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Investigation of the Molecular Response in Blood and Skin of Belugas in Response 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 Aquarium 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 overall study includes sample collection of blood and skin from belugas, 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 was drawn from the fluke vessels for both live and subsistence hunted whales and was preserved in PAXgene RNA tubes. Pieces of skin (0.5-1 cm) were 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 were also utilized (Table 1). Stressor samples include archived PAXgene blood samples before, during and after out-of-water events (OWE) from Aquarium whales. In addition, samples from belugas before and after transport as well as those from wild belugas obtained after chase and capture (subsistence hunts and live capture-release) were also utilized.

Total RNA is extracted by using either PAXgene Blood RNA kit (Qiagen) or by RNAzol protocol depending on the method of RNA preservation for blood. Skin samples are 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 is assessed via spectrophotometry and agarose gel electrophoresis. RNA concentration and purity (A260/A280 ratio) are measured by using Take3 micro-volume plate of BioTek Epoch Microplate Spectrophotometer. RNA samples are also run on agarose gels to assess their integrity. cDNA sequences are synthesized by using QuantiTect Reverse Transcription Kit (Qiagen). The mRNA sequences are 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 by using QuantiTect SYBR-Green Kit (Qiagen). The housekeeping genes (GAPDH and S-9) are used to normalize the expression of each biomarker. For each target and reference gene, amplification efficiencies were calaulated from the slope of the standard curves according to the formula $E = 10^{(-1/slope)}$. Relative quantification of each target gene normalized to reference genes, and subsequent data analysis are carried out by GenEx 6.0.1 software (MultiD Analyses AB). Normality of data is assessed in all variables with probability plots. Repeated measures analysis of variance (ANOVA) is used to evaluate the changes in the expression of bio-markers in blood before, during and after the stressor paradigm from Aquarium experiments. Correlations are used to explore the relationship between the expression of bio-markers in paired skin and blood samples from wild belugas.

WORK COMPLETED

Objective 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
The total number of archived and field-collected blood and skin samples are listed in Table 1. In addition to the blood and skin samples collected from wild belugas in Bristol Bay and Point Lay, AK, monthly blood samples have been collected from Aquarium belugas and archived. Moreover, post-mortem skin samples have been collected from 2 Aquarium whales post mortem and preserved in RNAlater, which were also utilized for testing of the designed primers and products (Table 1).

Total RNA isolation and quality/quantity assessment from blood samples were completed for all Out of Water Events (OWE) samples and 2008, 2009, 2012 and 2014 subsistence-hunted whales and 2014 wild-live capture whales, in addition to archived samples collected from Aquarium whales (Table 1). Skin RNA extractions were completed for 2014 subsistence-hunted whales, 2014 wild-live capture whales, and the Aquarium whales. The samples that had >20ng/ul of RNA yield and >2.0 absorbance ratio of A260/A280 were included in the downstream analysis. For the samples that fell outside of this range, duplicates were also processed whenever available to obtain RNA templates of sufficient yield and quality.

Table 1. Archived and recently collected blood and skin samples for the current study. Number of individual whales are indicated in the columns, except for OWE and monthly samples where the number of individuals are indicated as "n".

	PAXgene blood	*RNAzol blood*	*RNAlater skin*	*Notes*
Wild-Live Capture				
2008	18	N/A	18	
2012	9		10	
2013	10		10	
2014	20		10	Blood and skin RNA extracted
Subsistence-Hunted				
2008	18	N/A		Blood RNA extracted
2009	12			Blood RNA extracted
2010	9		9	
2012	10		10	Blood RNA extracted
2013	15		15	
2014	16		16	Blood and skin RNA extracted
Captive-Stressor				
Transport/Social	20 (n=7)	20 (n=4)		
Social/No Transport	N/A	15 (n=3)		
Out of Water Events (OWE)	40 (n=3)	7 (n=3)		Blood RNA extracted
Archived monthly samples from Aquarium belugas	26 (n=4)	N/A	2	Blood and skin RNA extracted

Objective 2: *To utilize archived tissues collected outside the proposed study to validate previously published primers for use in blood and skin from belugas.*
In addition to the previously proposed molecular markers, additional biomarkers were also investigated for their established roles as immune/stress response markers in both blood and skin samples. The published DNA sequences for belugas (Noel et al, 2014; 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) were used to design primer sequences. Whenever a published sequence was not available for belugas, or if the primer sets could not be successfully validated, published sequences for cetaceans and other related species were downloaded from GenBank, aligned by using the Clustal Omega software, and new sets of primers were desigined for the consensus sequence of the alignment by using Primer3 software. Seventeen sets of primers have been tested and validated in blood while fourteen sets for skin (IL2, IL4 and IL13 could not be amplified in skin). A subset of the amplified qPCR products were also sent to Eurofins Genomics for custom DNA sequencing in order to confirm that the amplification of the target biomarker.

Objective 3: To apply molecular methods to describe and quantify changes in the expression of immunological "stress" markers in peripheral blood from Aquarium and wild belugas.
The gene expression analysis of OWE blood samples was carried out for 3 Aquarium whales that participated in a prior ONR effort (#N00014-11-1-0437). The markers used for this sample set included IL-1β, IL2, IL10, IL12, IL18, IFNγ, COX2, TNFα, TGFβ, TLR4, Nr3c1 and Hspa1l. The gene expression analysis of wild belugas was carried out on subsistence-hunted whales for 2012, and on a subset of live capture-released whales for 2014 by using IL2, IL10, IL12, IL18, TLR4, Nr3c1 IFNγ, Hspa1l and Ahr genes.

Objective 4: To apply molecular methods to describe and quantify the expression of immunological markers in skin.
Gene expression analysis of skin samples obtained from subsistence-hunted whales for 2014, and for 2 Aquarium whales was carried out for the genes IL-1β, IL10, IL12, TGFβ, Hspa1l, Ahr, Nr3c1and COX2. The amplification of the low-copy genes (IL2, IL4, IL13) was also attempted, but amplification of these low-copy genes in skin has not been successful to date.

RESULTS

Objective 1:
The total number of blood and skin samples processed up to date including the archived samples are listed in Table 1. The total RNA values obtained from four Aquarium belugas and wild belugas showed consistent values for RNA purity, as calculated by the absorbance ratio of A260/A280 ranging between 2.010 and 2.340. The total RNA yields ranged from 0.85 to 11.5 µg. These results demonstrate that sufficient amounts of high-quality RNA can be obtained from PAXgene and RNAzol preserved blood samples and RNAlater preserved skin samples to be used in downstream applications of gene expression analysis.

Objective 2:
The amplification efficiencies for tested genes ranged from 0.97 to 1.10 with an R^2 value of 0.95 to 1.00. The cDNA input amounts varied between 1-50ng depending on the initial copy number of the gene products. All of the genes were successfully amplified in blood samples, however, the amplification of the low-copy genes (e.g., IL2, IL4, IL13) have been problematic in skin samples. Additional experiments for optimization of reaction conditions for low-copy genes in skin are in progress in order to obtain reliable quantifications.

Objective 3:
Aquarium belugas (OWE samples)
All of the biomarkers were successfully quantified, however the relative expressions of IL18, TLR4, Nr3c1 did not show significant regulation in relation to the Aquarium whales. For low-copy genes in which a reliable gene expression quantification cannot be achieved (e.g., IL2, IL4, IL13), new sets of primers were designed and tested on a live-capture released beluga sample to verify successful amplification. Further gene expression analysis of the complete sample sets are in progress.

Gene expression analysis of blood samples obtained before, during, and after OWE events have been carried out for 3 Aquarium belugas on the available samples for the different time points. Gene expression analysis for OWE samples was carried out with IL2, IL10, IL12, IL18, IFNγ, TGFβ, TLR4, Nr3c1, IL-1β and TNFα genes. Among these genes, Nr3c1, IL-1β and TNFα did not show differential

regulation throughout the course of OWE events. However, when normalized against their baseline values, over 2-fold (>0.4 in log scale) gene expression changes were observed for the IL12, IL18, TLR4, IFNg and IL2 genes, all showing an upregulated response. Whale-1 showed the largest upregulation in IL12 gene expression at 30min while on the beach (Figure 1), corresponding to over a 6-fold change relative to baseline values (>0.8 in log scale). TLR4 gene expression also followed a similar trend in this data set. TGFβ was upregulated only at 30min, whereas IL10 was not significant. Although not as pronounced, the other two whales also showed upregulation for IL12 gene at the 30min time point (>0.3 in log scale, results not shown). IL2 and IFNg gene upregulation were most pronounced for the other two whales 1hr post-release following the OWE event whereas these two genes did not show significant changes in their expression in Whale-1's OWE samples even at the 30min time point.

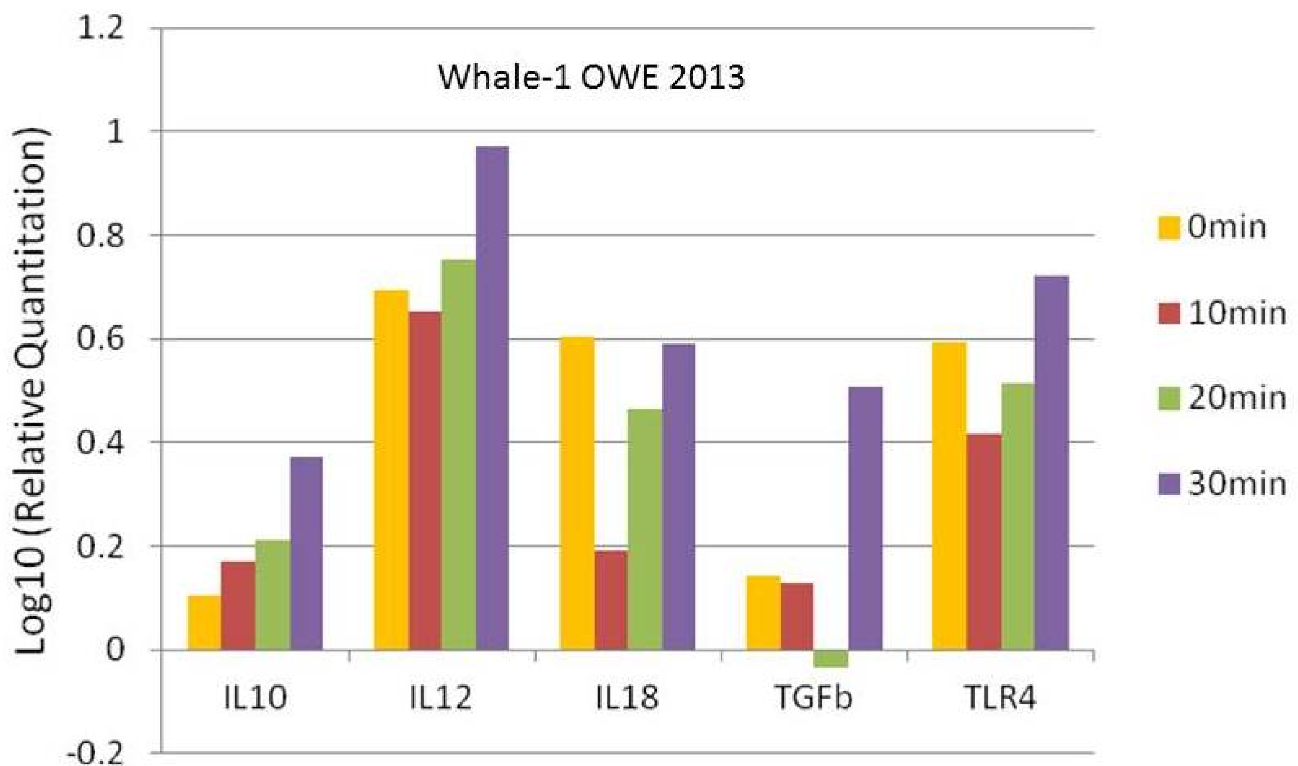

Figure 1. Relative quantification analysis results for Kela OWE blood samples normalized against the baseline value. The values are indicated in the y-axis in log-scale and the genes tested are indicated in the x-axis.

Wild belugas (Subsistence-hunted whales in Point Lay)
Gene expression changes in blood samples from 10 subsistence-hunted whales from Point Lay, AK from the year 2012 were identified for IL12, IL10, IFNγ, Hspa and Ahr (Figure 2). Among the genes tested, the anti-inflammatory IL10 gene showed downregulation in all individuals relative to Aquarium belugas (Figure 2A), and Ahr showed the highest overall upregulation (Figure 2B). Apart from being an ecotoxicological response marker, Ahr has also recently been identified with its role in inflammatory response (Hanieh, 2014). While pro-inflammatory cytokines IL12 and IFNγ were upregulated in most individuals, Hspa11 gene showed variable responses when compared to Aquarium belugas.

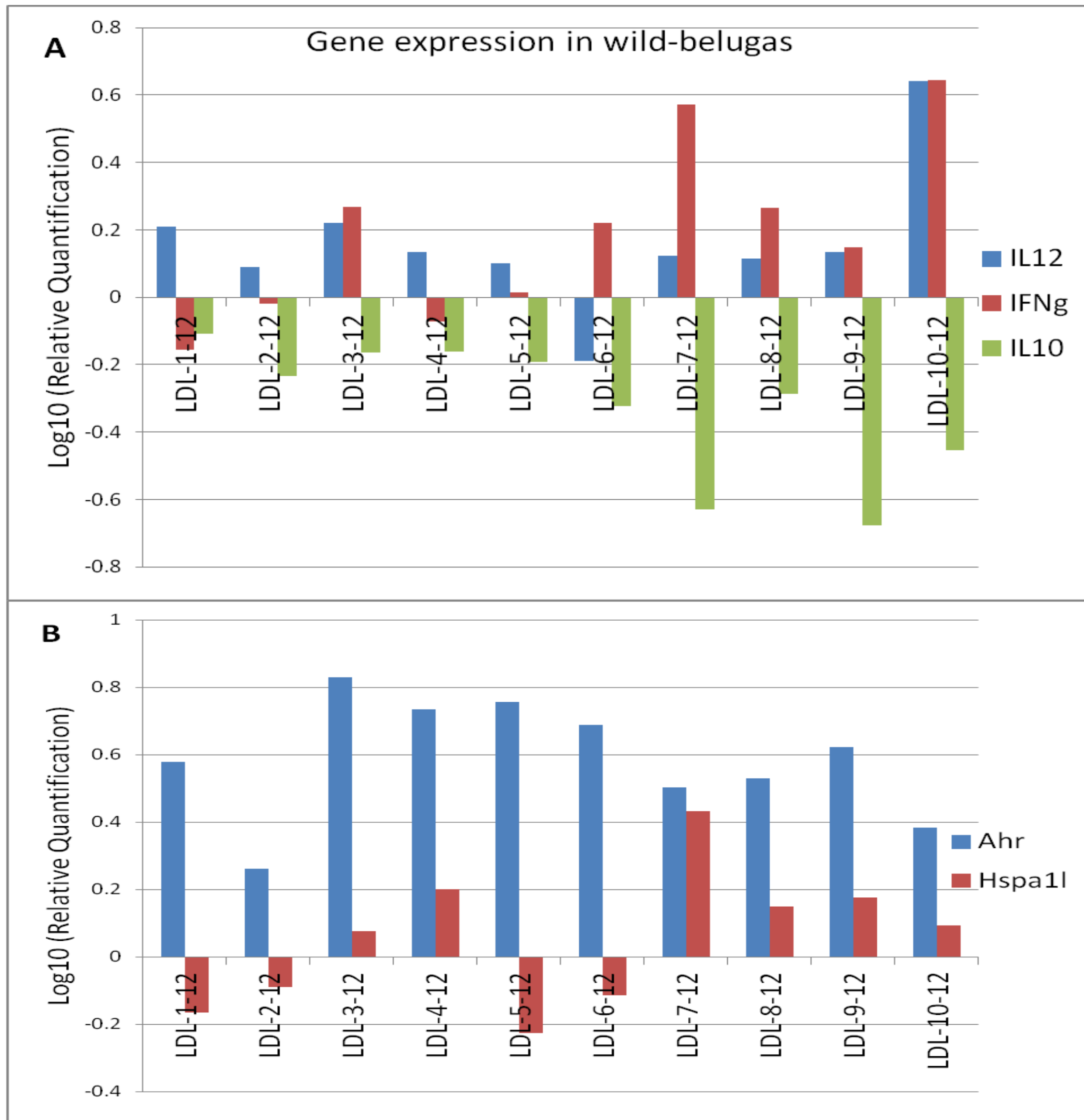

Figure 2. Relative quantification analysis results for subsistence-hunted wild beluga samples relative to Aquarium whales. The values are indicated in the y-axis in log-scale and the individual whales tested are indicated in the x-axis.

Objective 4:

Among the markers tested for skin samples obtained from wild belugas, Ahr, Hspa1l, IFNγ, IL10 and IL12, Nr3c1 and TGFβ genes were consistently amplified in all samples. An initial assessment of blood and skin samples analyzed together was carried out for Ahr, Hspa1l, IFNγ, IL10 and IL12 genes. Preliminary analysis of a subset of blood and skin data set by using raw Ct values normalized against a housekeeping gene (delta Ct) showed comparable amplifications in both tissue types (Figure 3).

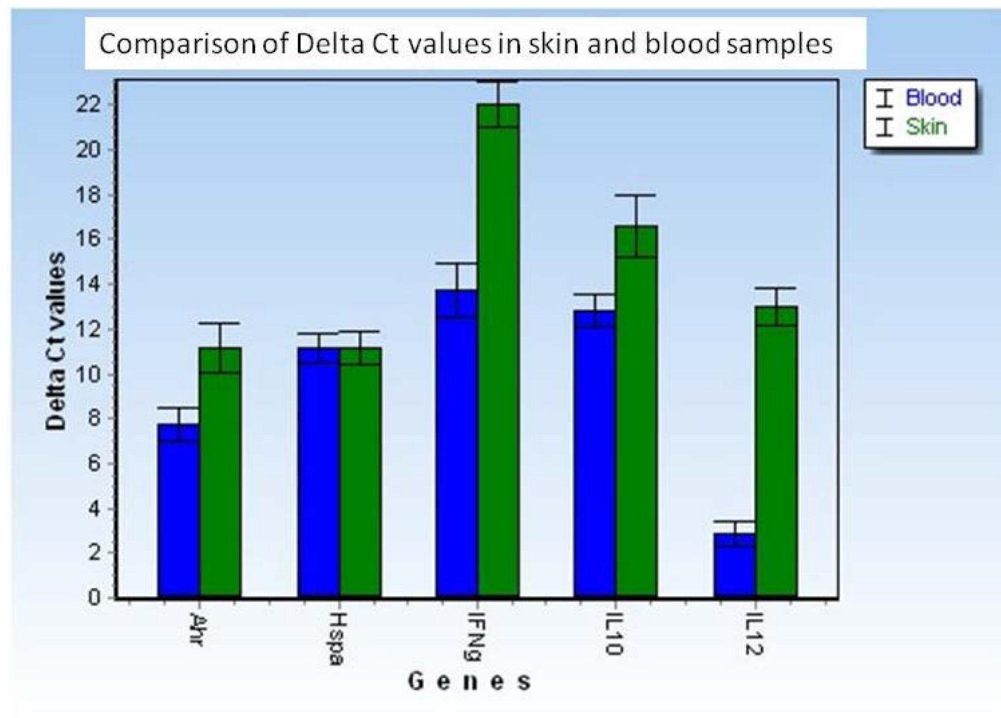

Figure 3. Comparison of real-time PCR amplifications of skin and blood samples. Cycle threshold (Ct) values were normalized with a reference gene to obtain Delta Ct values.

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 also assesses 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.

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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