INVESTIGATION OF THE EFFECTS OF REPEATED CHASE AND ENCIRCLEMENT ON THE IMMUNE SYSTEM OF SPOTTED DOLPHINS (*STENELLA ATTENUATA*) IN THE EASTERN TROPICAL PACIFIC

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JULY 2002

Report completed under contract 40ABNF112369 for the
Southwest Fisheries Science Center
National Marine Fisheries Service, NOAA
8604 La Jolla Shores Drive
La Jolla, CA 92037

ADMINISTRATIVE REPORT LJ-02-35C

ABSTRACT

The immune system, which protects the body from potential infectious agents, is critical for health maintenance and survival. Stress has been shown to have effects on the immune system, and may ultimately increase susceptibility to disease. Therefore, as part of the Chase Encirclement Stress Studies (CHESS), the effects of repeated chase and encirclement on the immune system of dolphins (*Stenella attenuata*) in the Eastern Tropical Pacific (ETP) were investigated. Lymphocytes were isolated from dolphin peripheral blood and labeled with monoclonal antibodies to cell surface proteins to measure specific lymphocyte subsets. In addition, lymphocytes were cultured and incubated with T and B cell dependent mitogens to measure proliferation. DNA damage was also assessed using the Comet Assay to visualize DNA strand breakage.

Lymphocyte percentages and absolute cell numbers were obtained for T cells, B cells, T helper cells, and class II+ cells in the first time capture and encircled dolphin group (n=51) and the repeat capture group (n=10). There was a significant increase in T cell percentages and a significant decrease in B cell percentages ($p<0.05$) in the repeat capture group vs. the first time capture group. Males in the first time capture group had significantly higher absolute class II+, T cells, and T helper cells ($p<0.05$) than females in the first time capture group. Mature dolphins had higher percentages of T and T helper cells with significantly lower percentages of B cells than immature dolphins ($p<0.05$). Absolute lymphocyte, class II and B cell counts were significantly higher in immature vs. mature animals ($p<0.05$). There were no significant differences in lymphocyte proliferation or DNA damage between the first capture group vs. the repeat capture group of dolphins, although a decrease in B cell function was observed in those animals repeat captured on day 3 vs. day 1. Confounding variables present in this study make it difficult to draw definitive conclusions; however, it is possible that changes in the lymphocyte percentages observed or decreases in lymphocyte function observed after 3 days recapture, may increase susceptibility to disease. This is the first report of lymphocyte subsets for this species as well as the first study of the investigation of the effects of a stressor – repeated chase and encirclement on the cetacean immune system.

INTRODUCTION

The immune system is the body's major defense against invading microorganisms (i.e. viruses, bacteria, protozoa, fungi and multicellular parasites). An intact and functioning immune system is critical for health maintenance and survival. It is now generally accepted that various stimuli, such as stress, as "perceived" by the brain could in turn effect immunocompetence, and an organism's ability to fight off infectious agents that may compromise the immune system, and ultimately result in disease or mortality. Therefore, in addition to measuring blood parameters (e.g. serum chemistries, blood cell counts, catecholamines, stress and reproductive hormones) in dolphins sampled during Chase Encirclement Stress Studies (CHESS) (Forney et al., 2002; St. Aubin, 2002), measurements of the immune system were also carried out to determine the effects of repeated chase and encirclement on immune competence of dolphins in the Eastern Tropical Pacific (ETP).

Evidence from a variety of disciplines supports the bi-directional communication between the nervous and immune systems (see Felten et al., 1987; Felten et al., 1993; Ader et al., 1995; Madden and Felten, 1995; and Madden et al., 1995 for review). While most of this evidence has been shown in humans and rodents, we have anatomical and experimental evidence for communication between the immune and nervous system in cetaceans. Anatomically, postganglionic sympathetic nerve fibers innervate cellular lymphoid compartments in primary and secondary lymphoid organs in the beluga, (*Delphinapterus leucas*) forming close associations with cells of the immune system (Romano et al., 1994). Moreover, measurements of catecholamines by high performance liquid chromatography reveal norepinephrine as the most abundant catecholamine in beluga lymphoid organs (Romano et al., 1994). In addition, tyrosine hydroxylase-positive (the rate-limiting enzyme in norepinephrine synthesis) nerve terminals were observed in close apposition with lymphocytes in the spleen at the electron microscopic level (Romano et al., *in press)*.

Functionally, ligand binding studies have shown the presence of beta adrenergic receptors on cetacean peripheral blood lymphocytes. Preliminary experiments investigating the second messenger, cyclic AMP, revealed increases in cyclic AMP after incubation of cetacean lymphocytes with norepinephrine and isoproterenol (a beta adrenergic agonist), suggesting a receptor mediated effect of norepinephrine and isoproterenol on cetacean lymphocytes (Romano, 1993 doctoral dissertation; Romano et al., *in press*). In addition, functional aspects of neural-immune interactions were investigated in cetaceans by using the *in vitro* mitogen proliferation assay to investigate the effects of norepinephrine (a mixed agonist), isoproterenol (a beta adrenergic selective agonist), and/or Neuropeptide-Y on lymphocyte proliferation. A significant decrease in proliferation was observed with isoproterenol at 10^{-5} compared to ISO at 10^{-6}-10^{-11} following stimulation with the B cell dependent mitogen *Salmonella tiphimurium*.

Given the above evidence for communication of the nervous and immune system in cetaceans and the evidence for stress and its effects on immunocompetence in other species, we investigated the effects of repeated chase and encirclement on the immune system of dolphins in the ETP. Although the immune system of cetaceans is not as well characterized as that of human and mouse, it is increasingly becoming an important area of investigation given its importance for marine mammal health. One of the major obstacles in characterizing the cetacean immune system is the lack of cetacean-specific reagents and assays to assess immune function. Although we currently lack the resources to fully assess immune function in cetaceans, we have proceeded with the limited reagents and assays we do have available to aid in understanding the effects of repeated chase and encirclement on the immune system of dolphins in the ETP. It is important to keep in mind that the reagents and assays used in this study are relatively recent and are still currently being characterized and used to establish baseline immune parameters for cetaceans maintained in captivity where external variables can be controlled. We have attempted to answer the question of the effects of repeated chase and encirclement on the immune system of dolphins in the ETP within the limits of the samples obtained, field logistics, and the reagents and assays available for investigating the dolphin immune system.

An independent scientific peer review of this work was administered by the Center for Independent Experts located at the University of Miami. Responses to reviewer's comments can be found in the Appendix.

METHODS

Field methods are described in Forney et al. (2002) and St. Aubin et al. (2002). The effects of repeated chase and encirclement on the immune system of spotted dolphins (*Stenella* *attenuata*) in the ETP were investigated by studying the: 1). Percentages/numbers of lymphocyte subsets (including T helper cells, T cells, B cells, MHC class II + cells) from first time chase and encircled dolphins vs. repeat chased and encircled dolphins by lymphocyte immunophenotyping and flow cytometry. 2). Degree of lymphocyte proliferation from first time chase and encircled dolphins vs. repeat chased and encircled dolphins by the mitogen proliferation assay. 3). Degree of DNA damage in white blood cells from first time chase and encircled dolphins vs. repeat chased and encircled dolphins by the comet assay.

Lymphocyte Immunophenotyping

Sample Preparation: Cryopreserved white blood cells were thawed quickly in a 37 degree C water bath and washed 2x in complete RPMI 1640 containing 10% fetal bovine serum and 200 mM L-glutamine. Cells were counted on a hemocytometer, with trypan blue exclusion as a measure of viability (viability > 90%). The cells were then washed 2x in Hank's balanced salt solution (HBSS), pH 7.2 and resuspended to a final concentration of 1.0×10^6 cells/ml for indirect immunofluorescence labeling.

Indirect Immunofluorescence: Lymphocytes were labeled with 50 µl of the following hybridoma supernatants for 30 min. at 4 degrees C: Q5/13, a monoclonal antibody to human MHC class II molecules which cross-reacts with dolphin counterparts (Romano et al., 1992); a monoclonal antibody against cetacean CD2 to label T lymphocytes, and CD21 to label B lymphocytes (De Guise et al., 2002); or SIM4 a monoclonal antibody to human CD4 which cross-reacts with cetacean CD4 to label T helper lymphocytes (De Guise, 1998). Hybridoma supernatant from the myeloma cell line P2X63-AG8.653 was used as a negative control. The cells were washed 3x with HBSS before incubation with fluorescein (FITC)-conjugated affinity purified goat anti-mouse $F(ab)'_2$ IgG (Immunotech) for 30 min. at 4 degrees C in the dark. Cells were washed 2x in phosphate buffered saline and resuspended in 250 µl of 1% paraformaldehyde for subsequent flow cytometry analysis.

Analysis: Samples were analyzed on an LSR flow cytometer (Becton Dickinson). Forward/side scatter plots were obtained for each subject. Lymphocytes were gated based on their size and low degree of granularity. Ten thousand gated events (cells) were analyzed by histogram statistics. Percentages of each lymphocyte subset were obtained for each individual dolphin. Absolute numbers for each lymphocyte subset were calculated based on total lymphocyte numbers (St. Aubin, 2002) for each dolphin (% lymphocyte subset x total lymphocytes/100 = absolute cell counts for subset). Statistical analyses were performed using one-way analysis of variance.

Lymphocyte Proliferation

Sample Preparation: Cryopreserved white blood cells were thawed quickly in a 37 degree C water bath and washed twice in complete RPMI 1640 containing 10% fetal bovine serum and 200 mM L-glutamine-Pen-Strep. The white blood cells were resuspended in 10 ml of media and layered over 5 mls of Histopaque 1077 (Sigma) to isolate the lymphocytes. The cells were centrifuged at 400xg for 30 min. The lymphocyte layer was removed and washed twice in complete RPMI 1640. Cells were counted on a hemocytometer, with trypan blue exclusion as a measure of viability (viability > 90%).

Mitogen Assay: Cells were resuspended at a final concentration of 1×10^6 cells/ml in complete RPMI-1640 containing 10% fetal bovine serum, 200 mM L-glutamine, 100 units/ml Penicillin/Streptomycin, 0.01 M Hepes, 0.001 M sodium pyruvate, non-essential Amino Acids, and 0.05 M 2-mercaptoethanol. One hundred microliters of cells were plated per well in a 96-well flat bottom tissue culture plate. One hundred microliters of the T cell dependent mitogen, Concanavilin A (ConA) (Sigma) and/or the B cell mitogen, Lipopolysaccharide 026:B6 (LPS) (Sigma) or media alone were added to each well in triplicate. Preliminary experiments tested various concentrations of ConA and LPS ranging from 0.625 µg/ml to 160 µg/ml to obtain dose response curves and the optimal and suboptimal doses of each mitogen for proliferation. In subsequent experiments, the optimal and three suboptimal doses were used to evaluate the proliferation response to ConA, or the optimal and two suboptimal doses were used to evaluate the proliferation response to LPS for each dolphin. A 72 hr incubation period was used based on previous experiments conducted in our laboratory for the bottlenose dolphin (Romano, unpublished). To control for interassay variability previously frozen cells from a bottlenose dolphin were thawed and included in each experiment. Cells were incubated for 72 hrs at 37 degrees C with 5% CO_2. After 72 hrs in culture, each well was pulsed with 50 µl of 0.4 µCi/ml of tritiated thymidine (ICN). Cells were harvested 18 hrs later onto fiberglass filters using a cell harvester (Skatron, Instruments). Filters were transferred to scintillation vials and 2 mls of CytoScint (ICN) scintillation counting fluid was added to each vial.

Analysis: The uptake of tritiated thymidine was measured on a beta counter (Beckman) and expressed as counts per minute (cpm). To obtain the degree of proliferation, the stimulation index (SI) was obtained for each individual dolphin at each mitogen concentration by dividing the average of the triplicate cpm of each well containing mitogen by the triplicate average cpm of the control wells in which no mitogen was added. Statistical analyses were performed using one-way analysis of variance.

DNA Damage Assessment

Sample Preparation: DNA damage assessment of white blood cells was carried out in collaboration with Dr. Scott Steinert, Computer Sciences Corporation, San Diego using the comet assay. The samples collected for DNA damage were thawed and pelleted at 2000xg for 2 min. and resuspended in 250 µl of 0.65% low melting agarose in PBS at 30 degrees C.

Comet Assay: Cells were transferred onto slides coated with 100 µl of 0.65% agarose in 40 mM Tris-acetate, 1 mM EDTA, pH 7.5 and allowed to gel. Slides were placed in lysing solution containing 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, 1% Triton X-100, and 10% DMSO, pH 10.0 for 1 hr at 4 degrees C. Subsequently, slides were washed 3x5 min. with distilled water and placed in a submarine gel electrophoresis chamber filled with 300 mM NaOH, 1mM EDTA. After a 15 min. incubation, electrophoresis was carried out at 300 mA, 25 V for 10 min. Slides were neutralized with 3x2 min. rinses in 0.4 M Tris, placed in ethanol for 5 min., and dried at 37 degree C for 20 min.

Analysis: DNA was stained with ethidium bromide (40 µl of 20 µg/ml). Slides were analyzed at 200x with an epifluorescent microscope (excitation filter 510-560 nm green light, barrier filter 590 nm) with an attached CCD camera and image analysis software. The nucleus diameter and length of any trailing DNA "tails" resulting from strand breakage were measured for each nucleus analyzed. Measurements were made in five sectors on each slide, and 5-10 nuclei were randomly counted in each sector. Comet optical density, percent DNA in the tail, and the tail moment were calculated by the image system. Statistical analyses were performed using one-way analysis of variance.

RESULTS

Lymphocyte Immunophenotyping

Cetacean-specific and cross-reactive monoclonal antibodies were used to label cell surface proteins on lymphocytes from dolphins sampled during CHESS. Table 1. lists the antibodies used, the specific protein recognized by each antibody, and the lymphocyte cell subset identified by labeling each cell surface protein. Monoclonal antibodies specifically raised against human CD4 and/or bottlenose dolphin CD2 and CD21 recognize similar proteins on spotted dolphin peripheral blood lymphocytes. Figure 1a. shows the forward/side-scatter plot of white blood cells from one individual dolphin sampled during CHESS. Forward scatter, (a measure of cell size) and side scatter, (a measure of the cell's internal complexity) enables the identification of the lymphocyte population. The lymphocyte population was gated for further analysis with fluorochrome labeled antibodies. Histogram profiles of dolphin lymphocytes labeled with antibodies to cell surface markers are shown in Figure1b. Histogram statistics were obtained for each peak.

This is the first report of T, B, and T helper lymphocyte percentages/numbers, MHC class II expression, and T/B and T/T helper cell ratios in spotted dolphins. Table 2. shows the mean, standard deviation, and range of lymphocyte subset percentages/absolute values, as well as ratios of T/B lymphocytes and T/T helper lymphocytes for 46 first time chased and captured dolphins and 10 repeat captured animals (recaptured 1-3 days). The 10 repeat capture dolphins are not represented in the first time chased and captured group. T cell percentages showed a significant increase in the repeat captured dolphin group ($p<0.05$), with B cells showing a significant decrease ($p<0.05$).

Given the smaller sample size of the recapture dolphin group, the data was further examined by matching gender, length, and girth of the 10 repeat dolphins with 20 first capture dolphins (Table 3). After matching, the 20 first capture dolphins were randomly selected. T cells and B cells from repeat captured dolphins showed a similar significant increase and decrease from first time captures at $p<0.05$. In addition, the total lymphocyte percentage showed a significant decrease, and the T/B cell ratio significantly increased ($p<0.05$) from first time captured dolphins to repeat captured dolphins.

Lymphocyte subsets from dolphins in both the first time chase and capture group and the repeat chase and capture group were further examined. Lymphocyte subsets from dolphins in the first time chase and capture group were analyzed for gender and maturity differences. Table 4 shows the mean, standard deviation, and ranges for lymphocyte subsets for males (n=22) vs. females (n=24) in the first capture group. Males showed significantly higher T cell counts and T helper cell counts than females ($p<0.05$), as well as higher MHC class II+ cell counts.

Differences in lymphocyte subset percentages and cell counts for mature vs. immature dolphins from the first time capture group are shown in Figure 2. The percentage of T and T helper cells is significantly higher in mature dolphins ($p<0.05$), while B cell percentages are significantly lower ($p<0.05$). Absolute counts were significantly higher for lymphocytes, class

II+ cells, and B cells in immature animals ($p<0.05$). Figure 3 shows the differences between mature vs. immature male lymphocyte subset percentages and absolute counts and Figure 4 shows differences for mature vs. immature female lymphocyte subset percentages and absolute counts. Immature males have significantly higher B cell percentages than mature males ($p<0.05$). Immature females have higher lymphocyte, class II+, and B cell counts than mature females, and higher percentages of B cells, but lower percentages of T and T helper cells ($p<0.05$).

Repeat captured dolphins were broken down into 1 day, 2 days, and 3 days recapture. Table 5. shows the lymphocyte subset values for 1 day, 2 days, and 3 days recapture. Initial values for one of the 1 day recaptured dolphins and the 2 day recaptured dolphin are shown. To examine the effects on lymphocyte subsets after 1 day recapture alone, the six 1 day recaptured dolphins were compared with the 46 first time captured dolphins (Table 6). T cell percentages remained significant at $p<0.05$.

Ranges of T, B, and T helper cells for spotted dolphins after chase and capture were similar to the ranges obtained for healthy bottlenose dolphins at rest (Romano, *unpublished*). Table 7. lists the ranges of these cell subsets for the two species. Lymphocyte percentages for other species including humans (taken from Tizard, 1996) are also listed for comparison.

Lymphocyte Proliferation

The lymphocyte proliferation assay was used to assess immune function in first time chased and encircled dolphins and repeat captured animals. The T cell dependent mitogen, Concanavilin A (ConA) and the B cell dependent mitogen lipopolysaccharide (LPS) were used to stimulate proliferation of dolphin peripheral blood T and B lymphocytes. Dose response curves were obtained for ConA and LPS to determine optimal and suboptimal concentrations for stimulation. The degree of proliferation is expressed as the stimulation index (SI) which is the ratio of the amount of radioactivity incorporated into cells that were incubated with mitogen vs. cells incubated without mitogen. Stimulation indices were obtained for each mitogen at 3-4 concentrations (depending on the number of cells available) from the first time captured set of dolphins and the repeat captured group. Table 8. shows the mean, standard deviation, and ranges for the SIs of ConA at 0.625, 1.25, 2.5, and 5.0 μg/ml and LPS at 40, 80, and 160 μg/ml. No significant differences in proliferation were observed between the first and repeat captured animals.

For further analysis, 20 of the first time captured dolphins were matched by gender, length and girth, and randomly selected for comparison with the 10 recaptured animals. Figures 5 and 6 show plots of the SI at different concentrations of ConA and LPS for each dolphin in the 20 first time capture group vs. the repeat capture group. No significant differences were found in lymphocyte proliferation after matching.

The first time captured dolphin group was examined further for gender differences in lymphocyte proliferation. Table 9 lists the mean, standard deviation, and ranges of the SI for males ($n=18$) and females ($n=17$) after stimulation with ConA and LPS. No significant differences in lymphocyte proliferation were found between males and females in the first time capture group. Furthermore, no significant differences were found in lymphocyte function between mature vs. immature animals (data not shown).

The repeat captured group was further examined by breaking down the number of days for recapture. Blood samples were available from initial and repeat captures for dolphins, D34 and D67. Figure 7 graphically shows the proliferative responses of these two dolphins at intial and repeat capture. The SI for dolphins recaptured at 1 day, 2 days, or 3 days is shown graphically in Figure 8a,b,c for ConA and in Figure 9a,b for LPS. The SIs for first time capture dolphins were also compared with the 6 dolphins in the 1 day recapture group (Table 10). No significant differences were found for lymphocyte proliferation between these two groups.

DNA Damage Assessment

DNA damage was assessed in white blood cells by measuring the degree of DNA strand breakage. Strand breakage was measured by the comet assay in the laboratory of Dr. Scott Steinert, Computer Sciences Corporation, San Diego. Results are expressed as percent DNA in the comet tail or as the tail moment which is the percent DNA in the tail multiplied by the tail length divided by 100. Figure 10a,b illustrates the comet schematically and as viewed under the fluorescent microscope. The mean, standard deviation, and ranges for the tail moment and the percent DNA in the Comet are shown in Table 11 for the first capture group of dolphins (n=51) and the repeat captured dolphins (1-3 days) (n=10) as well as 1 day repeat captured dolphins (n=6). The comet values for 20 matched dolphin in the first capture group vs. the repeat captured dolphins, and males vs. females in the first time capture group are also shown in Table 11. Table 12 shows DNA damage assessments for the repeat capture group broken down into 1 day, 2 days, or 3 days of recapture. No significant differences were seen in any of the groupings for DNA damage.

DISCUSSION

The effects of stress on the immune system are well documented (Schorr and Arnason, 1999; Sgoutas-Emch, 1994; Kelley, 1980; Keller et al., 1991). Studies have shown, for example, various stressors to increase or decrease Natural Killer Cell function, primary and secondary antibody responses, delayed type hypersensitivity reactions, neutrophil function, lymphocyte subsets, and lymphocyte proliferation. The actual effects on the immune system depend on the type of stressor, its intensity and duration, as well as the individual's response to the stressor. While values for hormones such as cortisol and aldosterone have been documented for cetaceans after "stress" e.g. after capture and restraint (Ortiz and Worthy, 2000; St. Aubin and Geraci, 1989), the effects of stress on the cetacean immune system have not been measured. Studies are currently being conducted to answer this question in the bottlenose dolphin and beluga (Romano, *unpublished*). This is the first report of immune measurements in the spotted dolphin as well as the first study of the effects of a stressor- repeated chase and encirclement, on dolphin immune function.

Lymphocytes are the primary cells involved in the immune response. There are several different types of lymphocytes, but there are two major categories- T cells and B cells. The role of B cells is to produce antibody that will destroy extracellular pathogens and their products. T cells are divided into two major subsets-T helper cells ($CD4^+$), cells that help the immune response and cytotoxic T cells ($CD8^+$) cells, cells that destroy host cells infected with viruses or other intracellular pathogens. While there are reports of lymphocytes subsets in cetaceans, (Romano et al., 1992; De Guise et al., 1997; DeGuise et al., 1998) it is only recently that cetacean-specific monoclonal antibodies for major lymphocyte populations have become available (Romano et al., 1999; De Guise, 2002). Using these recently developed tools, lymphocyte subsets were measured in dolphins after repeated chase and encirclement. The subsets measured include MHC class II+ cells, CD2 + cells (T cells), CD21+ cells (B cells), and

T helper cells. Unfortunately, CD8 (a marker on cytotoxic T cells) and the CD4/CD8 ratio (a measurement often used to determine immune status) weren't able to be measured since there is no marker for dolphin CD8 currently available. While these markers were specifically raised against bottlenose dolphin cell surface proteins, they cross-react with similar proteins on spotted dolphin lymphocytes suggesting similar characteristics and conserved epitopes of these molecules.

The numbers and proportions of leukocytes in the blood reveal information on the distribution of these cells in the body and immune "status" (Dhabhar et al., 1995). The classic stress leukogram has been documented in cetaceans (leukocytosis, neutrophilia, eosinopenia, and lymphopenia) after capture (St. Aubin and Geraci, 1989) and after transport (Medway and Geraci, 1964). Peripheral blood lymphocytes of spotted dolphins showed significant changes in subset percentages between first time captures and repeat captures. Increases in T cell percentages and decreases in B cell percentages were observed in the repeat capture group. Lymphocyte percentages from these subsets remained significant after matching 20 dolphins in the first time capture group with dolphins from the repeat capture group, with the addition of a significant decrease in lymphocyte percentages and an increase in the T/B cell ratio. Increases in the T/B cell ratio are consistent with an in increase in T cells and a decrease in B cells. These changes in lymphocyte percentages may have significant consequences for the ability of the immune system to respond to potential or ongoing challenges (Dhabhar et al., 1995).

However, the results need to be interpreted with caution, since dolphins in the repeat capture group were not represented in the first time group, and the number of total lymphocytes as well as numbers of lymphocytes in each subset is highly variable from individual to individual. Moreover, the physiological response to repeat chase and encirclement will vary from individual to individual. Some animals may have become conditioned or habituated to repeat chase and capture as well. The lack of significance for changes in absolute numbers could reflect this high degree of variability and the small sample size of the repeat capture group. It's interesting to note that the ranges of T cells, B cells, and CD4+ T cell percentages for first time chased and encircled dolphins are similar to normal ranges for healthy bottlenose dolphins at rest (Romano *unpublished*) (refer to Table7). However, percentages obtained for first time chased and encircled dolphins are not considered "baseline" for this population and so comparisons are difficult to make.

It has been reported that exercise, as a stressor, initiates an increase in lymphocytes with more profound effects on T cells than B cells (Simon, 1991). There are reports of a decrease in the T helper/T suppressor ratio due to an increase of CD8+ T cells (Lewicki et al., 1988; Berk et al., 1986). Hoffman-Goetz and Pedersen (1994) report consistent patterns of lymphocyte reactivity with increases in total lymphocytes, T and B cells, and a decrease in the CD4/CD8 ratio. These changes return to normal levels as early as 15 min. to 2 hrs after exercise ceases (Robertson et al., 1981). However, the stress of long-term physical exercise such as running a marathon brought about increased leukocyte counts, and an increase in T cells two days after the run, and a significant decrease in B cells (Gmunder et al., 1988). Moreover, restraint stress induced rapid changes in all leukocyte populations examined with B cells showing a greater stress-induced decrease than T cells (Dhabhar et al., 1995).

There are also reports in the stress literature of T cells, T helper cells, and B cells increasing or decreasing depending on the type of stressor, its duration, and intensity (Gmunder et al., 1988; Landmann et al., 1984; Sgoutas-Emch et al., 1994; Dhabhar et al., 1995). Baseline measurements as well as levels after exposure to different stressors, intensities, and duration need to be established for cetaceans. This study has provided lymphocyte subset percentages for spotted dolphins after first time chase and encirclement as well as repeat chase and encirclement. However, the changes observed should be interpreted with caution. Baseline levels or first time capture samples from the same repeat chased and captured dolphins are needed before any definitive conclusions can be made. Moreover, the previous chase and capture history of the "first time capture" group is unknown. Current studies in our laboratory are carrying out similar measurements of immune function on bottlenose dolphins before and after various stressors. In this situation, we will be able to obtain a blood sample before exposure to the stressor to determine effects on dolphin health and the immune system.

MHC class II molecules were investigated since they indicate T cell activation in some species, and have unique expression on bottlenose dolphin peripheral blood lymphocytes (Romano et al., 1992). MHC class II molecules are generally expressed on B cells and antigen presenting cells. Human T cells generally do not express MHC class II but become MHC class II+ when activated (Kaufman et al., 1984). In the bottlenose dolphin however, MHC class II is constitutively expressed on dolphin T cells, as it is in some ungulates and carnivores (Romano et al., 1992). Previously, measurements of MHC class II have only been carried out in dolphins maintained in captivity. Measuring MHC class II expression in dolphins in the ETP further confirms the constitutive expression of MHC class II molecules on dolphin T lymphocytes. In fact, the ranges for MHC class II expression for dolphins in the ETP (42-83%) closely resembled the ranges of semi-domesticated bottlenose dolphin (53-95%). It's important to keep in mind however, that the MHC class II percentages for *Stenella* were after chase and encirclement. Baseline values for *Stenella* are not available for comparison, and so we cannot conclude if 42-83% MHC class II expression is what is normally expressed or if these percentages were the result of chase and capture. It is interesting to note that MHC class II+ cells were significantly higher in males from the first capture group as well as T cells and T helper cells. Significant differences have not been found between males and females in these lymphocyte percentages in resting healthy bottlenose dolphin (Romano, *unpublished*). These differences may reflect differences in hormonal basal levels or differences as a result of the chase and encirclement. Morever, changes were seen between mature and immature dolphins reflecting possible influences of age on the immune system.

The ability of lymphocytes to proliferate in response to antigen is integral for a functioning immune system. The *in vitro* lymphocyte proliferation assay measures the ability of lymphocytes to proliferate in response to mitogens and is often used as a measure of immune function. This assay is well documented for cetaceans (De Guise et al., 1996; Mumford et al., 1975; Colgrove, 1978; Romano, 1993) and has been used to understand the effects of PCBs, DDT, and heavy metals on lymphocyte function *in vitro* (Lahvis et al., 1995; De Guise et al., 1996). The mitogen proliferation assay has also been used to assess the effects of stress on immune function in other species (Keller et al., 1991; Minton and Blecha, 1990; Weisse et al., 1990). Measures of decreased proliferative responses have been measured after stress, but as with lymphocyte subsets, decreases or increases in proliferation depend on the type of stressor, its intensity and duration.

Exercise itself can effect lymphocyte function. The proliferation response was reported to be suppressed immediately after exercise (Oshida et al., 1988) but returned to normal levels within 24 hours. Hoffman-Goetz and Pedersen (1994) report reponses to ConA decrease but responses to LPS increase after strenuous exercise. However, Wong et al., (1992) report no change in lymphocyte responses after intense exercise in horses. In this study, we saw no significant changes in proliferation responses between the first capture group of dolphins compared to the repeat capture group. This remained to be the case when we tried to control for age and gender by matching dolphins in the first capture group with dolphins in the repeat capture group. Due to the nature of the immune response, the variation in proliferative responses from one individual to the next is quite high. Differences may be seen if the same individuals were in the first capture group and the repeat capture group. Only two dolphins that were in the first time capture group were also represented in the repeat capture group. No striking differences were seen in D34 from initial to repeat capture in lymphocyte proliferation responses to ConA except at 5 ug/ml. The stimulation index was much lower at this concentration upon repeat capture. A slight decrease was observed in the stimulation index for D67 after repeat capture. However, other concentrations were not carried out due to lack of cells.

In terms of mitogenic responses, spotted dolphins respond to the T cell dependent mitogen, ConA and the B cell dependent mitogen, LPS (although minimally). Mitogens that showed responses in bottlenose dolphin (Romano, unpublished) and other cetacean species (De Guise, et al., 1996) were used for spotted dolphins. If additional samples were available, a more comprehensive study could be carried out to determine the best mitogen to use for B cell proliferation in spotted dolphins. For the most part however, spotted dolphin lymphocytes gave similar proliferative responses as described for other cetacean species.

Although no significant differences were observed in lymphocyte proliferation between the first capture group of dolphins and the repeat captured animals, these observations may not reflect actual effects since the repeat capture group had a small number of individuals. Moreover, it should be kept in mind that this measurement is highly variable not only between different individuals but the response may be variable for one individual from one point in time to the next. However, one interesting trend is the apparent decrease in B cell function in the repeat capture group on day three compared to the animals recaptured on day one, suggesting that changes in immune function may not be observed until days or weeks after the initial stressor.

The maintenance of DNA integrity is critical to all living organisms; therefore, efficient and intricate mechanisms exist to protect genetic material. Significant stresses (oxidative stress, environmental stressors) eventually result in increases in observable cellular DNA damage. The comet assay provides information on DNA damage in individual cells. The degree of strand breakage and the frequency and types of cells with significant levels of DNA damage is indicative of the severity of the stress (see Steinert et al., 1998 for details). The comet assay has been used to study oxidative stress including that associated with exercise as well as the genotoxic effects of pollutants on DNA integrity at a cellular level (Moller et al., 2001; Valverde et al., 2001). In dolphins, the comet assay has been used to study the genotoxic effects of Methyl-Mercury and PCB's on DNA integrity in peripheral blood lymphocytes in the bottlenose dolphin (Taddei et al., 2001; Betti and Nigro, 1996).

Through collaborative efforts with Dr. Scott Steinert, Computer Sciences Corporation, studies have been initiated to evaluate DNA integrity in white blood cells of the dolphin. Baseline measures are currently being carried out in bottlenose dolphins and will be followed by sampling after various "stressors". Dolphins in the ETP were evaluated for DNA damage or strand breakage in white blood cells after repeated chase and capture. No significant differences were seen in matched or unmatched first time chase and captured dolphins vs. repeat capture dolphins. Measurements of DNA damage for dolphins in the ETP were in the range of levels obtained for normal healthy bottlenose dolphin at rest. The time course of DNA damage after stress needs to be determined in dolphins. It could be that the time point for seeing damage occurs later than the time of sampling for dolphins in the ETP. Perhaps DNA damage is not induced in this cetacean species (or cetaceans in general) at high enough levels for detection. Further validation of this assay as it relates to cetaceans needs to be carried out. It would be interesting to specifically look at immunoreacitivity of markers for oxidative stress in the skin of both live biopsied and necropsied dolphins (Dizon et al., 2002; Southern et al., 2002) and compare with results from the comet assay and/or specifically look at the same markers for oxidative stress by immunohistochemistry on dolphin white blood cell smears.

In summary, the immune data from CHESS reveal some changes in lymphocyte subset percentages from the repeated chase and capture group of dolphins with no change in immune function. The changes observed in lymphocyte subset percentages in the ETP dolphins are similar to other studies that have investigated stress effects on the immune system (Gmunder et al., 1988; Dhabhar et al., 1995). It's important to keep in mind that we are only looking at a short period of time after repeated chase and capture. More dramatic changes in immune function may be observed days or weeks after the initial stress. Also, deleterious effects may be observed on the immune system if the stressor was to persist and the animals were chased and encircled repeatedly. If perturbations of the immune system were to persist, it is likely that susceptibility to infectious agents would increase. We are also not comparing the same individual animals from the first time capture group to the repeat capture group. If this were possible, there may be more significant differences observed in immune parameters. Also, habituation and conditioning to repeat chase and encirclement is a confounding variable that needs to be considered. Gender and age may also influence responses to chase and encirclement. Moreover, some of the significant differences observed may be due to species-specific differences and more related to exposure of the dolphins to microorganisms in their environment.

During this study, we gained basic information on the immune system of a cetacean species that we knew nothing about, as well as preliminary results as to the effects of repeated chase and encirclement on the immune system. In addition to the limited measurements we have made on immune function for this species, archived serum samples from these animals will enable the quantification of immunoglobulin classes as well as antibody titers to various infectious agents, such as brucella, erysipelas and morbillivirus. Archived RNA samples will be evaluated to investigate cytokine expression levels. Most importantly, immunologic data where appropriate will be compared and correlated with studies of CHESS to help in determining the overall health and population effects of dolphins that are repeatedly chased and captured in the Eastern Tropical Pacific.

ACKNOWLEDGMENTS

The authors thank the CHESS team for their dedicated and cooperative efforts in obtaining blood samples from dolphins in the Eastern Tropical Pacific, as well for the data analyses that are pertinent to this study. The authors especially thank Drs. David St. Aubin (Mystic Aquarium), Roger Geertsema (U.S. Navy Marine Mammal Program), and Karin Forney (Southwest Fisheries Science Center). We thank Dr. Jeffrey Stott (UC Davis) for the CD2 and CD21 antibodies, Dawn Crover (UCSD) for technical assistance, and Drs. Stephen Reilly and Robert Brownwell (Southwest Fisheries Science Center). This work was supported by NMFS (contract # 40-AB-NF-108447) and the Office of Naval Research (grant # N00014-00-1-0041).

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Table 1. Antibodies used to label dolphin lymphocyte subsets.

Antibody	*Cell Surface Protein*	*Lymphocyte Subset*	**Reference:**
F21F.4 (Cetacean)	CD21	B Cell	De Guise et al., 2002
F21C.2 (Cetacean)	CD2	T Cell	De Guise et al., 2002
SIM 4 (Human)	CD4	T Helper Cell	De Guise et al., 1997
Q5/13 (Human)	MHC Class II	Class II+	Romano et al., 1992

Table 2. Lymphocyte subset percentages and absolute values after first capture and recapture (1-3 days). Recaptured dolphins are not represented in the first capture data set.

		First Capture				**Recapture (1-3 days)**				
Cell Type	**units**	**n**	**Mean**	SD	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
Class II + Cells	%	46	89.59	± 9.72	41.49 - 97.43	10	92.22	± 4.05	92.07 - 97.60	ns
T Cells	%	46	66.55	± 9.15	42.24 - 82.86	10	74.13	± 6.19	64.75 - 83.09	p<0.05
T Helper Cells	%	46	39.63	± 10.00	20.85 - 58.47	10	44.67	± 6.02	35.00 - 53.09	ns
B Cells	%	46	19.31	± 9.58	3.23 - 46.85	10	12.64	± 3.18	7.90 - 16.76	p<0.05
Total Lymphs	%	46	23.77	± 9.68	3.00 - 42.00	10	18.50	± 5.68	13.00 - 32.00	ns
Class II + Cells	cells/mm^3	46	2206	± 1274	200 - 4781	10	1600	± 311	1084 - 2123	ns
T Cells	cells/mm^3	46	1554	± 734	176 - 4004	10	1280	± 229	902 - 1596	ns
T Helper Cells	cells/mm^3	46	900	± 396	125 - 2157	10	772	± 171	552 - 1024	ns
B Cells	cells/mm^3	46	557	± 583	7.0 - 2935	10	221	± 85	123 - 390	ns
Total Lymphs	cells/mm^3	46	2430	± 1331	213 - 7440	10	1737	± 350	1255 - 2410	ns
T/T Helper Ratio		46	1.7	± 0.2	1.4 - 2.1	10	1.7	± 0.2	1.4 - 2.0	ns
T/B Ratio		46	4.8	± 4.0	0.9 - 25.6	10	6.3	± 2.2	4.1 - 10.2	ns

Table 3. Lymphocyte subset percentages and absolute values of the 10 recaptured dolphins matched with 20 first capture dolphins of similar gender, length and girth.

		First Capture				Recapture (1-3 days)				
Cell Type	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	Range	**p**
Class II + Cells	%	20	91.48	± 5.13	75.60 - 96.48	10	92.22	± 4.05	92.07 - 97.60	ns
T Cells	%	20	66.87	± 8.42	49.91 - 78.48	10	74.13	± 6.19	64.75 - 83.09	$p<0.05$
T Helper Cells	%	20	40.24	± 8.56	24.49 - 55.33	10	44.67	± 6.02	35.00 - 53.09	ns
B Cells	%	20	18.93	± 8.35	8.79 - 39.45	10	12.64	± 3.18	7.90 - 16.76	$p<0.05$
Total Lymphs	%	20	24.73	± 8.36	13.00 - 40.00	10	18.50	± 5.68	13.00 - 32.00	$p<0.05$
Class II + Cells	cells/mm^3	20	2330	± 1409	879 - 6960	10	1600	± 311	1084 - 2123	ns
T Cells	cells/mm^3	20	1635	± 813	704 - 4004	10	1280	± 229	902 - 1596	ns
T Helper Cells	cells/mm^3	20	951	± 412	486 - 2157	10	772	± 171	552 - 1024	ns
B Cells	cells/mm^3	20	563	± 649	123 - 2935	10	221	± 85	123 - 390	ns
Total Lymphs	cells/mm^3	20	2520	± 1469	1089 - 7440	10	1737	± 350	1255 - 2410	ns
T/T Helper Ratio		20	1.7	± 0.2	1.3 - 2.1	10	1.7	± 0.2	1.4 - 2.0	ns
T/B Ratio		20	4.3	± 2.0	1.3 - 8.7	10	6.3	± 2.2	4.1 -10.2	$p<0.05$

Table 4. Lymphocyte subset percentages and absolute values of males and females from the first capture group of dolphins.

		First Capture									
		Males				Females					
Cell Type	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
Class II + Cells	%	22	91.77	± 4.66	75.60 - 97.01	24	87.60	± 12.50	41.49 - 97.34	ns
T Cells	%	22	66.26	± 9.38	48.83 - 78.48	24	66.81	± 10.70	42.50 - 82.86	ns
T Helper Cells	%	22	38.64	± 8.90	23.75 - 55.33	24	40.54	± 9.46	20.85 - 58.47	ns
B Cells	%	22	20.61	± 9.92	7.93 - 39.50	24	18.11	± 9.30	5.80 - 46.85	ns
Total Lymphs	%	22	25.71	± 8.65	9.00 - 42.00	24	22.00	± 10.40	3.00 - 42.00	ns
Class II + Cells	cells/mm^3	22	2589	± 1367	675 - 4428	24	1855	± 1095	200 - 4781	$p<0.05$
T Cells	cells/mm^3	22	1788	± 777	579 - 4004	24	1340	± 633	176 - 2048	$p<0.05$
T Helper Cells	cells/mm^3	22	1029	± 428	318 - 2157	24	783	± 330	125 - 1433	$p<0.05$
B Cells	cells/mm^3	22	672	± 659	64 - 2935	24	451	± 496	6.9 - 2361	ns
Total Lymphs	cells/mm^3	22	2795	± 1428	801 - 7440	24	2096	± 1167	213 - 5040	ns
T/T Helper Ratio		22	1.7	± 0.2	1.4 - 2.1	24	1.7	± 0.2	1.3 - 2.0	ns
T/B Ratio		22	4.2	± 2.4	1.2 - 7.4	24	5.3	± 5.0	0.9 - 25.6	ns

Table 5. Lymphocyte subset percentages and absolute values for dolphins recaptured at 1, 2, or 3 days.
* a (-) indicates no data available.

		Lymphocyte Subsets (%)					Lymphocyte Subsets (cell counts)						
	ID	**Class II+**	**T Cells**	**T Helper**	**B Cells**	**Lymphs**	**Lymphs**	**Class II+**	**T Cells**	**T Helper**	**B Cells**	**T/B**	**T/T Helper**
1 day	**67- initial**	90.36	67.66	37.48	19.81	19.00	1615	1459	1093	605	320	3.4	1.8
Recapture	**67.512**	94.78	75.32	46.64	11.74	18.00	1458	1382	1098	680	171	6.4	1.6
	193.508	95.67	75.35	38.81	11.72	23.01	1714	1640	1291	665	201	6.4	1.9
	203.504	94.59	75.53	50.84	16.76	19.00	1349	1276	1019	686	226	4.5	1.5
	209.510	94.41	69.61	48.25	15.24	32.00	1984	1873	1381	957	302	4.6	1.4
	215.509	86.38	71.91	43.96	12.96	13.01	1255	1084	902	552	163	5.6	1.6
	242.514	97.60	82.92	49.52	8.15	15.00	1875	1830	1555	929	153	10.2	1.7
2 days	**34-initial**	94.77	74.65	46.83	16.06	14.00	1806	1712	1348	846	290	4.6	1.6
Recapture	**34.501**	88.09	66.22	42.50	16.17	20.00	2410	2123	1596	1024	390	4.1	1.56
3 days	**244.515**	86.04	64.75	35.00	13.10	13.00	2009	1729	1301	703	263	4.9	1.85
Recapture	**245.517**	92.60	83.09	53.09	-	16.00	1760	1630	1462	934	-	-	1.6
	257.516	92.07	76.62	38.04	7.90	16.00	1552	1429	1189	590	123	9.7	2.1

Table 6. Lymphocyte subsets (percentages and absolute values) for dolphins recaptured 1 day vs. 1st time capture.

		Lymphocyte Subsets (%)					Lymphocyte Subsets (cell counts)						
	ID	**Class II+**	**T Cells**	**T Helper**	**B Cells**	**Lymphs**	**Class II+**	**T Cells**	**T Helper**	**B Cells**	**T/B**	**T/T Helper**	**Lymphs**
1 day Recapture	**67.512**	94.78	75.32	46.64	11.74	18.00	1382	1098	680	171	6.4	1.6	1458
	193.508	95.67	75.35	38.81	11.72	23.01	1640	1291	665	201	6.4	1.9	1714
	203.504	94.59	75.53	50.84	16.76	19.00	1276	1019	686	226	4.5	1.5	1349
	209.510	94.41	69.61	48.25	15.24	32.00	1873	1381	957	302	4.6	1.4	1984
	215.509	86.38	71.91	43.96	12.96	13.01	1084	902	552	163	5.5	1.6	1255
	242.514	97.60	82.92	49.52	8.15	15.00	1830	1555	929	153	10.2	1.7	1875
	AVG	93.91	75.11	46.34	12.76	20.00	1514	1208	745	203	6.3	1.6	1606
	SD	3.87	4.51	4.39	3.02	6.81	317	244	161	56	2.1	0.2	296
	Range	86.40-97.60	69.61-82.92	38.81-50.84	8.15-16.76	13.01-32.00	1084-1873	902-1555	552-957	153-302	4.5-10.2	1.4-1.9	1255-1984
1st time	**AVG**	89.59	66.55	39.63	19.31	23.77	2206	1554	900	557	4.8	1.7	2430
Capture	**SD**	0.72	9.15	10.00	9.58	9.68	1274	734	396	583	4.0	0.2	1317
N=46	**Range**	41.49-97.43	42.24-82.86	20.85-58.47	3.23-46.85	3.00-42.00	200-4781	176-4004	125-2157	7-2935	0.9-25.6	1.4-2.1	213-7440
	p	ns	P<0.05	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 7. Lymphocyte subset percentages in peripheral blood reported for other species (taken from Tizard, 1996). Values for Bottlenose and Spotted dolphins are listed for comparison.

	% T Cells	**% B Cells**	**% CD4+**	**% CD8+**	**CD4/CD8**
Bovine	45 - 53	16 - 21	8 - 31	10 - 30	1.53
Sheep	46 - 64	11 - 50	8 - 22	4 - 22	1.55
Pigs	45 - 57	13 - 38	23 - 43	17 - 39	1.4
Horses	38 - 66	17 - 38	56	20 - 37	4.75
Dogs	46 - 72	7 - 30	27 - 33	17 - 18	1.7
Cats	31 - 89	6 - 50	19 - 49	6 - 39	1.9
Human	70 - 75	10 - 15	43 - 48	22 - 24	1.9 - 2.4
Dolphin (bottlenose)	53 - 95	5 - 42	18 - 56	-	-
Dolphin (spotted) (after chase and encirclement)	42 - 83	3 - 47	21 - 58	-	-

*a (-) indicates no data available

Table 8. Lymphocyte proliferation expressed as the stimulation index (SI) for the concentrations indicated of ConA and LPS for first capture and recapture (1-3 days) dolphins. Recapture dolphins are not represented in the 1st capture data set.

		First Capture				**Recapture (1-3 days)**				
ConA	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
0.625 mg/ml	SI	35	287	$\pm$217	49 - 900	10	377	$\pm$266	37 - 808	ns
1.25 mg/ml	SI	35	365	$\pm$211	95 - 823	10	414	$\pm$238	58 - 727	ns
2.5 mg/ml	SI	33	403	$\pm$223	86 - 804	9	479	$\pm$237	70 - 791	ns
5.0 mg/ml	SI	24	383	$\pm$188	112 - 818	8	407	$\pm$220	71 - 733	ns

		First Capture				**Recapture (1-3 days)**				
LPS 026:B6	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
40 mg/ml	SI	21	3.82	$\pm$2.20	1.00 - 9.10	6	3.01	$\pm$1.50	1.30 - 5.52	ns
80 mg/ml	SI	25	3.80	$\pm$3.05	0.62 - 12.69	6	4.00	$\pm$2.67	1.02 - 7.72	ns
160 mg/ ml	SI	26	2.93	$\pm$2.76	0.63 - 13.11	7	2.74	$\pm$2.47	0.74 - 7.90	ns

Table 9. Lymphocyte proliferation expressed as the stimulation index (SI) for the concentrations indicated of ConA and LPS for males and females from the first capture set of dolphins.

First Capture

		Males				Females				
ConA	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
0.625 µg/ml	SI	18	311	±240	68 - 900	17	261	±193	49 - 559	ns
1.25 µg/ml	SI	18	383	±230	103 - 823	17	346	±193	95 - 659	ns
2.5 µg/ml	SI	18	411	±241	130 - 804	17	393	±207	98 - 735	ns
5.0 µg/ml	SI	13	427	±204	112 - 719	11	331	±161	115 - 631	ns
LPS 026:B6	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
40 µg/ml	SI	12	4.18	±2.30	1.40 - 9.09	9	3.36	±2.09	0.99 - 6.91	ns
80 µg/ml	SI	15	4.39	±3.65	0.62 - 12.68	10	2.90	±1.61	0.99 - 5.01	ns
160 µg/ ml	SI	15	3.65	±3.39	0.62 - 13.11	11	1.95	±1.07	0.79 - 4.37	ns

Table 10. Lymphocyte proliferation expressed as the stimulation index (SI) for the concentration indicated of ConA and LPS for dolphins recaptured 1 day vs. first time captures.

		First Capture				Recapture (1 day)				
ConA	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
0.625 µg/ml	SI	35	287	±217	49 - 900	6	357	±197	176 - 715	ns
1.25 µg/ml	SI	35	365	±211	95 - 823	6	411	±196	161 - 716	ns
2.5 µg/ml	SI	33	403	±223	86 - 804	5	500	±178	266 - 756	ns
5.0 µg/ml	SI	24	383	±188	112 - 818	5	454	±197	220 - 734	ns
		First Capture				**Recapture (1 day)**				
LPS 026:B6	**units**	**n**	**Mean**	**SD**	**Range**	**n**	**Mean**	**SD**	**Range**	**p**
40 µg/ml	SI	21	3.82	±2.20	1.00 - 9.10	4	3.79	±1.16	3.05 - 5.51	ns
80 µg/ml	SI	25	3.80	±3.05	0.62 - 12.69	4	5.37	±2.09	3.15 - 7.72	ns
160 µg/ ml	SI	26	2.93	±2.76	0.63 - 13.11	4	3.90	±2.82	1.49 - 7.90	ns

Table 11. DNA damage assessment expressed as Tail Moment and % DNA in comet tail from first capture and recapture dolphins(1-3 days). Male and female values are shown for first time capture animals and for first time captures vs. 1 day repeat values. Recaptured dolphins are not represented in the first capture data set.

	First Capture				Recapture (1-3 days)				
	Mean	SD	Range	n	Mean	SD	Range	n	p
Tail Moment	0.58	+0.31	0.1 - 1.6	51	0.42	+0.23	0.2 - 1.0	10	ns
% DNA in Comet	3.90	+1.10	2.0 - 7.4	51	3.74	+1.04	2.5 - 5.6	10	ns

	Recapture (1-3 days)				Recapture (1day)				
	Mean	SD	Range	n	Mean	SD	Range	n	p
Tail Moment	0.42	+0.23	0.2 - 1.0	10	0.35	+0.14	0.2 - 0.5	6	ns
% DNA in Comet	3.74	+1.04	2.5 - 5.6	10	3.67	+ 1.04	2.5 - 5.3	6	ns

	Males				**Females**				
	Mean	SD	Range	n	Mean	SD	Range	n	p
Tail Moment	0.58	+0.31	0.2 - 1.6	24	0.57	+0.32	0.1 - 1.6	27	ns
% DNA in Comet	3.80	+1.04	2.5 - 7.4	24	3.99	+1.16	2.0 - 5.8	27	ns

Table 12. DNA damage expressed as Tail Moment and % DNA in comet tail from repeat captures (1-3 days).

	ID	**TM**	**SEM**	**%DNA in comet**	**SEM**
1 day	**67-initial**	0.3	0.1	3.7	1.3
Recapture	**67.512**	0.3	0.1	3.0	0.8
	193.508	0.2	0.1	3.2	0.8
	203.504	0.5	0.3	4.5	1.4
	209.510	0.5	0.4	3.5	1.4
	215.509	0.2	0.1	2.5	0.7
	242.514	0.4	0.2	5.3	1.3
2 days	**34-initial**	0.6	0.2	4.9	1.3
Recapture	**34.501**	0.3	0.2	2.9	1.2
3 days	**244.515**	0.3	0.1	3.3	1.0
Recapture	**245.517**	0.5	0.2	3.6	1.0
	257.516	1.0	0.4	5.6	1.6

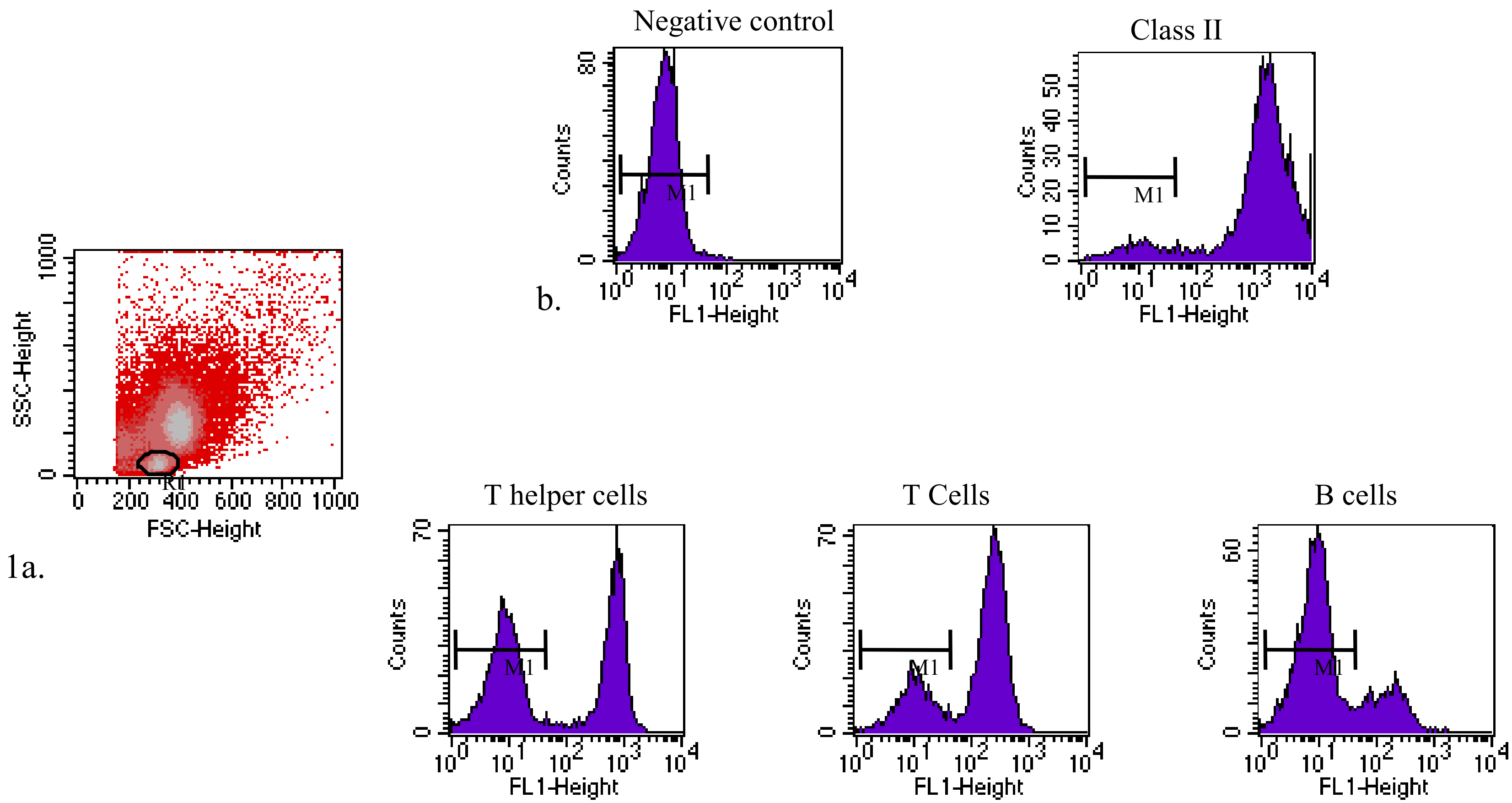

Figure 1a. Forward scatter (cell size) vs. Side scatter (cell complexity) plot of white blood cells from one individual dolphin (D34) sampled in the ETP. Lymphocytes are gated (circle) based on their small size and low degree of complexity. 1b. Histograms showing flow cytometry analysis of dolphin (D34) lymphocytes. Cell number is indicated on the vertical axis vs. a log scale of fluorescence on the horizontal axis. The marker M1 indicates the negative population of cells that did not label with antibody. The cell population recognized is indicated at the top of each histogram. Histogram statistics reveal the percentage of positively stained cells for each lymphocyte subset labeled.

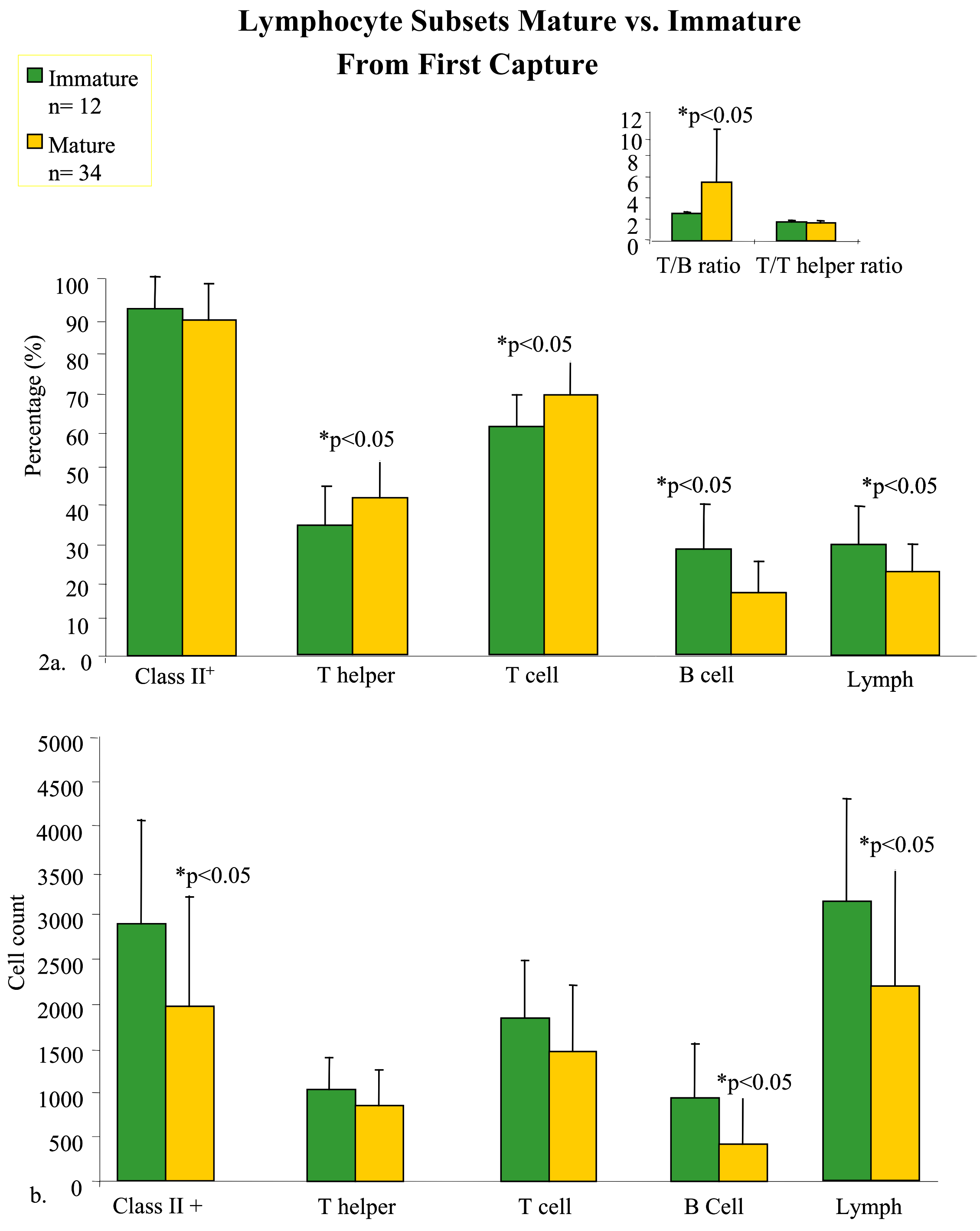

Figure 2a and b. Lymphocyte subset percentages (a) and absolute counts (b) of Mature vs. Immature dolphins from the first time capture group. Significant differences are indicated between mature vs. immature dolphins with an (*) at $p<0.05$.

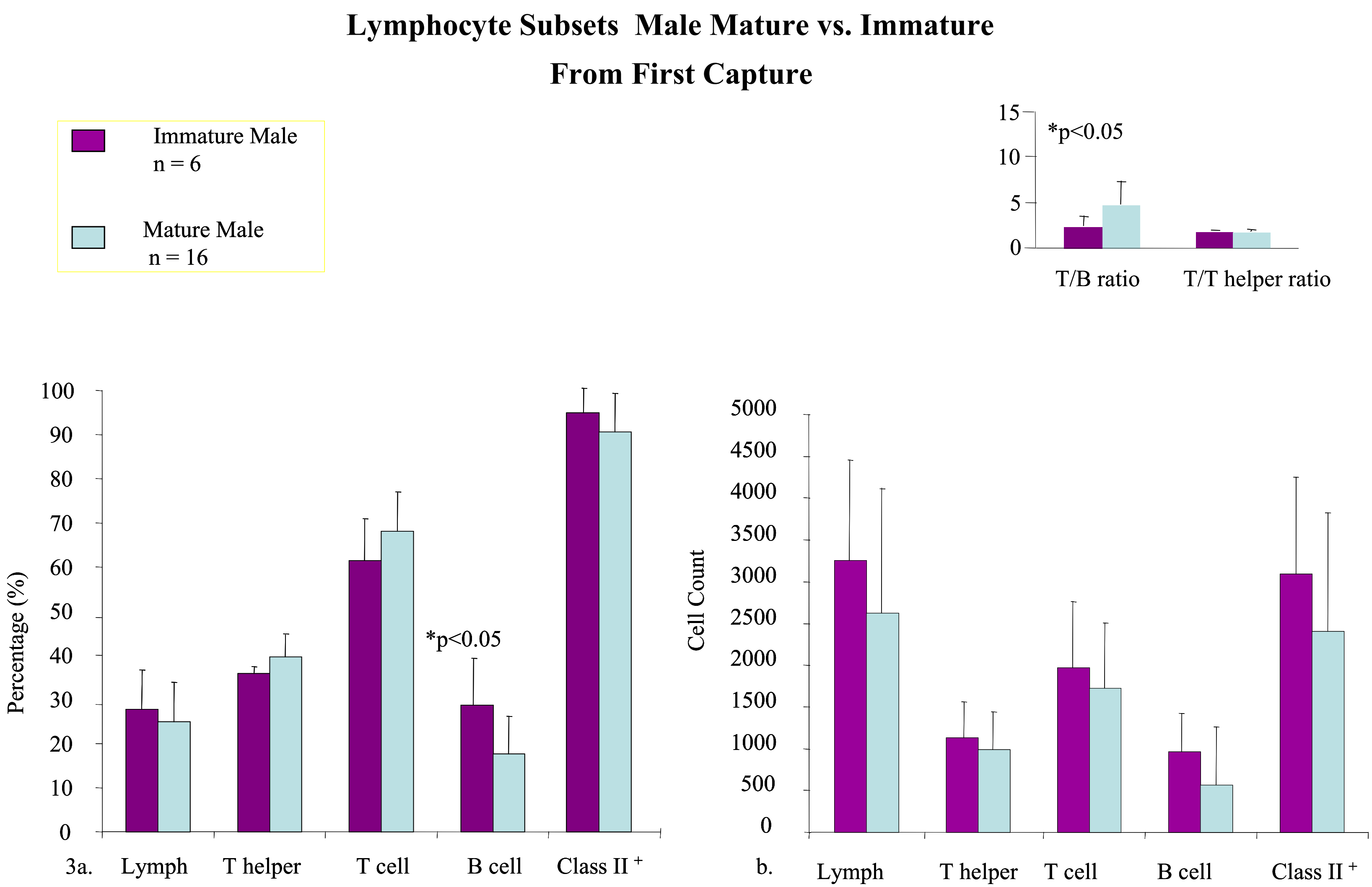

Figure 3a and b. Lymphocyte subset percentages (a) and absolute counts (b) of Mature males vs. Immature males from the first time capture group. Significant differences are indicated between mature vs. immature male dolphins with an (*) at $p<0.05$.

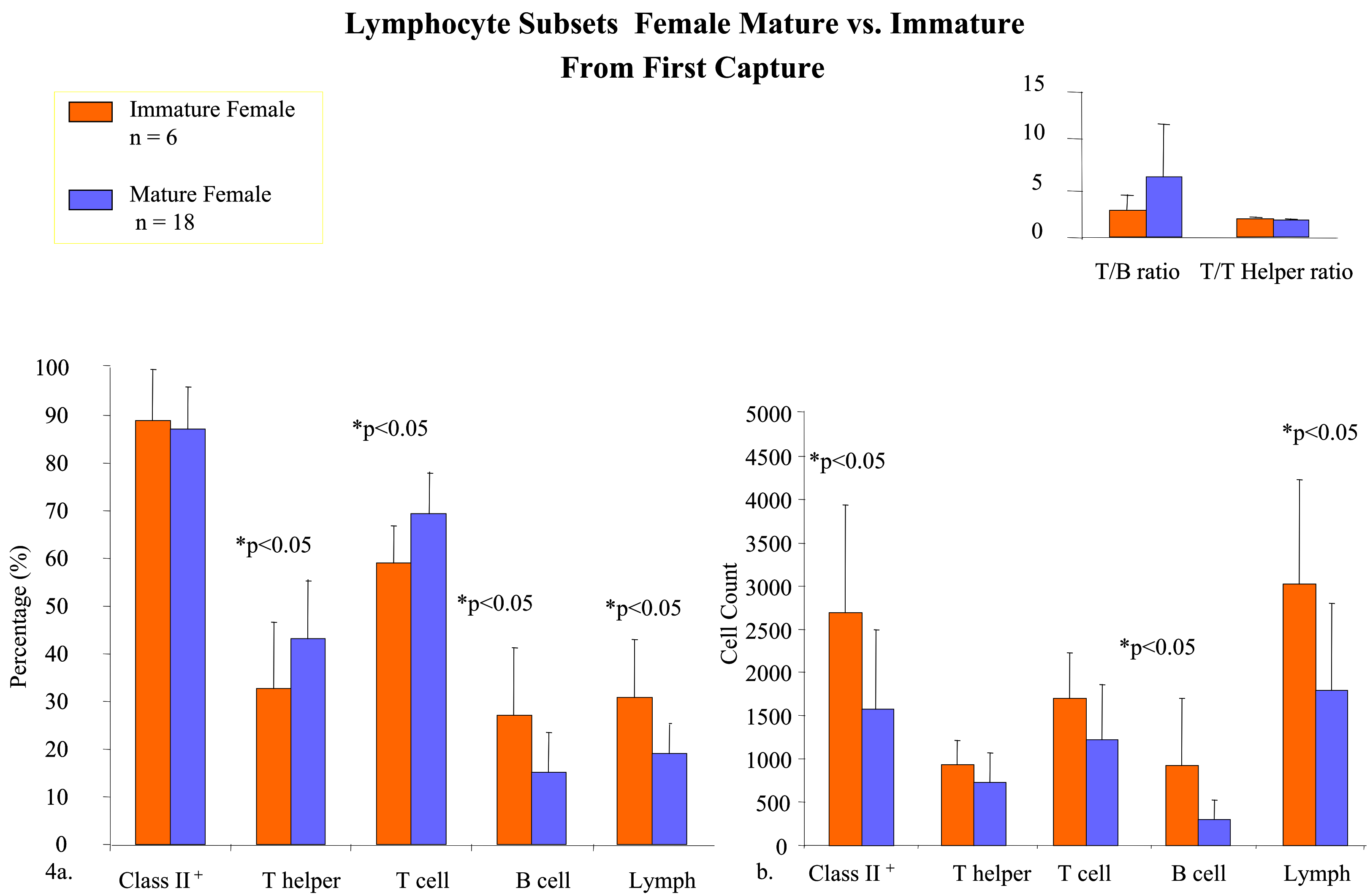

Figure 4a and b. Lymphocyte subset percentages (a) and absolute counts (b) of Mature females vs. Immature females from the first time capture group. Significant differences are indicated between mature females vs. immature females with and (*) at $p < 0.05$.

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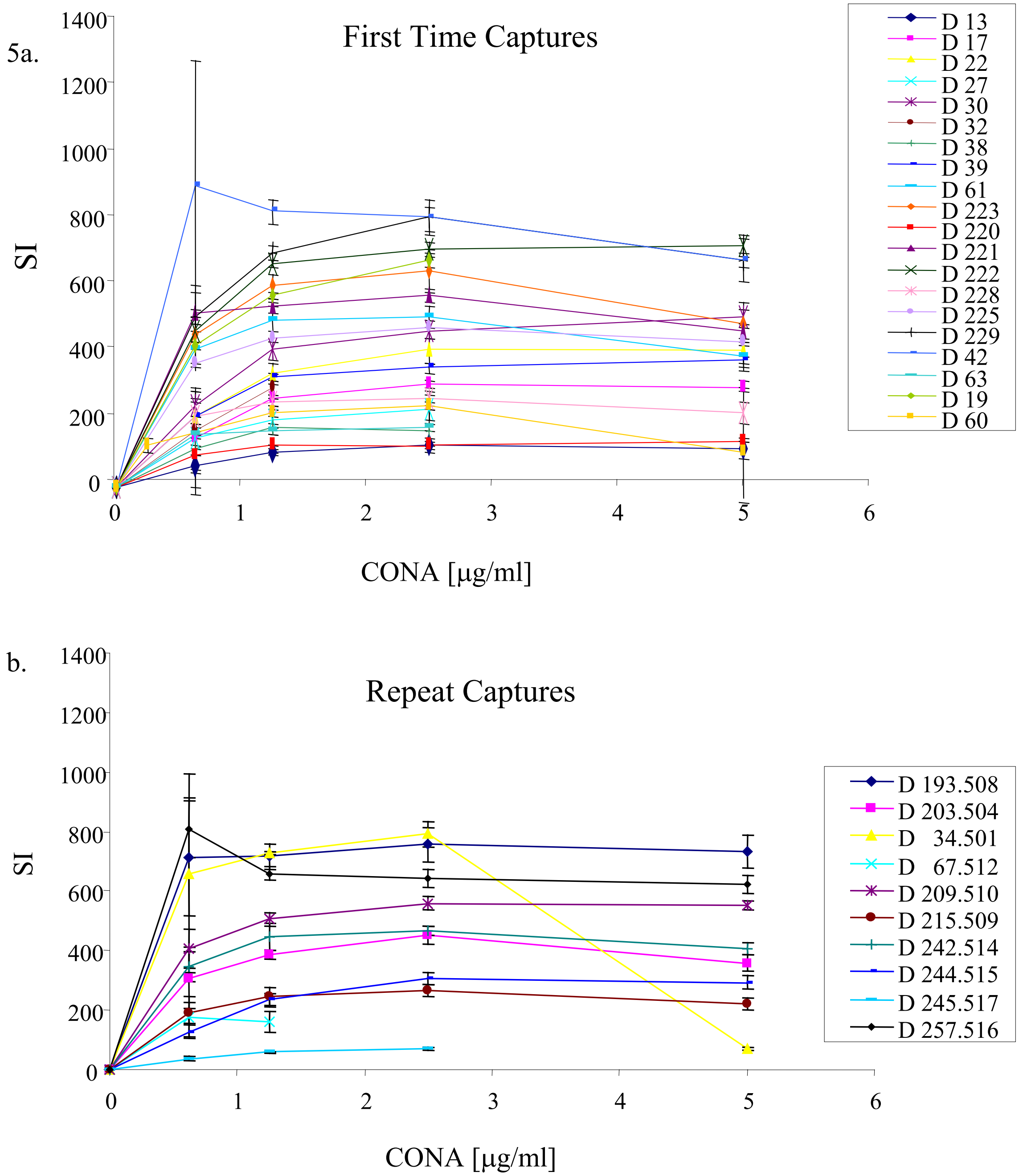
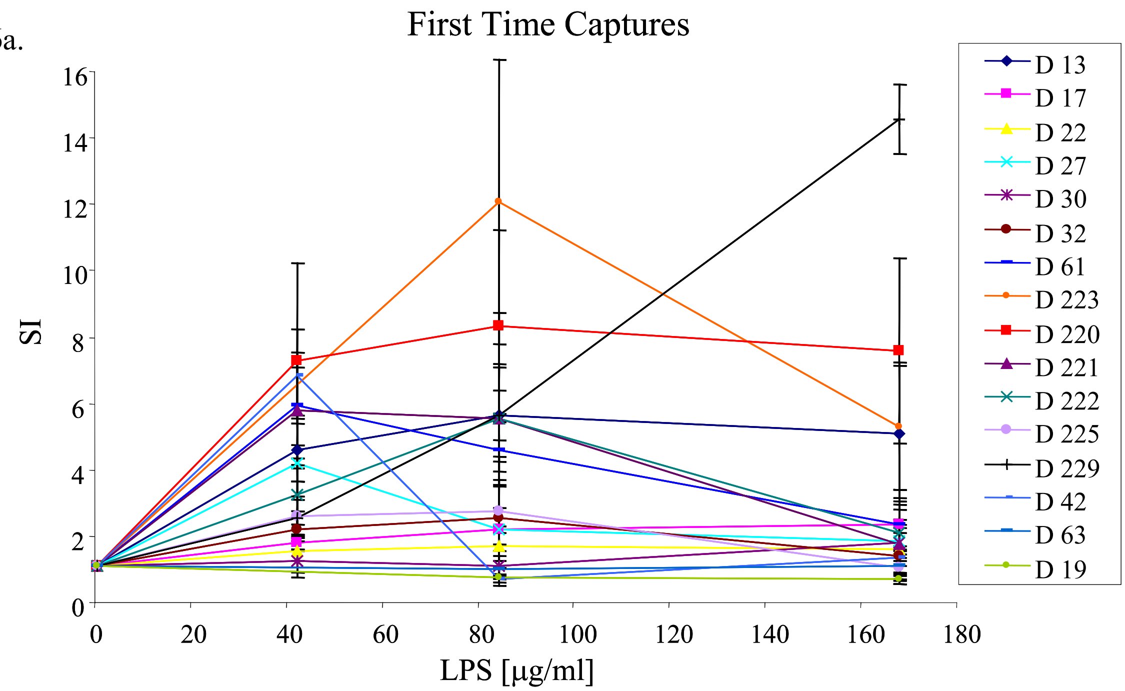


Figure 5. Plots of the stimulation indices (SI) for optimal and suboptimal concentrations of the T cell dependent mitogen ConA (mg/ml) from 20 first time captured dolphins (a) and the 10 repeat captured dolphins (b).

6a.



b.

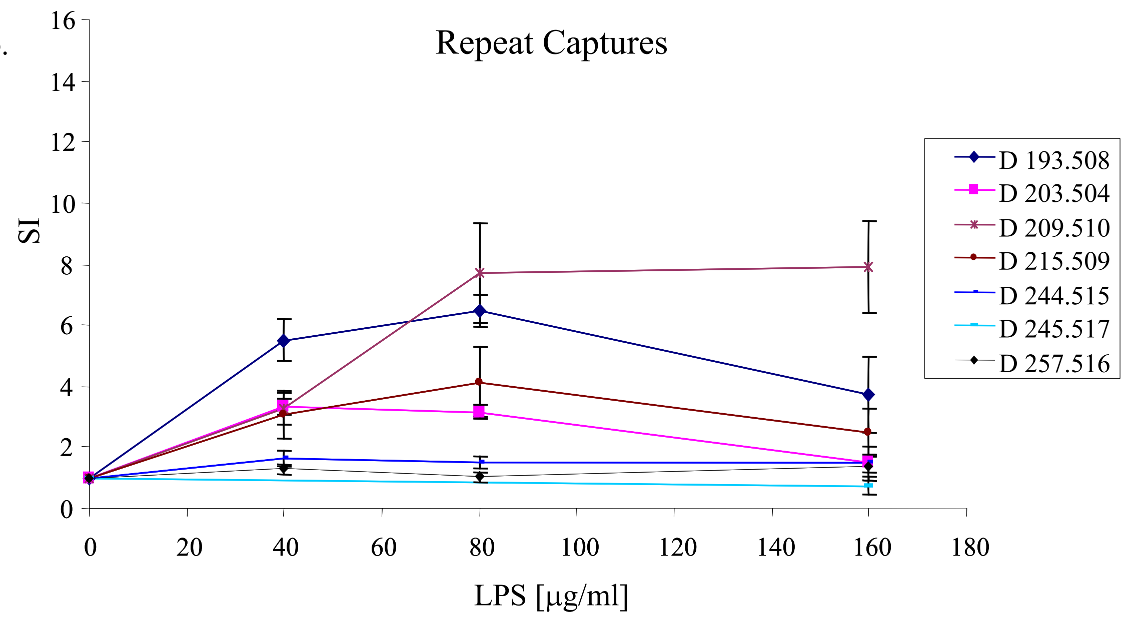


Figure 6. Plots of the stimulation indices (SI) for optimal and suboptimal concentrations of the B cell dependent mitogen LPS (µg/ml) from matched first time captured dolphins (a) and the repeat captured dolphins (b).

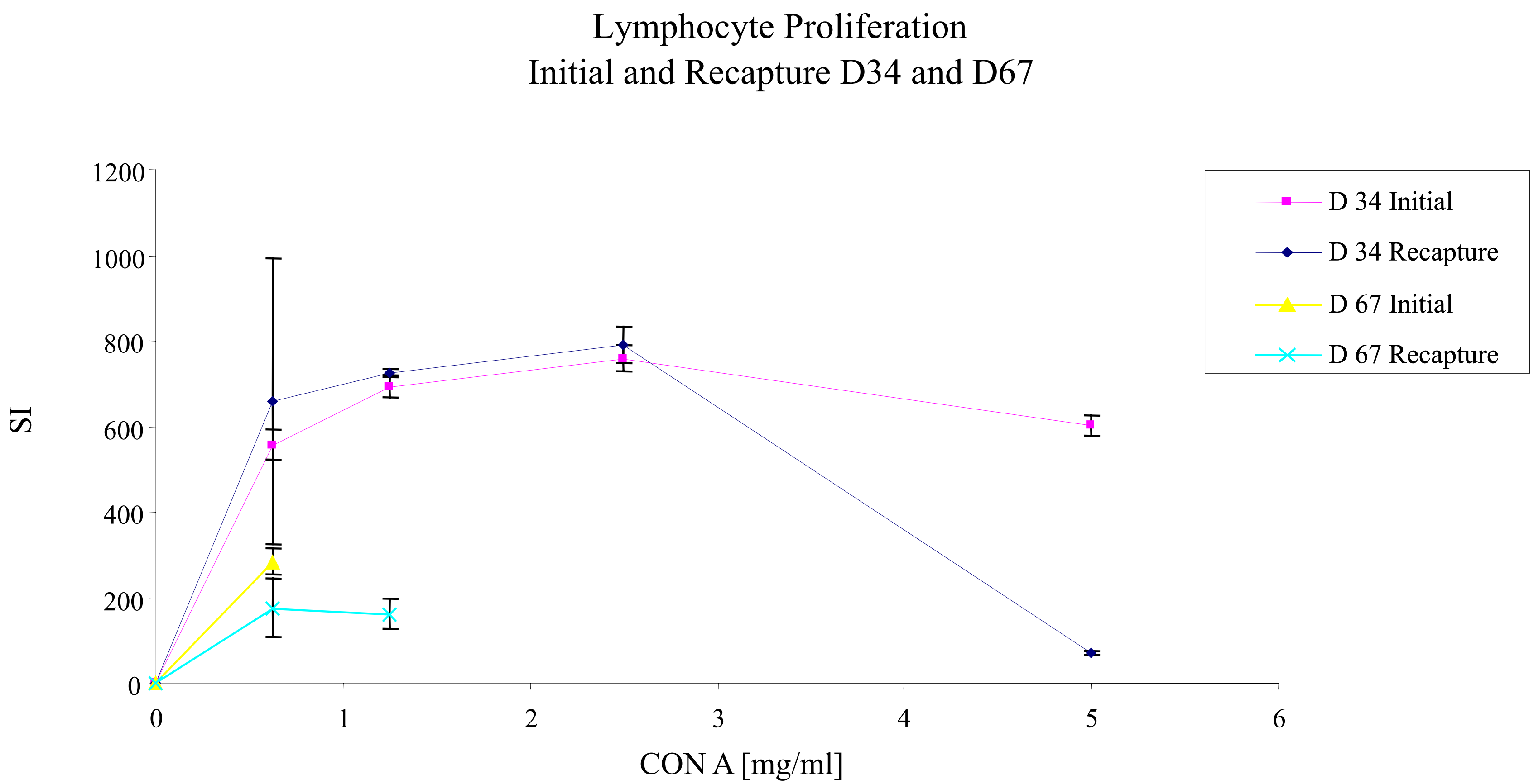


Figure 7. Plots of the stimulation indices (SI) for optimal and suboptimal concentrations of the T Cell dependent mitogen ConA showing initial capture values and repeat capture values for D67 and D34.

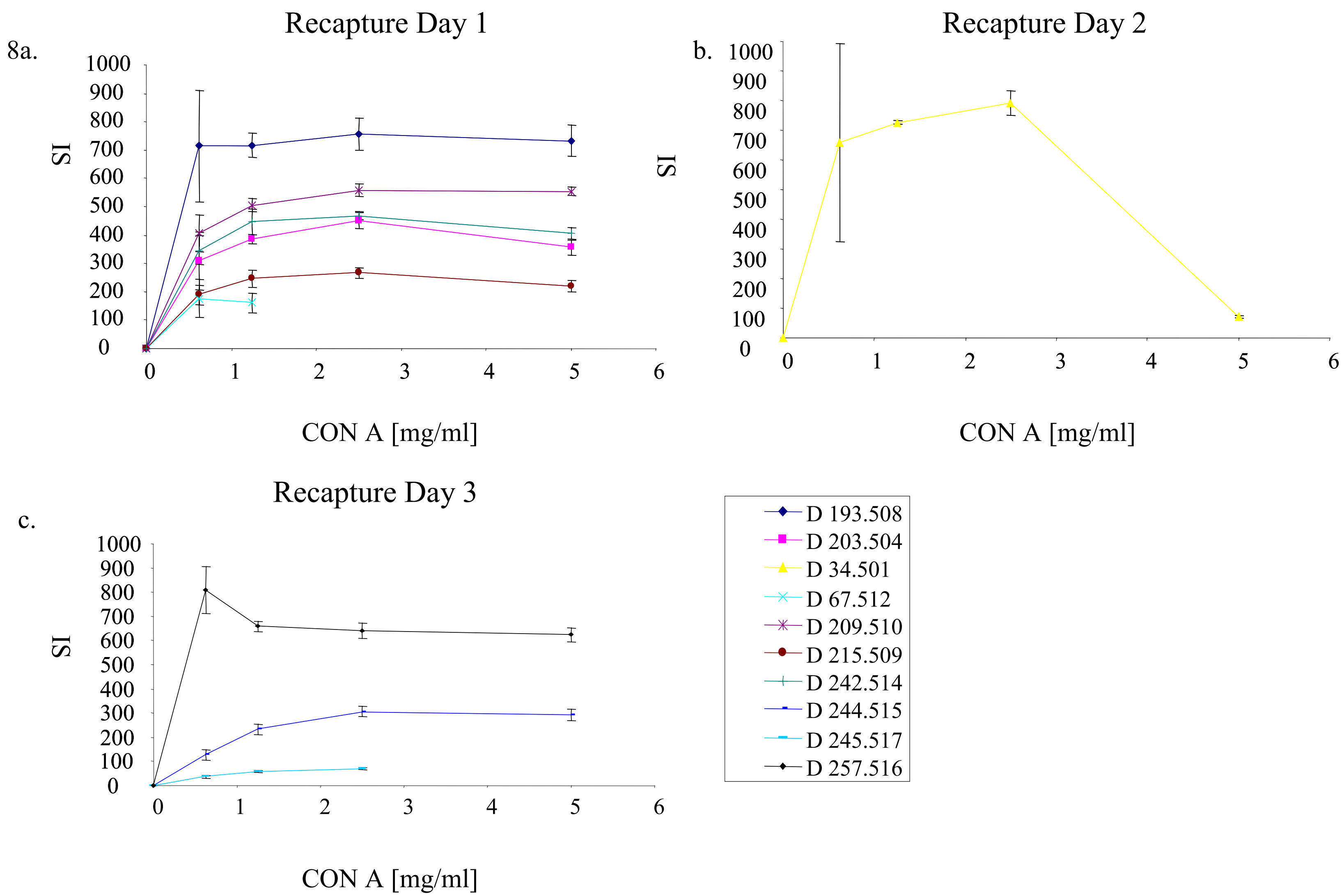


Figure 8. Plots of the stimulation indices (SI) for optimal and suboptimal concentrations of the T cell dependent mitogen ConA (μg/ml) from dolphins at 1 day recapture (a), 2 days recapture (b) and 3 days recapture (c).

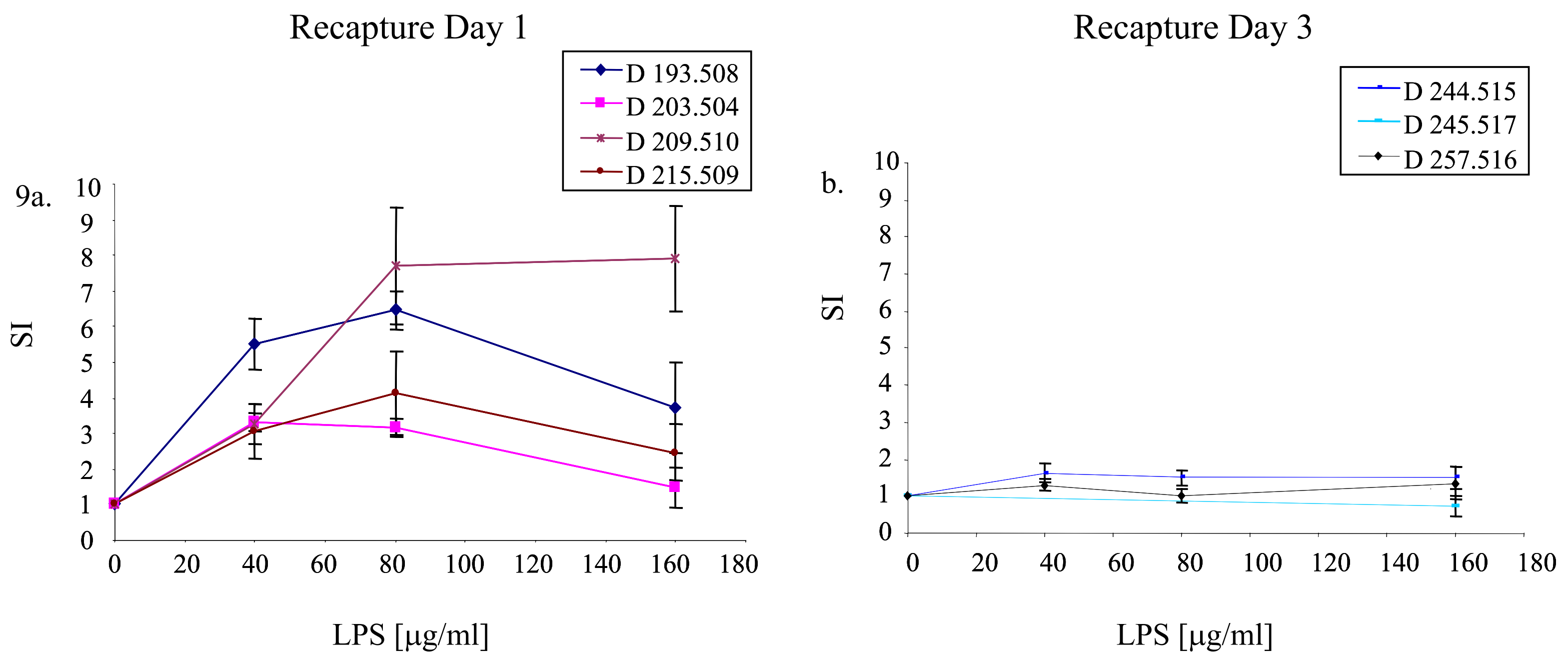
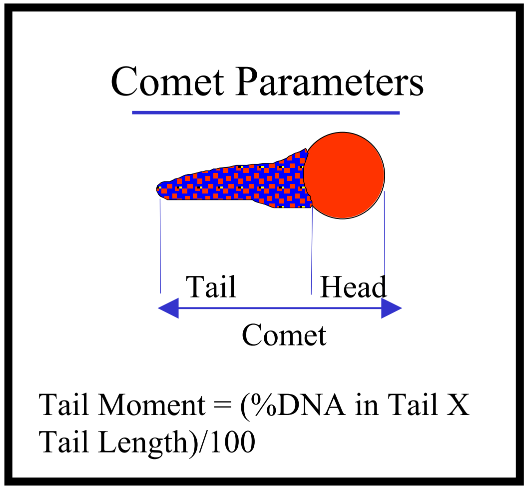
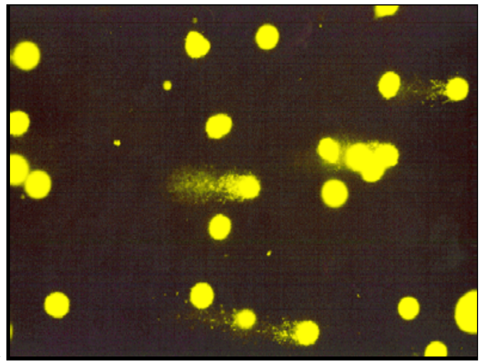


Figure 9. Plots of the stimulation indices (SI) for optimal and suboptimal concentrations of the B cell dependent mitogen LPS (µg/ml) from dolphins at 1 day recapture (a) or 3 days recapture (b).

10a. 

b. 

Average Tail Moment	St. Dev.	Range	N
1.7 mm	± 1.2	0.2 - 5.0	32

Baseline values for the bottlenose dolphin, *Tursiops truncatus.*

Figure 10. The comet assay was used to assess DNA damage in dolphin white blood cells. Comet parameters on shown schematically in (a), while (b) shows a comet (head and tail) surrounded by intact cells as visualized under the fluorescent microscope (figure provided by S. Steinert, CSC Biomarker Laboratory). Baseline values for bottlenose dolphin, *Tursiops truncatus* are shown.

APPENDIX

Investigation of the Effects of Repeated Chase and Encirclement on the Immune System of Spotted Dolphins (*Stenella attenuata*) in the Eastern Tropical Pacific, Romano et al.

Revisions/Responses to Reviewer's Comments

Sylvain De Guise

1. The reason only 5-10 nuclei are counted per sector in the comet assay is because that is the number that the researchers who carry out the Comet assay on a regular basis found necessary for statistical strength and level of effort.
2. The cell viability was >90% and only viable cells were used in the assays. Percentage of cell viability was added to the methods section on pages 6 and 7.
3. Control cells were used to assess the validity of the tests and to account for daily inter-experiment variability as already stated on page 8.
4. Four concentrations of ConA were used and 3 concentrations of LPS. This was added in the methods section on Page 8 and was already addressed in the Results section on page 13.
5. Figures were added to address differences in mature vs. immature dolphins (Figures 2,3,4).
   Moreover, this was added to the results (Page 11-12) and discussion (Page 19).
6. The CD4 monoclonal that wasn't used in this study was removed from Table 1.
7. The total number of lymphocytes was used.
8. Rationale for using class II was to see if it is constituituively expressed on *Stenella* as in *Tursiops* as stated on page 18 and 19 (second paragraph). The sentences talking about "continuous activation" were deleted to avoid confusion, and reference to Class II expression for ungulates and carnivores was indicated.
9. DNA damage frequency in certain subsets of cells was not investigated here. All white blood cells were assessed together and so only strand breakage in white blood cells is reported in the results.
10. Table 8 was left in since it is not redundant, but Table 9 was removed because it was redundant with figures 2 and 3. Table 11 was removed as recommended.
11. The unknown previous chase/capture history of the first capture group was added to the discussion (Page 18)
12. The decrease in proliferation to LPS was added to the discussion (Page 21)
13. The proliferation results from the 2 dolphins in which we had an initial sample and repeat sample were added to the discussion (Page 20)
14. The criteria that was used to select 20 dolphins to match gender and size with 10 recaptured dolphins were matching genders, and matching both lengths and girths within 5 cm. They were equally matched (2 first capture for each recapture animal).

Responses to Daniel Martineau

No Changes/Responses

Responses to Gregory Bossart

No recommended changes. The points made are already mentioned in the text.

-ex. "glimpse of an extraordinarily complex, dynamic, and multifaceted system-individual variation, variation of the type of stressor, intensity and duration of the stressor and desensitization of the stressor, species-specific variation, flux of health and physiologic factors including age, sex, and reproductive status" (addressed in text: Pages 22-23;20-21;16-17)

Responses to Rudy Ortiz

1. The question posed: "Were the observed differences due to the chase-induced exercise or to the restraint of the animals?" cannot be answered. Although by immediately taking a blood sample before other procedures cut down on the restraint time.
2. I am not sure how one can measure the "intensity" of the chase/capture.
3. Individual variation due to the animal's habituation to the procedures and its conditioning were added to the discussion as a confounding variable (Pages 16-17 and 22).

Responses to Janet Mann (received review on Tuesday, April 23)

1. The "repeat-capture" group wasn't compared with itself using a Wilcoxon Matched-Pairs or a Fisher Matched-Pairs Randomization test, since the primary author isn't familiar with these tests. However, if time allowed can find someone who knows how to run them.
2. Blood sampling times/methodologies are addressed in other CHESS document. Methodologies are described starting with collected, frozen sample.
3. The title was changed- longirostris was removed.
4. Immature vs. mature male and female immune differences were added as figures.
5. The possibility that the "first capture" dolphins may not have truly been first captures was addressed in the discussion (Page 18; Paragraph 1)