers and assays from different tissue matrices of the beluga to aid in assessing health in aquarium and wild whales

C. Introduction and background

Over the past years our laboratory has focused on developing reagents, adapting and validating assays, and exploring non-invasive methodologies for assessing the physiological response to stressors and immune function and/or overall health in cetaceans. Belugas have been a primary focus given the Aquarium's collection animals and our collaborative efforts in the field to study wild belugas. The Aquarium whales allow for controlled studies in that we know general information such as diet, environmental quality, health history, etc. and that they are trained for sample collection. Moreover, the aquarium setting allows for opportunistic sampling before, during and after scenarios that are likely to bring about a physiological response that can be measured which helps directly in validating new biomarkers and assays. Examples of some of the opportunistic scenarios include: obtaining weights in the stretcher, veterinary procedures on the Arctic Coast beach, transport of whales from one facility to another or within the aquarium, introduction to new environments, and introduction to new social groupings/interactions.

The gold standard for gaining information on the physiological health and response of cetaceans to various challenges has been blood. There is still a lot of information to obtain from blood including new biomarkers and technologies that have yet to be explored. However, it is difficult and sometimes not feasible to get blood samples from wild whales. In addition to blood, our laboratory has been investigating the potential use of other tissue matrices that require less invasive sampling. These tissue matrices include blow or breath condensate, saliva, feces and skin biopsies* (*wild whales).

We propose to continue investigation of these tissue matrices by including other biomarkers, refining current methodology and introducing new methodologies. It is proposed that blood, blow, saliva, feces and skin scrapings be obtained from the whales resident at Mystic Aquarium during routine monthly blood draws and before, during and after opportunistic scenarios described above. Samples will also be obtained from wild belugas through live capture-release health assessments or from subsistence harvested whales.

D. Brief summary of proposed research. (Note if the study would be done in house or in the field.)

The overall purpose of the proposed research is to continue investigations of biomarkers and assays in blood and less non-invasive tissue matrices that will contribute to assess health status in aquarium and wild belugas. This study entails the collection of blood, saliva, feces, skin scrapings and blow from resident belugas at Mystic Aquarium during routine monthly blood draws and before, during, and after scenarios likely to bring about a physiological response. These scenarios include but are not limited to the

following: obtaining weights in the stretcher, veterinary procedures on the Arctic Coast beach (out of water examinations), transport of whales from one facility to another or within the aquarium, introduction to new environments, and introduction to new social groupings/interactions. Routine baseline samples will be collected monthly. In addition, similar samples will be collected from wild belugas during live-capture release health assessments in Bristol Bay and Point Lay, AK as well as from harvested belugas in Point Lay. In general, molecular biomarkers and assays, microbiome, hormones and immune markers/assays will be the primary targets in blood and the other tissues.

E. Specific goals of the proposed research.

The specific goals for each tissue matrix are:

Blow

- Further validation of assay by determining a dilution marker
- Detection of additional hormones such as thyroid hormone
- Initial studies of microbiome
- Gene expression of immune markers

Skin Scrapings and biopsies* (*from wild belugas)

- Determine feasibility for use in molecular and microbiome studies
- Utilize developed probes for skin biopsies on skin scrapings

Feces

- Continuation of cortisol/corticosterone analysis
- Other hormones
- Initial microbiome studies

Saliva

- Review and modify collection methods
- Determine if EIA is feasible or if need Mass Spectrometry

Blood

- Use to compare with above tissue matrices
- Hormones, immune function, routine hematology and serum chemistries

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

The interactions between the immune, nervous, and endocrine systems are remarkably complex, yet understanding these interactions is critical to understanding the impacts of environmental and anthropogenic challenges on health. Understanding the complexity of

these systems and mechanisms of interactions require the use of living biological systems. The methodologies used to assess immune system parameters and biomarkers in diverse tissue matrices have been and continue to be developed in the research laboratory at Mystic Aquarium. The results of the project will aid in the further development of non-invasive techniques and information used to provide indices to assess health of wild and aquarium cetacean populations.

B. Justify the choice of species to be used.

Chronic activation of the stress response and concomitant decline in immunocompetence stemming from numerous anthropogenic challenges (e.g., pollution, fisheries interactions, reduction in food resources, introduction of novel pathogens, noise, boat traffic, and climate change) are hypothesized to increase the susceptibility of cetacean populations to infection and disease (Van Bresse *et al.* 2009; Romano *et al.*, 2002, 2004). Several emerging infectious diseases responsible for mass mortality events and reproductive decline have been reported in cetaceans in recent years (Reif *et al.* 2009; Van Bresse *et al.* 2009). Cetaceans inhabiting extreme latitudes, e.g., beluga whales (*Delphinapterus leucas*), may be especially vulnerable given the multifaceted impacts of rapid climate change and oil and gas activities in those regions (Jenssen 2006). Understanding the effects of anthropogenic challenges on cetacean health requires the identification of biomarkers and assays to determine the effects of various stressors on health.

C. Identify and justify the number of animals to be used.

This study proposes to use the beluga whales housed at Mystic Aquarium. Using the maximum number of belugas available is important to maximize sample size. The number of wild animals from live capture release studies is usually about 10 per year with harvested animals ranging from 10-25 whales per year.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

Monthly sampling

Blood, blow, saliva, feces and skin scrapings will be collected at the time of research monthly blood draws on Aquarium whales. Methodology for collecting blood and feces is by routine and established veterinary protocols at Mystic Aquarium. Collection of blow and saliva were piloted under the PI's prior IACUC entitled: "Investigation of the Physiological Responses of Belugas to "Stressors" to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health". Blow collection techniques have been established through these prior studies (Thompson *et al.*, 2014). Saliva collection has also been tested but may need minor modification due to dilution effects. Skin scrapings are a new addition to the sampling regime. Similar to published methods for dolphins (Bechsoft *et al.*, 2015), a rubberized scraper is proposed to be used to collect samples of epidermal skin cells from belugas under behavioral control. Belugas will be desensitized to the instrument first. Proposed sampling involves running the rubber edge of the ice scraper along each side of the animal once using single, not overly forceful strokes. The location right below the dorsal ridge is targeted for this purpose (Figure 1) at

each side of the animal. The skin sample will then be transferred to 15ml conical tubes filled with RNAlater solution for genetic analysis.

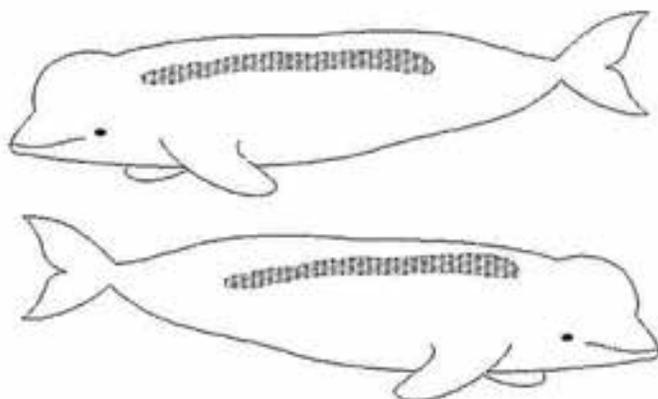


Figure 1. Outline of a beluga (*Delphinapterus leucas*) indicating the sampling location on the animal's left and right side by using a non-invasive rubberized scraper to collect samples of sloughed skin cells.

Before, During and after "Events" Sampling

Samples (described above) will also be collected during events such as before, during and after obtaining weights in the stretcher, veterinary procedures on the Arctic Coast beach (out of water examinations), transport of whales from one facility to another or within the aquarium, introduction to new environments, and introduction to new social groupings/interactions. The types of samples and tissue matrices will depend on the feasibility of obtaining these samples depending on the scenario. Ideally, baseline samples of all tissue matrices (as feasible) would be collected 1-2 days prior to the event, and at time points during and after the event. Time points during will be variable depending on feasibility and the specific scenario. Consultation with trainers and veterinary staff will occur to determine the best sampling options for the scenario. Ideal time points after the event are 1-3 hours, 24 and 48 hours. All samples should be collected the same approximate time of day given the diurnal changes in hormones; however, experiments can be included that test AM vs. PM samples in different tissue matrices if necessary.

Where appropriate, samples will be immediately centrifuged, aliquoted, and archived for subsequent analyses, and biomarker/assay validation. Hematological measures, complete blood cell counts and serum chemistries are requested in order to have this general important clinical information for each animal for that specific day and time.

- B. Will a test substance be administered?** No If yes,
 1.) What is the name of the compound?

2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes.

C. Will manual, chemical and/or mechanical restraint of the animal be necessary? Yes, mechanical restraint **If yes, explain the method of restraint.** A portion of the samples collected (event samples) will occur in conjunction with the scheduled weighing of the belugas, out of water examinations in conjunction with staff training and development or veterinary procedures and/or transport. Weight measurements require the belugas to be restrained in a canvas stretcher and suspended out of the water from a crane. Blood, blow, saliva, skin scrapings and fecal samples will be collected while the belugas are out of water or in the stretcher immediately after weighing as feasible. All other samples (i.e., before and after the weighing events) will be collected under behavioral control.

D. Indicate the level of pain the proposed procedure is expected to cause.

a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.

b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.

c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery of procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

E. Describe the method of euthanasia to be used if this were to become necessary

The method of euthanasia to be used if necessary would follow Mystic Aquarium's Program of Veterinary Care that is fully compliant with the American Veterinary Medical Association's standards.

F. Does the proposed research involve surgery? No

If yes, go to Addendum II. Surgical Protocol

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

The study animals are housed at Mystic Aquarium in appropriate conditions. The belugas are already trained or currently receiving training as part of routine husbandry behaviors for all necessary procedures, e.g., blood, saliva, blow, and fecal collection. The whales will need to be desensitized for the skin scrapings.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours. There is a veterinarian available or on call 24/7.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics? YES

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include 1.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

Dr. Tracy Romano has a PhD in neurobiology and anatomy from the University of Rochester in Rochester, N.Y. Before coming to Mystic Aquarium she carried out research at the U.S. Navy Marine Mammal Program in collaboration with the Scripps Research Institute in San Diego, Calif. The focus of her research was the impact of environmental stressors on the health of bottlenose dolphins. She subsequently established a research laboratory at the Navy facility dedicated to marine mammal neuroimmunology.

She has been at Mystic Aquarium since 2004 and is currently the VP of Research & Chief Scientist. Currently, her research focuses on studying the health of beluga whales in the wild and in the aquarium setting. She regularly travels to Point Lay, Alaska, to collect blood samples to monitor the belugas' health and to help determine whether climate change, oil and gas exploration, and/or other factors are having an impact on their well-being. This data will be compared to other beluga populations such as the endangered beluga population in Cook Inlet, Alaska, with the belugas at Mystic Aquarium serving as a baseline. All of this data will help to better understand belugas' health and how they respond to environmental change, including human-related factors.

CV available upon request.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

Dr. Jen Flower is currently the staff Veterinarian at Mystic Aquarium and will oversee all procedures and sampling. CV available upon request.

Kristine Magao, Assistant Curator oversees the training of the whales and the staff taking care of the whales on a daily basis. She will oversee the procedures and any necessary training related to the project. CV available upon request.

Drs. Maureen Driscoll, Ebru Unal and Laura Thompson are research fellows at Mystic Aquarium and are Co-PIs on this research. CVs available upon request.

REFERENCES

Beschoft, T., A.J. Wright, J.J. Weisser, J. Teilmann, R. Dietz, M. Hansen, E. Bjorklund, and B. Styrihave. 2015. Development of a new research tool for use in free-ranging cetaceans: recovering cortisol from harbor porpoise skin. *Conservation Physiol.* 3:1-9.

Jenssen, B.M. 2006. Endocrine-disrupting chemicals and climate change: A worst-case combination for arctic marine mammals and seabirds? *Environ. Health Perspect.* 114: 76-80.

Reif, J.S., Peden-Adams, M.M.; Romano, T.A.; Rice, C.D.; Fair, P.A.; Bossart, G.D. 2009. Immune dysfunction in Atlantic bottlenose dolphins (*Tursiops truncatus*) with lobomycosis. *Med. Mycol.* 47: 125-135.

Romano, T.A., Keogh, M.J.; Schlundt, C.; Carder, D.; Finneran, J. 2004. Anthropogenic sound and marine mammal health: Measures of the nervous and immune systems before and after intense sound. *Can. J. Fish. Aq. Sci.* 61: 1124-1134.

Romano, T.A.; Olschowka, J.A.; Felten, S.Y.; Quaranta, V.; Ridgway, S.H.; and Felten, D.L. 2002. Immune Response, Stress, and Environment: Implications for Cetaceans. In: *Cell and Molecular Biology of Marine Mammals*. C.J. Pfeiffer (ed). Krieger Publishing Co., Inc. pp. 253-279.

Thompson, L.A., Spoon, T.R., Geortz, C.E., Hobbs, R.C., Romano, T.A. 2014. Blow collection as a non-invasive method for measuring cortisol in the Beluga (*Delphinapterus leucas*). *PLoS*. 9(12).

Van Bresseem, M.; Raga, J.A.; Di Guardo, G.; Jepson, P.D.; Duignan, P.J.; Siebert, U.; Barrett, T.; de Oliveira Santos, M.C.; Moreno, I.B.; Siciliano, S.; Aguilar, A.; Van Waerebeek, K. 2009. Emerging infectious diseases of cetaceans worldwide and the possible role of environmental stressors. 86: 143-157.

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

Not Applicable

1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).

2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?

3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.

4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.

5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.

ADDENDUM II. SURGICAL PROTOCOL

Not applicable

1.) Have alternatives to the surgical procedure been considered and found to be inappropriate? Explain.

2.) Describe with reasonable detail the surgical procedures to be used and where it is to be performed. Include the name of the person who will be performing the surgery and that person's level of expertise in this area. (Note: All survival surgery must be performed using aseptic technique.)

3.) Will the surgery be major or minor?

4.) Will multiple survival surgeries be performed OR is there more than one major operative procedure from which the animals are allowed to recover? (Note: More than one major operative procedure is NOT allowed without prior approval from the IACUC.)

5.) What method of anesthesia will be used; who will supervise the administration of anesthesia and how will the anesthetic depth be monitored?

6.) Describe the pre- and postoperative care that will be provided. Include names of anesthetics, analgesics, antibiotics, dose and route of administration and duration.

STATEMENT OF ASSURANCE

Based upon available information, the alternatives to the use of live animals for the purpose of this study have been considered and found to be inappropriate. It is the responsibility of the Principal Investigator to assure that the protocols outlined here for the humane care and treatment of the animal are followed. The Mystic Aquarium reserves the right to discontinue the study if the health of the animal or the safety of the staff is jeopardized. Any necessary changes in the study design will be approved by the Institutional Animal Care and Use Committee (IACUC) prior to implementation. The Principal Investigator has reviewed the qualifications of all personnel involved in the study and found them to be adequate.

To the best of my knowledge, the animal research described in this protocol review does not unnecessarily duplicate previous experiments.

Tracy Remond 07/12/16
Principal Investigator Date

The Attending Veterinarian has reviewed the proposed protocols for animal use and has found them to be acceptable.

Allison Tuttle, DVM, Dipl. ACZM 08/08/16
Attending Veterinarian Date

IACUC

Comments or Conditions of Approval: Per Dr. Tuttle: At least initially, until determined otherwise by VP Biological Programs, skin scrapings will be collected by a veterinarian.

N/A _____
Signature of IACUC Chair Date

Approval Period: 8/8/2016 - 8/8/2017 Protocol # 16006
(Must be reviewed annually)

Re-Approval Date 10/30/2017 Signature of IACUC Chair _____

Re-Approval Date 2/19/2019 Signature of IACUC Chair see attached

Re-Approval Date _____ Signature of IACUC Chair _____



MYSTIC
AQUARIUM

ANNUAL RE-APPROVAL
BY THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
(IACUC)

Project Title: Determination of biomarkers and assays from different tissue matrices of the beluga to aid in assessing health in aquarium and wild whales

Principle Investigator: Dr. Tracy Romano, Mystic Aquarium

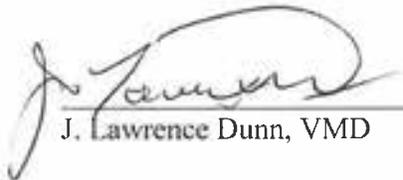
Project Number: 16006

Date Originally Approved: 8/8/2016

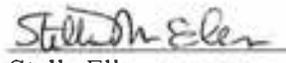
The attached materials are provided as an annual update to an IACUC approved research project. The PI is requesting re-approval with no modifications to the original project protocols. IACUC members are asked to carefully review this request. *The signature of the IACUC member signifies re-approval for one year.*

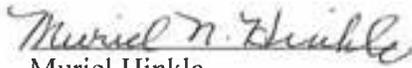
IACUC Members:

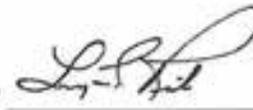
_____ Date _____
Tracy Romano, Ph.D.
Committee Chairman


J. Lawrence Dunn, VMD

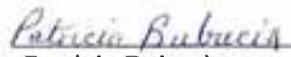
Date 10/30/17

 Date 10/30/17
Stella Elbaum

 Date 10/30/17
Muriel Hinkle

 Date 10-30-17
Larry Rivarde

 Date 10/30/17
Allison Tuttle, DVM, Diplomat ACZM

 Date 10/30/17
Patricia Bubucis

 Date 10/31/17
Gayle Sirpenski
IACUC Secretary

 Date 30/11/17
Toni O'Connell



IACUC REVIEW OF PROPOSED AMENDMENTS TO PREVIOUSLY APPROVED PROTOCOL

Project Number: 16006

Project Title: Determination of biomarkers and assays from different tissue matrices of the beluga to aid in assessing health in aquarium and wild whales

Principle Investigator: Tracy Romano, PhD

Date Originally Approved: 8/8/2016

Description of Amendment: (Attach additional pages if necessary)

The following four additions to the existing IACUC are being requested:

1) Increase frequency of blow sampling in beluga whales resident at Mystic Aquarium

In addition to monthly samples of blow per the original protocol, weekly blow samples (1-2 days per week as feasible, up to four blow plates each) are requested from the whales resident at Mystic Aquarium. These samples will be used to: 1) to determine and validate a suitable marker of dilution which can be measured in beluga blow samples and used to standardize hormone measurements 2) to validate measurement of thyroid hormones T3 and T4, in blow samples for monitoring responses to stressors and health status and 3) to determine the feasibility and utilization of genetic and transcriptomic techniques to characterize molecular markers of health in blow samples.

Moreover, one day per spring, summer and fall (3 days total) paired blood and blow samples are requested at one time point in the morning and one time point in the afternoon, in order to examine diurnal variation of hormones in parallel with urea as a dilution factor.

2) Sampling as feasible (saliva, blow, blood, feces, skin scrapings) throughout the training period and stationing of belugas resident at Mystic Aquarium on a hydraulic lift and being lifted out of the water for purposes of veterinary examinations, etc.

In addition to the original scenarios described in the original approved IACUC proposal (obtaining weights in the stretcher, veterinary procedures on the Arctic Coast beach (out of water examinations), transport of whales from one facility to another or within the aquarium, introduction to new environments, and introduction to new social groupings/interactions) sampling before and after training sessions with the new hydraulic lift is requested.

Mystic Aquarium will be installing a hydraulic lift in the Arctic coast exhibit where the belugas reside in order to be able to get the whales out of the water in a more efficient manner. As with anything new to the whales, desensitization and training are necessary so the whales will station on the lift and remain stationary and calm while the lift slowly moves up out of the water. The lift itself and the training procedure while the whales are getting used to the lift provide an opportunistic "stressor" in which we expect the whales to (especially the first few times) to mount a physiological response e.g. increase in cortisol. Opportunities such as the lift allow for ground truthing hormone assays, and verification of measurements and timing of hormones in different tissue matrices (blood, blow, feces, saliva, skin scrapings).

Specifically, paired blood and blow samples are requested prior to isolating whales in the pool containing the lift, and then again after the whales have gone through the procedure of being lifted out of the water. Following return of the whales to the exhibit, blow sampling is requested to continue behaviorally every 5 minutes for the first 20 min and every 10 minutes thereafter up to 2 hours. Raw cortisol values and cortisol correction by urea will be compared with blood cortisol in order to verify detection of an increase in cortisol in response to a known stressor, as well as determine timing in which cortisol returns to baseline levels. Feces will be collected prior to the lift sessions and behaviorally at time points after the session as feasible (1-6 hours, 24 and 48 hours). Saliva and skin scrapings will be collected as feasible.

3) Collection of blow with a pole containing a collection device on free-swimming whales at different locations in the Arctic Coast exhibit and from a small boat placed in the exhibit

In order to take the first steps in transitioning blow collection to free swimming belugas in the wild, proof of concept will be carried out on Aquarium whales. The feasibility of collecting blow samples from free swimming whales will be tested on belugas resident at Mystic Aquarium, using a hand-held fiber glass or titanium pole (approximately 6ft in length) with an attached collecting device (e.g. petri dish 90mm or 150 mm in diameter). In collaboration with whale husbandry staff, a location in the exhibit will be identified where whales are likely to surface for a breath given their known swim patterns (e.g. female consistently surfacing to breath by large window at underwater viewing) or if needed a simple swim pattern will be trained.

Collection of samples will be attempted by the following: 1) a person holding the pole over the exhibit at the surface of the water on a ladder 2) a person holding the pole from a small boat (the boat similar to what has been used in special presentations to the public). The pole will be held approximately 6-16" from the blowhole as the whale surfaces. Blow collection attempts will occur opportunistically when whales surface at appropriate distances. Blow samples will be assessed for quality based on volume recovered as well as hormone and urea measurements to determine impact of water contamination if any.

4) In collaboration with Canadian colleagues, to assist in collecting blow from free swimming whales utilizing the proof of concept above

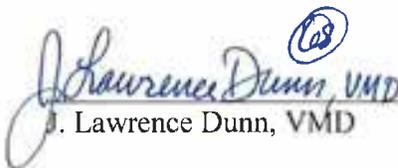
Following successful blow collection from aquarium whales, attempts will be made to transition collection to wild free swimming whales in order to look at the

feasibility of blow collection and measuring hormones (cortisol, thyroid hormones, reproductive hormones), urea as a dilution factor, and molecular components (DNA for sex determination and gene expression of immune targets). Our Canadian colleagues have had some experience in this regard and have indicated that the belugas in the Churchill River in the summer are curious and will approach boats within the reach of a hand-held sampling pole. Under their authorizations, we will collaborate with them in collecting blow samples from free swimming whales in Churchill, and apply the technology we have developed at Mystic Aquarium to determine hormone and molecular components. A small zodiac boat with a designated captain will station in the area the whales congregate and sample collection will be attempted from those whales that approach the boat. The same pole, used on Aquarium whales will be used to collect from wild free-swimming whales. This will be in collaboration and authorization with Canadian colleagues from the Department of Fisheries and Oceans, Canada.

The attached materials reflect proposed changes to an approved research project. IACUC members are asked to carefully review this request for an amendment and provide comments. ***The signature of the IACUC member signifies approval of the amendment as written.***

IACUC Members:

_____ Date _____  ^{DVM, Dipl ACZM}
Tracy Romano, Ph.D. Allison Tuttle, DVM, 01/24/18
Diplomate ACZM

 ^(CS) Date 1/23/2018  Date 1-24-18
J. Lawrence Dunn, VMD Larry Rivarde

_____ Date _____ attached page Date 1/23/2018
Muriel Hinkle Stella Elbaum

attached page Date 1/25/2018 see attached email Date 1/23/2018
Toni O'Connell Patricia Bubucis

 Date 1/24/2018
Gayle Sirpenski

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The attached materials reflect proposed changes to an approved research project. IACUC members are asked to carefully review this request for an amendment and provide comments. *The signature of the IACUC member signifies approval of the amendment as written.*

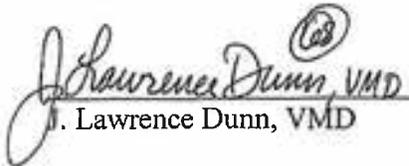
IACUC Members:

Tracy Romano, Ph.D.

Date _____

 ^{DVM, Dipl ACZM}
Allison Tuttle, DVM,
Diplomate ACZM

Date 01/24/18

^(C)
J. Lawrence Dunn, VMD

Date 1/23/2018


Larry Rivarde

Date 1.24.18

Muriel Hinkle

Date _____

attached page Date 1/23/2018
Stella Elbaum

attached page
Toni O'Connell

Date 1/26/2018

see attached email Date 1/25/2018
Patricia Bubucis


Gayle Sirpenski

Date 1/24/2018



MYSTIC
AQUARIUM

**REVIEW OF PROPOSED AMENDMENTS
BY THE INSTITUTIONAL ANIMAL CARE
AND USE COMMITTEE (IACUC)**

Project Title: Determination of biomarkers and assays from different tissue matrices of the beluga to aid in assessing health in aquarium and wild whales

Principle Investigator: Tracy Romano, PhD

Project Number: 16006

Date Originally Approved: 8/8/2016

The attached materials reflect proposed changes to an approved research project. IACUC members are asked to carefully review this request for an amendment and provide comments. *The signature of the IACUC member signifies approval of the amendment as written.*

IACUC Members:

F. Fatima Matos Date 2/19/19 n/a Date _____
Fatima Matos Tracy Romano, Ph.D.
Committee Chairman

J. Lawrence Dunn Date 2/9/19 Larry Rivarde Date 2.11.19
J. Lawrence Dunn, VMD Larry Rivarde

Allison Tuttle, DVM, Diplomate ACZM Date 2/8/19 _____ Date _____
Allison Tuttle, DVM, Diplomate ACZM Patricia Bubucis

_____ Date _____ _____ Date _____
Jennifer Flower, DVM, Diplomate ACZM Toni O'Connell

Stella Elbaum Date 2/8/19 _____ Date _____
Stella Elbaum Gerald Sonnefeld, Ph.D.

Gayle Sirpenski Date 2/8/2019
Gayle Sirpenski
IACUC Secretary

Project No. 14006



**Institutional Animal Care and Use Committee
RESEARCH PROPOSAL and PROTOCOL REVIEW
By
Designated Reviewer**

Animal Welfare Act, Part 2, Subpart C, Section 2.31 (d)(2) ... "If full Committee review is not requested, at least one member of the IACUC, designated by the Chairman and qualified to conduct the review, shall review those activities and shall have the authority to approve, require modifications in (to secure approval), or request full committee review of any of those activities. If full Committee review is requested for a proposed activity, approval of that activity may be granted only after review at a convened meeting of a quorum of the IACUC."

Designated Reviewer: Dr. J. Lawrence Dunn

Project Title: Quantifying masked hearing in belugas with anthropogenic noise from Cook Inlet

Reviewer Comments/Recommended modifications:

I recommend having supervision by both training and vet staff during all levels above 115 dB to make certain that Kela is not adversely impacted by high amplitude noise. If they note that there is no adverse reaction on the first few trials at each 5 dB increase, no additional supervision at that level would be required. If I am available, I could do some of the vet supervision for this the study.

Reviewer Decision: Approve Require Modifications Full Committee Review

Signature of Reviewer: J. Lawrence Dunn, VMD ^(CDS) **Date** 8/28/2014

Signature of IACUC Chair: Tracy Romond **Date** 8/29/14



MYSTIC
AQUARIUM

Project No. 14006

RESEARCH PROPOSAL and PROTOCOL REVIEW

MYSTIC AQUARIUM
Sea Research Foundation, Inc.
55 Coogan Blvd.
Mystic, CT 06355

Introduction

Mystic Aquarium is committed to research and promotes the use of its collection for the purpose of enhancing knowledge of aquatic animals through scientific investigation.

The following proposal materials are designed to address all pertinent issues regarding the use of live animals for scientific study at Mystic Aquarium. Individuals interested in conducting research must provide this information to satisfy the requirements of the Aquarium's Staff Research Committee and Institutional Animal Care and Use Committee (IACUC). It is the function of these groups to ensure that all protocols comply with the regulations and standards of the United States Department of Agriculture, as well as standards established by Sea Research Foundation. Information should be specific and comply with the format as presented.

Submission and questions regarding the preparation of this proposal should be directed to:

Gayle Sirpenski, Secretary, IACUC
(860) 572-5955 ext. 108
gsirpenski@searesearch.org

I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Manuel Castellote
National Marine Mammal Laboratory, AFSC, NOAA Fisheries Service, 7600
Sand Point Way NE, Seattle, WA 98115.
Tel. (206)526-6866
Email: manuel.castellote@noaa.gov

B. Title of proposed research

Quantifying masked hearing in belugas with anthropogenic noise from Cook Inlet

C. Introduction and background

It is highly likely that for Cook Inlet Belugas (CIB) sound plays a crucial role in navigation, feeding and communication because of the high turbidity of their habitat. Because sound is so important to them, changes in ambient noise levels may have a significant impact on their ability to thrive. Man-made (anthropogenic) sources of noise in Cook Inlet (CI) include large and small vessels, aircraft, oil and gas drilling, marine seismic surveys, pile driving, and dredging (Blackwell and Greene 2002). Anthropogenic noise may have a variety of impacts on marine mammals. Perhaps one of the most basic, but poorly understood, impacts is masking, or hiding, of acoustic signals by overwhelming background noise.

An understanding of the extent of masking by anthropogenic noise is crucial for regulating industrial underwater noise emissions and properly managing these activities. A few studies have examined masking with cetaceans both psychophysiological (Burdin et al. 1973, Johnson 1968, Johnson 1971, Johnson et al. 1981, Au and Moore 1990, Thomas et al. 1990, Erbe and Farmer 1998) and electrophysiologically (Supin and Popov 1990, Supin et al. 1993). Most of these looked at high frequency signal discrimination; only three (Burdin et al. 1973, Johnson et al. 1981, Erbe and Farmer 1998) provided masked hearing data at frequencies below 10 kHz, where industrial noise prevails. Therefore, masking has generally been studied with the signal to detect being a pure tone, and the masker being either a pure-tone or designed, broad-band (white) and temporally consistent noise. These studies have provided valuable information on the basic characteristics of the animal auditory filter, such as the relationship between the amplitude, frequency, and frequency bandwidth of signal and masker. However, as mammalian hearing is highly nonlinear and depends on both frequency and temporal structure of signal and noise, results from pure tone experiments cannot be superposed to predict the masking of complex communication signals by noise. To date, only one masked hearing study (Erbe and Farmer 1998) used structured noise (icebreaking noise) on a captive beluga. A data gap is clearly identified showing the need to study low-frequency masking with signals occurring in cetacean habitats.

The study by Erbe and Farmer (1998) used psychophysiological methods (behavioral audiogram) to obtain masked thresholds. However, since then, there have been substantial advances in the design of rapid, non-invasive physiological investigations (e.g., auditory evoked potentials-AEPs) of marine mammal hearing. This proposal aims to collect AEPs to document baseline hearing and masked hearing thresholds of captive belugas when exposed to anthropogenic noise, in different angles, recorded in Cook Inlet beluga critical habitat.

This proposal is a continuation of a previously funded study. As part of a Cook Inlet beluga acoustic monitoring study, calibrated wideband sound recordings of different sources of anthropogenic noise have been collected in Cook Inlet during the period 2010-2012. Also, AEP audiograms have been collected in 7 temporarily restrained belugas from the Bristol Bay population in September 2012 to confirm the validity of captive beluga hearing studies. This field work has been supported in part, by The Mystic Aquarium.

D. Brief summary of proposed research. (Note if the study would be done in house or in the field.)

We propose to quantify the hearing sensitivity of 2 belugas at Mystic Aquarium under normal conditions (baseline hearing), that is just exposed to the background noise of the facilities. This data will be used to obtain the audiogram of each individual. Once this dataset is collected, we will repeat the procedure under a controlled noise exposure experiment. The whales will be exposed to low levels of human-produced noise previously recorded in Cook Inlet beluga critical habitat. In all cases, hearing will be measured rapidly and non-invasively using AEPs. Common sources of anthropogenic noise in this region are commercial ship noise (cargos, tankers, tug boats towing barges, etc.) outboards from small crafts, dredge operations noise and airplane overpass noise. One noise source type will be projected in each trial with precise control of the received level at the stationed beluga. Sound levels will be measured at the beluga. AEP collection under this context will allow addressing the level of acoustic masking generated by each tested type of anthropogenic noise source. Belugas are readily exposed to multiple anthropogenic noise sources. This exposure is likely to increase in the near future as human activities expand in their habitat. The impacts of anthropogenic noise on beluga hearing are poorly understood. Our results will significantly expand our knowledge on: 1) the masking effects of several of the most common sources of noise in beluga habitat, 2) our knowledge of noise coping mechanisms of belugas, and 3) our knowledge of the severity of the effects of noise in beluga hearing. These results will be valuable for defining industrial underwater noise emission regulations in critical beluga habitat and help establish the correct management of these activities. The fact that anthropogenic noise sources used in this study have been recorded in Cook Inlet beluga critical habitat must be highlighted as the Cook Inlet population is currently endangered and a recovery plan must be drafted within the next months. Results from this study would greatly benefit the recovery plan and proposed recovery actions.

E. Specific goals of the proposed research.

- 1) Collection of baseline and masked beluga hearing thresholds from 2 captive belugas, using anthropogenic noise sources recorded in Cook Inlet (including: commercial vessel noise, outboard motor noise, dredging, commercial airplanes and jetfighters).
- 2) Quantification of masking (frequency range and dB magnitude) caused by the different noise sources projected.
- 3) Quantification of the directional hearing abilities and directivity index of belugas exposed to noise projected from 3-5 different angles (front, back, sides, above, below).

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

To better understand what marine animals hear and how their hearing may be affected by underwater noise we plan to employ AEPs. Traditional behavioral tests (following a go-no go paradigm) can be reliable means to examine hearing ability. However, they often required years of training and months to collect data. AEPs provide similar data but in a rapid (minutes), non-invasive manner.

Modeling hearing data can only provide estimates of hearing ranges in mammals. It does not provide direct measures of the frequencies and thresholds of what animals hear and how types and direction of noise can affect their hearing. Common hearing models are based on terrestrial mammal data; extrapolations are particularly difficult for marine species for which we know little. Extrapolations cannot be confirmed without our empirical measures. AEP techniques and controlled exposure experiments are the only means by which cetacean hearing thresholds can be measured and masking quantified in this diverse array of species.

B. Justify the choice of species to be used.

This increasing activities and anthropogenic noise in the Arctic will likely impact sound-sensitive species such as beluga whales. Unfortunately, understanding how anthropogenic noise impacts belugas is impeded by a poor understanding of the (i) auditory frequencies and sensitivities of most belugas, (ii) the methods belugas may have to reduce noise and (iii) how these animals receive sound.

There are currently only 3 published beluga audiograms. Measuring hearing in two Mystic belugas would provide an important contribution to the overall understanding of basic beluga hearing. Furthermore, this would be only the second masked hearing experiment in this species and the first to examine industrial noise from a critical habitat. Such data are novel, provide a direct comparison to effects in the wild, and establish valuable new information on the effect of typical noise sources in beluga habitat. Further, these data would be vital for the protection and management of critical beluga whale habitat, and a means to relate to an endangered population (the CIB) without actually involving those individuals. This study would significantly increase our information on the effects of anthropogenic noise in the hearing of this critical Arctic species.

C. Identify and justify the number of animals to be used.

We are aware of the complexity of the training involved to allow this type of data to be collected. At least 2 belugas (*Delphinapterus leucas*) would be desired to be trained and tested. However if training conditions and time availability allow testing just one individual, this study will still be valuable. Adding a second individual would be very beneficial because the study would be able to describe intrinsic (natural) variability in hearing and masking effects by the same anthropogenic noise stimuli.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

Belugas will need to be trained to voluntarily position themselves in a station and maintain that position with minimal movement for a period of at least 3 minutes. This task will need to be repeated multiple times per session. Because of the unfused neck vertebrae, some belugas tend to move their head while in station if they are not conditioned to contact a target with their melon, therefore head alignment might also need to be trained.

It generally takes ~50-60 seconds for one record and the beluga must remain in station, with minimal movement, although allowed to breathe if necessary, during that period. Multiple records will be needed to complete the sampling, which can take ~30-70 min. This can be broken up across trials (i.e., 2-5 min stationing), training sessions (e.g. feeding sessions through the day), and days (e.g. more than one day to complete the audiogram of one whale). The longer the animal can hold in station the fewer number of times it will be required to repeat it and less time will be needed to obtain its audiogram.

In order to maintain the position, beluga stations are typically designed to help the animal in this task. One method is to use a surface target where the beluga must place its melon. This gives the animal a spatial reference and impedes the movement of the head while holding the station. This technique is typically easier to train as it only requires extending the time of a beluga to stay in the target, as most belugas are already trained to attend targets. A preferred alternative is to use an underwater hoop of sufficient diameter for the beluga to insert his head. The hoop allows the whale to maintain its station without the need to compensate with fluking or pectoral fin movements, reducing its body movement to a minimum. The data collection allows the whale to be floating, partially exposed to the air (except for the lower jaw), thus the hoop can be placed at the surface to allow the whale to breathe while on station. This method, as with the surface target technique, allows much longer station holdings than a submerged hoop.

Instrumentation of the whale will be three soft silicone suction cups placed in the beluga, one attached near the melon the second near the beginning of the dorsal ridge and the third posterior of the maximum girth. Each cup includes a passive surface electrode that will be coated with conductive gel and attached with suction. The whale must be desensitized to the suction cup and cable contact.

The whale will be exposed to two different types of sound stimuli. The sound stimulus to measure hearing thresholds that consists of 20 ms sinusoidally amplitude modulated tone followed by 30 ms break of no sound between tones, presented as a 20/s tone-burst. Sound level will gradually increase from very low intensities up to the threshold of hearing. The second type of sound will be anthropogenic noise recordings from Cook Inlet, Alaska. These will consist of several types of noise sources (commercial ship noise, outboard noise, dredge operations noise and airplane overpass noise. These noises will be projected at intensities tolerated by belugas that will never exceed the Level A harassment threshold of 180 dB re 1 μ Pa established by NMFS as the onset of temporary threshold shift for cetaceans. Noise exposure will be continuously monitored and can be stopped at any time if veterinary or training staff requests it.

The amount of continuous time belugas will be exposed to anthropogenic noise will be decided by the trainers, based on the time the belugas can hold in station

(approximately 3 to 5 minutes). Noise projection will stop as soon as the beluga is ready to leave the station following the trainer command. Noise will not be projected between trials or sessions.

The study requires collecting AEP data in four different conditions in the following sequence:

- 1) Baseline hearing data (AEPs in normal background noise) until the audiogram is completed (1-2 data sessions).
- 2) Same AEP collection of data but with the concurrent exposure of anthropogenic noise projected 1 meter in front of the animal (in line with his longitudinal body axis) until AEP collection is complete (1-4 data sessions).
- 3) Same AEP collection of data but with the concurrent exposure of anthropogenic noise projected 1 meter off to the side (90° from the midline axis) (1-4 data sessions).
- 4) Same AEP collection of data but with the concurrent exposure of anthropogenic noise projected 1 meter directly behind the animal (1-4 data sessions).

Thus to complete a test of one type of anthropogenic noise interference will require 4 to 14 data sessions of 2-5 minutes each or between 20 and 50 minutes of station time per whale. Additional sessions with anthropogenic noise projection at angles of 45° and 135° as well as directly above and below will be examined if captivity training conditions and available time with the animals and staff allow. To some extent these experiments will be adaptive and data will be reviewed between sessions and during breaks to assure data quality and allow modifications to make efficient use of available time.

**B. Will a test substance be administered? If yes,
1.) What is the name of the compound?**

No, N/A

2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes.

N/A

C. Will manual, chemical and/or mechanical restraint of the animal be necessary? If yes, explain the method of restraint.

No

D. Indicate the level of pain the proposed procedure is expected to cause.

a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.

- b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.
- c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery of procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

E. Describe the method of euthanasia to be used if this were to become necessary

N/A

**F. Does the proposed research involve surgery?
If yes, go to Addendum II. Surgical Protocol**

N/A

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

The study animals are housed at Mystic Aquarium. We will perform the study in their facilities. Mystic research and training staff will decide the best location in their facilities to perform the study and the only modifications will be the installation of a target or hoop and the temporary presence of underwater transducers hanging from their cables at different locations around the beluga during the training and the data collection. Belugas will need to be desensitized to the suction cup and cable contact as well as to the exposure of two types of sound stimuli: burst-tones to measure hearing that will be projected 1 meter from the rostrum tip of the beluga following its body longitudinal axis and anthropogenic noise recordings from Cook Inlet, Alaska, projected 1 meter from different angles around the animal. Belugas will need to be trained to hold station with minimal body movement for at least 3 minutes while exposed to these different sound stimuli and repeat this action multiple times per session. Trainers will need to be present at all times during data collection and will decide when to stop the sound stimuli exposures or reduce the intensities of anthropogenic noise exposures if belugas show discomfort or disruptive behaviors.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours.

A Mystic veterinarian, trainer or researcher will always be present during the study.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics?

Yes.

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include 1.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

Manuel Castellote (P.I.) – 13 years of experience with beluga whale acoustics and behavior in aquarium facilities (L'Oceanografic of Valencia, Spain; WCS New York Aquarium) and Arctic waters (USA, Russia, Norway). Peer reviewed publications on beluga acoustics, hearing, behavior and anthropogenic noise effects on cetaceans. Member of the NMFS Cook Inlet Beluga Recovery Panel as bioacoustics specialist. Currently working at the NMFS National Marine Mammal Laboratory as odontocete acoustics specialist and involved in anthropogenic noise regulations for both the USA and European Union.

2005-2009 Ph.D. program on Biodiversity and Conservation. University of Valencia, Spain. PhD thesis (Psychobiology Dept., University Complutense of Madrid, Spain): Migratory patterns, population identity and noise impact in fin whale (*Balaenoptera physalus*) communication in the western Mediterranean Sea. PhD co-direction: Dr. Christopher W. Clark (Cornell U., USA) and Dr. Fernando Colmenares (U. Complutense of Madrid, Spain). Honors degree cum laude.

1997-2000 M.S. in Zoology. U. Complutense of Madrid, Spain. Master's Thesis: "invertebrate fauna of a Mediterranean submarine cave". Honors degree. Directed by Dr. Jesús Benito and Dr. Fernando Pardos.

1992-1997 B.S. in Biology. U. Complutense of Madrid, Spain.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

T. Aran Mooney (co-P.I.) – 10 years experience with AEP procedures including working on stranded odontocetes, beluga whales, review papers on AEP methods, designing portable AEP systems and mysticete AEP systems. He has 17 peer-reviewed papers on bioacoustics and hearing, half of those using AEP methods (and 2 more in press). One paper is on the hearing of beluga whales using AEPs.

Ph.D., Department of Zoology, Marine Biology specialization 2004-2008
University of Hawaii at Manoa, Honolulu, Hawaii
"Temporal effects of sound on the odontocete auditory system: an electrophysiological study"
Advisor: Paul Nachtigall

M.S., Department of Zoology, Marine Biology specialization University of Hawaii at Manoa, Honolulu, Hawaii "How acoustically enhanced gillnets reduce odontocete bycatch" Advisor: Paul Nachtigall	2001-2003
B.S. in Biology, magna cum laude, Spanish minor University of New Hampshire, Durham, New Hampshire	1996-2000

Roderick Hobbs - 22 years experience with marine mammal field research including aerial and vessel surveys, biopsy, photo id, tagging and health assessment. Primary duties are population assessment of the Cook Inlet beluga . Have conducted workshops and coauthored papers on safe handling and health assessment of small cetaceans and serve on the IACUC of the National Marine Mammal Laboratory of the Alaska Fisheries Science Center.

1992 PhD. Graduate Group in Ecology, University of California Davis specialized in population biology.

1975 BA Division of Biological Sciences University of California Davis

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).

N/A

2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?

No pain or distress is expected in association with the AEP collection. AEPs are collected non-invasively from odontocetes, including belugas, in many situations such as strandings, laboratories, rehab centers and display facilities. The same method is a standard hearing test method for human infants (Burkhard et al. 2007).

Discomfort to the animal causing display of disruptive behaviors could occur when belugas are exposed to high intensity levels of anthropogenic noise, but also unexpected sounds. We plan to use published literature and experience in noise-exposure experiments to avoid such responses (Southall et al. 2008; Mooney et al. 2009a; Mooney et al. 2009b). Further, during training, sound levels will be increased gradually to allow the animal(s) to habituate to the condition. If they occur, we anticipate that these behaviors might only be detected during the training period, while belugas are desensitized to low-level noise and sound levels. These responses would help define the maximum allowed in the study and would be novel themselves. In these cases, the animal can easily leave station and decrease noise exposures. At no time do we expect the noise to reach levels of physiological or auditory damage. Veterinary and training staff will be evaluating these tests and therefore the exposure will be immediately terminated if discomfort or disruptive behaviors are observed. During data collection, noise levels will never exceed the maximum defined during training and therefore discomfort or disruptive behaviors are not expected.

Belugas will be constantly monitored before, during and after procedures where acoustic stimulus is used (AEP collection and controlled noise exposure experiment). Monitoring will be made by the researchers, attending veterinarian, or training staff. If the animal displays any evidence of distress, the procedures will be immediately terminated.

3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.

N/A

4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.

N/A

5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.

Statement for Acoustic Research IACUC:

With regards to the acoustic research, there is not currently an IACUC in place for this project, as it will only be conducted if the permit is granted. IACUC protocol #14006 *Quantifying masked hearing in belugas with anthropogenic noise from Cook Inlet* was originally approved in 2014 and completed in 2015. This was included to demonstrate our track record and expertise in this area. According to Dr. Manuel Castellote, Alaska Fisheries Science Center, NOAA Fisheries and Dr. Aran Mooney, Woods Hole Oceanographic Institute, they were able to accomplish the objectives of this study in part due to the “impressive research training” and the “solid support and flexibility by the trainers and husbandry staff”. Our prior successful collaborations with these researchers are the basis for our plans to review a second IACUC proposal that will support the important research goals of our permit application. We expect to have the approval in place shortly after our next IACUC Committee meeting and no later than Q4 2019.

Sirpenski, Gayle

Project 14006

From: Manuel Castellote - NOAA Affiliate <manuel.castellote@noaa.gov>
Sent: Sunday, November 15, 2015 11:48 PM
To: Magao, Kristine; Sirpenski, Gayle; Romano, Tracy; Macha, Laurie; Richard, Carey; Anderson, Paul; Thompson, Laura; McElroy, Kate; crd90@comcast.net; Lena Donnarumma
Cc: Aran Mooney
Subject: Big thank you!

Dear Mystic aquarium friends,

Aran and I wanted to send this thank you message to all of you. Our 9 days of sampling ending last week have been a real success. We have now completed all the data we needed to accomplish the objectives of this study. We completed a total of 17 sessions where the trainers proved an outstanding control of Kela and the boys. You guys have achieved an impressive research training level with this whale. We are so glad this sampling has been such a success. We wanted to make sure you all are well aware of the fantastic level of training build up on Kela. But our sampling success has not just been possible because of Kela's performance, also, and equally important, because of the solid support and flexibility by the trainers and husbandry staff that accommodated all our ongoing requests while sampling the whale. Longer layout durations, never ending changes in the session schedule, new noise projections to Kela, pool recordings after the sessions, and many other little annoyances that allowed us optimizing the sampling and time spent at Mystic. Thank you VERY much Kathryn, Kate and Kristine who spent quite a bit of time breaking capelin into little bits to prolong Kela's layouts! and special thanks go to interns Chris DeRosa and Lena Donnarumma for their consistent support handling speakers and taking session notes.

We will spend the next months processing and analyzing data to prepare a couple of papers for publication from this study. We will make sure you all receive the final drafts before submission for review. Also, we will keep an eye on funding opportunities to submit more proposals and continue our research collaboration. We definitely need to capitalize on Kela's excellent trained behavior!

Kind regards,
Manolo and Aran

--
Manuel Castellote, PhD
Cetacean Assessment and Ecology Program
National Marine Mammal Laboratory
Alaska Fisheries Science Center/NOAA
7600 Sand Point Way N.E. F/AKC3
Seattle, WA 98115-6349
(206) 526-6866 (voice)
(206) 526-6615 (fax)

PROJECT # 17007



Mystic Aquarium
a division of Sea Research Foundation Inc.
55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT/SPECIMEN REQUEST

APPLICANT:

Name - Dr. Valeria Vergara

Address - 845 Avison Way, Vancouver BC V6G 3E2

Telephone/Mobile: (604)659 3452 E-mail valeria.vergara@ocean.org

- **PROJECT TITLE: Photogrammetry Validation with Beluga Whales under Human Care**

- **SCIENTIFIC OBJECTIVES:**

The main objective of this study is to provide periodic photogrammetry data (from a fixed camera at a known height) and morphometric data (measurements and weights) from known individuals (at various Aquariums) to inform photogrammetry studies of wild beluga populations. The data will aid in developing a minimally invasive method to assess body condition in beluga whales in the wild using photographs taken with Unmanned Aerial Vehicles.

- **PROCEDURE / SPECIMEN NEEDS** (Append detailed research and sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. **Provide FedEx account number if samples are to be shipped**):

Photogrammetry protocol:

Photos from a fixed known height (minimum of 6 m) above each of the beluga whales in this study will be used to determine how body condition can be estimated using photogrammetry. Ideally, the photos would need to be taken at least once per month (ideally bi-weekly).

To approach as closely as possible the conditions encountered in the wild when flying an Unmanned Aerial Vehicle (UAV) above belugas, the photos will be taken when each beluga whale swims directly under the camera, hoping to avoid the curved position that belugas tend to adopt when stationed.

The images will be collected with a micro 4/3 camera (either an Olympus E-PM2, 16 MP RAW files, or a Sony Alpha A5000, 20 MP RAW files), with a fixed focal lens yielding a 50 mm equivalent field of view. The camera will be mounted on an extended pole held at least 6 m above the surface of the water. Multiple images will be taken as each whale

PROJECT #. _____

swims under the camera. Training the whales to swim from Point A to Point B in a straight line under the camera would facilitate obtaining successful pictures but opportunistically photographing the whales as they swim in the exhibit can work too, as indicated by other photogrammetry validation studies of cetaceans under human care.

A minimum of 20 photos are required in order to extract the best 3 photos to estimate length and widths at pre-determined landmarks, using measurements in pixels that are scaled to true size.

Morphometrics and Weights:

Monthly morphometrics (width, circumference, and length taken with a measuring tape and/or a caliper) and quarterly weights will be obtained in order to estimate any changes in body condition with time/season.

The morphometric measurements would ideally be obtained on the same week as the overhead photos are taken (i.e. not necessarily during the same session). The whales will be asked to station in a location indicated by the trainer. A measuring tape and/or large animal caliper can be used to measure the straight length of the animal and the circumference at 6 different landmarks (see Figure 1). Three repeated measurements will be conducted for length and at least 2 widths per landmark (ideally three, if time allows) to assess the individual measurement error.

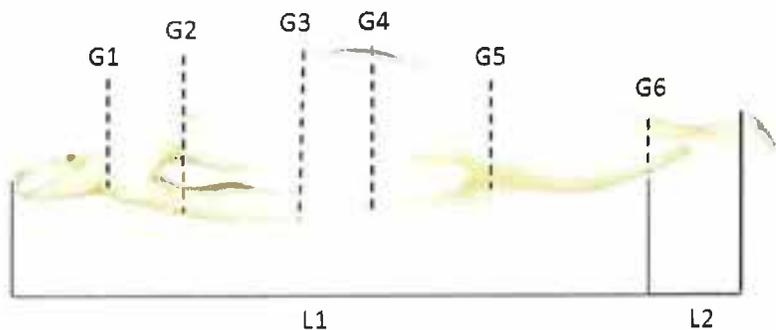


Figure 1: Landmarks for assessment of body condition

G1: Neck, G2: Behind pectoral fins, G3: Origin of dorsal ridge, G4: Center of dorsal ridge, G5: Urogenital, G6: Mid-peduncle, L1: Tip of rostrum to mid-peduncle, L2: peduncle to fluke notch

- **PROPOSED TIME FRAME FOR PROJECT** (include dates if possible):
December 2017 to December 2018
- **Attach NMFS or FWS authorization if applicable.**

Can request be met as part of routine / scheduled handling? NO

Able to pay for staff OT, lab costs, special sampling equipment, NO

PROJECT #. _____

shipping costs, if required?

Requires intrusive procedure ¹ not performed as part of a routine medical examination. (If yes, applicant must complete form for approval by IACUC). NO

¹ Any procedure involving a break in or cutting of the skin or equivalent, insertion of an instrument or material into an orifice, introduction of a substance or object into an animal's environment that is likely either to be ingested or to contact and affect directly an animal's tissues, or a stimulus directed at an animal that may involve a risk to health or welfare or that may have an impact on normal function or behavior.

TERMS AND CONDITIONS

- Specimens will be used only for the purposes described above, and will not be redistributed or sold; any excess material will be returned to the Aquarium, or destroyed in a manner acceptable to both the Applicant and the Aquarium.
- The Applicant recognizes that there may be zoonotic hazards associated with handling biological materials, and hereby releases and holds harmless the Mystic Aquarium and Sea Research Foundation, its officers, trustees, employees and agents, from any and all claims, demands, actions, and causes of actions whatsoever on account of or in connection with any aspect of this request for biological materials.
- The Applicant must submit an annual report of findings prior to project renewal. Applicants will submit a full proposal and summary report every third year for ongoing projects.
- The Applicant will acknowledge Mystic Aquarium in any publication, abstract, manuscript report or presentation arising from this project,
- **The Applicant will allow Mystic Aquarium to review a draft of any report based on the analysis of specimens or data originating from the Aquarium.** Abstracts, manuscripts, reports, power point presentations, posters, etc. must be submitted to the Mystic Aquarium Research Review Committee for review and approval no later than 2 weeks prior to submission/presentation deadline.
- The Applicant will provide the Aquarium with an electronic copy of any publication dealing with the specimens or data requested.
- Failure to comply with the above terms and conditions will jeopardize future collaborations with Mystic Aquarium.

Applicant Signature

17 October 2017

Date

VANCOUVER AQUARIUM, AN INITIATIVE OF OCEAN WISE CONSERVATION ORGANIZATION

Sponsor / Advisor's Signature*

Name/Title/Affiliation

Date

* (Required for Applicants who are not University faculty, research scientists, or health professionals)

Return to: Gayle Sirpenski, Animal Management Specialist
Research and Animal Care
Phone: 860.572.5955 x 108

Return electronic copy to: gsirpenski@mysticaquarium.org

=====

APPROVAL Projects must be re-approved annually.

10/18/17

PROJECT # _____

VP of Biological Research

Date

Curator (if applicable)

Date

Allison Outte, DVM, Dipl. ACZM 10/18/17
VP of Biological Programs Date

Comments or Conditions for Approval:

Anniversary Date: 10/18/2018

Re-approval Date: _____ Signature: _____

PROJECT/SPECIMEN REQUEST

COMMENTS FROM REVIEWERS

VP of Biological Research

Date:

Comments:

VP of Biological Programs

Date:

Comments:

Curator OR Other designated reviewer

Date:

Comments:

PROJECT #. **17007**



PROJECT AMENDMENT / RENEWAL REQUEST

APPLICANT:

Name - Dr. Valeria Vergara
Address - 845 Avison Way, Vancouver BC V6G 3E2

Telephone - (604)659 3452 E-mail - valeria.vergara@ocean.org

ORIGINAL PROJECT TITLE: **Photogrammetry Validation with Beluga Whales under Human Care**

- DATE OF ORIGINAL APPROVAL: 10/18/2017
- ANNIVERSARY DATE: 10/18/2018
- PROJECT UPDATE: (Attach description of progress/ challenges to date)
- AMENDED PROCEDURE / SPECIMEN NEEDS (Attach detailed description of changes to original research and/or sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. Provide FedEx account number if samples are to be shipped):
- PROPOSED TIME FRAME FOR AMMENDED / RENEWED PROJECT (include dates if possible):

Check here if request is for renewal only with NO changes to previously approved protocol. *Attach summary of project findings/presentations/publications during previous year.*

Terms and Conditions of original project approval will apply.

	10/30/2018
Applicant Signature	Date
Marine Mammal Research Scientist	Ocean Wise Conservation Association (VancouverAquarium)
Title	Affiliation

Return to: Animal Management Specialist
 Research and Veterinary Services
 Phone: 860.572.5955 Ext. 108
 gsirpenski@mysticaquarium.org

PROJECT #. **17007**

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Reviewer Comments:

Conditions of Approval:

Add new measurement
caudal blowhole to dorsal ridge

APPROVAL:

Tracy Remond 10/31/18
VP Biological Research Date

Allison Duff 10/31/18
VP Biological Programs Date

Curator or Husbandry Supervisor (if required) Date

Anniversary Date: _____

***Projects must be re-approved annually.
Project updates or reports are due with request for renewal.***



MYSTIC AQUARIUM

Project No. 15004

RESEARCH PROPOSAL and PROTOCOL REVIEW

MYSTIC AQUARIUM
Sea Research Foundation, Inc.
55 Coogan Blvd.
Mystic, CT 06355

Introduction

Mystic Aquarium is committed to research and promotes the use of its collection for the purpose of enhancing knowledge of aquatic animals through scientific investigation.

The following proposal materials are designed to address all pertinent issues regarding the use of live animals for scientific study at Mystic Aquarium. Individuals interested in conducting research must provide this information to satisfy the requirements of the Aquarium's Staff Research Committee and Institutional Animal Care and Use Committee (IACUC). It is the function of these groups to ensure that all protocols comply with the regulations and standards of the United States Department of Agriculture, as well as standards established by Sea Research Foundation. Information should be specific and comply with the format as presented.

Submission and questions regarding the preparation of this proposal should be directed to:

Gayle Sirpenski, Secretary, IACUC
(860) 572-5955 ext. 108
gsirpenski@searesearch.org

I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Laura Thompson, PhD
Postdoctoral Fellow
Mystic Aquarium
55 Coogan Blvd, Mystic CT
860 572 5955 x155
lthompson@searesearch.org

Tracy Romano, PhD (Postdoctoral fellow Advisor)

Mystic Aquarium
55 Coogan Blvd, Mystic CT
860 572 5955 x155
tromano@searesearch.org

B. Title of proposed research

Evaluation of Non-lethal effects of N₂ bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury.

C. Introduction and background

Introduction:

In recent decades, there have been several reports of pathologies which resemble decompression sickness (DCS) in stranded marine mammals. The species tend to be deep divers and the stranding events occur in close proximity either temporally or spatially to major anthropogenic activities such as naval sonar exercises (e.g. Jepson *et al.*, 2003). In addition, gas bubbles have been reported in live stranded dolphins (Dennison *et al.*, 2012) and several species of marine mammal which were by-caught at depth and pulled rapidly to the surface (Moore *et al.*, 2009). As a result, questions have arisen as to the actual risk marine mammals face while diving, and there is increasing concern over the role of anthropogenic stressors in compromising adaptations and facilitating injury and disease in these animals.

Background:

It is widely known that marine mammals possess many physiological and behavioral adaptations to the challenges associated with diving (reviewed in Kooyman *et al.*, 1981), yet cellular adaptations have received little attention, with no studies focused on immune responses. However, in humans the development of dive related injury and disease is linked to changes in immune function (Brenner *et al.*, 1999). The development of decompression sickness for example is linked to complement activation (Montcalm-Smith *et al.*, 2007; Ward *et al.*, 1987), leukocyte activation and aggregation (Nyquist *et al.*, 2004) and inflammatory processes (Ward *et al.*, 1987; Nyquist *et al.*, 2004; Barack and Katz, 2005). Aggregation of leukocytes in tissues, such as the lung and liver, accompanied by cell activation are also a marker of DCS in pigs (Nyquist *et al.*, 2004).

Such changes in immune function may occur in response to direct or indirect effects of N₂ bubbles. These may include damage to the endothelium which results in the release of chemical factors that initiate inflammatory response, denaturation of proteins, or recognition of the bubble itself as a foreign surface. The presence of bubbles, however, does not explicitly lead to DCS symptoms (Barack and Katz, 2005) and susceptibility is difficult to predict. 'Silent' or asymptomatic bubbles have been reported in human divers and risk of DCS development can be further modulated by various factors including moderate to intense exercise prior to a dive (Wisloff *et al.*, 2004) or 'pre-tuning' of the immune system (Kayar *et al.*, 1997).

It has long been thought that marine mammals are not subject to decompression related injuries. However, the occurrence of nitrogen supersaturation and development of decompression sickness has been reported in breath hold diving humans (Wong, 2006; Schipke *et al.*, 2006) and gas bubbles may form under particular circumstances in marine mammals (Hooker *et al.*, 2012), specifically where characteristics of the dive do

not allow appropriate gas washout (Paulev, 1967; Ferrigno and Lundgren, 2003; Wong, 2006). Tissue nitrogen loads have been estimated to reach up to 300% supersaturation for the deep diving sperm whale (Jepson *et al.*, 2003) and 200% for bottlenose dolphins, beaked whales and blue whales (Houser *et al.*, 2001). In addition, modelling efforts have predicted bubble growth following repeated dives, with short surface intervals, to depths shallower than that at which lung collapse occurs (Zimmer and Tyack, 2007). Should bubbles be more common than previously thought, adaptive non-responsiveness of the immune system has been suggested to be one mechanism by which marine mammals avoid the development of injury (Fahlman *et al.*, 2006).

Results of prior research in our lab (Thompson, Doctoral Dissertation, 2014) suggests that pressure *per se* can alter the response of beluga and phocid immune cells to an immunologic challenge, and that anthropogenic activity may affect marine mammal health by 1) triggering a physiological stress response during diving or 2) altering dive behavior. During 'baseline' conditions, belugas displayed decreased phagocytic activity as well as lymphocyte proliferation immediately following *in vitro* pressure exposures, lending some support to theory of a less reactive immune system. However, this response was altered (i.e. pressure induced increases in function) during stressor conditions and in wild belugas following chase and restraint (i.e. stressor exposure). Additionally, the phagocytic response of phocid granulocytes to pressure changed as health improved over a course of rehabilitation following stranding. Thus, health status, the presence of stressors and dive behavior may all be important factors in determining the physiological response of marine mammals to the presence of N₂ bubbles.

While there is currently no direct evidence of N₂ bubbles occurring under normal conditions, questions remain as to the presence and prevalence of bubbles, and under what circumstances bubbles may be symptomatic vs. silent. It is possible that marine mammals more commonly face conditions which threaten bubble formation, and DCS injury in humans, than previously thought. Stranding and animal death have been reported as consequences of bubbles in some cases, however there may also be a graded response to bubble formation which does not inevitably lead to such outcomes (Houser *et al.*, 2010). The identification of spinal injuries possibly due to dysbaric osteonecrosis in sperm whales (Moore and Early, 2004; Hellier *et al.*, 2011) for example, suggests chronic sub-lethal consequences of a deep dive lifestyle. Thus, this project aims to investigate the potential consequences of bubble formation in marine mammals in terms on initiating inflammatory responses.

Additionally, methodology for collecting blow samples from belugas, and validation of a commercial enzyme immunoassay (EIA) to measure cortisol in blow as a non-invasive means to monitor stress responses has been carried out (Thompson *et al.*, 2014). Blow sampling has also been used for measuring reproductive hormones such as testosterone and progesterone in bottlenose dolphins (*Tursiops truncatus*) (Hogg *et al.*, 2005), humpback whales (*Megaptera novaeangliae*) and north Atlantic right whales (*Eubalaena glacialis*) (Hogg *et al.*, 2009), as well as for monitoring bacterial communities (Acevedo-Whitehouse *et al.*, 2010) and genetics (Frere *et al.*, 2010). This methodology is valuable in that it provides a means to collect physiological information from animals that cannot be handled directly (e.g. free swimming whales, large whale species, or endangered species). The proposed work provides an excellent opportunity to investigate the presence and detectability of complement proteins in blow samples simultaneously with blood. If complement activation plays a role in determining risk of decompression related injury and inflammatory responses, and we are able to monitor changes in complement components in blow, this would provide a tool to investigate risk of DCS in large, free swimming deep diving animals such as beaked whales and sperm whales.

D. Brief summary of proposed research. (Note if the study would be done in house or in the field.)

The major purpose of this project is to investigate the potential non-lethal effects of gas bubbles on immune function in marine mammals and address the hypothesis that a less reactive immune system serves a protective role against the development of DCS. Additionally we aim to investigate blow (exhaled breath condensate) sampling as a means to monitor complement activation and potential risk of DCS-like pathologies.

To do this, blood samples will be collected from marine mammals (belugas, harbor seals, harp seals and grey seals) to investigate the response of immune cells and the complement system to *in vitro* exposures to nitrogen bubbles. For seals, stranded animals will be targeted and blood samples will be drawn during health examinations at the time of admit to rehabilitation and again pre-release. For belugas, blood samples will be drawn in conjunction with monthly clinical draws. In addition, blood draws following stationary and active dive behaviors are proposed in order to investigate the role of activity modulation on immune responsiveness. Blow samples will also be collected from belugas at Mystic Aquarium during the same sessions as blood samples in order to investigate the presence of complement protein markers. Blood and blow samples will also be collected from belugas during field efforts in Point Lay and Bristol Bay, AK, and will be sought from belugas and pinnipeds housed at other facilities.

The majority of this work would be done in-house, including blood and blow sampling from belugas and blood sampling from stranded pinnipeds admitted to the rehabilitation program. Lab work, including *in vitro* exposure of blood samples to nitrogen bubbles, immune function assays and complement protein measurements will be done in house.

E. Specific goals of the proposed research.

- 1) *Measure phagocytosis and respiratory burst, as well as granulocyte activation, in marine mammal blood samples following in vitro exposure to N₂ bubbles.* Blood samples from belugas and pinnipeds will be exposed *in vitro* to N₂ bubbles and the immune response will be evaluated. Marine mammal granulocyte activity is not expected to change significantly in response to N₂ bubbles.
- 2) *Monitor changes in complement components (complement activation) in marine mammal blood samples following in vitro exposures to N₂ bubbles.* Complement activation will be measured in blood samples from belugas and pinnipeds with and without *in vitro* exposures to N₂ bubbles. Exposure to N₂ bubbles is not expected to result in complement activation in marine mammals.
- 3) *Evaluate the role of exercise modulation on the response of immune cells and complement activation to in vitro exposures to N₂ bubbles.* Blood and blow samples will be collected from belugas following trained dive behaviors including 1) a stationary dive and 2) an active swimming dive. Activity during a dive is expected to result in altered immune responses and complement status.
- 4) *To validate the presence of complement proteins in blow for potential use in monitoring immune status in belugas.* Blow samples collected from belugas will be used to investigate the presence of complement proteins in exhaled condensate. The degree of complement activation in blow is expected to reflect activation in blood samples indicating that blow could be a useful tool for evaluating immune activity in response to diving and other factors.

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

Fresh blood samples will be necessary for this study in order to assess the proposed immune functions (e.g. phagocytosis) without the confounding effects of archiving, storage and thawing on the target cells (granulocytes) and for validation experiments for complement activation assays. Fresh blood samples will also be necessary to determine if archived samples are appropriate for retrospective studies looking at complement proteins in serum.

The use of animals for blow collection is necessary, as the purpose of that aim is to expand the potential physiological information that can be gained from blow sampling as a non-invasive tool in conservation and health research.

Finally, the physiological responses to exercise (activity) cannot be mimicked in a non animal model, but may be important in determining the effects of a dive on immune function and ultimately animal health. Thus it is necessary to use voluntary participation in trained behaviors (active underwater swimming, diving to a stationary target) which result in normal physiological adjustments to evaluate the importance of such behavior in modulating immune reactivity.

B. Justify the choice of species to be used.

This project is a continuation of previous work which measured immune function following *in vitro* pressure exposures in belugas. Belugas are very capable divers, capable of diving to over 1000m and have been reported to a wide variety of dive behaviors and profiles. Additionally, we have baseline immune function data from these individuals and so can build upon previous research.

The phocid species targeted for this project are also capable of dives to several hundred meters. While these dives are not as extreme as the deepest reported to belugas, they do present physiological challenges. Additionally, it may be argued that these animals are potentially more or less susceptible to negative effects of altered dive behavior if they are not as conditioned to deep and extreme dives. The variety of dive ability provides important information as to whether risk of dive-related injury or disease is species specific, i.e. related to dive ability.

C. Identify and justify the number of animals to be used.

For *in vitro* assessments of immune function and complement responses to N₂ bubble exposure, and measurement of complement in blow, samples will be collected from the three belugas resident at the Mystic Aquarium, with additional samples being sought from partner institutions which also house belugas (total aquarium belugas targeted n=10). For these experiments blood and blow samples will also be obtained during field efforts in Bristol Bay and Point Lay, AK and 10 animals are targeted for each location for each field season (total n=20 for Bristol Bay, n=20 for Point Lay).

Stranded pinnipeds (harbor seals, harp seals, grey seals) will be sampled as opportunity allows, based on stranding occurrences, animal condition etc. The target sample size for stranded animals over two seasons in 10 individuals.

For purposes of looking at activity modulation, only animals resident at Mystic Aquarium are targeted at this time (n=3). Permission is being sought from Shedd and Sea World to include their animals (housed at Mystic) within this study.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

BLOOD DRAWS

Beluga blood samples will be drawn by veterinary staff from the superficial fluke vessel. Sampling will occur under behavioral participation and positive reinforcement during husbandry sessions. This sampling will occur once per month per animal, in conjunction with clinical blood draws, for the duration of the project. Additional blood draws per month will occur in conjunction with trained dive behaviors.

For stranded seals in rehabilitation, blood will be drawn from either the epidural vertebral sinus or the plantar venous plexus during manual restraint of the animal by trained staff. This will occur in conjunction with clinical blood draws once at the time of admit and again pre-release.

BLOW COLLECTION

Blow collection from belugas is done with behavioral participation of the whales. Belugas place their head on the exhibit beach and exhale once to clear the blowhole of exhibit water. A petri dish with nylon membrane cover is held inverted over the blowhole for four repeated exhales to collect condensate. Blow collection will occur in conjunction with monthly blood draws, as well as with additional blood sampling following dive behaviors.

DIVE BEHAVIORS

Two dive behaviors will be trained in the belugas residing at Mystic Aquarium for this research; 1) stationary dive and 2) active dive. For the stationary dive individuals will be asked to submerge to a target and remain stationary (i.e. not swimming) for a duration of 3-5 minutes. For the active dive, individuals will be asked to submerge and swim either in a perimeter pattern around the pool, or back and forth through a target hoop for a duration of 3-5 minutes. Blood and blow samples will be obtained from animals either immediately following the dive behavior or approximately 1 hr following the dive behavior. For each dive behavior and post sampling time, two replicates will be targeted, for a total of 8 sessions per whale.

B. Will a test substance be administered? No If yes,

1.) What is the name of the compound?

2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes.

C. Will manual, chemical and/or mechanical restraint of the animal be necessary? If yes, explain the method of restraint. No

D. Indicate the level of pain the proposed procedure is expected to cause.

a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.

b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.

c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery of procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

E. Describe the method of euthanasia to be used if this were to become necessary

F. Does the proposed research involve surgery? No
If yes, go to Addendum II. Surgical Protocol

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

The belugas at Mystic Aquarium are housed in the outdoor Arctic Coast exhibit and would remain there for this study. Stranded pinnipeds will be housed in the seal rescue clinic at the Mystic Aquarium.

No special animal care requirements or behavioral conditioning is required for the pinnipeds for this study. For the belugas, training of stationary and active dive behaviors are required for objective 3. This behavior will require new behaviors for two whales, while the third has previous trained underwater swimming behaviors that can be built upon. Preliminary discussions have been carried out with husbandry supervisors, and have agreed that these requirements are feasible.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours.

There are veterinarians on site at least 6 days per week, and on 24 hour call.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics?

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include 1.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

Laura Thompson, PhD: Dr. Thompson brings experience in marine mammal immunology, with particular focus in relation to diving physiology and the effects of anthropogenic stressors such as noise on marine mammal health. This project will support her postdoctoral work and is a follow-on from her PhD funded work supported by ONR. She will be the PI on this project with close mentorship by Dr. Tracy Romano.

Laura Thompson holds a first class degree with honors BSc from Queen Mary College, University of London. For her undergraduate thesis she focused on phenotyping different subsets of lymphocyte in two contrasting populations of wild bottlenose dolphins along the east coast of the US, which she worked on under the supervision of Dr. Tracy Romano. Following graduation Laura worked within the marine mammal and sea turtle rehabilitation program at the Mystic Aquarium, before returning to the research department as a technician investigating the prevalence of exposure to marine origin *Brucella* in marine mammal species. Laura obtained a PhD in Oceanography from the Department of Marine Sciences at the University of Connecticut in May 2014. Her dissertation work focused on investigating the response of marine mammal immune cells to increased pressure (i.e. simulated dives) and the potential for an additional stressor to alter that response. Laura has experience with a variety of laboratory techniques, including sterile technique, PCR and cell culture, as well as specific training and experience with immune function assays such as phagocytosis which have been proposed for this work. She is also familiar with protocols in endocrinology, including ELA's and has previously developed and validated a methodology for measuring cortisol in beluga blow.

Tracy Romano, PhD: Expertise in marine mammal neuroimmunology and impacts of environmental and anthropogenic stressors on aquatic animal health. She will be Co-PI on this project with prime responsibility of mentoring Dr. Laura Thompson (postdoctoral fellow) and ensuring the project is executed successfully.

Tracy Romano graduated with a Bachelor's of Science degree from Saint Michael's College in Winooski, VT. In 1986 she was awarded a fellowship from the American Society for Engineering Education to pursue her graduate studies in neuroimmunology and in 1993 received a PhD in neurobiology and anatomy from

the University of Rochester in Rochester, N.Y. She was awarded a National Research Council fellowship to carry out her postdoctoral studies with the U.S. Navy Marine Mammal Program in collaboration with the Scripps Research Institute in San Diego, C.A. The focus of her research was characterization of the marine mammal immune system with subsequent focus on the impact of environmental stressors on the health of bottlenose dolphins. She subsequently established a research laboratory at the Navy facility dedicated to marine mammal neuroimmunology. Some of her accomplishments include the cloning and characterization of dolphin CD4, investigation of sound as a stressor on cetacean health, contributions to a study mandated by Congress to investigate the impact of the tuna fishery on dolphin health, and the establishment of an anatomical link between the cetacean nervous and immune systems. In May 2004, Tracy moved her research program to Mystic Aquarium, a division of Sea Research Foundation, Inc. to lead the Aquarium's research department as the vice president of research. In 2006, she was promoted to senior vice president of research and zoological operations, overseeing the animal collection and programs in animal care and husbandry, research, animal rescue and exhibits. In 2008, she was a finalist for the Women of Innovation Awards by the Connecticut Technology Council and in 2012 was inducted in the Academic Hall of Fame for her Alma mater. Currently, her primary research focuses on studying the health of beluga whales in the wild and in the aquarium setting. She regularly travels to Point Lay, Alaska, to participate in live capture release studies and is responsible for collecting and analyzing blood samples for health assessment given future impacts of climate change, pollutants and oil and gas exploration.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

Allison Tuttle, DVM, Diplomate ACZM; Allison D. Tuttle obtained her DVM in 2002 from North Carolina State University and went on to complete her internship in aquatic animal medicine at Mystic Aquarium, and residency in zoological medicine with an aquatic health management focus at North Carolina State University. Dr. Tuttle is specialty-boarded with the American College of Zoological Medicine. She has over 12 years of experience in aquatic animal health and currently serves as the Senior Director of Animal Care & Veterinary Services at Mystic Aquarium and directs Mystic Aquarium's Animal Rescue Program. Dr. Tuttle will oversee and advise on all blood sampling proposed for this study.

Barbara Mangold, DVM; Barbara Mangold obtained her DVM from Virginia-Maryland Regional College of Veterinary Medicine. She has trained in both small animal medicine and surgery, and completed her residency in zoo medicine at the Wildlife Conservation Society/Bronx Zoo. She has worked at the Bronx Zoo, Disney's Animal Kingdom and consults for the Maritime Aquarium in Norwalk. Dr. Mangold will participate in blood draws for this study.

Veterinary Intern; Mystic Aquarium offers an internship in aquatic animal medicine to graduates of AVMA accredited veterinary colleges. Interns will be involved with blood draws for this study.

Kristine Magao; Kristine Magao graduate from Eastern Connecticut State University with a BA in Psychology in 1997. She has been working with Mystic Aquarium since 1997, spending the past 18 years as a professional animal trainer with supervisory and managerial positions and responsibilities. Currently, Kristine oversees the beluga and pinniped programs, assuming responsibility for the development of staff and animal training programs. She will oversee training behaviors proposed for this research.

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

- 1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).**

- 2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?**

- 3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.**

- 4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.**

- 5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.**

STATEMENT OF ASSURANCE

Based upon available information, the alternatives to the use of live animals for the purpose of this study have been considered and found to be inappropriate. It is the responsibility of the Principal Investigator to assure that the protocols outlined here for the humane care and treatment of the animal are followed. The Mystic Aquarium reserves the right to discontinue the study if the health of the animal or the safety of the staff is jeopardized. Any necessary changes in the study design will be approved by the Institutional Animal Care and Use Committee (IACUC) prior to implementation. The Principal Investigator has reviewed the qualifications of all personnel involved in the study and found them to be adequate.

To the best of my knowledge, the animal research described in this protocol review does not unnecessarily duplicate previous experiments.

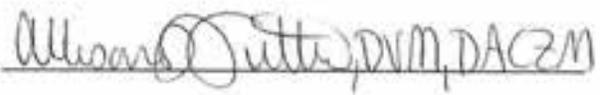


Principal Investigator

3 Feb 2015

Date

The Attending Veterinarian has reviewed the proposed protocols for animal use and has found them to be acceptable.



Attending Veterinarian

3 Feb 15

Date

=====

IACUC Comments or Conditions of Approval:

Reviewed by Designated Reviewer. See attached approval with comments.



Signature of IACUC Chair

2-12-15

Date

Approval Period: 2/12/15 - 2/12/16 Protocol # 15004
(Must be reviewed annually)

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Project No. 15004



MYSTIC
AQUARIUM

**Institutional Animal Care and Use Committee
RESEARCH PROPOSAL and PROTOCOL REVIEW**

**By
Designated Reviewer**

Animal Welfare Act, Part 2, Subpart C, Section 2.31 (d)(2) ... "If full Committee review is not requested, at least one member of the IACUC, designated by the Chairman and qualified to conduct the review, shall review those activities and shall have the authority to approve, require modifications in (to secure approval), or request full committee review of any of those activities. If full Committee review is requested for a proposed activity, approval of that activity may be granted only after review at a convened meeting of a quorum of the IACUC."

Designated Reviewer: Mike Osborn

Project Title: *Evaluation of Non-lethal effects of N2 bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury.*

Reviewer Comments/Recommended modifications:

See attached comments.

Reviewer Decision: Approve Require Modifications Full Committee Review

Signature of Reviewer: *[Signature]* **Date** 2-11-15

Signature of IACUC Chair *Tracy Romond* **Date** 2-12-15

Evaluation of non-lethal effects of N2 bubbles on Marine Mammal health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury

Designated Reviewer: Mike Osborn

REVIEWER COMMENTS/RECOMMENDED MODIFICATIONS:

After review of the proposed project I recommend approval of the project with the following considerations to maintain behavioral cooperation with the beluga whales. Kristine Magao (Assistant Curator of beluga whales and pinnipeds) will oversee the training, with use of positive reinforcement operant conditioning, to best maintain cooperation throughout the proposed project. The following considerations should be followed to best maintain this objective:

1. Target a maximum of 1 dive/blood per month for Laura's project (this will give us 2 blood draws/month total— 1 for clinical/in-house research purposes and 1 for Laura's project). Kristine would recommend that during the data collection phase of this project, we carefully look at the monthly bloods we are currently giving to research.

Example:

Schedule a monthly blood for each whale at the beginning of the month to include clinical/research. If the desired amount of blood is not achieved for research purposes, we give 2 options:

- a. We forego research blood for that month and continue with Laura's project for the month

Or

- b. We forego Laura's project for the month and try for a research blood later in the month

2. We can potentially do 2 dive/blood sessions per month for Laura if her samples can be obtained as part of our typical research draw. If the blood sample can be "shared" we will need to understand the total volume required to meet each specific animal's ability to hold duration for a blood draw.

Other considerations from Kristine

1. Juno bloods more than 2x/month, should be vetted by veterinarians due to his history with phlebitis
2. Recommend that we avoid breeding season for data collection for this project



55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT AMENDMENT / RENEWAL REQUEST

APPLICANT:

**Laura Thompson, PhD
Postdoctoral Fellow
Mystic Aquarium
55 Coogan Blvd, Mystic CT
860 572 5955 x155**

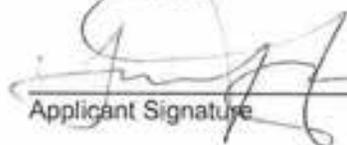
ltompson@mysticaquarium.org

- ORIGINAL PROJECT TITLE: **Evaluation of Non-lethal effects of N2 bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury**
- DATE OF ORIGINAL APPROVAL: **Project approved in Feb 2015 but was on hold pending funding. Funding received in late May 2015 and project began in June 2015.**
- AMENDED PROCEDURE / SPECIMEN NEEDS (Attach detailed description of changes to original research and/or sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. Provide FedEx account number if samples are to be shipped):
- PROPOSED TIME FRAME FOR AMMENDED / RENEWED PROJECT (include dates if possible):

Check here if request is for renewal only with NO changes to original protocol.

Attach summary of project findings/presentations/publications during previous year.

Terms and Conditions of original project approval will apply.

 _____
Applicant Signature Date 20 April 2016

Post Doctoral Researcher _____
Title Affiliation Mystic Aquarium

Return to: Animal Management Specialist
Research and Veterinary Services
Phone: 860.572.5955 Ext. 108
gsirpenski@mysticaquarium.org

PROJECT # 15004

=====

Conditions of Approval:

APPROVAL: Projects must be re-approved annually.

n/a - Dr. Romano is Co-PI

VP of Biological Research Date

Allison Dutte, DVM, Dipl. ACZM 20 April 2016

VP of Biological Programs Date

Anniversary Date: 4/20/2017

PROJECT # 15004



55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT AMENDMENT / RENEWAL REQUEST

APPLICANT:

**Laura Thompson, PhD
Postdoctoral Fellow
Mystic Aquarium
55 Coogan Blvd, Mystic CT
860 572 5955 x155**

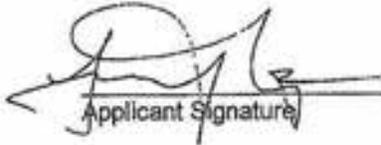
ltompson@mysticaquarium.org

- ORIGINAL PROJECT TITLE: **Evaluation of Non-lethal effects of N2 bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury**
- DATE OF ORIGINAL APPROVAL: **2/12/2015**
- AMENDED PROCEDURE / SPECIMEN NEEDS (Attach detailed description of changes to original research and/or sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. Provide FedEx account number if samples are to be shipped):
- PROPOSED TIME FRAME FOR AMMENDED / RENEWED PROJECT (include dates if possible): **3/31/17 — 3/31/18**

Check here if request is for renewal only with NO changes to original protocol.

Attach summary of project findings/presentations/publications during previous year.

Terms and Conditions of original project approval will apply.


Applicant Signature

2 June 2017
Date

Postdoctoral Fellow
Title

Mystic Aquarium
Affiliation

Return to: **Animal Management Specialist
Research and Veterinary Services
Phone: 860.572.5955 Ext. 108
gsirpenski@mysticaquarium.org**

PROJECT # 15004

Conditions of Approval:

APPROVAL: Projects must be re-approved annually.

Tracy Romano 6-6-17
VP of Biological Research Date

Allan Dittus 6/6/17
VP of Biological Programs Date

Anniversary Date: 6/6/2018

PROJECT # 15004



55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT AMENDMENT / RENEWAL REQUEST

APPLICANT:

Laura Thompson, PhD
Postdoctoral Fellow
Mystic Aquarium
55 Coogan Blvd, Mystic CT
860 572 5955 x155

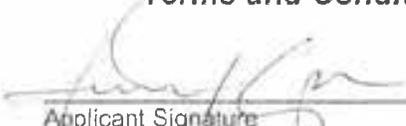
ltompson@mysticaquarium.org

- ORIGINAL PROJECT TITLE: **Evaluation of Non-lethal effects of N2 bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury**
- DATE OF ORIGINAL APPROVAL: **2/12/2015**
- AMENDED PROCEDURE / SPECIMEN NEEDS (Attach detailed description of changes to original research and/or sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. Provide FedEx account number if samples are to be shipped):
- PROPOSED TIME FRAME FOR AMMENDED / RENEWED PROJECT (include dates if possible):

Check here if request is for renewal only with NO changes to original protocol.

Attach summary of project findings/presentations/publications during previous year.

Terms and Conditions of original project approval will apply.

 14 May 2018
Applicant Signature Date

Research Fellow Mystic Aquarium
Title Affiliation

Return to: Animal Management Specialist
Research and Veterinary Services
Phone: 860.572.5955 Ext. 108
gsirpenski@mysticaquarium.org

PROJECT # 15004

Conditions of Approval:

APPROVAL: Projects must be re-approved annually.

Tracy Roman 05/15/18
VP of Biological Research Date

Allison Dittie, DVM, Dpl. ACZM 05/15/18
VP of Biological Programs Date

Anniversary Date: 5/15/2019



MYSTIC AQUARIUM

55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT AMENDMENT / RENEWAL REQUEST

APPLICANT:

Laura Thompson, PhD
Postdoctoral Fellow
Mystic Aquarium
55 Coogan Blvd, Mystic CT
860 572 5955 x155
lthompson@mysticaquarium.org

- ORIGINAL PROJECT TITLE: **Evaluation of Non-lethal effects of N2 bubbles on Marine Mammal Health and the Potential Role of Immune Activity in Facilitating the Development of Dive Related Injury**
- DATE OF ORIGINAL APPROVAL: **2/12/2015**
- AMENDED PROCEDURE / SPECIMEN NEEDS (Attach detailed description of changes to original research and/or sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. Provide FedEx account number if samples are to be shipped):
- PROPOSED TIME FRAME FOR AMMENDED / RENEWED PROJECT (include dates if possible):

Check here if request is for renewal only with NO changes to original protocol.

Attach summary of project findings/presentations/publications during previous year.

Terms and Conditions of original project approval will apply.


Applicant Signature

10 April 2019
Date

Research Fellow
Title

Mystic Aquarium
Affiliation

Return to: **Animal Management Specialist**
Research and Veterinary Services
Phone: 860.572.5955 Ext. 108
gsirpinski@mysticaquarium.org



Conditions of Approval:

APPROVAL: Projects must be re-approved annually.

Tracy Romond 4/26/19
VP of Biological Research Date

Allison Tuttle, DVM, Dipl. ACZM 4/26/19
SVP of Biological Programs Date

Anniversary Date: 4/26/2020



**MYSTIC
AQUARIUM**

Project No. 18018

RESEARCH PROPOSAL and PROTOCOL REVIEW

MYSTIC AQUARIUM
Sea Research Foundation, Inc.
55 Coogan Blvd.
Mystic, CT 06355

Introduction

Mystic Aquarium is committed to research and promotes the use of its collection for the purpose of enhancing knowledge of aquatic animals through scientific investigation.

The following proposal materials are designed to address all pertinent issues regarding the use of live animals for scientific study at Mystic Aquarium. Individuals interested in conducting research must provide this information to satisfy the requirements of the Aquarium's Staff Research Committee and Institutional Animal Care and Use Committee (IACUC). It is the function of these groups to ensure that all protocols comply with the regulations and standards of the United States Department of Agriculture, as well as standards established by Sea Research Foundation. Information should be specific and comply with the format as presented.

Submission and questions regarding the preparation of this proposal should be directed to:

Gayle Sirpenski, Secretary, IACUC
(860) 572-5955 ext. 108
gsirpenski@searesearch.org

I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Maureen Driscoll, PhD
Mystic Aquarium
55 Coogan Blvd
Mystic, CT 06355
860.572.5955 x.153
mdriscoll@mysticaquarium.org

B. Title of proposed research

Microbiome Surveillance: A Noninvasive Tool to Measure Beluga Whale Health

C. Introduction and background

Beluga whales live in the arctic and subarctic where they face numerous challenges, including changing ice and weather patterns, declines in prey availability, pollution, noise, tourism, shipping traffic, and oil and gas exploration. Changes in ice patterns and increased water temperatures pose an increased risk of emerging pathogens. As a sentinel species, belugas are indicators of environmental health. Infectious diseases and emerging pathogens pose serious risks to cetaceans, and pathogenic organisms have been linked to several mass mortality events. The critically endangered Cook Inlet beluga population for example, could suffer catastrophic consequences from an infectious disease outbreak. In addition, belugas and other cetaceans under human care are susceptible to respiratory infections, a cause of mortality for these animals. Numerous microbiome studies have shown that microbial communities play critical roles in host nutrition, immune function, and protection from invading pathogens. However, marine mammal microbiome research is lacking. This study will aim to characterize the skin, oral, gastrointestinal, vaginal, and respiratory microbiome of beluga whales under human care to identify vital core microbial populations that serve as indicators of beluga health. Results will offer comparison data and novel methods to better understand the health of wild belugas and identify emerging microbial threats to animal and human health.

D. Brief summary of proposed research. (Note if the study would be done in house or in the field.)

This study will be conducted using beluga whales housed at Mystic Aquarium's Arctic Coast Exhibit. This study will provide critical baseline information regarding variations in the beluga oral, skin, gastrointestinal, vaginal, and respiratory microbiomes. The overall goal of microbiome surveillance is to improve management and health outcomes of belugas under human care and in the wild. Monitoring the microbiome of Aquarium belugas can help determine whether a common bacterial community exists among these animals, and if a shift in the core community is indicative of a compromised health state. Such data will provide information on beneficial bacterial strains that provide protection from pathogens. The data will also contribute to the long-term goal of monitoring wild beluga populations, with the ultimate goal of mitigation measures for management and conservation.

E. Specific goals of the proposed research.

1. Establish methods for collecting samples for microbiome analysis from beluga whales.
2. Establish methods for extracting DNA from samples collected from belugas for microbiome analysis.
3. Document bacterial community profiles of beluga skin, oral, gastrointestinal, vaginal, and respiratory microbiomes in belugas using next-generation sequencing technologies.
4. Identify "core" bacterial communities necessary for beluga health.
5. Correlate bacterial community profiles with clinical assessments and other health measures to show that microbiome analysis can be used as a noninvasive technique to assess health in beluga whales.

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

Gathering baseline data on the bacterial populations commonly found in aquaria belugas may help to identify pathogenic microorganisms capable of causing disease, and categorize protective strains essential for beluga health. These data can then be combined with other health assessment parameters such as traditional blood testing. Additionally, the use of a relatively noninvasive technique paired with microbiome surveillance has the potential to become an alternative method to monitor beluga health when traditional sampling (i.e. blood collection, skin biopsy) is not feasible.

B. Justify the choice of species to be used.

The ultimate goal of the project is to apply these methods to free-ranging belugas.

C. Identify and justify the number of animals to be used.

Mystic Aquarium is home to one juvenile male and one adult female beluga. Samples will be obtained from both whales to provide baseline microbial profiles from each sex and different age classes.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

Aquarium belugas will be trained to station to accept swab sampling for microbiome studies. The beluga microbiome will be characterized and monitored including oral, blowhole, skin, vaginal and gastrointestinal tract locations. Samples will be collected twice weekly, as microbiomes naturally shift (in number and type of bacteria present) over the course of a lifetime of the host. It is unknown what factors influence changes in beluga whale microbiomes, however microbiome shifts have been associated with breeding, molt, illness, dietary changes, and environmental factors in other vertebrates.

Oral: A sterile swab will be placed inside the mouth and used to sample/swab the oral cavity. The swab will be stored at -80°C until DNA can be extracted.

Blowhole: A sterile swab will be gently placed inside the nostril or blowhole. The swab will be stored at -80°C until DNA can be extracted.

Skin: A sterile swab will be used to rub the surface of the skin on the dorsal ridge. The swab will be placed at -80°C until DNA can be extracted.

Vaginal: A sterile swab will be placed ½" inside the vaginal opening. The swab will be stored at -80°C until DNA can be extracted.

Gastrointestinal tract: GI samples will be obtained by placing a sterile swab into the anal opening. The swab will be stored at -80°C until DNA can be extracted.

B. Will a test substance be administered? No If yes, 1.) What is the name of the compound?

2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes. N/A

B. Will manual, chemical and/or mechanical restraint of the animal be necessary? If yes, explain the method of restraint. No

D. Indicate the level of pain the proposed procedure is expected to cause.

- a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.
- b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.
- c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery or procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

E. Describe the method of euthanasia to be used if this were to become necessary N/A

**F. Does the proposed research involve surgery? No
If yes, go to Addendum II. Surgical Protocol**

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

Mystic Aquarium belugas are housed at the Arctic Coast exhibit. The belugas will remain on exhibit serving as ambassadors for their species.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours.

The beluga whales at Mystic Aquarium receive routine veterinary care from three full-time veterinary staff. A veterinarian is on call 24 hours.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics?

Yes

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include 1.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

1. Education

Degree	Major/Field of Study	Institution and Location	Year Received
B.S.	Biology	Salem State University, Salem MA	2000
Ph.D.	Cell & Molecular Biology	University of Rhode Island, Kingston RI	2010

2. Relevant Experience

Job Title	Role	Affiliation	Location (City, State, Country)	Dates (MM/YY- MM/YY)
Research Fellow	Collect, process, and analyze samples (blow, feces, saliva, blood) from marine mammals	Mystic Aquarium	Mystic CT USA	06/15-present
Postdoctoral Fellow	Routinely carried out DNA and RNA extractions from bacteria, mammalian tissues (feces, skin, brain, liver, adipose tissue), and cell cultures for use in downstream applications such as PCR and real-time qPCR. Carried out a microbiome study to assess the effect of Bisphenol A on the gut microbiota of rats using training received as a graduate student. Was trained by Brown University researcher to carry out bioinformatics to assess microbial communities.	University of Rhode Island	Kingston, RI USA	04/10-08/14

3. Summary of Experience

Procedures	Experience Metrics: Estimated Number of Animals, Hours/Months/Years, Species, and Age Class
Oral swab	Regularly collect saliva samples from two aquarium belugas for the past two years.
Sample - Blood	Assist with monthly blood collection from 2-4 aquarium belugas for the past three years.
Sample - Exhaled air	Regularly collect respiratory blow samples from two aquarium belugas for the past three years. Train other staff in collection techniques.
Sample - fecal	Assist with collecting fecal samples from two aquarium belugas for the past year.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

Mystic Aquarium veterinary and beluga husbandry staff will be assisting with this study.

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

- 1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).**

- 2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?**

- 3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.**

- 4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.**

- 5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.**

STATEMENT OF ASSURANCE

Based upon available information, the alternatives to the use of live animals for the purpose of this study have been considered and found to be inappropriate. It is the responsibility of the Principal Investigator to assure that the protocols outlined here for the humane care and treatment of the animal are followed. The Mystic Aquarium reserves the right to discontinue the study if the health of the animal or the safety of the staff is jeopardized. Any necessary changes in the study design will be approved by the Institutional Animal Care and Use Committee (IACUC) prior to implementation. The Principal Investigator has reviewed the qualifications of all personnel involved in the study and found them to be adequate.

To the best of my knowledge, the animal research described in this protocol review does not unnecessarily duplicate previous experiments.

[Handwritten Signature]

11.5.2018

Principal Investigator

Date

The Attending Veterinarian has reviewed the proposed protocols for animal use and has found them to be acceptable.

[Handwritten Signature] MS
DIP. ACVIM

11/6/18

Attending Veterinarian

Date

=====

IACUC Comments or Conditions of Approval:

[Handwritten Signature]

11/7/18

Signature of IACUC Chair

Date

Approval Period: 7 Nov 2018 - 6 Nov 2019 Protocol # 18018
(Must be reviewed annually)

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

PROJECT #. 12001



MYSTIC
AQUARIUM

Mystic Aquarium
55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT/SPECIMEN REQUEST

APPLICANT:

Name - Becky Sartini/Justin Richard

Address – University of Rhode Island
Kingston, RI

Telephone – (401) 874-2667 FAX – (401) 874-7575 e-mail bsartini@uri.edu;
jt_richard@my.uri.edu

- PROJECT TITLE: Evaluating the use of blow for reproductive hormone and genetic analysis in belugas (*Delphinapterus leucas*)
- SCIENTIFIC OBJECTIVES:
 - Establish blow (exhale) sampling as a source of reproductive hormones (testosterone, progesterone, and estrogen) for analysis
 - Recover nuclear DNA from blow samples for use in analyzing genetic relationships
 - Perform testes ultrasounds to monitor reproductive seasonality in males
- PROCEDURE / SPECIMEN NEEDS (Append detailed research and sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. **Provide FedEx account number if samples are to be shipped**):

Matching blow ^{how much?} and blood samples would be collected from all 4 belugas once a month for one consecutive year. All collections would be made with the voluntary cooperation of the animal. Blow samples would be collected on a Petri dish with nylon stretched over the top, identical to the method currently being used by Mystic research staff. Blow samples would be collected during blood draw training sessions, but all animals would not need to be sampled on the same day. One ml of plasma for each sampling event would be sufficient for this project. Blow and plasma samples will be stored at -80° C until analysis.

Because sample volumes may be too small (<100 µL) to use enzyme immunoassays, liquid chromatography-mass spectrometry (LC-MS) will be utilized to detect the presence and concentration of hormones in blow samples (Hogg et al. 2005, Hogg et al. 2009). For consistency, the same methodology will be used for blood samples. Testosterone, progesterone, and estrogen presence or absence will be determined for each sample based on the known molecular weights of each hormone. Hormone concentrations will be determined by comparison to internal standards of commercially available hormone isotopes (Hogg et al.

PROJECT #. _____

2005). Hormone concentrations in blood and blow will be correlated, and these levels will then be compared with the known gender and reproductive condition of the animal sampled.

To evaluate the reproductive condition of each animal, information from ultrasound exams would be included in the project. Ultrasounds would not need to be performed on the same day as blow/blood sampling. Data already being collected by veterinary staff would be used to define estrous cycle stages; no additional ultrasound exams of the female belugas would be required. To monitor the male belugas, twice monthly testes ultrasounds would be performed. Both testes would be measured in the same training session. Once the ultrasound operator is familiar with each animal, the measurements can generally be performed in 90 seconds or less on each side. Ideally, both animals would be sampled during the same session. Sessions would be scheduled at any time of day at the convenience of the husbandry staff. If deemed appropriate, Justin Richard would perform all of the testes ultrasound exams with the Aquarium's ultrasound machine.

Using methodology described in Frere et al. (2010), genomic DNA will also be isolated from blow samples. Blow has yielded similar DNA concentrations as blood samples (10 ng/ μ l) (Frere et al. 2010). DNA from blood and blow will be extracted using a Qiagen DNeasy blood and tissue kit and amplified using standard PCR protocols. The amelogenin gene, which is Y-chromosome specific and known for a high degree of gender-dependent polymorphism, will be amplified from male samples and sequenced. This gene has been proposed as a marker for gender determination and for evaluating genetic relationships between cetacean populations, and specific primers have already been developed (Mace and Crouau-Roy 2008). Sequences will be evaluated as markers for use in population genetics studies in belugas.

The use of LC-MS to analyze blow samples for this project means that fewer exhales will be necessary to acquire sufficient volume in each sample. This methodology may also allow for simultaneous analysis of reproductive hormones and stress hormones, which are already being investigated at Mystic Aquarium. To evaluate this possibility and to perform genetic analysis, a number of blow samples would be collected independently of blood samples. The number of samples required will depend on the success of the laboratory methodology, but because the samples can be frozen until analysis, sampling would be at the convenience of the husbandry staff.

- PROPOSED TIME FRAME FOR PROJECT (include dates if possible): Approximately 3 years, to begin after project approval and as animal training allows.
- Attach NMFS or FWS authorization if applicable.

Can request be met as part of routine / scheduled handling?	YES	<u> X </u>	NO	<u> </u>
Able to pay for staff OT, lab costs, special sampling equipment, shipping costs, if required?	YES	<u> </u>	NO	X <u> </u>
Requires intrusive procedure ¹ not performed as part of a routine medical examination. (If yes, applicant must complete form for approval by IACUC).	YES	<u> </u>	NO	X <u> </u>

¹ Any procedure involving a break in or cutting of the skin or equivalent, insertion of an instrument or material into an orifice, introduction of a substance or object into an animal's environment that is likely either to be ingested or to contact and affect directly an animal's tissues, or a stimulus directed at an animal that may involve a risk to health or welfare or that may have an impact on normal function or behavior.

PROJECT #. _____

TERMS AND CONDITIONS

- Specimens will be used only for the purposes described above, and will not be redistributed or sold; any excess material will be returned to the Aquarium, or destroyed in a manner acceptable to both the Applicant and the Aquarium.
- The Applicant recognizes that there may be zoonotic hazards associated with handling biological materials, and hereby releases and holds harmless the Mystic Aquarium and the Sea Research Foundation, its officers, trustees, employees and agents, from any and all claims, demands, actions, and causes of actions whatsoever on account of or in connection with any aspect of this request for biological materials.
- The Applicant must submit an annual report of findings prior to project renewal. Applicants will submit a full proposal and summary report every third year for ongoing projects.
- The Applicant will acknowledge Mystic Aquarium in any publication, abstract, manuscript report or presentation arising from this project,
- **The Applicant will allow Mystic Aquarium to review a draft of any report based on the analysis of specimens or data originating from the Aquarium.** Abstracts, manuscripts, reports, power point presentations, posters, etc. must be submitted to the Mystic Aquarium Research Review Committee for review and approval no later than 2 weeks prior to submission/presentation deadline.
- The Applicant will provide the Aquarium with an electronic copy of any publication dealing with the specimens or data requested.
- Failure to comply with the above terms and conditions will jeopardize future collaborations with Mystic Aquarium.

Becky Pan 1/10/12 ASSISTANT PROFESSOR
 Applicant Signature Date UNIVERSITY OF RI

Sponsor's Signature _____ Name/Title/Affiliation _____ Date _____
 Sponsor
 (Required for Applicants who are not University faculty, research scientists, or health professionals)

Return by FAX to: Animal Management Specialist
 Research and Veterinary Services
 Fax: 860.572.5972 Phone: 860.572.5955 x 108
 Return electronic file to: gsirpenski@searesearch.org

=====

APPROVAL Projects must be re-approved annually.

Jacy Ronard 2/15/12 [Signature] 2-15-12
 Director of Research Date Curator Date

Allison Tuttle 02-15-12
 Staff Veterinarian Date

Comments or Conditions for Approval: Per Dr. Tuttle, 1/3 portion of project can begin but limit to 1 animal/session until behaviors are stronger. Collection of molting blow and plasma will be provided when we are able to increase frequency and duration of blood draws.
 Anniversary Date: 2/17/2013

Re-approval Date: _____ Signature: _____

PROJECT #. 12001



MYSTIC AQUARIUM

Mystic Aquarium

a division of Sea Research Foundation Inc.
55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT/SPECIMEN REQUEST

APPLICANT:

Name - Justin Richard

Address - Mystic Aquarium, Mystic, CT

Telephone - (860) 383-3955 e-mail jt_richard@my.uri.edu

- **PROJECT TITLE:** Project #12001, originally approved 2/17/12, last amendment to protocol was May 2013, last renewal was Feb 2015.
Evaluating the use of blow for reproductive hormone and genetic analysis in belugas (*Delphinapterus leucas*)
- **SCIENTIFIC OBJECTIVES:**
This project aims to develop minimally invasive research tools for belugas. Reproductive hormone concentrations will be measured in blow (exhale) collected from trained animals to monitor reproductive physiology. With the development of these assays completed, the focus has shifted toward applying this methodology toward monitoring the attainment of sexual maturity in the male beluga whales housed at Mystic Aquarium. Hormone levels measured in blow will be correlated to breeding behavior in the group. DNA isolation and amplification has been successfully developed, and the focus is shifting toward RNA isolation to establish the utility of blow samples for gene expression studies.
- **PROCEDURE / SPECIMEN NEEDS** (Append detailed research and sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods.
Provide FedEx account number if samples are to be shipped):

With method development completed, a reduction in sampling is requested relative to the previous approval (May 2013):

Blow Sampling	Ultrasound exams (males only)	Behavioral Observations
1x/week	1x/week	5 hours/week (public side only)

Blood sampling and female ultrasound exams are no longer required to meet the project's goals. Less frequent blow sampling is also required. Behavioral observations will continue to be made by Justin Richard or trained undergraduate students that have

PROJECT #. _____

been trained by Justin Richard and have completed a Mystic Aquarium volunteer orientation conducted by human resources.

Additionally, with Justin Richard's impending graduation from the University of Rhode Island in April of 2016, a shift in PI is also requested.

- PROPOSED TIME FRAME FOR PROJECT (include dates if possible):

February 2016 – February 2017

- **Attach NMFS or FWS authorization if applicable.**

Can request be met as part of routine / scheduled handling? YES X NO

Able to pay for staff OT, lab costs, special sampling equipment, shipping costs, if required? YES NO

Requires intrusive procedure ¹ not performed as part of a routine medical examination. (If yes, applicant must complete form for approval by IACUC). YES NO X

¹ Any procedure involving a break in or cutting of the skin or equivalent, insertion of an instrument or material into an orifice, introduction of a substance or object into an animal's environment that is likely either to be ingested or to contact and affect directly an animal's tissues, or a stimulus directed at an animal that may involve a risk to health or welfare or that may have an impact on normal function or behavior.

PROJECT # 12001



**MYSTIC
AQUARIUM**

55 Coogan Blvd.
Mystic, CT 06355-1997

PROJECT AMENDMENT / RENEWAL REQUEST

APPLICANT:

Name - Justin Richard

Address - University of Rhode Island

Kingston, RI

Telephone - (860) 383-3955 e-mail jt_richard@uri.edu;

ORIGINAL PROJECT TITLE:

Evaluating the use of blow for reproductive hormone and genetic analysis in belugas
(*Delphinapterus leucas*)

Revised title: Investigating beluga whale reproductive biology using minimally-invasive techniques

- DATE OF ORIGINAL APPROVAL: 17 February 2012, last renewal Feb 2016
- AMENDED PROCEDURE / SPECIMEN NEEDS (Attach detailed description of changes to original research and/or sampling protocol. If applicable, list specific amount of specimen requested, storage / shipping methods. Provide FedEx account number if samples are to be shipped):
- PROPOSED TIME FRAME FOR AMMENDED / RENEWED PROJECT (include dates if possible): Oct 2017. – Sep 2019

Check here if request is for renewal only with NO changes to original protocol.
Attach summary of project findings/presentations/publications during previous year.

Terms and Conditions of original project approval will apply.


Applicant Signature

28 Sep 17
Date

Past Doctoral Fellow University of Rhode Island
Title Affiliation

Return by FAX to: Animal Management Specialist
Research and Veterinary Services
Fax: 860.572.5972 Phone: 860.572.5955 Ext. 108
gsirpensi@mysticaquarium.org

PROJECT # 12001

Scientific Objectives:

The objective of this study is to capitalize on the unique research opportunities afforded by the aquarium to longitudinally study the reproductive physiology and behavior of the belugas at Mystic Aquarium to better describe the reproductive biology of this species. This study also aims to develop non-invasive methods of collecting demographic information from a free-ranging species of whale. Reproductive hormones will be assayed in blow (exhale) samples. Hormone concentrations assayed in blow will be correlated with behavioral observations and ultrasonographic evaluation of reproductive organs to monitor the reproductive physiology of individuals over time. DNA will also be acquired from blow, blood, and skin for use investigating a molecular aging method based on epigenetic changes to the DNA. This technique would make it possible to resolve genetic relationships in wild belugas and allow for a molecular investigation of mating strategies in this species based on hypotheses developed from the study of belugas at Mystic Aquarium.

Specimen Requests:

All sample collection or behavioral observations will be performed by JR or aquarium interns or volunteers who have completed volunteer orientation conducted by human resources and trained by JR and under the guidance and discretion of the beluga whale training staff. Ultrasound exams will only be performed by JR.

Blow sampling, all animals: using established methods, up to 1x/week.

Ultrasounds, males only: using established methods, 2x/month.

Sloughed skin: collected by rubbing a gloved finger along "sloughy" regions of the animal, typically on the pectoral flipper, up to 1x/week, although once sufficient sample has been collected to perform the molecular experiments, collection frequency will decrease or cease.

Whole blood: subsampled from blood collected for routine husbandry purposes, collected into a small purple top tube. Needs will likely be met in a single sampling event, although resampling may be requested a maximum of twice during the study period.

Behavioral observations: conducted from the public side of the exhibit, up to 5 hours per week. As already established, observers will wear an ID badge to facilitate interactions with exhibit hosts, and research volunteers will attend a volunteer orientation hosted by aquarium staff.

Opportunistic sampling of stranded cetaceans: For live stranded cetaceans, 5 exhales would be collected in a 50 ml polypropylene conical tube (provided by investigators) in the field during the assessment of the animal by veterinary or stranding staff if possible. This tube would be labeled with the species and sex of the animal (if determined), as well as the date of collection. The tube can then be frozen at -80°C as soon as possible upon returning to the aquarium. If the animal subsequently dies, a small (approximately 1 cm square) skin sample would be collected upon necropsy and frozen at -20°C . For dead stranded cetaceans, a small (approx 1 cm square) skin sample would be collected upon necropsy and frozen at -20°C .

*New
Samples
Requested*

PROJECT # 12001

Requested changes relative to previous renewal

Skin and blood sampling

Scientific progress since project approval:

Validation of the measurement of reproductive hormones in blow samples for assigning reproductive status of the beluga:

Richard et al. 2017. Testosterone and progesterone measurements in blow samples are biologically relevant in belugas (*Delphinapterus leucas*). *General and Comparative Endocrinology* 286: 183-193.

Determination of reproductive seasonality in male belugas using ultrasound:

Richard et al. 2017. Seasonal variation in testes size and density detected in belugas (*Delphinapterus leucas*) using ultrasonography. *Journal of Mammalogy* 98(3): 874-884.

Validation of DNA isolation from blow samples for use in molecular research:

Richard et al. 2017. Assessing the quantity and downstream performance of DNA isolated from beluga (*Delphinapterus leucas*) blow samples. *Aquatic Mammals* 43(4): 398-408.

Validation of RNA isolation and amplification of immune function genes from blow samples:

Richard et al. in preparation: Evaluating beluga (*Delphinapterus leucas*) blow samples as a potential diagnostic for immune function gene expression within the respiratory system

PROJECT # 12001

Conditions of Approval:

APPROVAL: Projects must be re-approved annually.

Tracy Romano 10/11/17 _____
Director of Research Date Director of Husbandry Date

Alison Outter, DVM, Dipl. ACZM 10/11/17
Staff Veterinarian Date

Anniversary Date: 10/11/2018

Re-approval Date: _____ Signature: _____

REVIEWER COMMENTS

Director of Research

Date: 10/12/17

Comments:

Tracy Romano

As long as we can ~~fulfill~~ ^{still} accommodate internal similar needs with this sample request.

Staff Veterinarian

Date:

Comments:

Director of Husbandry

Date:

Comments:

Other Comments:

From Kristine Magao 10/1/2017 - Looks fine

PROJECT # 12001
Conditions of Approval:

APPROVAL: Projects must be re-approved annually.

Tracy Romond 10/23/18 _____
Director of Research Date Director of Husbandry Date

Almond Duttler, DVM, Dipl. ACZM 10/23/18
Staff Veterinarian Date

Anniversary Date: 10/23/2019

Re-approval Date: _____ Signature: _____



Dear Gayle,

Thanks so much for your help with project requests for #12001 “Investigating beluga whale reproductive biology using minimally-invasive techniques.” Scientifically, the key findings of the project so far are that blow samples can be used to assess reproductive status in belugas, that single-exhale blow samples can be used for molecular analyses, and that testes size varies seasonally in male belugas. Additionally, manuscripts are in preparation that demonstrate correlations between reproductive physiology and behavior, and that blow samples can be used to evaluate immune function within the respiratory tract. I am also nearing completion on the laboratory analyses needed to evaluate the possibility of aging a beluga by measuring epigenetic changes to DNA, and hope to publish this work in 2019. A full list of publications and conference presentations that have resulted from our collaboration is listed below. This work has also had tremendous value as an educational opportunity for aspiring scientists. Since 2013, a total of 35 undergraduate students have contributed to the project for at least one semester, learning to apply ethological, physiological, or molecular research approaches, and engaging in their own scientific inquiry in the process. Opportunities created by Mystic Aquarium have thus played an important role in the development of many future researchers.

I greatly appreciate all of Mystic’s support and look forward to many more years of fruitful collaboration!

Publications

Richard JT, Robeck T, Osborn S, Naples L, McDermott A, LaForge R, Romano T, and Sartini BL. 2017. Testosterone and progesterone concentrations in blow samples are biologically relevant in belugas (*Delphinapterus leucas*). *General and Comparative Endocrinology* 246: 183-193. DOI: 10.1016/j.ygcen.2016.12.006

Richard JT, Schmitt T, Haulena M, Vezzi N*, Dunn JL, Romano T, and Sartini BL. 2017. Seasonal variation in testes size and density detected in belugas (*Delphinapterus leucas*) via ultrasonography. *Journal of Mammalogy* 98(3): 874-884. DOI:10.1093/jmammal/gyx032 *Undergraduate

Richard JT, Schultz KL*, Goertz C, Hobbs RC, Romano T, and Sartini BL. 2017. Assessing the quantity and downstream performance of DNA isolated from beluga (*Delphinapterus leucas*) blow samples. *Aquatic Mammals* 43(4): 398-408. DOI: 10.1578/AM.43.4.2017.398 *Undergraduate

Manuscripts in Preparation

Richard JT, Desfosses R*, Romano TA, and Sartini BL. For spring, 2019. Minimally invasive physiological correlates of intersexual association in belugas (*Delphinapterus leucas*). *Undergraduate

Richard JT, Schultz KL*, Unal E, Romano TA, and Sartini BL. For spring, 2019. Evaluating beluga (*Delphinapterus leucas*) blow samples as a potential diagnostic for immune function gene expression within the respiratory system. *Undergraduate

Scientific Presentations to Professional Audiences

Richard J, Desfosses R, Romano TA, and Sartini BL. 2017. Minimally invasive physiological correlates of intersexual association in belugas (*Delphinapterus leucas*). *The Society for Marine Mammalogy's 22nd Biennial Conference on the Biology of Marine Mammals, Halifax, Nova Scotia, Canada*. “Speed talk” oral presentation.

Richard J, Robeck T, Osborn SD, McDermott A, Naples LM, Romano TA, and Sartini BL. 2016. Biological validation of progesterone measurements in beluga (*Delphinapterus leucas*) blow samples. *International Association for Aquatic Animal Medicine Conference, Virginia Beach, VA. Formal Oral Presentation.*

Richard J, Romano TR, and Sartini BL. 2015. Testosterone concentrations in blow correlate with testosterone concentrations in blood in belugas (*Delphinapterus leucas*) and are biologically relevant. *International Association for Aquatic Animal Medicine Conference, Chicago, IL. Formal Oral Presentation.*

Richard J, Schultz KL*, and Sartini BL. 2015. Determining the number of exhales necessary for the application of minimally-invasive blow sampling to molecular analyses in wild belugas (*Delphinapterus leucas*). *Alaska Marine Science Symposium, Anchorage, AK. Poster Presentation.* *Undergraduate

Richard J, Romano TR, and Sartini BL. 2013. Minimally invasive molecular gender determination in belugas (*Delphinapterus leucas*) from a single-exhale blow sample. *Proceedings of the 44th Annual Conference of the International Association for Aquatic Animal Medicine, Sausalito, CA. Poster Presentation.*

Richard J and Sartini BL. 2013. Developing whale “snot” as a research tool. *University of Rhode Island Research and Innovation Showcase. Poster Presentation.*

Schultz KL*, Richard J, and Sartini BL. 2014. Molecular analysis of beluga whale (*Delphinapterus leucas*) mtDNA haplotypes from single-exhale blow samples. *Greater Atlantic Region Marine Animal Stranding Network Conference, Groton, CT. Poster Presentation.* *Undergraduate

Submitted Abstracts for The 2nd International Workshop on Beluga Whale Research and Conservation

Richard J. Use Your Melon: Engaging Undergraduates in the Process of Science by Exploring Beluga Facial Expressions.

Richard J, Desfosses R, Romano TA, and Sartini BL. Minimally Invasive Physiological Correlates of Intersexual Behavior in Belugas.

Thanks again!



Justin

Justin Richard, PhD
Department of Fisheries, Animal and Veterinary Science
University of Rhode Island
Kingston, RI



MYSTIC AQUARIUM

Project No. 19001

RESEARCH PROPOSAL and PROTOCOL REVIEW

MYSTIC AQUARIUM
Sea Research Foundation, Inc.
55 Coogan Blvd.
Mystic, CT 06355

Introduction

Mystic Aquarium is committed to research and promotes the use of its collection for the purpose of enhancing knowledge of aquatic animals through scientific investigation.

The following proposal materials are designed to address all pertinent issues regarding the use of live animals for scientific study at Mystic Aquarium. Individuals interested in conducting research must provide this information to satisfy the requirements of the Aquarium's Staff Research Committee and Institutional Animal Care and Use Committee (IACUC). It is the function of these groups to ensure that all protocols comply with the regulations and standards of the United States Department of Agriculture, as well as standards established by Sea Research Foundation. Information should be specific and comply with the format as presented.

Submission and questions regarding the preparation of this proposal should be directed to:

Gayle Sirpenski, Secretary, IACUC
(860) 572-5955 ext. 108
gsirpenski@mysticaquarium.org

I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Laura Thompson, PhD
Research Fellow
Mystic Aquarium, 55 Coogan Blvd, Mystic CT 06355
(860)572-5955 x155
lthompson@mysticaquarium.org

B. Title of proposed research

Importance of Coagulation Pathways in Determining the Impact of Nitrogen Bubbles in Dive and Non-Dive Adapted Mammals

C. Introduction and background

Introduction:

New questions concerning the natural occurrence of nitrogen gas bubbles in marine mammals during diving have arisen from renewed interest in marine mammal dive physiology, driven by reports of gas emboli and tissue damage resembling decompression sickness (DCS) in several species (Jepson *et al.*, 2003; Dennison *et al.*, 2012; Moore *et al.*, 2009). If gas bubbles do occur more commonly than previously thought, it is possible that marine mammals have evolved a mechanism of protection to avoid damage under normal circumstances; damage which is linked to immune and inflammatory activity in humans. However, few studies have considered dive adaptation of the marine mammal immune system and more effort is needed to understand the relationship between diving, immune function and dive related injury and the potential for anthropogenic activities to compromise such adaptations.

Background:

Reports of gas emboli and tissue damage resembling decompression sickness in several species of marine mammals, such as deep diving beaked whales which stranded in close proximity to naval sonar exercises (Jepson *et al.*, 2003), have renewed interest in marine mammal dive physiology and susceptibility to dive related injury.

While a recent study was unsuccessful in identifying bubble formation following repetitive dive bouts in bottlenose dolphins (Houser *et al.*, 2010), bubbles have been reported in bi-caught dolphins and seals (Moore *et al.*, 2009) and modelling efforts have predicted bubble growth following repeated dives, with short surface intervals, to depths shallower than that at which lung collapse occurs (Zimmer and Tyack, 2007). Moreover, tissue nitrogen loads have been estimated to reach 200-300% supersaturation for bottlenose dolphins, sperm whales, beaked whales and blue whales (Houser *et al.*, 2001). If nitrogen gas bubbles occur in diving marine mammals more commonly than previously thought, it is possible that these animals possess specific adaptations, which protect them from injury under normal conditions.

An important determining factor in the development of injury from gas bubbles is involvement of the immune system via activation of inflammatory pathways, particularly through the alternative complement pathway. Previous efforts within our laboratory have demonstrated a decrease in certain immune cell functions in belugas following increased pressure exposures, which differs from humans, but also varies with characteristics of pressure exposure and the presence of additional stressors (ONR Award # N00014-13-1-0768; Thompson and Romano, 2015). Recent work (ONR Award # N00014-15-1-2203) also suggests that the marine mammal complement cascade (an initiator of inflammation) is not activated, as measured by changes in the protein C5a, in response to 30 minute exposures to nitrogen bubbles at a flow rate of 0.5 ml/min; conditions which have been reported to significantly increase C5a in humans (Bergh *et al.*, 1993). This information supports the hypothesis that a less reactive immune system may be one mechanism through which marine mammals may be protected from dive related injury and disease under normal conditions

The mechanism behind why marine mammal complement may be less sensitive to bubbles is unknown. While our current studies indicate there may be little direct activation of complement, C5a is a shared product of all activation pathways and further work is needed to describe activity in the alternative pathway specifically as this is the pathway implicated in DCS development. It is possible there is not a connection between early activation of the alternate pathway and the terminal pathway, which may prevent unwanted inflammatory damage, or that activation of the complement system must occur through indirect effects of bubbles in marine mammals.

Preliminary *in vivo* studies also support the idea that there may be additional influencing factors at play. A single beluga whale trained to perform either stationary (submerged at a target) or active (swimming while submerged) dives displayed increases in C5a following these behaviors, as well as large increases in C5a in response to nitrogen exposures, suggesting that activity level or breath hold itself may play a role in the response of complement during diving. This may be an important consideration as changes in dive behavior have been reported in marine mammals following exposure to anthropogenic stressors, such as noise. While decompression sickness itself may not develop, there may be similar sub-lethal consequences.

Indirect activation of the complement system can also occur through endothelial damage, platelet aggregation (del Conde *et al.*, 2005) or through activation of the coagulation cascade (Barack and Katz, 2005). Platelet aggregation in response to decompression or at the bubble-blood interface has been noted to be of importance in the development of decompression injury in rats (Pontier *et al.*, 2008) and humans (Philp 1974). In addition, epinephrine has been reported to augment platelet aggregation in the presence of gas bubbles (Thorsen *et al.*, 1989). Killer whale (Patterson *et al.*, 1993) and northern elephant seal (Field and Tablin, 2012) platelets have been reported not to display decompression induced platelet aggregation, though the response of platelets to gas bubbles has not been tested. It is possible that marine mammal platelets do not aggregate at the bubble surface under normal circumstances, but under conditions of increased stress, are more susceptible to activation in the presence of bubbles.

Coagulation Factor XII (also termed Hageman Factor) can be activated by the presences of gas bubbles, and subsequently activate the alternative complement pathway (Kitano *et al.*, 2001; Radziwon *et al.*, 2015). Interestingly, cetaceans lack Factor XII as well as other coagulation factors and this has been suggested to play a role in their insensitivity to potential gas bubbles under normal circumstances (Ridgway, 1986). This lack of Factor XII has been reported for dolphins and killer whales (Lewis *et al.*, 1969; Robinson *et al.*, 1969). While longer clotting times have been noted in belugas, the presence or absence of Factor XII has not been measured directly for this species, to the best of our knowledge.

In contrast, some pinniped species are very deep divers, such as the northern elephant seal (*Mirounga angustirostris*) but the presence or lack of Factor XII in these animals is also unconfirmed, and they have similar or even shortened clotting times as compared to terrestrial species and humans (Gulland *et al.*, 1996). Elephant seals have also been noted to be prone to disseminated intravascular coagulation (DIC; Gulland *et al.*, 1996; 1997), a clotting disease that has been related to decompression sickness in humans (Holland, 1969). Yet these animals spend

the majority of their time at sea, making repeated dives to great depths. Thus, the relationship between clotting, inflammation and dive related injury in this species is of particular interest.

Few studies of coagulation or immune function in the context of diving have been carried out for marine mammals, with our laboratory being the first to look at cellular response of marine mammal cells and complement to pressure and nitrogen bubble exposures. Despite the description of hemorrhage patterns similar to those driven by DIC in beaked whales which stranded in the Canary Islands following Naval sonar activities, the role of clotting processes in the development of these injuries was undetermined (Joint Interim Report Bahamas Marine Mammal Stranding Event of 15-16 March 2000, Dec 2001, NOAA, ONR).

More work is needed to understand the mechanisms behind complement activation, or lack thereof, in marine mammals and the role of complement and coagulation during diving in these animals. The goal of this project is to continue research initiated within our lab focused on the dynamic relationship between diving and immune responses in marine mammals, leading to a better understanding of the physiological consequences of human disturbances.

D. Brief summary of proposed research. (Note if the study would be done in house or in the field.)

The purpose of this project is to describe the potential role of direct vs. indirect effects of nitrogen gas bubbles on immune responses in marine mammals in order to better understand the development of dive related injury in these animals and the potential health effects of human disturbance. This project will continue our investigation of the complement response to nitrogen bubbles during diving, as well as investigate the relationship between coagulation pathways and inflammation using both *in vitro* approaches and *in vivo* physiology. We aim to evaluate whether specific adaptation of coagulation pathways (e.g. lack of Factor XII) plays a role in determining inflammatory damage and the sensitivity of marine mammals to decompression sickness injury.

To do this, blood samples will be collected from belugas and northern elephant seals to continue investigating the response of the complement system to *in vitro* exposures to nitrogen bubbles, and evaluate the role of coagulation pathways in regulating this response. For belugas, blood samples will be drawn in conjunction with monthly clinical draws. Blood samples paired with specific dive behaviors will also be drawn either in conjunction with monthly draws or as separate sampling session as deemed appropriate by Mystic Aquarium veterinarians. Blood samples will also be collected from belugas during field efforts in either Point Lay, or Bristol Bay AK as feasible. Additional samples from aquarium maintained belugas will be sought from other institutions. For elephant seals, animals that have been admitted to The Marine Mammal Center, Sausalito, CA for rehabilitation will be targeted. Blood samples will be obtained from successfully rehabilitated individuals prior to release as part of routine health sampling. Human blood samples will be purchased from Biological Specialty Corporation for direct comparison with marine mammals in order to provide context of dive related injury and disease, and ensure that results are not an artifact of experimental design but reflect true differences in physiology between dive-adapted and non-diving mammals. All experimental measures will be carried out at the Mystic Aquarium laboratories at UConn Avery Point.

E. Specific goals of the proposed research.

1) Investigate activity in the alternative complement pathway in marine mammals and humans in response to *in vitro* nitrogen gas bubble exposures

A prior ONR effort (Award # N00014-15-1-2203) measuring changes in C5a following N₂ bubble exposures suggests no activation of the terminal shared complement pathway. Though Bergh *et al.*, (1993) showed similar nitrogen exposures results in significant increases in C5a in humans; no directly comparable data is available. Because the alternative pathway is associated with development of decompression sickness in humans, specific proteins involved with this pathway will be used to determine activity in response to N₂ bubbles using commercially available reagents. Function of this pathway may also be determined through hemolytic assays. It is hypothesized that no significant activation signal of the alternate complement pathway will be detected following N₂ bubble exposures in belugas and elephant seals. Activation of the alternative pathway following stationary and active dive behaviors will also be assessed with and without *in vitro* nitrogen exposures. In order to be confident that results represent true physiology, the same experimental setup will be run using human samples purchased from Biological Specialty Corp. for comparison.

2) Determine whether marine mammal platelets aggregate in response to *in vitro* nitrogen gas bubble exposures.

Human platelets have been reported to aggregate at the bubble surface (Pontier *et al.*, 2008; Thorsen *et al.*, 1989). In contrast, we expect that beluga platelets will not show the same degree of platelet aggregation in response to nitrogen bubbles. Elephant seals however, which may be prone to disseminated intravascular coagulation (DIC) conditions particularly in conjunction with parasitic infection (Gulland *et al.*, 1997) may show high levels of platelet aggregation. In order to determine platelet aggregation, platelet rich plasma (PRP) will be produced from beluga and elephant seal blood samples and exposed to N₂ gas bubbles *in vitro*. Platelet aggregation at the bubble interface may be assessed either through a reduction in platelet count (Softeland *et al.*, 1994) obtained through use of a HM2 hematology analyzer, or using a microplate reader (Fratantoni *et al.*, 1990; Bednar *et al.*, 1995; Chan and Warner, 2012).

3) Characterize coagulation factors in beluga and elephant seals; in particular confirm presence or absence of Factor XII and describe species differences.

SDS Page and Western Blots will be used to identify coagulation factors in beluga and elephant seal samples. Identification of proteins will be confirmed through protein sequencing. In addition, function of coagulation pathways will be evaluated using an iSTAT analyzer to measure activated clotting time (ACT), prothrombin consumption (PT) and activated partial thromboplastin generation time (aPPT) in belugas and elephant seals throughout the entire study period. We expect belugas will lack Factor XII and Fletcher factor, similar to other cetacean species. While the presence of coagulation factors has not been measured directly in pinnipeds, to the best of our knowledge, we expect these factors will be present in elephant seals due to their clotting ability (Gulland *et al.*, 1996; 1997). Results of characterization of coagulation factors in marine mammals will be compared to published accounts of the human coagulation cascade. Differences in the coagulation cascade (e.g. the occurrence of coagulation factors, coagulation activity) are expected between beluga, elephant seals and humans.

4) Investigate the connection between coagulation and complement activation in marine mammals vs. humans and the role this may play in dive related disease.

In vitro manipulation of coagulation cascades are expected to impact complement activation. If Factor XII is absent in belugas as expected (to be confirmed in Aim 3), it will then be introduced to beluga samples *in vitro* in order to trigger activation of the intrinsic coagulation pathway. For humans, activity of Factor XII will be inhibited *in vitro*. Elephant seal samples will also be exposed to Factor XII inhibition, if they are found to retain this factor (Aim3). The effects of artificial activation or inhibition will be assessed by measuring complement activity (C5a, Factor B and hemolytic assays), platelet aggregation and coagulation function (e.g. ACT, aPPT) as described for aims 1, 2 and 3 in belugas, elephant seals and humans.

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

Belugas have been the focus of previous research efforts within our laboratory investigating the relationship between dive physiology and health in marine mammals. Belugas are good models for dive physiology research as they are capable of deep dives to over 1000m and can hold their breath for up to 25 minutes. Given that belugas are professionally maintained at Mystic Aquarium, this is a unique opportunity to obtain samples under behavioral control. Our laboratory also routinely participates in field based live capture-release health assessments of two beluga stocks in Alaska.

Elephant seals regularly dive to over 1500m and can stay submerged for up to an hour. They are commonly used as model pinniped for dive studies and so are ideal for our proposed work. Each year 80-100 elephant seals strand along the coast of California and are admitted to The Marine Mammal Center, Sausalito, CA for rehabilitation, providing access to sampling in conjunction with routine health assessments.

Fresh blood samples from belugas and elephant seals will be necessary for this study in order to carry out the proposed assessment of coagulation and complement activation in marine mammals, as there is no commercial source for the samples we are targeting. The use of training for belugas is integral for exploring the effects of physiological responses to breath-hold and activity during a dive, which cannot be mimicked in a non-animal model.

B. Justify the choice of species to be used.

This project is a continuation of previous work (Mystic Aquarium IACUC protocol #15004) which began looking at the complement response of belugas and harbor seals to nitrogen bubble exposures *in vitro*. Belugas are very capable divers, capable of diving to over 1000m and have been reported to use a wide variety of dive behaviors and profiles. Access to blood samples

utilizing animals resident at Mystic Aquarium provide a unique opportunity to study this species and validation of several methods proposed in this study have already begun for belugas.

As a pinniped comparison, northern elephant seals have been a model for studies on marine mammal dive physiology as they regularly dive to over 1500m for as long as an hour. Elephant seals regularly strand and are admitted to the Marine Mammal Center in Sausalito, CA for rehabilitation providing access to blood samples in conjunction with clinical blood draws as The Marine Mammal Center routinely draws blood from animals before release.

Human blood samples will be purchased from Biological Specialty Corporation for use as a positive control for this work, as they retain Factor XII and are known to be sensitive to DCS. Furthermore, there is literature connecting specific changes in complement proteins or coagulation activity to the occurrence of DCS for humans. Thus, comparing results from humans to belugas and elephant seals will provide context for data interpretation in relation to DCS risk.

C. Identify and justify the number of animals to be used.

Three belugas are projected to be resident at Mystic Aquarium during the proposed project period of June 2019-May 2022 and will be targeted for blood sampling in conjunction with routine clinical health assessments occurring once per month for the duration of this study. Additional blood samples from whales resident at Mystic Aquarium will be targeted before (pre) and after (post) specifically trained stationary and active dive behaviors in order to investigate the impact of breath hold and activity level on complement and coagulation. One animal is already trained in dive behaviors, and would require only training for a longer duration. Beluga blood samples will be drawn by veterinary staff from the superficial fluke vein. Power analyses for a two-sided test, assuming a 5% significance level and 90% power suggests a minimal sample size of four. Increasing this number to nine decreases noise, meaning there is increased likelihood that a true difference between treatments would be detectable. Thus additional samples from belugas at other institutions are being sought in order to increase confidence in the analysis and interpretation of data for a total of up to 10 aquarium belugas for this study.

Blood samples from wild belugas will be obtained during established collaborative health assessment efforts (Permit numbers pending). Up to 10 individuals will be targeted per season (Total of 20 individuals over 2 seasons), and samples will be drawn from the superficial fluke veins. Samples will be drawn immediately upon restraint of an animal for assessment (pre), and again following full assessment and/or tagging (post) as possible.

Up to 15 elephant seals per season (total of 45 over three years) will be targeted for sampling. This number of animals will provide 90% power and a signal/noise ratio near one, at a significance level of 5%. This will increase the likelihood that a significant difference between treatments, even if small, will be detected. This may also provide enough individuals to compare values between males and females, depending on the cohort that strands each year. Elephant seals will be sampled immediately before release from rehabilitation at The Marine Mammal Center,

as healthy animals are the target for initial studies. These samples will be drawn from the epidural vertebral sinus, and will coincide with a final health assessment clinical draws.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

Blood Draws

Beluga blood samples will be drawn by veterinary staff from the superficial fluke vessel. Sampling for aquarium whales will occur under behavioral participation and positive reinforcement during husbandry sessions. This sampling will occur once per month per animal, in conjunction with clinical blood draws for the duration of the project. Additional blood draws will occur in conjunction with trained dive behaviors following approval by veterinary staff. For dive behaviors, blood is requested before (pre) and after (post) each dive. No more than a single dive behavior will occur per day, meaning no more than two sticks will occur per animal per day. One dive behavior will be targeted per month, meaning no more than three blood draws will occur per animal per month. In the case that extra sticks are not feasible, dive behaviors will be coordinated with monthly clinical draws.

Elephant seal samples will be obtained by trained veterinary staff in conjunction with pre-release clinical assessment in healthy animals. Blood will be drawn from the epidural vertebral sinus. Initial assessments may occur onsite at The Marine Mammal Center, while experimental blood samples will be shipped to Mystic Aquarium for full analysis. Comparisons between fresh and shipped blood samples will be carried out in order to rule out the impacts of shipping.

Up to, but no more than 50ml of blood will be drawn per sampling stick per animal for this study. This will include samples drawn into multiple tube types for hormone analysis (NaHep), complement activity (SST), platelet function (NaCitrate) and hematology (EDTA).

Dive Behaviors

Two dive behaviors will be trained in belugas residing at Mystic Aquarium for this research: 1) stationary dive and 2) active dive. For the stationary dive, individuals will be asked to submerge to a target and remain stationary (i.e. not swimming) for a duration of 3 or 5 minutes. For the active dive, individuals will be asked to submerge and swim between targets set throughout the exhibit pools for a duration of 3 or 5 minutes. Blood samples will be obtained from animals prior to and immediately following each dive behavior. Each dive behavior (active and stationary) for each duration (3 and 5 minutes) will be performed twice, for a total of eight dive sessions per whale per year.

B. Will a test substance be administered? No If yes, 1.) What is the name of the compound?

2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes. N/A

C. Will manual, chemical and/or mechanical restraint of the animal be necessary? If yes, explain the method of restraint. No

D. Indicate the level of pain the proposed procedure is expected to cause.

a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.

b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.

c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery or procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

E. Describe the method of euthanasia to be used if this were to become necessary N/A

F. Does the proposed research involve surgery? No
If yes, go to Addendum II. Surgical Protocol

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

The belugas at Mystic Aquarium are housed in the outdoor Arctic Coast exhibit and would remain there for this study. Training of stationary and active dive behaviors are required. These behaviors will require new training for 1-2 whales to perform both dives types for each duration, and continued training for one whale to reach the duration of 5 minutes.

Stranded elephant seals are housed in outdoor rehabilitation pools at The Marine Mammal Center, Sausalito CA that provide access to pools and dry haul outs, as well as daily observation and access for staff. No special care or behavioral conditioning is required.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours.

There are veterinarians on site at Mystic Aquarium 7 days per week and on 24-hour call for 7 days per week. Veterinarians are also on site at The Marine Mammal Center 7 days per week, with 24 hours on-call.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics?

N/A

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include one.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

Laura Thompson, PhD: Dr. Thompson brings experience in marine mammal immunology with particular focus in relation to the connection between diving physiology and marine mammal health. This project is a continuation of her previous work investigating the response of the immune system to nitrogen gas bubbles. She will be the PI on this project.

Laura Thompson holds a first class degree BSc with honors from Queen Mary College, University of London. For her undergraduate thesis, she focused on phenotyping different subsets of lymphocyte in two contrasting populations of wild bottlenose dolphins along the east coast of the US. She obtained her PhD in Oceanography from the Department of Marine Sciences at the University of Connecticut in May 2014. Her dissertation work focused on investigating the response of marine mammal immune cells to increased pressure (i.e. simulated dives) and the potential for an additional stressor to alter that response. Laura has experience with a variety of laboratory techniques, including sterile technique, PCR and cell culture, as well as specific training and experience with immune function assays and protocol in endocrinology. For the past 4 years, she has been working towards evaluating the complement (inflammatory) response of marine mammals to nitrogen bubbles *in vitro*, and has begun preliminary investigation of the effects of activity during diving.

Tracy Romano, PhD: Expertise in marine mammal neuroimmunology and impacts of environmental and anthropogenic stressors on aquatic animal health. She will be Co-PI on this project with prime responsibility overseeing progress and mentoring Dr. Laura Thompson.

Tracy Romano graduated with a Bachelor's of Science degree from Saint Michael's College in Winooski VT. In 1986, she was awarded a fellowship from the American Society for Engineering Education to pursue her graduate studies in neuroimmunology and in 1993 received a PhD in neurobiology and anatomy from the University of Rochester in Rochester, NY. She was awarded a National Research Council fellowship to carry out her postdoctoral studies with the U.S. Navy Marine Mammal Program in collaboration with the Scripps Research Institute in San Diego, CA. The focus of her research was characterization of the marine mammal immune system with subsequent focus on the impact of environmental stressors on the health of bottlenose dolphins. She subsequently established a research laboratory at the Navy facility dedicated to marine mammal neuroimmunology. Some of her accomplishments include the cloning and characterization of dolphin CD4, investigation of sound as a stressor on cetacean health, contributions to a study mandated by Congress to investigate the impact of the tuna fishery on dolphin health, and the establishment of an anatomical link between the cetacean nervous and immune systems. In May 2004, Tracy moved her research program to Mystic Aquarium, a division of Sea Research Foundation, Inc. to lead the Aquarium's research department as the vice president of research. In 2008, she was a finalist for the Women of Innovation Awards by the Connecticut Technology Council and in 2012 was inducted in the Academic Hall of Fame for her Alma mater. Currently, her primary research focuses on studying the health of beluga whales in the wild and in the aquarium setting. She regularly travels to Point Lay, Alaska, to participate in live capture release studies and is responsible for collecting and analyzing blood samples for health assessment given future impacts of climate change, pollutants and oil and gas exploration.

Cara Field, PhD, DVM: Expertise in marine mammal medicine, platelet function and coagulation in elephant seals. Dr. Field will serve as Co-PI on this project, with prime responsibilities being provision of elephant seal samples, guidance on coagulation studies and interpretation of results and contributions to scientific publications.

Dr. Cara Field graduated with a Bachelor of Science degree in physiology from the University of California, Davis in 1993. She continued on to complete a dual DVM/PhD program at UC Davis in 2005. Her dissertation research entailed characterizing northern elephant seal blood platelets and their response to changes in temperature and pressure, as may occur over periods of diving. Dr. Field was a post-doctoral research fellow at Mystic in 2006-2007, and in 2008-2009 where she worked on developing and running diagnostic techniques for marine origin *Brucella* and conducting original research around *Brucella*. She completed a veterinary internship at Mystic Aquarium in 2007-2008 and worked as a veterinarian for the Audubon Nature Institute in New Orleans and Georgia Aquarium before joining The Marine Mammal Center in Sausalito, CA in 2014.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

Jennifer Flower, DVM, MS, ACZM Diplomate: Chief Veterinarian who will oversee and advise on all blood sampling proposed for this study. Dr. Flower has 8 year's experience in marine mammal veterinary medicine and is a diplomate of the American College of Zoological Medicine with an aquatic animal health focus.

Barbara Mangold, DVM: Barbara Mangold obtained her DVM from Virginia-Maryland Regional College of Veterinary Medicine. She has trained in both small animal medicine and surgery, and completed her residency in zoo medicine at the Wildlife Conservation Society/Bronx Zoo. She has worked at the Bronx Zoo, Disney's Animal Kingdom and consults for the Maritime Aquarium in Norwalk, CT. Dr. Mangold will participate in blood draws during this study.

Veterinary Intern, DVM: Mystic Aquarium offers an internship in aquatic animal medicine to graduates of AVMA accredited veterinary colleges. Interns will be involved with blood draws for this study.

Carey Richard: Assistant Curator of Pinnipeds at Mystic Aquarium will oversee all training progress for proposed dive behaviors. Carey has a B.S. in marine biology and a minor in psychology. She has extensive experience with marine mammal training, including 5 years working with belugas at Mystic Aquarium.

Lindsey Nelson: Senior Trainer of Cetaceans and Pinnipeds will be involved with progressing the training of proposed dive behaviors. Lindsey has a B.S. in marine biology and has been working with belugas at Mystic for 11 years. She has been integral to preliminary studies utilizing these trained behaviors in a single whale.

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

Check if N/A X

- 1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).**

- 2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?**

- 3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.**

- 4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.**

- 5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.**

ADDENDUM II. SURGICAL PROTOCOL

Check if N/A

1.) Have alternatives to the surgical procedure been considered and found to be inappropriate? Explain.

2.) Describe with reasonable detail the surgical procedures to be used and where it is to be performed. Include the name of the person who will be performing the surgery and that persons level of expertise in this area. (Note: All survival surgery must be performed using aseptic technique.)

3.) Will the surgery be major or minor?

4.) Will multiple survival surgeries be performed OR is there more than one major operative procedure from which the animals are allowed to recover? (Note: More than one major operative procedure is NOT allowed without prior approval from the IACUC.)

5.) What method of anesthesia will be used; who will supervise the administration of anesthesia and how will the anesthetic depth be monitored?

6.) Describe the pre- and postoperative care that will be provided. Include names of anesthetics, analgesics, antibiotics, dose and route of administration and duration.

STATEMENT OF ASSURANCE

Based upon available information, the alternatives to the use of live animals for the purpose of this study have been considered and found to be inappropriate. It is the responsibility of the Principal Investigator to assure that the protocols outlined here for the humane care and treatment of the animal are followed. The Mystic Aquarium reserves the right to discontinue the study if the health of the animal or the safety of the staff is jeopardized. Any necessary changes in the study design will be approved by the Institutional Animal Care and Use Committee (IACUC) prior to implementation. The Principal Investigator has reviewed the qualifications of all personnel involved in the study and found them to be adequate.

To the best of my knowledge, the animal research described in this protocol review does not unnecessarily duplicate previous experiments.

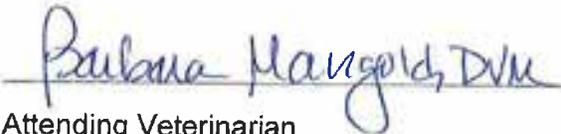


Principal Investigator

1/25/2019

Date

The Attending Veterinarian has reviewed the proposed protocols for animal use and has found them to be acceptable.



Attending Veterinarian

1/29/2019

Date

=====
IACUC Comments or Conditions of Approval:

See attached comments



Signature of IACUC Chair

01/29/2019

Date

Approval Period: 1/29/2019 - 1/29/2020 Protocol # 19001
(Must be reviewed annually)

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Comments from IACUC Committee

1/25/2019 From Larry Dunn: Wouldn't it be useful to include trials with a known DCS sensitive and factor XII positive species as presumed positive controls? Also check your grammar in para 1 as there is a place where subject and verb are not in agreement.

Tracy's response to Larry (TR): Yes. We are proposing to include human blood samples for this purpose as 1) they are Factor XII positive and 2) they are sensitive to DCS and 3) there is literature connecting specific changes in complement proteins or coagulation activity to the development of DCS for humans. Blood samples will be purchased from a commercial source as described in section D; we have added a statement also to section IIB to make this more apparent.

1/25/2019 From Pat Bubucis: Looks good to me. Should be interesting. My diving physiology learnings are ancient and understanding of coagulation cascades is minimal so following question may be silly. Should post-dive changes in peripheral blood be comparable to those in internal circulation? Looks like beluga samples will be peripheral while elephant seal samples might not be. Location of sampling is clearly specified only for belugas in section II C paragraph 3, and specified for both belugas and seals in III A.

TR: The location of elephant seal draws has been added to section II C paragraph 3. The location of blood draw does differ between belugas and elephant seals for this project, however we do not expect the location of sampling to impact our *in vitro* experiments or data analyses for this project. For elephant seals all experimental conditions will be carried out *in vitro*, and will be compared with baseline beluga samples obtained during monthly health checks, without specific dive activity. Post-dive changes, following specific dive behaviors, are only being investigated in belugas and these will only be compared with baseline beluga results.

1/26/2019 From Gerry Sonnefeld: The proposed protocol entitled "Importance of Coagulation Pathways in Determining the Impact of Nitrogen Bubbles in Dive and Non-Dive-Adapted Mammals" contains good justification for the use of animals. Pain and discomfort are minimized. The proposed project addresses important scientific and health issues. On this basis, I support approval of the proposed protocol, but recommend that the following issues be addressed:

1) the number of beluga whale subjects is small (3). The possible benefits to animals would be enhanced if power analyses of the beluga study, as well as the elephant seal study, were provided to show that the number of animals proposed for use would be likely to provide statistically significant differences if differences occur. This would also demonstrate the proposed statistical analyses of the data to enhance the possibility of success in the use of animals.

TR: We acknowledge that a sample size of 3 is small. We are seeking to increase this number by asking for additional blood samples from belugas housed at other institutions. A power analysis for a two sided test, assuming 5% significance level and 90% power to reduce the chance of not detecting a response suggests that n=4 is the smallest samples which should be used, however there would be a lot of noise and the difference between treatments would need to be large in

order to be detected. By increasing the sample size to 9, this noise is reduced by half. Thus we are aiming for 10 individual belugas. The goal of 15 elephant seals per season also reduces the noise, increasing the likelihood that an actual difference will be detected. A brief description has also been added to justification section IIC.

2) The proposed protocol includes the participation of the attending veterinarian in the proposed project. If this is the case, should veterinary approval and oversight for the project fall on an alternate veterinarian?

TR: Participation of the attending veterinarian, and veterinary staff, is required to ensure safety of animals and success of sampling during blood draws. The close routine monitoring of animals by the attending veterinarian will be relied upon for approval of extra sampling surrounding dive behaviors, as they are familiar with the history of the individual animals involved and any potential risk of multiple samplings.

1/26/2019 From Toni O’Connell: Would the nitrogen content in the water for the Mystic Aquarium belugas influence blood sample results compared to belugas sampled in Alaska? If differences in the nitrogen content between the two groups environment could influence the blood sample results, would that information need to be included in the study protocol? If “No” is the answer, then I approve this.

TR: **No.** Water nitrogen content is not an influencing factor here. All nitrogen exposures will be done *in vitro* to simulate the formation of gas bubbles in blood. For a diving animal, the source of this nitrogen would be inspired air. In addition, the response we are interested in is the physical response to a bubble surface. The effect of the composition of blood gas bubbles is not a focus of this study.

1/27/2019 From Fatima Matos: Interesting study. A couple of suggestions to clarify: Number of animals in Section IIC: number of belugas residents at MA (currently 2, not 3. So, perhaps just say ‘a total of 10 belugas from MA and other aquarium institutions’)?

TR: The proposed project period for this work is June 2019 through May 2022, during which time there will be 3 belugas resident at Mystic Aquarium. This project aims to utilize access to each of these animals. The wording of Section IIC has been changed to reflect this.

Frequency of blood sampling in Section IIIA: It seems that the frequency of blood sampling is greater in belugas (including 2 samples from wild belugas, pre/post?) while there will be only one pre-release blood sampling from elephant seals (is this because of the method of blood sampling in these animals?)

TR: Similar to belugas, blood collection for elephant seals will occur in conjunction with routine clinical health checks which The Marine Mammal Center carries out at the time of animal admit to rehabilitation and again at release. The frequency of these routine draws limits the frequency of sampling for our work. Additional blood draws may be feasible if The Marine Mammal Center deems them necessary for clinical purposes, however, since we are aiming for healthy

animals these samples may not be appropriate for our studies, as inflammation and coagulation respond to injury and disease or may be affected by medication.

1/28/2019 From Allison Tuttle:

Please note the caveat that we will need to run this new project for approval by the organizations that own the belugas not owned by Mystic. For Juno, this is SW, and for 3rd whale Natasha (coming soon, please keep confidential internally for now) this is WCS. I will reach out to these organizations for the approvals once we have an approved IACUC for this project internally.

TR: So yes, as far as my understanding we were going to ask for approval for use of Juno and Natasha once approved.

One clarification for Tracy- please define, between this IACUC and the other Romano/Thompson/Unal/Driscoll IACUC approved projects, what the total expectation/request for # of whale blood draws is on a monthly basis? I.e. does Dr. Thompson's request of up to 50 ml 3X per month wrap the other projects into this or are there going to be additional blood draw event requests on top of this? I am less concerned with volume requested than number of needle punctures for blood draws. I am, in general, supportive of this project (good science and well justified) but I would like to be sure I understand the total number of blood draw events per month being requested for the whales for all research projects together prior to giving my official approval. (For those new to the committee, it is important to oversee the number of blood draws for the whales as we have seen phlebitis with frequent needle punctures for blood draws in the past.)

TR: The highest number of sticks requested per animal per month is 3 (one per month (with other research sampling, plus pre and post dive). The 3 per month are not for the entirety of the project however, and would only be 8 months of the project per animal, beginning in or after July 2020. So, it would be 1 blood draw per month for the entire study period, plus 2 sticks per month for 8 months out the study period. The breakdown of the number of dive behaviors sampled is discussed in section IIIA.

In the event that 2 additional sticks are not feasible, we would like to propose combining dive behaviors with clinical blood draws. This would reduce sticks during those 8 months to twice/month (pre and post dive behavior).

1/29/2019 From Dr. Jen Flower: Thank you to Tracy and Laura for their responses and comments, this project is certainly very interesting and a worthwhile endeavor. My one remaining question would be, if the health of the beluga's flukes would only allow for the typical once monthly blood sampling – how would this affect the results of this project? I.e. would they still be able to achieve the desired information/data from utilizing once monthly blood sampling on Mystic beluga? The addendum only addresses circumstances that would reduce 3 monthly blood sticks to 2, but not the possibility of reducing the 3 requested monthly blood sticks to 1 during the 8 months of study period, which is also a likely possibility based on the whale's intermittent phlebitis challenges.

Thank you for any clarification that can be provided,
Jen Flower, DVM, MS, Dipl. ACZM
Chief Clinical Veterinarian

1/29/2019 From Laura Thompson In the event that we could only do one stick per month, I would propose the approach we have previously done for Kela which is having the whale perform a dive behavior and then taking the monthly clinical draw after the behavior. The results from that would count as the “post dive” and would then need to be compared with monthly baselines that were collected without the dive behaviors, i.e. during an earlier part of the study for example.

However because so much can vary with immune responses, it is ideal to have a pre and post sample the same day. Additionally, we could spread out the dive behavior collection over a longer period so that individual whales would not have multiple sticks per month in 8 consecutive months. For example, we could target dive behaviors with multiple sticks every other month over a 15 month period. This would be from July 2020 through Sept 2021 to fit within the timeline of the project funding.

Laura



**MYSTIC
AQUARIUM**

Project No. 11002

RESEARCH PROPOSAL and PROTOCOL REVIEW

MYSTIC AQUARIUM
Sea Research Foundation, Inc.
55 Coogan Blvd.
Mystic, CT 06355

Introduction

Mystic Aquarium is committed to research and promotes the use of its collection for the purpose of enhancing knowledge of aquatic animals through scientific investigation.

The following proposal materials are designed to address all pertinent issues regarding the use of live animals for scientific study at Mystic Aquarium. Individuals interested in conducting research must provide this information to satisfy the requirements of the Aquarium's Staff Research Committee and Institutional Animal Care and Use Committee (IACUC). It is the function of these groups to ensure that all protocols comply with the regulations and standards of the United States Department of Agriculture, as well as standards established by Sea Research Foundation. Information should be specific and comply with the format as presented. Questions regarding the preparation of this proposal should be directed to:

Gayle Sirpenski, Secretary, IACUC
(860) 572-5955 ext. 108
(860) 572-5972 FAX
gsirpenski@mysticaquarium.org

I. GENERAL INFORMATION

A. Name, title, address, phone, e-mail of the Principal Investigator.

Greg Marshall
Principal, Marshall Innovation
2309 Cheshire Ln
Alexandria, VA 22307
571-251-6668; marshallinnovation1@gmail.com

B. Title of proposed research

Beluga Animal Borne Imaging tests

C. Introduction and background

Crittercam is an animal-borne imaging and data collection system, deployed on wild animals to collect visual data in situations where other observation techniques are not possible or would likely affect the animals being observed. There have been more than 700 deployments of Crittercams on more than 80 marine and terrestrial species. Crittercam has been able to document never before seen behaviors or provide new insights into such areas of investigation as foraging, inter- and intra-specific interactions, habitat use and activity budgets. Crittercams (aka Animal Borne Imaging Systems – “ABIs”) provide critical visual insights into the unseen behavior and ecology of cryptic species.

Marshall Innovation is currently in discussion with Dr. Tracy Romano and collaborators regarding deployments of Animal Borne Imaging systems (ABIs) on beluga whales in Cook Inlet, Alaska. Whenever possible, it is valuable to conduct test deployments with captive animals before working with a relatively new species. This provides the opportunity to confirm the equipment and technique will be able to achieve the research goals before investing significant funds and time in wild deployments. While Marshall has great experience and has refined the instruments through many wild deployments, new environments and animals can sometimes pose surprising challenges.

The presence of captive and trained belugas at the Mystic Aquarium provides a great opportunity to conduct preliminary tests before attempting wild deployments.

D. Brief summary of proposed research.

Suction cups have been used very successfully to attach ABIs to a variety of cetaceans. Different types of suction cups have been used, each with its advantages and drawbacks. Also, because ABI is a visual tool, the angle of view provided by the attachment is important to achieving the research goals.

We would like to take advantage of the controlled conditions and trained beluga whales at Mystic Aquarium to do a variety of fairly short test deployments of a selection of suction cups/mounts and in several different locations on the whales' bodies to determine the best attachment method and location. We will look at the attachment stability, the field of view and also observe the animal's reactions to the different cups and placements.

We propose to conduct 5-7 ABI deployments on test animal(s). Most would be fairly short, 30min or less, possibly with a longer deployment up to 2hrs.

Suction cup mounts have been used successfully in the past for ABI deployments on cetaceans, including belugas. Those cups were:

- Smaller – flattened diameter 16cm – has a wider lip and can more easily adapt to a curved or contoured surface.
- Larger – 23cm diameter – provides a more stable attachment.

Both were soft silicone rubber. We would now like to expand testing to include still smaller suction cup mounts which we feel are appropriate for the smaller Animal Borne Imaging systems we anticipate using in future wild animal field deployments.

As with previous test deployments, a narrow, high-density foam 'cradle' sits atop a central stem on the cup. The ABI rests along this cradle, held in place by plastic tie wraps whose edges are hidden (either in the foam or under tape).

In air, the ABI plus suction cup assemblies weighs approximately 0.5kg, though it is slightly buoyant in water.

The typical deployment position for ABIs on cetaceans is on the dorsal surface posterior to the blowhole. The exact placement varies a bit due to field deployment conditions or the skin surface of a particular animal. It is possible that a lateral mounting could provide a more informative field of view, so we would also attempt to try mounts on test animal sides, above and probably slightly posterior to the pectoral fin.

We would like to test up to three cup mounts, one at a time, first in the dorsal position, then in the lateral placement. In each case we would be looking for any reaction from the animal and mount stability. Observed behavior will be recorded and reviewed with ABI video to evaluate the field of view from each mount and position.

We have had good success with suction cup mounts in the past, with some ABIs remaining attached to wild animals for more than 24hrs, through breaches and aggressive interactions between large cetaceans. If the mounts do fall off prematurely in these tests, they will float and can easily be retrieved with the kind of long-handled dip net routinely used at the facility. In only one case in past tests where an animal played with a prematurely detached Crittercam. This occurred with a killer whale. The whale picked up a Crittercam in its mouth and played 'keep-away' with it for several minutes before allowing the trainer to have it. The Crittercam was not damaged nor the whale injured.

This work can likely be completed within a matter of days.

E. Specific goals of the proposed research.

To determine the optimal ABI mounting method and placement on a beluga whale for future study of wild beluga behavior.

II. JUSTIFICATION FOR ANIMAL USE

A. Justify the use of animals and choice of methodology (explain why non-animal models are not appropriate.)

In preparation for instrument deployment on wild animals, there are always quirks of animal behavior that can affect the success of an instrumentation project and cannot be simulated in lab testing. Conducting captive tests before working with wild individuals can greatly increase the probability of success in field research.

The visual data that ABIs provide can reveal behaviors and details that no other instrument can. ABIs have been deployed using a suction cup mount on eleven cetacean species, from blue whales to bottlenose dolphins, with no evidence of lasting

effect at the attachment site or significant (or in most cases, observable) alteration of behavior.

B. Justify the choice of species to be used.

The wild research to be conducted is with beluga whales, so it is most effective to test with belugas. Though there is a significant history of Crittercam deployments on other cetacean species, it is possible that some aspect of belugas physiology or 'personality' would not be adequately analogous to any of these previous species.

C. Identify and justify the number of animals to be used.

A single animal should be sufficient to conduct the desired tests, though two or more would be better to ensure possible individual variances are accounted for. Trainers will select which individual(s) to use and whether it might be preferable to spread the activities among multiple animals.

III. DESCRIPTION OF ANIMAL CARE AND USE PROCEDURES

A. Describe in sequence, the proposed use of the animals.

Suction cup mounts for ABI will be evaluated on test animals.

Animals will be positioned by the trainer within arm's reach and with its dorsal surface above the water. The first mount will be gently pressed into place on the dorsal surface, posterior to the blowhole. If the animal is cooperative, this should take no more than 20sec. The animal will then be observed while being allowed to move freely about its habitat. If the animal shows no distress and the mount appears to be stable, the trainer will engage the animal in more energetic activities, such as fast swimming and rolling (assuming it had not done so on its own). The overall duration of the attachment should be no more than 30min. The team will observe animals for evidence of odd behavior and mount stability.

Animals will be recalled to the platform and the mount removed. Animals will be requested to present lateral surfaces and the mount will be set in place roughly above and slightly posterior to the pectoral fin. Animals will again be released to swim about the habitat. After approximately 30 minutes the instrument will be removed.

This process will be repeated with other variants of the suction cup mount. An additional deployment may be conducted (with the most promising mount/position) lasting up to 2hrs.

Suction cup mounts normally have an automatic release incorporated for wild deployments, but for the purposes of these tests, animals will be recalled by the trainer and the unit removed by hand. If during any test, the mount looks unstable or an animal appears in any way bothered by the instrument, the trainer can recall the animal and the instrument removed.

Samples of the single cup mounts ~~may~~ will be sent ahead of time for de-sensitization procedures.

- B. Will a test substance be administered? NO** If yes,
 1.) What is the name of the compound?
 2.) Indicate all routes of administration and describe the dosage regimen. Include frequency, amounts of volumes.

- C. Will manual, chemical and/or mechanical restraint of the animal be necessary? If yes, explain the method of restraint. NO**

- D. Indicate the level of pain the proposed procedure is expected to cause.**

a.) No pain or distress will occur OR the proposed procedures are expected to cause no more than momentary or slight pain or distress.

b.) The procedure is expected to cause more than momentary or slight pain or distress, but the pain or distress will be alleviated or minimized by the use of appropriate anesthetics, analgesics and/or tranquilizers.

c.) The procedure is expected to cause more than momentary or slight pain or distress that will not be relieved due to scientific necessity OR cannot be relieved because no appropriate drugs are available to alleviate the pain or distress.

Note: ANY surgery or procedure which requires anesthesia is considered by the USDA to be painful, with pain alleviated by anesthesia.

If b. or c. are checked, go to Addendum I. Considerations of Painful Procedures.

- E. Describe the method of euthanasia to be used if this were to become necessary
 NA**

- F. Does the proposed research involve surgery? NO**
If yes, go to Addendum II. Surgical Protocol

G. Where are the study animals to be housed and are conditions appropriate for the species involved. Describe any special animal care requirements of behavioral conditioning that will be provided or needed.

Tests will be conducted in the facilities housing the belugas at Mystic Aquarium. Mystic staff will determine the optimal location within those facilities.

Samples of the suction cups to be used as ABI mounts may be sent to Mystic ahead of time to allow trainers to desensitize animals and gauge initial reaction to attachment.

H. Describe the emergency medical care available and if there is a veterinarian on call 24 hours. Ref.: Dr. Rogers.

I. Has the attending veterinarian been involved in the choice of animal use procedure and the choice of anesthetics, analgesics and antibiotics? NO

IV. QUALIFICATION OF PERSONNEL

A. Describe the qualifications of the principal investigator. Include 1.) formal education, 2.) relevant general experience in the performance of procedures in the species studied, 3.) specific training and experience in the performance of non-routine, invasive, and surgical procedures in the species studied.

M.S. Marine Sciences Research Center (MSRC), SUNY Stony Brook, NY, 1988, Marine Environmental Science.

B.A. Georgetown University, Washington D.C., 1981, Political Science (International Relations) and Biology minor.

Marshall is the inventor of Crittercam (Animal-Borne Instrument, ABI) and has conducted hundreds of deployments of these devices on diverse species over the last 30 years. Marshall has experience working with belugas, having conducted test deployment of Crittercam with Mystic belugas previously as well as subsequent research with belugas (as well as several other species of cetaceans) in the wild.

B. Identify and describe the qualifications of all personnel who will be performing animal-related support functions associated with the experimental protocol.

Dr. Tracy Romano of Mystic Aquarium will be collaborating in planning and conducting these tests.

Mystic beluga trainers will manage and monitor all animal procedures.

ADDENDUM I. CONSIDERATION OF PAINFUL PROCEDURES

Check if N/A **X**

- 1.) Address why alternative procedures either do not exist or were rejected. Provide the source and methods used to determine that alternatives are not available (i.e. database search with key words, etc.).**

- 2.) How will pain or distress be monitored or assessed and what criterion will be used to determine when administration of pain medication is necessary?**

- 3.) Identify measures to be taken to alleviate pain or distress. Include drugs, dosage, route of administration, and expected duration.**

- 4.) If anesthetics or analgesics will not be used to minimize pain or distress, provide scientific justification as to why these drugs must be withheld.**

- 5.) If more than momentary pain or distress cannot be effectively alleviated with appropriate medication, provide scientific justification as to why the procedure must be performed.**

ADDENDUM II. SURGICAL PROTOCOL

Check if N/A

1.) Have alternatives to the surgical procedure been considered and found to be inappropriate? Explain.

2.) Describe with reasonable detail the surgical procedures to be used and where it is to be performed. Include the name of the person who will be performing the surgery and that persons level of expertise in this area. (Note: All survival surgery must be performed using aseptic technique.)

3.) Will the surgery be major or minor?

4.) Will multiple survival surgeries be performed OR is there more than one major operative procedure from which the animals are allowed to recover? (Note: More than one major operative procedure is NOT allowed without prior approval from the IACUC.)

5.) What method of anesthesia will be used; who will supervise the administration of anesthesia and how will the anesthetic depth be monitored?

6.) Describe the pre- and postoperative care that will be provided. Include names of anesthetics, analgesics, antibiotics, dose and route of administration and duration.

STATEMENT OF ASSURANCE

Based upon available information, the alternatives to the use of live animals for the purpose of this study have been considered and found to be inappropriate. It is the responsibility of the Principal Investigator to assure that the protocols outlined here for the humane care and treatment of the animal are followed. The Mystic Aquarium reserves the right to discontinue the study if the health of the animal or the safety of the staff is jeopardized. Any necessary changes in the study design will be approved by the Institutional Animal Care and Use Committee (IACUC) prior to implementation. The Principal Investigator has reviewed the qualifications of all personnel involved in the study and found them to be adequate.

To the best of my knowledge, the animal research described in this protocol review does not unnecessarily duplicate previous experiments.

[Signature] 2/13/19
Principal Investigator Date

The Attending Veterinarian has reviewed the proposed protocols for animal use and has found them to be acceptable.

[Signature] 2/18/19
Attending Veterinarian DVM, MS, Dipl. ACVIM Date

IACUC

Comments or Conditions of Approval: Per Dr. Tuttle, animals that are known ingestors of foreign objects may be excluded. During time of tag attachment, Husbandry Staff will observe animals; Nets in place to retrieve a dislodged device.

J. Fatima Matos 2/19/19
Signature of IACUC Chair Date

Approval Period: 2/19/19 - 2/18/20 Protocol # 11002
(Must be reviewed annually)

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____

Re-Approval Date _____ Signature of IACUC Chair _____