

## *Mystic Responses – Take Numbers and Additional Questions*

**File No. 22629 Comments and clarifying questions received during the public comment period on the permit application take numbers and additional questions on the permit application sent to Mystic Aquarium on March 4, 2020. *Mystic Aquarium's responses received March 16, 2020.***

**Comment 1:** It has been recommended counting a **maximum of one take per day regardless of the number of samples taken in a day**, for consistency with other permits (NMFS' standard practice). Understanding that these studies depend on trained behaviors, and the voluntary participation of the whales dictates the number of samples in a given day (which may vary from day to day), we (NMFS) allowed flexibility with your application in counting takes as the total number of samples. To the best of your ability, please clarify the maximum number of "takes" under the paradigm where multiple samples in a day = 1 take. Below are excerpts from the permit application. Please clarify/edit as appropriate. **NMFS' recommended take clarifications in black bold. *Mystic Aquarium's clarifications in blue.***

### **Study 1 (Neuroimmunological Response to Environmental and Anthropogenic Stressors) Methods**

#### ***Blood samples***

Whales behaviorally present their tail flukes and station for blood collection (up to 55 mls collected using a sterile 21-g butterfly catheter, well below the acceptable volume threshold for blood collection in mammals). ***In belugas the volume threshold is reported to be 8% of total blood volume according to Ridgway, personal communication. It is accepted in terrestrial mammals that 10% of blood volume can be collected, without replacement fluids giving a 2 weeks recovery time (Diehl et al., 2001). This would be presumed to be greater for marine mammals, as they have a greater blood volume to mass ratio (Castellini et al, 2010; Ridgway et al., 1984). The smallest of the whales proposed for transport is presently 306 kg; the 55 mls collection is well below 1% of blood volume.*** Collection is proposed at 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 or **24 takes/whale/year** as well as reagent and assay development. Note these samples will also contribute to objectives in Studies 2 and 5. 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. 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 will result in 72 blood samples per year or 1 session per day/12 sessions/3 novel training exercises/and or social interactions = **36 takes per whale/year**.

**Blood Sampling Comment 1(a): Is it possible to predict how many sessions could be conducted in a given day to give a maximum number of takes per year?**

**Mystic Response 1(a):** For all of the above scenarios, 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).

**Blood Sampling Comment 1(b): What is the maximum number of blood samples that could be taken on a beluga whale in a given day for any/all of these events?**

**Mystic Response:** The maximum number of blood samples proposed for any study in a given day for any/all of these events is 5 blood samples.

**Blood Sampling Comment 1(c): Is the following parameter consistent with your blood sampling protocols: Minimizing the number of needle insertions per blood collection site (e.g., no more than 2 insertions per site)?**

**Mystic Response 1(c):** Minimizing the number of needle insertions per blood collection site (e.g. no more than 2 insertions per site) is consistent with Mystic Aquarium's blood sampling protocols.

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### ***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).

#### Initial Transition to Collection of Breath for Free-Ranging Belugas

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.  $\geq 80\mu\text{l}$ ) 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.

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**.

**Breath Sampling Comment 1(d): What is the maximum number of breath samples that could be taken from an individual beluga on a given day for any/all of the research events?**

**Mystic Response 1(d):** 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).

#### *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**).

#### *Fecal Samples*

Whales will voluntarily lay out 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 3 takes/whale/year**). **NMFS Correction: In this case, there would be 12 takes/whale/year. 4 OWE over 3 days = 12 takes.**

#### *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**.

### **Study 5 (Diving Physiology)**

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 samples/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).

**Diving Physiology Comment 1(e): Will blood and breath samples all be collected in one day? If not, how many days will sampling be conducted for each (blood and breath)?**

**Mystic Response 1(e):** 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.

**Diving physiology Comment 1(f): Could blood and breath for Study 5 be taken on the same day/time as blood and breath samples for Studies 1 and 2? If yes, what could be the maximum number of samples in a day?**

**Mystic Response 1(f):** 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.

**Additional Comments/Questions on Permit Application:**

**Comment 2: Please provide a description of how imported beluga whales will be introduced to the current belugas at Mystic and what immediate actions would be taken if the animals were not compatible.**

**Mystic Response 2:** These details are addressed in response to Round 2 of comments.

The following is an example of the degree to which Mystic Aquarium will provide and ensure that the highest level of animal welfare is maintained throughout an acclimation period and would be used to introduce the 5 imported belugas to the resident belugas:

- When the belugas arrive, one beluga at a time will be carefully lowered into the holding habitat in its stretcher. A hydraulic lift will be raised up to ~3-4 ft. of water to provide a smooth, slow and controlled entry into the habitat. The stretcher will be lowered into the water; animal care professionals will assist in lowering the sides of the stretcher so the beluga can comfortably swim out of the stretcher and into the habitat.
- Animal care staff will be positioned around the pool to provide any assistance or guidance.
- Once the beluga is observed swimming, the lift will slowly be lowered to provide more depth in the habitat. This will be continued until the lift is completely in the lowest position giving the beluga the full depth of the habitat. This process will be used for each beluga until all 5 belugas are swimming in the large holding habitat.
- Animal care staff will monitor the belugas constantly recording behavior, respirations, defecation and any other notable behaviors.
- After ~ 1hr. post transport, the familiar trainer(s) from the beluga's originating facility will attempt to offer food. From prior experience with beluga transports, belugas usually will begin eating immediately, indicating they are comfortable in their environment.
- For the first 2-4 days the belugas will be held in the holding habitat, while teaching each beluga to shift reliably between medical habitat and holding habitat, allowing for behavioral observations, and the building of other foundational behaviors. At this time,

the belugas will have visual and acoustic exposure to Mystic Aquarium's resident belugas with the ability to interact through clear acrylic gates. This provides a safe introduction and starts to build a familiarity between the two groups.

- While building the foundation of shifting, the resident belugas will be brought to the acrylic gates under stimulus to allow controlled visual introductions, building positive reinforcement history between the beluga groups.
- After approximately 4 days, or when behavior indicates appropriate time to advance, the resident females will be introduced to the 5 new belugas and monitored by staff. This will be carried out by bringing each female into the medical habitat, under stimulus – closing the gate, and opening the medical habitat gate and bringing the resident female into the larger holding habitat with 5 new belugas.
- If the 5 new belugas are shifting between medical and holding habitats, then the 5 new belugas will be introduced to the main habitat, with the resident male and females restricted to the holding habitat. The 5 new animals will be allowed to explore the large main exhibit and to become familiar with the new surroundings and habitat.
- If baseline behavior is observed, then the same introduction process will ensue with the resident male beluga.
- Once all belugas are introduced, a variable social grouping will be maintained on a regular basis giving all the belugas the ability to thrive, elicit normal behavior, and be in a stimulating enriching environment.
- This plan and the beluga's condition are monitored constantly and will be adjusted as needed for optimal animal acclimation and introduction.

This introduction schedule is flexible and can be advanced or extended based on the behaviors that each beluga is eliciting. Study of behavior will be conducted daily via ethograms. This process has been done successfully in the past with the introduction of new belugas into the social group while quantifying behavior and physiological responses (Spoon and Romano, 2012).

**Comment 3(a): What situations would lead to needing to quarantine the animals (if deemed necessary after transport), the actual quarantine methods, and duration of quarantine. Although the risk is low, the methods should still be described.**

**Mystic Response 3(a):** As detailed in the application, considering the closed nature of the Marineland collection, health screenings of the belugas proposed for import will be conducted prior to transport, both to ensure they are healthy for transport and to ensure the health and well-being of the whales presently at Mystic Aquarium. This will allow for the most optimal acclimation for the incoming animals. Quarantine of the incoming animals is thus not anticipated.

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.

**Comment 3(b): Similar to the question regarding whether the acoustic studies would be considered intrusive research, please confirm that the veterinary staff at Mystic Aquarium have determined that all other proposed studies do not pose undue risk to the health or welfare of the public display animals and therefore, an additional permit should not be required.**

**Mystic Response 3(b):** In addition to the AEP study, 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.

**Comment 4: Please confirm that the personnel listed in the application for transport, including attending veterinarian, are still proposed to accompany the animals for transport. If not, please let us know any changes to the personnel.**

**Mystic Response 4:** The personnel listed in the application are still proposed.

**References provided as part of Mystic's responses:**

Castellini, M.A., Baskurt, O., Castellini, J.M., and H.J. Meiselman. 2010. Blood Rheology in Marine Mammals. *Frontiers in Physiology* 1:146.

Diehl K., Hill R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J., and C. Vorstenbosch. 2002. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J. Appl. Toxicol* 21: 15-23.

Ridgway, S.H., Bowers, C.A., Miller, D., Schultz, M.L., Jacobs, C.A. and C.A. Dooley. 1984. Diving and blood oxygen in the white whale. *Can. J. Zool.* 62:2349-2351.

Spoon, T.R., and T.A. Romano. 2012. Neuroimmunological response of beluga whales (*Delphinapterus leucas*) to translocation and a novel social environment. *Brain. Behav. Immun.* 26(1):122-131.

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