Anthropogenic sound and marine mammal health: measures of the nervous and immune systems before and after intense sound exposure

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Abstract: Anthropogenic sound is a potential stressor for marine mammals that may affect health, as has been demonstrated in other mammals. Therefore, we have initiated investigations on the effects of intense underwater sounds on nervous system activation and immune function in marine mammals. Blood samples were obtained before and after sound exposures (single underwater impulsive sounds (up to 200 kPa) produced from a seismic water gun and (or) single pure tones (up to 201 dB re 1 μPa) resembling sonar "pings" from a white whale, *Delphinapterus leucas*, and a bottlenose dolphin, *Tursiops truncatus*, to measure neural–immune parameters. Norepinephrine, epinephrine, and dopamine levels increased with increasing sound levels and were significantly higher after high-level sound exposures (>100 kPa) compared with low-level sound exposures (<100 kPa) or controls (P = 0.003, 0.006, and 0.020) for the white whale. Alkaline phosphatase decreased over the experimental period ($P < 0.001$), while γ-glutamyltransferase increased over the experimental period ($P < 0.001$). Significant neural–immune measurements for the dolphin after exposure to impulsive sounds included an increase in aldosterone (P = 0.003) and a decrease in monocytes (P = 0.006). Neural–immune changes to tonal sound exposures were minimal, although changes were observed in multiple neural–immune measures over time.

Résumé : Les sons d'origine d'anthropique constituent des stress potentiels pour les mammifères marins qui peuvent affecter leur santé, comme c'est le cas chez les autres mammifères. Nos avons donc initié des travaux pour voir les effets de sons sous-marins intenses sur l'activation du système nerveux et la fonction immunitaire chez les mammifères marins. Nous avons prélevé des échantillons de sang chez un béluga, *Delphinapterus leucas*, et un grand dauphin, *Tursiops truncatus*, avant et après des expositions à des sons (des impulsions sonores uniques sous-marines pouvant atteindre 200 kPa) produits par un canon sismique à eau et (ou) des tons purs uniques (pouvant atteindre 201 dB re μPa), qui ressemblent à des « pings » de sonar, afin de mesurer les paramètres neurologiques et immunitaires. Chez le béluga, les concentrations de norépinéphrine, d'épinéphrine et de dopamine s'accroissent toutes en fonction du niveau du son et elles sont significativement plus élevées après une exposition à un son de haute intensité (>100 kPa) qu'après une exposition à un son de basse intensité (<100 kPa) et plus élevées que chez les témoins (P = 0,003, 0,006 et 0,020). La concentration de phosphatase alcaline a décru pendant la durée de l'expérience ($P < 0,001$), alors que celle de la gamma glutamyl transférase a augmenté ($P < 0,001$). Parmi les mesures significatives de changements neurologiques et immunitaires chez le grand dauphin après une exposition à des sons impulsifs, signalons une augmentation de l'aldostérone (P = 0,003) et une diminution des monocytes (P = 0,006). Les changements neurologiques et immunitaires lors d'expositions à des sons tonals sont minimaux, bien qu'on observe des changements de plusieurs paramètres neurologiques et immunologiques sur une période temps plus longue.

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Introduction

Within the last decade, there has been increasing concern regarding the potential effects of anthropogenic (human-generated) sound on the marine mammal auditory system and the impact that these sounds may have on the navigational, foraging, reproductive, and hearing capabilities of cetaceans. Anthropogenic sounds of concern include those associated with dredging and construction, oil and gas drilling, geophysical surveys, sonars, transportation, explosions, and oceanographic research (Richardson et al. 1995). Observational studies have shown whales deviating from normal migratory paths to avoid human-generated noise as well as behavioral changes associated with exposure to anthropogenic sound (Frankel and Clark 2000; Miller et al. 2000; Morton and Symonds 2002). Moreover, it has been proposed that cetacean strandings have been the result of human-made noise (Frantzis 1998; Malakoff 2001; US Department of Commerce and US Navy 2001). There is considerable debate about the actual impact of sound on marine mammals. Scientific data and knowledge of the physiological and health effects of loud sound exposure on marine mammals are lacking (Popper et al. 2000).

Anthropogenic sound is a potential "stressor" for marine mammals. Not only can loud or persistent noise impact the auditory system of cetaceans, it may impact health by bringing about changes in immune function, as has been shown in other mammals (Raaij et al. 1996; Spehner et al. 1996; Van Archana and Namasivayam 2000). Although the effects of sound on the cetacean auditory system and behavior have been investigated (Au et al. 1999; Finneran et al. 2000; Schlundt et al. 2000) and continue to be investigated, only one study has looked at human-generated noise as a stressor by investigating plasma epinephrine and norephinephrine levels. In this single study, Thomas et al. (1990) measured epinephrine and norepinephrine levels as indicators of physiological stress after exposing white whales to playbacks of noise from an oil drilling platform. To date, no studies of cetaceans have measured autonomic nervous system and neuroendocrine activity with immune function before and after noise exposure.

The catecholamines (norepinephrine, epinephrine, and dopamine) are among the first molecules released from the adrenal medulla and sympathetic nerves as an initial response to stress ("fight or flight reaction") (see Fowler (1995) and Young and Landsberg (1998) for review). In addition, hormones from the hypothalamic pituitary axis initiate release of gluco- and mineral-corticoids from the adrenal cortex. Cortisol is the primary glucorticoid that has been identified and studied in marine mammals. However, aldosterone (a mineralcorticoid normally not considered part of the stress response in most mammals) has been implicated in playing a major role in the stress response in marine mammals (St. Aubin et al. 1996; Ortiz and Worthy 2000; St. Aubin and Dierauf 2001). Both the catecholamines and glucocorticoids, which are released in response to stressors such as noise (Tafalla and Evans 1997; Van Raaij et al. 1997; Muchnik et al. 1998), have been shown to affect the immune system by bringing about changes in numbers and distribution of white blood cells including specific subsets of lymphocytes (e.g., T cells, T helper cells, cytotoxic T cells, and B cells) as well as changes in immune function (Sgoutas-Emch et al. 1994; Dhabhar et al. 1995, 1996) in both animals and humans.

Measurements of the nervous and immune systems such as those described above, before and after stressors, especially anthropogenic sound, are difficult to carry out in cetaceans. However, through collaborative efforts with the US Navy Marine Mammal Program, we were able to obtain blood samples from a white whale, *Delphinapterus leucas*, and a bottlenose dolphin, *Tursiops truncatus*, during a series of experiments designed to understand the impact of anthropogenic noise on cetacean hearing. These experiments were designed to measure temporary elevations in the hearing threshold, or temporary threshold shifts (TTS), in marine mammals exposed to intense underwater sound. Using reagents and assays currently available (some developed specifically for cetaceans), we were able to measure plasma catecholamines (norepinephrine, epinephrine, and dopamine), cortisol and aldosterone, and specific lymphoid cell subsets (T cells, B cells, T helper cells, and MHC class II cells) in cetaceans before and after exposure to single underwater impulsive sounds produced from a seismic water gun (Finneran et al. 2002) and brief tones resembling sonar pings (Finneran et al. 2001). It is hypothesized that exposure to these sounds may bring about changes in the above neural–immune parameters. Routine serum chemistries, hematology, and complete blood cell counts were also evaluated to monitor health status throughout the duration of the experiment. The purpose of this study was to investigate anthropogenic noise as a "stressor" and potential impacts on the marine mammal immune system. The studies are intended to contribute to an understanding of the effects of sound on marine mammal health.

Methods

Subjects and samples

Subjects consisted of one female white whale, designated MUK (age 32 years), and one male bottlenose dolphin, designated BEN (aged 36 years). Hearing thresholds were measured in each subject before and immediately after exposure to intense sound. TTS was defined as a 6-dB or larger difference between pre- and post-exposure thresholds. MUK was exposed to single underwater impulses produced by a seismic water gun (see Finneran et al. (2002) for a detailed description of the experimental design and results). Impulse peak pressure levels ranged from approximately 8 to 200 kPa or 198–226 dB re 1 μPa peak pressure. BEN was exposed to water gun impulses (44–207 kPa or 213–226 dB re 1 μPa peak pressure) and 1-s, 3-kHz tones with sound pressure levels ranging from 130 to 201 dB re 1 μPa (Finneran et al. 2001). Control sessions were also conducted with each subject where hearing thresholds were measured before and after a "mock" sound exposure (i.e., no intense sound was presented). For tests with the water gun, two test sessions were conducted each day: one control and one exposure. The order (control or exposure) of the tests was randomized from day to day. Tonal experiments were conducted one session (either control or exposure) per day.

The subjects were trained to voluntarily and on signal present their tail flukes for blood collection. The hearing studies and all blood collection for this study followed a protocol approved by the Institutional Animal Care and Use Committee under the guidelines of the Association for the Accreditation of Laboratory Animal Care. Thirty-five millilitres of blood (drawn on sodium heparin, EDTA, or without additive)

was collected before testing began, 1 h after exposure to the sounds or 1 h after a control (no sound), and 24 h after the sound exposure or control. One hour was the soonest that the blood could be collected after the sound exposure or control so as not to interfere with the subsequent hearing tests. Blood was placed on ice and taken to the laboratory for immediate processing.

Blood processing

Clotted whole blood and the blood containing sodium heparin were centrifuged at 1600*g* for 10 min at 10 °C in an IEC centrifuge (Centra GP8R). Plasma and (or) serum from each tube was removed and aliquoted into 1-mL cryovials (Sarstedt Inc, Newton, North Carolina, USA), frozen on dry ice, and stored at –80 °C until analysis. The white blood cells were harvested and the mononuclear cells were isolated as described below.

Lymphocyte isolation

Dolphin mononuclear cells (lymphocytes and monocytes) were isolated from heparinized whole blood using a density gradient. Briefly, 10 mL of blood was diluted 1:2 with RPMI 1640 supplemented with 5% fetal bovine serum plus 200 mmol L-glutamine·L^{-1}, pen-strep and layered on 10 mL of Histopaque 1077 (Sigma-Aldrich Corp., St. Louis, MO 63178, USA). Tubes were centrifuged at 400*g* for 30 min at room temperature. The recovered mononuclear cell layer was washed twice with media and then incubated with 0.17 mol ammonium chloride·L^{-1} for 10 min at room temperature to lyse the red blood cells. The lymphocytes were counted on a hemocytometer, with trypan blue exclusion as a measure of viability. The cells were then washed twice in Hank's balanced salt solution and resuspended to a final concentration of 0.5×10^6 cells·mL^{-1}.

Indirect immunofluorescence

Lymphocytes were labeled with 50 μL of the following monoclonal supernatants for 30 min at 4 °C: Q5/13, a monoclonal antibody to human class II molecules that cross-reacts with dolphin class II molecules (Romano et al. 1992), a monoclonal antibody against cetacean CD2 and CD21 (De Guise et al. 2002), SIM4, a monoclonal antibody to human CD4 that cross-reacts with cetacean CD4 (De Guise et al. 1997), and a cetacean-specific monoclonal antibody to CD4 (Romano et al. 1999). Monoclonal supernatant of the myeloma cell line P2X63-AG8.653 was used as a negative control. The cells were washed three times with Hank's balanced salt solution before incubation with fluorescein isothiocyanate conjugated affinity purified goat anti-mouse $F(ab)'_2$ IgG (Immunotech, Westbrook, ME 04092, USA) for 30 min at 4 °C in the dark. Cells were washed twice in phosphate-buffered saline and resuspended in 500 μL of 1% paraformaldehyde for subsequent flow cytometry analysis.

Analyses

Catecholamines

Dolphin plasma previously frozen and stored at –80 °C was submitted to ARUP Laboratories (Salt Lake City, Utah) for catecholamine analyses. Norepinephrine, epinephrine, and dopamine were quantified using high-performance liquid chromatography with electrochemical detection after alumina extraction. Briefly, 2.0 mL of dolphin plasma was added to 30 mg of alumina (Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA) in a conical centrifuge tube containing 200 μL of working internal standard and 1.0 mL of Tris buffer. After vigorous shaking, the tubes were centrifuged at 1300*g* for 5 min. The supernatant was removed and the alumina washed twice with 1.0 mL of high-performance liquid chromatography grade water. One hundred and fifty microlitres of 0.1 M phosphoric acid was added to the alumina and vortexed for 30 s to extract the catecholamines. The supernatant was removed after centrifugation at 1300*g* for 2 min. Fifty microlitres of extract was injected onto a plasma catecholamine analytical column (Bio-Rad). Quantitation was determined by comparing peak height ratios of norepinephrine, epinephrine, and dopamine to an internal standard in the unknown sample with the corresponding ratios in the plasma calibrator sample.

Cortisol and aldosterone

Hormone assays for serum cortisol and aldosterone were carried out in duplicate using commercially available radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, Calif.). Hormone test kits were validated for dolphins by limiting dilution. Samples from each testing period (baseline, exposure, or control, 24 h later) were run together. Intraassay variability was less than 5% for both cortisol and aldosterone, while interassay variability was less than 10% for cortisol and less than 15% for aldosterone. The sensitivity or "minimal detectable dose" of the cortisol assay was 0.01 μg·dL^{-1} and 0.26 pg·mL^{-1} for aldosterone.

Complete blood cell counts and serum chemistries

Two millilitres of serum and 2 mL of EDTA blood were submitted to Quest Diagnostics (San Diego, Calif.) for serum chemistry analysis and complete blood cell count determinations. The US Navy Marine Mammal Program routinely submits dolphin samples to Quest Diagnostics for evaluation, and there is a long history of quality control for health screening of the animals.

Lymphocyte subsets

Samples were analyzed on an LSR flow cytometer (BD Biosciences, San Jose, California, USA). Forward/side scatterplots were obtained for each subject. Lymphocytes were gated based on their size and low degree of granularity. Ten thousand gated events were analyzed by histogram statistics.

Statistics

A multivariate analysis of variance (MANOVA) was used to evaluate the effects of sound (impulsive and tonal) on neural–immune measurements. Owing to the low number of data points for each sound exposure, the data were pooled into a low impulsive sound exposure group (peak pressures = 8.2, 20.2, 58.6, and 87.2 kPa) and a high impulsive sound exposure group (peak pressures = 116, 118, 143, 160, and 198 kPa) for MUK. However, for BEN, both sound exposure data for the water gun experiments (peak pressures = 146, 207 and 220 kPa) and the tonal experiments (sound pressure level = 180, 190, 196, 198, 200, and 201 dB re 1 μPa) were each evaluated as one sound exposure group because of the limited number of exposures. Neural–immune measurements were used as the dependent variables, while the sound exposure levels no (control), low, or high (for MUK) or control versus exposure (for BEN) were used as the independent variable in

Table 1. Descriptive statistics (including the number of measurements in each group (*N*) and the range, minimum (Min.), maximum (Max.), means, and SD) for significant ($P < 0.05$) neural–immune measures for MUK in the control, high sound exposure, and low sound exposure groups.

Variable	Group	*N*	Range	Min.	Max.	Mean	SD	*P*
Norepinephrine ($pg \cdot mL^{-1}$)	Control	5	322	763	1085	925.20	150.81	0.003
	High	6	503	947	1450	1223.00	184.93	
	Low	5	410	461	871	692.60	176.76	
Epinephrine ($pg \cdot mL^{-1}$)	Control	4	21	17	38	31.25	9.91	0.006
	High	6	55	40	95	62.17	18.45	
	Low	3	25	5	30	21.00	13.89	
Dopamine ($pg \cdot mL^{-1}$)	Control	5	45	37	82	52.60	17.29	0.020
	High	6	65	49	114	82.67	23.88	
	Low	5	29	37	66	49.60	11.52	
Alkaline phosphatase ($U \cdot L^{-1}$)	Control	5	19	59	78	66.80	7.26	<0.001
	High	6	8	63	71	67.33	2.87	
	Low	5	14	74	88	81.20	5.93	
GGT ($U \cdot L^{-1}$)	Control	5	4	18	22	20.20	1.64	0.002
	High	6	3	17	20	18.83	1.17	
	Low	5	4	15	19	17.00	1.41	
MCV (fL)	Control	5	3	180	183	182.00	0.99	0.019
	High	6	2	181	183	182.00	0.66	
	Low	5	2	182	184	183.00	0.54	

the MANOVA. The effect of the order of exposures for the water gun experiments (whether the experimental exposure or control was given first) was also investigated in the MANOVA. Order was not found to have a significant effect and so was excluded in further analyses.

Univariate ANOVAs were also conducted and group differences (for MUK) were determined using Tukey's post hoc pairwise comparison. Regression analysis was run on those variables that were significant to determine if neural–immune measurements were linearly increasing or decreasing with sound exposure. The date of the measurements was also considered in the analysis, since the experimental groups and the date of the experiments were correlated. Therefore, several analyses of covariance (ANCOVAs) were performed using the date of the experiment as a covariate and experimental group as a factor with each significant neural–immune measurement as the dependent variable and then assessing the differences among the groups.

The changes from baseline to 24 h later were calculated for those neural–immune measures that showed significant effects in the experiment. These differences were viewed as a measurement of the long-term effects of the experiment, which were then compared for each experimental condition using a nonparametric ANOVA (the Kruskal–Wallis test). Regression analysis of the baseline and 24-h data was carried out to see if these values changed over the duration of the experimental period.

Results

MUK seismic water gun experiment

Measurements of the nervous and immune systems including catecholamines, hormones, and lymphocyte subsets were investigated to determine the effects of loud sound on cetacean health. Complete blood cell counts, hematological parameters, and serum chemistries, routinely measured by marine mammal veterinarians, were also measured to ensure that these parameters were in the normal ranges for the experimental subjects before initiation of the experiment and during the duration of the experimental period. A MANOVA revealed significant differences between the groups (Pillai's trace, $P = 0.038$). Univariate analyses showed significant differences among the control, low, and high sound exposure groups for MUK for the following neural–immune measurements: catecholamines (norepinephrine, epinephrine, and dopamine), mean cell volume (MCV), alkaline phosphatase, and γ-glutamyltransferase (GGT) (Table 1).

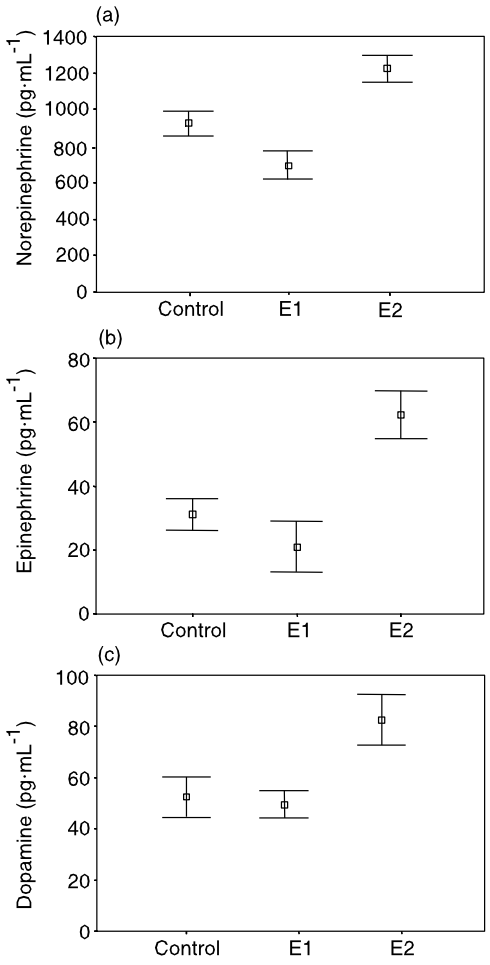
Tukey's post hoc tests revealed that mean norepinephrine, epinephrine, and dopamine levels increased significantly immediately after a high-level sound exposure but not after a low-level sound exposure (Fig. 1). Mean norepinephrine levels increased by 337.75 $pg \cdot mL^{-1}$, mean epinephrine levels increased by 30.92 $pg \cdot mL^{-1}$, and mean dopamine levels increased by 37.42 $pg \cdot mL^{-1}$ after high-level sound exposures.

Regressing catecholamine levels on sound levels showed that all three increased significantly with increasing sound levels ($P = 0.021$, 0.012, and 0.021, respectively) (Fig. 2). Each increase in 10 kPa of sound impulse corresponded to a mean increase of 22.1, 2.1, and 1.9 $pg \cdot mL^{-1}$ in norepinephrine, epinephrine, and dopamine levels, respectively.

Statistically significant differences among the control, low, and high sound exposure groups were also observed in the mean levels of MCV, alkaline phosphatase, and GGT. However, when date was included as a covariate in an ANCOVA model, these hematological and serum chemistry constituents no longer showed significant differences between the sound exposure groups, unlike the catecholamines (Table 2); date accounted for more variability than the sound level. MCV and alkaline phosphatase decreased over the experimental period ($P = 0.004$ and < 0.001, respectively), whereas GGT increased over the experimental period ($P < 0.001$) (Fig. 3).

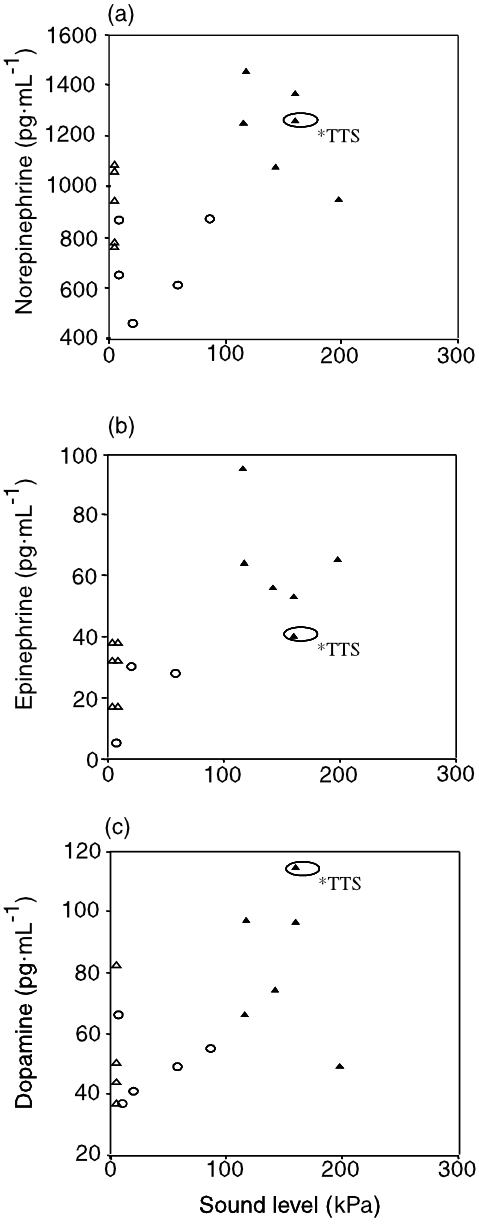
In addition to samples collected after sound exposure, blood samples were also taken before any testing was initiated, as a baseline, and then 24 h after the sound exposure or control

Fig. 1. Boxplots of the means ± SE of plasma (*a*) norepinephrine ($N = 5$), (*b*) epinephrine ($N = 5$), and (*c*) dopamine ($N = 6$) for the three experimental groups (Control, E1 (low sound exposure), and E2 (high sound exposure)) for MUK. Norepinephrine levels were higher in E2 than in E1 ($P = 0.003$) and E2 levels were higher than the control ($P = 0.036$). Epinephrine levels were higher in E2 than in E1 ($P = 0.009$) and E2 levels were higher than the control ($P = 0.28$). Dopamine levels were higher in E2 than in the control ($P = 0.24$).



to determine if significant changes in neural–immune measures returned to baseline levels. The change from baseline to 24 h after the exposures (control, low, and high) was calculated for the catecholamines, MCV, alkaline phosphatase, and GGT. No significant effects were observed after 24 h for any of the variables, except alkaline phosphatase. The average alkaline phosphatase levels decreased from the baseline to 24 h later in the control (average decrease of 2.3 $U \cdot L^{-1}$, where U is the quantity of enzyme that will catalyze the reaction of 1 μmol of substrate per minute) and low sound exposure (average decrease of 1.4 $U \cdot L^{-1}$) groups but increased in the high sound exposure group (average increase of 2.2 $U \cdot L^{-1}$). The control group was not significantly different from the low sound exposure group, but the high sound exposure group was significantly higher than the control and the low sound exposure group ($P < 0.05$).

Fig. 2. Regression analysis showing increasing levels of (*a*) norepinephrine ($P = 0.021$, $R^2 = 0.324$), (*b*) epinephrine ($P = 0.012$, $R^2 = 0.453$), and (*c*) dopamine ($P = 0.021$, $R^2 = 0.327$) with increasing sound levels. The data point corresponding to a temporary threshold shift (*TTS) in hearing for MUK is circled. Corresponding groups of the individual data points: circles, low sound exposure; solid triangles, high sound exposure; open triangles, control.



Regression analysis of each variable over time of the experimental period from the baseline and 24-h groups showed that alkaline phosphatase, triglycerides, MCV, blood urea nitrogen, percent T helper cells, and creatine all declined over the study period in either the baseline or 24-h data, while GGT increased significantly over the study period in the baseline data (Table 3).

Table 2. ANCOVA results of neural–immune measures for MUK.

Neural–immune response variable	Explanatory variable	*P*
Dopamine	Date	0.307
	Control vs. low	0.335
	High vs. low	0.022*
	High vs. control	0.025*
Epinephrine	Date	0.353
	Control vs. low	0.232
	High vs. low	0.018*
	High vs. control	0.050*
Norepinephrine	Date	0.310
	Control vs. low	0.054
	High vs. low	0.002*
	High vs. control	0.017*
MCV	Date	0.186
	Control vs. low	0.631
	High vs. low	0.763
	High vs. control	0.189
Alkaline phosphatase	Date	0.050
	Control vs. low	0.299
	High vs. low	0.264
	High vs. control	0.990
GGT	Date	0.035
	Control vs. low	0.543
	High vs. low	0.739
	High vs. control	0.124

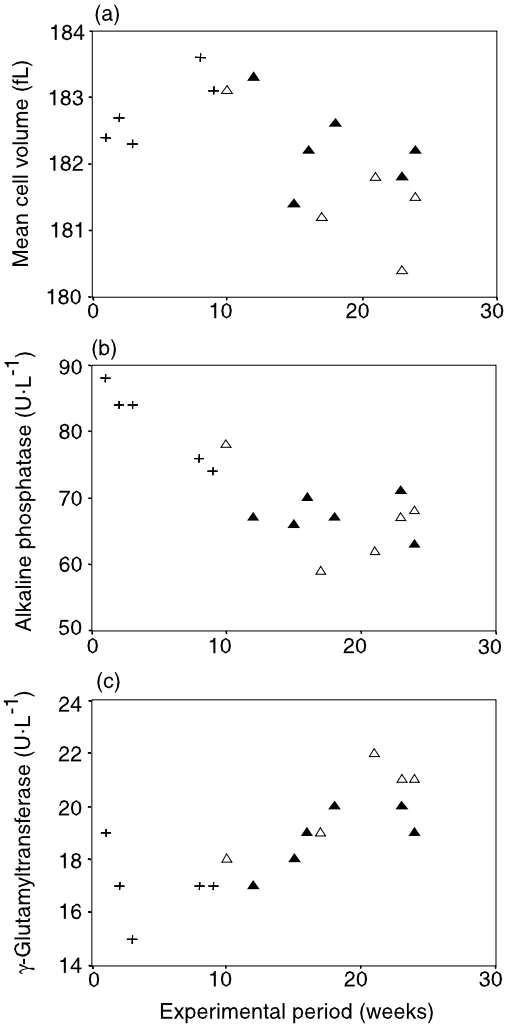
Note: Significant group differences ($P < 0.05$) for each neural–immune response variable after including date as a covariate in the analysis are indicated with an asterisk.

BEN water gun experiment

The same neural–immune measures that were used as dependent variables for MUK were used similarly for BEN, with the session type (control or exposure) as the independent variables in univariate ANOVA analyses. Only two neural–immune measurements, aldosterone and monocytes (absolute count), showed significant differences ($P = 0.003$ and 0.006, respectively) between the experimental and control groups for the open-water experiment (Fig. 4). Neural–immune parameters that showed significant differences for MUK did not show significant differences for BEN.

As with MUK, since the experimental groups and the date of the experiment are correlated, an ANCOVA was performed on the variables that were significant in the ANOVA. When date of the experiment was used as a covariate and the differences between the two groups assessed, the experimental group continued to have significantly higher aldosterone values than the control group ($P < 0.001$) and significantly lower monocyte counts ($P = 0.015$). Aldosterone levels were significantly higher in the experimental condition than in the control, with a mean difference of 50.48 $pg \cdot mL^{-1}$, when adjusting for an overall increase in aldosterone levels over the experimental period. Absolute monocyte counts were significantly lower in the experimental condition than in the control with a mean difference of 192.00 cells.

Fig. 3. Regression analysis showing decreasing levels of (*a*) mean cell volume and (*b*) alkaline phosphatase and increasing levels of (*c*) GGT over time for MUK. Corresponding groups of individual data points: plus signs, low sound exposure; solid triangles, high sound exposure; open triangles, control.



BEN tone experiment

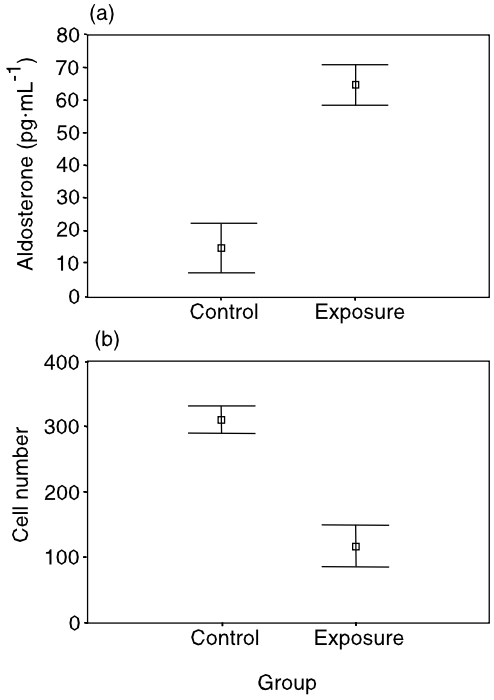
Dopamine and mean corpuscular hemoglobin showed significant differences between the control and exposure groups for BEN in the tone sound experiment using ANOVA ($P = 0.052$ and 0.027, respectively). However, when time was used as a covariate in an ANCOVA to assess differences between the two groups, dopamine was no longer significant ($P = 0.099$) and mean corpuscular hemoglobin only slightly significant ($P = 0.050$). There were no significant differences for those neural–immune measures that were significant for MUK or for aldosterone and absolute monocyte counts as were found significant for BEN during the open-water experiments. Regression analysis of the baseline neural–immune measurements over the experimental period showed increases over time in absolute T cells, class II+ cells, T cell / T helper cell ratio, red blood cells, hemoglobin, and lymphocyte percentage. Cortisol, norepinephrine, white blood

Table 3. Regression analysis of baseline and 24-h neural–immune measures for MUK over time.

Neural–immune response variable	Variable	Slope	P	R^2
Alkaline phosphatase	Baseline data	–0.81	<0.001	0.811
GGT	Baseline data	0.15	0.002	0.426
Triglycerides	Baseline data	–1.57	0.030	0.249
MCV	24-h data	–1.35	0.017	0.345
Blood urea nitrogen	Baseline and 24-h data	–0.371, –0.30	<0.001, 0.001	0.727, 0.551
T helper cells (absolute no.)	Baseline	–0.000009	0.042	0.249
T helper cells (%)	Baseline	–0.0000003	<0.001	0.600
Class II+ (%)	Baseline	–0.0000001	0.002	0.491
T cell / T helper cell ratio	Baseline	0.00000006	<0.001	0.665
Creatine	Baseline and 24-h data	–0.008, –0.007	0.007, 0.011	0.358, 0.363

Note: Significant ($P < 0.05$) results are shown.

Fig. 4. Group differences (Control versus exposure) of (*a*) serum aldosterone ($P = 0.003$) ($N = 3$) and (*b*) absolute monocyte counts ($N = 4$) for BEN in the water gun study. The boxplots indicate mean ± SE aldosterone and absolute monocyte number.



cell counts, neutrophil counts, platelets, and MCV decreased over the study period (Table 4).

Table 4. Regression analysis of baseline change over time for BEN during the tone sound exposure experiment.

Neural–immune response variable	Slope	P	R^2
Class II+ (absolute no.)	36.471	0.018	0.341
T cells (absolute no.)	24.948	0.047	0.253
T cell / T helper cell ratio	0.056	0.011	0.380
Red blood cells	0.014	0.031	0.273
Hemoglobin	0.058	0.004	0.432
Lymphocytes (%)	0.572	0.020	0.312
Cortisol	–0.257	0.001	0.540
Norepinephrine	–36.112	<0.001	0.776
White blood cells	–0.138	0.013	0.344
MCV	–0.256	0.002	0.495
Platelets	–6.442	0.006	0.407
Neutrophils (absolute no.)	–0.134	0.006	0.406

Note: Significant ($P < 0.05$) results are shown.

Discussion

"Stress" is a concept that is difficult to define and attempts in defining stress have brought about much controversy. However, it is generally agreed that stress can be described as a state of threatened homeostasis (Moberg 1985). Stress can be beneficial, initiating responses that promote a homeostatic state, or harmful, degrading the homeostatic state, depending on its duration and intensity. The response to stress, whether it is physical, psychological, or environmental, begins with cognitive processes in the brain that send information through descending pathways by stimulation of the hypothalamic pituitary axis and (or) sympathetic nervous system. Centers in the hypothalamus control pathways of the autonomic nervous system as well as the release of factors that stimulate the release of hormones from the pituitary (Moberg 1985; Fowler 1995; Elenkov et al. 2000). Epinephrine is released from specialized cells in the adrenal medulla with release of norepinephrine from postganglionic sympathetic nerve terminals in the periphery. Adrenocorticotropin releasing hormone is released from the pituitary, which signals the release of glucorticoids, such as cortisol, from the adrenal cortex. The temporal release of these hormones depends on many factors (e.g., type of stressor, duration, and intensity). Furthermore, evidence from a variety of disciplines supports the bidirectional communication between the nervous and immune systems (see Madden et al. (1995), McEwen et al. (1997), and Dhabhar (2002) for review). An anatomical pathway between the brain and immune system has been shown in cetaceans as well as in terrestrial mammals in which postganglionic sympathetic nerve fibers innervate cellular compartments of lymphoid organs, establishing close associations with cells of the immune system (Romano et al. 1994, 2002). Moreover, noradrenergic nerve terminals were identified closely abutting lymphocytes at the electron microscopic level in the cetacean spleen, adrenergic receptors were identified on cetacean peripheral blood lymphocytes, and functional changes in the lymphocyte proliferation response were observed

when cetacean lymphocytes were incubated with isoproterenol, a beta adrenergic agonist (see Romano et al. (2002) for review). The evidence for communication between the nervous and immune systems suggests that stressors such as loud sound may have an effect on the immune system and the ability to defend the body against foreign invaders. To date, there have been no investigations of sound as a "stressor" as in other mammals, with implications for effects on marine mammal health. In this study, the effects of seismic impulses and sonar pings on various measurements of the nervous and immune systems in cetaceans were investigated to provide preliminary information in this regard.

This study identified several neural–immune measurements that may be implicated as indicators of stress in the white whale and bottlenose dolphin that were either released acutely or changed over time during the experimental period. The catecholamines (norepinephrine, epinephrine, and dopamine) were the primary neural–immune measurements that showed changes in response to high-level impulsive sound in the white whale. Similarly, plasma and urinary levels of epinephrine and norepinephrine, to a lesser degree, have been shown to increase in response to noise stress in other animals (Schmid et al. 1989; Van Raaij et al. 1997; Muchnik et al. 1998) and humans (Testa et al. 1994; Tafalla and Evans 1997). However, it should be recognized that responses are not consistent among studies and can depend on many factors including the duration and intensity of the sound, the species and strain of the animal, and the individual's response to the sound stressor and the amount of control that an individual has over the stressor (Irwin et al. 1989; Sudo and Miki 1993; Peters et al. 1998). Our findings of increased catecholamine levels after high-level sound in the white whale differ from the findings of Thomas et al. (1990) in which playbacks from an oil drilling platform showed no significant changes in catecholamine levels or behavior in white whales after sound exposure. Differences in the type of sound (oil drilling versus simulated underwater explosion), intensity and duration of the sound, the individual's response, and the surrounding circumstances of the individual's environment may attribute to these differences. Moreover, the differences in methodology used to measure catecholamines may also attribute to the differences in the results obtained. High-performance liquid chromatography with electrochemical detection for quantifying levels of plasma catecholamines used in this study allowed for improved sensitivity, selectivity, and precision compared with radioenzyme assays, which were used in the prior study.

Furthermore, Miksis et al. (2001) measured increases in the heart rate of the bottlenose dolphin in response to playbacks of conspecific vocalizations. Increases in heart rate from baseline were observed in the dolphin with playbacks of jaw clap threats and signature whistles but not in response to tank noise. Heart rate is a measurement of the autonomic nervous system and increases suggest activation of the autonomic nervous system, similar to increases in catecholamines as found in the white whale. Although there were no observed increases in catecholamines after impulsive sound exposure for the bottlenose dolphin as there were for the white whale, it cannot be concluded with confidence that there were no significant effects for these hormones, given the small sample size for BEN in the water gun experiments. Measurements of catecholamines and heart rate in the same animal before and after sound exposures warrant investigation and future studies.

The catecholamines function as neurotransmitters and hormones and play important roles in maintaining homeostasis, yet very little information is available in regard to cetaceans. J.R. Geraci (Mystic Aquarium and the Institute for Exploration, Mystic, CT 06355, USA, and Baltimore Aquarium, 501 East Pratt Street, Baltimore, MD 21202, USA, personal communication) measured catecholamine levels in white whales after chase and capture with levels far exceeding those observed in MUK after high-level sound exposure. The catecholamines are among the first molecules released in response to "stress" and are short-lived in most mammals (Fowler 1995; Young and Landsberg 1998). Given the short half-life of catecholamines, it was surprising that catecholamine levels remained high in MUK 1 h after sound exposure. However, studies of repeated chase and encirclement with a purse-seine net on spotted dolphins in the Eastern Tropical Pacific showed elevated catecholamine levels several hours after initiation of the chase (St. Aubin 2002), with dopamine showing unusually high levels when compared with terrestrial species. Although we observed increases in catecholamine levels with increasing sound exposure, the TTS observed in MUK was associated with the highest levels of dopamine but not necessarily the highest levels of epinephrine and norepinephrine. Given all of the above observations, it is possible that the synthesis and metabolism and (or) sources and mechanisms of action of catecholamines may be different in cetaceans than in terrestrial mammals. This is highly likely, given the pressor effects of catecholamines and their influence on the vasculature and circulation (Young and Landsberg 1998) and the unique aspects of the circulatory and vascular system (e.g., thoracospinal rete, large portal system, large posterior vena cava, and countercurrent heat exchangers) and diving adaptations in cetaceans (Slijper 1962; Williams et al. 1999; Reynolds et al. 2000).

Other changes (in addition to the catecholamines) observed in this study for the white whale included a decrease over time in the MCV, a decrease in alkaline phosphatase, and an increase in GGT. MCV is a hematological parameter, and alkaline phosphatase and GGT are enzymes involved in many tissue and organ functions (i.e., kidney, liver, heart, bone, skeletal muscle, and pancreas). It is difficult to determine whether these serum concentrations were changing over time, independently of the testing conditions, or if there were physiological changes that occurred in the high-level exposure group that persisted over time and affected the control measurements. The measurements from the baseline and 24 h after exposure groups showed the same trends over time, which could support either theory. However, when effects were measured as the difference in the 24-h and baseline data, only alkaline phosphatase showed significant effects of the high-sound levels. It is important to consider, however, that alkaline phosphatase can be affected by a number of factors including age, nutritional status, and health status (Fothergill et al. 1991; McBain 2001).

The bottlenose dolphin, BEN, showed different changes in neural–immune parameters than the white whale. BEN showed significant increases in aldosterone and a decrease in absolute

monocyte levels after the impulsive loud sound exposure. Aldosterone is a mineralcorticoid released from the adrenal cortex and has been implicated as one of the primary stress hormones in cetaceans and may be a more sensitive indicator of stress than cortisol (Thomson and Geraci 1986; St. Aubin and Geraci 1989; St. Aubin and Dierauf 2001). Monocytes are cells of the immune system that originate in the bone marrow and migrate via the peripheral blood to organs and tissues where they differentiate into macrophages that are capable of phagocytosis and intracellular digestion of invading microorganisms. Their numbers have been shown to decrease as a consequence of stress (Weisse et al. 1990; Dhabhar et al. 1995, 1996). Therefore, the increase in aldosterone and the decrease in absolute monocyte levels after the impulsive loud sound exposure for BEN can be expected; however, this needs confirmation with additional data points. Moreover, the very small sample size for this data set (four experimental observations and three control observations) does not allow us to detect small to moderate effects of the experimental conditions for all neural–immune measurements tested. Furthermore, the small data set did not allow for examination of measurements over time, although it was observed that the levels of aldosterone and monocytes returned to baseline levels within 24 h.

While there was only one slight observed difference in neural–immune measures between exposure groups for BEN in the tone sound experiment, there were significant changes over time for multiple neural–immune parameters. It is interesting to note that the catecholamine levels and the cortisol levels as well as total white blood cells including neutrophils were high in the first few weeks of the experiment but steadily decreased over the experimental period with no significant differences in the experimental and control conditions. The confounding variable of the new testing environment (the pool versus the open-water environment) may play a role in these changes; however, there was a run-in period for the dolphin to get used to the pool for 4 months before the loud sound exposure sessions and control sessions were run 1 month before sound exposure sessions. Regardless of the run-in period, there were observations of a stress response in the beginning of the experimental period with higher neutrophils, cortisol, and catecholamines and lower lymphocyte percentages and subsets that decreased or increased appropriately as the experimental period advanced. It should be kept in mind that these results could reflect "anticipation" of the pool and experimental procedures, with an adaptation to the new experimental conditions over time.

The limitations of this study are recognized. Although the TTS experiments offered an opportunity to obtain blood samples before and after sound exposure, the neural–immune study was compromised to accommodate the primary TTS study. For instance, the shortest time period that a blood sample could be obtained postexposure was 1 h so as not to interfere with the subsequent auditory test after the sound exposure. Because of experimental limitations, only two cetaceans were used in these studies, which were of different species. The fact that these animals are housed in San Diego Bay, an environment with harbor activity and noise, may affect the perception of noise as a stressor in these animals. The pool condition for BEN, while offering a more "quiet" environment for the hearing tests, added a confounding variable (a new environment and testing setup) to the neural–immune study. Moreover, because a number of measurements were considered in this study, it is possible that some statistically significant differences are spurious. However, we find additional evidence that some of these measurements (such as the catecholamines in the whale) are indicators of stress, since they are significantly associated with the sound level and continued to show significant differences when adjusting for the date of the experiment. It should be kept in mind that further studies are needed to verify these results with more animals from both species.

Despite the limitations and constraints in investigating the effects of sound on the health of marine mammals, there is a recognized need for such studies (Richardson et al. 1995; Popper et al. 2000; US Department of Commerce and US Navy 2001). This is the first attempt to investigate the effects of sound on the cetacean nervous and immune systems. Studies such as this one, utilizing cetaceans kept under human care and carried out under controlled experimental conditions, offer valuable information regarding sound exposure for cetaceans in the wild (with recognized limitations). Specific examples of anthropogenic sound sources that have brought about controversy and questioning as to effects on marine mammals include (*i*) ATOC (acoustic thermometry of ocean climate) experiments involving the transmission of low-frequency sounds over ocean basins to study global ocean temperatures, (*ii*) SURTASS LFA sonar, a long-range low-frequency sonar signal that allows the US Navy to detect and find quiet submarines, (*iii*) "ship-shock" trials of US Navy ships that called for detonations, (*iv*) LFAS, a low-frequency active sonar system operated by NATO, and (*v*) LWAD (littoral warfare advanced development sea test) designed to test technologies with active acoustic components for the US Navy including utilization of midrange-frequency sonar. Moreover, mass strandings of multiple cetacean species over the past few years have occurred during similar time periods and geographic locations as the testing of the above, e.g., Bahamas stranding event of March 2000. Experiments such as this neural–immune study carried out in conjunction with auditory experiments such as the TTS study on cetaceans kept under human care will provide a guideline as to the hearing and nervous and immune system effects of various types of anthropogenic sound on cetaceans. Our studies and those of Schlundt et al. (2000) and Finneran et al. (2002) show that these studies can be done safely without long-term detriment to the animals. A full workup of live stranded as well as beached and expired cetaceans during future stranding events is required to provide additional information on these events and their causes. Testing the auditory system and obtaining blood samples to carry out neural–immune measures on live stranded animals will provide real-time data on wild cetaceans. A complete necropsy workup of beached cetaceans including sampling of heart, adrenals, and lymphoid organs will provide information on the state of autonomic nervous activation and immune status.

Future studies will be directed at measuring similar and additional neural–immune parameters (some currently being developed) after longer duration simulated sonar pings, after multiple simulated sonar pings, and after actual shipborne tactical sonar on US Navy dolphins. This study is the first

attempt to investigate the autonomic nervous system and immune systems after loud sound exposure in cetaceans in helping to understand the effects of anthropogenic sound on marine mammal health.

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