

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 project N000141110432 is to utilize novel biomarkers to examine whether significant sublethal responses to sonar-type sounds occur in bottlenose dolphins exposed to such sounds. The collaborators will use 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 are 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 μ Pa. 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 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. 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: Our original intention was to conduct clinical chemistry analyses on samples sent to us by collaborator Dr. Dorian Houser. However, the sample volumes were very small, and after dividing the samples for biomarker analyses of various types, insufficient sample volume remained to conduct the clinical chemistry at Mote Marine Laboratory. Therefore we shall simply rely on clinical chemistry data acquired on site by Navy personnel and Dr. Houser. The decision to prioritize biomarker assays over clinical chemistry analyses was discussed with and approved by the ONR Program Manager, Michael Weise.

Immune function and acute phase markers: For assays of immune function and acute phase markers, prepared kits will be acquired from Bio-Rad. Immune function will be assessed using a 27-cytokine kit, and acute phase responses will be evaluated using a 4-plex kit. The assays will be done using a Luminex Bio-Plex 3D. This instrument combines existing technology in flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry to assess up to 500 different analytes simultaneously.

Metabolomic biomarkers: Non-targeted metabolomics will be conducted using direct infusion Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS). Previously, we developed and utilized this approach for measuring a diverse range of metabolites in several different sample types. Spectra will be acquired in triplicate (per sample) from a 96-well plate using the SIM stitching method, from m/z 70 to 590 in both ion modes. The resulting spectra will be processed using in-house software and analyzed using multivariate statistics (e.g., principal components analysis, partial least squares discriminant analysis) in order to discover metabolic biomarkers that change concentration in response to varying levels of sound exposure.

Targeted metabolite analysis will be conducted using a highly sensitive, specific and quantitative approach implemented on an LC triple quadrupole mass spectrometer. Targeted profiling will focus on the measurement of steroids and steroid-like molecules (including stress hormones such as cortisol) known to play a role in the stress responses of marine mammals. We will optimize our extraction protocols and then implement LC-MS/MS analyses on our Thermo Fisher Scientific TSQ Vantage triple quadrupole mass spectrometer. This will involve 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: We will conduct ELISAs using a DSX Automated Plate Processor. In the assays being conducted for AMH, inhibin A, and inhibin B, standards, controls, and serum samples will be incubated in microtitration wells coated with the appropriate antibody. A set of AMH, inhibin A, or inhibin B standards will be used to plot standard curves of absorbance vs. hormone concentration, and samples will be run in duplicate. Based on the standard curves, the concentration of AMH, inhibin A, or inhibin B in the dolphin serum samples will be calculated.

WORK COMPLETED

This component of the report describes activities and accomplishments between October 2011 and September 2012.

I. Overall accomplishments

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

Status: completed. The samples were provided to Mote Marine Laboratory in year one of the study by Dr. Houser. The samples were shipped to the United Kingdom under a blanket permit held by personnel at the National Marine Fisheries Service-Southwest Fisheries Science Center. Particular thanks go to Dr. R.L. Brownell, Ms. Siri Hakala, Agent Rhyon Wilcox, Dr. Paul Jepson, and Mr. Rob Deaville for facilitating the process.

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

Status: completed.

Task 3: Conduct assays.

Status: underway. See discussion below under “**Analyses**”.

Task 4: Conduct data interpretation and synthesize results.

Status: underway. The anticipated timeframe for this task was August 2012-November 2012. Delays have occurred with the project, as follows: 1) the grant was initiated by ONR three months later than anticipated; 2) making arrangements for sending samples to the UK under a proper CITES permit took longer than anticipated; and 3) delays occurred in hiring a post doctoral scientist at University of Birmingham. These issues have been discussed with ONR project manager Weise who remains comfortable that the project, while delayed slightly, remains generally on track.

Task 5: Coordination and communication of results.

Status: to be completed. The anticipated timeframe for these components was December 2012-February 2013. For reasons discussed under Task 4 (above) we anticipate a more realistic completion date of mid-2013. As above, the ONR Program Manager, Michael Weise, has discussed this with the PI and co-PI during a site visit in July, 2012, and is comfortable with a modestly delayed completion date.

II. Analyses

As directed by ONR, this section of the report deals with processes and technical accomplishments. Although we present some “results”, the formal “**Results**” section of this report is intended to highlight meaningful or significant results, interpretations, and conclusions.

I. University of Birmingham:

As introduced above, two strategies have evolved in metabolomics – targeted, quantitative profiling of a pre-selected class of metabolites, and non-targeted, semi-quantitative fingerprinting of a large number of metabolites providing a “systems level” view of metabolism. Both approaches have unique advantages for assessing the metabolic health of dolphins and are being used in this study.

Staffing: In April, 2012, a search for a postdoctoral scientist to work on this project at the University of Birmingham was completed successfully with the hiring of Dr. Gregory Genta-Jouve. Dr. Genta-Jouve started work on 30 April 2012 and has been an outstanding contributor to date.

Targeted profiling: Quantification of the selected steroids (Appendix 1) is being established using UHPLC-MS/MS. The separation is realized with a Thermo Fisher UltiMate 3000 Rapid Separation LC System combined with a TSQ Vantage triple quadrupole mass spectrometer. The identification and quantification of different steroids is being achieved using multiple reaction monitoring (MRM) with a specific transition for each compound.

Untargeted profiling: Two different approaches will be used for the non-targeted profiling: (1) direct infusion Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (DIMS) will be conducted on a LTQ FT Ultra instrument using an established University of Birmingham pipeline; and (2) to further complement and expand on the targeted steroid studies above, non-targeted LC-MS analyses will also be conducted on the FT-ICR mass spectrometer.

Results: We decided to start with the targeted analysis developing the quantification protocol for the selected steroids. In this analysis two sets of parameters required optimization, the liquid chromatography conditions and the mass spectrometer's parameters.

i. Liquid chromatography conditions: Using optimized UHPLC conditions we have obtained the chromatogram presented in Appendix 2. All compounds are well resolved except corticosterone and 11-deoxycortisol. The LC flow rate (for that chromatogram) was lowered to decrease the pressure of the system due to a valve problem. Thermo Fisher has recently sent the necessary replacement valves, which have been installed, and targeted steroid analyses are now underway. Using a higher flow rate will increase the resolving power and these two compounds should be separated enough to enable accurate quantification.

ii. Mass spectrometer parameters optimization: Several ion source parameters can be changed in order to increase the sensitivity and the reproducibility of the analyses. As explained in the previous section, we are using MRM to identify and quantify each compound. All the transitions used to quantify the compounds are described in Appendix 3. All the steroids are quantified using their deuterated analogues.

In order to test the accuracy of our protocol, we will compare our results with results obtained using the protocol developed by Dr. Angela Taylor in the group of Prof. Wiebke Arlt, Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham. This collaboration will also facilitate the comparison of steroid metabolism between marine mammals and humans.

Planned Studies: Next steps include: a) determination of the MS transitions for deuterated standards; b) measurement of the ca. 60 dolphin samples, including statistical analyses; and (c) non targeted profiling starting with the implementation of the LC-MS pipeline, i.e., finding the optimal LC conditions, optimizing the MS parameters, implementing chromatogram alignment software (XCMS), and measurement of samples and statistical analyses. The implementation of the non targeted profiling is planned to begin in the next few weeks (Appendix 4).

II. Mote Marine Laboratory

The samples from the various tested dolphins have been analyzed for both fertility potential and for a range of cytokines using the methods described above (see **PLANNED APPROACH**). A few samples are being re-analyzed but the results to date are presented in Appendices 5-25.

Results

i. Fertility assays: We conducted assays for three peptide hormones that can provide insights in mammals with regard to fertility potential and changes therein: anti-Müllerian hormone (AMH), inhibin B, and inhibin A. Although we have not proceeded to the point at which we have conducted thorough analyses and interpretations of the data, some general observations with regard to the assay results are as follows:

Pre, test, and post exposure values for AMH for the various dolphins appear in Appendices 5-7. It is clear that AMH values in male and female dolphins are quite different, as we would expect based on assessments of AMH in free-ranging dolphins. It is also apparent that the mean values for AMH for pre-exposure dolphins, recently tested dolphins, and post-exposure dolphins are not very different. Thus, the exposures to sonar-type sounds did not appear to affect AMH levels.

Pre, test, and post exposure values for inhibin B for the various dolphins appear in Appendices 8 and 9. The results for individual dolphins are quite variable, both with regard to pre, test and post exposure levels and with regard to the concentration values of inhibin B. Mean values for female dolphins appear to slightly increase during testing and post exposure.

Pre, test, and post exposure values for inhibin A for the various dolphins appear in Appendices 10-12. The values for inhibin A are generally more consistent than those for inhibin B for particular animals pre, test and post exposure. However, the mean values for females suggest a slight decrease in inhibin A values during and following exposures. Some individual dolphins of either sex have unusually high values of inhibin A.

Cytokines: Using the Luminex, we conducted assays for 27 different cytokines. Of those 27, 16 cytokines (Appendix 13) provided potentially-meaningful responses and information. Appendices 14-25 provide data regarding pre, test, and post exposure levels of the various cytokines for specific dolphins tested in this study. Inasmuch as (a) there is considerable variability in response among individuals, and (b) we have not completed all the assays for all dolphins, we shall only provide general comments and observations at this point:

- ❖ Responses varied among individual dolphins
- ❖ Most dolphins exposed to sonar-type sounds demonstrated an increase in levels of IL-1ra (inflammatory response); however, dolphins D4, D9, D19, and D21 showed the opposite trend.
- ❖ A response that was shown by more than one dolphin exposed to sonar-type sounds was a decrease in PDGF-BB (expression of growth factor).
- ❖ Several tested dolphins showed an increase in IL-6 (immune response).
- ❖ Eotaxin levels rose in some of the tested dolphins (eosinophils/inflammatory response).

We will soon complete the analyses of cytokines for all tested dolphins, and will also complete the assays of acute phase response proteins. The interactions among cytokines are complex, and once all the data are compiled, we will conduct a careful interpretation of the results and their implications for dolphins exposed to sonar-type sounds.

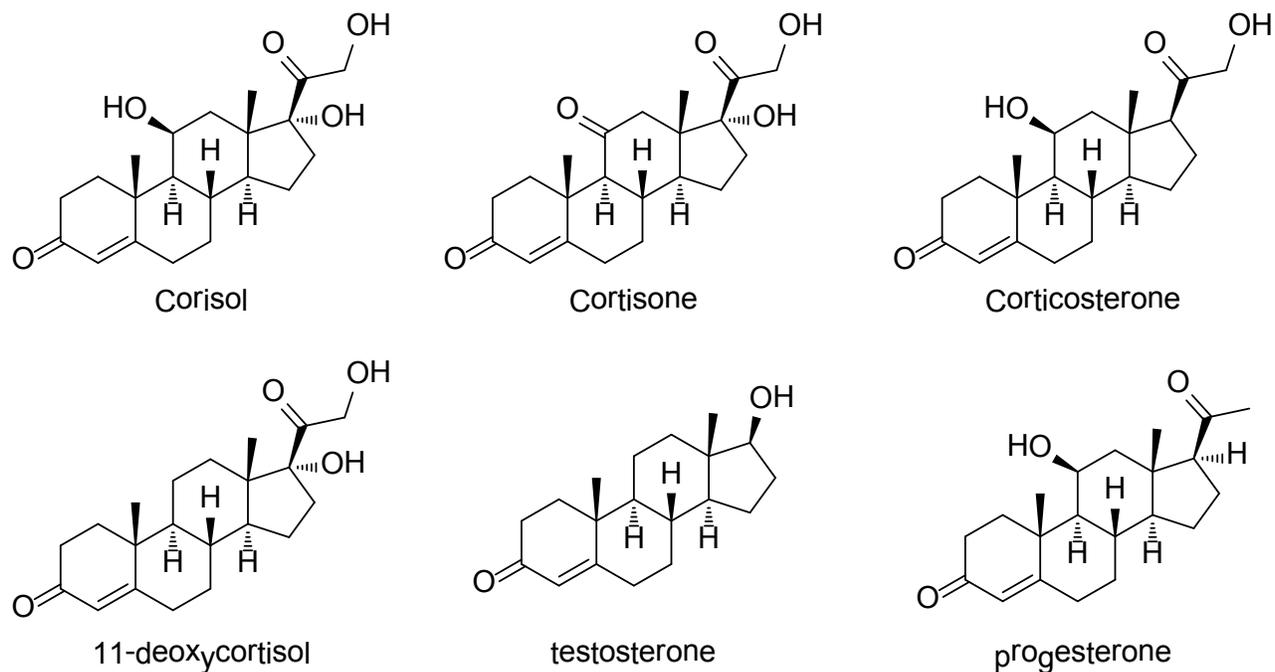
RESULTS

Inasmuch as we are still conducting a number of assays, it is premature to provide definitive study results. Nonetheless, to date we feel it is clear that changes in fertility potential for dolphins associated with testing were modest, whereas certain assays of cytokine levels suggest that the exposure tests appeared to elicit in several individuals sublethal changes with regard to inflammatory response, growth factors, and generalized immune response. At this time, the level of sound exposure has not been taken into consideration. Sound exposures varied from near-ambient to being within several hundred meters of a 53C sonar operating at a nominal source level of 235 dB re 1 μ Pa. The received sound pressure level will be incorporated into upcoming analyses.

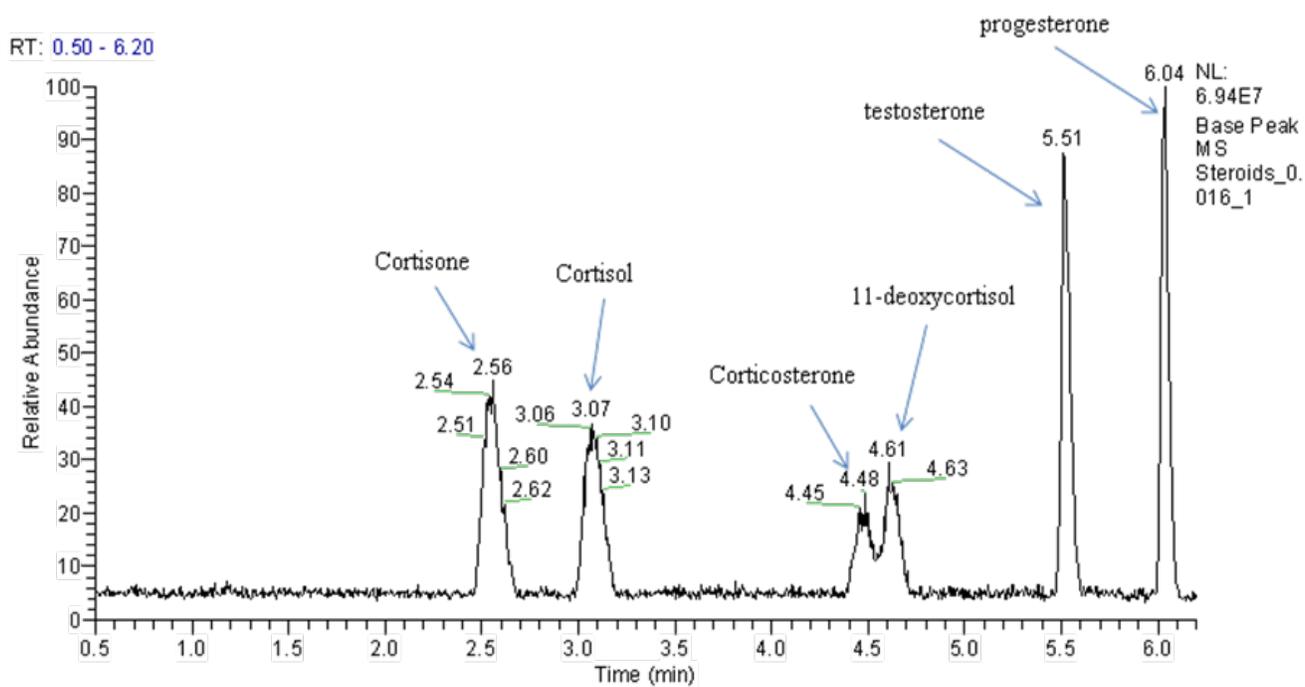
IMPACT/APPLICATIONS

The conduct of this study will provide a number of basic and applied benefits with regard to science and management, as follows: provide development of new tools for understanding the biology of bottlenose dolphins and other marine mammals and provide a clarification of the real extent of sublethal harm.

SUPPLEMENTAL MATERIALS/APPENDICES



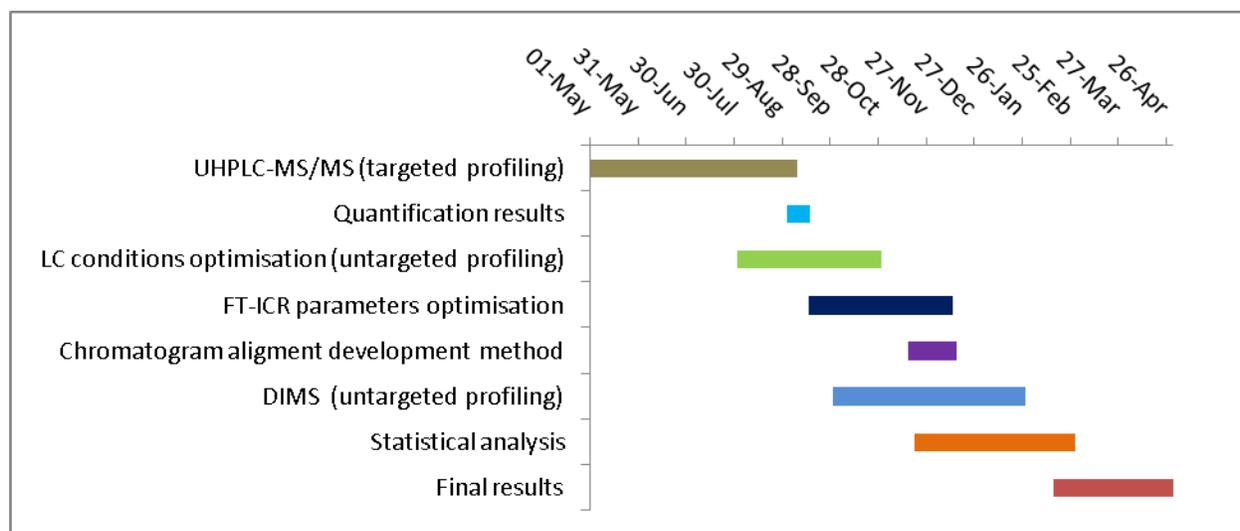
Appendix 1: Selected steroids for the UHPLC-MS/MS quantification



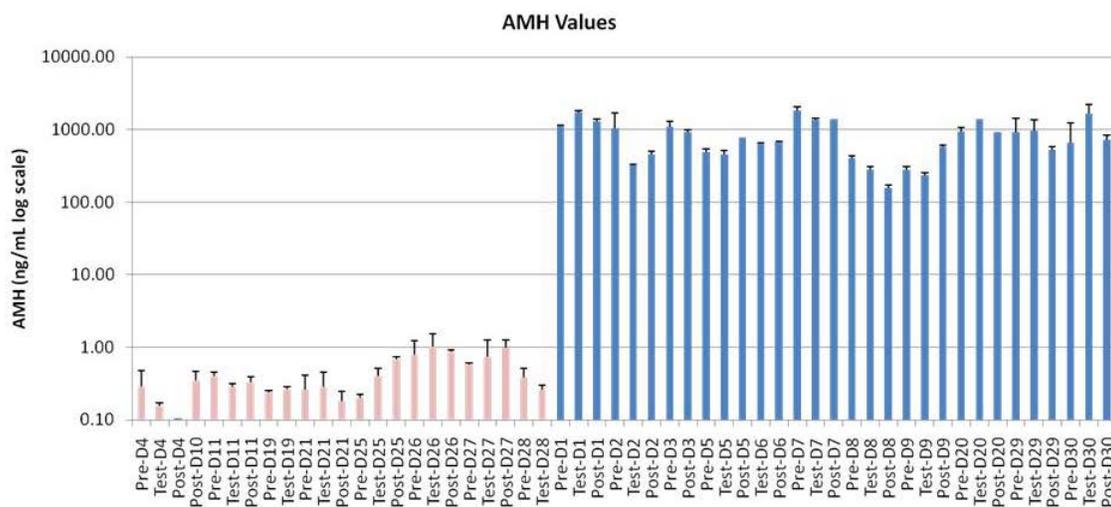
Appendix 2: UHPLC chromatogram using a Kintex C₁₈ 1.7 μ .

Appendix 3: Table showing steroid mass transitions used to identify and quantify steroids in sample matrix.

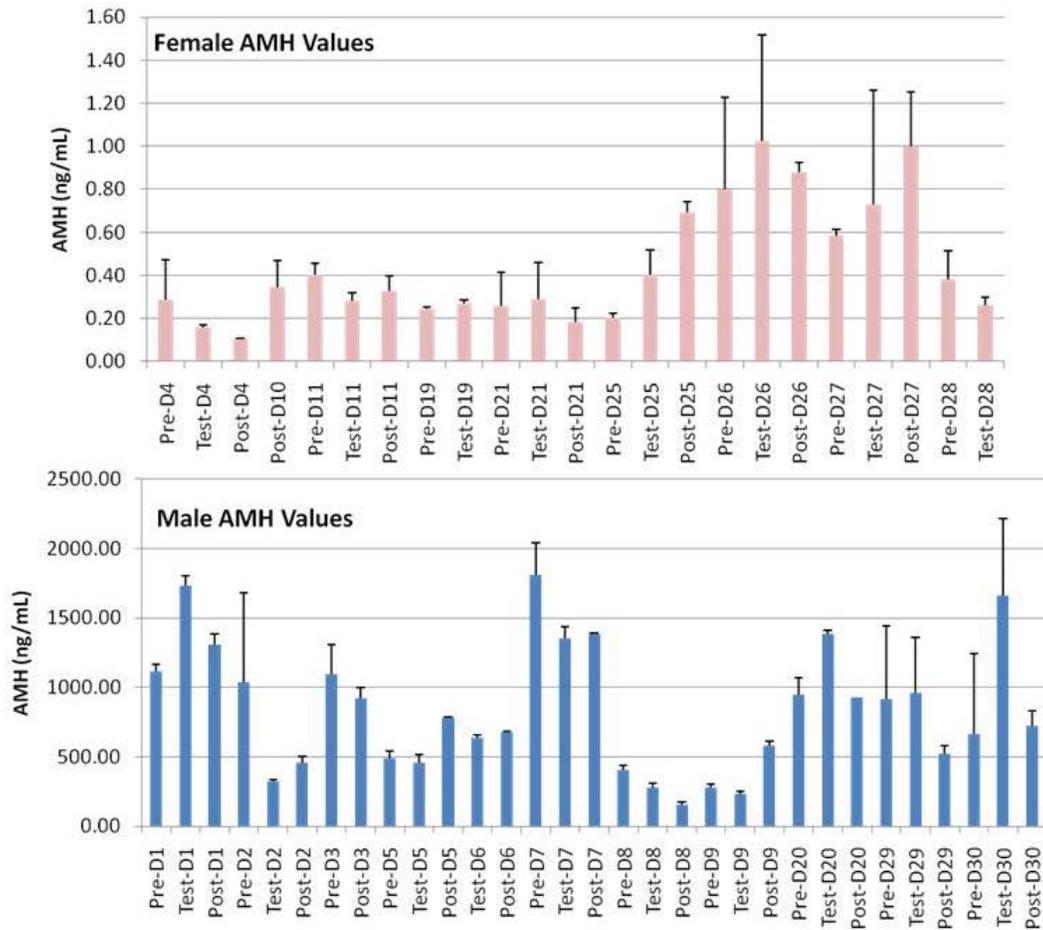
Steroid	Quantifier ion	Collision energy
Cortisone	361.3>163.1	20
Cortisol	363.2>121.1	21
Corticosterone	347.3>329	10
11-deoxycortisol	347.3>97.1	24
Testosterone	289.2>97.1	22
Progesterone	315.2>97.1	18



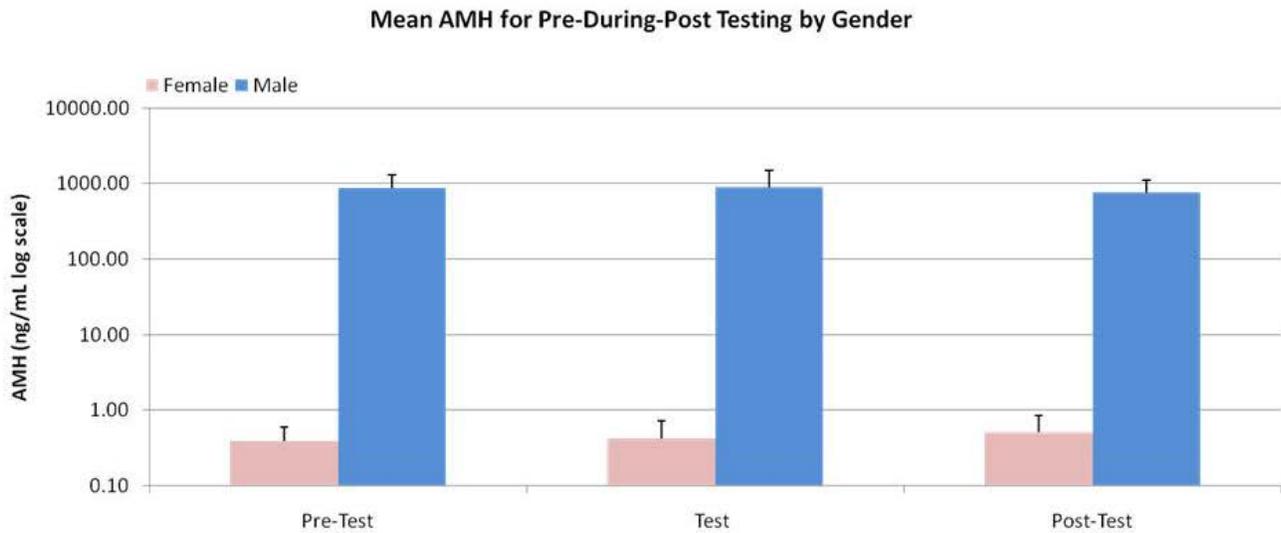
Appendix 4: Anticipated schedule for University of Birmingham activities.



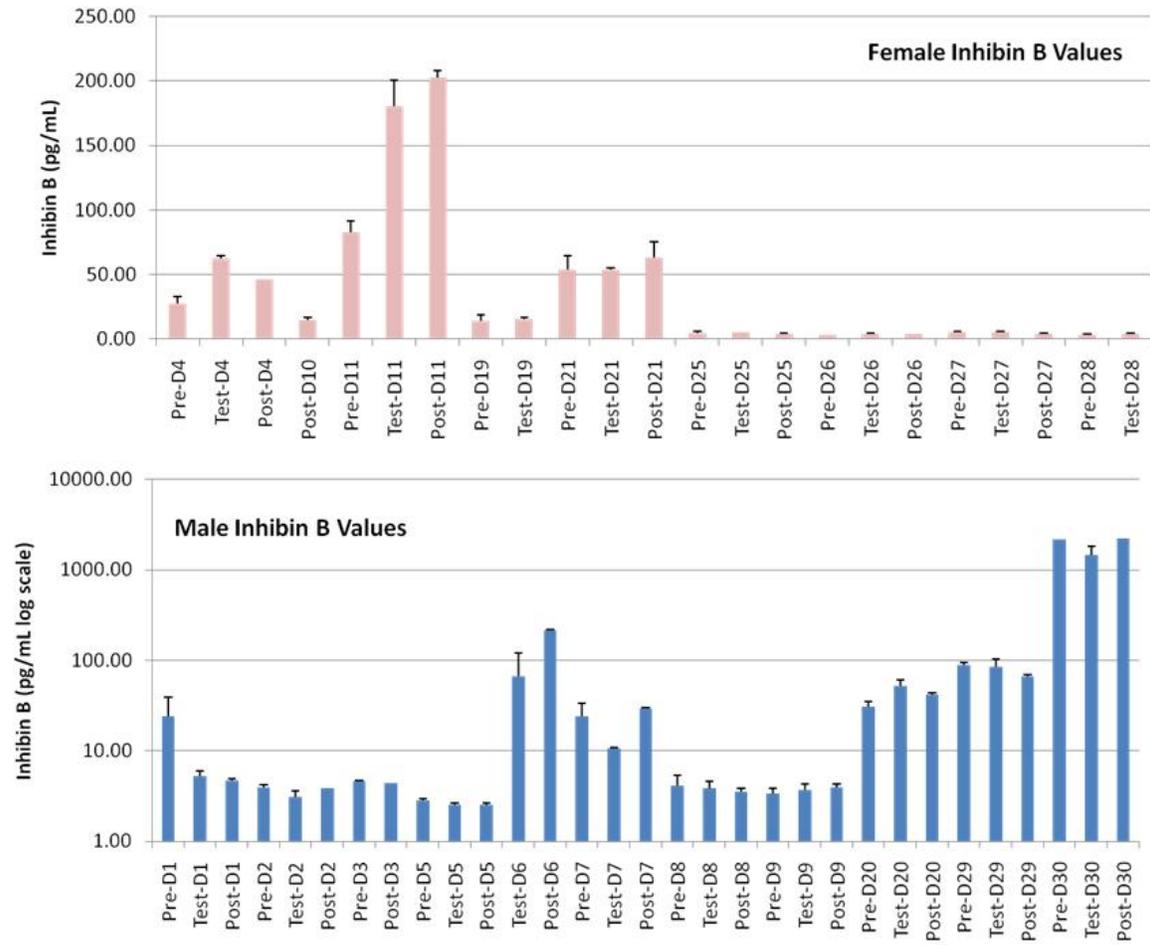
Appendix 5: Pre, test and post exposure values for AMH in tested dolphins. Values for males appear on the right side of the graph in blue; values for females are in pink on the left side of the graph.



Appendix 6: Pre, test and post exposure values for AMH in tested dolphins.

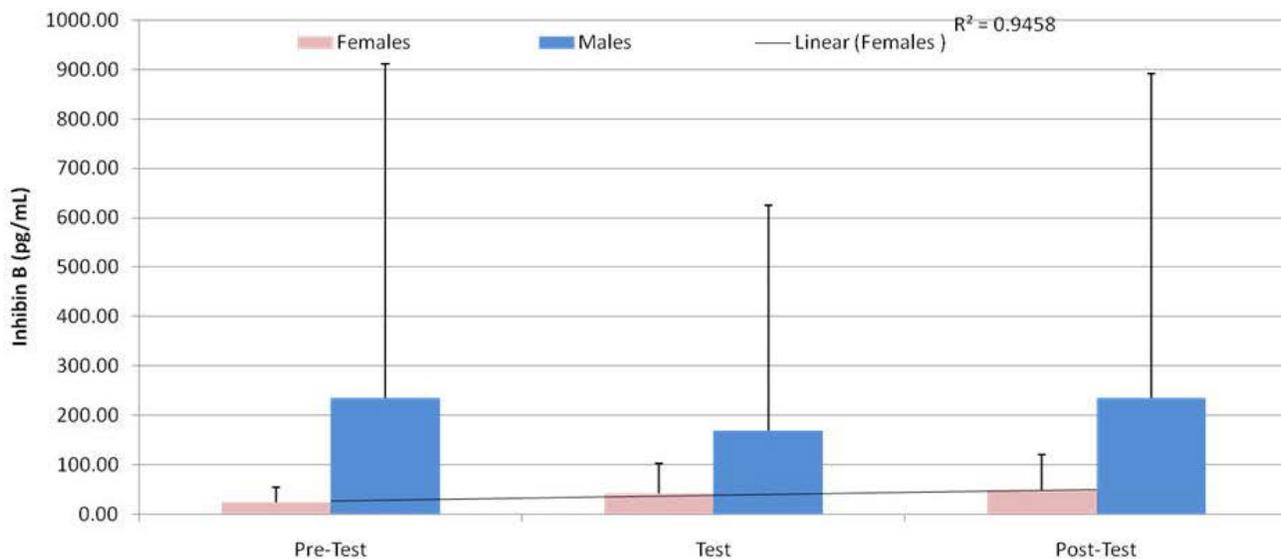


Appendix 7: Mean pre, test and post exposure values of AMH for male and female dolphins tested in this study.



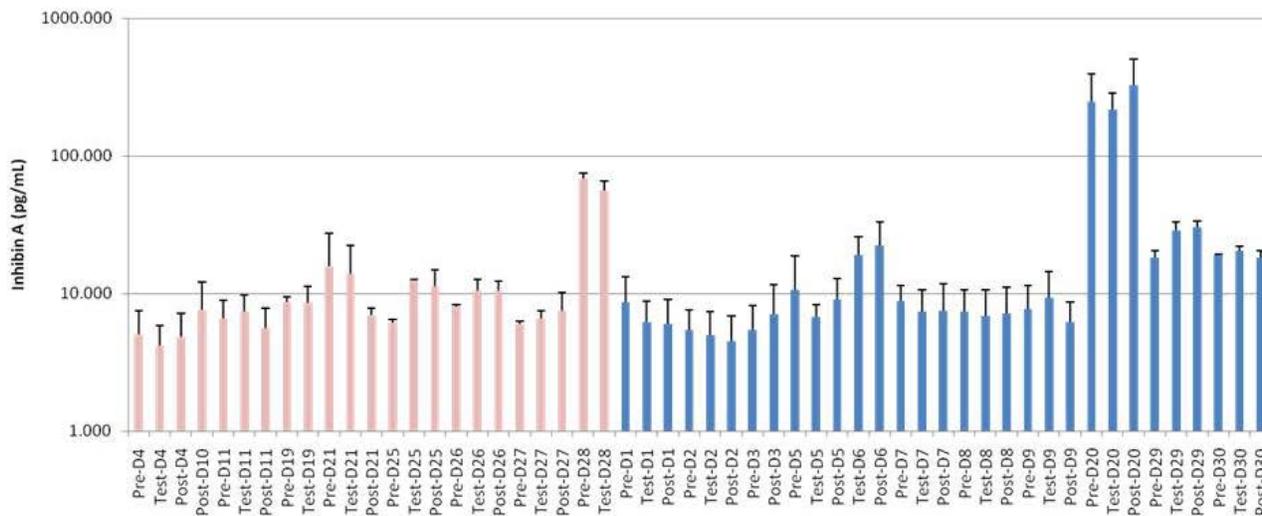
Appendix 8: Pre, test and post exposure values for inhibin B in tested dolphins.

Mean Inhibin B Pre-During-Post Testing by Gender

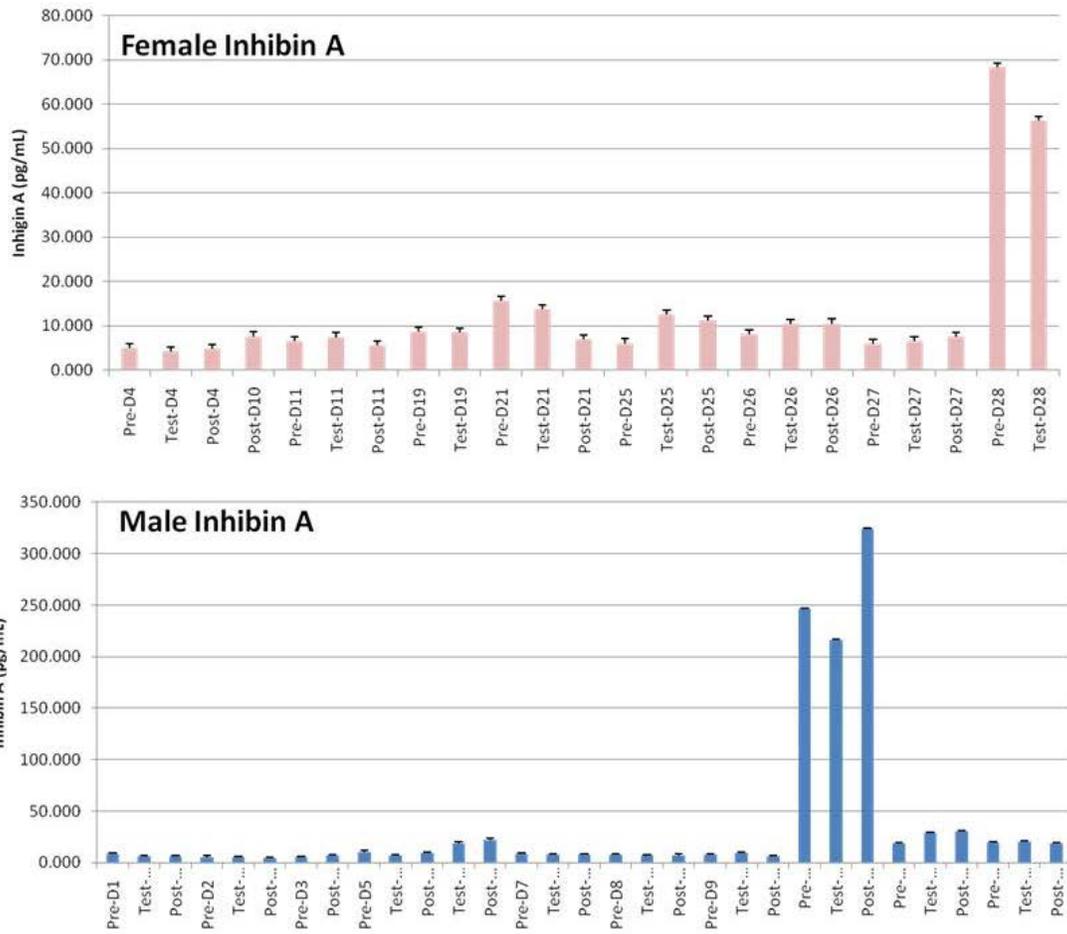


Appendix 9: Mean pre, test and post exposure values of inhibin B for male and female dolphins tested in this study.

Inhibin A Values

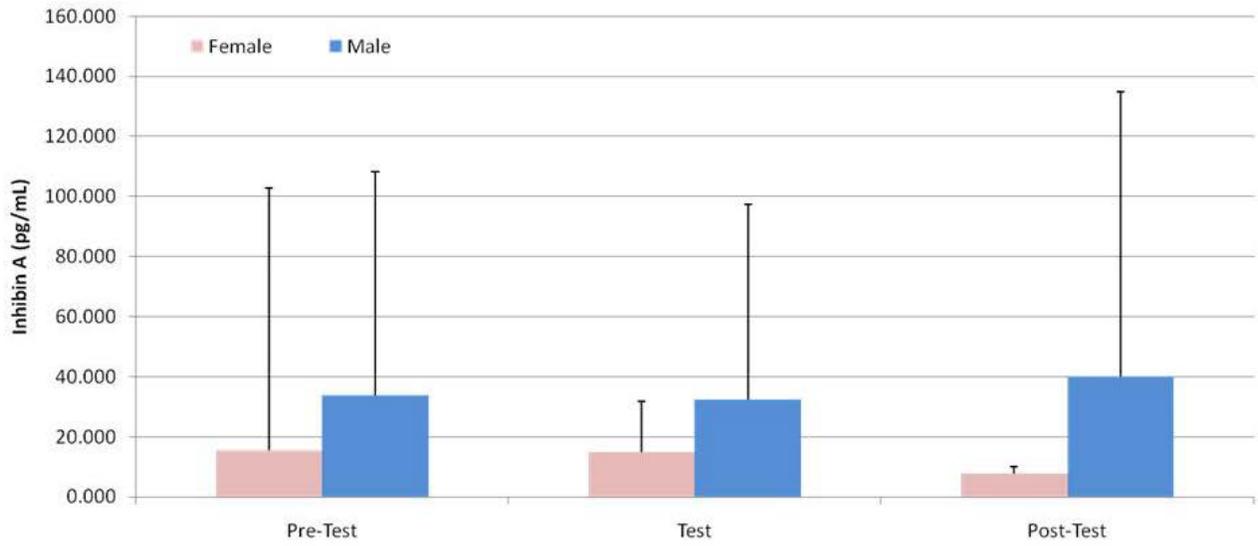


Appendix 10: Pre, test and post exposure values for inhibin A in tested dolphins. Values for males appear on the right side of the graph in blue; values for females are in pink on the left side of the graph.



Appendix 11: Pre, test and post exposure values for inhibin A in tested dolphins.

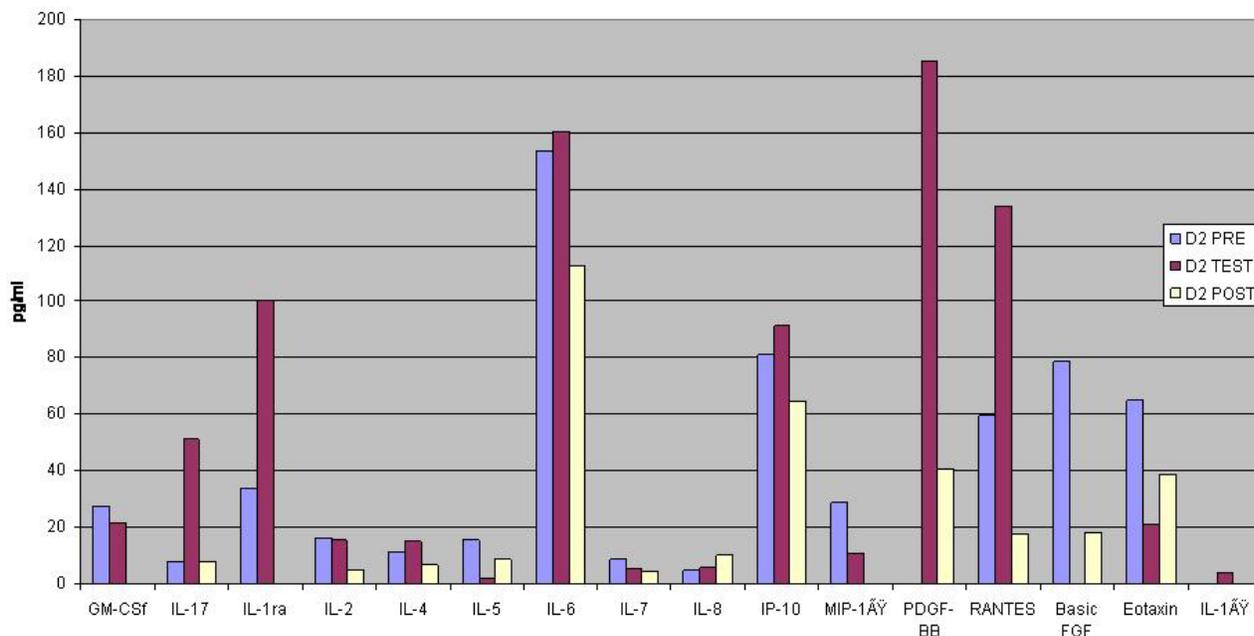
Mean Inhibin A for Pre-During-Post Testing by Gender



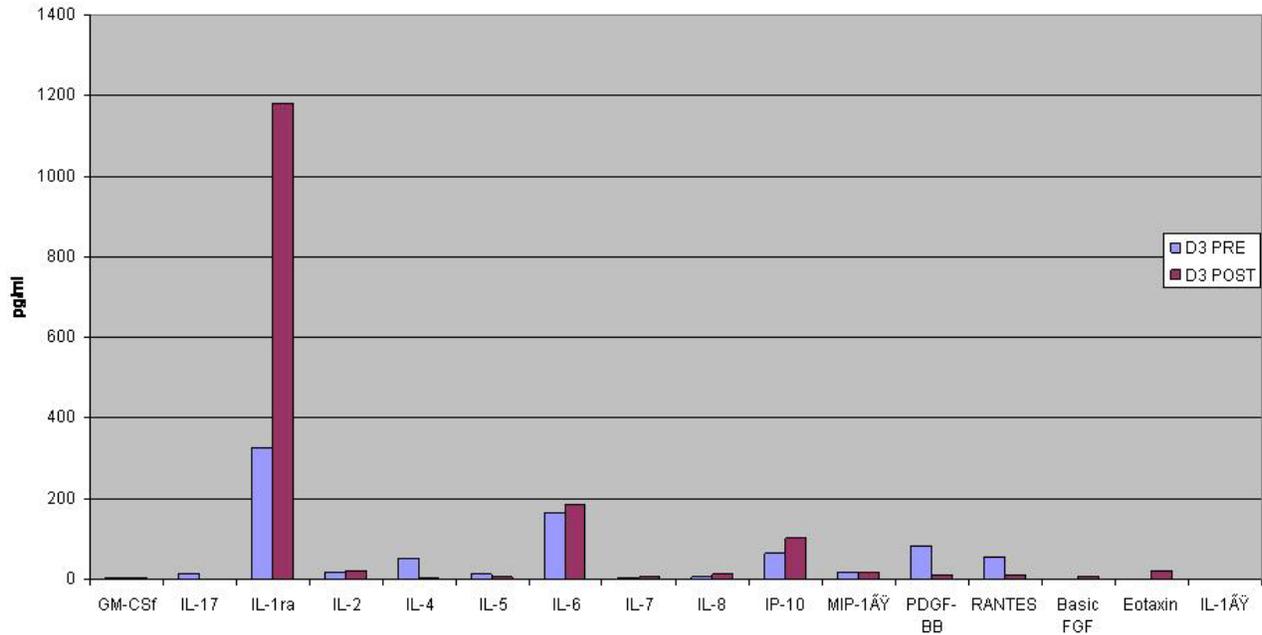
Appendix 12: Mean pre, test and post exposure values of inhibin A for male and female dolphins tested in this study.

Appendix 13: Cytokines tested that appear to provide meaningful information with regard to possible effects of sonar-type sounds on dolphins.

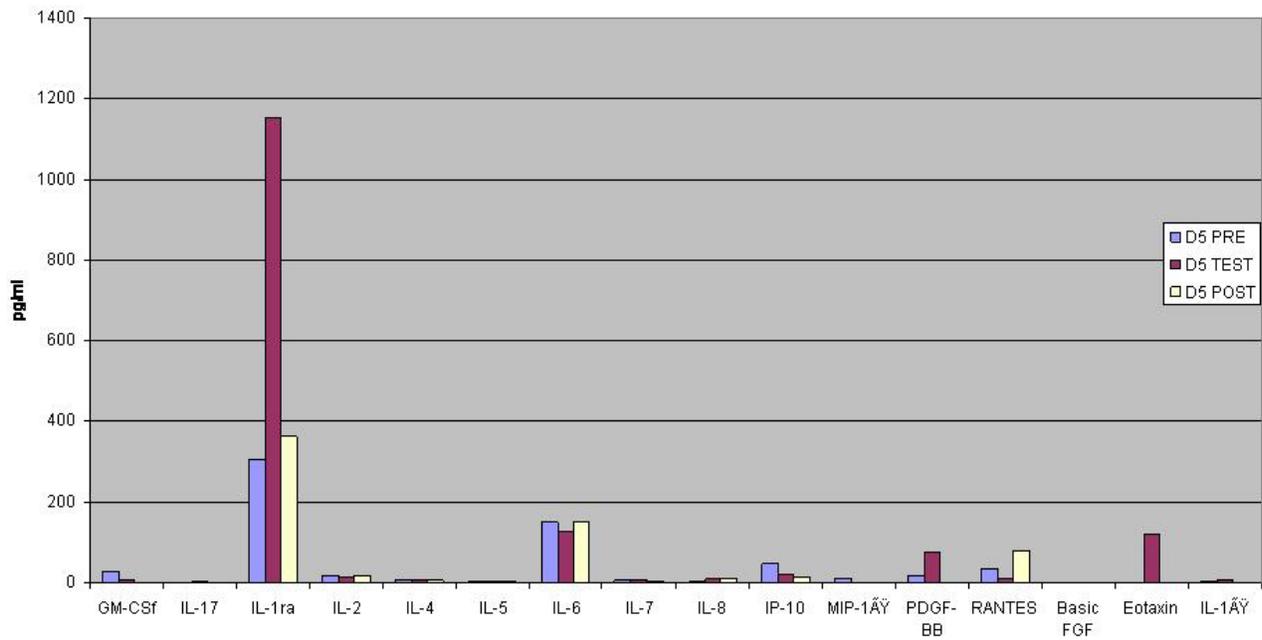
- GM-CSf: granulocyte macrophage colony stimulating factor
- IL-17: interleukin 17, involved in inducing and mediating proinflammatory responses
- IL-1ra: interleukin 1 receptor antagonist, inhibits the proinflammatory effect of interleukin b
- IL-2: interleukin 2, regulates growth and function of T cells
- IL-4: interleukin 4, induces B-cell and T-cell proliferation, inflammation, and wound repair
- IL-5: interleukin 5, promotes B-cell growth and immunoglobulin secretion
- IL-6: interleukin 6, stimulates immune response
- IL-7: interleukin 7, promotes T-cell and B-cell maturation
- IL-8: interleukin 8, induces chemotaxis in neutrophils, and is a proinflammatory mediator
- IP-10: interferon gamma-inducible protein10, secreted in response to IFN-y (interferon y); anti-tumor activity and chemo-attractant for T-cells
- MIP-1 β : macrophage inflammatory protein 1 beta, influences immune responses for inflammation
- PDGF-BB: platelet derived growth factor family, associated with unregulated expression of growth factor
- RANTES: stands for Regulated upon Activation, Normal T-cell Expressed and Secreted, member of the interleukin 8 superfamily; active role in recruiting leukocytes
- Basic FGF: basic fibroblast growth factor, mediates formation of new blood vessels, reducing tissue death
- Eotaxin: promotes recruitment of eosinophils during inflammation
- IL-1 β : interleukin 1 beta (also called catabolin), produced by activated macrophages as Important mediator of an inflammatory response



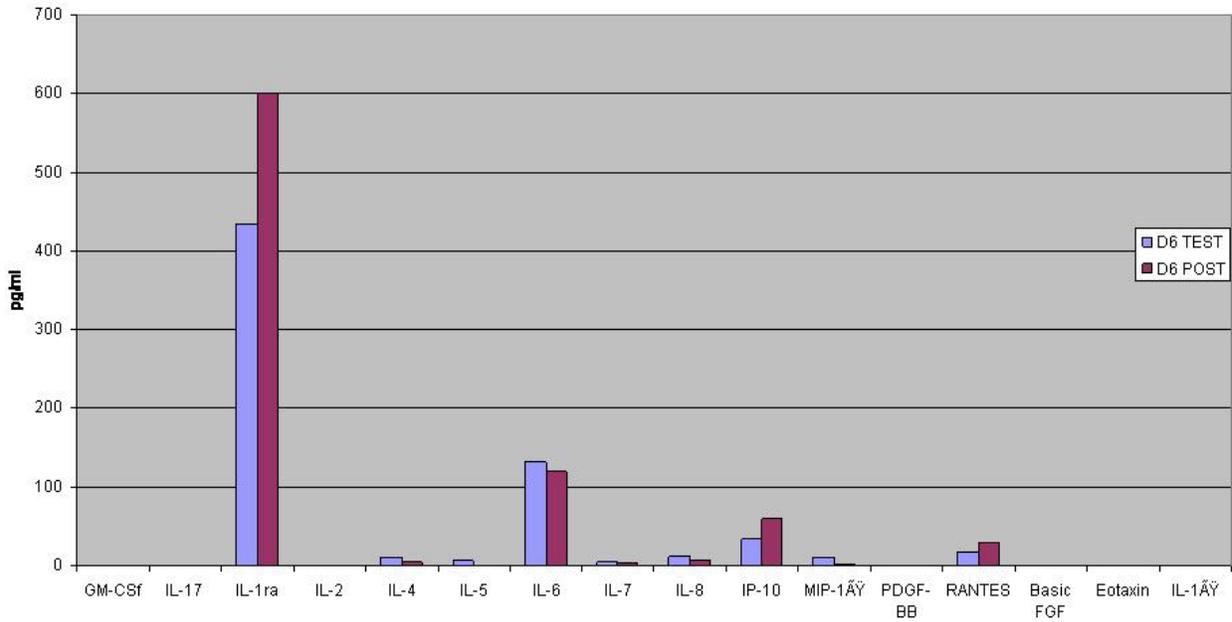
Appendix 14: Male D2 immune function responses pre, test and post exposure.



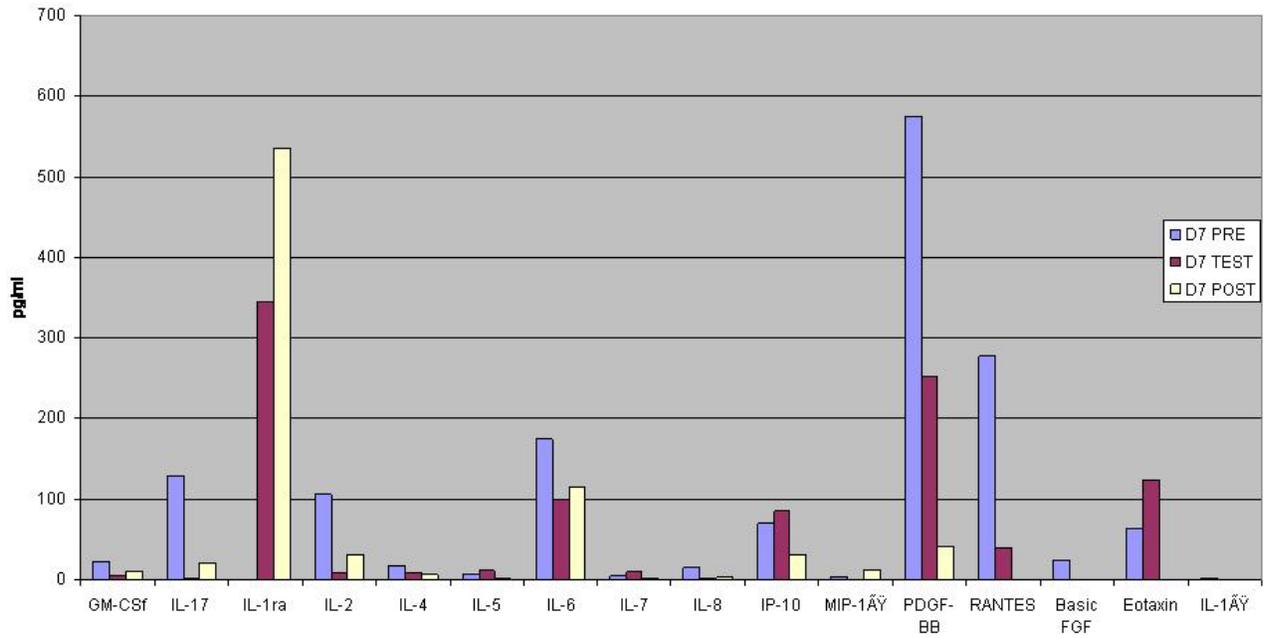
Appendix 15: Male D3 immune function responses pre and post exposure.



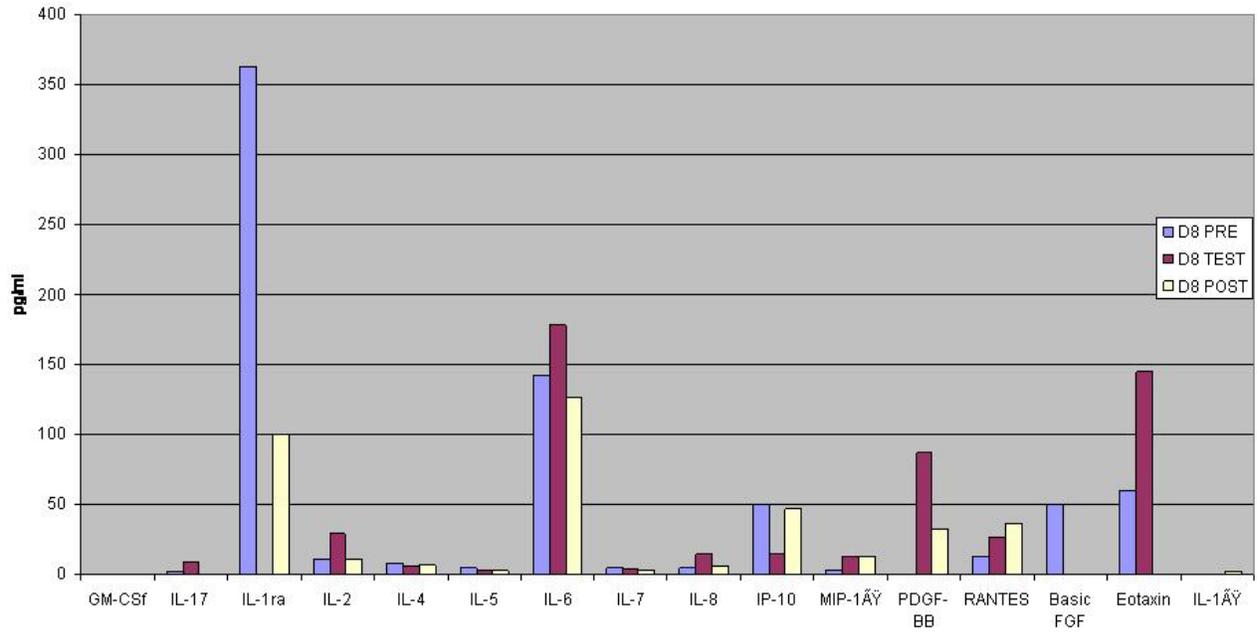
Appendix 16: Male D5 immune function responses pre, test and post exposure.



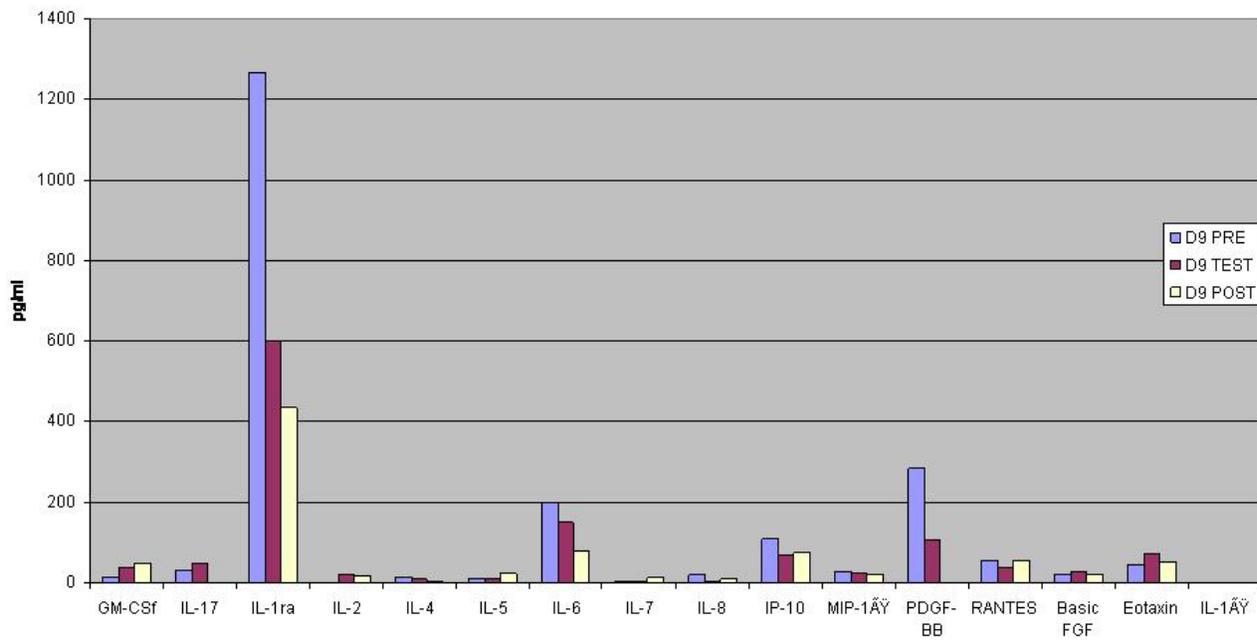
Appendix 17: Male D6 immune function responses test and post exposure.



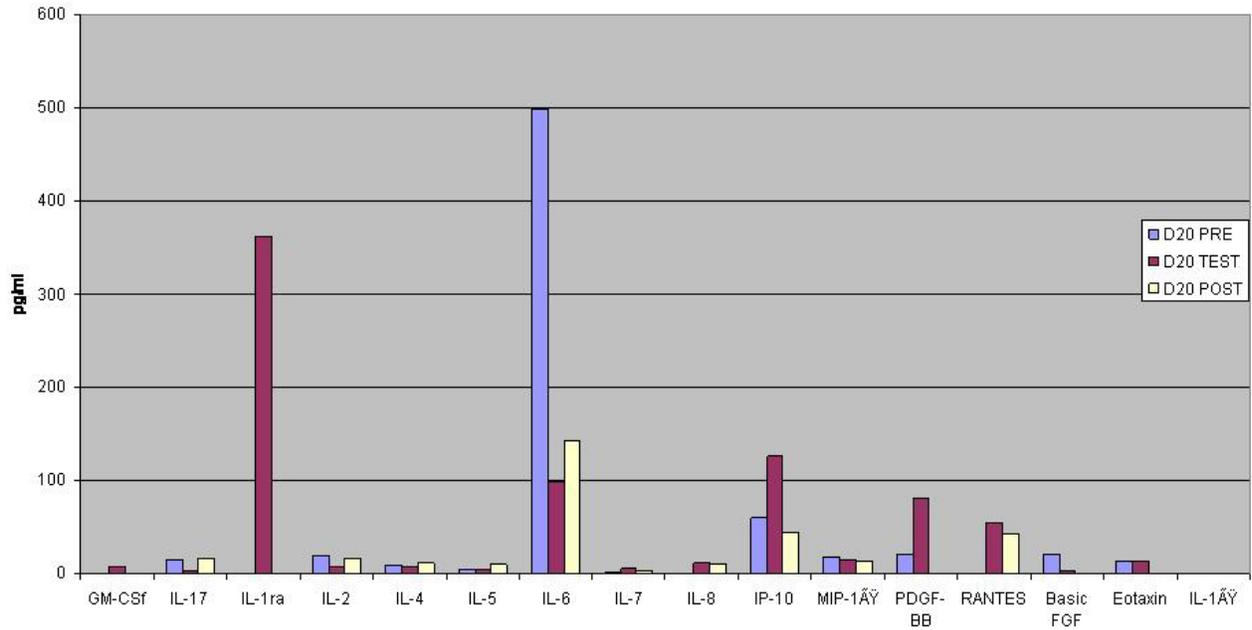
Appendix 18: Male D7 immune function responses pre, test and post exposure.



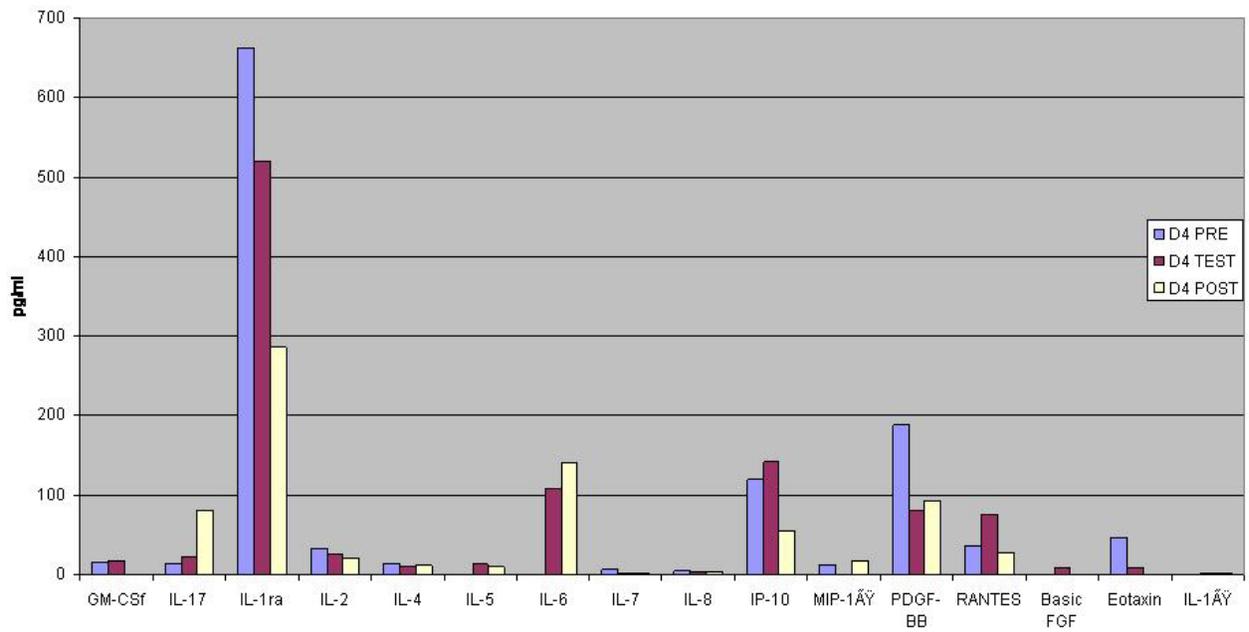
Appendix 19: Male D8 immune function responses pre, test and post exposure.



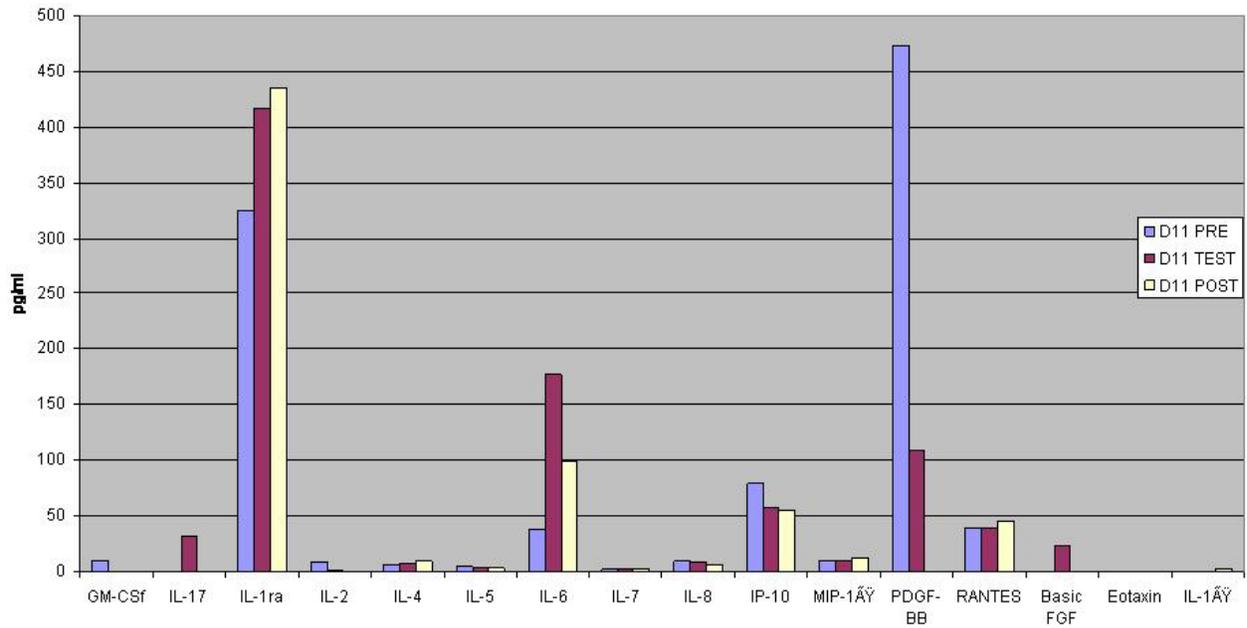
Appendix 20: Male D9 immune function responses pre, test and post exposure.



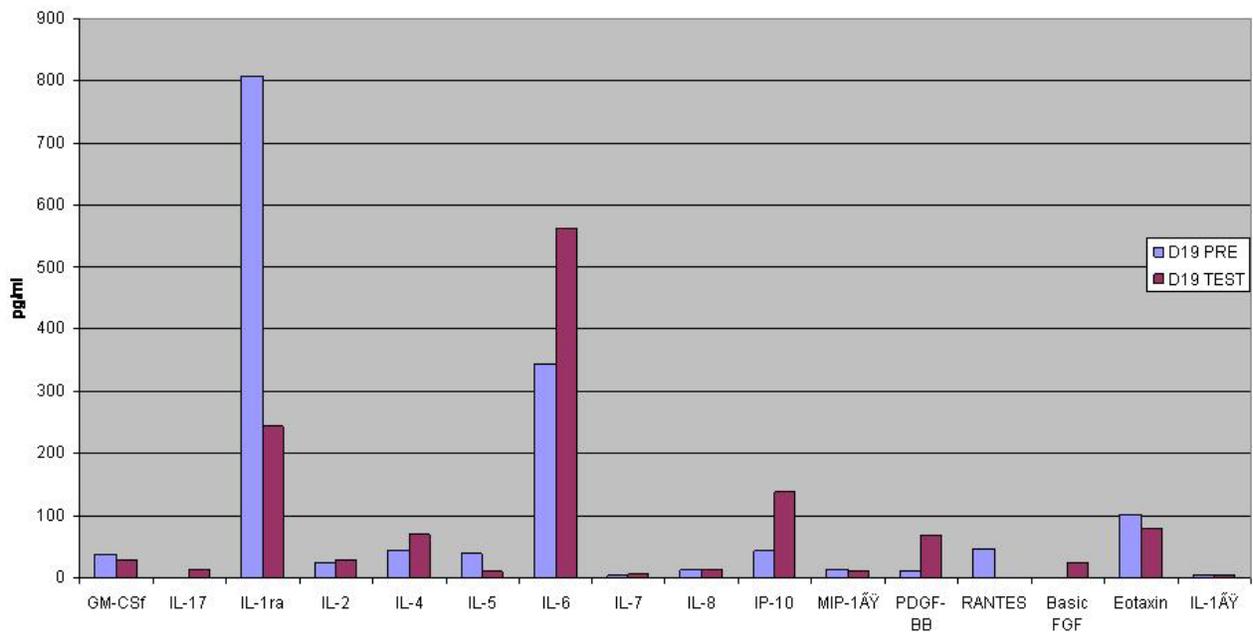
Appendix 21: Female D20 immune function responses pre, test and post exposure.



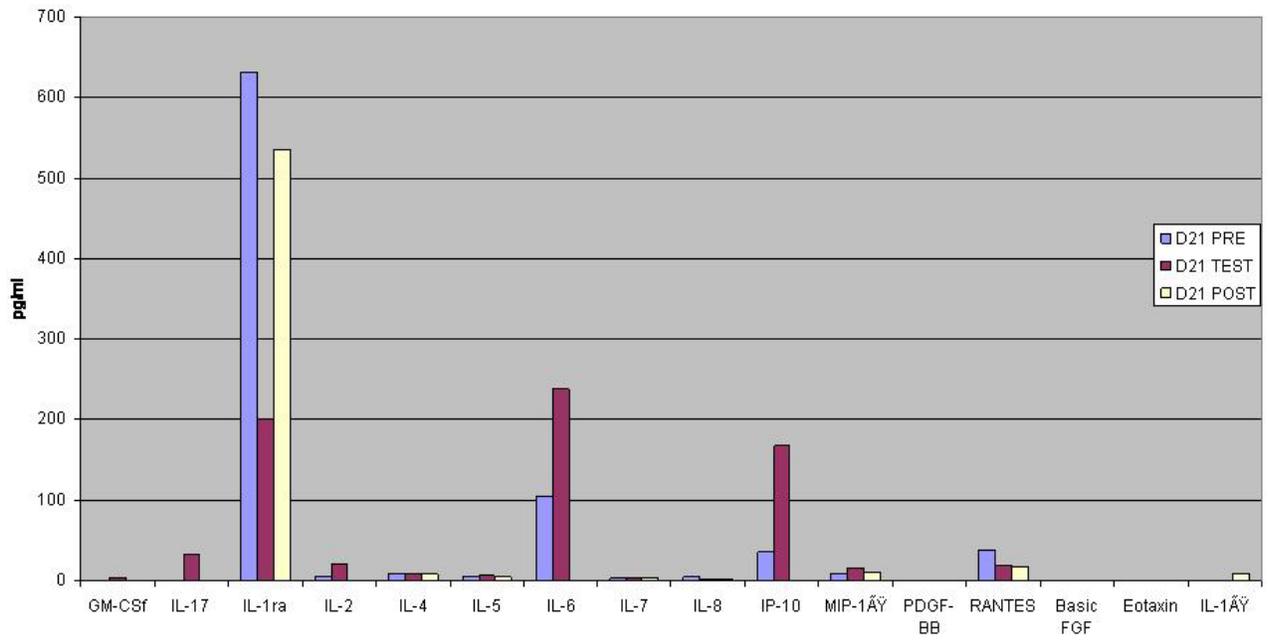
Appendix 22: Female D4 immune function responses pre, test and post exposure.



Appendix 23: Female D11 immune function responses pre, test and post exposure.



Appendix 24: Female D19 immune function responses pre and test exposure.



Appendix 25: Female D21 immune function responses pre, test and post exposure.