Investigation of the Molecular Response of Belugas to "Stressors"

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LONG-TERM GOALS

The overall goal of this project is to characterize the beluga immune response at the molecular level utilizing archived and fresh blood and skin samples collected in association with different stressors. In the long term, selected biomarkers found to be informative will be utilized in skin potentially eliminating the need for blood sampling from wild populations.

OBJECTIVES

The specific objectives are listed as follows:

1. To assess the quality and quantity of total RNA from blood and skin samples collected in the field from wild belugas, and compare with samples collected and archived from Aquarium belugas.
2. To utilize archived tissues collected outside the proposed study to validate previously published primers for use in blood and skin from belugas.
3. To apply molecular methods to describe and quantify changes in the expression of immunological "stress" markers in peripheral blood from captive and wild belugas.
4. To apply molecular methods to describe and quantify the expression of immunological markers in skin.
5. To correlate the biomarkers in blood from the proposed study to the findings of the previous ONR funded project (N00014-11-1-0437).

APPROACH

The approach for the study includes the sample collection, isolation of total RNA followed by quality and quantity assessment, cDNA synthesis through reverse transcription, and amplification of mRNA sequences by using sequence-specific primers by real-time quantitative PCR.

For sample collection, blood will be drawn from the fluke vessels for both live and subsistence hunted whales and will be preserved in PAXgene RNA tubes. Pieces of skin (0.5-1 cm) will be taken from subsistence hunted whales from consistent locations along the dorsal ridge, or collected from live-capture released belugas in association with satellite tagging or skin biopsies. Archived samples from prior collections will also be utilized. Stressor samples include archived PAXgene blood samples before, during and after Out-of-water events from Aquarium whales. In addition samples from belugas before and after transport or introduction to new social groupings as well as those from wild belugas obtained after chase and capture (subsistence hunts and live capture-release) will be analyzed.

Total RNA will be extracted by using either PAXgene Blood RNA kit (Qiagen) or by RNAzol protocol depending on the method of RNA preservation for blood. Skin samples will be processed according to the protocol provided with the Aurum™ Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, Hercules, CA). The quantity and quality of total RNA will be assessed via spectrophotometry and formaldehyde agarose gels. RNA concentration and purity (A260/A280 ratio) will be measured. RNA samples will also be run on agarose gels to assess integrity. cDNA sequences will be synthesized by using QuantiTect Reverse Transcription Kit (Qiagen). The mRNA sequences will be amplified on a 7300 Real Time PCR System (Applied Biosystems) utilizing designed primers from cetacean published sequences and/or from conserved regions of targeted biomarkers. The house-keeping genes (GAPDH and S-9) will be used to normalize the expression of each biomarker and the Expression Index (EI) will be calculated as described in Fonfora et al (2008). Data analysis will be carried out by Systat 10TM (Systat Software, Inc, Point Richmond, CA, USA). Normality of data will be assessed in all variables with probability plots. Repeated measures analysis of variance (ANOVA) will be used evaluate the changes in the expression of bio-markers in blood before, during and after the stressor paradigm from Aquarium experiments. Correlations will be used to explore the relationship between the expression of bio-markers in paired skin and blood samples from free-ranging belugas.

WORK COMPLETED

Sample collection

The current project was awarded funding in June of 2014. In June-July additional blood and skin samples were collected for assessment on free-ranging belugas during two separate field collections that took place in Point Lay and Bristol Bay, Alaska. A total of 32 PAXgene blood tubes were collected from 16 belugas during native subsistence hunts in Point Lay, AK within 1-4 hr postmortem. Blood was also taken for routine health assessment tests. Morphometrics and natural history data were collected for each animal. A total of 30 skin samples were collected from 15 of the subsistence hunted whales at the same time, from the same whale to enable paired skin/blood samples and analyses.

Blood and skin samples were collected from a total of 10 live-captured released belugas in Bristol Bay in August-September, 2014 during health assessments. Blood samples were collected directly in PAXgene tubes and kept at room temperature for 2-24 hours, and then stored frozen at -20°C. Two samples were collected for each of the pre- and post-exam procedures, making a total of 40 blood samples. The pre-exam samples were collected right after the whale was captured before performing any additional procedure. The post-exam samples were collected following the tag placement, size measurements and other health assessment procedures, approximately 1-2 hours after the animal was captured. The blood samples were then shipped back to the Aquarium on dry ice and were stored at -20°C for further processing. Paired skin and blood samples were collected from all 10 whales.

Moreover, arrangements have been made to participate in Point Lay (June-July, 2015) and Bristol Bay (August-September, 2015) field studies to collect samples from 10 to 15 belugas from each site. For the subsistence hunted belugas in Point Lay, two blood and two skin samples will be collected per animal. For the live captured belugas in Bristol Bay, four blood (two pre- and two post-exam) and two skin samples will be collected.

Total RNA extractions
The samples that were collected during monthly blood draws from the Aquarium belugas were used for initial assessment of the total RNA quality and quantity. The samples were stored at -20°C, except for one sample which was processed the same day without freezing (blood sample taken from Kela on 9/23/2014). The Qiagen PAXgene Blood RNA Kit was used to extract RNA from the blood collected into PAXgene tubes. The RNA was extracted using the kit protocol and the RNA concentration and purity (A260/A280 ratio) was measured by using the Take3 micro-volume plate of BioTek Epoch Microplate Spectrophotometer.

The current list of molecular markers proposed in the project were revisited and another literature search was performed in order to identify additional molecular markers (Table 2). Some of the selected primer sequences of these proposed bio-markers have already been published for belugas (Sitt et al, 2008; St-Laurent and Archambault, 2000), or for other marine mammal species such as harbor seals and harbor porpoises (Müller et al, 2013; Fonfara et al, 2008). Whenever a published set of primers are not available for belugas, published sequences for cetaceans and other related species were downloaded from GenBank and aligned by using the Clustal Omega software. Sets of primers were designed for the consensus sequence of the alignment by using Primer3 software for the genes TLR4, IL-1β, IL-6, HSP70, and HP and for the housekeeping gene GAPDH (Table 2).

RESULTS

Preliminary analysis of RNA Quantity and Purity
The total RNA extraction protocol by using Qiagen Blood RNA Kit was tested on blood samples obtained from four aquarium belugas following the optimization of the protocol. The RNA quantity and the purity values were subsequently measured by using the BioTek Microplate Spectrophotometer (Table 1).

Table 1. RNA concentration, total RNA quantity, and the RNA purity values for the Mystic Aquarium belugas.

Beluga	Sampling Date	RNA Concentration (ng/µL)	Total µg	RNA Purity (A260/A280)
Juno	6/5/2014	20.981	1.68	2.107
Kela	9/23/2014	67.086	5.37	2.126
Naku	3/6/2014	10.622	0.85	2.121
Naluark	8/27/2014	23.213	1.86	2.125

The ratio of spectrophotometric absorptions at 260 nm vs 280 nm is commonly used to assess the purity RNA. A ratio of ~2.0-2.2 is generally accepted as "pure" for RNA. The total RNA values obtained from four in-resident belugas showed consistent values for RNA purity, as calculated by the

absorbance ratio of A260/A280 (Table 1). For these belugas, the total RNA yields ranged from 0.85 to 5.37 µg, which are well over the minimum RNA amounts required by the reverse transcription kits. The QuantiTect Reverse Transcription Kit (Qiagen) is optimized for efficient and sensitive cDNA synthesis from 10 pg to 1 µg of RNA. These preliminary results demonstrate that sufficient amounts of high-quality RNA can be obtained from PAXgene blood samples to be used in downstream applications of gene expression analysis.

Primers for gene expression analysis
An updated set of primer sequences that will be used in gene expression analysis are listed in Table 2. Toll-like receptor 4 (TLR4) gene has been proposed as a new molecular marker to be used in this study. Being a part of vertebrate innate immune system, Toll-like receptors are located at the direct interface between the host and the microbial environment and act as a first line of defense being expressed in response to pathogens in skin and blood of animals (Shen et al, 2012). Another proposed gene, IL-1β is a cytokine which is an important mediator of the inflammatory response produced primarily by monocytes and macrophages (Dennis and Armchambault, 2001).

The search for additional molecular markers enabled us to investigate the published DNA and protein sequences from cetaceans and other related species within the context of their immune responses. The information gained with this initial search will be beneficial for further analysis and interpretation of the gene expression data. It is also important to note that the current list of proposed molecular markers is not exclusive and many more markers can potentially be determined by using the protocols that are suggested in this study.

IMPACT/APPLICATIONS

This study will contribute to our understanding of stress physiology in marine mammals as a first step towards a better understanding of the effects of sound on marine mammals. Specifically, this proposal will add to our understanding of the variation of multiple biomarkers following exposure to different stressors in both free-ranging and Aquarium belugas. It will address technical needs including the application and validation of molecular techniques such as RT-PCR as a method to quantify stress and immune markers in different matrices including blood and skin. This study will also assess the integrity of samples collected in a field setting, including blood and skin from capture-released and subsistence hunted whales. The use of skin samples as an alternate to blood collection to assess stress and immune markers will be investigated in this study. Skin sampling is proposed as an alternative to blood in order to eliminate the additional stress and difficulty imposed through capture and handling of wild whales and for obtaining potential health information which would be obtained from biopsy darting of free ranging whales. The analysis of paired blood and skin samples will aid in identifying the molecular signatures in response to stressors and may provide for a means of assessing stress and and health status in free ranging whales.

Table 2. Primer sequences to be used for the gene expression analysis by real-time PCR in the blood and skin of belugas.

Gene	Primer	Sequence (5'to 3')	Amplicon length (bp)	Melting temperature (°C)
TLR4	Forward	CCTTTTCTGGGCTATCAAGTTTAC	191	59.6
	Reverse	ATTAGAAAGATCCAAGTGCTCCAG		60.2
IL-1β	Forward	TTCGTGCAAGGAGATGAAAGTAAC	197	61.8
	Reverse	TCGACGCTATTCTTGATTTCTGTC		61.9
IL-2	Forward	CATGCCCAAGAAGGCTACAGAATTG	173	64.6
	Reverse	CTTGTTTCAGATCCCTTTAGTGTCAA		61.4
IL-4	Forward	GCATGGAGCTGCCTGTAGAAGACG	185	68.0
	Reverse	CTTCATTCACAGAACAGGTCATGTTTG		63.1
IL-6	Forward	AGTATGAGGGTGATAAGGGAAGCA	199	62.3
	Reverse	CTTCGCAGGATGAGGATAATCTTT		62.0
IL-10	Forward	CAAGCCTTGTCGGAGATGATCCAG	143	66.3
	Reverse	CGATGACAGCGCCTCAGCCGC		70.4
IL-13	Forward	CAGAAGGCACCCCTGTGCAATGG	133	68.1
	Reverse	CAGCATCCTCTGGGTCCTTTGGAT		66.3
IL-18	Forward	GAGGATATGCCTGATTCTGACTGTTC	175	64.6
	Reverse	GGAGGACTCATTTCCTTAAAGGAAAG		63.0
TNFα	Forward	CCAGAGGGAAGAGTTCCCAACTG	121	66.3
	Reverse	GAGCACTGAGGTTGGCTACAACAT		64.6
IFNγ	Forward	GGAGAATTGGAAAGATGAGAGTGAC	132	62.9
	Reverse	CTGGATCTGCAAATCATCTACCGGAATTTG		66.0
TGFβ	Forward	GGTGGAAAACAGCAACGAAATCTATG	183	63.0
	Reverse	GTATTTCTGGTACAGCTCCACGTG		64.6
HP	Forward	GGGTCATGACAATACAACAAAAGC	197	61.8
	Reverse	AATCTTTTGAAGGTAGGCAGATGG		61.9
HSP70	Forward	CAGATCGACGTAACCTTTGACATC	196	62.1
	Reverse	CAGCAATTTTCTCTCTCTGGACCT		62.4
GAPDH	Forward	AGTATGACAACCACCTCAAGATCG	218	61.6
	Reverse	AGTAGAAGCAGGGATGATGTTCTG		61.0
S-9	Forward	GAGCTGAAGCTGATCGGCGAGTA	145	66.3
	Reverse	GCATTGCCTTCAAACAGACGCCGC		68.0

RELATED PROJECTS

Investigation of the Physiological Responses of Belugas to "Stressors" to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health, T.Romano, T. Spoon and S. Lamb (ONR # N00014-11-1-0437). A portion of the samples for the project described above were obtained from this related project.

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