Mystic Responses – Objectives, Justification, and Methods

File No. 22629 Comments received during the public comment period on the permit application justification/objectives and methods sent to Mystic Aquarium on February 13, 2020. Mystic Aquarium's responses received March 11, 2020.

Comment 1: Study 1 (Neuroimmunological response to environmental and anthropogenic stressors)
Application p. 26 – The application states: "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." The application also states: "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." Please clarify whether blood sampling calves less than 2 months of age will or will not be included for research purposes.

Mystic Response 1: Calves less than 2 months of age will not be handled for the sole purpose of research. Calves less than 2 months of age will only be handled if medically necessary, as deemed by the veterinarian; however, will not be sampled for research purposes.

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

Comment 2(a): Application pp. 4 and 32 – Commenters noted that fecal samples would be taken via swabs, except for collection of feces from calves taken directly from the water column, until trained for swabs. It is not clear why collection of feces from the water column was not included for older animals, as this is how samples are collected in the field. Commenters stated that anal swabs are only applicable to free-ranging beluga whales directly handled and that in-water collection methods could be tested for application to field studies (e.g., effects of pollutants and other water contaminants).

Mystic Response 2(a): The Commenters' methods for fecal sample collection are inaccurate. Fecal samples will be collected from the 5 whales in two different ways depending on the specific study: 1) Anal swabs will be used for microbiome studies (see methods for Study 6 in the permit application) 2) Soft plastic tubing (i.e. catheter tubing) will be used to collect fecal samples for hormone studies (see methods for Study 2 in the permit application). Swabs are used for microbiome studies to minimize contamination with other microbes which would provide erroneous results for microbiome investigations. Catheter tubing is used to collect fecal samples for hormones to maximize efficiency, ensure proper identification of fecal samples for each whale and maintain sample integrity. In order to ground truth the use of feces in detecting and quantifying hormones, biological validation needs to occur including longitudinal hormone data which requires samples to be collected on a regular basis. Collection of "opportunistic" fecal samples is not reliable and would produce data gaps and unpublishable results. Moreover, a staff person would need to be monitoring for defecation on a regular and consistent basis which isn't feasible. Much needed groundwork and study is necessary before transition to wild whales. Methods for collecting feces in the water column

for belugas will be carried out once techniques and assays have been validated. As stated in the permit only "opportunistic" sampling will occur by chance if any calves are born during the duration of the permit.

Comment 2(b): Application p. 30 – Regarding the use of a small boat to collect breath samples, commenters stated that sample size is largely immaterial for a methodology test and this could be conducted with the three whales already at Mystic.

Mystic Response 2(b): The commenters are missing the fact that transition of this technique to the wild will indeed include more than 3 whales at a time. We need to determine an efficient methodology for sampling multiple whales closely at the same time with optimal processing for each sample so samples are high quality (as it would be in the field). Moreover, different aged whales and individual whales will respond differently. Given the cohort of even 8 whales, some may not participate due to behavioral difference and individual preference to participate or not.

Comment 3: Study 4 (Photogrammetry)
Application p. 8 – It was noted that the following objectives primarily describe conducting the methodologies, not the scientific objectives of carrying out the study:

- 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

The objectives of the study instead appear to be to compare photogrammetry images to actual morphometric measurements/masses to ground truth whether inferences made from photogrammetry images in the wild are accurate. Please amend the scientific objectives as necessary.

Mystic Response 3: The Scientific objective will be amended to include:

- 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

Comment 4: Study 5 (Diving physiology)
Application pp. 9, 40, and 53 – Commenters state the *in vitro* laboratory methods do not need live whales and that the *in vivo* studies have little if any applicability to free-ranging populations. They state that beluga whales have a maximum recorded dive of 900 m, may dive to 600 m once a day, and routinely dive to depths of 10 to 50 m (see Rose et al., 2017). Commenters state that diving to 2 m is highly unlikely to result in physiological *diving* responses relevant to free-ranging beluga whales and a 10-min breath hold would lead to physiological responses of limited applicability to free-ranging animals.

Mystic Response 4: Dive physiology studies in cetaceans are limited, yet the majority occur in animals under human care, including in aquariums. In a PubMed search specifically for 'beluga dive physiology' only 6 references were returned: two of these used samples from deceased animals (Noren and Suydam, 2016; Bisaillon *et al*., 1988) while the remaining 4 utilized aquarium belugas and include two of our own studies (Noren *et al*., 2018; Fago *et al*., 2017; Thompson and Romano, 2015, 2016;). Other studies of beluga dive physiology occurred using trained open water animals in the US Naval Marine Mammal Program (e.g. Ridgway *et al.*, 1984), but this program no longer houses belugas. Overall, dive physiology research appears to have favored pinnipeds (both in human care and the wild), or the far more commonly held species, bottlenose dolphins (summary in Ponganis, 2015), all of which speaks to the need for beluga specific dive physiology studies. The current lack of feasibility to obtain a blood sample from a wild whale in deep water, let alone the difficulty in resampling the same individual before and after a specific dive, currently renders aquariums the only practical setting in which the proposed beluga research can take place. Our proposed research, however, does move towards tools which would transition such studies to the wild in the future.

In response to the comment: *In vitro methods do not require live whales*

In vitro methods require live whales to dive and to provide a blood sample. The blood sample may be taken before or after a stationary or active dive or at rest. The blood sample contains the target immune cells needed for the study and needs to be processed and analyzed immediately with special equipment i.e. pressure chamber designed specifically for bringing biological tissues to a specified pressure. If samples aren't processed and subjected to the pressure chamber immediately, the results will be inconclusive.

In response to the comment: *In vivo studies have little if any applicability to free-ranging populations*

In vivo physiological studies are absolutely applicable to free-ranging belugas. Proposed *in vivo* studies are aimed at understanding the role of breath-hold, dive duration and dive activity level on immune function. Breath-hold is a necessity of all dives for belugas in the wild as well as in the Aquarium setting and has direct application to wild belugas. While Mystic Aquarium's habitat is 5m deep, comparable shallow dives of <5m are often reported in wild beluga populations (Goetz *et al*., 2012; Laidre *et al*., 2017; Heide-Jorgensen *et al*., 1998; Vacquie-Garcia *et al*., 2019). In addition, changes in the duration of dives or activity during dives has been reported for wild marine mammals in response to human activities or a changing environment. For example, bottlenose dolphins increase dive durations in the presence of boats (Constantine *et al*., 2004) and belugas are diving deeper and longer to feed as prey shifts in response to melting ice and warming surface waters (Hauser et al., 2018). Additionally, belugas in Bristol Bay have been observed to end milling or feeding behaviors at the water surface and submerge for longer periods of time in response to approaching boats, in some cases swimming at increased speeds as a potential avoidance behavior (Thompson, *personal observation 2014, 2016).* Furthermore, Cook Inlet belugas display increased diving in response to pile driving activity (Kendall and Cornick, 2015). These studies demonstrate that changes in dive durations and activity level are occurring for wild beluga populations. With continued rise in water

temperatures, loss of sea ice and increased accessibility, human presence will likely increase, as will these behavioral responses. By studying whales in the Aquarium setting using specifically designed dive behaviors, we create a framework for understanding 1) basic adaptation of immune responses to diving, and 2) potential changes in physiology which would leave wild animals more susceptible to disease, or dive-related injury. Without this research, there is no reference for interpretation of data for wild whales.

In response to comment: *Beluga whales have a maximum recorded dive of 900 m, may dive to 600 m once a day, and routinely dive to depths of 10 to 50 meters (Rose et al., 2017). Commenters assert that diving to 2 m is highly unlikely to result in physiological diving responses relevant to free-ranging beluga whales.*

The maximum recorded dive for belugas in the literature that the investigators are aware of is from Citta *et al*. (2013), which indicates that belugas are capable of diving to over 1000m, with the deepest dive observed at 1160m. However, while linked, behavior is not physiology; just because these animals *can* dive deep is not evidence that they must. It is thought that most dives for belugas are much shallower, with a large amount of variability. Dives up to 50m have been reported to be common among belugas (Vacquie-Garcia *et al*., 2019). Belugas in the Beaufort and Chukchi seas often dive to a few hundred meters, though dive behaviors show some variation by region (Citta *et al.*, 2013; Hauser *et al.*, 2015). Due to the coastal nature of several populations, dive depth is often constrained by bathymetry, such as belugas in Svalbard that predominately make shallow dives to <5m (Vacquie-Garcia *et al*., 2019). Hauser *et al*., (2015) also found that belugas along the continental shelf, often dove to the bottom which is approximately 100m. It is too extensive to detail all of the variability in beluga diving, though it is clear that they have great capability to reach a variety of depths, and do so in different habitats (Watt *et al*., 2016; Hauser *et al.*, 2017, 2018). Locations where deep dives occur naturally have been linked to foraging on the bottom in deep water (Martin and Smith, 1992; Citta *et al.*, 2013; Hauser *et al.*, 2016), or navigation under ice; two conditions which are alleviated in aquaria. Nonetheless, the combination of Aquarium *in vivo* (shallow dives, dive duration and activity) and *in vitro* (simulation of deep dives) are intended to help build a framework of 'normal' immune responses that occur within beluga's common dive range.

The fact that commenters assert that diving to 2m is highly unlikely to result in physiological diving responses relevant to free-ranging belugas demonstrates a lack of understanding of dive physiology. The mammalian dive response involves physiological adjustments which serve to conserve oxygen throughout a dive, including bradycardia (slowed heart rate) and vasoconstriction of peripheral vessels to conserve blood flow to sensitive organs, which are regulated through nervous and neuroendocrine changes such as release of catecholamines (Foster and Sheel, 2005).

Dive responses occur at various intensities among mammalian species and are based on dive characteristics. These responses are also important in allowing extended duration dives regardless of depth. Depth is not a prerequisite for initiating a physiological dive response and is observed during apnea (i.e. breath hold) and facial submergence, which both initiate the dive responses in humans (Foster and Sheel, 2005). Moreover, apnea in air or with only the face submerged results in bradycardia in pinnipeds (Kaczmarek et al., 2018). Drivers of the dive

response in cetaceans (whose blowholes are submerged the majority of the time) have been studied less, including belugas. However, a recent study at Mystic measured increased catecholamines in a single beluga following 3.5-minute stationary submerged breath-holds at approximately 2m depth. Studies in the harbor porpoise have demonstrated bradycardia during stationary dives at a depth of 1m, which varied based on breath hold duration (Elmegaard *et al.*, 2016) and suggest that blowhole submergence may be a more important driver of the dive response (Elmegaard *et al.*, 2019).

Moreover, it has been well documented that belugas in the wild regularly make shallow dives, which are comparable to those in aquarium settings. For example, researchers have recorded mean dive depths of 1.6-6.7 m in Cook Inlet belugas (Goetz *et al.*, 2012; Laidre *et al.*, 2017), while a tag study of six belugas near Devon Island, Canada showed animals spent 20-39% of their time at depth less than 5m (Heide-Jorgensen *et al*., 1998), and belugas near Svalbard reportedly dive to <5m approximately 60% of the time (Vacquie-Garcia *et al.*, 2019). Currently, Mystic Aquarium is the only research facility in the world studying immune function of marine mammals during diving, and all current knowledge on this subject comes from recent studies within our laboratory (Thompson, 2014; Thompson and Romano, 2015, 2016, 2019). Furthermore, any information on the relationship between diving and health, regardless of diving depth, is important new information on the relationship between belugas, their physiology and environment.

In response to comment: *a 10 min breath hold would lead to physiological responses of limited applicability to free-ranging animals*

This is the opinion of the commenters with no basis in science or understanding of beluga dive behavior, and general marine mammal physiology. Dive responses occur at various intensities according to the conditions of the dive. Mystic has already documented increases in catecholamines (important drivers of vascular regulation, and splenic contraction during diving) in a beluga following dives of only 3.5 minutes in duration. The premise that these dives would not be applicable to free-ranging animals is false. Most natural dives are expected to be within an animal's aerobic dive limit (duration at which oxygen stores become depleted) in order to protect against damage from the products of anaerobic metabolism. Based on physiological measures, this duration is estimated to be 8-10 minutes in belugas (Shaffer *et al.*, 1997). While the longest reported dive for belugas that we are aware of is 31.4 minutes (Vacquie-Garcia *et al.*, 2019), studies conducted over several decades, report average dive durations ranging from 1.1-18 minutes (Ridgway *et al.*, 1984; Martin *et al.*, 1998; Martin and Smith, 1999; Martin *et al.*, 2001; Goetz *et al.*, 2012; Citta *et al.*, 2013; Vacquie-Garcia *et al.*, 2019). A 10- minute dive is within the mid-range of these durations, and is specifically applicable to belugas in Cook Inlet, that have average dive durations between 1 and 7 minutes (Goetz *et al.*, 2012; Laidre *et al.*, 2017). While concern may be that belugas will need to dive longer while foraging (Hauser *et al., 2015*), the 'normal' relationship between dive physiology and immune function needs to be determined before we can assess the potential impacts of longer dives.

Comment 5: Study 6 (Microbiome)

Application p. 10 – Commenters question the applicability of the microbiome study to free-ranging populations, stating differences in environments (whales in an enclosure with treated, fresh city water and fed frozen, thawed fish versus wild whales in a natural environment) would likely result in differences in the microbiomes. The commenters see value in the study when the goal is to address health and welfare of captive animals but see no value if the purpose is to understand the role of microbiomes in *free-ranging* beluga health. They recognize the value of developing methodologies to study microbiomes that can be used on samples taken in field studies.

Mystic Response 5: We are aware of the limitations of science and respectful of the peer-review publishing process to hold us accountable for conclusions. Our requirement in obtaining a permit is to propose research that will contribute significantly to the field of beluga health. Later, in publications, it will be our responsibility to draw reliable conclusions. We have a solid track record at publishing important and valid data and that will be true for this research as well.

The ultimate purpose of this research objective is to understand the role of microbiomes in wild animal health; however, in order to understand microbiomes of belugas in the wild, a reference starting point is needed under controlled conditions to work out optimal sampling protocols, processing, archiving of samples and methodologies to be able to better interpret the results. As the commenters point out we will obtain data from whales in which we know their diet, the source of their food, and the chemical and microbial make-up of the water they are housed in, all of which will be valuable information when comparing the microbiome of wild whales.

Microbiome studies on beluga whales are lacking. Conducting microbiome studies on aquarium whales will help identify core bacterial groups that are necessary for beluga health without confounding variables from wild whales. Use of aquarium belugas will help fast-track identification of these core groups. Recent studies on humpback whales demonstrate that microbiome analysis can be applied to different groups, as populations from four distinct geographic locations show that they share a core skin microbiome (Apprill *et al.*, 2014).

The factors associated with being housed under human care or in the wild does not explain a large portion of variation in microbiomes (McKenzie *et al.*, 2017). The variable that explains the highest amount of variation in marine mammal microbiomes is host taxonomy at the genus level (McKenzie *et al.*, 2017). Studies focused on the gastrointestinal (GI) microbiome of several marine mammals found that the bacterial composition is shaped by age, diet, and phylogeny (Erwin *et al.*, 2017, Nelson *et al.*, 2013, Sanders 2015). There is currently a lack of information on the effect of diet and the effect of the environment on the microbiomes of marine mammals, including cetaceans (Bik *et al.*, 2016). Belugas housed at Mystic Aquarium are housed in a controlled environment, which offers the opportunity to measure the inter-specific and inter-individual variability of their microbiota independently from environmental variability (Apprill *et al.*, 2017).

Comment 6: Study 7 (Behavior and reproduction)

Comment 6(a): Application p. 12 and 68 – Commenters state that the hypothesis does not appear to be a hypothesis, but rather a statement ("Changes in behavior and physiology will occur in belugas before, during and after the breeding season, throughout pregnancy and after birth.") Please amend the hypothesis as necessary.

Mystic Response 6(a): The hypothesis statement will be amended as follows: "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".

Comment 6(b): Regarding the statement in this study that "breeding is a natural behavior and will be allowed to occur", commenters state that there are many natural beluga whale behaviors that are prohibited in captivity, including foraging and feeding on live prey, diving greater than 5–8 m, migrating, and choosing social partners. They state that preventing breeding, "which is seasonal and intermittent (Steinman et al., 2012), seems a minor additional stressor, compared to the stress that already results from the prevention of these other, *daily* natural behaviors."

Mystic Response 6(b): Belugas demonstrate a wide variety of natural behaviors such as foraging, diving, sleeping, migrating, social grouping, molting, play, mating/breeding/courtship, calving, nursing, spy hopping, tail waving, tail slapping, jaw clapping and bubble blowing.

Breeding/Social Grouping

The statement by the commenters that breeding is only a minor stressor which is seasonal and intermittent is inaccurate. There are a multitude of behaviors that encompass breeding and what a beluga experiences during these events. Behaviors and activities associated with breeding include social grouping, play, courtship, mating, calving, nursing and calf rearing (Hill *et al.*, 2014, 2015, 2019; Robeck *et al.*, 2005)

Moreover, the social grouping, breeding, calving, and nursing portion of the beluga's life cycle encompasses up to 3 years and cannot be described as a minor event to the beluga whale (Russell *et al.* 1997; Hill *et al.* 2013; Robeck *et al.* 2005).

While captive belugas can demonstrate most of the behaviors listed above freely within their habitat, the commenters point out that there are a few behaviors such as foraging/feeding on live fish, diving deeply, and migrating that are difficult to mimic in an aquarium setting. This comment seems irrelevant to the questions of the submitted research permit and we maintain that the animals under our professional care have optimal welfare, including an enriching and supportive habitat environment. Additionally, there are many benefits to a managed setting for belugas including a lack of exposure to toxins, no predators, and being offered a nutritionally complete diet and 24/7 veterinary care.

Mystic Aquarium has an enrichment program that allows belugas to demonstrate natural behaviors in their managed habitats. Mystic Aquarium's enrichment program provides the belugas the opportunity to satisfy their behavioral needs through creative physical and mental stimuli, and a dynamic, variable environment. The details below describe the facts and

importance of each behavior (diving, foraging, migrating) and how Mystic Aquarium addresses the ability for captive belugas to express these behaviors:

Diving/Foraging

While belugas have been documented at various depths, from extremely shallow waters to deep trenches, most dives are not that deep, and belugas are often described as a shallow-water coastal species (Leatherwood and Reeves, 1983). As detailed above, average dive durations range from 1.1- 18 minutes, which indicates that the species does not need to dive to extreme depths (Ridgway *et al.*, 1984; Schreer and Kovacs, 1997; Heide-Jørgensen *et al.*, 1998; Martin and Smith, 1999; Martin *et al.*, 2001; Reidenberg and Laitman, 2002; Schreer and Kovacs, 1997; Goetz *et al.*, 2012; Citta *et al.*, 2013; Vacquie-Garcia *et al.*, 2019). The biggest variable effecting the diving behavior of a beluga is habitat and location of prey, therefore diving is tied closely to foraging and food availability (Heide-Jørgensen, *et al.*, 2001). If belugas do not need to forage, they would not perform the dive behavior; therefore, there is no need for them to conduct deep dives in managed care.

Belugas are relatively slow swimmers and therefore hunt for food in ways that yield the highest nutritional reward with the lowest energy expenditure. For example, in the Cook Inlet, belugas wait in shallow waters to catch salmon as they swim by, not requiring great depth, long dives, or excessive energy (Balsiger, 2003). The depth and length of dive is highly influenced by the area belugas inhabit (versus belugas seeking out a specific type of area or depth) and is related to prey availability and reduced forage time (Martin and Smith, 1999). Thus, diving greater than 16 – 26 feet is not a critical behavior to beluga well-being. The belugas at Mystic Aquarium engage in diving to 16.5 ft. daily but do not have the pressure for survival to hunt for their food as each animal is provided with optimal nutrition.

Mystic Aquarium takes the importance of expressing of species-specific behaviors into consideration and several processes are in place to ensure the belugas can engage in these behaviors. First, the belugas at Mystic Aquarium are observed to regularly utilize their entire habitat, swimming both shallowly at the surface and diving down to the bottom of the habitat (16.5 ft). They are also trained in behaviors that encourage retrieving items from the bottom of the pool, swimming the perimeter of the habitat, swimming at varying depth, and swimming with variable lengths of time for dive mimicry, research and exercise. The foraging/feeding behavior at Mystic Aquarium is encouraged through daily changes in food delivery, presentation, frequency, timing, task-oriented devices (feeder balls, ice/fish devices), novelty of foods, variety of foods, and other items that elicit natural foraging behavior while also providing the optimal nutrition. Live food delivery is not a preferred practice at Mystic Aquarium presently due to the risk of introducing harmful parasites and bacteria to the beluga whales; it is much safer and healthier for the belugas to eat human-grade, optimally prepared frozen-thawed seafood.

Migrating

Beluga whales are migratory, although some beluga populations are resident in defined areas and do not migrate, spending winter months offshore in waters associated with pack ice (COSWIC, 2004), then shifting to their summering grounds when sea ice recedes in the spring to warmer coastal estuaries, bays, and rivers where they give birth to their calves (Smith et *al.*, 1992; Wursig *et al.*, 3rd Edition). Mystic Aquarium belugas are maintained in an outdoor habitat

and are exposed to the natural variation in the daylight cycle, seasonal changes in air temperature, and seasonal changes in water temperature, all environmental factors they would experience in the wild. Additionally, male and female belugas are managed together in the population without contraceptives and therefore experience natural hormonal changes.

The commenter's remarks on whales needing the ability to choose a social partner supports the import of these whales, as the individual whales proposed for import were chosen with a goal of optimizing social groupings for both the whales proposed for import and the whales presently at Mystic Aquarium. Also, increasing the number of animals in the Mystic Aquarium habitat provides more choice with regards to social selection.

Comment 7: Study 8 (Testing of prototype telemetry and imaging devices before deployment on wild whales)

Comment 7(a): Application p. 13 – Commenters state that this work can be conducted adequately on the three whales at Mystic Aquarium or with whales at Marineland, and it is unclear why it is necessary to conduct this activity on a larger sample size when Mystic successfully conducted a previous test with only one whale.

Mystic Response 7(a): It is anticipated that differences in size (including blubber thickness), age, sex, maturity status and whales at different growth states will result in differences in optimal location for placement of the telemetry devices on the body, duration of attachment and behavioral effects. We do not know if all the whales will respond the same. Testing on different and variable whales will allow for the most conservative measures in ensuring deployment success of the telemetry devices as well as data collection.

Moreover, the epidermal skin composition where the suction cups will be attached may differ between males and females and different life stages. It has been previously shown that epidermal growth factor, growth hormone, and thyroid hormones can significantly influence the mitotic activity of epidermal cells, resulting in differences in epidermal turnover rates (St. Aubin *et al.*, 1990). Since these parameters are strongly dependent on age, sex and health status, the design will need to be optimized to cover as many epidermal structural differences as possible before deployment on wild whales.

The reason why only one whale was tested with a camera prior is because only one whale was trained to test the device.

Comment 7(b): Application p. 13 – Like the objectives for the photogrammetry study, commenters stated that the objectives for this study (below) do not appear to answer science-based, bona fide questions.

- 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
- To test the ability of the device to stay on by having the whale swim, dive and breach

- To observe any change in behavior and any physical effects from the suction cup after the device is deployed
- To observe how long the device will stay on

Please amend the scientific objectives as necessary.

Mystic Response 7(b): We cannot anticipate new tag technologies or devices that will be developed with the need for testing and the questions that they will address. Below the objectives have been rephrased as questions although the objectives for all studies in the permit application are listed as statements, not questions.

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?
How long will the telemetry device stay on the whale? Does diving, breaching, swimming impair the device to remain on the whale?

Is there any change in behavior or any physical effect from the suction cup attachment after the device is deployed?

Comment 8: Determination of Sample Size/Take numbers:

Comment 8(a): Application p. 15 – Commenters requested clarification as to why Mystic did not request to conduct all activities on 8 adults/juveniles, in addition to the calves, to include the animals currently residing at Mystic under public display status. In addition to the AEP study (see comments sent previously), has the attending veterinarian approved all of the research on the animals held for public display as non-intrusive (i.e., a procedure that would not constitute a risk to the health or welfare of the captive animal)? If yes, can you provide documentation for the public display animals?

Mystic Response 8(a): The veterinary staff at Mystic Aquarium have determined that all the proposed studies in the permit are non-intrusive and do not pose undue risk to the health or welfare of the public display animals. Therefore, the three whales currently residing at Mystic Aquarium were not included in the permit. If there is concern regarding an individual whale at the time of the research study due to a clinical or behavioral concern, the whale would not participate in the study until the behavior or clinical concern was resolved.

Regarding the AEP study, this was discussed both internally between veterinary and research staff as well as with the IACUC. We have attached the minutes of the IACUC meeting where this project was discussed. This documents that the veterinarian and Designated Reviewer, Dr. Dunn, was informed and determined that this project was not a health risk to the animals. Dr. Dunn's approval was included with the prior approved and current IACUC protocols submitted with the application (see Appendix 5, page 27) and involve the current public display whales at Mystic Aquarium. (Note: Mystic submitted the full minutes of the IACUC meeting on May 28, 2020.)

Comment 8(b): Application pp. 15, 16, 19, 21, 60, and 76 – Commenters state that the request to increase the sample size to six whales is not accompanied by any analysis that shows six

whales would be sufficient to increase the power of any statistical tests applied to the data. The application refers to the "opportunity for statistical conclusions" and that six whales "may allow for statistical significance and stronger data more applicable to wild belugas," and other similar statements. It appears no power analysis has been conducted (or if it has, it found six or eight whales little better three or one). The commenter asserts that Mystic Aquarium's argument that importing the five whales is critical is undermined by their statements regarding statistical conclusions. Please provide additional justification for the desired sample size of a minimum of six whales.

Mystic Response 8(b): While there is no question that a larger sample size will be more powerful statistically, it is not usually feasible to achieve the desired sample sizes while studying large animals especially marine mammals. Obtaining five additional whales is an effort to increase the sample size of whales for our research studies. A sample size of 6 is better than a sample size of 3. With a sample size of 6, the confidence interval will be less than half of what we would get with a sample size of 3, as seen in Figure 1 below (Burgess, 2014). In this figure, it is shown that confidence intervals in terms of multiples of standard deviation rapidly tightens until sample size reaches approximately 6. Even though we would certainly aim for larger sample sizes whenever feasible and possible, we are confident that a minimum sample size of 6 will allow an acceptable range of statistical significance for the purposes of the proposed studies.

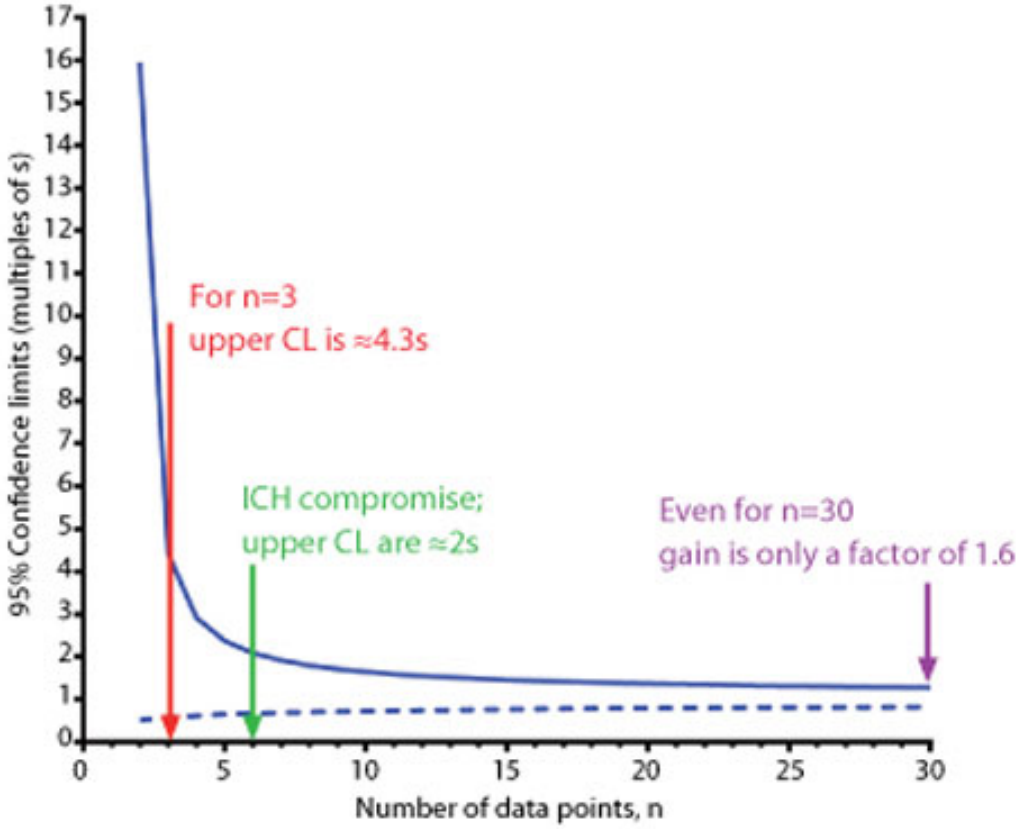

***Figure 1**: Effect of the number of samples (n) on the 95% confidence interval of the standard deviation expressed as multiples of itself (Burgess, 2014).*

Determining power *a priori* is imprecise, and results will not reflect the actual power of tests following data collection. However, exploratory *a priori* Power tests were conducted for sample sizes of 3, 6 and 8 whales using G*power 3.1.9.4, estimating medium effect sizes and utilizing a set alpha of 0.05 (Table 1). An increase in power is observed for all tests from 3 to 6 to 8 individuals, though the increase for paired analyses remains low. A common target power is 0.8, though a range of 0.5 to 0.9 may be considered acceptable (McDonald, 2014), and results for ANOVA testing fall within this range. Using this a priori approach, for T-tests, 34 animals would be required for a power or 0.8, and 18 animals would be required for a power of 0.5. However, T-tests were initially designed for use with small sample sizes, and replicates within each animal are intended to help mitigate efforts and reduce variability in standard deviations of data in effort to increase power (Student 1908; Fay and Gerow, 2013). The actual effect of this however, is unknown until data is collected and analyzed.

*Table 1: Results of a priori power testing for sample sizes of 3,6, and 8 whales using G*power 3.1.9.4. Assumptions were made for a medium effect size (0.5 for T-test and Wilcoxon non-parametric test; 0.25 for ANOVA). Alpha (α) was set at 0.05. Actual power will vary based on calculated means and standard deviations.*

	Number of Individuals		
Statistical Test	n=3	n=6	n=8
T test	<.1	0.15	0.232
Wilcoxon (non-parametric)	0.145	0.269	0.343
ANOVA (repeated measures)	0.302	0.575	0.721

We are aware of the limitations of science and sample numbers and respectful of the peer-review publishing process to hold us accountable for conclusions. Our requirement in obtaining a permit is to propose research that will contribute significantly to the field of beluga health. Later, in publications, it will be our responsibility to draw reliable conclusions. We have a solid track record at publishing important and valid data with limited samples sizes and that will be true for this research as well.

Comment 8(c): Given Georgia Aquarium currently has five whales and has entered into a cooperative agreement to conduct the proposed research, commenters asked why can't these whales be used to achieve the desired sample size of six whales?

Mystic Response 8(c): There are no plans to conduct this research at Georgia Aquarium. With the permit and agreement between Georgia Aquarium and Mystic Aquarium, the priority of the 5 imported whales is conservation research *at* Mystic Aquarium. Only in the case of a needed move for animal welfare, which is unlikely, has Georgia Aquarium agreed to continue the research.

As described in detailed responses to Round 2 of comments, most of the studies proposed in the permit, call for in-depth animal training, dedicated personnel and resources, and, most importantly, researchers on site to ensure the research is being conducted properly, in a consistent manner and moved forward in an efficient manner. Prioritization and efficiency of

research training is guaranteed at Mystic Aquarium based on the long-term commitment of the facility to beluga research and conservation. Careful consideration and attention are needed for sample collection. Without staff members dedicated to this, quality and consistency of sample collection cannot be guaranteed as has been determined from prior experience.

As illustrated by the training time budgets in responses to Round 2 of comments, the amount of dedicated staffing and time per day to accomplish all training associated with data collection for the 8 proposed research studies is extensive. It is unrealistic to expect Georgia Aquarium to dedicate this much time, money, and effort to external research projects when they have their own priorities with their own whales as well as the five proposed whales that will reside at Mystic Aquarium. However, *only if and when* the animal(s) must be moved as a contingency for animal well-being and to maintain the right cohort for optimal social grouping has Georgia Aquarium agreed to participate in the research. Realistically, if there is a contingency, most of the training will have occurred, and the studies could continue without a substantial time investment for Georgia Aquarium. Moreover, it isn't feasible for our husbandry and research teams to leave their homes and families and move to Atlanta nor to start at time zero to validate protocols and establish a fully equipped research laboratory, not to mention the continued need for personnel to take care of and continue with research studies on our belugas at Mystic Aquarium. However, *only* in a contingency situation, would it be necessary for Mystic Aquarium to send staff to Georgia Aquarium to get research there in motion in collaboration with the Co-PI from Georgia.

Mystic Aquarium is a world leader in beluga whale research and is the only beluga holder in the US to maintain a research license under the USDA to conduct bona fide research on our animal collection. The amount of resources, including staff hours, dedicated training time during the workday, and unobstructed habitat space needed to condition animals to participate to the extent required for the proposed research to occur in these voluntary, non-invasive behaviors is extensive and will be accommodated for at Mystic Aquarium.

Comment 8(d): Application p. 19 – Regarding limiting "confounding factors," commenters stated that if whales are transported to Georgia Aquarium, there would be confounding factors from differences in enclosures/environments and personnel. As included in the previous set of comments sent to Mystic for response, the commenters state that the work could be done at Marineland. They state that this would save transportation costs, increases sample size, improve the welfare of the whales at Marineland, reduce the risks of import, and remove any confounding factors.

Mystic Response 8(d): As stated previously, there are no plans to conduct this research at Georgia Aquarium. With the permit and agreement between Georgia Aquarium and Mystic Aquarium, the priority of the 5 imported whales is conservation research *at* Mystic Aquarium. Only in the case of a needed move for animal welfare, which is unlikely, has Georgia Aquarium agreed to continue the research. In the unlikely event of a contingency for animal welfare, Mystic Aquarium will need to start at time zero to validate and optimize protocols, invest in the proper and validation of equipment at Georgia and be fully away of all potential confounding factors. We are aware of the limitations of science and respectful of the peer-review publishing process to hold us accountable for conclusions. Our requirement in obtaining a permit is to

propose research that will contribute significantly. Later, in publications it will be our responsibility to draw reliable conclusions. We have a solid track record at publishing important and valid data and that will be true for this research as well.

The research proposed cannot be carried out at Marineland for the reasons already detailed in Round 2 responses. Transportation costs have no bearing on this research permit application. We will continue to seek samples to increase sample size from other facilities that are feasible to obtain (e.g. breath) but given the constraints, issues and challenges described in detail in responses to Round 2 comments we cannot rely on this. There are no risks of import as described in the detailed responses to Round 2 comments; the accredited beluga-holding institutions have expertise and experience moving whales and can do so in a safe, controlled manner. Regarding animal welfare, the beluga whale habitat has been identified by the Animal Care Committee at Marineland as at capacity. Moreover, additional calves have recently been born. It is not reasonable to think the welfare of belugas at Marineland would be improved if the 5 whales stayed there, given that they are at capacity of their habitat with the inability to provide full and focused clinical, behavioral and enriching attention on individual whales. Moving five whales out of the Marineland habitat would ensure those five whales are given the individual care and attention they deserve while making additional room for the newly born calves to develop and grow. There are no confounding factors moving the whales to Mystic Aquarium for the research; as described in detail in Round 2 responses to comments, there are numerous confounding factors in conducting the research in other facilities. Having these animals at Mystic Aquarium, and remaining at Mystic Aquarium, is the best way to remove confounding factors and the ideal situation for the proposed research.

Comment 9: Anticipated Effects on Animals
Application p. 57 – The application states that no mortalities have occurred due to Mystic Aquarium's research program, which has been ongoing since 1999. Commenters state that four beluga whales have died at Mystic Aquarium since 1999 and additional information (i.e., necropsy reports) should be provided to substantiate this claim. Do you have additional information to support the claim that there have been no research-related mortalities?

Mystic Response 9: There have been no research-related mortalities to public display animals at Mystic Aquarium. Mortalities are reported to NMFS within 30 days, as required by the Marine Mammal Protection Act. In each case, the information submitted on the Marine Mammal Data Sheet accurately described the cause of death and remains part of the animals' permanent record in the National Inventory of Marine Mammals. At the time of their deaths none of the animals were included in a research project that required anything of them other than what is considered a non-invasive sample collection, as is done for health monitoring purposes.

Comment 10: Captive Information

Comment 10(a): Application p. 65 – The pool depth is described as varying "from just a few inches to 16.5 feet *to mimic a wild environment*." The commenter states that this statement is demonstrably false and that the depths of the tanks at Mystic Aquarium in no way "mimics a wild environment" for beluga whale populations anywhere in their circumpolar distribution.

Mystic Response 10(a): Water Levels/Depths/Contours/Substrate
Beluga whales inhabit very diverse habitats in the Arctic, from shallow, brackish, coastal zones, estuaries and river mouths, to deep ocean basins and trenches (Laidre *et al.*, 2008; Martin, 1996; Leatherwood and Reeves, 1983). Studies on Cook Inlet belugas showed that belugas spent the entire time in the inlet from September to January averaging depths from 8.5 to 17 feet (Laidre *et al.*, 2017). Belugas spend more time in extremely shallow water and have been caught stranded on shore with a tidal change, wait for the tidal change to un-strand, then swim away demonstrating the time spent in shallow waters is a common stance (Martin, 1996).

Cook Inlet features varying depths, contours, and substrates that include sand, mud, pebbles, and rocks. Mystic Aquarium's Arctic Coast beluga habitat was designed after the Cook Inlet habitat. The Arctic Coast habitat has depths varying from a few inches to 16.5 feet with large boulders, pebbles, gravel and rocks built into the bottom surface of the pool, like Cook Inlet, allowing the belugas opportunity to engage in deeper dives as well as frolic in the shallower areas. Belugas are also unique such that they go through an annual molt which is thought to be triggered by environmental cues such as temperature and salinity (St. Aubin *et al.*, 1990). The Arctic Coast provides a shallow area (~4 ft. deep) with loose cobble rubbing stones that promotes a critical and natural behavior of rubbing to facilitate the sloughing of old skin (Solntseva, 1995; Smith *et al.*, 1992).

The habitat is enhanced with naturalistic décor, an island, and a set of waterfalls that cascade down off the rock face into the shallows to create the appearance of the region along the Cook Inlet. The pool walls and bottom are dynamic and contain contours and shelves to resemble river bottoms that are shaped by the tidal currents coming in and out of the Cook Inlet. There is a swim-through arch, large boulders, and steep vertical walls that add to the complexity of the habitat, providing enrichment, opportunities to hide and self-separate, and resemble areas around Cook Inlet. The depth of the habitat is not a detriment to the belugas welfare as it is shown in literature that the primary factor that drives belugas for deep diving is food availability, and they do not have pressures to feed in our care (Leatherwood and Reeves, 1983; Schreer and Kovacs, 1997; Heide-Jørgensen *et al.*, 1998; Reidenberg and Laitman, 2002; Heide-Jørgensen *et al*., 2001). If prey items remain in shallow water, belugas will not choose to dive deep for food, spending less energy for greater nutritional return. The belugas at Mystic Aquarium engage in diving to 16.5 ft. daily but do not have the pressure for survival to hunt for their food as each animal is hand fed, which then mitigates the argument that they need to dive deeper than 16.5 ft. The details described above of the exhibit do, in fact, mimic several areas of the Cook Inlet, one of the habitats of beluga whales, found in the areas surrounding Anchorage, AK in the USA.

Comment 10(b): Application p. 66 – According to the application, the water temperature maintained at Mystic Aquarium is never colder than 50° F (10° C); commenters state that this is more similar to summer water temperatures in parts of the Arctic and that beluga whales in the winter are often found in water at 0° C. Thus, commenters state that the facilities provided to captive belugas do not mimic natural conditions, which is one reason why captive research results, particularly physiological and microbiome results, are of restricted value to free-ranging populations and must be judiciously applied, with suitable caveats.

Mystic Response 10(b): We are aware of the limitations of science and respectful of the peer-review publishing process to hold us accountable for conclusions. Our requirement in obtaining a permit is to propose research that will contribute significantly. Later, in publications it will be our responsibility to draw reliable conclusions. We have a solid track record at publishing important and valid data and that will be true for this research as well.

Water Temperature

The water temperature in the Arctic Coast habitat varies on a seasonal basis to provide the environmental stimulus to the belugas in a similar fashion to that of the Cook Inlet belugas. The Cook Inlet beluga, a non-migratory species, is found in coastal and nearshore areas in the southwestern part of Cook Inlet, Knik Arm, nearshore areas in the southwestern part of the inlet, and Kachemak Bay, all these are areas surrounding Anchorage (temperatures may vary and be higher in the nearshore areas) (Marine Mammal Commission). From November through March the Arctic Coast drops in temperature in conjunction with the ambient air temperature, no heating of the water is conducted, yielding temperatures below 40°F, contrary to what is stated in the above comment. A table below details the average recorded temperatures and the minimum recorded temperature for the Arctic Coast habitat, which are similar to what wild belugas experience.

Month	Ave Arctic Coast Temp (°F)	Min. Arctic Coast Temp (°F)
January	44.6	33.8
February	46.4	35.6
March	51.8	41.0
April	55.4	50.0
May	53.6	50.0
June	55.4	51.8
July	57.2	53.6
August	57.2	53.6
September	57.2	51.8
October	55.4	51.8
November	53.6	48.2
December	48.2	41.0

Comment 11: Transport

Application p. 71 – Commenters noted that the application instructions request the *name* of the transportation company to be used for the importation, yet the application only provides the type of aircraft and indicates "a trucking company" will be used. Do you have the name of the transportation companies you propose to use at this time?

Mystic Response 11: As required by law, information on the exact transportation companies will be provided prior to transport to the Permits Division. Exact logistics for the transport are still being determined.

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