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FINAL REPORT
Biomarkers to Assess Possible Biological Effects on Reproductive Potential, Immune Function, and Energetic Fitness of Bottlenose Dolphins Exposed to Sounds Consistent with Naval Sonars

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LONG-TERM GOALS

The overarching goal of the project was to utilize novel biomarkers to examine whether significant sublethal responses to sonar-type sounds occur in bottlenose dolphins exposed to such sounds. The collaborators used immune function markers, acute phase proteins, fertility potential assays, and targeted and non-targeted metabolomics to investigate samples collected from trained dolphins before exposure to simulated mid-frequency sonar signals, immediately after exposure, and one week post-exposure.

OBJECTIVES

Today's molecular technologies, in the form of biomarkers, provide avenues by which scientists can directly measure biologically significant responses such as changes in reproductive potential, immune system function, acute phase responses, and energetic fitness. The objectives of our collaborative study were to: (a) acquire appropriate samples for analysis, (b) conduct R&D to ensure that available assays can measure useful parameters, (c) design experiments in which animals are humanely exposed to stressors and an extensive suite of biological effects are monitored, (d) conduct chemical assays that rigorously adhere to QA/QC requirements; and (e) interpret the results of assays with regard to what they do or do not mean in terms of potential biologically significant effects on individuals and populations.

APPROACH

Sampling protocols: In fall 2009, U.S. Navy scientists (SSC Pacific) exposed 30 Navy dolphins maintained in San Diego, California, to sounds that are consistent with those produced by mid-frequency active (MFA) sonar. The signals (~3.5kHz) produced received levels ranging from 115 to 185 dB re 1 μPa. Subjects received up to 10 exposures within a 5 minute period, depending on their willingness to participate. All appropriate permits and IACUCs were in place prior to the exposure testing. A longitudinal experimental design was used for the exposure studies, such that individual dolphins served as their own controls, thereby increasing the power of the analyses for the discovery of

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new predictive biomarkers of stress. This entailed collection of a series of serum and plasma (heparin and EDTA) samples from each individual animal subjected to the acoustic exposure. All samples were collected with the voluntary participation of the subject dolphin. For 18 dolphins used in the tests, there are pre-test samples, samples taken immediately following the exposure session, and samples taken approximately one week after the exposure. For one of the subjects, only the samples taken immediately following and one week following the exposure exist.

Clinical Chemistry: Clinical chemistry data were acquired on site by project collaborator, Dr. Dorian Houser.

Immune function and acute phase markers (conducted at Mote Marine Laboratory): For assays of immune function and acute phase markers, commercial kits were used Immune function was assessed using a 13-cytokine kit, and acute phase responses were evaluated using a 4-plex kit. The assays were done using a Luminex Bio-Plex 3D, an instrument that combines existing technology in flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry to assess up to 500 different analytes. simultaneously.

Metabolomic biomarkers (conducted at University of Birmingham): Non-targeted metabolomics was conducted using UHPLC liquid chromatography Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS). A standard method was developed for rapid analysis of the samples using a gradient of H_2O/MeOH with 1% formic acid. The mass spectra were recorded from m/z 100 to 1000 in both positive and negative ion modes. The resulting chromatograms were then processed using XCMS (alignment and peak picking). The data were processed with in-house software and analyzed using multivariate statistics (e.g., principal components analysis [PCA], partial least squares discriminant analysis [PLS-DA]) in order to discover metabolic biomarkers that change concentration in response to varying levels of sound exposure.

Targeted metabolite analyses were conducted using a highly sensitive, specific and quantitative approach implemented on an LC triple quadrupole mass spectrometer. Targeted profiling focused on the measurement of steroids and steroid-like molecules known to play a role in the stress responses of marine mammals. We optimized our extraction protocols and then implemented LC-MS/MS analyses on our Thermo Fisher Scientific TSQ Vantage triple quadrupole mass spectrometer. This involved optimizing the ionization conditions, selecting appropriate multiple reaction monitoring (MRM) transitions, as well as optimizing the internal standards used for metabolite quantification.

Fertility potential assays (conducted at Mote Marine Laboratory): We conducted ELISAs using a DSX Automated Plate Processor. In the assays conducted for AMH, inhibin A, and inhibin B, standards, controls, and serum samples were incubated in microtitration wells coated with the appropriate antibody. A set of AMH, inhibin A, or inhibin B standards was used to plot standard curves of absorbance vs. hormone concentration, and samples were run in duplicate. Based on the standard curves, the concentration of AMH, inhibin A, or inhibin B in the dolphin serum samples was calculated.

WORK COMPLETED

Task 1: Provide archived serum samples to co-investigators in the United Kingdom.
Status: Completed.

Task 2: Ensure that instrumentation access and service contracts are in place.
Status: Completed.

Task 3: Conduct all biomarker assays.
Status: Completed. The analyses were completed by mid-2013 at both the University of Birmingham and Mote Marine Laboratory.

Task 4: Conduct data interpretation and synthesize results.
Status: Completed. The PIs for the study and University of Birmingham postdoctoral scientist Dr. Gregory Genta-Jouve, met in August 2013 for two full days. During that time, they (a) reviewed the laboratory results and statistical analyses thereof; (b) discussed the implications of the data; (c) considered ways by which experimental design could be improved in the future to provide more meaningful information for science and management; and (d) strategized about proposals for extending the research using an improved experimental design.

Task 5: Coordination and communication of results.
Status: Generally completed, but ongoing. On behalf of the group, Genta-Jouve made two presentations of the metabolomics results in mid-2013, at the following conferences: (a) 9th Annual Conference of the Metabolomics Society, 1-4 July, Glasgow, Scotland, UK; and (b) 7th Journées Scientifiques du Réseau Français de Métabolomique et Fluxomique, 10-12 June, Amiens, France. The project specified that complete results of the study would be presented at the 20th Biennial Conference on the Biology of Marine Mammals, in Dunedin, New Zealand, in December 2013. The abstract submitted by the group was accepted for an oral presentation by Reynolds. The conference occurs after the project ends in September 2013. Further, publication of the results in the peer-reviewed literature is anticipated in the near future, as the PIs are considering the most appropriate journal to which to submit. Hence, "communication of results" has begun but is not completed.

RESULTS

Analyses attempted to assess relationships: a) among results of pre-exposure, immediate post-exposure and delayed post exposure samples; and b) and among individuals exposed to various intensities of sound. Small sample sizes and a range of variables (age, size, sex, reproductive condition, medical treatments) made it difficult to identify statistically significant trends and relationships, although some assays showed excellent promise.

Metabolomics: A targeted approach focused on a single class of metabolites often reported to be good indicators of stress in mammals. We developed a highly sensitive UHPLC-QQQ method to quantify six steroids of interest (i.e. cortisone, cortisol, corticosterone, 11-deoxicortisol, testosterone and progesterone). Each compound was identified using both retention time and multiple reactions monitoring (MRM). The targeted approach did not yield results that showed any apparent trends or relationships (see supplemental results for cortisol).

The untargeted approach led to more interesting findings. Statistical analysis (PLS-DA) of the pre, test and post samples (i.e., samples taken before the sound exposure, immediately following the sound exposure, and one week after the sound exposure) yielded a robust model to discriminate dolphin samples before and after exposure to the simulated sonar signal. Univariate statistical analyses of the data suggested that several tens of compounds were involved in the stress response. It should be

noted that the identity of many of those compounds is unknown, pending further study. Some of the analytes that contribute to the observed differences appear to be steroids.

Whereas our non-targeted metabolomics approaches can detect metabolites that indicate perturbations in energy metabolism and energetic fitness --such as Krebs cycle intermediates and metabolites involved in glycolysis-- our computational analyses did not highlight any of these metabolites as contributing to the metabolic separations between pre-test and sound-exposed dolphins. Consequently, based solely on this metabolic view of energetic status, we did not detect any indication of an energetic perturbation.

Cytokines and acute phase proteins: The sampling regime was designed to address goals of a different study, and sampling one hour and one week after exposure to sounds is not optimal for capturing changes in cytokines in blood. Nonetheless, certain trends seem apparent, even though statistically significant relationships were not identified. Those trends include:

(a) C-reactive protein (CRP) and haptoglobin generally increased with the magnitude of the stressor;

(b) increases in CRP generally should follow increases in interleukin 6 (IL 6), and although we did not observe this, lack of verification may relate to sampling intervals;

(c) cytokine expression was generally greatest in animals > 20 years of age, and was especially prevalent in males approaching 40 years old;

(d) those individuals that already express higher levels of cytokines pre-test also tended to express greater increases in test and post-test samples (i.e., those individuals that are already stressed in some way responded more than those that are not inherently stressed);

(e) the category of cytokines that seems to be expressed most in the older animals is the Th2 group (IL 4 and IL 10), consistent with what is found in stressed humans;

(f) the oldest dolphins in the study were males. Males showed a trend in cytokine expression for pre-test-post animals that could be related to age or existing vulnerability of immune function. Females did not show such a trend either due to age of the individuals or for unknown sex-related attributes; and

(g) principal components analysis showed that cytokine profiles for older male animals are different from those of younger animals of either sex.

Fertility potential: The analyses showed that certain expected trends (e.g., AMH levels are greater in males than in females) occurred. However, the small sample sizes and range of demographic variables generally made it difficult to identify trends associated with the exposures.

IMPACT/APPLICATIONS

The study provided a number of useful observations, as well as productive next steps for assessing effects of sound, and perhaps other stressors on marine mammals. The most salient observations with regard to assessing responses to stress in marine mammals include the following:

1) Marine mammals have fundamental similarities to humans and other terrestrial mammals, but certain adaptations (e.g., associated with a dive response) make it inappropriate to assume that

all physiological and biochemical responses to stress exhibited by humans will apply to marine mammals;

2) Traditional markers of stress (e.g., cortisol) do not appear to be optimal when assessing responses by dolphins to acute noise-related stress;
3) The biomarker analyses we used provided definite results and offer promise as tools to assess effects of stress on important biological functions of dolphins and other marine mammals. Small sample sizes and a range of demographic variables precluded acquisition of statistically significant results;
4) Sampling regimes and experimental design are critical to acquiring meaningful results due to the sometimes-transitory availability of particular markers in the blood stream. The sampling regime used in this study was not optimal for assessing cytokines.

We are encouraged by the results of our study, especially in comparison with the limitations associated with targeted analyses using traditional stress markers such as cortisol. We recommend the following be done to further validate our approaches:

1) Conduct the same study again, but with larger sample sizes and a different sampling regime. The latter would ensure that sampling was done at appropriate time intervals after exposure (i.e., 0, 4, 8, 12, 24, 48, 72, 96, and 120 hours after testing) to capture any changes that occur in cytokine levels.
2) Conduct similar studies using chronic sound stress, rather than acute sound stress.
3) A very important step involves challenging dolphin cell lines to: a) validate responses at the RNA level, (b) develop dolphin-specific antibodies for "the marine mammal community" to use to assess immune function and changes therein, and (c) use a variety of real-world challenges (e.g., oil, dispersant, cold) to assess whether there are stressor-specific profiles of cytokine expression.
4) Inasmuch as blood is difficult to acquire from many species of marine mammals, including deep diving species of particular interest with regard to effects of sound, it would be useful to investigate the possibility of using a suite of available biomarkers to assess stress-related changes in other matrices that are easier to acquire (e.g., exhalations, feces).

Altogether, we believe that our study provides evidence that traditional markers of stress may be inadequate for characterizing acute stress responses to sound exposures and the suite of biomarkers we used in this study offers real promise following further study. Clear, empirical demonstrations of sublethal impacts of stressors on critical biological functions for marine mammals will be of vital importance for status determinations of affected stocks and for focused, effective and cost-effective mitigation

SUPPLEMENTAL MATERIALS/APPENDICES

ABSTRACTS

ABSTRACT ACCEPTED FOR SPOKEN PRESENTATION AT 20TH BIENNIAL CONFERENCE ON THE BIOLOGY OF MARINE MAMMALS, 9-13 DECEMBER 2013, DUNEDIN, NEW ZEALAND

Proteomic and metabolomic biomarkers indicate sublethal effects of simulated sonar signals on bottlenose dolphins, *Tursiops truncatus*

John E. Reynolds, Wiebke Artl, Kristina Deak, Grégory Genta-Jouve, Dorian S. Houser, Angela Taylor, Mark R. Viant, and Dana L. Wetzel

Human activities introduce a range of sounds into the marine environment, but sublethal effects of underwater noise on marine mammals are not well understood. One category of anthropogenic sound that has received particular attention is sonar associated with military activities. The objective of this study was to use a range of biomarkers to characterize stress responses of 19 bottlenose dolphins exposed, under controlled conditions, to mid-frequency active (MFA) sonar-type signals (~3.5kHz) with received levels ranging from 115 to 185 dB re 1 μPa. Subjects received up to 10 exposures within a 5 minute period, depending on their willingness to participate. Blood/plasma samples were collected from dolphins prior to, immediately following, and one week after exposure to the sounds. Proteomic analyses using ELISAs focused on 27 cytokines, 4 acute phase proteins, and 3 peptides associated with fertility. Changes in fertility potential associated with testing were minimal, whereas certain assays of cytokine levels suggested sublethal changes with regard to inflammatory response, growth factors, and generalized immune response for exposed dolphins. A targeted metabolomic approach developed a highly sensitive UHPLC-QQQ method to quantify cortisone, cortisol, corticosterone, 11-deoxycortisol, testosterone, and progesterone levels, but did not yield significant results associated with exposures. However, a non-targeted metabolomic approach used UHPLC coupled with FT-ICR mass spectrometry to yield a robust model that discriminated samples before and after exposures. Univariate statistical analyses suggested that several tens of compounds were involved in the stress response. These results will promote better understanding of biologically significant effects of sound exposure on marine mammals, and possibly other taxa. This, in turn, may facilitate improved effectiveness and efficiency of efforts to minimize risks of certain types of anthropogenic noise.

ABSTRACT OF SPOKEN PRESENTATION AT THE 9th ANNUAL CONFERENCE OF THE METABOLOMICS SOCIETY, 1-4 JULY, GLASGOW, SCOTLAND.

A SIMILAR ABSTRACT BY THE SAME AUTHORS WAS ACCEPTED AS A POSTER PRESENTAION AT THE 7th JOURNÉES SCIENTIFIQUES DU RÉSEAU FRANÇAIS DE MÉTABOLOMIQUE ET FLUXOMIQUE, 10-12 JUNE, AMIENS, FRANCE

Application of both targeted and untargeted metabolomics approaches to assess potential biological effects of simulated sonar signals on bottlenose dolphins.

Grégory Genta-Jouve, Dorian S. Houser, Angela Taylor, Dana Wetzel, John E. Reynolds, Wiebke Artl and Mark R. Viant

The effects of underwater noise associated with naval activities have retained the attention of the scientific community throughout the last decade. Multiple studies have reported the impact of such sounds on marine mammals. In a search for a deeper mechanistic understanding of the effects of underwater noise on marine mammals, and to explore potential biomarkers of the stress response, we have employed a metabolomics approach to characterise the stress response of bottlenose dolphins induced by simulated sonar signals.

Two approaches were used. A targeted approach focused on a single class of metabolites often reported to be good indicators of stress in mammals. An untargeted approach investigated new biomarkers of stress in dolphin serum. During the first part of the study, we developed a highly sensitive UHPLC-QQQ method to quantify six steroids of interest (i.e. cortisone, cortisol, corticosterone, 11-deoxicortisol, testosterone and progesterone). Each compound was identified using both retention time and multiple reactions monitoring (MRM). The untargeted approach was run on a UHPLC system coupled to a FT-ICR mass spectrometer in order to acquire very high resolution mass spectra.

While the targeted approach did not yield significant results, the untargeted approach led to more interesting findings. PLS-DA analysis of the different class of samples pre, test and post (i.e. before the sound exposure, immediately following the sound exposure, and one week after the sound exposure) yielded a robust model to discriminate dolphin samples before and after exposure to the simulated sonar signal. Univariate statistical analyses of the data suggested that several tens of compounds were involved in the stress response.

Identification of these biomarkers will improve our ability to identify and understand biologically significant effects of sound exposure on marine organisms and especially marine mammals. This should improve the effectiveness and efficiency of efforts to minimize the risks of anthropogenic noise.

FIGURES

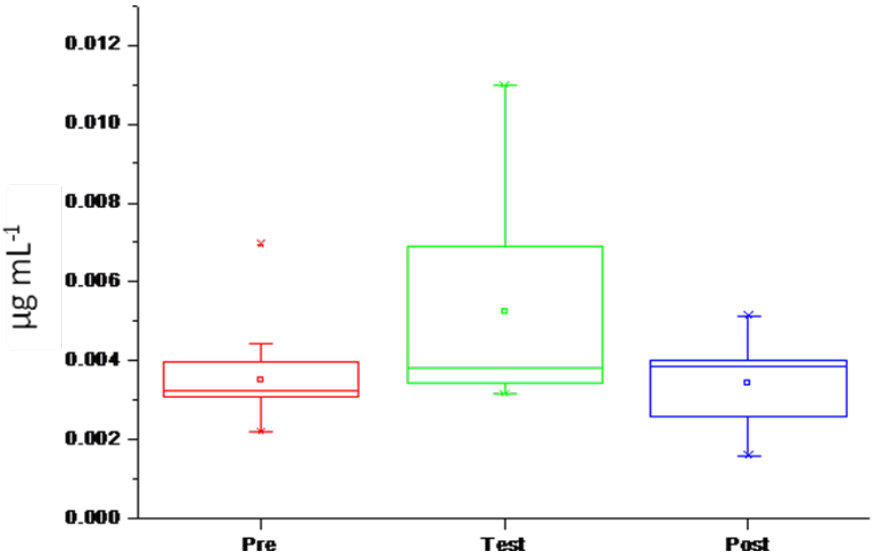

Figure 1: Cortisol concentration in the samples. There is no significant difference in the pre, test, and post samples ($p > 0.05$).

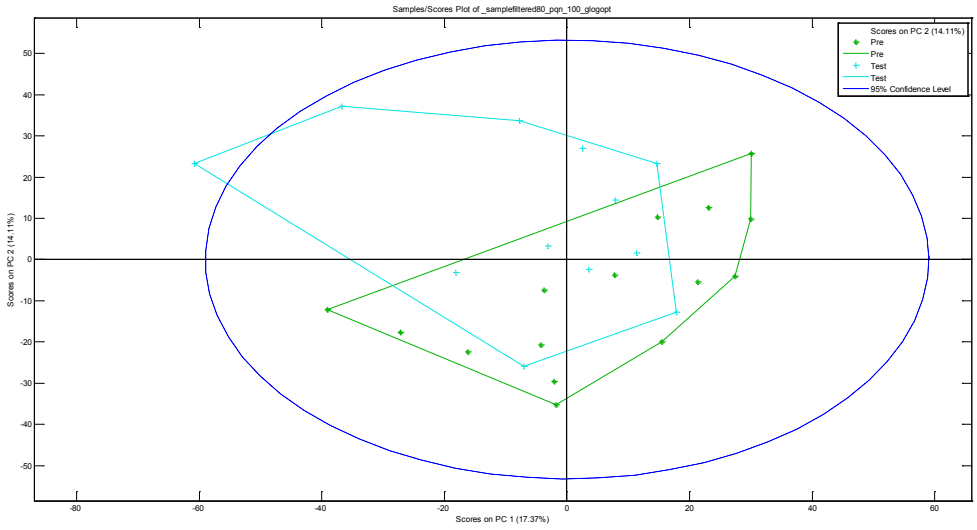

Figure 2: Principal components analysis (PCA) of the PRE and TEST samples; this analysis does not provide good separation.

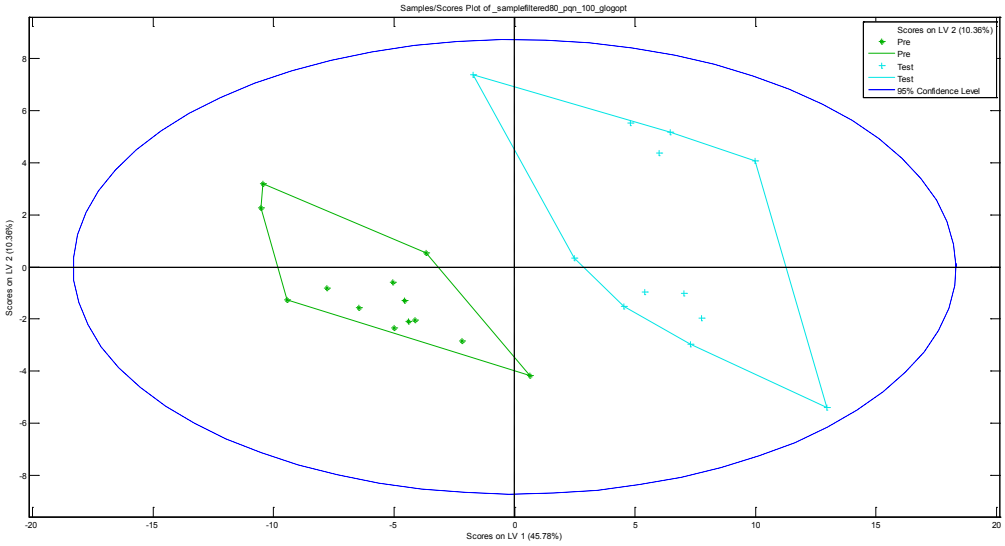

Figure 3: Partial least square discriminant analysis (PLS-DA) of the PRE and TEST samples ($p < 0.001$), the model was cross-validated by random permutations 1000 times and indicates good separation of the two sample types.

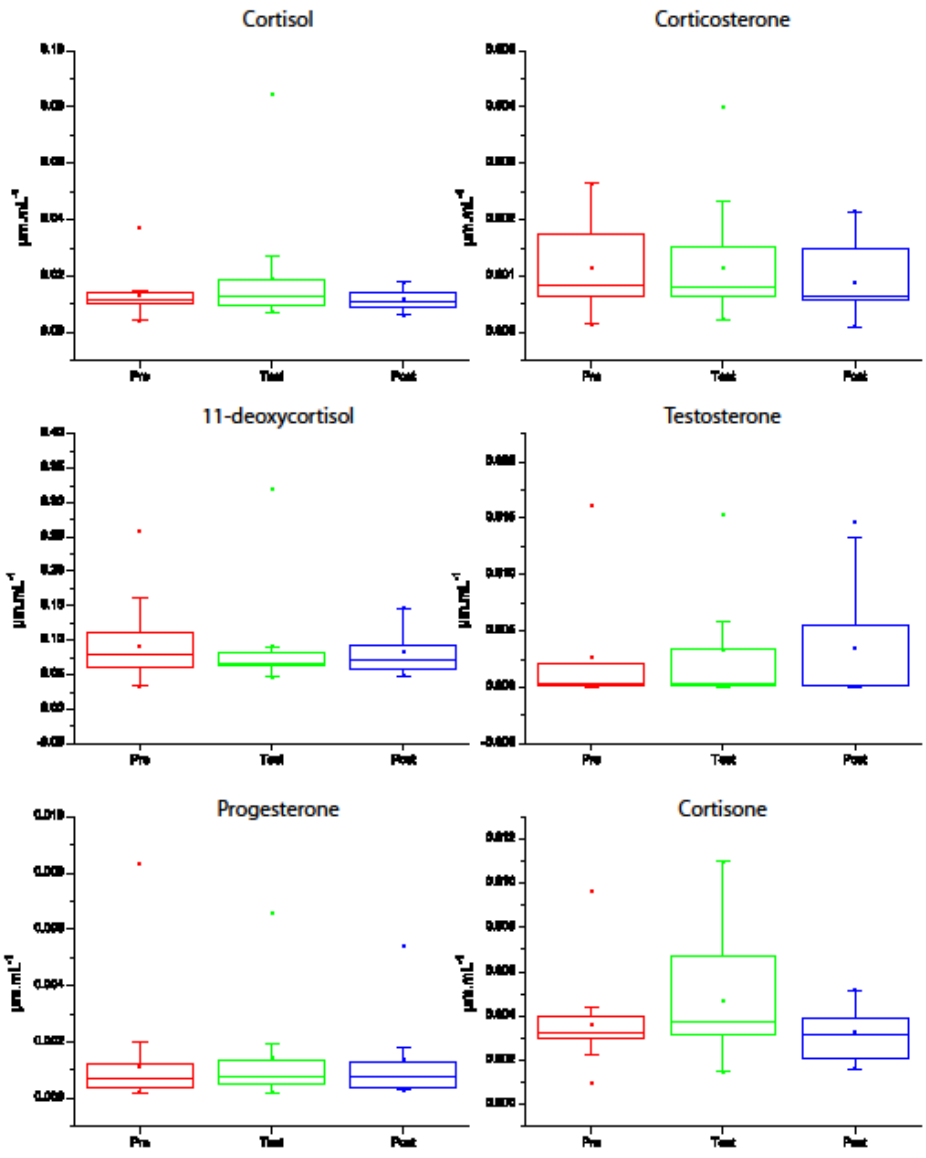

Figure 4: Concentrations of the targeted compounds by MRM.

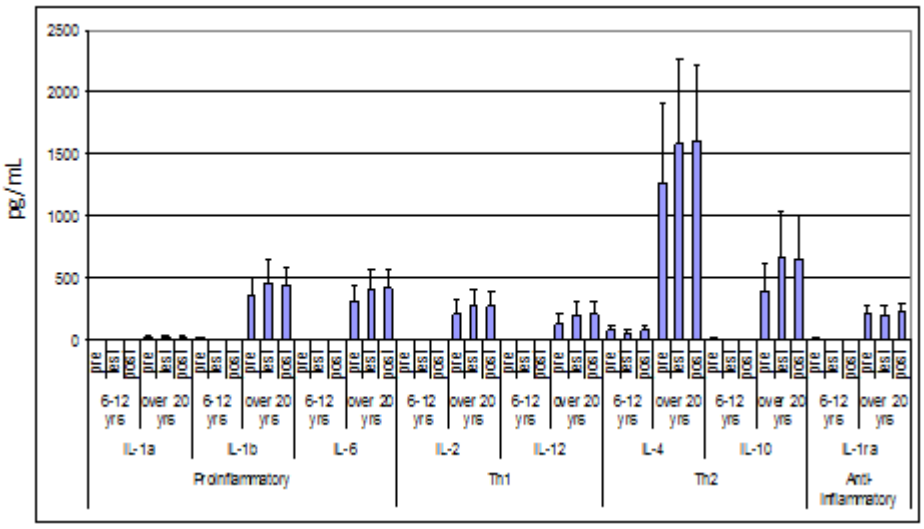

Figure 5. Cytokine immunoassay illustrating increased expression in dolphins over the age of twenty.

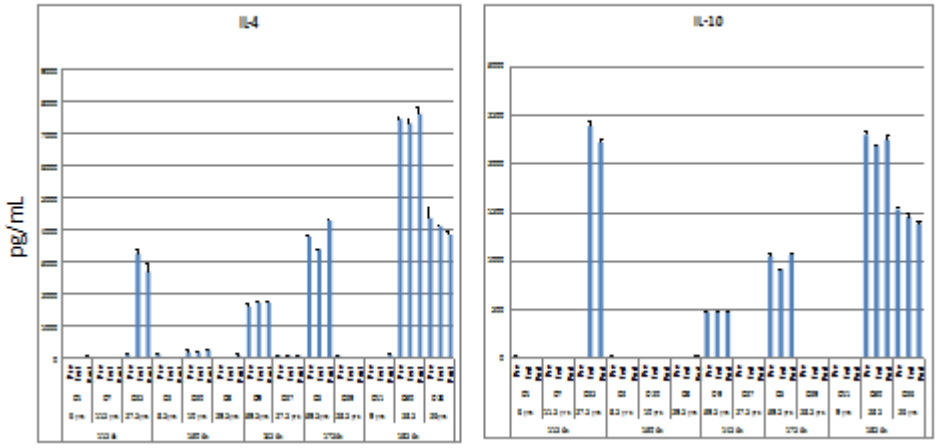

Figure 6. The cytokine expression predominantly in older dolphins.

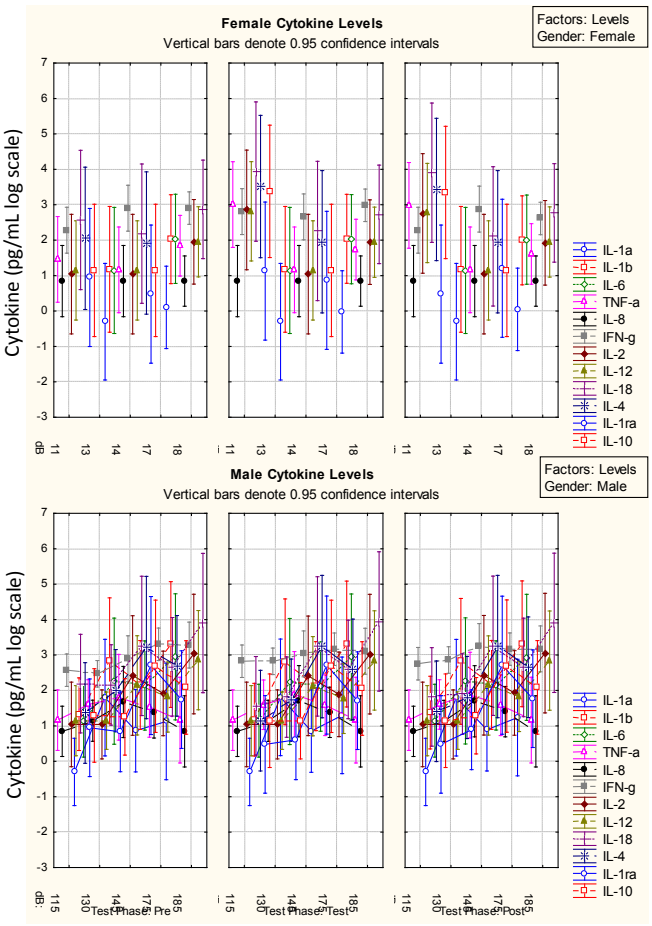

Figure 7: Pre, test, and post results of cytokine assays for female (top) and male dolphins. Although no statistically significant relationships occur, there is a slight trend for increasing cytokine levels with increasing dB in the males.

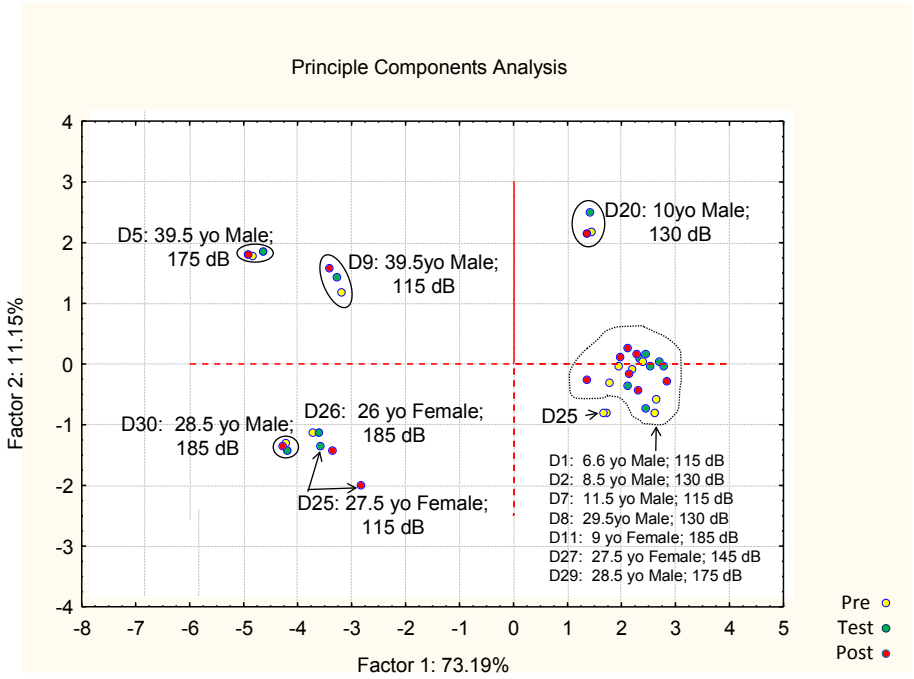

Figure 8: Principal components analysis demonstrates that 73.19% of the variability in cytokine profiles has age (> 26 years old) as a contributing factor, although a few older dolphins cluster in the lower right quadrant with younger animals.

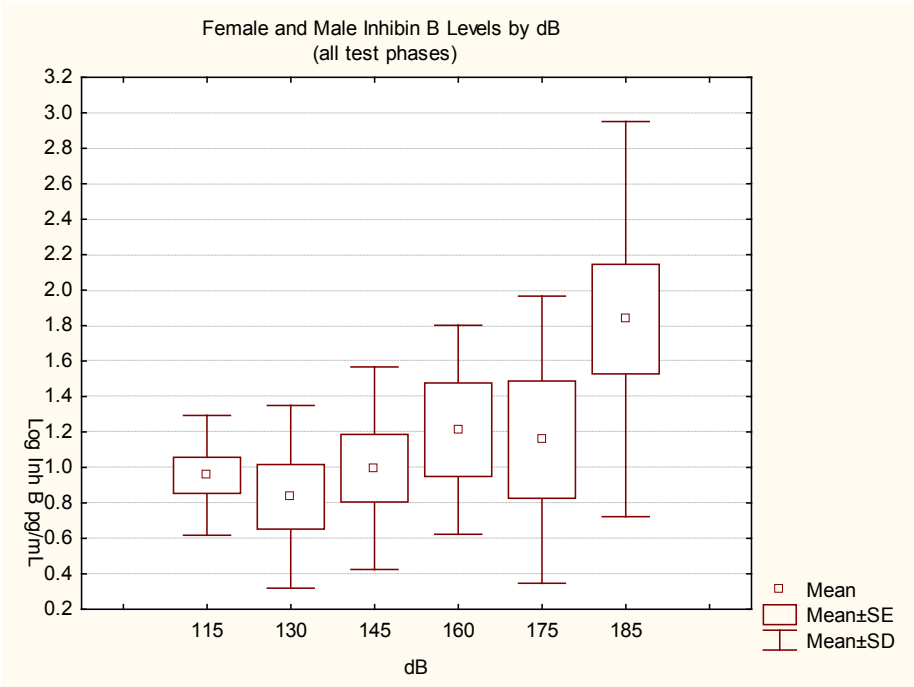

Figure 9: Although differences in fertility hormone levels associated with all sound levels were not significant, inhibin B levels were elevated for the cohort exposed to 185dB sounds.

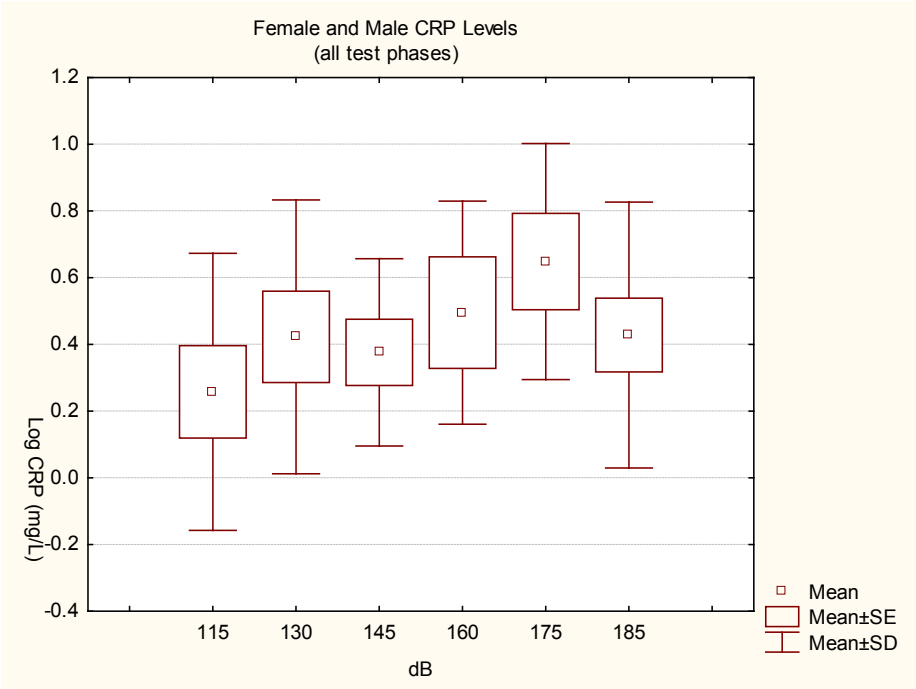

Figure 10: Levels of C-reactive protein generally tended to increase with increasing sound level exposures, but unexpectedly declined for animals exposed to 185 dB sounds.