Project Information

File Number #22629 **(Final Revised)**

Bold text in body reflects clarifications to the application following the public comment period

****Project Title***

Health-related Research on beluga whales conducted at Mystic Aquarium to contribute knowledge and inform management and recovery of wild beluga populations, including the ESA-endangered Cook Inlet and MMPA-depleted Sakhalin Bay-Nikolaya Bay-Amur River beluga populations.

****Project Status***

New

Previous Federal or State Permit #

NMFS Parts Permit #21966 (Issued 3/30/2018)
Scientific Research/Enhancement Permit #42-1642 (Expired 10/15/2007)
Scientific Research/Enhancement Permit #42-1908 (Expired 1/31/2014)

****Permits Requested***

MMPA Research permit

****Where Will the Activities Occur?***

Transport of animals will occur from Marineland, Niagara Falls, Ontario, Canada to Mystic Aquarium, Mystic, CT USA. Research activities will occur at Mystic Aquarium, 55 Coogan Boulevard, Mystic, CT 06355 USA. Mystic Aquarium has a formal partnership agreement with Georgia Aquarium that includes research, animal exchanges, education and PR initiatives (See Appendix 1 Georgia_Mystic_Partnership). If deemed in the best interest of an individual beluga or the US beluga population for social, health, or welfare reasons, animals may be moved to Georgia Aquarium (Georgia Aquarium, 225 Baker Street NW, Atlanta, GA USA) which has agreed to participate in the described research studies and sample collections that can be conducted as part of the routine health monitoring of the belugas housed at the aquarium. Georgia Aquarium is an accredited facility with experience in the care of belugas.

****Research Timeframe***

The requested duration of the permit is for a 5-year period: January 1, 2020 through December 31, 2025.

****Sampling Season/Project Duration***

Import is proposed to occur in January 2020. This permit is for continuous marine mammal health related research conducted on a daily basis at Mystic Aquarium. Research will occur continuously;

there is no specific season or duration of the research. To gain the maximum knowledge that can be applied for beluga conservation, we are requesting the 5-year maximum for the permit. Training for research activities is already part of the Mystic Aquarium husbandry protocol and will occur on a daily basis during regular scheduled training sessions.

***Abstract**

We request to import 5 aquarium-born belugas (*Delphinapterus leucas*) from Marineland Canada to Mystic Aquarium for the purpose of conducting scientific health-related research over a 5 year period that will contribute knowledge and inform management and recovery of beluga populations in the wild, including the ESA-endangered Cook Inlet and MMPA-depleted Sakhalin Bay-Nikolaya Bay-Amur River beluga populations. Given the escalating pace of environmental and anthropogenic changes in the Arctic, it is critical to act as soon as possible. We are prepared to identify causes, measure impacts, and gather important data to help with the recovery of Cook Inlet, Sakhalin Bay-Nikolaya Bay-Amur River and other beluga populations. Certain information can only be gained from studying belugas under controlled conditions. It is essential for Aquarium scientists to collaborate with wildlife biologists and managers in order to utilize our collective expertise and resources. Through this type of collaborative research we can further our understanding of beluga biology and make strides in population recovery and conservation. The research includes investigations on 1) the neuroimmunological response to environmental and anthropogenic stressors, 2) the development of novel non-invasive techniques to assess health in free-ranging, stranded and endangered belugas, 3) the hearing and physiological response to anthropogenic sound, 4) morphometrics to inform photogrammetry studies, 5) diving physiology, 6) microbiome, 7) behavior and reproduction, and 8) testing of prototype telemetry and imaging devices before deployment on wild whales.

Project Description Page

***Project Purpose: Hypothesis/Objectives and Justification**

Study 1 (Neuroimmunological Response to Environmental and Anthropogenic Stressors)

Specific Purpose: To continue to develop and ground truth biomarkers, reagents and diagnostics in belugas under controlled conditions in an aquarium setting in order to assess and monitor health and interpret findings in wild whales

Hypothesis: Anthropogenic and environmental stressors elicit physiological responses, which may affect the immune system or health in marine mammals.

Objectives:

- To characterize and investigate the beluga nervous and immune systems
- To continue to develop and evaluate hormone, immune system assays and reagents, and diagnostics for health assessments in belugas
- To investigate the effects of anthropogenic and environmental stressors on the nervous and immune systems (i.e. health) of belugas under controlled conditions to better interpret findings in wild belugas

Justification and Summary of published findings related to the objectives:

Anthropogenic and environmental stressors continue to increase and pose significant threats to marine mammals. These stressors include but aren't limited to emerging pollutants, changes in climate resulting in shifts in prey availability, changes in predator/prey relationships, emerging pathogens, and increases in noise due to dredging and construction, shipping, oil and gas exploration and drilling, sonars, geophysical surveys and oceanographic research (Bossart, 2011; Letcher *et al.*, 2010; Moore and Huntington, 2008; Van Bressem *et al*., 2009; Kyhn *et al*., 2014).

Our laboratory was the first to characterize an anatomical link between the nervous and immune system in cetaceans and specifically the beluga (Romano et al., 1994; 2002) whereby hormones and neurotransmitters released during stress could have an effect on the immune system and an animal's ability to fight off infection and disease. In order to characterize the nervous and immune system in cetaceans, specific reagents and assays were developed, adapted and standardized in our laboratory utilizing the beluga and bottlenose dolphin as representative cetaceans (Romano et al., 1992, 1999; Mancia et al., 2007; Keogh et al., 2011; Peden-Adams et al., 2012; Spoon and Romano, 2012; Levin et al., 2014).

We now have a 'tool box" to help assess health in belugas but continued development of reagents and assays are needed (e.g. additional markers for lymphocyte subsets, natural killer cell functional assays, etc.). Moreover, our laboratory developed marine specific diagnostic assays to detect disease and pathogens such as *Brucella* (bacteria that cause reproductive failure) in marine mammals (Meegan et al., 2010, 2012). These tests have been useful in the management of marine mammals under professional care as well as investigating the prevalence in wild counterparts.

In order to study the physiological response to stressors and impact on the immune system in marine mammals, we have studied and are continuing to conduct controlled studies on belugas under professional care as well as live capture released cetaceans and subsistence hunted belugas (Spoon and Romano, 2012; Romano et al., 2004; Bossart et al., 2008; Reif et al., 2009; St. Aubin et al., 2013; Fair et al., 2014; Thompson et al., 2014). Additional studies similar to the above are needed to increase the number of whales being investigated as well as further provide information on the health impacts of anthropogenic and environmental stressors on belugas.

The continued development of reagents and assays, including diagnostic assays, to detect disease and pathogens will enable us to monitor the health of wild beluga populations. Importantly, through application of developed reagents and assays, the health of different beluga populations can be compared with the ESA-endangered Cook Inlet (CI) and the MMPA-depleted Skahalin Bay-Nikolaya Bay-Amur River beluga populations i.e. in the event of live-capture release studies, strandings (live and dead), and/or the application of non-invasive techniques (see Study 2) to collect samples from free-ranging whales. Moreover, continued research and development and validation of these "tools" will help with interpretation of health measurements obtained from other tissues such as skin and breath. Overall, these studies will contribute to the Cook Inlet Beluga Whale Recovery Plan including Action #s 4 ("Increase efforts to identify and monitor individual CI belugas, coordinating photo-identification, stranding data, genetic studies, and body condition assessments via biopsy samples of skin and blubber"); #6 ("Conduct regular biopsy surveys of CI belugas to monitor changes in condition and reproductive success in relation to environmental changes"); #25 ("Using currently available information, compare data on diseases from CI belugas

with other beluga populations to determine if there are abnormal levels or atypical types of disease agents present in Cook Inlet affecting CI belugas") and #26 ("Determine types and sources of disease agents identified to be of concern specifically to CI belugas and assess management actions targeted at mitigating the disease agents") identified in the Cook Inlet Beluga Whale Recovery Plan.

The health information gained through the development and validation of health-related reagents and assays and monitoring the physiological response to challenges in a controlled setting will help enable interpretation of health measurements/biomarkers in wild whales. Moreover, these reagents and assays will contribute to information and interpretation of data from individual Cook Inlet belugas through skin biopsy studies (Action #4) as well as contribute to monitoring changes in body condition and reproductive success in relation to environmental changes (Action #6). Continued diagnostic assay development and validation for pathogens and disease agents can be applied to "compare data on diseases from CI belugas with other beluga populations to determine if there are abnormal levels or atypical types of disease agents present in Cook Inlet affecting CI belugas" (Action #25). Hand in hand with pathogen exposure is immune system function and comparing immune status of CI belugas with other beluga populations will help determine if CI belugas are immunocompromised. These studies would help reveal the types of disease agents specific to each population including CI belugas (Action #26). Once the pathogens are identified, sources can be investigated and mitigation management action put into place. This study also sets the groundwork for investigating the physiological response to stressors (e.g., noise) that can be incorporated into experimental paradigms (e.g., see Study 3). Knowledge gained from our proposed research carried out under controlled conditions in an aquarium setting will benefit the ESA-listed Cook Inlet beluga and MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide. As described above, the research proposed will contribute significantly to understanding the basic biology of belugas. Through our research studies we will gain information on belugas' physiological response to stressors and impact on the immune system and health.

In order to achieve these objectives, we propose to take blood samples as described in the Project Description (methods) and take table.

Study 2 (Development of novel non-invasive techniques to assess health in free-ranging, stranded and endangered belugas)

Specific Purpose: To validate the utilization of less invasive tissue matrices (i.e. breath, saliva, feces and skin) in belugas under controlled conditions in an aquarium setting and transition to assess and monitor health in wild belugas

Hypothesis: Hormones, immune and molecular components important to health and physiology can be measured in tissue matrices such as breath, saliva, feces and skin and used to assess and monitor health in wild belugas and/or during controlled experimental studies.

Objectives:

- To develop and validate assays in the laboratory to detect stress, metabolic and reproductive hormones, and immune and molecular components in beluga breath, saliva, feces and skin
- To biologically validate the use of beluga breath, saliva, feces and skin for assessing and monitoring health in belugas
- To determine optimal sampling methods for transition and application of non-invasive techniques to wild belugas

Justification and Summary of published findings related to the objectives:
Our laboratory has developed and is continuing to develop, adapt and validate assays for detecting hormones (stress e.g. cortisol; metabolic e.g. thyroid hormone; reproductive e.g. progesterone) and immune components in tissue matrices such as blood, saliva, breath, feces and skin (Richard et al., 2017; Thompson et al., 2014; Flower et al., 2015; Fair et al., 2014; Unal et al., 2018). Blood is the "gold standard" but requires hands-on the animal and restraint, unless the behavior is trained, to obtain a sample. We aim to transition these less invasive techniques as feasible to free ranging belugas as has been done in other cetaceans (Hunt et al., 2013; Rolland et al., 2005; Acevedo-Whitehouse et al., 2010). Testing of sample collection methodology under controlled conditions with whales at Mystic Aquarium, for example for breath collection, will help determine the number of breaths needed to detect a hormonal or immune signal, how high the collecting device needs to be from the blowhole and improve the methodology for collecting samples from wild whales whether with a Unmanned Autonomous Vehicle (UAV) or drone or utilizing a pole (with the collecting device attached) from a boat and/or when collecting from stranded belugas.

Moreover, these novel non-invasive techniques have application to controlled research investigations on belugas such as the hearing studies described under Study 3. For example, instead of having to halt hearing studies to obtain a blood sample (Romano et al, 2004), a breath sample can easily and efficiently be obtained so as to not interfere or interrupt the hearing study, reflecting more accurate measurements specific to the noise exposure.

This study contributes to Action #45 ("Refine research techniques, evaluate alternatives, and implement research methods which minimize harassment, harm, and general adverse impacts on CI belugas") and Actions #4, 6 ("Increase efforts to identify and monitor individual CI belugas, coordinating photo-identification, stranding data, genetic studies, and body condition assessments via biopsy samples of skin and blubber" and "Conduct regular biopsy surveys of CI belugas to monitor changes in condition and reproductive success in relation to environmental changes") in the Cook Inlet Beluga Whale Recovery Plan. Development and validation of non-invasive health monitoring techniques on aquarium belugas can be transitioned to monitor health and reproductive status in wild belugas (e.g. validation of blow sampling for application to live stranded Cook Inlet belugas and free ranging belugas in general) (Action #45).

Continued determination and validation of molecular biomarkers in skin from studies on aquarium belugas can be compared with skin biopsies obtained and received from other beluga populations (for which the PI has already obtained from prior studies) (NMFS Permit Number 21966) and will add information for "monitoring individual CI belugas" which can be integrated with "photo-

identification, stranding data, genetic studies and body condition assessments" (Action #4). Skin gene expression studies can be utilized to "monitor changes in condition and reproductive success in relation to environmental changes" (Action #6).

Moreover, the novel non-invasive techniques that prove to be successful can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to take breath**, skin, feces, blood,** and saliva samples as described in the Project Description (methods) and take table.

Study 3 (Hearing and Physiological Response to Anthropogenic Sound)

Specific Purpose: To investigate the impact of anthropogenic sound on beluga hearing and physiology in belugas under controlled conditions in an aquarium setting in order to increase knowledge on the masking and physiologic effects of noise common to beluga habitats and to help define industrial underwater noise emission regulations for management of these activities

Hypothesis: Certain anthropogenic sound sources will increase beluga hearing thresholds and elicit a physiological response that can be measured and quantified.

Objectives:

- To collect baseline and masked beluga hearing thresholds from 5 belugas using a variety of sound sources particularly recordings of anthropogenic noise from Cook Inlet (e.g. commercial shipping, outboard motors, airplanes, pile driving, etc.) and to collect AEPs on offspring of calves of whales listed in this permit in order to monitor hearing development via methodology described below.
- **To quantify the frequencies which are masked (if they are masked), and the difference (in dB) between baseline thresholds and masked thresholds.**
- To quantify directional hearing abilities of belugas to noise projected from 3-5 different angles **in order to address how masking release could be achieved by a beluga, simply by turning its head away from the noise.**
- To collect exhaled blow (breath condensate) before and after sound exposure to obtain information on the physiological response of belugas to anthropogenic noise

Justification and Summary of published findings related to the objectives:
Increasing sound levels in the ocean are of concern for marine mammals and are associated with but not limited to dredging and construction, shipping, oil and gas exploration and drilling, sonars, geophysical surveys and oceanographic research. Noise can impact hearing, navigational abilities, foraging and reproduction especially in cetaceans (Putland *et al.*, 2017; Halliday *et al.*, 2017); however, it is also known that noise can bring about a physiological response that may ultimately impact immune function, metabolism and growth, reproduction and health (Archana and Namasivayam, 2000; Spehner et al., 1996; Van Raaij et al., 1996; Broucek, 2014) as has been demonstrated in other mammals. Importantly, our controlled studies on beluga and bottlenose

dolphin responses to impulsive and tonal sounds demonstrated autonomic nervous system activation and changes in immune components (Romano et al., 2004).

Cook Inlet belugas, given that their critical habitat is concentrated near Anchorage, the largest urban area in Alaska, are exposed to a wide variety of potential noise stressors including fishing, mining, dredging, military operations, oil and gas development, air and water transportation, and residential and industrial shore development. Many of these activities are intensified during the main foraging season of belugas when ice is absent (May-October).

Working with our collaborators who are acoustic experts, preliminary proof-of-concept hearing studies were carried out on one beluga whale at Mystic Aquarium under controlled conditions. The whale was trained to station and accept suction cup electrodes to measure non-invasive auditory evoked potentials (AEPs). An audiogram was obtained to compare with wild belugas. Moreover, a controlled noise exposure experiment was carried out in which the whale was exposed to low levels of anthropogenic noise previously recorded in the Cook Inlet beluga critical habitat (i.e. shipping and pile driving noise) at levels well below the regulatory acoustic exposure thresholds indicating significant masking in beluga hearing sensitivity, exceeding 20 dB from baseline hearing. This level of masking has the potential to compromise vital functions such as foraging. Previous acoustic monitoring data shows these noise levels regularly occur in Cook Inlet belugas critical habitat. Moreover, AEPs carried out on whales in Bristol Bay showed similar audiograms to that of the beluga at Mystic Aquarium. This work is currently in preparation for publication.

We are proposing masking hearing experiments thus measuring hearing during the noise exposure. We will be measuring for a ca. 20 dB increase in thresholds (due to masking) during the exposure, but not afterwards. However, it's important to measure hearing before and after to ensure that temporary threshold shift (TTS) does not occur and to determine the amount of masking that did occur during the noise exposure. This differs from a TTS study in (a) the level of noise projected and (b) typically in a TTS study, hearing is not measured during the noise exposure. Also note, TTS and masking both result in increased thresholds, but typically differ in the auditory mechanisms that drive that increase. But in masking, once the noise is off, hearing should be at the 'normal' or baseline levels. There is no recovery time in masking as there is in TTS. We will use the user spreadsheet tool (NMFS 2018) to define a maximum SELcum based on the noise source characteristics and duration of exposure. The spreadsheet results will guide us to make sure we will never reach TTS. Furthermore, we will measure hearing just after the end of the exposure trial to make sure there was no TTS induced in any of the trials.

We have shown this experimental paradigm will work and propose to continue these studies with additional belugas, utilizing various anthropogenic sound sources, **quantifying the frequencies which are masked (if they are masked), and the difference (in dB) between baseline thresholds and masked thresholds**, quantifying directional hearing abilities of belugas **in order to address how masking release could be achieved by a beluga, simply by turning its head away from the noise** and adding in the collection of blow samples before and after sound exposure to measure the physiological impacts of sound.

This study contributes to Action #30 ("Describe the acoustic characteristics of different anthropogenic noise sources in Cook Inlet and rate the potential acoustic impacts from each type of noise source on CI belugas") in the Cook Inlet Beluga Whale Recovery Plan, by quantifying the masking effects in beluga hearing from noise sources in Cook Inlet (e.g. pile driving, shipping). The results from this study can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to conduct auditory evoked potential (AEP) measurements (baseline audiograms and masked hearing sessions) and breath samples as described in the Project Description (methods) and take table.

Study 4 (Photogrammetry Body Condition Studies)

Specific Purpose: To utilize belugas under controlled conditions in an aquarium setting to help inform, validate and interpret photogrammetry of wild belugas

Hypothesis: Belugas under controlled conditions at Mystic Aquarium can be utilized to help ground truth photogrammetry data obtained from belugas in the wild particularly in regards to body condition and pregnancy status (if animals naturally breed, see Study 7 below).

Objectives:

- To obtain photographs of belugas as they swim underneath a camera at a minimum of 6 m in height
- To obtain a series of morphometric measurements that coincide with the photographs
- To obtain body weights that coincide with the photographs and measurements
- To provide these data to biologists conducting photogrammetry studies on wild belugas especially endangered beluga populations
- **Demonstrate feasibility of detecting seasonal changes in beluga body condition using photographs and body measurements**
- **Should a pregnancy occur, demonstrate feasibility of detecting pregnancy using photographs and body measurements**
    - **Determine at what stage of pregnancy morphometric changes are detectable by photogrammetry**

Justification and Summary of published findings related to the objectives:
Photogrammetry, a technique that uses quantitative measurements from photographs has been utilized to collect measurements on cetaceans such as length, growth and body condition in order to infer health information on individual cetaceans, or to determine group abundance and structure (Webster et al., 2010; Cheney et al., 2017; Jaquet, 2006). More recently, less expensive Unmanned Autonomous Vehicles (UAVs) have been utilized rather than aerial surveys to monitor cetacean populations at risk (Durban et al., 2018). Photogrammetry with UAVs has recently been used or is in development for monitoring beluga populations such as Cook Inlet and St. Lawrence Estuary belugas. We are currently participating in a study to provide longitudinal photogrammetry and morphometric data from two known individual belugas (an older female and a younger male) 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 UAVs. We propose to continue obtaining photographs and morphometric measurements on additional belugas at different life stages for body condition and potentially pregnant females, if belugas were to incidentally breed, in order to contribute to development, validation and interpretation of photogrammetry on wild belugas.

This study contributes to Action #14 ("Monitor body condition of living and deceased CI belugas to assess the presence/absence of nutritional distress or nutritional-related mortalities, and determine the percentage of necropsied CI with mortalities attributed to nutritional distress") and Action #5 ("Determine annual mortality and reproductive rates of CI belugas") in the Cook Inlet Beluga Whale Recovery Plan, by providing morphometric measurements paired with photographs taken from above aquarium whales in order to provide standardized measurements on belugas of known body condition and pregnancy status for interpretation and validation of photogrammetry protocols on wild belugas. The results from this study can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to take photographs, morphometrics, and weights, as described in the Project Description (methods) and take table.

Study 5 (Diving Physiology)

Specific Purpose: To investigate the role of the immune system in the diving physiology of cetaceans and susceptibility to decompression sickness in marine mammals utilizing belugas under controlled conditions in an aquarium setting.

Hypothesis: We hypothesize that, as with other physiological systems, the marine mammal immune system possesses specific adaptations to diving, which allow for repeated and prolonged dives to great depth without suffering from dive-related injuries or disease as humans and other terrestrial mammals experience. It is expected, however, that this regulation can be interrupted by the presence of additional stressors leaving marine mammals susceptible to dive-related disease, such as decompression sickness.

Objectives:

- To understand how the marine mammal immune system may be adapted to challenges associated with diving, including depth (pressure), dive duration and activity level (swimming vs. gliding)
- To correlate markers of health or stress with immune activity during diving
- To describe the role immune activity may play in protection from, or facilitation of, dive related injuries (e.g. decompression sickness) in cetaceans
- To develop the use of blow sampling for gaining immune information from free ranging (free diving) cetaceans

Justification and Summary of published findings related to the objectives:
Our lab is the first to investigate immune responses in marine mammals in the context of diving, with interest in describing the role of immune activity in the development of, or protection from, dive related injury and disease (Thompson and Romano, 2015; Thompson and Romano, 2016).

Protocols for evaluating immune functions, such as phagocytosis and lymphocyte proliferation, were modified to incorporate exposure to *in vitro* changes in pressure, temperature or exposure to nitrogen gas bubbles. Additional protocols for measuring immune activation utilizing commercially available antibodies or reagents have been validated for use with marine mammals for this work, including activation of granulocytes via CD11b detection (Thompson and Romano, 2015), activation of lymphocytes via expression of IL2R (Thompson and Romano, 2016), and activation of inflammatory responses through increases in the complement protein C5a.

Our work is the first to show 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 (Thompson and Romano, 2015). Additionally, we have provided the first evidence supporting the theory that decreased sensitivity of the immune system during diving may be one way marine mammals avoid dive related injuries, such as decompression sickness, under normal conditions (Thompson and Romano, 2015; Thompson and Romano, 2016*).*
Results of our current work suggest that the complement system is less sensitive to the presence of nitrogen bubbles in belugas and harbor seals, as compared with humans, but that health status again may play an important role in determining the complement response. In addition, this work suggests that belugas and harbor seals (i.e. species with different dive abilities) may have different thresholds in terms of nitrogen bubble size or number, for initiating an immune response. Diving physiology studies with belugas under controlled conditions at Mystic Aquarium will enable further investigation of the immune system's role in dive related injury for cetaceans while enabling comparison with other marine mammals i.e. pinnipeds.

This study contributes to Action #45 ("Refine research techniques, evaluate alternatives, and implement research methods which minimize harassment, harm, and general adverse impacts on CI belugas") in the Cook Inlet Beluga Whale Recovery Plan, by investigating diving physiology under controlled conditions on aquarium whales with known diving parameters and conditions and/or simulating dives (i.e. exposing blood cells to pressure in vivo) and determining changes in cellular response. This study will contribute to our understanding of the potential impact on the health of belugas whose dive patterns are altered or interrupted by anthropogenic activities. The results from this study can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to take blood and breath samples as described in the Project Description (methods) and take table.

Study 6 (Microbiome)

Specific Purpose: To investigate the microbial communities of belugas under controlled conditions at Mystic Aquarium in conjunction with clinical health status, season, sex and age

Hypothesis: We hypothesize that beluga whales harbor specific microbial core communities that are necessary for physiological health, and changes or disruptions in these communities can be an indicator of illness, disease, or chronic stress.

Objectives:

- To document the bacterial composition of the oral, respiratory, skin, vaginal, and gastrointestinal microbiomes of belugas
- To document differences in microbial communities based on age, sex and environmental parameters
- To compare the microbiome profiles with available clinical data in order to identify any changes that can be correlated to illness or disease

Summary of published findings related to the objectives:

The term microbiome refers to the collection of microorganisms that live in or on a host species. A microbiome occupies a specific niche, such as the skin or gut, each harboring different bacterial communities. These bacteria make up the normal flora of the host, and help protect them from invading pathogens. Microbiomes have also been shown to play critical roles in host nutrition, immune function, and can even affect brain development (Diaz et al., 2011; Hooper, 2009, Nelson et al., 2013). A microbiome naturally shifts (in number and type of bacteria) over the course of the lifetime of the host. However, disruptions in a microbiome can lead to a dysbiosis that can alter host health (Hanning and Diaz-Sanchez, 2015; Apprill et al., 2017; Holmes et al., 2011). These finding have led to an increase in microbiome research, however most of these studies have focused on human or terrestrial animals. In fact, there have been very few microbiome studies focused on marine mammals (Nelson et al., 2015).

Initial studies of bottlenose dolphins (Venn-Watson et al., 2008; Johnson et al., 2009) helped to show that marine mammal microbiomes are surprisingly diverse. Culture-based techniques have been used to characterize the gastrointestinal microbiome of dolphins and other cetaceans (Shaefer et al., 2009; Morris et al., 2011). Respiratory microbiomes of dolphins have been studied using blow, or respiratory exhalate (Lima et al., 2012) and have identified several pathogens. The epidermal microbiome of baleen whales has been characterized using skin swabs from free-ranging whales (Apprill et al., 2014) and has shown that the skin microbiome is distinct from the microbes found in the surrounding aquatic environment. Recently, the microbiomes of dolphins and sea lions were compared using samples collected from five body sites, which showed that microbiomes vary with both species, body site, and geographical location (Bik et al., 2016). We supported a recent study that included preliminary investigations of the skin microbiome of belugas under controlled conditions at Mystic Aquarium (Keller et al., Submitted) which demonstrated a low diversity of skin bacteria on the beluga. Marine mammals, such as belugas are indicators of ocean fitness, and more comprehensive studies are needed to fully understand the role that microbiomes play in the health of these animals. Further investigation of belugas under controlled conditions at Mystic Aquarium will enable further characterization of the beluga microbiome and the role it plays in beluga health.

This study contributes to Action #26 ("Determine types and sources of disease agents identified to be of concern specifically to CI belugas and assess management actions targeted at mitigating the disease agents") in the Cook Inlet Beluga Whale Recovery Plan, by collecting and characterizing microbes in different tissues matrices in aquarium belugas under controlled conditions and correlating with sex, season, age and health status which can serve as a reference for investigations of microbes on stranded live or dead Cook Inlet belugas. The results from this study can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to take swabs (skin, blowhole, anal, oral, vaginal) as described in the Project Description (methods) and take table.

Study 7 (Behavioral and Reproduction Studies)

Specific Purpose: To provide information on breeding, pregnancy, successful birth and calf rearing, and the critical early life stages from belugas in an aquarium setting under controlled conditions that may engage in reproduction as part of their natural behaviors, that will assist in informing wildlife biologists and management of outcomes that are more favorable for population recovery should pregnancy and birth occur

Hypothesis:. **It is hypothesized that** changes in behavior and physiology will occur in belugas before, during, and after the breeding season, throughout pregnancy, and after birth.

Objectives:

- To monitor the behavior of belugas before, during and after the breeding season, and during any incidental pregnancy, birth and throughout calf development
- To monitor the physiology of belugas including hormone levels, size and activity of reproductive organs, before, during and after the breeding season, and throughout any incidental pregnancy and after birth

Justification and Summary of published findings related to the objectives:
Mystic Aquarium belugas were instrumental in the reproductive research that led to artificial insemination in belugas maintained under professional care (Robeck et al., 2005; Robeck et al., 2010). In order to contribute to that research, belugas were trained for ultrasound of reproductive organs, as well as blood and urine collection which is currently being used to monitor reproductive status. This research contributes not only to our understanding of beluga reproduction in an aquarium setting, but advances knowledge towards understanding reproduction of belugas in the wild. Moreover, we have contributed to studies that investigate and monitor seasonal fluctuations in testes size and reproductive hormones under controlled conditions to contribute to knowledge on male beluga reproduction (Richard et al., 2016; Richard et al., 2017). Data was collected on belugas throughout the entire year gaining insight on baseline rhythms and seasonal fluctuations of the reproductive system which is challenging to understand in wild belugas. In addition, we have contributed to studies investigating beluga vocalizations and behavior before, during and after the breeding season (Justin Richard, Dissertation 2016).

While beluga whale reproduction is not the purpose of the proposed research, breeding is a natural behavior and will be allowed to occur. Artificial insemination will not be used and contraception will not occur unless medically necessary for the health and wellbeing of an individual beluga. In the event the subject whales become pregnant, we propose to continue to contribute knowledge and information regarding beluga reproduction on belugas under controlled conditions in an aquarium setting. We propose to monitor behavior and reproductive organs as well as hormones before, during and after the breeding season on belugas in different reproductive states. Moreover, if females were to get pregnant, data would be collected throughout the pregnancy, as feasible, as well as throughout birth and calf rearing, providing meaningful information regarding critical early life stages that would help advise wildlife biologists and inform management on factors that lead to more favorable pregnancy and birth rates for population recovery. In the event a participating beluga is lactating, the beluga may participate in the research described above.

Any progeny born to belugas as part of this research would be incorporated into the research program as described in the Take Table and Project Descriptions and closely monitored for health and wellbeing. The proposed research will be done under voluntary behavioral cooperation and it is unlikely that a beluga calf will provide voluntary physiological research samples at a young age. In the absence of behaviors allowing for physiological data collection, behavioral data that can be collected via observation will be generated. Additionally, physiological samples (blood, blow, feces, saliva, measurements, photos, etc.) for research may be collected in conjunction with handling of any progeny for necessary veterinary examinations, as determined by the attending veterinarian and described in the Take Table and Project Descriptions; however, calves will not be handled expressly for the purposes of research. The progeny will be trained to participate in research over time and may contribute to the research sampling under behavioral control as they are trained to do so. Progeny would be kept with the dam until naturally weaned. Following weaning, the progeny would be cared for at Mystic Aquarium or, if deemed in the best interest of the individual beluga or the US beluga population for social, health, or welfare reasons, may be cared for at Georgia Aquarium. Examples of scenarios that could necessitate moving animals include meeting the social needs of the animals, medical reasons, or to ensure optimal animal management.

This study contributes to our overall knowledge on beluga breeding, birthing and calf rearing, and the critical early life stages and can contribute important information in regards to characteristics and needs for successful reproduction. Moreover, reproductive data collected on aquarium whales can be compared with wild populations. Actions #5, #15, #16 ("Determine annual mortality and reproductive rates of CI belugas"; "Analyze the existing collection of CI beluga teeth to determine if age at first reproduction for female CI belugas can be determined, and assess if there has been a significant change in this parameter over time"; "Review available data which may provide information about calving rate (population-wide) or calving interval (individual belugas), and assess whether either of these parameters is correlated with prey abundance") in the Cook Inlet Beluga Whale Recovery Plan all address reproduction. This study can contribute basic information on beluga reproduction under controlled conditions with potential to gather data and basic biological information and increase our knowledge on reproduction in general and potentially "reproductive rates", "age at first reproduction", "calving rates and intervals" in parallel with known diet types and caloric intake. The results from this study can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to take vaginal swabs, conduct behavioral observations (video monitoring), ultrasound, blood**, and breath samples** as described in the Project Description (methods) and take table.

Study 8 (Testing of prototype telemetry and imaging devices before deployment on wild whales)

Specific Purpose: To test new telemetry and imaging devices for wild cetaceans on belugas under controlled conditions at Mystic Aquarium before deployment on wild whales and dolphins.

Hypothesis: Belugas under controlled conditions at Mystic Aquarium will allow for testing of new telemetry and imaging devices including optimal body placement, size, duration (how long the device will stay on), as well as noting any changes in animal behavior with the device in place.

Objectives:

- To collaborate with researchers and engineers as needs arise to test new telemetry and imaging devices adhered via suction cups or other non-invasive mechanisms on belugas under controlled conditions at Mystic Aquarium
- To train whales to station for placement of the device on different locations of the body and to help determine suitable size **(i.e., Where is the best location on the body for a certain type of telemetry device and what is the appropriate size that serves the electronics but also is optimal for the whale?)**
- To test the ability of the device to stay on by having the whale swim, dive and breach **(i.e., Does diving, breaching, swimming impair the device to remain on the whale?)**
- To observe any change in behavior and any physical effects from the suction cup after the device is deployed **(I.e., Is there any change in behavior or any physical effect from the suction cup attachment after the device is deployed?)**
- To observe how long the device will stay on **(i.e., How long will the telemetry device stay on the whale?)**

Justification and Summary of published findings related to the objectives:
Technology for tracking and sensing devices deployed on marine mammals for measuring environmental parameters (temperature, salinity, location, etc.) as well as activity (dive frequency, depth, movements, etc.) for marine mammals is constantly being improved and perfected and can be quite expensive (LaPlanche et al., 2015; Nowacek et al., 2016; Hauser et al., 2014, 2015). It is often helpful to deploy and test some of these devices on a cetacean in a controlled environment before deploying on a wild cetacean. In collaboration with one of our scientists-in-residence, Mystic Aquarium tested a camera on one resident beluga to gain as much information as possible before deploying on a wild beluga. One whale was trained to station to receive the camera which was adhered via suction cups. Tests were run to determine the optimal placement on the body. Two different sized prototypes were tested. Moreover, adherence was tested utilizing different sizes and configurations of suction cups to determine which worked the best. Observations were made while the whale was swimming and then after the whale was asked to carry out more rigorous activity such as breaching and increasing swim speed. Given results from the testing on the beluga,

the engineers are currently modifying the camera and it will then be further tested on the beluga. Mystic Aquarium proposes to continue to support testing of important devices on belugas under controlled conditions in an aquarium environment that will be beneficial to track and monitor cetaceans in the wild.

Tracking and monitoring belugas in the wild especially given environmental changes and anthropogenic challenges is becoming more and more critical for collecting important information on whales and the information gained can contribute to their conservation and management. New technologies allow for recording noise, vocalizations, diving depth, frequency and finer movements in addition to providing location information. The ability to test, deploy and refine telemetry devices and cameras on aquarium whales before deploying on wild whales enables a greater success rate on wild whales. Topics in the Cook Inlet Beluga Whale Recovery Plan that can benefit from these studies include population monitoring, reduction in prey, noise, habitat loss or degradation, unauthorized take, and cumulative effects of multiple stressors. Moreover, the results obtained from this study can also be applied to study the MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide.

In order to achieve these objectives, we propose to deploy external devices with suction cups as described in the Project Description (methods) and take table.

Determination of Sample Size/Take numbers:

Justification on the frequency and number of samples collected

Frequency and number of samples described in the Project Description below for all studies and listed in the Take Table were determined based on current research sampling efforts demonstrating feasibility and take into account animal welfare, behavior and husbandry first and foremost, while maximizing volume and/or mass of biological samples, observations and experimental sessions for research. Frequency and number of samples, observations and experimental sessions for research are determined with consultation and recommendations from Mystic Aquarium's veterinary and animal husbandry staff with final approval and oversight from the Aquarium's Institutional Animal Care and Use Committee. Relevant sampling schedules surrounding transport, out of water events, training and dive behaviors are designed to determine the time course of endocrine and immune changes in response to each physiological challenge as well as determine the time course to return to baseline conditions. Frequency of sampling for these events as well is based on demonstrated feasibility through prior and ongoing studies conducted at Mystic Aquarium.

The samples to be taken from the 5 belugas from Marineland plus 1 beluga currently owned by Mystic Aquarium upon which IACUC-approved research is currently conducted will allow for a minimum sample size of N=6 whales, which enables a more robust sample size with opportunity for statistical conclusions to be drawn from the proposed studies. The 1 beluga owned and currently at Mystic Aquarium is held under public display and is not included under the proposed permit.

It is estimated that a maximum of two calves would be born during the 5 year permit, as only two of the belugas proposed for import are adults. We propose inclusion of these calves in some of the research initiatives detailed in this permit in order to maximize the information learned about

belugas at this stage as well as throughout development, which is rarely studied. Calves are at a particularly vulnerable life stage, and it is critically important to maximize information regarding their physiology and behavior to better understand calf survival in the wild. The ability to study calves throughout their growth and development also allows for gaining information at different life stages and the potential impact of anthropogenic and environmental stressors on the health of young wild belugas.

Ownership of any calves born to the imported whales will be determined by a written breeding loan agreement. Mystic Aquarium will be the owner of all of beluga Qila's odd-numbered offspring (first, third, etc.), with the owner of the sire owning all even numbered offspring (second, fourth, etc.). For offspring of belugas Mira, Kharabali, and Havana, Georgia Aquarium will be the owner of all odd-numbered offspring, and Mystic Aquarium the owner of all even-numbered offspring, without regard to the fact that they born to different dams. If belugas not owned by either Mystic Aquarium or Georgia Aquarium breed with these imported belugas, an agreement between Mystic Aquarium, Georgia Aquarium, and the third party institution would be created.

As discussed below in methods, calves will not have the behavioral repertoire to participate in research under behavioral control. Thus, research samples on calves will be collected opportunistically in conjunction with the handling of calves for health assessment for some projects as defined in the Take Table. Calves will not be handled for the sole purpose of research; all data that will contribute to research will be collected as part of the calf health assessment process.

The 2 additional belugas currently on loan to Mystic Aquarium for public display purposes (and any other belugas that may be transported to Mystic Aquarium from other U.S. facilities over the duration of the permit) will also contribute to the non-intrusive research while they are under Mystic Aquarium's care; however, any beluga whales held for public display purposes would not be covered under this research permit. Research would be conducted on those whales in accordance with the NMFS regulatory definition of "intrusive research."

Mystic Aquarium currently has 3 belugas in its care- an older female (approximately 38 years of age) for which it maintains ownership, as well as a mature male (17 years of age) and female (approximately 38 years of age) which are on loan. The fact that Mystic Aquarium only owns one beluga which is an older animal limits the ability of Mystic Aquarium's research team to ensure long term continuity of research on belugas under controlled conditions with robust sample sizes at our facility. Even with 2 additional whales on loan in its care, 3 animals do not provide a robust sample size for the studies proposed. Mystic Aquarium collaborates with other AZA accredited aquaria that maintain belugas under professional care, including Georgia Aquarium, and has requested and received beluga samples for research in the past. However, the sample types were limited in scope (i.e. breath samples, blood samples, ultrasounds). Moreover, over the past 2 years in some cases, half of the blood volume requested was received to supplement baseline information for dive physiology studies and none of the samples requested for additional blood paired with breath samples, samples to address the number of exhales and diurnal patterns of hormones in blow were received. Samples are often limited to a small number of whales and limited in frequency of sampling due to personnel constraints and limited time available given other priorities in other animal care and training programs. While obtaining beluga samples from other facilities helps to increase sample size for some studies (i.e., those that are easier to obtain, such as breath

samples), those research studies that take ample training time and a more in-depth effort for sampling have not been feasible. It has not been feasible for other facilities to alter their institutional priorities to train whales for hearing or diving regimes in diving physiology studies for example.

Mystic Aquarium is unique in that it is the only US aquarium that cares for belugas and maintains a Research license under the USDA to conduct bona fide research on our animal collection. This demonstrates Mystic Aquarium's commitment to research with research as a pillar of its mission since opening its doors in 1973. It is essential to Mystic Aquarium's beluga conservation research initiative to have a critical mass of belugas under care at Mystic Aquarium, under similar conditions. This will allow the necessary data collection for the benefit of wild populations. Given our institutional collaboration with Georgia Aquarium (See Appendix 1 Georgia_Mystic_Partnership) and their support of research sampling in the past (i.e. breath, blood, ultrasounds), Georgia Aquarium will support continued research on these imported belugas at their facility, with samples sent to Mystic Aquarium, should it be deemed in the best interest of a beluga or the US beluga population to move the belugas for social, health, or welfare purposes.
The five additional whales from Marineland to be cared for and participate for the primary purpose of research at Mystic Aquarium will enable a minimum sample size of 6 belugas (5 transported belugas plus the current 1 whale owned by Mystic Aquarium) which will enable a more robust sample size and thus more significant conclusions to be drawn for the studies proposed. Therefore, Mystic Aquarium is proposing to bring 5 additional belugas to Mystic Aquarium for the research purposes described above.

In the spirit of shared mission, Georgia Aquarium has pledged support of beluga research being conducted at Mystic Aquarium as they have done in the past with various aspects of the program. Mystic Aquarium does not have the resources to fund this initiative alone and, in compliance with our lending institution, must engage in a lease in lieu of a loan agreement. In this, a lease scenario, Georgia will own three of the belugas. Mystic Aquarium will own two of the belugas. Georgia Aquarium-owned belugas will be on loan to Mystic Aquarium for the duration of this permit such that all belugas would be housed under care at Mystic Aquarium for research purposes for the duration of the project and beyond. The only exception to this would be if relocation is necessary for the best interest of an individual beluga or the US beluga population for social, health, or welfare purposes; therefore, in this case, care would shift for that individual at Georgia Aquarium and the research would continue at Georgia Aquarium.

Proposed *bona fide* research:
Mystic Aquarium's prior research studies have been published in peer reviewed scientific journals (See Appendix 3 Publications) and it is expected that our continued research will also result in peer reviewed scientific publications. The results from our studies will contribute to baseline knowledge of belugas including information on the physiological response to environmental and anthropogenic stressors, health, hearing, diving physiology, behavior, and reproduction. Additional beluga whales for our research studies will provide a larger sample size and cohort of belugas (males and females; different ages) and thus a greater ability to draw conclusions which have direct application to conservation and management efforts (see explanations above). Moreover, additional belugas will allow for larger scale testing of technologies for transition to wild belugas such as photogrammetry, blow collection, biomarkers in skin, testing of new cameras

and telemetry devices and hearing ranges of different aged belugas to help establish regulations of anthropogenic noise in areas where belugas reside. Reproductive studies will provide information on breeding, pregnancy, successful birth and calf rearing and the critical early life stages of belugas that will assist wildlife biologists and management on outcomes that are more favorable in regards to pregnancy, birth rates and population recovery.

In addition to a USDA research license, Mystic Aquarium has a dedicated team of scientists who focus on beluga research, behavior, and conservation. Mystic Aquarium research laboratories are located near the campus of Mystic Aquarium, in Groton, CT (University of CT, Avery Point), facilitating the ability of our researchers to be present for sample collection, as needed to inform the research with immediate processing, archiving or analyses. Having a critical mass of belugas in one location close to the laboratory and the expertise of Mystic Aquarium scientists, and trained similarly will minimize confounding factors and facilitate a maximal amount of data collected. It is critical that we continue to study belugas in aquarium collections in order to benefit endangered and depleted beluga populations, and to maintain the continued health and sustainability of currently stable populations. There is critical information that can only be gained from belugas maintained under professional care. There are many unknowns and confounding variables when studying wild belugas that can be overcome by studying animals under professional care. Benefits include: (1) Known information on natural history, health history, environmental parameters and diet (2) Training of whales for biological samples under behavioral control with no apparent stress (3) Training of animals to participate in controlled study designs.

If we do not have the ability to study belugas under controlled conditions in aquarium settings, the pace and extent of our research contributions to beluga whale health described above and knowledge gained to help inform management and recovery of depleted and endangered populations would decrease substantially. While field work is and has been a focus of our research, it is necessary to ground-truth methods and data in a controlled setting in order for accurate interpretation of samples from wild belugas to be possible. Having the ability to test methodology and create data sets of baseline parameters for belugas in aquariums under known conditions greatly facilitates the maximal amount of information gained from a project on wild belugas. Furthermore, developing non-invasive techniques in a controlled setting allows for more hands-off assessment when applied to wild belugas.

Mystic Aquarium has well established collaborations with wildlife biologists who work in the field studying belugas as well as Native Alaskans who rely on belugas for food security, as well as medicinal, spiritual and social needs. The PI has worked with the North Slope Borough Department of Wildlife Management and in collaboration with Native Alaskans participating in research on Chukchi Sea Belugas since 1994. Most of the pioneering work on the immune system in belugas was made possible by collecting tissues from subsistence hunts (Romano et al., 1992, 1993, 1994, 1999, 2002). The reagents and assays developed from this research were able to be applied to live capture release studies in tandem with tagging studies on Chukchi Sea belugas as well as Bristol Bay whales (Flower et al., 2015; Thompson et al., 2014; Unal et al., 2018). Data from both of these populations is available for comparison with Cook Inlet belugas and other populations as feasible. Findings have been reported at the Alaska Beluga Whale Committee as health of belugas is a major concern and our research has been presented to NOAA at multiple workshops with the most recent at the Cook Inlet Beluga Research Methods Workshop

(November, 2017) and the Cook Inlet Beluga Management, Research and Partnership Opportunities meeting (April 2018). Mystic Aquarium scientists have also conducted hands-off, observational studies in belugas that summer in Cunningham Inlet, Somerset Island, Inuvut, Canada to determine how the changing environment may be impacting belugas and their use of Cunningham Inlet (Anderson et al., 2017).

Importantly, we have worked closely with Native Alaskan and Native American youth with primary focus on promoting beluga sustainability and research. In collaboration with the North Slope Borough Department of Wildlife Management, we have conducted a nationally recognized educational and cultural exchange program in which Native Alaskan and Native American youth work side by side with the PI and scientists in the field collecting research samples on belugas and then travel to Mystic Aquarium for continued hands-on scientific learning. Activities include interaction with our beluga whales and learning about their natural history, care and training, observing how we take research samples from our trained whales and then taking the samples to the research lab to process them and learn about what we are learning from the samples, a field trip to our local marine environment, a workshop on careers and other educational opportunities the aquarium offers. Our local tribal youth also participate and time is spent at the Mashantucket Pequot Museum and Research Center where both groups talk about and exchange their culture. Mashantucket Pequot youth have travelled with the PI to the Arctic for the past few years. Our relationship with the native communities and our commitment to native youth demonstrates our long standing dedication to the cultural and environmental importance of belugas in the wild, their conservation and sustainability.

Given declining beluga populations and the escalating pace of environmental and anthropogenic changes in the Arctic, it is critical to do all we can now and as soon as possible to ensure the sustainability of belugas in the wild. As described above, we are prepared to identify causes, measure impacts, and gather important data to help with the recovery of Cook Inlet belugas and other beluga populations such as the MMPA-depleted Sakhalin Bay-Nikolaya Bay-Amur River beluga population, while ensuring healthy populations remain stable. Many of the threats defined in the ESA recovery plan for Cook Inlet are also likely factors in the productivity problems that lead to a Depleted finding under MMPA. In the same way that our work addresses the ESA threats, it enables investigation of these same factors with minimally-invasive methods on wild whales in Depleted populations.

Mystic Aquarium was proud to host the 2nd International Workshop on Beluga Research and Conservation on March 12-14, 2019 at Mystic Aquarium. Under the direction of PI Romano, the workshop brought together beluga stakeholders and collaborators to focus on research and conservation needs for belugas. The research proposed in this permit helps to address some of those needs brought forward by scientists, natives and management from the US, Canada and beyond. The program book for the workshop is attached. (See Appendix 2 Beluga Workshop Program)

Our program has great capacity for beluga research and increasing the numbers of animals will increase the samples size which will greatly enhance the strength of the data allowing for research to approach statistical significance. We propose this permit as a continuation of our already in-progress beluga research and the proposed permit will allow for strengthening the research and making it more robust. It is our hope that other institutions will become involved over time further increasing the number of samples. A great example of this is Mystic Aquarium's *formal*

institutional collaboration with Georgia Aquarium (See Appendix 1 Georgia_Mystic_Partnership) in which Georgia Aquarium has committed to support sampling on any of the belugas identified on this permit that would need to be moved to their facility if deemed in the best interest of an individual beluga or the US beluga population (for social, health, or welfare reasons), allowing for a contingency should these whales need to be moved without disruption to or compromise of the research. The research that would continue at Georgia Aquarium is identical to the research at Mystic Aquarium, without exception. The animals would continue to participate in all eight proposed research studies. The PI will train Georgia Aquarium personnel for sample and data collection to support all of the studies included in this permit. Behaviors to be trained for this research include presentation of tail flukes and allowing a needle to be inserted for blood collection, exhaling on signal, opening mouth and allowing a swab to occur, opening blowhole and allowing a swab to occur, voluntary stationary layouts and allowing a fecal tube to be inserted into the anus or swabs into the anus or vagina, voluntary stationary layouts allowing an ultrasound to be placed on the body, voluntary stationary layouts allowing suction cups to be placed on body either for electrode placement for hearing studies or the testing of novel telemetry devices, directed swimming to support photogrammetry and diving studies, allowing a flexible tape measure to be gently wrapped around the body to allow measurements, and allowing the skin to be rubbed and swabbed. Samples will be shipped to Mystic Aquarium for analysis. A great benefit to having a formal collaborative research partner in Georgia Aquarium is having the dynamic flexibility to move the belugas there as needed for benefit of an individual beluga or the population for social, welfare, or health reasons with no compromise to the data collection process or research goals.

For **ESA-listed and MMPA-depleted** species, also:

- Discuss why your project must involve ESA-listed or MMPA-depleted species.

Conducting research on belugas with genetics in common with a later-designated Depleted stock lineage may offer more direct insight into basic biology helpful in restoring the wild stock. Studies on the immune system, reproductive biology and behavior, and endocrine and auditory systems would be directly applicable. These studies would also serve as “reference” should the feasibility of studying these whales in the wild occur through future studies. Studying whales from a later-designated Depleted stock also adds another basis for comparison with ongoing studies and samples we have collected previously. We will be able to compare results from these whales to others under professional care and also with wild belugas from Bristol Bay, Chukchi Sea, St. Lawrence, and Cook Inlet. Preliminary investigations on these beluga populations indicates differences in their immune function (e.g. lymphocyte subset counts higher in Bristol Bay whales vs. Chukchi Sea whales) and gene expression in skin (Bristol Bay samples cluster together and form a distinct group vs. Chukchi Sea Whales (Unal et al., 2018). Samples relating this work to Cook Inlet and St. Lawrence belugas are only beginning to be collected through recent collaborations as skin biopsy samples have been obtained from both Cook Inlet and St. Lawrence belugas to compare with gene expression of Bristol Bay and Chukchi Sea whales. Comparisons between belugas under controlled conditions and those from a later-designated Depleted stock lineage is a unique opportunity that rarely presents itself. This is an informative comparison because genetics and environment (geographic location) may influence health and fitness (Fair et al, 2017; Pagan et al., 2018). The ability to study the differences will allow full characterization of the immune system for belugas belonging to depleted stock and comparison with stable populations such Chukchi Sea or Bristol Bay. Hearing studies applied under controlled conditions

on a later-designated Depleted stock lineage will allow for audiograms and masking studies specific to belugas from a Depleted stock lineage and comparison with whales from other populations. The ability to collect photogrammetic data from a later-designated Depleted stock will serve for direct application for photogrammetry studies on wild belugas from this stock. Monitoring reproduction via hormones, behavior, and reproductive organs from a later-designated Depleted stock will allow for increased knowledge on reproduction directly on this Depleted stock and, if calves are born, on the birthing and calf rearing process. A direct example of the value of studying representative whales from a certain population is the case of Tyonek, an orphaned beluga calf from the critically endangered Cook Inlet beluga population that was rescued and rehabilitated. This also offered the unique opportunity to study the immune system, endocrine system, gene expression, hearing, and body condition and growth from a Cook Inlet beluga calf (see Romano et al., 2019 report to NOAA). The ability to study this Cook Inlet beluga throughout its development is key and will contribute to our understanding of the critically endangered Cook Inlet belugas. The ability to study belugas under controlled conditions from a later-designated Depleted stock lineage will do the same for the MMPA-depleted Sakhalin Bay-Nikolaya Bay-Amur River beluga population and contribute to our knowledge on their immune and endocrine systems, body condition and growth, and reproductive status, etc.

In addition, the belugas identified form the only ideal cohort available for the research purposes of this application: all are captive born and have been captive since birth. All are trained or trainable in sampling behaviors and they reside in the largest captive population in the world. We are intently aware that at least one parent of each of these individuals was captured from the wild over a decade or more ago from the Sea of Okhotsk stock that was subsequently designated 3 years ago as Depleted under MMPA. Our research will turn the historical genetic connection with the Depleted stock into the benefit of findings that contribute significantly to the critically important research needs of that and other wild stocks of belugas. Moreover, the movement of these captive-born whales to Mystic Aquarium will create space at Marineland for the wellbeing of their belugas and to accommodate the imminent calving expected as a result of confirmed pregnancies at Marineland.

As mentioned above, Mystic Aquarium collaborates with other AZA accredited aquaria that maintain belugas under professional care and has requested and received beluga samples for research. These samples have been limited to a small number of whales and limited in frequency of sampling due to personnel constraints and limited time available given other priorities in other animal care and training programs. While obtaining beluga samples from other facilities helps to increase sample size for some studies (i.e. those that are easier to obtain such as breath samples), those research studies that take ample training time and a more in-depth effort for sampling are not feasible at other locations including Marineland of Canada and most U.S. facilities. It has not proven feasible to ask other facilities to shift their institutional priorities to train whales for hearing or diving physiology studies, for example. Thus, Mystic Aquarium is seeking to import 5 additional animals from Marineland Canada to reach a minimum N of 6 whales for the research, to allow for a greater likelihood of data achieving statistical significance (one whale currently owned by Mystic in addition to the 5 whales to be imported =6). The other 2 belugas currently under care at Mystic Aquarium will also participate in the proposed research; however, will do so outside the scope of this permit. Since Mystic Aquarium does not own these animals or have an official research partnership with the facilities that own these animals, their continued presence at

the facility cannot be assured for the full duration of this project. Importing these whales is critical, as Mystic Aquarium is seeking to maintain an established population of belugas to ensure continuation of our necessary biological research that will increase general knowledge on beluga whales and inform management plans and recovery for wild belugas including the ESA-listed Cook Inlet DPS and the MMPA-depleted Sea of Okhotsk stock.

The five additional whales from Marineland to be cared for and participate for the primary purpose of research at Mystic Aquarium will enable a minimum sample size of 6 whales (including the whale owned by Mystic Aquarium) enabling a more robust sample size and the possibility for statistical conclusions to be drawn for the studies proposed.

- Discuss how your project will, as applicable:
  - contribute to the objectives identified in the species' recovery or conservation plan or otherwise respond to recommendations of a scientific body charged with management of the species;
  - contribute significantly to understanding the basic biology or ecology of the species; and/or
  - contribute significantly to identifying, evaluating, or resolving conservation problems.

As described in the studies listed above, the significant knowledge gained from our proposed research carried out on belugas under controlled conditions at Mystic Aquarium will benefit the ESA-listed Cook Inlet beluga and MMPA-depleted belugas from the Sakhalin Bay-Nikolaya Bay-Amur River in addition to beluga populations world-wide. As described above, the research proposed will contribute significantly to understanding the basic biology of belugas. Through our research studies we will gain information on belugas' physiological response to stressors and impact on the immune system and health. Importantly, we will be able to transition techniques to measure the response to anthropogenic stressors non-invasively (such as breath) in free ranging whales and monitor health in wild populations. Techniques such as these will "minimize harassment, harm and general adverse impacts on Cook Inlet Belugas" as populations are being monitored and assessed.

Our molecular studies on skin biomarkers can be applied to Cook Inlet Beluga skin biopsies to look at specific cellular pathways that may be compromised specific to Cook Inlet belugas when compared to healthy, thriving beluga populations. Our health studies have contributed and will continue to contribute to live capture-release beluga health assessments and assist with interpretation of data on wild belugas. Controlled studies with Mystic Aquarium belugas have provided baseline ranges of blood parameters, hormones, immune function, and other measures in which biological sampling was carried out behaviorally, without any apparent stress. Moreover, experimental paradigms using belugas under controlled conditions have enabled us to put hormone levels and immune function measurements in context for live capture-released belugas (e.g., cortisol levels after sound exposure vs. live capture-release vs. behavioral blood collection).

Hearing studies will help to increase knowledge on the masking and physiologic effects of noise common to beluga habitats, help define industrial underwater noise emission regulations and help establish management of these activities. Photogrammetry studies conducted on belugas at Mystic Aquarium will help inform, validate and interpret photogrammetry applied to wild belugas with

accurate scores of body condition and pregnancy status for population monitoring. Moreover, we will pilot new telemetry devices and cameras which will be used to track whales and monitor their behavior in relation to the changing environment. Information gained from whales at Mystic Aquarium on breeding, pregnancy, successful birth and calf rearing and the critical early life stages will assist in informing wildlife biologists and managers on outcomes that are more favorable for population recovery.

The research described above addresses the recommendations and objectives for research in the Cook Inlet Beluga Recovery Plan (NOAA, 2016). Through our research we contribute to increased knowledge on the identified "Threats of High Relative Concern" in the Recovery Plan including "Cumulative and Synergistic Effects of Multiple Stressors" and "Noise". Our research on health includes "Disease Agents" identified as a "Threat of Medium Relative Concern" in the Recovery Plan. Our research on disease exposure specifically addresses Brucella, a bacterium that causes reproductive failure. In addition to investigating exposure to Brucella, its presence in post-mortem tissues and monitoring the whales for exposure over time, more research is needed to determine how Brucella evades the immune system and its mode of transmission. Studies can be conducted after obtaining blood samples from whales and isolating immune cells for experiments in vitro to help answer some of these questions making samples from healthy individuals equally valuable in this pursuit as Brucella-infected animals. We are also able to conduct research to address "Threats of Low Relative Concern" in the Recovery Plan for pollution with in vitro studies investigating the impact of different types of pollutants on the beluga immune response.

Project Description

Activity 1: Import of 5 Belugas from Marineland Canada to Mystic Aquarium

Brief Overview:

Whales will be transported from Marineland, Canada to Mystic Aquarium, Mystic, CT, for the purposes of research. This is likely to occur in 2 separate transports (3 whales on one flight, 2 whales on the other flight) within a week period based on available airplane size and the number of personnel that are allowed to attend the flights (to ensure that there is at least 1 experienced staff member per beluga on each transport). Incidental to the research, display of the beluga whales in the Arctic Coast habitat at Mystic Aquarium will occur. The movement of these captive-born whales to Mystic Aquarium will create space at Marineland for the wellbeing of their belugas and to accommodate the imminent calving expected as a result of confirmed pregnancies at Marineland.

Methods:

Mystic Aquarium has extensive experience transporting belugas including large scale moves involving multiple facilities. Transports between facilities generally include a combination of ground and air transport, with air transport as the major mode of transportation. Truck transportation generally serves to move animals to and from airports for air transportation. Air transport with a reputable transport company with a history of live animal transports is used. A cargo configured AirBus A300 (or a jet craft of appropriate size to handle load), pressurized, will fly the beluga from Hamilton International Airport (Ontario, Canada) to Bradley International

Airport (Hartford, CT). We will utilize a licensed broker to ensure appropriate paperwork is in place for the transports. We will apply for a USFWS Designated Port Exception Permit (Form 3-200-2) and request permission to use the Canadian Hamilton International Airport in Ontario, Canada located approximately 55 miles (~1 hour drive time) from the Marineland property to arrive directly at the Bradley International Airport in Hartford, CT. Animals will be loaded on board a chartered jet aircraft for a flight approximately 2 hours in length. Transport plans are designed to minimize time and number of layovers.

Each beluga will be placed into its own transport cradle approximately 30-60 minutes prior to departure from Marineland Canada. The belugas will be supported in a stretcher when in transport cradles. Individual beluga cradles meet and exceed standards set forth by the International Air Transport Association (IATA) and the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS), which regulate the transport of animals. The cradles are 16' or 18' long, depending on the length of the beluga being transported, and are steel reinforced, with a plywood wall, and of fiber glass construction with a fiber glassed coated interior, lined with 1" closed cell foam padding and covered with a custom beaded vinyl liner. These are then filled with fresh water to approximately 26 inches, a level below the blowhole of the whale. There are steel cross braces with chains for suspending beluga stretcher poles. Beluga cradles will be secured with web strapping, onto a flatbed truck and transported to the Hamilton International Airport in Ontario, Canada (approximately 55 miles/1 hour drive time) where they will be loaded on board a chartered jet aircraft for a flight to Bradley International Airport in Hartford, CT, USA. During all truck transportations there are two trained and experienced husbandry attendants safely secured via safety harnesses to the transport carriers who are secured on top of the baffles on either end of the carrier to monitor water temperature, behavior, and wet the beluga's exposed dorsal surface with ladles. On the aircraft, the transport cradle is placed on a 20' 'slim-line' aircraft pallet (provided by the airline) and cribbed on wood construction pallet of 2'x 12's running the length of the pallet and connected via 2' x 12' cross pieces. The cradle is set on cross pieces to distribute load. During the entire transport there are at least 3 trained and experienced personnel accompanying the animal, at least one of whom is a licensed veterinarian with extensive beluga whale experience who can administer emergency medications and provide emergency medical care if necessary. The attending veterinarian will bring a medical kit containing adequate equipment for medical situations including sedatives as well as water/ice to cool animals as needed. While in air transit, attendants don dry suits and alternate, as needed, from outside the transport cradle or stand inside the cradle to assist with calming, handling, or administration of any medical treatment needed. The general monitoring routine during air transit is as follows:

- Feeding not anticipated during transport
- Constant air and water temperature monitoring
- Adjustment of cabin temperature as needed to be maintained at 50 – 60^{o} Fahrenheit
- Cabin pressure will be maintained by the flight crew at or below 5000' ASL
- Attendants will keep the belugas' backs moist, adjust positioning of stretcher as needed, and log data
- Temperature of the water in the transport cradle (as measured with a thermometer suspended in the water) and respiration rates of the belugas will be monitored and logged during the transport

Upon arrival at Bradley International Airport in Hartford, CT., the transport cradle and beluga will be transferred to a flatbed truck for truck transport to Mystic Aquarium (approximately 68 miles/ 1h 15min drive time). Once at Mystic Aquarium, the beluga will be crane lifted with an IC-200 Broderson crane out of the transport cradle, placed on a beluga transport cart, wheeled onto the Arctic Coast habitat, crane lifted out of the transport cart and placed into the medical pool (containing a false-bottom beluga lift) to be released from the stretcher. The transport vehicles will have a police escort to ensure maximum safety.

30,000 lb. / 15 ton fork trucks and cranes under contract from commercial equipment companies will be used at Marineland Canada, Niagara Falls International Airport, Bradley International airport, and Mystic Aquarium. Fork trucks will be used to transfer the beluga cradle from the ground to all flatbed trucks and from flatbed trucks to the ground.
Support vehicles will be used on both ground transport segments to carry additional staff and equipment as needed.

Beluga Transport Unit Specifications	Exterior (inches)			~ Weight Dry (lbs.)	~ Weight animal/stretcher/water/pallet, cribbing, & animal (lbs.)
	L	W	H		
16’	192	60	71	3,114	~13,000
18’	216	65	66	3,630	~17,000

Estimated length of Time To Complete Transport:

Action	Location	Approximate Duration
Stretcher Beluga	Marineland	30 minutes
Load Beluga in Transport Cradle/flatbed	Marineland	30 minutes
Marineland to Hamilton International Airport	Ontario, Canada	Up to 1.5 hours
Clear Customs and Load Beluga onto Aircraft	Hamilton International Airport, Ontario, Canada	2 hours
Flight: Hamilton International Airport to Bradley International Airport	Ontario, Canada to Hartford, CT	2 hours (est.)
Arrive Bradley International Airport	Bradley International Airport, Hartford, CT	
Unload from Plane and onto Trucks	Bradley International Airport, Hartford, CT	1.5 hours
Drive: Hartford to Mystic	Mystic, CT	1.5 hours
Unload beluga/move to med pool/release	Mystic Aquarium	30 minutes
	Total Time	Est. 10 Hours

Activity 2: Research

All studies are planned to be conducted in the beluga habitat at Mystic Aquarium. If it is deemed in the best interest of an individual beluga or the US beluga population for social, health, or welfare reasons, the belugas may be moved to Georgia Aquarium, an accredited organization with experience in the care of belugas. Georgia Aquarium is a collaborative partner of Mystic Aquarium and has committed to providing samples on any belugas in this proposed research should a move to their facility. This collaboration will ensure dynamic flexibility for the management of these animals without compromise to research endeavors or sampling. This arrangement will ensure the optimal welfare of all belugas while also ensuring the research goals are met. The research activities described below will contribute and increase the collective knowledge on belugas and inform management and recovery of depleted and endangered beluga populations, as described above.

The studies described below will be purposefully-conducted independently in order to maintain the voluntary behaviors of the animals.

Study 1 (Neuroimmunological Response to Environmental and Anthropogenic Stressors) Mystic IACUC #13007 and #16006

Brief Overview:

Blood samples will be obtained in order to characterize and monitor the nervous and immune systems of the whales (as well as reproductive hormones for Study 7). In addition, blood samples will also be collected opportunistically before and after events that we anticipate will elicit a physiological response which will help biologically validate assays, reagents and health measurements.

Methods:

Blood Samples
Whales behaviorally present their tail flukes and station for blood collection (up to **55** mls **max** collected **per day** using a sterile 21-g butterfly catheter, well below the acceptable volume threshold for blood collection in mammals) twice a month to characterize and monitor the nervous and immune systems (Routine Blood Samples: 2x month x 12 months/per year = 24 samples per year/**24 takes/whale/year** for use in characterization of the nervous and immune systems, as well as reagent and assay development. Note these samples will also contribute to objectives in Studies 2 and 5. **The maximum number of blood samples proposed for any study in a given day for any/all of these events is 5 blood samples (175 mls max).** For all blood draws, the tail flukes will be cleaned with isopropyl alcohol and 0.2% chlorhexidine solution prior to needle insertion.

Blood will be collected from calves under gentle handling restraint for research only in conjunction with blood collection for needed health assessments. Calves will not be sampled for health assessments, unless medically necessary, until 2 months of age; therefore, this is the earliest that calf blood samples would be collected for research. Calves will be gently handled by staff experienced with working with beluga calves at the surface of the water and the flukes will be held

gently to avoid movement. Up to 20 mls of blood will be collected from calves at each sampling. For neonatal belugas, blood will be collected from the ventral peduncle using a 21-23- gauge sterile butterfly catheter or straight needle, no more than twice a month for the purposes of this study. (2X/month = 24 samples/year/calf). As the calves grow, blood would be solely collected from the ventral flukes, using a sterile 21-g butterfly catheter. For all blood draws, the peduncle or tail flukes will be cleaned with 0.2% chlorhexidine solution and isopropyl alcohol prior to needle insertion.

Samples will also be collected opportunistically that will help characterize and measure the physiological response to challenges such as transport, out of water events, veterinary examinations, novel social interactions and training exercises, as described below:

- Blood will be collected at 4 time points throughout the day, 4x per year seasonally to investigate circadian rhythms of hormones and immune function. Blood samples for diurnal variation assessment: 4 time points per day x 4 days/per year (one in each season) = 16 samples per year or **4 takes per whale/year.**
- For transport events, blood will be collected once before transport and immediately after transport before the whale enters the new habitat. Thereafter, blood will be attempted behaviorally at 12 and 24hrs post transport. Blood samples before and after transport: 4 time points (1 baseline **(approximately 2-24 hours prior to transport),** 1 upon arrival, 2 post- transport **(12 and 24 hours)** – under behavioral control) = 4 samples per transport or **3 takes per whale/year.**
- For other out of water events (OWE) such as being lifted out of the water via a hydraulic lift for weights or veterinary examinations, blood will be sampled before **(30 min – 24 hours prior)**, one time point during and attempted at 3 time points **(1, 2, and 4 hours)** post (OWE) under behavioral control.
  Blood samples for OWEs associated with being lifted out of the water via a hydraulic lift for weights or veterinary examination: 5 time points x 4 OWE (one in each season) = 20 samples per year **or 8 takes per whale/year**.

- Blood samples will be obtained before and after novel vs control learning/training sessions to monitor the physiologic response to novel tasks and learning, and/or novel social interactions. Two blood samples (1 before and after training sessions) x 12 sessions (6 control and 6 experimental) x 3 novel training exercises/and or social interactions (6 control and 6 experimental) x 3 samples per year or 1 session per day/12 sessions/3 novel training exercises/and or social interactions **= 36 takes per whale/year**.

For all of the above, plus those described under Study 5 (16 blood samples per whale per year or 8 takes per whale per year) blood sampling will not exceed 152 samples per year per whale or 83 takes per whale/year, all done on a voluntary basis under behavioral control (except for when out of water for OWE events and arrival upon transport).

Catecholamines and stress hormone levels will be measured by High Performance Liquid Chromatography and radioimmunoassay as in previous studies (Romano et al., 2004; St Aubin et al., 2013; Thompson et al., 2014). The immune system will be evaluated by flow cytometry, utilizing marine mammal-specific antibodies that we and our colleagues have developed (Romano et al., 1992, 1999, 2004; De Guise et al., 2002; Spoon and Romano, 2012; Bossart et al., 2008) to quantitate lymphocyte subsets. Functional assays will include lymphocyte proliferation,

phagocytosis and respiratory burst, natural killer cell function, and ELISA for Brucella antibodies (Meegan et al., 2010; Keogh et al., 2011; Spoon and Romano, 2012; Romano et al., 2002; DeGuise et al., 1997). Molecular biology, tissue culture, and protein chemistry will be used to develop new "tools", reagents and assays for investigating the marine mammal immune system and its response to stress and the environment.

Immunophenotyping: Antibodies against cetacean T and B lymphocytes will be used to quantitate and monitor lymphocyte populations. Isolated beluga peripheral blood lymphocytes will be washed 3x with Hank's Balanced Salt Solution (HBSS), pH=7.4 before incubation with dolphin or beluga-specific monoclonal or polyclonal antibodies. Cells will be re-suspended at a concentration of 0.5-1x10^6 cells per ml for each antibody incubation, and antibodies will be titrated for optimal working concentrations. After a one hour incubation with the primary antibody, cells will be washed in HBSS 3x before a 1 hour incubation with mouse Ig-specific secondary antibody conjugated with fluorescein isothiocyanate or phycoerythrin. For double labelling studies, alantibodies will be labelled directly with fluorochromes to permit 2 color fluorescence of 2 monoclonal antibodies at the same time. Cells will be washed twice in phosphate buffered saline and fixed in 1% paraformaldehyde. The lymphocyte population will be gated and analyzed on a flow cytometer. Histograms statistics will be obtained.

Natural killer cell function of peripheral blood lymphocytes**:** Target cells (YAC-1 and/or K-562) (1x10^6 cells/ml) will be labeled with 3mM DIO (3, 3-dioctadecyloxacarbocyanin) for 20 min at 37°C in the dark. Target cells will be washed twice in media and re-suspended in 10 ml medium at a final concentration of 1x10^5 cells/ml. Marine mammal effector cells (PBMC) will be isolated from peripheral blood, washed and brought to a concentration of 1x10^6 cells/ml. Effector cells and labeled targets will be mixed at ratios of 100:1, 50:1, 25:1, and 12.5:1 and incubated at 37°C for 6 hours. Cells will be washed and re-suspended in 500 ul PBS containing 50 ug/ml Propidium Iodide (PI). Cells will be read on a flow cytometer utilizing FL1 (for DIO) and FL2 for PI. Percent cytoxicity will be calculated and compared among experimental groups. This assay can be adapted to measure cytoxicity of CD8+ cells as well as Natural Killer cells. Other methodologies for measuring cytotoxicity will also be explored (e.g. MTT). The more traditional chromium release natural killer cell assay will be compared with the technologies developed above on cetacean cells for comparison, especially in regards to sensitivity.

Lymphocyte Blastogenesis of peripheral blood lymphocytes: Isolated beluga mononuclear cells will be cultured with varying concentrations of the T cell dependent mitogens, Con A and PHA and the B cell dependent mitogens STM and LPS for 24-96 hours. For each mitogen, the optimal experimental conditions have been or will be been determined by evaluation of dose-response curves. After 24, 48, 72, and 96 hours, cells are pulsed with BrdU a thymidine analog. Cells will be fixed and lysed for colorimetric detection of BrdU incorporated into new DNA produced during each cycle of cell division. Stimulation indices will be calculated by comparing optical density values for cells exposed to mitogens as compared with a control group (i.e. no mitogen exposure).

Phagocytosis and Respiratory burst activity of neutrophils and monocytes: Briefly, bacteria e.g. *Staphylococcus aureus* will be grown-up in Tryptic Soy Broth, heat killed, brought to a concentration of 1x10^9/ml in PBS and labeled with 100 ug/ml of propidium iodide. Thirty microliters of the PI-labelled *S. aureus* will be incubated with 100 ul of beluga whole blood and

placed in a shaking incubator for 10 min at 37°C. Twenty-five microliters of 5 uM (2'7'-dichlorofluorescein diacetate) is then added and incubated for 5-30 min in a shaking water bath at 37°C. Red blood cells are lysed by adding 2 mls 0.17M Ammonium Chloride 0.01M Tris 0.001M EDTA solution, pH 7.4. Cells are then washed in PBS and fixed in 1% paraformaldehyde. Cells will be read on a flow cytometer utilizing FL1 (for DCFDA) and FL2 for PI. The percent phagocytosis and the mean fluorescence for respiratory burst will be collected on 10,000 events (neutrophils and/or monocytes) and percentages compared between experimental groups.

Catecholamine and hormone assessment: Plasma will be analyzed for several monoamines and metabolites, including norepinephrine, epinephrine and dopamine, by High Performance Liquid Chromatography with electrochemical detection. Adrenocorticoids (including aldosterone, which has been shown to be elevated during periods of stress in odontocetes), cortisol and ACTH will be measured by radioimmunoassay or enzyme immunoassay (Romano et al., 2004; St Aubin et al., 2013; Thompson et al., 2014). Thyroid hormone and reproductive hormones as measures of metabolic and reproductive health will be measured via enzyme immunoassay (Flower et al., 2015; Richard et al., 2017).

Study 2 (Development of novel non-invasive techniques to assess health in free-ranging, stranded and endangered belugas) Mystic IACUC #16006

Brief Overview: Whales will be trained for blow, saliva, fecal, and skin scraping collection in order to develop and validate non-invasive methods for assessing health in belugas with transition for health monitoring in wild whales. Methodologies for hormone analysis, molecular biomarkers, and/or immune components will be validated both in the laboratory and biologically. Collection methods for blow will be investigated to inform transition to wild belugas.

Methods:

Breath Samples
Breath samples will be collected behaviorally by having the whale exhale into an open petri dish covered with a nylon membrane held 3-4" over the blowhole for a duration of 2-4 exhales. The membrane will be placed in a 15 ml conical tube and centrifuged (4000 rpm, 30 min, 4°C) and the expelled condensate transferred to a sterile cryovial and stored at -80°C until further processing in the laboratory (see Thompson et al, 2014 for details).

<u>Initial Transition to Collection of Breath for Free-Ranging Belugas</u>
a) Determination of maximum distance from blowhole that will provide adequate volume and signal for hormones and molecular targets. Whales will station with their heads on the exhibit beach and the breath collection plate will be held (as a start) at 2, 4, 8, 12, 16, 24, 30 and 36 inches above the blow hole. Sessions will be repeated 3 times. Following collection, samples will be assessed by volume recovered. Samples with adequate volume for hormone assays (i.e. ≥80ul) will be assayed for cortisol and urea (as standard protein) to determine quality of the sample. Adjustments in distance will be made according to results obtained and tests repeated if necessary.

b) Test of collection methods within aquarium setting prior to field collection

In order to take the first steps in transitioning breath collection to free swimming belugas (or toothed whales in general) in the wild, a proof of concept will be carried out on whales under controlled conditions at Mystic Aquarium. The feasibility of collecting breath 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 90 mm 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 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 and 2) a person holding the pole from a small boat. The pole will be held approximately 6-16 inches above the blowhole as the whale surfaces. Breath collection attempts will occur opportunistically when whales surface at appropriate distances. Breath 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.

For assay development, validation and measurement, breath samples will be collected twice per week (Breath Samples: 2x per week x 50 weeks/yr x 3 plates per session = 300 samples/year or **100 takes per whale/year)**. Breath collection is defined as either passive collection of respiratory exhale or 3-4 forceful chuffs given voluntarily into a collection vessel.

In addition, breath will be sampled at 4 time points throughout the day and at a minimum of 4x per year seasonally to investigate circadian rhythms of hormones and immune function (Breath samples for diurnal variation assessment: 4 time points per day x 4 days/per year (one in each season) = 16 samples per year or **4 takes per whale/year**.

For transport, breath will be collected **approximately 30 min-24 hours** before transport and then at two hour intervals throughout the transport passively, without restraint, followed by collection upon arrival and post transport under behavioral control at 1, 2, 4, 6, 12 and 24 hours (Breath samples before, during and after transport: 13 time points (1 baseline, 2, 4, 6, 8, 10 hr during transport, 1 upon arrival, 1, 2, 4, 6, 12, 24 hr post transport = 13 samples) per transport or **3 takes per whale/ year**.

Concurrent with blood, saliva, and feces, breath samples will be collected from out of water events associated with being lifted out of the water via a hydraulic lift for weights or veterinary examination. OWE breath samples will be collected **approximately 30 min-24 hours** before and at 30 min during the OWE followed by various time points such as 30 min, 1, 2, 4, 6, 12 and 24 hours post OWE (Breath samples for OWEs: 9 time points x 4 OWE (one in each season) = 36 samples per/whale/year or **3 takes/OWE = 12 takes/whale/year.**
Breath samples will be collected before and after novel training sessions and/or novel social interactions. Breath samples for novel training exercises and/or novel social interactions: 2 samples (1 before and after training session) x 12 sessions (6 control and 6 experimental) x 3 novel training exercises and/or social interactions = 72 samples per whale/year **or 1 session per day/12 sessions/3 novel training exercises/and or social interactions = 36 takes per whale/year**.

Breath samples will be collected in order to initiate transition to collect blow on wild whales. Breath samples will be collected at 2, 4, 8, 12, 16, 24, 30 and 36 inches from the blowhole to determine the greatest distance with detectable biomarkers. **Samples will be collected during the same sampling session and in close approximation in order to avoid confounding effects of diurnal changes in hormones or exposure to stimuli between sessions. Up to four heights will be tested in a single day (each with 1 sample per height). All samples will be collected within a period of approximately 3 minutes.** Breath Transition: 8 samples x 3 replicates over 2 days = 48 samples each whale/year **or 6 takes/year/whale**. Six samples collected utilizing a pole from the beach and from a boat (6 breaths x 2 sampling methods= 12 samples/whale/year **or 12 takes/whale/year**). **Total = 60 samples/whale/year or 18 takes per whale/year.**

The maximum number of breath samples per day in the proposed studies is 12 (Transport and post transport sampling- without the 24-hour time point).

For calves, breath samples will be passively collected when beluga calves are handled for health assessment purposes starting at 1 week of age by holding the open petri dish 3-4" over the blowhole while the calf exhales up to 5 times. This will occur a maximum of once weekly (1X/week x 50 weeks = 50 samples/year/calf).

The commercially-available serum cortisol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) validated in our laboratory will be used to measure cortisol levels in cetacean blow (Thompson et al., 2014). Commercially-available serum hormone EIA kits will be validated in our laboratory to measure stress, metabolic and reproductive hormone levels in marine mammal blow/breath. The kits will be validated using standard validation protocols including tests for parallelism using serial dilutions to detect the presence of substances that interfere with antibody binding, spiking tests with known standards to compare observed and expected values, determination of sensitivity limits using serial dilution, and calculation of intra-assay coefficients of variation (Simontacchi et al., 1999; Amaral, 2010).

Saliva Samples

Belugas will voluntarily open their mouths for saliva collection with a designated swab. For characterization and monitoring studies saliva will be collected twice per week (saliva samples 2x per week x 50 weeks/year = 100 samples/year or **100 takes/whale/year**).

Saliva will also be sampled at 4 time points throughout the day, at a minimum of 4x per year seasonally to investigate circadian rhythms of hormones and immune function (Saliva samples for diurnal variation assessment: 4 time points per day x 4 days/per year (one in each season) = 16 samples per year or **4 takes per whale/year**.

For transport, saliva will be collected **approximately 30 min-24 hours** before transport and immediately after transport, followed by collection at 1, 2, 4, 6, 12, and 24 hours post transport. (Transport saliva samples: 8 time points /transport = 8 samples per transport) **or 3 takes per year/whale**).

Saliva samples for out of water events associated with being lifted out of the water via a hydraulic lift for weights or veterinary examination will be collected **approximately 30 min -24 hours** before the OWE and at 30 min, 1, 2, 4, 6, 12 and 24 hours post OWE (Saliva samples for out of

water events associated with being lifted out of the water via a hydraulic lift for weights or veterinary examination: 8 time points x 4 OWE (one in each season) = 32 samples per year **or 12/takes/whale/year**.

Saliva samples will be collected before and after novel training sessions and/or novel social interactions (Saliva samples for novel training exercises: 2 samples (1 before and after training session) x 12 sessions (6 control and 6 experimental) x 3 novel training exercises and/or social interactions = 72 samples per year) **or (or 1 session per day/12 sessions/3 novel training exercises/and or social interactions = 36 takes per whale/year**.

For calves, saliva will be collected under gentle handling at the water surface by a person experienced in calf handling. Saliva will be obtained by gently opening the mouth by inserting a gloved hand into the side of the mouth, and then a swab introduced to collect the saliva. Calves will be sampled at a maximum of once weekly during handling for health assessment beginning at 2 months of age (1X/week x 40 weeks = 40 samples/calf/year in the first year; then 1X/week X 50 weeks = 50 samples/calf/year beginning at an age of 2 years).

For all animals, saliva will be collected by wiping the roof and upper gum line of the mouth with a collection swab comprised of an inert polymer (Salimetrics, LLC, State College, PA, USA) while exercising caution not to contact pooled water at the back of the mouth. The collection swab will be placed into the supplied collection tube and kept on ice until it can be centrifuged (4000 rpm, 30 min, 4^{o}C) to remove the saliva from the swab. Saliva will be transferred to a sterile cryovial and stored at -80^{o}C until processing.

Commercially-available serum hormone EIA kits will be validated in our laboratory to measure stress, metabolic and reproductive hormone levels in beluga saliva. The kits will be validated using standard validation protocols including tests for parallelism using serial dilutions to detect the presence of substances that interfere with antibody binding, spiking tests with known standards to compare observed and expected values, determination of sensitivity limits using serial dilution, and calculation of intra-assay coefficients of variation (Simontacchi et al., 1999; Amaral, 2010).

Fecal Samples

Whales will voluntarily layout and accept a tube for collection of feces. Fecal samples will be collected twice weekly for characterization and monitoring purposes (2x per week x 50 weeks = 100 samples/year/whale **or 100 takes/year/whale**).

Fecal samples will also be collected **approximately 30 min - 24 hours** before transport with subsequent attempts made at 6, 12, 24, 36 and 48 hours post transport (Fecal samples before and after transport: 6 time points (1 baseline, 6, 12, 24, 36 and 48hr post transport = 6 samples per transport **or 3 takes/whale/year**).

Fecal samples will be collected in association with out of water events e.g. being lifted out of the water via a hydraulic lift for weights or veterinary examination. Samples will be collected **approximately 30 min - 24 hours** before the OWE and post OWE with sampling attempts made at 12, 24, 36 and 48 hours (Fecal samples associated with OWEs: 5 time points x 4 OWE (one in each season) = 20 samples per year/whale **or 12 takes/whale/year**).

Non-intrusive fecal sampling will not exceed **115** takes per year per whale.

Feces will be collected by using a 5-mm diameter lubricated soft plastic tube inserted approximately 15 cm into the rectum. After collection, the fecal samples will be placed in 8 mL cryovials or plastic bags and kept on ice before being stored at -80°C until processing.
Calves will not be restrained for fecal collection. Calves will be monitored and, when they defecate in the pool, feces may be collected opportunistically without touching the calf. Feces may be collected up to 2X/week by this free catch methodology. (2 samples/week x 50 weeks = 100 samples/calf/year).

To control for varying moisture content, fecal samples will be freeze-dried, pulverized, and sifted to remove larger, undigested food particles (Wasser et al., 2000). Glucocorticoids will be extracted from the dried feces using 90% methanol (Wasser et al., 2000). Commercially-available EIA kits for will be validated in our laboratory to measure stress, metabolic and reproductive hormone levels in beluga feces. The kits will be validated using standard validation protocols including tests for parallelism using serial dilutions to detect the presence of substances that interfere with antibody binding, spiking tests with known standards to compare observed and expected values, determination of sensitivity limits using serial dilution, and calculation of intra-assay coefficients of variation (Simontacchi et al., 1999; Amaral, 2010).

Skin scrapings
Skin scrapings will be collected for transcriptomic analysis from consistent locations on the whale (i.e. along the dorsal ridge on either side of the midline, the flukes, the head region (near the blowhole), or ventrally on either side. Skin scrapings will be collected up to 4x per week, **all on different days** (Non- intrusive sampling-Skin scrapes 4x per week x 50 weeks = 200 samples/year). Non-intrusive skin sampling will not exceed 200 takes/year/whale.

For calves, skin scraping samples would be collected opportunistically when calves are handled for health assessment beginning at 1 week of age. This will occur a maximum of once weekly (1X/week x 50 weeks = 50 samples/year/calf). Unlimited samples may be collected opportunistically as skin is shed naturally during the molt.

Similar to published methods for dolphins (Bechsoft et al., 2015), a rubberized spatula will 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 spatula along the skin of the whale once using single, not overly forceful strokes. Skin scrapings will be preserved in five volumes of RNAlater® solution aliquoted into 2.0ml cryovials. Samples will be kept at 4°C for one day then transferred to -80°C until processing for RNA extraction.

RNA will be extracted by using AurumTM Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, Hercules, CA), and RNA samples will be cleaned by using RNeasy® Mini and Minelute kits. RNA concentration and purity (A260/A280 ratio) will be measured by using Epoch™ Microplate Spectrophotometer. Following RNA quantification and quality-control analysis, strand-specific mRNA libraries will be prepared by using TruSeq® Stranded mRNA Library Preparation kits (Illumina), indexed for different individuals. RNA transcriptome sequencing will be carried out at the Genome for Center Innovation at University of Connecticut on Illumina NextSeq® 500

instrument by using its 150 cycle (High Output) sequencing kit with a paired-end protocol. Following the assessment of reads for quality and trimming, the reads will be mapped to the published reference beluga genome (Jones et al, 2017) with Bowtie2 (Langmead et al, 2012). Transcript assembly, abundance estimates, and differential expression analysis will be carried out by using Cufflinks (Trapnel et al, 2010), followed by functional annotations using Blast2GO suite (Conesa et al, 2005) and the metabolic pathway analysis by using KEGG Pathway Database (Kanehisa, 2000).

Study 3 (Hearing and Physiological Response to Anthropogenic Sound) Mystic IACUC #14006

Brief Overview: Belugas will behaviorally station and receive suction cup electrodes for collection of auditory brainstem evoked potentials (AEPs) under baseline and sound exposure conditions to examine the effect of different sound sources on hearing. Blow samples will be collected before and after experimental sessions to study the physiological response to different sounds.

Methods: Beluga hearing will be collected in sessions lasting 45 minutes each. Each session will include several frequencies, and each frequency sampled constitutes a trial. Each trial involves the beluga voluntarily stationing with minimal movement except for breathing for up to 3-5 minutes. **Baseline hearing sessions involve AEP collection, and masked hearing trials involve concurrent noise playback and AEP collection. During each session, the sampled beluga will have 3 silicone suction cups containing electrodes** coated with conductive gel gently placed on the body. The suction cups will be placed near the melon, near the beginning of the dorsal ridge and posterior to the maximum girth. Calves of whales listed in this permit will be tested for hearing and monitored throughout development starting at 4 weeks of age. Calves will be held at the surface in the arms of husbandry personnel for suction cup electrode placement and baseline AEPs.

Background noise at the Mystic Aquarium beluga facilities station where previous hearing research has been completed is 102.12 dB re 1micro Pa (100 Hz to 50 kHz range). Non-target animals will be gated or moved to separate pools. The acoustic power of the proposed underwater speakers (both source level and directionality of the projection driven by the size of the speakers and the wattage) is not strong enough to exceed the baseline background noise levels of the facilities in adjacent pools, where the rest of the belugas will be housed while the experiment is ongoing. If the speaker is projecting noise towards this station, which is away from any connecting gate to other pools, the indirect noise of the projection will be largely attenuated before it propagates into the adjacent pools (it is not simple spherical or cylindrical spreading, as there is no direct path from the speaker to the gates, and the gates are a confined funneled space for noise to propagate through).

Hearing will be tested using standard AEP procedures: **Recording AEPs is a well-established procedure. We will conduct AEP hearing tests following Castellote et al., 2014; Mooney et al., 2018, (for belugas) and other AEP odontocete studies. We first need to establish baseline audiograms for these animals. This will include testing from 4-180 kHz. Likely frequencies will include: 4, 5.6, 8, 11.2, 16, 22.5, 32, 45, 54, 80, 100, 120, 128, 150 and 180 kHz. These are**

typically octave and half-octave increments. On occasion we may use finer increments, such as quarter-octaves, if there is a need to understand a particular part of an audiogram better.

For the masking study we expect to conduct hearing measurements on the same increments as above, but we do not expect to go as high in frequency, thus this will be a limited frequency range. As masking typically occurs in critical bands (but we must confirm this for each anthropogenic noise) we do not expect to measure much higher than an octave above the maximum higher ⅓ band of the noise. This would be 22 kHz with respect to the commercial ship noise. Going just higher than this we expect the auditory measurements to not go higher than 32 kHz for all other anthropogenic sounds.

Clicks will be used in addition to pips. This is standard for audiometry to ensure animals hear and a response can be generated, because these pulses span a broad range of frequencies.

Sessions can last 45 minutes and will include several trials that last 3-5 minutes each. A maximum of 3 sessions per day are requested. Noise playback will occur only during the trial periods in each session. The number of AEP trials completed in each session, and consequently sound-on times is dependent on the animal's hearing ability. If an animal only hears to 50 kHz, fewer trials will be needed to complete the sampling compared to if an animal hears up to 150 kHz. The number of trials for a particular animal to acquire an audiogram (based on wild belugas that hear very, very well) was ca. 60-80. There are 1000 sweeps (tone pip or click series) per record (trial). That can change with certain methods of objective response detection used in the ANSI AEP hearing standards (ANSI 2018). On the high side, that would be ca. 80,000 sweeps per audiogram. Although all of these are at, near, or below threshold, in approximately 20% of them the animal cannot hear (and in that case the animal does not generate an AEP response). We estimate about 5 frequencies per masking study, so about 40 records and 40,000 tone pips for each set of hearing measurements.

Using a sound stimuli based on a sinusoidally amplitude modulated tones (SAM) of specific frequencies and amplitudes, whales will be exposed to two types of sound projections: 1) synthetic tones designed to test specific frequencies at specific amplitudes and 2) real sound sources recorded in the field (e.g. ship noise, pile driving, etc.)

Using tones as masking sounds will allow for exploring what frequencies are more sensitive to masking. Using real recordings of noise sources will allow for exploring how structured sound signals impact hearing.

An underwater speaker system will be used at 0.1 to 0.5 m depth and minimum distance to target whale of 1 m (frequency response 200 Hz to 23 kHz, maximum output level of 180 dB re 1 µPa @ 1m). Signal duration and duty cycle will depend on the tolerance level of the trained whale to remain on station, with maximum exposure in each trial always determined by the trainer. The sound stimulus to measure hearing thresholds consists of 20 ms modulated tones followed by 30 ms break, presented as a 20/s tone burst. Sound levels will gradually increase from low intensities up to hearing threshold, thus tend to be inherently low amplitude and often below detectable levels by the whale. AEP hearing data will be collected during normal background noise and noise-exposure sessions.

The second set of sounds will be anthropogenic noise recordings such as those from Cook Inlet, AK. Noise sources include recordings from: Commercial ship, Dredging, Jet aircraft (commercial or military non-fighter) (recorded underwater), Jet aircraft—military fighter (recorded underwater), Outboard motor (small skiffs, rafts), and Pile driving (impact hammer method) at different distances from 400m – 2 km. In all cases, pile strikes maintain their fast energy rise and short duration characteristic of impulsive signals (Castellote et al., 2018) (Table 1). Potential and desired noise sources include some of those recently reviewed in the paper led by CoPI Castellote et al., 2018 (Anthropogenic Noise and the Endangered Cook Inlet Beluga Whale, *Delphinapterus leucas*: Acoustic Considerations for Management, MFR, 2018. doi.org/10.7755/MFR.80.3.3) which is the most comprehensive published review and characterization of noise in beluga habitats. Measurements of the hearing and physiological response to such noise sources is critical to address noise impacts on belugas. Impulsive sources, particularly pile driving, is an important noise disturbance in beluga habitat, and thus it is a desired type of noise to include in this research. Noise exposure in all experiments will be designed to avoid reaching TTS levels based on the metrics described by the 2018 Revision to: Technical Guidance for Assessing the Effects of Anthropogenic Sound on Marine Mammal Hearing (NMFS, 2018). These include cumulative sound exposure (SEL_{cum}) including a weighting factor adjustment, and peak values (dB PK). AEPs will not exceed 5 full audiograms/whale/year under normal background noise conditions. Noise-related AEP hearing tests will follow each noise exposure session. Anthropogenic noise exposures described above will be projected 1 m in front of the whale (0 degrees), but other sessions will be done with the speaker at 1 m and 90 degrees from the animal midline; and **4-5** m directly behind the whale **(180 degrees) simulating a beluga turning its head (or its body in the case of 180 degrees) away from the noise source**. Exposure of the sound stimuli and fatiguing noises will be limited to a maximum of 3 sessions of 45 min each per day, with at least a rest day included every 3 consecutive days of sessions in experiment schedules. All playbacks on a given day will use the same type of noise source, impulsive or non-impulsive, to simplify the calculation of accumulation of exposure. It is important to note that even if the session could last 45 minutes, the speaker will only be active when the whale is on station **for each trial sampling**, and thus the amount of time with the beluga positioned on station and exposed to playbacks will always be much shorter than 45 minutes. We expect that exposure during the session will not exceed 15 minutes in total based on previous beluga playback training experience. A maximum of 3 **masking** sessions per day is planned, but may be reduced to 2 or 1 sessions per day, or the session duration will be shortened, as dictated by the belugas. Masked hearing experiment schedules will be designed to avoid reaching TTS levels following the NOAA acoustic guidelines (NMFS, 2018), and as explained below.

With regards to source levels, the aim of the research is to quantify masking, thus Temporary Threshold Shift (TTS) is not a desired outcome of our noise exposures. Because we do not have all the session designs already defined (source of noise, duration of exposure **trials**, source level, angle of exposure, etc.) we will calculate the SEL_{cum} over the full exposure duration of all **trials** planned **in up to 3 sessions** per day (total playback time from **all trials of** each session accumulated over 24h) once designed, to make sure we never reach TTS thresholds at 1 m (whale station). As a precautionary approach, we will set a maximum SEL_{cum} equivalent to 10 dB below the TTS threshold. Following the NOAA Technical Guidance for Assessing the Effects of Anthropogenic Sound on Marine Mammal Hearing (Version 2.0) (NMFS, 2018), beluga whales fit into the mid-frequency (MF) cetaceans hearing functional group, and thus their TTS threshold

is 178 dB (SEL_{cum}) for non-impulsive sources, 170 dB (SEL_{cum}) for impulsive sources, and 224 dB (PK), therefore our maximum exposure at 1 m will never exceed 168 dB (SEL_{cum}) for non-impulsive sources, 160 dB (SEL_{cum}) for impulsive sources over the 24 hours (including all sessions within a day) or 214 dB PK. Because our maximum SL cannot exceed 180 RMS dB (transducer physical limitation), it will be impossible to exceed 214 dB PK under any session design, therefore peak values will not be considered for TTS risk. **We will follow the TTS definition from NMFS Technical guidance of at least 6 dB shift in threshold and hearing will be measured approximately 1 minute after the end of the exposure trial. Using the user spreadsheet tool (NMFS 2018), we will calculate what exposure is expected to trigger TTS, which we will make sure is never reached in any exposure trial. In the unlikely event of detecting TTS at any frequency tested just after an exposure trial, we will stop the study, reassess what was the actual SELcum reached for that whale that day, and define how much we need to increase our buffer to avoid further TTS. We are currently proposing to set a buffer of 10 dB below the onset of TTS identified in the user spreadsheet tool (NMFS 2018).Two playback session scenarios are presented below, as an example of the application of the TTS -10dB threshold limit (=160 dB for impulsive and 168 dB for non-impulsive).**

Example 1: impact hammer pile driving playback with a SL of 157.2 dB (SEL for 1 strike)

- 2 sessions in 24 h **including several trials per session**
- Exposure duration is 3 minutes (i.e., **3 trials of 1 minute as** total time of whale on station within a 45-minute playback session). These exposure times will be calculated for each session or day and exposure time is/will be controlled by trainer holding the whale on station..
- Interval between strikes 3 s

Based on these characteristics, the total number of strikes exposed to the whale in 24 h will be 60 x 2 = 120. The unweighted SEL_{cum} for the total amount of strikes exposed to the whale corresponds to: 157.2+10x(log10(60x2))=178 dB. [Note: the spectra data collected in Cook Inlet will be different than the spectra of the recording projected through a transducer into an enclosed facility. For simplicity purposes and to avoid extended calibration efforts in the facility,simplified weighting is being used.]

Based on a weighting factor adjustment of 2 kHz as recommended in the user spreadsheet tool (NMFS, 2018) for impact pile driving, the correction factor corresponds to -19.7 dB, thus the weighted SEL_{cum} will be 178-19.7=158.3 dB

This value is lower than our buffer. Therefore, as far as that only 2 sessions are run with a maximum of 3 minutes of exposure on station (1 m from transducer) per session, the 10 dB buffer to the TTS threshold will be maintained (or essentially matched in this case).

Example 2: Ship noise exposures with SL of 143 dB (SPL)

- 2 sessions in 24 h **including several trials per session**
- Exposure duration is 12 minutes and 45 seconds (i.e., **3 trials of 3 minutes and 1 trial of 3 minutes and 45 seconds, as** total time of whale on station while playback within the 45 minute session)

Based on these characteristics, the total exposure time to the whale in 24 h will be 765s x 2 = **1530**s. The unweighted SEL_{cum} for this time of exposure corresponds to: 143+10x(log10(765x2))=174.8 dB

Because NMFS (2018) does not provide a default weighting factor for ship noise, this will be calculated by calibrating this specific noise source playback at the whale station directly and the obtained spectrum will be used to calculate a new weighting factor. For the example, a weighting factor adjustment is used - 2.5 kHz as recommended in the user spreadsheet tool (NMFS 2018) for vibratory pile driving (the closest of the available examples for a continuous source), that is -16.8 dB, thus the weighted SEL_{cum} will be 174.8-16.8=158.0 dB.

This is lower than our 168 dB non-impulsive buffer. Therefore, as far as only 2 sessions are run with a maximum of 12 minutes and 45 seconds of exposure on station (1 m from transducer) per session, the 10 dB buffer to the TTS threshold will be maintained.

When conducting the AEP measurements, the recent 2018 marine mammal ANSI standards will be taken into account; however they will not be relied upon entirely as those measurements are explicitly for audiograms, i.e., to enable comparisons of audiograms between animals and species. Co-PI Mooney was on the team for writing those standards and in general the standards will be applied when appropriate. Because experimental, masking studies are being addressed, AEP measurements might be tweaked to improve electrophysiological signal-to-noise, or for other similar reasons (Supin and Popov, 2007; Mooney et al., 2015).

Baseline hearing sessions: 5 sessions/year/whale.

Masked hearing sessions: up to 3 sessions per day, 6 days per week, 2 weeks **per month, for 3 months per year**, totals = 108 **sessions**/year/whale (**36 takes/whale/year**).

AEPs will not exceed 113 **sessions**/whale/year (Baseline + Masked) (**41 takes/whale/year**).

For offspring calves of the belugas listed in this permit, baseline hearing sessions to monitor and record hearing will be tested monthly beginning at 4 weeks of age for the first 6 months in order to assess hearing development (1X/month X 6 months = 6 hearing sessions in the first year of life). Beginning at age 2, an annual hearing session will occur to monitor hearing over time (1 session/calf/year, over the next 5 years.

Blow samples will be collected voluntarily before and after each sound exposure session (Breath samples for physiological response to anthropogenic sound: Breath samples will be collected behaviorally by having the whale exhale into an open petri dish covered with a nylon membrane

held 3-4" over the blowhole for a duration of 2-3 exhales (with no more than 10 breath sample attempts/whale/day). The membrane will be placed in a 15 ml conical tube and centrifuged (4000 rpm, 30 min, 4°C) and the expelled condensate transferred to a sterile cryovial and stored at -80°C until further processing in the laboratory as described in methods for Study 2 above.

Baseline hearing sessions: 5 sessions/year/whale x 2 breath samples (1 before and 1 after) = 10 breath samples per year.

Masked hearing sessions: up to 3 sessions per day x 6 days per week x 2 weeks **per month, for 3 months per year** = 108 **sessions**/year x 2 breath samples (1 before and after) **=** 216 breath samples per year.

Study 4 (Photogrammetry Body Condition Studies) Mystic IACUC #17007

Brief Overview: Belugas will be trained to swim underneath a camera and to station for morphometric measurements and weights. A series of photographs and measurements for each whale will be provided to researchers conducting photogrammetry on wild belugas in order to help develop, ground-truth, and interpret photogrammetry on wild belugas.

Methods:

Belugas will be trained to swim from Point A to Point B, underneath a camera (Sony 35 mm OSS Lens and Sony A5100 Digi Cam Body) with a fixed focal lens mounted 6 m above the surface of the water. Multiple photographs will be taken as the whale swims underneath the camera. Thirty photographs will be taken in total during training sessions over the course of a month each month. (Non-intrusive sampling-Photogrammetry: 30 photographs/month x 12 months/year = 360 takes/year) which will provide at least 3 photographs that are appropriate for photogrammetry measurements. Non-intrusive photogrammetry sampling will not exceed 360 takes per year per whale. For up to 2 pregnant females, 10 photographs/week will be taken each week (X52 weeks/year) to yield 520 takes/whale/year.

Belugas will be trained to station once per week on the same day as when the aerial photographs are taken. A measuring tape will be used to measure the straight length of the beluga, the distance from the caudal blowhole to the start of the dorsal ridge and girth at 6 different landmarks. The width at each landmark will be measured. Three repeated measurements will be conducted for length and widths per landmark to assess the individual measurement error. Morphometrics will be taken monthly (Morphometrics: 1 set of morphometrics taken per month X 12 months = 12 sets of morphometrics/whale/year). For pregnant females, morphometrics will be taken at 1 set of morphometrics/whale/week X 52 weeks/year = 52 sets of morphometrics/pregnant whale/year.

Weights will be taken quarterly on each beluga as part of their routine health monitoring **(4 weights/whale/year = 4 takes/whale/year)**. Weights will also be provided to the research team to support this project, but weights will not be taken solely for the purposes of research. Beluga weights will be conducted by behaviorally gating belugas to the medical pool of the Mystic Aquarium beluga whale habitat. The medical pool is outfitted with a hydraulic lift that will be raised up to create a depth of 3-3.5 feet of water. Experienced beluga training staff will enter the

water and guide the beluga to the side of the pool. A stretcher custom made for belugas that is attached to a hanging scale will be lowered via crane into the center of the pool. One side of the stretcher will be disconnected and submerged in water allowing the beluga to be guided into the stretcher. Once the beluga is in place, the disconnected side of the stretcher will be re-attached, securing the beluga in the stretcher. The positioning will be checked to ensure that the animal is safely secured and the stretcher is lifted from the water with a crane. Water will drain from the stretcher and the weight will be recorded. The stretcher will then be lowered back into the water and the opened so the beluga can swim out. Staff will assist in clearing the pectoral flippers as needed from the stretcher openings to ensure safe exit of the beluga from the stretcher to the pool. Once the beluga has exited the stretcher, the stretcher will be lifted out of the pool with the crane. A veterinarian will observe the beluga at all times during a weight and for a minimum of 30 minutes following the weight procedure.

For offspring calves of the whales listed in this permit, morphometrics are proposed to be collected at a maximum of 1X/week to occur opportunistically when calves are being handled for health assessment purposes which includes growth measurements. (1 set of measurements/week X 50 weeks = 50 takes per year per calf collected at times of handling for health assessment) Weights will be recorded weekly for the first 2 months of life and then weight frequency will be monthly up to 1 year of age. Thereafter, weights will occur every three months for a total of 18 weights and measurements per year for the first year of life and then 4 weights and measurements each subsequent year. If a calf opportunistically swims underneath the camera set-up, a photograph will be taken but the calves will not be trained to swim underneath the camera for this purpose.

Photographs and morphometric data will be provided to researchers conducting photogrammetry on wild belugas.

Study 5 (Diving Physiology) Mystic IACUC #15004 and #19001

Brief Overview: Blood and breath samples will be obtained under behavioral control from belugas either under baseline conditions or after different dive activities and the cells exposed to pressure and/or nitrogen bubbles in the laboratory followed by immune function assessment in order to investigate the role of the immune system in cetacean diving.

Methods:
In order to investigate the relationship between the immune system and dive behavior, blood (up to 30 mls) and breath samples will be obtained from belugas after baseline or different dive activities and depths. Blood samples for diving physiology: 2 dive activities (1 stationary dive, 1 active dive) x 2 durations x 2 blood samples (1 before and after the dive taken from tail flukes) x 2 repetitions = 16 blood samples/whale/year **and 8 takes/whale/year**.

Breath samples for diving physiology: 2 dive activities (1 stationary dive, 1 active dive) x 2 durations x 3 breath samples x 2 repetitions = 24 breath samples per year **= 8 takes/whale/year. Each dive behavior and the replicates will occur on a separate day. Both blood and breath samples associated with each dive behavior will be collected on the same day for a total of 8 separate days of sampling per year.**

Depending on the particular need for each study and animal behavior, blood sampling for Study 5 can be combined with sampling for Studies 1 and 2. This would result in no more than 2 blood samples per whale in a single day. In combination with Study 2, no more than 5 breath samples per whale per day would occur.

Immune function testing will occur in samples exposed to simulated dives e.g. changes in pressure, temperature or nitrogen bubbles as well as following actual dive behaviors in trained whales. Hormone analyses will be used to describe changes in catecholamines and cortisol which may accompany natural diving, and will be analyzed with measures of immune function. Calves will not participate in this study; however some of the blood samples collected opportunistically when blood is being collected for health monitoring purposes for Study 1 may be utilized for in vitro dive simulated studies (i.e. subjecting blood samples to pressure followed by immune function tests).

Hormone Analyses

Hormone analyses will occur as describe under study 1. Briefly, catecholamines, such as epinephrine and norepinephrine will be assessed via High Performance Liquid Chromatography (Thompson and Romano, 2016). Cortisol will be assessed via enzyme immunoassay (Spoon and Romano, 2012).

Immune Activity

Phagocytosis and respiratory burst will be assessed via flow cytometry utilizing propidium iodide labelled Staphylococcus aureus and DFCDA, as described above under study 1. Activation of inflammatory responses will be assessed by detecting changes in complement proteins in serum (Bergh et al., 1993; Kirschfink and Mollnes, 2003). For example, data from our laboratory show an increase in C5a following *in vitro* activation can be measured in belugas and harbor seals using a commercially available ELISA based kit from MyBiosource.com (unpublished data from our laboratory). Additional measures of complement activation will be developed through modification and validation of existing reagents and protocols. The relationship between inflammation and coagulation (clotting) will also be investigated. The presence or absence of clotting factors will be confirmed in both cetacean and pinniped species and correlated with measures of inflammatory activation. New protocols will be developed for *in vitro* manipulations in order to test this relationship between coagulation and complement activation.

Simulated Dive Challenges

To investigate the specific effects of high pressure, or varying rates of compression and decompression (ascent and descent), blood samples will be loaded into a stainless steel benchtop pressure chamber and mineral oil used to bring the chamber to the desired pressure for the duration of exposure (Field and Tablin, 2012; Thompson and Romano, 2015; Thompson and Romano, 2016). Irrigation tubing around the pressure chamber will also allow for manipulation of temperature within the chamber during simulated dives and thus can be used to investigate the effects of temperature. The response of beluga immune cells or inflammatory processes to nitrogen bubbles will be investigated by introducing nitrogen gas to blood or serum samples via 3/8inch tubing and a modified pipette tip. The flow of gas will be manipulated to alter the number of bubbles and the size of bubbles being introduced to samples prior to immune function measures.

In vivo Measures of Dive Physiology

Belugas will be trained to perform specific dive behaviors prior to sample collection in order to make *in vivo* measures of dive related changes in hormones and immune activity. These dives may include varying duration, activity level, and to some degree, depth (Shaffer et al., 1997; Fahlman et al., 2008). Dive durations will not exceed 10 minutes, which is well within the breath hold capacity of belugas (Martin and Smith, 1999; Citta et al., 2013). Underwater videography and photography using an underwater pole camera may be employed to monitor activity of individuals during the performed behaviors.

Breath as a Non Invasive Tool

Breath will be collected from belugas behaviorally as described previously. A protocol for measuring complement activation by detection of specific proteins, such as C5a using a commercial ELISA based kit, has been validated for use with beluga serum by measuring increases in C5a following exposure to zymosan which is known to activate the complement responses (Ward *et al*, 1987). This kit will also be used to analyze beluga breath samples. Measures of C5a in blow will be compared with measures in matched blood samples for biological validation. Additional validation methods include running proteomic analysis on individual samples to identify the array of immune proteins which are present in blow, to confirm the presence of C5a and identify further specific proteins which display changes in concentration following specific situations such as *in vivo* dive behaviors (Fumagalli et al., 2012; Mucilli et al., 2015).

Study 6 (Microbiome) Mystic IACUC #18018

Brief Overview: Belugas will behaviorally accept swab sampling for microbiome studies. The beluga microbiome will be characterized and monitored including oral, blowhole, skin, vaginal and gastrointestinal tract locations in order to determine the microbial communities in conjunction with clinical health status, season, sex and age. For calves, swab samples will be collected opportunistically when calves are handled for health assessment. Calves will be gently handled at the surface of the water by a person experienced in handling beluga calves. Swabs will be gently inserted to the appropriate location as described below while calves are being handled for health assessment.

Methods:

Belugas will be trained behaviorally to accept swab sampling for Microbiome Studies. Sampling will occur twice per week for the following:

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 (Oral swabs: 2x per week x 50 weeks = 100 samples/year).

Nasal/blowhole: A sterile swab will be gently placed inside the blowhole. The swab will be stored at -80°C until DNA can be extracted (Blowhole swabs: 2x per week x 50 weeks = 100 samples/year).

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 (Skin swabs: 2x per week x 50 weeks = 100 samples/year).

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 (Vaginal swabs: 2x per week x 50 weeks = 100 samples/year).
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 (Anal swabs: 2x per week x 50 weeks = 100 samples/year).

Non-intrusive sampling for swabs will not exceed 100 takes per whale per year for each of the following: oral, blowhole, skin, vaginal, anal.

Calves will be handled at a maximum of 1X/week for swab sampling for microbiome research. Skin swabs will begin to be collected at 1 week of age (1X/week x 50 weeks = 50 samples/calf/year). Oral, blowhole, vaginal, and anal swabs will begin to be collected at 2 months of age (1X/week x 40 weeks = 40 samples/year in the first year; beginning at age 2 1X/week x 50 weeks = 50 samples/calf/year).

Next Generation Sequencing (NGS) targeting the small subunit (SSU) ribosomal RNA (16S rRNA) gene is a high throughput tool that can be used to document the bacteria that reside within or on a marine mammal host. This technique can help identify microbes that may be common to both marine and terrestrial animals, or those unique to marine mammals (Hanning and Diaz-Sanchez, 2015).

DNA Extractions

Genomic DNA will be extracted directly from swabs or fecal samples using the MoBio FastDNA™ SPIN Kit for Feces (MP Biomedicals; Santa Ana, CA) per the manufacturer's directions. Extracted DNA will be used as a template for amplification of the 16S rRNA gene.

Next-generation sequencing (NGS)

Assessment of microbial community membership will be carried out using NGS of 16S ribosomal RNA (rRNA) genes using a MiSeq platform (Illumina; Hayward, CA). This sequencing method is a well-established method for studying microbiomes that allows analysis of the entire microbial community within a sample. The V3-V4 hypervariable regions will be amplified using the purified genomic DNA followed by the addition of sequencing adaptors and index tag sequences to the amplicons using the Nextera XT Index kit (Illumina). Resulting amplicons will be purified before use as a template for massively paired-end sequencing using a 600-cycle MiSeq reagent kit.

Bioinformatics

Raw data will be filtered for quality and checked for chimeric sequences. Read files will be generated using the MiSeq Reporter software and forward and reverse sequence reads will be aligned. The assembled sequencing data will be analyzed using QIIME open-source bioinformatics pipeline (Caporaso et al., 2010). Sequences will be clustered into operational taxonomic units (OTUs) and taxonomic assignment will be conducted using a BLAST search against the Greengenes database. Weighted Unifrac analyses will be performed to calculate the pairwise distances between the bacterial communities, and principal component analysis (PCA) will be applied to visualize the results.

Study 7 (Behavioral and Reproduction Studies) Mystic IACUC #12001

Brief Overview: Behavioral observations and monitoring of reproductive organs via ultrasound images and hormones will be obtained before, during and after the breeding season. Should breeding occur incidental to this permit, similar data will be collected throughout pregnancy, birth and calf development as feasible in order to increase the collective knowledge about beluga reproduction, pregnancy, birth, and calf development.

Methods:

Quantitative behavioral assessments will be carried out on belugas before, during and after the breeding season (Jan-Apr) using an ethogram as used in Richard, 2016 *doctoral dissertation* and video recordings. Behaviors of interest in the ethogram will be adapted depending on the life stage of interest. Observation sessions will be conducted 4-6 times weekly (Non-intrusive sampling- Behavioral Observations: 6 Observational sessions per week x 50 weeks=300 takes/year); Non-intrusive sampling- Video monitoring: 6 video sessions per week x 50 weeks =300 takes/year) with sessions occurring both in the morning and afternoon. Each session will last for up to 30 minutes. Quantification of behaviors will be carried out using behavioral analysis software e.g. J-Watcher software (Anderson et al., 2017). Calves may also be monitored via behavioral ethogram and video to assess calf behavior and social development. Non-intrusive behavioral observations and video monitoring will not exceed 300 takes per whale per year.

Ultrasound exams will be carried out on belugas trained to lay out and to accept an ultrasound convex 3.5 MHz probe externally over the reproductive organs. Exams will be carried out as in Richard et al., 2016 once or twice per month in July through December and twice per month in the months of January through June (Non-Intrusive sampling- Ultrasound: 4x/month x 12 months/year = 48 takes/year). Total testicular volume in males and follicular size in females will be measured. Non-intrusive ultrasound sampling will not exceed 48 takes per year per whale. Should a female beluga become pregnant, assessment of maternal reproductive condition and fetal monitoring via ultrasound will occur once weekly (as behavior allows) throughout gestation. Fetal monitoring will include heart rate, organ development, fetal activity level, and fetal measurements including bi-parietal distance, thoracic width, and body length. Monitoring of the dam includes monitoring of uterine, ovarian, and placental health. Thus, for a pregnant beluga, non-intrusive ultrasound sampling will not exceed 52 takes per year per whale.

Testosterone, estrogen and progesterone will be measured and monitored in the blood and breath of males and females at time points before, during and after the breeding season within 24 hours of ultrasound exams whenever possible (to coincide with bimonthly sampling as described in Study 1). Briefly, blood samples will be centrifuged at 2000 *x g* for 10 minutes at 10˚ C and serum will be stored at -80˚ C until assay.
Blood samples will be assayed for testosterone using an EIA previously validated for use with beluga serum and plasma (Richard et al., 2016). Serum will be sent to the Animal Health Diagnostic Laboratory at Cornell University for estrogen and progesterone measurements. Blow samples will be assayed for reproductive hormones according to Richard et al., 2017.

Study 8 (Testing of prototype telemetry and imaging devices before deployment on wild whales) Mystic IACUC #11002

Brief Overview: Prototypes for telemetry and imaging devices adhered with suction cups will be tested on belugas to determine optimal placement on the body, size, adherence, and behavioral response. Once tested on whales under controlled condition, the technology can be transferred to deployment on wild whales.

Methods:

Belugas will behaviorally station and accept telemetry or imaging devices. not to exceed dimensions and weights described below. Devices such as the Animal Borne Imaging (ABI) systems adhere via a non-invasive attachment such as soft silicone rubber suction cup which forms a narrow, high-density foam 'cradle'. The ABI system rests along this cradle. Suction cup mounts have been used successfully in the past for ABI deployments on cetaceans, including belugas. Those cups were small, flattened, 16cm diameter and the larger systems, 23cm diameter. Out of water the suction cup assembly weighed approximately 0.5kg, though it was slightly buoyant in water. Testing will be expanded to include smaller suction cup mounts than used previously which are appropriate for the smaller ABI systems anticipated for use in future wild beluga field deployments. (3 sessions per week x 50 weeks= 150 takes/year).

Potential areas on the body to be tested include the upper portion on either side of the dorsal ridge and behind the head before the start of the dorsal ridge. Trials will be conducted to test optimal placement on the body, evaluation of the size of the device, the ability of the device to stay attached after belugas breach or increase swim speed, the amount of time the device stays on, as well as determination of any behavioral changes of the whales after deployment. Engineers and researchers will have an opportunity to improve, modify and retest devices before deployment on wild whales. Devices can also be tested for collecting data while deployed on the whales under controlled conditions at Mystic Aquarium. Deployment of the cameras with non-invasive attachments will not exceed 150 takes per whale per year. Prototype telemetry and imaging devices will not be tested on calves.

- **Non-target species and conspecifics**: Indicate the estimated number and type of non-target species that may be encountered in your study area annually, and whether and how they may be incidentally harassed, captured, or otherwise affected. This includes but is not limited to conspecifics as well as other marine mammals and ESA-listed species such as sea birds and sea turtles.

No wild animals or non-target species will be encountered or harassed. During sampling, conspecifics in the habitat would not be affected by the research.

Explain how you will avoid them or minimize impacts to them (e.g., not in area during time of study; would not approach closer than 100 meters; would halt operations until non-target species moved out of study area).

No non-target species will be encountered or harassed in the wild.

- For ESA species designated by DPS, specify the DPSs that are likely to be encountered.

No animals from an ESA DPS will be encountered in the wild.

- If takes to non-target animals may occur, include these on separate rows in the Take Table to include incidental take (e.g., harassment or capture) of non-target conspecifics or other species.

Takes to non-target animals will not occur as part of this project. In general, conspecifics are not affected by another animal performing a voluntary behavior. The training program is managed such that behaviors are not forced or pushed, which eliminates a concern for negative interactions and aggression between animals associated with voluntary behaviors. Belugas participating in the research will be cared for in a managed environment where incidental take would be impossible. When acoustic studies are conducted, conspecifics will be gated to another pool to limit proximity to the sound generated. Behavior of conspecifics will be monitored throughout all studies, and the collection of samples ceased if the conspecifics display any signs that could indicate they are being adversely affected by the sample collection.

Project Supplemental Information

Status of the Affected Species

- As applicable, indicate the status of the species or stock as follows:
  - ESA - threatened or endangered;
  - MMPA - depleted or strategic; and
  - Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) - Appendix I, I, or III

ESA – The beluga Cook Inlet Distinct Population Segment (DPS) is Endangered
MMPA – Within the US, NOAA identifies 5 stocks, (Beaufort Sea, Bristol Bay, Cook Inlet, E. Bering Sea, E. Chukchi Sea) all of which are protected under the MMPA and the following stocks are depleted: Cook Inlet stock. The Russian stock (Sakhalin Bay, Nikolaya Bay-Amur) is depleted under MMPA
CITES - Appendix II throughout range

All belugas included in this permit are aquarium-born; none of the belugas included in this permit were wild-collected. Belugas have name identifications of Qila, Frankie, Havana, Kharabali, and Mira (see Take Table for animal details). All belugas included in this permit have known dams that were wild collected in the Sea of Okhotsk, Russia more than 10 years prior to the designation of that population as depleted. The population of sires at the facility of origin includes belugas collected from the Barents Sea, Russia, a non-depleted population, or the Sea of Okhotsk, Russia, as detailed in the take table. A male named Andre, initially collected from Barents Sea, is the definitive sire for Mira, Frankie, and Havana. A male named Kodiak, who was initially collected from the Sea of Okhotsk, Russia prior to its designation as a depleted population, is the sire of Kharabali. Marineland reports that, based on behavioral records, the sire for beluga Qila is likely

to be Andre because he was the dominant male, and dam Isis was the dominant female at that time. This whale also shows some similar traits with other animals that Andre sired. Additionally, Marineland, based on behavioral records, indicates a male named Belyi would be the next most likely sire for Qila. A genetic test ruled out Belyi as the sire for Qila but the genetic test to confirm Andre as the sire was inconclusive; Andre is listed as a possible sire using the genetic analysis, but this was not definitively confirmed. (See Appendix 4 Genetic Test Results).

Lethal Take

- If authorization for serious injury[1] or mortality[2] (euthanasia/intentional[3] or accidental/unintentional) is proposed:
  - What activities could result in mortality?

None of the research activities proposed will result in serious injury or mortality. The beluga whales involved in this project will be trained to allow the collection of research samples (blow, saliva, feces, blood, measurements, ultrasound, hearing, etc., as described above) using operant conditioning and trained behaviors during routine husbandry and veterinary activities.

  - Explain why it's not feasible to use other methods that won't result in mortality.

Not applicable; none of the research activities proposed will result in serious injury or mortality.

  - If authorization for mortalities of ESA-listed or MMPA-depleted species is proposed, explain how the research will directly benefit the species or fulfill a critically important research need.

Not applicable; no authorization for mortalities is proposed.

  - What is the maximum number of animals of each species/DPS and age class that could be seriously injured, unintentionally die, or be euthanized annually? Over the life of the permit?

None of the research activities proposed will result in serious injury or mortality. No euthanasia is proposed as part of this permit's activities.

  - Justify the number of mortalities.

No mortalities should occur related to research activities proposed.

  - How is euthanasia decided, conducted, and who conducts it?

[1] A serious injury is an injury that will more likely than not result in mortality.

[2] Caused by the presence or actions of researchers including but not limited to deaths or serious injuries sustained during capture and handling, while attempting to avoid researchers or escape capture, or resulting from infections related to intrusive procedures such as sampling or tagging. This does **not** include a fetus if a pregnant female dies.

[3] This includes unintentional euthanasia for humane reasons (e.g., due to serious injury during research).

Euthanasia of animals would be required only for the purposes of alleviating animal suffering when an animal is found to have a condition for which no treatment is possible that causes pain and distress. Euthanasia is not anticipated as needed in conjunction with the activities proposed in this research permit; however, while unlikely, animals could possibly develop disease or other medical issues unrelated to the research project that could warrant a decision of humane euthanasia in the best interest of the animal. In the extremely unusual situation where an animal is experiencing irreversible pain and distress, a decision of euthanasia may be made by Dr. Allison D. Tuttle, DVM, Diplomate ACZM, with approval by responsible party, Dr. Stephen Coan. Any euthanasia required would be carried out by Dr. Allison D. Tuttle, DVM, Diplomate ACZM, or Dr. Jennifer E. Flower, DVM, MS, Diplomate ACZM. Euthanasia would comply with American Veterinary Medical Association (AVMA) and Association of Zoos and Aquariums (AZA) standards.

- What are the protocols for necropsy and carcass disposal?

In the unlikely event of a mortality or euthanasia, complete necropsy is conducted. Necropsy is conducted by ACZM-boarded veterinarians and, when possible, in conjunction with a veterinary pathologist. Necropsy occurs in the designated necropsy room on the Mystic Aquarium campus or in the designated necropsy room in the Pathobiology department at the University of Connecticut in Storrs, CT. Samples are collected for histopathology to determine cause of death and carcasses are disposed of via incineration.

- What are the protocols for disposition of dependent pups or calves if lactating females may die as a result of your actions?

No animals are anticipated to die as a result of research activities.

Anticipated Effects on Animals

Because Mystic Aquarium utilizes operant conditioning to train animals to participate in their own health care behaviors and research behaviors, restraint is not needed to accomplish the proposed research. Research samples are collected in conjunction with routine health sampling and, because research is performed under behavioral control, the participation of the whales is voluntary. Whales are not required to participate and there is no negative consequence if they do not participate. Due to this, there is minimal, if any, negative effect on target animals. Because research will be conducted only in the controlled aquarium environment, there is no risk of negative consequence to non-target animals. Conspecifics are not affected negatively when other whales are asked to do behaviors. Behaviors that are not performed willingly are not pushed such that there is no creation of any anxiety or frustration for the animal or between animals. Veterinarians are kept apprised of the status of each whale daily and have the authority to cease sampling for research at any time if this is in the best interest of a beluga's health and/or wellbeing. The following is the expected result from belugas participating in the research under behavioral control (note, mitigation for auditory studies is included in the methods section for that study):

Study #1, 5, 7 Blood:

Collection method: Following a 0.2% chlorhexidine scrub and alcohol rinse to clean the target area, a veterinarian will insert 21 g butterfly needle into the blood vessel on the beluga's flukes, presented voluntarily under behavioral control, and draw desired amount of blood (Gulland et al, 2018).

S^D Behavior Criteria: Trainer presents cue to animal to present tail flukes. Flukes are stabilized on habitat beach while they are cleaned in preparation for blood collection and then blood is collected by a trained veterinarian.

% of way Animals Respond:

- 98% criteria is met (beluga maintains position 1 – 7 minutes)
- 1% criteria not met (beluga pulls flukes away during blood collection)
- 1% criteria not met (beluga refuses to present flukes)

Physiological effects on Animal: If animal pulls away during a behavioral fluke present, superficial abrasions on flukes can occur and needle insertion site can be mildly irritated due to increased movement of the needle compared to a normal draw.

- Short-term effects: Mild short-term irritation on the flukes could occur.
- Long-term effects: None anticipated from a physical perspective.
- Time for wound healing/recovery time: Complete healing of the site of needle stick with 21 g butterfly needle typically occurs in a maximum of 30-40 days (Geraci et al, 1987), generally less, approximating 7-14 days.
- Worst case scenario: From a behavioral perspective, the beluga could develop an aversion to the behavior and become unwilling to participate in the behavior. From a medical perspective, the worst case scenario is that the blood collection site develops local infection and inflammation called phlebitis, resulting in local irritation and prolonged healing times, and, while uncommon, possibly requiring oral antimicrobial medication.
-

Study #2, 3, 5, 6, 7 Respiratory Exhale/Blow:

Collection method: A trained individual will hold a sterile petri dish above beluga's blowhole to capture mucous/aerosol released on the beluga's voluntary exhalation (Gulland et al, 2018).

S^D Behavior Criteria: Trainer taps lightly next to blowhole with flat, open palm. Beluga exhales forcefully (1 exhale/tap) until bridged.

% of way Animals Respond:

- 95% criteria is met (forceful exhales each time)
- 2% criteria not met (weaker exhales)
- 3% criteria not met (exhales get weaker upon repeated cues from trainer or animal does not perform the exhale)

Physiological effects on Animal: Beluga exhales forcefully out of blowhole; extreme repetitive sampling in a single day presents low risk for upper respiratory irritation; however, sampling is limited to prevent this from occurring.

- Short-term effects: None seen at frequency requested

- Long-term effects: None seen at frequency requested
- Time for wound healing/recovery time: N/A

Risk of behavioral Breakdown: This is a low risk behavior for breakdown or loss.

- Worst case scenario: Beluga will not voluntarily exhale. No anticipated consequences to the belugas' health or wellness are anticipated.

Study #2 Saliva:

Collection method: A trained individual will hold a collection swab firmly and then gently rub collection swab on the beluga's upper palate to absorb saliva while the beluga voluntarily opens its mouth (Hogg et al, 2005).

S^D Behavior Criteria: Trainer requests beluga to open mouth. Beluga keeps an open mouth while trainer gently rubs sterile gauze on upper palate for ~5-10 seconds.
% of way Animals Respond:

- 95% criteria is met (beluga opens mouth and allows saliva collection)
- 5% criteria not met (beluga pulls away during saliva collection)

Physiological effects on Animal: None

- Short-term effects: None seen
- Long-term effects: None seen
- Time for wound healing/recovery time: N/A- no wounds are expected from this behavior
- Worst Case Scenario: Beluga will not willingly perform the behavior. No consequences to the beluga health or wellness are anticipated.

Study #2 Fecal:

Collection method: The beluga will voluntarily lay out at the water surface with its ventral side up. A trained individual will insert a small soft plastic tube (5 mm diameter, 60 cm long) that has been coated with sterile lubricant into beluga's anus, insert approximately 15 cm and stop if any resistance is felt, block the open end of tube that is outside of the anus with finger to create suction and slowly withdraw tube from within the anus, collecting any fecal matter that is present (Gulland et al, 2018).

S^D Behavior Criteria: Trainer presents cue to beluga to lie ventrally in water. Beluga allows trainer to hold peduncle above water while a second trainer dries anal slit, inserts the lubricated tube into anal opening, and removes tube from anal opening.
% of way Animals Respond:

- 99% criteria is met (beluga allows fecal collection and stays in position until bridged)
- 1% criteria not met (beluga refuses to do ventral layout)

Physiological effects on Animal: While no negative effects are expected at the frequency proposed, with repetitive sampling, skin surrounding the anal opening could become mildly irritated.

- Short-term effects: While there are typically no effects from the behavioral fecal collection as described, it is possible for the skin around the opening of the anus to

become mildly and superficially irritated. This typically resolves without need for treatment in a few days' time.

- Long-term effects: No long-term effects are expected, as the behavior is stopped if any mild irritation develops.
- Time for wound healing/recovery time: As discussed under short-term effects, any mild irritation that develops is expected to resolve in a couple of days.
- Worst case scenario: Behaviorally, the worst case scenario is that the beluga either pulls away from trainer during fecal collection or will not allow fecal collection. Physically, the worst case scenario is the short-term mild irritation, as described above. Should this be noted, veterinarians will direct cessation of sampling until the irritation in that area has resolved.

Study #2, 6 Skin Rubbing or Swabbing:

Collection method: Beluga's skin naturally sloughs making it easy to get sloughed skin samples by gently rubbing a rubberized spatula or sterile swab on the belugas body area of selected sampling (Bechshoft et al, 2015) while beluga is in a voluntary layout. Skin scrapings will be collected from consistent locations on the whale (i.e. along the dorsal ridge on either side of the midline, the flukes, the head region (near the blowhole), or ventrally on either side of the body.

S^D Behavior Criteria: Trainer presents cue to beluga to present body part to allow for gently rubbing of skin with various objects for skin scraping.
% of way Animals Respond:

- 99% criteria is met
- 1% criteria not met (beluga refuses to do the behavior)

Physiological effects on Animal: None. As skin sloughs naturally, there is no consequence to collecting this sample.

- Short-term effects: None seen at this frequency
- Long-term effects: Non seen at this frequency
- Time for wound healing/recovery time: N/A
- Worst case scenario: Beluga will not allow the swab or spatula to be rubbed on its skin. No expected consequences to belugas are expected.

Study #3 Hearing Study:

Collection method: The beluga lays out voluntarily at the surface of the water and three electrode sensors are placed with suction cups along beluga's back; one posterior to blowhole, one mid-back, and one anterior to dorsal ridge. An underwater speaker is placed in a predetermined location (1 m) relative to beluga's head and plays assigned noises through speaker via MP3 player.

S^D Behavior Criteria: Trainer asks beluga into dorsal layout parallel to beach and second trainer places suction cups in respective locations along beluga's back. Third person holds speaker underwater in desired location while researchers play sound and monitor brain activity.
% of way Animals Respond:

- 96% criteria is met (beluga stays still at surface of water while suction cups are attached and sounds are played underwater)

- 3% criteria not met (beluga sinks body and does not allow suction cups to be attached)
- 1% criteria not met (beluga refuses to lay out)

Physiological effects on Animal: Beluga is exposed to various decibels and frequencies of noise which are not expected to cause any damage to hearing or anxiety to whales.

- Short-term effects: Beluga may refuse to layout or swim away at presence of speaker if sound is undesirable
- Long-term effects: None seen at this frequency
- Time for wound healing/recovery time: N/A; no negative effects on the beluga are expected.
- Worst case scenario: Beluga will not lay out at surface of water to perform behavior, or beluga spooks during sounds played and swims away not completing the study. It is not anticipated that any negative physical effects on belugas would occur from this research.

Study #4 Photogrammetry (Aerial Photos):

Collection method: A camera is affixed to a pole at a height of 6 meters. The beluga swims voluntarily slowly at the surface of the water under the camera to allow for photographs to be taken.

S^D Behavior Criteria: The trainer asks the beluga into a dorsal layout at the surface of the water parallel to the beach and then slowly walks down beach alongside beluga as it swims at the surface.

% of way Animals Respond:

- 99% criteria is met (beluga swims slowly at the surface of the water parallel to beach and directly under camera)
- 1% criteria not met (beluga swims too quickly, too deep under surface of water, or not under the camera)

Physiological effects on Animal: None- animal is being asked only to swim at a slightly slower than usual speed for this behavior.

- Short-term effects: No negative effect is noted from asking animals to swim in a directed fashion
- Long-term effects: No long term negative effect is noted from asking animals to swim in a directed fashion repeatedly
- Time for wound healing/recovery time: No wounds are anticipated to occur from swimming slowly in a directed fashion.
- Worst case scenario: Beluga's surface swim becomes too fast or deep or beluga will not do the surface swim. No negative effects on the animal are anticipated aside from possible behavior refusal.

Study #4 Photogrammetry (Morphometrics & Weight):

Mophometrics

Collection method: While the beluga is in a voluntary layout at the surface of the water, fabric measuring tape is either gently wrapped around different areas of beluga's body for girth measurements or draped down different areas of body for length measurements.

S^D Behavior Criteria: Girth measurements: Trainer asks beluga into a dorsal layout at surface of water and wraps fabric measuring tape around base of peduncle, then manipulates it around 6 specific locations on the body. Length measurements: The trainer asks the beluga into a ventral layout at surface of water and places end of fabric measuring tape at the tip of beluga's rostrum and drapes it down body to obtain 2 length measurements. Lastly, the trainer asks beluga into a dorsal layout at surface of water and places fabric measuring tape posterior to blowhole and stretches it down the back to just anterior to dorsal ridge for a third length measurement.

% of way Animals Respond:

- 97% criteria is met (beluga lays out still at surface of water and allows for all measurements to be taken)
- 2% criteria not met (beluga sinks body or breaks out of layout)
- 1% criteria not met (beluga will not voluntarily lay out)

Physiological effects on Animal: No effects on the animal are typically noted from measuring length and girth with a soft tape. If animal were to spook during measurement and break, it is possible that sides of measuring tape may cause minor superficial abrasions on the body.

- Short-term effects: No negative effects on the belugas are expected from the collection of morphometrics.
- Long-term effects: No negative effects on the belugas are expected from the collection of morphometrics even when done frequently over time.
- Time for wound healing/recovery time: No wounds are anticipated to occur as a result of morphometric assessment; however, in the unlikely event that minor superficial abrasions occur as a result of the tape, these would likely heal within a few days to a week without treatment required.
- Worst case scenario: Beluga may not allow the behavior which makes determination of diet based on morphometrics difficult; in this instance, visual assessment of animal condition would be accomplished to inform diet management.

Weights:

Weights will be taken quarterly on each beluga as part of their routine health monitoring. Weights will also be provided to the research team to support this project, but weights will not be taken solely for the purposes of research.

Collection method: Beluga weights will be conducted by behaviorally gating belugas to the medical pool of the Mystic Aquarium beluga whale habitat. The medical pool is outfitted with a hydraulic lift that will be raised up to create a depth of 3-3.5 feet of water. Experienced beluga training staff will enter the water and guide the beluga to the side of the pool. A stretcher custom made for belugas that is attached to a hanging scale will be lowered via crane into the center of the pool. One side of the stretcher will be disconnected and submerged in water allowing the beluga

to be guided into the stretcher. Once the beluga is in place, the disconnected side of the stretcher will be re-attached, securing the beluga in the stretcher. The positioning will be checked to ensure that the animal is safely secured and the stretcher is lifted from the water with a crane. Water will drain from the stretcher and the weight will be recorded. The stretcher will then be lowered back into the water and the opened so the beluga can swim out. Staff will assist in clearing the pectoral flippers as needed from the stretcher openings to ensure safe exit of the beluga from the stretcher to the pool. Once the beluga has exited the stretcher, the stretcher will be lifted out of the pool with the crane. A veterinarian will observe the beluga at all times during a weight and for a minimum of 30 minutes following the weight procedure. If a beluga is moving too much in the stretcher such that there is risk for injury, the procedure will be stopped.

S^D Behavior Criteria: Experience trainer uses hand targets and their hands to gently guide beluga to the appropriate location at the edge of the pool and into and out of the stretcher. Where necessary, additional experienced training staff can provide gentle direction with their hands on the beluga's sides for clarity.

% of way Animals Respond:

- 97% criteria is met (belugas allow for shifting into medical pool under stimulus control and allow for manual direction and being guided into the stretcher, and stays still for lifting the stretcher from water).
- 2% criteria not met (beluga refuses to gate into medical pool) and no weight is accomplished.
- 1% criteria not met (beluga refuses to be guided by trainer around the medical pool and into the stretcher and/or actively avoids trainers) and no weight is accomplished.

Physiological effects on Animal: No effects on the belugas are typically noted from stretcher weight procedures. If the beluga were to make large movements in the stretcher, it is possible that parts of body (flukes, pectoral flippers) could sustain minor superficial abrasions or bruising.

- Short-term effects: No negative effects on the belugas are expected from stretcher weight procedures.
- Long-term effects: No negative effects on the belugas are expected from quarterly stretcher weight procedures.
- Time for wound healing/recovery time: No wounds are anticipated to occur as a result of stretcher weight procedures; however, in the unlikely event that minor superficial abrasions occur as a result of impact with stretcher poles, these would likely heal within a few days to a week without treatment required.
- Worst case scenario: Beluga may not allow the behavior which makes determination of weight difficult; in this instance, behavioral axillary girths in combination with visual assessment of animal condition would be accomplished to monitor beluga condition.

Study #5 Dive Physiology - Stationary Target:

Collection method: A circular target affixed to a pole is submerged underwater to a depth of 6'10". The beluga takes a breath and targets rostrum to circle slowly for 5 minutes, which is well within the beluga's natural ability for breath holding (Citta et al., 2013; Mortin and Smith, 1999). Following this behavior, blood and blow are collected from the beluga.

S^D Behavior Criteria: With beluga at station, trainer inserts circular target into the water at appropriate depth and the beluga targets to it for a fixed amount of time. A second trainer may insert fish into the water column to reinforce animal during the duration of the target. After the behavior, the trainer asks the beluga to participate in a blood draw as well as breath collection.

% of way Animals Respond:

- 98% criteria is met (beluga stays on target for full duration and participates in biological sampling following behavior)
- 1% criteria not met (beluga stays on target for full duration and may participate in less invasive biological sampling such as blow or saliva, but not blood)
- 1% criteria not met (beluga breaks off target and surfaces prior to desired duration)

Physiological effects on Animal: The potential effects of the animal are only from the post-target sampling, as described in the above sections.

- Short-term effects: No negative effects are anticipated from this behavior.
- Long-term effects: No long term effects are anticipated from performing this behavior.
- Time for wound healing/recovery time: Swimming on a target does not cause wounds. For possible wounds associated with post-target sampling, see relevant sections above.
- Worst case scenario: Beluga will not target underwater for desired duration and swims away. No negative effects on the beluga are expected.

Study #5 Dive Physiology - Active Swim:

Collection method: Trainers insert target poles at various locations around the habitat. The beluga will actively swim to each target while staying underwater for up to 5 minutes, which is greatly lower than the threshold for breath holding in belugas (Citta et al., 2013; Mortin and Smith, 1999). Following this behavior, blood and blow are collected from the beluga voluntarily under behavioral control.

S^D Behavior Criteria: With beluga at station, trainer submerges a long target pole into the water. The beluga takes a breath and targets rostrum to target pole, at which point another trainer inserts second target pole at an alternate location and splashes the surface of the water. The beluga swims underwater to touch second target pole, at which point an additional target pole is inserted and water is splashed. The beluga will travel underwater from target to target for 5 minutes. After the behavior, the trainer asks the beluga to participate in a blood draw as well as forced exhale collection.

% of way Animals Respond:

- 90% criteria is met (beluga remains underwater while swimming to targets for full duration and participates in biological sampling following behavior)
- 8% criteria not met (beluga surfaces while swimming and does not reach desired duration, but still participates in biological sampling following behavior)
- 2% criteria not met (beluga will not complete the behaviors)

Physiological effects on Animal: As belugas can hold their breath underwater for periods much longer than 5 minutes, no negative effects on the belugas are anticipated with this behavior. See potential effects from post-behavior sampling above.

- Short-term effects: No negative effects are anticipated from performing this behavior.
- Long-term effects: No long-term effects are anticipated from performing this behavior.
- Time for wound healing/recovery time: Swimming underwater does not cause wounds; see above for wound healing times for post-sampling behaviors.
- Worst case scenario: Beluga will not complete the behavior or completes the behavior incorrectly. This would cause no harm to the beluga.

Study #6 Blowhole Swab:

Collection method: The beluga will hold open the blowhole voluntarily. Beluga's blowhole will remain open while a sterile tipped applicator is inserted and gently rubbed along the internal mucosa for a few seconds.

S^D Behavior Criteria: Trainer presents cue for beluga to peer head upon habitat beach. With a gentle touch of two fingers on front of melon, trace fingers back in straight line towards beluga's blowhole. Beluga will open blowhole and it will remain open while trainer or second trainer/vet inserts a sterile tipped applicator into blowhole and gently rubs it along the mucosa.

% of way Animals Respond:

- 98% criteria is met (beluga stays in position and allows insertion of applicators)
- 1% criteria not met (beluga breaks out of position and closes blowhole)
- 1% criteria not met (beluga refuses to perform behavior)

Physiological effects on Animal: No negative effects on the belugas are expected at the proposed sampling frequency. In rare cases or with repetitive sampling, the mucosa inside the blowhole could become mildly irritated.

- Short-term effects: No negative effects on the belugas are expected.
- Long-term effects: No negative effects on the belugas are expected.
- Time for wound healing/recovery time: No wounds occur as a result of blowhole swabs and, though unlikely, it is possible that mild irritation could develop if the beluga were to move during swabbing. Should mild irritation occur, sampling will be ceased and the irritation would be expected to resolve in 2-3 days' time.
- Worst case scenario: Beluga does not open blowhole. No negative effects on the belugas beyond potential refusal of the behavior are anticipated.

Study #6 Anal/Vaginal Swab:

Collection method: With the beluga in a voluntary layout at the surface of the water with ventral side up, insert sterile tipped plastic stick swab 1-2 cm into beluga's anus or vagina.

S^D Behavior Criteria: Trainer presents cue to beluga to lie ventrally at surface of water. Beluga allows trainer to hold peduncle above water while a second trainer dries anal slit or vaginal opening, inserts swab into either opening for a couple of seconds to collect sample, and removes swab from either opening.

% of way Animals Respond:

- 98% criteria is met (beluga stays in position and allows insertion of swab)
- 1% criteria not met (beluga breaks out of layout during swab attempt)
- 1% criteria not met (beluga will not layout)

Physiological effects on Animal: At frequency proposed by this research, no negative effects on the belugas are anticipated. On rare occasion, if a whale were to break during the behavior, mild, superficial irritation at the orifice of application could occur.

- Short-term effects: No effects anticipated at the proposed frequency of collection.
- Long-term effects: No long-term effects on the belugas are anticipated.
- Time for wound healing/recovery time: Should mild, superficial irritation occur, continued sample collection would not be attempted and this would be expected to resolve without requiring treatment within 2-3 days' time.
- Worst case scenario: Beluga either breaks out of layout during swabbing, or will not layout. Should mild irritation occur, veterinarians would direct cessation of sample collection attempts and the irritation would be expected to resolve in 2-3 days without requiring treatment.

Study # 7 Reproductive Ultrasound:

Collection method: With the beluga in a voluntary layout, the ultrasound probe is presented on lateral, dorsal, or ventral sides of beluga's body so that the reproductive organs can be scanned.

S^D Behavior Criteria: Trainer drags back of hand on surface of water in direction in which beluga will layout.

% of way Animals Respond:

- 95% criteria is met (layouts successful and ultrasound conducted)
- 3% criteria not met (body sinking underwater during layouts, ultrasound not conducted)
- 2% criteria not met (beluga will not layout)

Physiological effects on Animal: None

- Short-term effects: None seen at this frequency; ultrasound examination dues not cause harm to the belugas.
- Long-term effects: None seen at this frequency; ultrasound examination does not cause harm to the belugas.

- Time for wound healing/recovery time: N/A- no wounds or damage to the belugas is expected.
- Worst case scenario: Beluga either breaks out of layout during ultrasound, or will not layout. This should not cause any harm to the beluga should a break in the behavior or refusal of behavior occur.

Study #8 Telemetry Devices Attached Via Suction Cups

Collection method: Using a voluntary behavior, a telemetry device intended for use in monitoring wild beluga populations that needs to be tested is placed on the beluga's back via non-invasive methods e.g. suction cups. A standby person is present with net to retrieve any device immediately should it fall off.

S^D Behavior Criteria: Trainer asks beluga into a dorsal layout at surface of water and places telemetry device on flat surface of back via suction cups. Trainer can then ask the beluga for a variety of behaviors to track/video its movement subsequent to the attachment.

% of way Animals Respond:

- 98% criteria is met (beluga allows for device to be placed on back and performs a variety of behaviors)
- 2% criteria not met (beluga sinks body and does not allow device to be attached)

Physiological effects on Animal: While it is unlikely that attachment of devices with suction cups would cause any negative effects on the animal, it is possible that suction cups could cause local minor skin irritation/inflammation.

- Short-term effects: Typically, no negative effects are seen from the attachment of a suction cup on the belugas' bodies. At most, local, mild skin irritation may occur.
- Long-term effects: No long term effects are anticipated from suction cup attachment.
- Time for wound healing/recovery time: While suction cups are not anticipated to cause any wounds on the animal, in the unlikely event that they cause local, mild skin irritation, this would be expected to resolve without treatment within a week.
- Worst case scenario: Short-term mild, local skin irritation at the site of the attachment of the suction cups is possible and is the worst-case scenario. In this event, further attachment would cease and the irritation would likely resolve without treatment within a week.

- Briefly summarize any **mortalities** that have occurred during the previous ten years of your permitted research using the same or similar techniques; include circumstances and cause of death.

No mortalities have occurred due to this research to date and work has been occurring since 1999.

- Discuss the anticipated **effects on the species or stock**, especially if mortalities or reproductive effects are possible. On what is your determination based?

No mortalities or reproductive effects are expected to occur as a result of this research; therefore, there is no anticipated effect on the species. There will be no effect on wild stock since the animals participating are aquarium-born and not of wild stock. Any progeny that may be born as a result of research activities would be maintained in captivity for the duration of their lives and would not be released to the wild. Marineland is at capacity and has provided an affidavit ensuring no subsequent collection of wild belugas as well. Additionally, Canadian Bill S-203 has been enacted to prohibit the import of additional belugas into Canada.

Humane[4] Take and Measures to Minimize Negative Effects

- **Humane determination**: Explain how you determined your methods involve the least possible degree of pain and suffering possible and why there are no feasible alternative methods to obtain the desired data or results.

Samples proposed for research in this permit are non-invasive or minimally invasive and are collected under behavioral control, as the samples mirror what is collected under behavioral control for the purpose of routine health monitoring to ensure the health and well-being of the belugas. The participation of the belugas in the research is thus voluntary (not forced). This allows for optimal sample collection without pain or distress to the belugas. Beyond this, research proposed has been evaluated and thoroughly vetted/approved by an Institutional Animal Care and Use Committee, a committee that ensures animal welfare during research sampling. Belugas are monitored daily and any abnormal behavior or any health concern is evaluated and treated, if necessary, without delay. Research behaviors may be paused as needed under veterinary or curator discretion for animal well-being.

Because of the biological nature of the research and the questions that need to be addressed, actual physiological samples from live belugas are essential. Using samples collected voluntarily, this research will further the collective understanding of beluga health and physiology and can be applied to the benefit of wild beluga conservation.

- Where an IACUC (Institutional Animal Care and Use Committee) review is required[5], to support a humane determination under the MMPA and compliance with the Animal Welfare Act, attach
  - the IACUC protocols submitted
  - any IACUC comments or recommendations
  - the signed IACUC approval (or status of approval)

See Appendix 5 IACUC Approved Protocols
Outline the various projects and their relation to the permit:

IACUC# 13007 – receipt of samples from the field
IACUC# 16006 – captive beluga health (blow, skin scrapings, feces, saliva, blood)
IACUC# 14006 – hearing studies

[4] Humane means using the method that involves the least possible degree of pain and suffering possible.

[5] Any marine mammal research that involves an invasive procedure, and which can harm or materially alter the behavior of the animals under study **requires an IACUC review and approval.** If an applicant does not have an IACUC, an alternate IACUC (e.g., of a Co-investigator or a local university/research institution) may be used.

IACUC# 17007 – photogrammetry
IACUC# 15004 and #19001 – diving studies
IACUC# 18018 – microbiome surveillance
IACUC# 12001 – reproductive biology
IACUC #11002 – testing prototypes telemetry devices

- **Mitigation and monitoring**: You may include mitigation and monitoring protocols here, or in the Project Description section or Anticipated Effects section above. If included in another section, simply reference the section where the following information is found:
- For each Take Action, Observe/Collect Method, and Procedure, describe your standard **mitigation to avoid or minimize the potential for adverse impacts** identified above.

- Describe your short- and long-term **post-procedure monitoring** protocols.

- If monitoring or mitigation is not feasible for specific procedures, species, situations, etc., explain why.

Factors that are used to evaluate effects of the behavioral research sampling on beluga behavior and welfare include:

- Difficulty of behavior to perform to collect samples
- Sampling methods – are they invasive or will they cause discomfort
- Sampling frequency – does it fall into the "safe" or recommended sampling amounts per day, etc.
- Training time – is there enough time to positively train a new behavior for sample collecting
- Are there incompatible behaviors for sampling methods at the same time therefore causing risk of loss of other behaviors
- Medical behaviors – will sampling methods or requests compete for important medical behaviors to monitor normal health parameters

Research samples are collected under behavioral control and the belugas' participation is thus voluntary. Only behaviors that are expected to be feasible for the belugas to learn and those that have minimal risk to their health and well-being are trained. The proposed research in this permit utilizes behaviors that are already trained in order to allow belugas to participate in their own health care on a voluntary basis as part of their routine veterinary preventative health protocol. As such, there is minimal impact of the research to the belugas.

Belugas are monitored by a trained and experienced husbandry supervisor or curator-level staff member and a licensed veterinarian at all times during sample collection. Belugas are also monitored continuously during the daytime and with hourly checks overnight, every day. Any abnormalities are immediately reported, assessed, and treated as warranted.

See also descriptions of effects on animals for more information.

- **Research Coordination**: Describe how you collaborate or coordinate with other researchers in your action area. Who are they? Explain how this will occur and how it

will minimize impacts. For example, will it involve sharing resources, samples or data; timing surveys to minimize disturbance, etc.?

Mystic Aquarium collaborates with other researchers and works to share samples obtained whenever possible for research studies. External researchers submit a sample request form which is reviewed by an internal committee that consists of experts in research, animal husbandry and animal care to determine if the research request can be accommodated. If research requests require invasive sampling an IACUC proposal must be submitted, reviewed, and requires approval for research to commence. Whenever possible, research samples are shared or experiments conducted to gather as much information as possible in order to limit unnecessary sampling and duplication of efforts.

Mystic Aquarium collaborates with other AZA-accredited aquaria that maintain belugas under professional care and has requested and received beluga samples for research. These samples have been limited to a small number of whales and limited in frequency of sampling due to personnel constraints and limited time available given other priorities in other animal care and training programs, as described above. Mystic Aquarium has contacted Marineland in the past to obtain biological and archived samples from their beluga collection; however, no research samples have been provided to date. Given Marineland's new management and desire to contribute to conservation research and education, it has expressed that it would be amenable to participating in conservation-related beluga research. However, Marineland is a for-profit institution and ownership could be transferred, which could place limitations on our access to their belugas. Additionally, there are many logistical constraints such as the lack of infrastructure for properly maintaining and archiving biological samples for quality control at Marineland (e.g. a -80 freezer), and the ability to transport samples that need analyses immediately over the border in a timely fashion due to the requirement for multiple permits. Time delays that can occur with shipping samples threaten the integrity of samples and compromise quality, perhaps resulting in inaccurate data or rendering the valuable samples useless. Additional confounding variables are also added when other facilities are collecting samples from whales and/or whales in different habitats with different environmental parameters which may impact results. Even though all facilities try to be consistent and follow protocols, a minor introduced change or variable may impact results. To minimize this and environmental variables, housing all the whales for the research studies at Mystic Aquarium, under the same environmental conditions, training regimes and sampling collection and processing is critical and will ensure the most accurate data possible. Moreover, Mystic Aquarium, as the lead research institution, has all equipment, labs, and staff at Mystic Aquarium. Even with the Marineland belugas or other aquaria that hold belugas participating in research, Mystic Aquarium needs a solid cohort of belugas on-site to develop and test research protocols that can be applied to the cohorts at other facilities.

For studies that can be conducted at another location, we will continue to seek samples from other aquaria that house belugas in order to increase sample size (i.e. those that are easier to obtain such as breath samples). Those research studies which take ample training time and a more in-depth effort for sampling have not been and are not feasible; it has not been feasible for other facilities to shift their institutional priorities to train whales for in-depth hearing or diving physiology studies, for example. It is our hope that other institutions will become involved over time further increasing the number of samples as feasible. A great example of this is Mystic Aquarium's

institutional collaboration with Georgia Aquarium (See Appendix 1 Georgia_Mystic_Partnership) in which Georgia Aquarium has committed to support Mystic Aquarium's beluga research studies by providing samples on the belugas listed on this permit if deemed in the best interest of an individual whale or the US beluga population for social, health, or welfare, reasons to move the animals to Georgia Aquarium. The 5 additional whales from Marineland to be cared for and participate for the primary purpose of research at Mystic Aquarium will enable a sample size of 6 whales (including the current 1 whale owned by Mystic Aquarium) enabling a more robust sample size and the potential for more statistically significant conclusions to be drawn for the studies proposed. Any additional belugas held at Mystic Aquarium for public display purposes will also be trained to participate in the proposed research to further enhance sample size for the duration they are located at the facility.

Attach a References File

- Attach a **bibliography** of references cited in this application. Referenced materials must be made available upon request, as needed for evaluation of the application, or preparation of any necessary ESA or NEPA analyses.

See Appendix 6 References

****Resources Needed to Accomplish Objectives***

- Explain how your expertise, facilities, and resources are adequate to accomplish your proposed objectives and activities.

- Attach copies of any relevant formal research proposals, contracts, grant awards, or letters of agreement that would demonstrate financial or logistical resources.

- Indicate the status of any other international, federal, state, or local authorizations you have applied for, secured, or will apply for.

Mystic Aquarium has an internal research team of five scientists dedicated to beluga research and conservation. Our team collaborates with scientists-in-residence, external scientists affiliated with Mystic Aquarium, (See Appendix 7 Scientists-In-Residence), and a cadre of beluga wildlife biologists, acoustics experts, social scientists and native community members.

Mystic Aquarium has well established collaborations with wildlife biologists who work in the field studying belugas as well as Native Alaskans who rely on belugas for food security, as well as medicinal, spiritual and social needs. The PI has worked with the North Slope Borough Department of Wildlife Management, and in collaboration with Native Alaskans participating in research on Chukchi Sea Belugas since 1994. Most of the pioneering work on the immune system in belugas was made possible by collecting tissues from subsistence hunts (Romano et al., 1992, 1993, 1994, 1999, 2002). The reagents and assays developed from this research were able to be applied to live capture release studies in tandem with tagging studies on Chukchi Sea belugas as well as Bristol Bay whales (Flower et al., 2015; Thompson et al., 2014; Unal et al., 2018). Data from both of these populations is available for comparison with Cook Inlet belugas and other populations as feasible. Findings have been reported at the Alaska Beluga Whale Committee as health of belugas is a major concern and our research presented to NOAA at multiple workshops

with the most recent at the Cook Inlet Beluga Research Methods Workshop (November, 2017) and the Cook Inlet Beluga Management, Research and Partnership Opportunities meeting (April 2018). Mystic Aquarium scientists have also conducted hands-off, observational studies in belugas that summer in Cunningham Inlet, Somerset Island, Inuvut, Canada to determine how the changing environment may be impacting belugas and their use of Cunningham Inlet (Anderson et al., 2017).

Importantly, we have worked closely with Native Alaskan and Native American youth with primary focus on promoting beluga sustainability and research. In collaboration with the North Slope Borough Department of Wildlife Management we have conducted a nationally recognized educational and cultural exchange program in which Native Alaskan and Native American youth work side by side with the PI and scientists in the field collecting research samples on belugas and then travel to Mystic Aquarium for continued hands-on scientific learning. Activities include interaction with our beluga whales and learning about their natural history, care and training, observing how we take research samples from our trained whales and then taking the samples to the research lab to process them and learn about beluga health from the samples; a field trip to our local marine environment; a workshop on careers; and other educational opportunities the aquarium offers. Our local tribal youth also participate and time is spent at the Mashantucket Pequot Museum and Research Center where both groups talk about and exchange their culture. Mashantucket Pequot youth have travelled with the PI to the Arctic for the past few years. Our relationship with the native communities and our commitment to native youth demonstrates our long standing dedication to the cultural and environmental importance of belugas in the wild, their conservation and sustainability.

Mystic Aquarium is unique among Association of Zoos & Aquariums (AZA) accredited beluga holders in that we have a USDA Research License (# 16-R-0031) to conduct bona fide research on our animal collection with a well-established IACUC. See Appendix 8 IACUC Overview. Funding from external grants as well as internal funds supports the research and sample analyses. See Appendix 9 Grant_Funding_History. We have fully functional research laboratories located at the University of Connecticut (15 minutes from the Aquarium) equipped for molecular biology, microbiology, immunology, endocrinology and marine mammal health related research and staff with expertise capable of completing the objectives. See Appendix 10 Resources_Mystic Aquarium for more detailed information.

Mystic Aquarium also collaborates with other AZA accredited aquaria that maintain belugas under professional care and has requested and received beluga samples for research. These samples have been limited to a small number of whales and limited in frequency of sampling due to personnel constraints and limited time available given other priorities in other animal care and training programs. Although scientific publications have resulted from this work (e.g. Spoon and Romano, 2012; Richard et al., 2016, 2017) samples were limited in scope and not as many samples were obtained as desired due to limitations in training time and personnel from the other facilities. While we will continue to seek samples from other aquaria to increase sample size for some studies (i.e. those that are easier to obtain such as breath samples), those research studies which take ample training time and a more in-depth effort for sampling are not feasible. The 5 additional whales from Marineland to be cared for and participate for the primary purpose of research at Mystic Aquarium will enable a sample size of 6 whales (5 imported plus the current 1 owned by Mystic Aquarium) enabling a more robust sample size with opportunity for statistical conclusions to be drawn for the proposed studies. The additional 2 belugas currently housed at Mystic Aquarium, but owned by other accredited beluga holding facilities, will participate in the proposed research

while they are housed at Mystic Aquarium; however, because they are present on a loan agreement, their presence cannot be assured for the duration of the permit.

Disposition of Tissue Samples

- Indicate the disposition of any remaining samples after your project is complete.
    - State whether samples will be consumed in analysis, destroyed, or exported back to facility/researcher
    - If applicable, list the name and location of the person or institution that will store/curate samples. Indicate if you will retain legal custody of the archived samples or if you wish to permanently transfer the samples once your project is complete.

It is anticipated that samples will be consumed in analysis and, if not, then will be appropriately banked at the Mystic Aquarium research operation site. Mystic Aquarium maintains sample archives at -80°C, -20°C, and -180°C and will retain legal custody of the samples.

Public Availability of Product/Publications

- Describe the end products of your proposed project and how they will be made available to the public.

Research will be published in peer reviewed scientific journals (e.g. Marine Mammal Science, Conservation Biology, etc.) and research presentations will be given at conferences (Society of Marine Mammalogy, Alaska Marine Science Symposium, Alaska Beluga Whale Committee, NOAA workshops, Association of Zoos and Aquariums, etc.).

Mystic Aquarium is visited by ~800,000 guests per year. Research will be shared with the public through graphics, presentations, classes, lectures, and social media. This will increase awareness of the issues facing wild beluga populations. This education and outreach is also in line with Action# 2 ("Create and support a CI Beluga Recovery Coordinator position") of the Cook Inlet Beluga Recovery Plan.

Research findings will be incorporated into our science-based Educational and Cultural Exchange Program for Native Alaskan and Native American youth as well as our National Science Foundation funded "Research Experiences for Undergraduates" where undergraduates from underrepresented groups such as Native Alaskans are targeted for research internship experiences.

Captive Information

If you will be working with animals in captivity (permanent or temporary), including removing animals from the wild into captivity and research or enhancement on captive or rehabilitating animals**,** address the following ***as applicable*** (explain if not applicable):

a) If removing animals from the wild, explain why removal is necessary and why you cannot obtain suitable animals from captive or rehabilitated stock.

No removal of animals from the wild is being proposed. Belugas proposed for research include only captive-born animals from Marineland of Canada.

b) If the source stock is to be beached/stranded marine mammals undergoing rehabilitation, indicate the name and location of the rehabilitation facility.

Not Applicable; animals proposed for research include captive-born animals only.

c) If the source stock is from animals already in captivity (other than animals in rehabilitation) indicate the name and location of the facility and, where possible, identify the specific animals (by NOAA ID number if applicable) to be involved in the proposed activity.

Belugas will be imported from Marineland of Canada, Niagara Falls, Ontario, Canada to Mystic Aquarium, Mystic, CT, USA for the purpose of research. The movement of these captive-born whales to Mystic Aquarium will create space at Marineland for the wellbeing of their belugas and to accommodate the imminent calving expected as a result of confirmed pregnancies at Marineland. The belugas proposed for import are: Qila (9 year old female), Frankie (7 year old male), Havana (4 year old female), Kharabali (5 year old female), and Mira (10 year old female). Research will occur on the Mystic Aquarium campus at 55 Coogan Blvd, Mystic, CT 06355. Additional research locations may include Georgia Aquarium and they have committed to continuation of research samples on any of the imported whales that may be transported to their facility if deemed in the best interest of an individual beluga or the US beluga population for social, health, or welfare reasons. Georgia Aquarium is located at 225 Baker Street NW, Atlanta, GA USA. Georgia Aquarium is an accredited facility with experience in the care of belugas. Should it become necessary or desirable to move belugas to another location besides Georgia Aquarium, authorization from NMFS would be sought. Any calves of imported belugas would remain with the dam at Mystic Aquarium (or Georgia Aquarium, should the dam need to be moved as described above) for at least the first three years of life. Calves less than three years of age will not be moved.

d) Attach a copy of any license or registration issued by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture, any outstanding variances granted, and the most recent APHIS inspection report.

Appendix11 USDA Licenses_Inspections

e) Attach the protocol forms submitted to the appropriate Institutional Animal Care and Use Committee (IACUC) established under the Animal Welfare Act (AWA), the IACUC approval, and any comments and recommendations of the IACUC.

Appendix 5 IACUC Approved Protocols

f) Attach a written statement from the responsible veterinarian or expert certifying that the facilities, methods of care and maintenance, and methods of transport will be adequate to ensure the well-being of the animals and will comply with all care and transport standards established under the AWA.
See Appendix 12 Veterinary Statement

g) Describe the care and maintenance of the animals, including a complete description of the facilities where they will be maintained. This includes but is not limited to:

- dimensions of the pools or other holding facilities
- number, sex, and age of animals by species to be held in each
- water supply, amount, and quality
- diet, amount and type
- sanitation practices.

The mission of Mystic Aquarium is to inspire people to care for and protect our ocean planet through conservation, education, and research. Since its founding in 1973, Mystic Aquarium has been a leader in global efforts to protect our ocean planet, while advancing the science of marine animal care. Mystic Aquarium has been engaged in the research and display of beluga whales since 1975 and its programs have contributed greatly to the body of knowledge known about beluga health, physiology, and conservation. Mystic Aquarium has the very highest standards for animal care and wellbeing and is accredited by the Association of Zoos and Aquariums (AZA), the Alliance of Marine Mammal Parks and Associations (AMMPA), the International Marine Animal Trainers Association (IMATA), and American Humane Association Humane Conservation.

The belugas referenced in this permit will be maintained in the Mystic Aquarium Arctic Coast beluga habitat with conspecifics and two species of phocids. While the goal of the permit is research, public display will be incidental. The current animal complement we propose to add these belugas to is a group consisting of: a 16-year old male beluga (NOA0006362), two 37-year old female belugas (NOA0001077 and NOA0000748), and periodically seals under care at Mystic Aquarium live within the exhibit, including a 4-year old female spotted seal (NOA0010324), a 1-year female spotted seal (NOA0010587) and the 6-7 year old female harbor seals (NOA0010117, NOA0010027, NOA0010008, and NOA0010009). In the event of a beluga transfer to Georgia Aquarium, the beluga would join the following complement of animals: currently two males ages 10 and 32 years (NOA0006607 and NOA0006731) and three females ages 11, 14 and 20 years (NOA0005856, NOA0006479, and NOA0006498).

The Arctic Coast Habitat is a 750,000 gallon, outdoor, closed system designed specifically for the research, care, and display of belugas. It is the largest outdoor beluga habitat in the United States. The habitat is enhanced by a naturalistic style, both above and below water to mimic the appearance of a region along the Arctic coast. The pool wall and bottom are dynamic in that they change in counter and shape throughout the habitat space. Pool depth varies from just a few inches to 16.5 feet to mimic a wild environment. Underwater outcroppings, an island with a swim-through arch, and a loose cobble rubbing bed add to the enrichment of the habitat. The main pool (165’L x 69’W x 16.5’Depth; ~590,000 gallons) is connected to the holding pool (40’ diameter x 13’ Depth; ~120,000 gallons) and medical pool (32’L x 24’W x 7’Depth; ~40,000 gallons) by a series of three sliding acrylic gates that can be open or closed as desired for animal management. An

additional sliding acrylic gate connects the holding pool to the medical pool for animal management. Each gate location has a channel to receive sectional bulkheads (water tights) that allow a pool to be drained without draining an adjacent pool. The medical pool contains a specially designed hydraulic false bottom (lift) that covers the bottom of the pool to aid in the rapid and safe handling of belugas, as needed for medical procedures. The lift functions by rising above the water level (and is able to stop at any point from the pool bottom to above the water level) to allow quick access and handling (less than one minute) of the whales to conduct medical procedures and move belugas in/out of the habitat for transports to other facilities. The lift consists of four vertical beams, a platform, 2 starboard baffles for the gate by the main pool, and a net/pvc baffle for the gate by the holding pool and has the capacity to lift 10,000 lbs.

The "beach" elevations between the main pool and the other two pools are raised such that the entire volume of the holding pool can be pumped into the main pool without overflowing it. This feature aids in water conservation during pool drains. The eastern end of the exhibit is fed by two sets of waterfalls that cascade down off of the rock face into the shallows.

Pool water is fresh city water from the local water company with Hi-Grade Evaporated salt brine added to a final salinity of 32-34 ppt. Saturated brine is stored in a 5,000 gallon vat and is pumped into the system in batches when replacement water for backwashes or pool drains causes the salinity to fall below set parameters.

Three 60 HP pumps circulate water through eight 5 foot diameter horizontal sand filters. System flow rate is ~9600 gallons per minute providing a total water turnover time of 78 minutes. The filters have a 13 foot total length and are backwashed as needed based on increases in pressure differential and decreases in system flow rates. Backwash is stored in a 12,000 gallon tank where it is reclaimed through filtration and ozonation. Reclaimed backwash water is monitored for turbidity and returned to the pool prior to the next backwash cycle if the turbidity is <2.0 NTU. Weekly coliform testing is run to ensure that proper sterilization of the water is being achieved. Coliforms are run by the membrane filtration method. Samples are incubated for 24 hours and values are recorded, in compliance with the Animal Welfare Act. In the unlikely event that a reading exceeds the set limit of 1000 MPN (most probable number), the system would be retested and, if it is still high, corrective actions would be immediately implemented under the direction of the Manager of Environmental Quality and/or Chief Clinical Veterinarian.

Soda ash and sodium bicarbonate are added to this system for pH control. Soda ash and water are replaced daily in a 40 gallon reservoir. Soda ash solution is metered into the filtered water headers at rates adjusted to maintain pH within accepted parameters. Sodium bicarbonate is batched to pool skimmers to assist with pH maintenance.

Aluminum sulfate (48%) is added via metered pump to the system to improvement water clarity following each scrub dive for approximately one hour.

Up to 30% of the system flow can be diverted from the filtered water headers to side stream loop where ozone is added through Mazzei injectors as it is pumped at ~380 gallons per minute to an ozone contact tower for contact time before returning to the pool. Ozone is added to decolorize the water and as a disinfectant eliminating the need for chlorination of the system. Water quality

determines the amount of ozone to be added and depends on a combination of parameters including water color, ORP readings, and dissolved ozone. ORP is monitored inline, and this is recorded 3X daily to ensure the appropriate ranges are achieved. Water returning to the pool from the ozone contact tower should have less than .04 mg ozone per L.
Salinity and pH are tested daily, run on probes located in the water quality lab. The probes are rinsed with reverse osmosis water between each sample and each probe is calibrated on a regular basis to ensure accuracy. Temperature is tested up to 3X/day, and is either recorded from inline temperature probes or by using a digital thermometer located at the sample port.
Ammonia, nitrite, nitrate, total alkalinity, phosphates, are tested on an as-needed schedule.

Water Quality Parameters:

<table>
<tr><th colspan="2">Parameters</th></tr>
<tr><td>Temperature</td><td>The water temperature varies on a seasonal schedule to mimic natural seasonal variation in the wild. The water is allowed to get as cold as it will naturally in the winter and with the following maximum temperatures year round:
<table>
<tr><td>Month</td><td>Max°F</td></tr>
<tr><td>January</td><td>50</td></tr>
<tr><td>February</td><td>50</td></tr>
<tr><td>March</td><td>52</td></tr>
<tr><td>April</td><td>52</td></tr>
<tr><td>May</td><td>56</td></tr>
<tr><td>June</td><td>59</td></tr>
<tr><td>July</td><td>59</td></tr>
<tr><td>August</td><td>59</td></tr>
<tr><td>September</td><td>59</td></tr>
<tr><td>October</td><td>56</td></tr>
<tr><td>November</td><td>56</td></tr>
<tr><td>December</td><td>52</td></tr>
</table></td></tr>
<tr><td>Salinity</td><td>32 - 34 ppt</td></tr>
<tr><td>pH</td><td>7.8 – 8.3</td></tr>
</table>

The Arctic Coast habitat is cleaned and maintained by Husbandry staff on a daily basis which includes cleaning from the surface and under water using SCUBA divers. High standards are maintained for providing high quality safety and animal care in compliance with USDA/APHIS and OSHA regulations, and accrediting organization standards.

Beaches are rinsed and disinfected utilizing a garden hose with a metered garden sprayer attached containing full strength Formula 10 (Quaternary Ammonia) set to 1.0 ounce per gallon. The disinfectant remains in contact with the surface of the beach for at least 10 minutes before being

thoroughly rinsed. Any standing water left after rinsing is removed by brushing water to an appropriate discharge location.

Any debris is removed daily utilizing a pool maintenance pole and a net and all waste is properly discarded. All poles and nets are disinfected after each use and stored in a designated location. Pool skimmers on Arctic Coast are located on the west side of the medical pool, the south side of the holding pool, and the west side of the main exhibit (in the public space) and covered with a fiberglass grate. All skimmers are checked routinely and any debris is removed and discarded. Exhibit glass is cleaned daily.

Belugas at Mystic Aquarium are fed a diet of frozen, thawed human quality fish, prepared according to the Animal Welfare Act requirements including high and low fat herring, capelin, squid, sardines, and anchovy. Fish types are selected based on the specific nutrient content, quality, availability, sustainability, price, and animal preference. Beluga food intake and caloric consumption are individually developed, monitored and recorded on a daily basis.

Target weights and girth measurements are established for each beluga based on:

- Species historical data
- Individual historical data
- Sex
- Age

Daily Caloric intake is established before the onset of each year based on:

- Individual historical data
- Time of year
- Body Condition
- Expected Growth rates
- Reproductive status
- Whether habitat is indoors/outdoors
- Air temperature
- Water temperature

Since each fish type provides different nutritive qualities and there is also great value to provide the belugas with a variable diet of fish, the daily diet and caloric intake for each beluga is formulated on fish variety, nutritive value, palatability and the Veterinarian's recommended % calories of fish type that can be consumed each day. The nutritive value of fish is obtained through proximate analysis that is run on each of the products that is purchased and fed to the belugas.

Target girth and caloric intake charts are made for each beluga and are used as reference throughout the year. Diet formulation is also based on body condition and is comprised of:

- Body weights, girth measurements, and caloric intake status
- Visual assessment of body condition
- Assessment of animals' degree of hunger drive during feeding sessions

Mystic Aquarium has a strong program of preventative medicine that ensures all animals, including belugas, are healthy and thriving. The Aquarium employs a large team of experienced trainers to provide for the daily husbandry care of the belugas, and a team of four veterinarians who can respond to any medical issues with immediacy. Mystic Aquarium has a state-of-the-art veterinary hospital and portable diagnostic equipment (ultrasound, radiograph, endoscopy, etc.) that can be brought to the habitat for pool-side diagnostics to avoid any need to move the belugas for this purpose.

The use of behavioral conditioning plays a vital role in the care of belugas at Mystic Aquarium (MA) and greatly supports our ability to deliver high quality animal care and provide for optimal welfare. The goal of the training program is to train the animals to participate in their own healthcare and research on a voluntary basis, as well as providing mental stimulation and enrichment for the animals. The Aquarium's behavioral conditioning program is designed and structured to meet the standards and guidelines established by the Association of Zoos and Aquariums, the Alliance of Marine Mammal Parks and Aquariums and International Marine Animal Trainers Association, emphasizing positive reinforcement utilizing standard operant conditioning.

Behavioral conditioning techniques are used to establish behaviors that assist with husbandry/medical care (i.e. blood samples, injections, fecal samples, mouth swabs, eye drops, etc.), environmental enrichment, exercise, and mental stimulation. Consistent and positive approaches to each behavior are the single most important aspect of maintaining a successful behavioral conditioning program and building trust with the animals.

Information on the facility and program of animal care at Georgia Aquarium is included as Appendix 13 GA_Facility Description.

h) Indicate whether a captive breeding program will be established and, if so, provide justification in accordance with the species conservation or recovery plan as applicable for enhancement activities. *For ESA-listed species,* indicate if you are willing to participate in a captive breeding program if requested by NMFS.

All of the proposed research activities within this permit provide important biological information on beluga whales. While beluga whale reproduction is not the purpose of the proposed research, reproduction is a natural behavior and will be allowed to occur under natural conditions. As described in the objectives and methods for Study #7 (Behavioral and Reproduction Studies), artificial insemination will not be used and contraception will not occur unless medically necessary for the health and wellbeing of an individual beluga. In the event the subject whales become pregnant, we propose to continue to contribute knowledge and information regarding beluga reproduction on belugas under controlled conditions in an aquarium setting (see Study #7 Behavioral and Reproduction Studies objectives and methods above).

Responsible management and production of offspring would allow for our research program to continue into the future, and will increase our understanding of maternal and calf physiology, which would be applied to the benefit of wild populations.

i) Indicate the disposition of captive animals at the termination of research or enhancement activities.

At the termination of research projects (i.e., at the end of the 5-year permit), belugas participating in the project would continue to reside at Mystic Aquarium or Georgia Aquarium. However, if deemed in the best interest of an individual beluga or the US beluga population for reasons as described above (i.e., social, health, or welfare of the animals), they may be moved to another professionally-accredited facility in the United States that has experience and expertise in the care of belugas.

j) If release of captive animals to the wild is proposed, state the length of time the animals will be held, no matter how temporary, and describe the protocols for the release, including post-release monitoring protocols. Include in the release protocol mitigation for the following:

- disease transmission between released animals and the wild population
- potential genetic exchanges between introduced and endemic stocks
- ability of the released animals to forage and protect themselves from predators
- elimination of behavioral patterns acquired during captivity that could prove detrimental to the released animals or the social structure of local populations.

Release of animals to the wild is not proposed.

Project Locations

- You will first describe where you plan to work. Then, for each location, you will use the Take Table to list the species you expect to encounter and the take procedures you will conduct.

Import/transport of animals will occur from Marineland of Canada, Niagara Falls, Ontario, Canada to Mystic Aquarium, Mystic, CT USA. Research activities will occur at Mystic Aquarium, 55 Coogan Boulevard, Mystic, CT 06355 USA. While not anticipated, if deemed in the best interest of an individual beluga or the US beluga population for social, health, or welfare reasons, they may be moved to Georgia Aquarium, 225 Baker Street, Atlanta, GA 30313 USA, an accredited facility with experience in the care of belugas. This collaboration ensures there dynamic flexibility for optimal animal management without compromise to the research projects proposed, as Georgia Aquarium will provide samples on these animals to Mystic Aquarium throughout the duration of this permit should belugas need to be moved.

- Add New Location: provide information about one (or more) study areas
  - General area (ocean basin)
  - State(s), as applicable.

- Enter Location Details, as applicable:

  - Latitude and longitude of your study area
  - Marineland CANADA: Latitude: 43.05 Longitude: -79.07

- Mystic Aquarium, Mystic, CT: Latitude : 41.37 Longitude: -71.95
- Georgia Aquarium, Atlanta, GA: Latitude: 33.76 Longitude: -84.40

- Attach File: Attach a high quality map(s) with the correct scale that clearly shows the location of your proposed activity and any environmental aspects of interest. If possible, include a shapefile, Google Earth kmz/kml, or ASCII text file with lat/long data and the associated basic metadata with your electronic application submission.

  See Appendix 14 Maps_Travel

1) **Transport**:

Mystic Aquarium has extensive experience transporting belugas including large scale transports involving multiple facilities. Transports between facilities generally include a combination of ground and air transport, with air transport being the major mode of transportation to minimize transport time. Truck transportation generally serves to move animals to and from airports for air transportation. Coordination of ground transport falls under the responsibility of Mystic Aquarium staff or collaborating facilities. Air transport with a reputable transport company with a history of live animal transports, is used. Transport plans are coordinated to minimize time and number of layovers.

a) **Mode(s) of transportation**: Describe the mode of transportation. Include a description of the vehicle or other platform used to transport animals.

Mode of transportation: Each beluga will be placed in a stretcher and then secured into its own transport cradle approximately 30-60 minutes prior to departure from Marineland of Canada. They will be transported via flatbed truck to the Hamilton International Airport (approximately 55 miles/ 1 to 1.5 hour drive time) where they will be loaded on board a chartered jet aircraft for the estimated 2 hour flight. Working with a licensed broker, port exemptions will be handled to ensure seamless transport. Upon arrival at Bradley International Airport in Hartford, CT., the transport cradle and beluga will be transferred to a flatbed truck for truck transport to Mystic Aquarium (approximately 68 miles/ 1h 15min drive time). Once at Mystic Aquarium, the beluga will be crane lifted out of the transport cradle, placed on a beluga transport cart, wheeled onto the Arctic Coast habitat, crane lifted out of the transport cart and placed into the medical pool (containing a false-bottom/beluga lift) to be released from the stretcher.

Transport vehicles – Flatbed trucks will be used to transfer the beluga between Marineland of Canada to the Hamilton International Airport and Bradley International Airport and Mystic Aquarium. The transport cradle will be secured to the truck bed via web strapping. Support vehicles will be used on either end to carry additional staff and equipment as needed.

Crane and Fork Trucks – 30,000 lb. / 15 ton fork trucks and cranes under contract from commercial equipment companies will be used at Marineland of Canada, Hamilton International Airport, Bradley International airport, and Mystic Aquarium. Cranes will be used to lift the beluga and stretcher in/out of the transport cradle. Fork trucks will be used to transfer the beluga cradle from the ground to all flatbed trucks and from flatbed trucks to the ground.

Aircraft – A cargo configured and pressurized AirBus A300 (or a jet craft of appropriate size to handle load), aircraft will fly the beluga from Hamilton International Airport to Bradley International Airport.

b) **The name of the transportation company, if applicable, and the qualifications of the common carrier to transport live animals**: If a contractor or other entity will do the transportation, enter information in the box. Otherwise, click on N/A.

An AirBus A300 (or appropriate sized jet to handle load) with previous experience in the air transportation of belugas will be chartered.
A trucking company will be used for the rental of 2 30,000/15 ton fork trucks and 1 IC-200 Broderson crane and for the rental of a backup low boy flatbed truck.
Contracted companies in Canada for flatbed truck, 30,000 lb. /15 ton fork truck and crane will be investigated.

c) **Maximum length of time from capture to arrival at destination**: How long will the animals be in transport?

The maximum transport time is estimated at 10 hours, as described above under Project Descriptions.

d) **Description of the container (e.g., cage, tank) used to hold the animal during transit**: Include the material of the container and its dimensions.

Belugas will be transported in individual cradles that meet or exceed IATA and USDA/APHIS standards.

Cradles utilized will be either 16’ or 18’ long, depending on the size of the beluga to be moved. The transport cradles are steel reinforced, plywood wall, fiber glass construction with a fiber glassed coated interior, lined with 1” closed cell foam padding and covered with a custom beaded vinyl liner. They will be filled with fresh water to approximately 26 inches, below the level of the blowhole. There are steel cross braces with 1 ton chain falls for suspending beluga stretcher poles. The transport cradle is placed on 20’ ‘slim-line’ aircraft pallet and cribbed on wood construction pallet of 2’x 12’s running the length of the pallet and connected via 2’ x 12’ cross pieces. Cradle is set on cross pieces to distribute load. Specifics for the transport units are described above under Project Description

e) **Any special care procedures (e.g., moisture, medicines) to be administered during transport**: How will the animals be cared for during transport?

During all truck transportations there are two experienced and trained husbandry attendants safely secured via safety harnesses to the transport carriers who sit on top of the baffles on the tail and head ends of the carrier to monitor water temperature, behavior, and wet the beluga’s exposed dorsal surface with ladles during the truck transit. During the entire transport there are at least 3

experienced and trained personnel accompanying the transport, at least one of whom is a licensed veterinarian with extensive beluga whale experience to provide care if necessary.

While in air transit, attendants will don dry suits and alternate, as needed, from outside the transport cradle or stand inside the cradle to assist with calming, handling, or assisting with administration of any medical treatment that is needed. The general monitoring routine during air transit is as follows:

- Feeding not anticipated during transport
- Constant air and water temperature monitoring
- Adjustment of cabin temperature as needed to be maintained at 50 – 60^{o} Fahrenheit
- Cabin pressure will be maintained by the flight crew at or below 5000' ASL
- Attendants will keep the belugas back moist, adjust positioning of stretcher, and log data
- Temperature and respiration rates will be monitored and logged during the transport

The attending veterinarian will bring a medical kit containing equipment and medications that could be needed, including sedatives for the rare event that the whales become agitated, as well as water/ice to cool down animals as needed.

f) **A statement as to whether the animals will be accompanied by a veterinarian or some similarly qualified person**: If so, give the name, affiliation, contact information for each person.

At least 3 qualified personnel will accompany the belugas on the aircraft, more if allowed by the aircraft regulations. At least one of the attendants will be a licensed veterinarian with extensive beluga experience to provide medical care if necessary.

On the ground transport, there will be a total team of 9 core people receiving the belugas, plus 3 additional people, including:

- Covered wagon/flatbed truck driver
- Fork Truck driver
- Large Equipment Coordinator With Experience Moving Belugas
- 2 Facilities staff (to help with strapping, unloading equipment etc.)
- Transport coordinator
- 1 licensed veterinarian with experience in beluga health assessment and management – to relieve vet coming on the plane
- 2 husbandry staff with experience in the assessment and care of belugas – to relieve the husbandry staff coming on the plane
- Safety Director
- 1 PR person
- 1 photographer/videographer

At Mystic Aquarium, 20 core people (some of the same as listed above) will be present to ensure successful unloading from truck to Cart/unloading from cart, including:

- Covered wagon/flatbed truck driver
- Crane driver
- Large equipment coordinator with experience in moving belugas
- Transport Coordinator
- 16 animal husbandry staff with experience in the care of belugas
- 2 or more licensed veterinarians with experience in the health assessment and management of belugas

As the belugas are transferred to the habitat at Mystic Aquarium, the following key individuals (some of the same as listed above) will be in place:

- Crane operator/large equipment coordinator with experience moving belugas
- Lift operator/transport coordinator with experience moving belugas
- 10 animal husbandry staff with experience in the care of belugas
- 2 standby divers
- 1 safety diver
- 2 or more licensed veterinarians with experience in the health assessment and management of belugas

Specific Roles:
Attendants: Curator of Marine Mammals and Birds, Laurie Macha, will be the transport coordinator, responsible for ensuring the transport is planned and executed. Laurie Macha has extensive experience in the transport of marine mammals, including belugas. Dr. Jen Flower, DVM, MS, Dipl. ACZM, will be the attending veterinarian overseeing the transport. Dr. Flower is board-certified in zoological medicine, and has extensive marine mammal medical and transport experience, including belugas. Dr. Flower will be responsible for all veterinary decisions during the transport and will be in communication with the veterinary teams in Mystic, CT and Marineland Canada throughout the transport.

Jennifer Flower, DVM, MS, Diplomate ACZM
Chief Clinical Veterinarian
Mystic Aquarium
55 Coogan Blvd.
Mystic, CT 06355

Laurie Macha
Curator of Marine Mammals and Birds
Mystic Aquarium
55 Coogan Blvd.
Mystic, CT 06355

3rd person to be determined- person selected will be experienced with beluga care and transport and trained for beluga transport.

More animal care staff may be included on the transport if allowed by the aircraft regulations.

g) **Destination**: Use the drop down list to select the destination. If your destination is not on the list, click on the "New Facility" button to add it. If the animals will be taken to a laboratory or aquarium, provide details of the location. If the animals will be released in another waterbody, provide details of the location.

Arctic Coast Beluga Habitat at Mystic Aquarium, 55 Coogan Blvd, Mystic, CT, USA.

h) **How will the animals be contained at the destination facility?**: Describe the containment system for the animals, quarantine procedures, and effluent treatment.

The belugas will be maintained in the Arctic Coast habitat which meets and/or exceeds the USDA/APHIS, AMMPA, and AZA enclosure requirements. A relative risk quarantine strategy is used: since belugas will be deemed healthy by way of pre-shipment testing and come from a closed environment, belugas will not be isolated for quarantine prior to introduction to the habitat to allow for a more rapid and seamless acclimation.

In the highly unlikely scenario where quarantine is necessary (ex. infectious disease is identified during or following transport), this can be accomplished in Mystic Aquarium's Wiederhold Veterinary Animal Health and Care center, where quarantine pools appropriate for belugas are available. In this situation, quarantine would be risk-based for the issues identified. Generally, the quarantine would last 30 days or until any disease identified is known to be completely resolved, but this would vary based on many circumstances. The length of quarantine is determined by a veterinarian board-certified by the American College of Zoological Medicine, who is highly qualified in this area.

i) **The final disposition of the animals**: Describe, for example, whether the animal will be released or retained in permanent captivity.

The belugas will be maintained in the beluga habitat at Mystic Aquarium or, if deemed in the best interest of an individual beluga or the US beluga population for social, health, or welfare reasons, belugas may be moved to Georgia Aquarium, an accredited facility with experience in the care of belugas, for reasons stated above.

Information on the transport of belugas from Mystic Aquarium to Georgia Aquarium, should that need to occur for the physical or social well-being of any of the belugas listed in this permit, is attached as Appendix 15 GA_Transport Plan.

National Environmental Policy Act (NEPA) Considerations

In addition to providing information on effects to the target and non-target species in other sections of the application, provide information as requested below on potential environmental effects to determine if your activity may be categorically excluded from the requirement to prepare an environmental assessment or environmental impact statement under NEPA. If you believe any of the criteria are "not applicable" you must explain why.

1) If your activities will involve equipment (e.g., scientific instruments) or techniques that are new, untested, or otherwise have unknown or uncertain impacts on the biological or physical environment, please describe the equipment and techniques and provide any information about the use of these in the natural environment. In addition, please discuss the degree to which they are likely to be adopted by others for similar activities or applied more broadly.

Our activities do not involve equipment or techniques that are new, untested, or have unknown or uncertain impacts on the biological or physical environment. One exception is novel telemetry devices that may have application to wild beluga monitoring; however, we do not know specifics about these devices at present. Before using novel telemetry devices, we will consult with NMFS regarding any addition NEPA considerations and obtain NMFS approval. All techniques and analyses proposed, including training and sampling from belugas maintained under professional care, are currently being used or may be adapted by other institutions and researchers.

2) Describe the physical characteristics of your project location, including:
   a. Whether you will be working in or near unique geographic areas including but not limited to Critical Habitat for endangered or threatened species, Essential Fish Habitat, National Marine Sanctuaries, Marine Protected Areas, State or National Parks, Wilderness Areas, Wildlife Refuges, Wild and Scenic Rivers, etc.
   b. Next, discuss how your activities could impact the physical environment in those locations, such as by direct alteration of substrate during use of anchoring vessels or buoys, erecting blinds or other structures, or ingress and egress of researchers, and measures you will take to minimize these impacts.
   c. Is there potential to cause direct or indirect physical, chemical or biological alterations of the waters or substrate, including loss of, or injury to, benthic organisms (e.g., sea grass, corals), prey species and their habitat, and other ecosystem components? Could your actions reduce the quality and/or quantity of Essential Fish Habitat? If so, please provide additional details below:

      - What is the degree of alteration (low, medium, high)?
      - Approximately how much area (square footage) of habitat/substrate (e.g., seafloor, estuary or river bed) will be disturbed?

The research proposed will be conducted in the beluga habitat at Mystic Aquarium only or if needed for beluga health and wellness, at another professionally-accredited beluga holding facility within the United States. Thus, there will be no impact to the wild environment or habitat, and there is no potential for any environmental alteration associated with this research (see discussion in Justification section above regarding assurances that no additional animals will be taken from the wild to replace the animals proposed to be imported).

To satisfy the requirement that the requested import "will not likely result in the taking of marine mammals or marine mammal parts beyond those authorized by the permit" (50 CFR 216.34(a)(7)), we have worked closely with new management at Marineland to assure compliance.

We have secured written assurance from Marineland (See Appendix 16 Marineland Assurance), now under new management, that they have neither the intention nor ability to acquire more beluga

whales by import or capture from the wild. Marineland's policy in this regard is reinforced by third-party review and accountability, and by practical necessity. The current population at Marineland is reproducing at a rate making further acquisitions unnecessary and undesirable. When Marineland last imported beluga whales (2008, under previous management), its captive population numbered 34 whales. That number is now 55. It expects 3 additional new births in 2019. None of the reproduction at Marineland is assisted in any way. The current management supports transfer of 5 belugas to Mystic Aquarium for research purposes, as they recognize research is a valuable need for preserving wild populations. In addition, they recognize the movement of these captive-born belugas to Mystic Aquarium will create space at Marineland for the wellbeing of the belugas housed at their facility and will help to accommodate the imminent calving expected as a result of confirmed pregnancies at Marineland.

Marineland has established and maintains an Animal Care Committee ("ACC") monitored under the zoo inspection program, which has directed Marineland's management towards appropriately limiting any further growth of the captive population and seeking distribution and rehousing of beluga whales to qualified institutions for research, conservation, education, and the preservation of genetic diversity. The ACC comprises in-house and external veterinarians, a community representative, and outside marine mammal academic experts in a diversity of expertise and thought exceeding the requirement of the law for the establishment and use of the committee (R.S.O 1990, c. A.22 s.17). As part of its mandate the ACC is seeking to expand and integrate research and conservation efforts with internationally recognized marine mammal institutions.

By exporting five belugas to Mystic Aquarium, Marineland is both implementing its ACC direction and also making possible the significant contribution to critically important research needs for belugas to be accomplished at Mystic Aquarium. Moving 5 belugas from Marineland to Mystic Aquarium will position these belugas under controlled conditions, like the other 3 belugas currently under Mystic Aquarium care and participating in the research, in proximity to our research laboratory and scientists to allow for optimized and standardized data collection. Ensuring a minimum of 6 belugas (5 imported belugas plus 1 beluga currently owned by Mystic Aquarium) is needed for informing management and recovery of depleted beluga populations, as described above, and may allow for statistical significance and stronger data more applicable to wild belugas.

Furthermore, Canadian national and provincial policy is enacting expanded prohibitions on the capture and import of wild whales and other policy ensuring that conservation is the only valid purpose for captive populations. The most relevant measure in Parliament (the bill S-203) has been approved. Another bill (C-68) is in the final stages of passage. The final form of either of these amendments to the Fisheries Act, Criminal Code, and the Wild Animal Trade Act would only further establish Marineland's operational policy for beluga conservation and education.

3) Briefly describe important scientific, cultural, or historic resources (e.g., archeological resources, animals used for subsistence, sites listed in or eligible for listing in the National Register of Historic Places) in your project area and discuss measures you will take to ensure your work does not cause loss or destruction of such resources. If your activity will target animals in Alaska or Washington, discuss measures you will take to

ensure your project does not adversely affect the availability (e.g., distribution, abundance) or suitability (e.g., food safety) of these animals for subsistence uses.

As the research proposed will occur only within the Mystic Aquarium beluga habitat or, if necessary for the health and wellness of the beluga(s), at Georgia Aquarium, an accredited facility within the United States with experience caring for belugas, and a collaborative research partner of Mystic Aquarium, there is no risk of destruction or loss of any important scientific, cultural or historic resources.

4) Discuss whether your project involves activities known or suspected of introducing or spreading invasive species, intentionally or not, (e.g., transporting animals or tissues, discharging ballast water, use of boats/equipment at multiple sites). Describe measures you would take to prevent the possible introduction or spread of non-indigenous or invasive species, including plants, animals, microbes, or other biological agents.

The proposed research will occur solely in the Arctic Coast habitat at Mystic Aquarium, or, if deemed in the best interest of an individual beluga or the US beluga population for social, health, or welfare reasons, at Georgia Aquarium, a professionally-accredited facility within the United States with experience caring for belugas and a formal research partner to Mystic Aquarium, and not in any wild environment. As research will only be occurring in an aquarium environment, there is no impact to or alteration of the wild physical environment or any important cultural, archeological, historic, or scientific resources. Furthermore, with research limited to the aquarium environment, there is no possibility for introducing or spreading invasive species to the wild environment.

Table of Personnel Duties:

Name/Affiliation	Role	Activities
Stephen M. Coan, PhD, Mystic Aquarium, Mystic, CT	Responsible Party	
Tracy Romano, PhD, Mystic Aquarium, Mystic, CT	Principal Investigator and Authorized Recipient	Supervise and perform all activities under the permit
Allison D. Tuttle, DVM, Dipl. ACZM, Mystic Aquarium, Mystic, CT	Co-investigator and Authorized Recipient	Oversee the beluga husbandry and veterinary program at Mystic Aquarium; oversee animal health and sampling procedures for all activities.
Jennifer Flower, DVM, MS, Dipl. ACZM, Mystic Aquarium, Mystic CT	Co-investigator and Authorized Recipient	Oversee animal health and sampling procedures for all activities
Laura Thompson, PhD, Mystic Aquarium, Mystic, CT	Co-investigator and Authorized Recipient	Breath and swab sampling

Name/Affiliation	Role	Activities
Ebru Unal, PhD, Mystic Aquarium, Mystic, CT	Co-investigator and Authorized Recipient	Breath and swab sampling
Maureen Driscoll, PhD, Mystic Aquarium, Mystic, CT	Co-investigator and Authorized Recipient	Breath and swab sampling
Manuel Castellote, PhD, University of Washington and Alaska Fisheries Science Center, NOAA Fisheries, Seattle, WA	Co-investigator	AEPs/Hearing Studies
Aran Mooney, PhD Woods Hole Oceanographic Institution, Woods Hole, MA	Co-investigator	AEPs/Hearing Studies
Greg Marshall, Marshall Innovation, Washington DC	Co-investigator	Critter Cam Studies (external camera attachment via suction cups)
Justin Richard, PhD, University of Rhode Island, Kingston, RI	Authorized Recipient	Breath samples for reproduction studies
Gayle Sirpenski, Mystic Aquarium, Mystic, CT	Primary Contact	