

Exxon Valdez Oil Spill State/Federal
Natural Resource Damage Assessment
Final Report

Biomarkers of Damage to Sea Otters in
Prince William Sound, Alaska,
Following Potential Exposure to Oil Spilled
from the *Exxon Valdez*

Marine Mammal Study 6-1
Final Report

Brenda E. Ballachey

U.S. Fish and Wildlife Service
Alaska Fish and Wildlife Research Center
1011 East Tudor Road
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Study History: Marine Mammal Study 6 (MM6), titled *Assessment of the Magnitude, Extent and Duration of Oil Spill Impacts on Sea Otter Populations in Alaska*, was initiated in 1989 as part of the Natural Resource Damage Assessment (NRDA). The study had a broad scope, involving more than 20 scientists over a three year period. Final results are presented in a series of 19 reports that address the various project components. An earlier version of this report was included in the November 1990 NRDA Draft Preliminary Status Report for MM6 ("Section 7 - Bioindicators of Damage from Oil Exposure to Sea Otters").

Abstract: This study was conducted to evaluate several biomarkers of genotoxic damage in sea otters that had potentially been exposed to oil spilled from the *Exxon Valdez*. Thirteen adult male sea otters were captured in eastern (unoiled) Prince William Sound, and 14 in western (oiled) Prince William Sound in September and October 1991. Blood lymphocytes, sperm and testicular cells were collected from the otters for flow cytometric analyses to measure: 1) DNA content of lymphocytes, 2) nuclear chromatin structure of sperm, and 3) subpopulations of cell types in the testis. Additionally, sperm cells were examined by light microscopy for morphological abnormalities. The DNA content of blood lymphocytes from sea otters in the oiled vs. unoiled areas was not significantly different, although there was greater variation among samples from the oiled area. One measure of sperm cell quality was poorer for male sea otters from the unoiled area, and may have been associated with differences in the age and breeding status of the two groups sampled. Other measures of sperm and testicular cells did not differ between areas. The percentage of sperm exhibiting morphological abnormalities (primarily of the midpiece and tail) was high (>80%) and similar in both areas. This study did not provide conclusive evidence of genotoxic damage associated with oil exposure in sea otters.

Key Words: biomarkers, *Enhydra lutris*, *Exxon Valdez*, flow cytometry, lymphocytes, oil spill, sea otter, sperm, testis.

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EXECUTIVE SUMMARY

This study was conducted to evaluate several biomarkers of genotoxic damage in sea otters that had potentially been exposed to oil spilled from the *Exxon Valdez*. Thirteen adult male sea otters were captured in eastern (unoiled) Prince William Sound, and 14 in western (oiled) Prince William Sound in September and October 1991. Blood lymphocytes, sperm and testicular cells were collected from the otters for flow cytometric analyses to measure: 1) DNA content of lymphocytes, 2) nuclear chromatin structure of sperm, and 3) subpopulations of cell types in the testis. Additionally, sperm cells were examined by light microscopy for morphological abnormalities. The DNA content of blood lymphocytes from sea otters in the oiled vs. unoiled areas was not significantly different, although there was greater variation among samples from the oiled area. One measure of sperm cell quality was poorer for male sea otters from the unoiled area, and may have been associated with differences in the age and breeding status of the two groups sampled. Other measures of sperm and testicular cells did not differ between areas. The percentage of sperm exhibiting morphological abnormalities (primarily of the midpiece and tail) was high (>80%) and similar in both areas. This study did not provide conclusive evidence of genotoxic damage associated with oil exposure in sea otters.

INTRODUCTION

Several thousand sea otters are estimated to have died acutely as a result of exposure to oil spilled by the *Exxon Valdez* (DeGange et al. 1994; Garrott et al. 1993). For surviving otters, there may be long-term detrimental effects from acute exposure to freshly spilled oil as well as from chronic exposure to petroleum hydrocarbons persisting in the environment. Assessing the impacts of hydrocarbon residues on the capacity of the sea otter population to recover to pre-spill levels is important to determine the full extent of the injury.

Sub-lethal exposure to oil, directly or through contaminated prey, may lead to accumulation of toxins and impairment of normal physiological processes in the otters. Pathological changes affecting long-term fitness may result. Recent biotechnical advances in evaluation of cellular components, including DNA, are applicable to damage assessment studies. Controlled laboratory studies and a more limited number of field studies have demonstrated the utility of these techniques for evaluating genotoxic injury following chemical exposures (McBee and Bickham 1990).

Flow cytometry is a relatively new and powerful technology for cellular analyses (Melamed et al. 1979). In general, flow cytometry involves labelling cells in suspension with a fluorescent dye which binds to the component of interest (e.g., DNA). The cells are then passed single file in a fluid stream through a laser beam. The fluorescent signals emitted from the cells upon laser excitation are detected by photomultiplier tubes and processed by a computer interfaced to the flow cytometer. Advantages of flow cytometry include rapid measurement of large numbers of cells per sample, unbiased selection of cells for measurement, multiparameter measurements per cell, and ease of counting subpopulations of cell types.

Nuclear DNA content can be a sensitive indicator of injury to developing cells from clastogenic contaminants in the environment. In a normal or unaffected animal, the DNA content (2c) of cells in the G1 stage of the cell cycle (such as blood lymphocytes) should be uniform. Chromosome aberrations induced by toxic chemicals, however, can lead to differences in DNA content among cells. Cells can be measured by flow cytometry and, for normal samples, the resulting frequency histogram of DNA content should have a very low coefficient of variation. In samples containing cells with abnormal DNA content, deviations are detected as an increase in the coefficient of variation, reflecting injury to the chromosomes. The efficacy of these measurements for detecting injury from environmental pollutants has been shown in wild rodents by McBee and Bickham (1988) and in turtles by Bickham et al. (1988).

Spermatozoa are another cell type in which injury to DNA can be assayed by flow cytometry. The developing spermatocytes in the testes are vulnerable to injury because they are rapidly dividing. If they are injured, this will be reflected in the mature, ejaculated spermatozoa. The Sperm Chromatin Structure Assay (SCSA) was developed to measure effects of toxic exposure on the stability of the sperm nuclear chromatin (Evenson et al. 1980). Studies on bulls, mice and rats have documented decreased chromatin stability (measured as the ratio of single-stranded to double-stranded DNA) following exposure to chemical agents (Ballachey et al. 1986, Evenson 1986, Evenson et al. 1985, 1989), and the SCSA is being tested as a routine assay to evaluate occupational exposures in humans (D.P. Evenson, Biomedical Diagnostics, Brookings, S.D., personal communication).

The SCSA has also been applied to evaluate fertility. A relationship between traditional measures of semen quality and stability of sperm chromatin has been clearly established and linked to decreased male fertility (Ballachey et al. 1987, 1988, Evenson and Melamed 1983, Evenson et al. 1980). That is, if an individual's sperm cells have less stable chromatin, then he is more likely to have impaired fertility. The SCSA thus provides an index of chemical damage to DNA of developing sperm cells and an indication of the relative fertility potential.

Morphology of sperm cells may also be affected by exposure to toxins. Measurement of samples by flow cytometry is far more rapid than scoring slides by light microscopy, and may be more sensitive for detection of damage. However, the proportion of sperm cells exhibiting abnormal morphology is an alternate indicator of genotoxic damage to sperm cells (Wyrobeck et al. 1983).

Further information on the process of spermatogenesis can be gained by flow cytometry of testicular cells. A DNA profile is obtained, quantifying subpopulations of cell types with differing DNA content, including tetraploids (4C), diploids (2C) and haploids (1C). Altered proportions of cell subpopulations may be indicative of deleterious effects of chemical agents. In severe cases of exposure to toxins, subpopulations of haploid cells (i.e., the developing spermatids) may be completely destroyed.

This study was conducted to evaluate potential genotoxic damage in male sea otters living in areas of western Prince William Sound (PWS) affected by the *Exxon Valdez* oil spill through flow cytometric analysis of blood lymphocytes, sperm and testicular cells. Sea otters from unoiled areas of eastern PWS served as a non-exposed control group.

OBJECTIVES

The objective of this study was to test the following hypotheses, based on flow cytometric analyses of blood lymphocytes, sperm and testicular cells from adult male sea otters:

1. Variation of DNA content in blood lymphocytes does not differ significantly between male sea otters living in eastern and western PWS.
2. DNA structure in sperm cells (measured by the stability of nuclear chromatin) does not differ significantly between male sea otters living in eastern and western PWS.
3. Spermatogenesis, measured by a DNA profile of the testicular cells, does not differ significantly between male sea otters living in eastern and western PWS.
4. Proportion of morphologically normal sperm cells, measured by light microscopy, does not differ significantly between male sea otters living in eastern and western PWS.

METHODS

Sea Otter Capture and Handling

Twenty-seven male sea otters were caught in unoiled areas of eastern PWS and oiled areas of western PWS between September 11 and October 4, 1991. Thirteen males were caught in Orca Inlet, EPWS. Fourteen males were caught in three areas of WPWS: Sawmill Bay on the east side of Evans Island, Prince of Wales Passage, and Channel Island in Montague Strait.

The sea otters were caught in tangle nets, transported to a support vessel, and sedated shortly thereafter. Otters were sedated with a combination of fentanyl citrate and azaperone. Blood was collected by jugular venipuncture, sperm cells by electro-ejaculation, and a testicular sample by fine needle aspiration. A premolar tooth was taken for age determination. Following collection of samples, which took approximately 30 minutes, the otters were given naloxone HCl to reverse the narcotic, and released from 1 to 2 hours later.

Blood Lymphocytes

Approximately 30 cc of blood was taken from each otter and processed to obtain separate fractions for hematology and serum chemistry (results of these analyses are presented by Rebar et al. 1993), and white blood cells. White blood cells were suspended in 1 ml of a cyroprotective media containing Ham's F10 cell culture media with fetal calf serum (18%) and glycerine (10%). The cells were refrigerated for several hours then frozen in liquid nitrogen. Samples were subsequently sent to LGL Ecological Genetics in Bryan, Texas, for measurement of blood lymphocyte DNA content.

Methods for flow cytometric analysis of blood lymphocyte DNA are presented in greater detail in Appendix A, a report submitted by Drs. J. Bickham and M. Smolen of LGL Ecological Genetics. Briefly, the assay involved staining the cells with propidium iodide, a DNA specific dye, and measuring the amount of emitted red fluorescence on 400-10,000 cells per sample. Measurements were taken with a Coulter Profile II flow cytometer. The variable of interest from this assay of lymphocytes is "HPCV" (half peak coefficient of variation), which quantifies the variation among cells within a sample in the amount of emitted fluorescence and, correspondingly, in the DNA content.

Sperm and Testicular Cells

Semen samples were collected by electro-ejaculation of the otters. A drop of the semen was fixed in a solution of paraformaldehyde/glutaraldehyde, and refrigerated for later morphological analysis of the sperm cells. The remaining sample was diluted approximately 1:1 with a small amount of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1mM disodium EDTA, pH7.4) and frozen in cryotubes in liquid nitrogen. Testicular cells were collected by fine needle aspiration biopsy using an 18 gauge needle attached to a syringe containing .5 ml HBSS (Hank's balanced salt solution), and fixed by mixing the aspirated cells and HBSS into 3 ml of a 96% ethanol solution.

Flow cytometric analyses of sperm and testicular cells were done at the laboratory of Biomedical Diagnostics, Brookings, South Dakota. Measurements were made with a

Cytofluorograf II (Ortho Diagnostic Systems, Westwood, MA) interfaced to an Ortho Diagnostics 2150 data handling system. Five thousand cells were measured per sample wherever possible. Some samples had very low concentrations and fewer cells were measured.

The assay on sperm involves treating the cells with a mild acid solution then staining with acridine orange, a dye which allows simultaneous measurement of double-stranded and single-stranded DNA content (Darzynkiewicz 1979). A normal, healthy cell will have negligible amounts of denatured, single-stranded DNA after the acid treatment. Increased amounts of single-stranded DNA reflect decreased structural stability of the chromatin, indicative of disturbances of spermatogenesis. For each cell, the relative amounts of double- and single-stranded DNA are measured. An index, α_t , is used to quantify the relative amounts of double and single-stranded DNA. A distribution of α_t values is obtained for each sample, with a corresponding mean ($X\alpha_t$) and standard deviation ($SD\alpha_t$). Higher values of $X\alpha_t$ and $SD\alpha_t$ are indicative of poorer quality sperm cells. An additional value, $COMP\alpha_t$, reflects the actual percentage of "abnormal" sperm cells in each sample, based on degree of DNA denaturation. Appendix B contains a more detailed explanation of the derivation and application of α_t , and greater detail on the flow cytometry assays described below for sperm and testicular cells.

The sperm samples were prepared for the SCSA as described by Ballachey et al. (1988). Samples were thawed and the concentration of sperm was quickly estimated with a hemocytometer count. Depending on the concentration, the sample was diluted as needed with TNE buffer. An aliquot of the diluted sample was mixed with an acid solution to improve dye uptake and potentially induce partial denaturation of the DNA in chromatin. After 30 seconds, a solution with acridine orange stain was added and samples were measured 3 minutes later by flow cytometry. Relative levels of double-stranded and single-stranded DNA in the nucleus of each cell were recorded. Values for each sample were processed by on-line software programs to obtain a frequency distribution and associated α_t statistics for each sample.

For flow cytometry of the testicular cells, the samples were spun down and the ethanol fixative solution was pipetted off. Pellets were washed in buffer, allowed to equilibrate and treated with RNAase. A propidium iodide staining solution was added to the sample, and flow cytometry measurements were made after 3.5 min. of staining. The relative DNA content of 5000 cells per sample was measured, providing a DNA profile. Proportions of tetraploid (4C DNA content), diploid (2C DNA content) and haploid (1C DNA content) cells were computed for each sample using software programs for that purpose. Lower values for percent haploid cells in the testis are indicative of disturbances of spermatogenesis.

Data Analysis

Comparisons between samples collected in eastern and western PWS were done by t-test. Results presented herein represent an exploratory data analysis designed to indicate which variables may be useful for measuring exposure to oil in future studies. The analyses for the different variables are not independent since all variables were measured on the same set of animals. Significance levels presented for each variable are on a per test rather than experiment-wise basis.

RESULTS

Blood Lymphocytes

Mean values for HPCV were 4.90 (n=11) and 5.06 (n=14) for eastern and western PWS blood lymphocyte samples, respectively (Table 1). These means did not differ significantly; however, variances (.56 and 2.31 for eastern and western groups, respectively) differed significantly ($P < .02$) between the two groups, with the sample from western PWS exhibiting greater variation.

Sperm Cells - Sperm Chromatin Structure Assay

Mean values for $X\alpha_t$, $SD\alpha_t$ and $COMP\alpha_t$ are presented in Table 1. Samples from eastern PWS were significantly higher ($P < .05$) in mean value of $X\alpha_t$ and $COMP\alpha_t$ than samples from the west. Semen collection attempts were not successful on all otters, and thus sample sizes for sperm were 7 for eastern PWS and 9 for western PWS, less than half of the sample size (20 per group) called for in the original study design.

Testicular Cells

No differences were noted among sea otters from eastern and western PWS in percentage of haploid, diploid or tetraploid cells. Mean values for testicular cell types are presented in Table 1. Percentage of haploid cells in the testis ranged from 33.4% to 81.5%.

Sperm Morphology

Sperm morphology was scored on only 7 semen samples, due to very low concentrations of sperm in many of the ejaculates. Percentages of morphologically abnormal sperm were high, ranging from 57% to 98%. The eastern and western mean values were similar (83.7% and 88.8%, respectively); these means did not differ significantly (Table 1).

DISCUSSION

The increased variability of HPCV values in the western sea otters in conjunction with the the lack of difference between mean HPCV values for eastern and western groups may indicate that any exposure to toxic petroleum residues has been at a low and/or variable level, so that not all individuals in the population show an effect. It also may be that the population is recovering following a relatively high initial exposure to oil; chronic exposure may have a less toxic effect. Individual sensitivity to genotoxic injury can also be expected to vary. The two highest HPCV values were observed in otters from WPWS, and two other values from the west were relatively high. Replicate analyses were not run on the samples, and thus experimental artifact cannot be ruled out as a factor in these results. No apparent health problems were observed when the otters were handled, and there was no correlation between the HPCV values and the α_t values obtained on sperm or testis cells. (See Appendix A for additional discussion of the results on blood lymphocytes).

For this study, samples were collected from the sea otters approximately 1.5 years after the spill, which is a relatively long time from the initial, and presumably most toxic, exposure. Collection of the samples much closer to the time of the spill would have been preferable, but was not an option. An assumption inherent in the design of the present study was that males living in oiled areas were exposed to oil, whereas those in unoiled areas were not. However, male otters in PWS have been observed to make relatively large movements (Garshelis et al. 1984), complicating our ability to make inferences about the uniformity of exposure levels.

Differences in α_t values between males from eastern and western PWS suggested that males in the eastern Sound actually have significantly poorer sperm cell quality. A likely factor in this difference is that capture of sea otters in eastern PWS was in a non-breeding area known to have high concentrations of males and very few females, whereas capture in western PWS targeted breeding areas, with relatively high concentrations of female sea otters. Fall is the season during which there is the greatest extent of breeding activity for sea otters (Garshelis et al. 1984). The group of males sampled from western PWS in this study would likely have included several breeding individuals; these animals, if producing sperm cells on a regular basis, may be expected to have higher quality semen samples compared to male otters that were not actively breeding. Younger males that are just becoming reproductively mature would also be expected to have poorer quality semen samples than reproductively mature males. In this study, the mean age of the two groups was relatively similar (5.5 years for eastern males and 6.1 years for western males, Table 1). However, the median ages were 3 and 6 years for eastern and western groups respectively, suggesting that differences in reproductive maturity may have contributed to variation in sperm cell quality between areas. Males otters from breeding areas in eastern and western PWS would have provided a more appropriate comparison of sperm cell quality, but restrictions on areas in which capture was allowed prevented sampling of breeding males from eastern PWS.

The slightly lower mean value for percentage haploid cells in the testis samples from males in eastern PWS also may reflect differences in breeding status of eastern vs. western males. The very low value of 33.4% was obtained on a sample from an old otter (16 years) caught in EPWS, and likely represents some type of testicular dysfunction associated with senescence. Percentage haploids was highly correlated with percentage diploids and tetraploids ($r^2 = -.89$ and $-.87$, $P < .0001$, respectively), as expected when measuring proportions.

The reason for high proportions of morphologically abnormal sperm in the samples from both sides of PWS have not been ascertained. Relatively low proportions of morphologically normal sperm have been reported in other canids (Howard 1993). In the sea otters, abnormalities were primarily of the midpiece and tail rather than of the sperm head, and may be associated with ejaculation of relatively immature sperm cells.

CONCLUSIONS

Evaluation of variation in blood lymphocyte DNA content (HPCV) in samples collected from adult male sea otters in oiled and unoiled areas of PWS indicated that mean HPCV did not differ by area, although HPCV values of the samples from the oiled area were more variable than those from the unoiled area. Assays on sperm and testicular cells did not

provide evidence of toxic exposure to those cells. This study did not provide conclusive evidence that otters potentially exposed to oil suffered genotoxic injury.

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Table 1. Means and standard deviations of measurements taken on adult male sea otters from Prince William Sound^a.

Value	Area		Level of Significance ^b
	East PWS	West PWS	
Lymphocytes-HPCV	4.90 ± 0.75 (n = 11)	5.06 ± 1.52 (n = 14)	ns
Sperm DNA ^c			
X α_t	237.2 ± 14.4	223.8 ± 9.9	P < 0.05
SD α_t	29.3 ± 6.9	24.6 ± 5.8	
COMP α_t	14.8 ± 6.6 (n = 7)	8.4 ± 5.4 (n = 9)	P < 0.05
Sperm Morphology			
% Abnormal	83.7 ± 23.1 (n = 3)	88.8 ± 5.4 (n = 4)	ns
Testis Cells			
% Haploid	69.5 ± 14.5	73.9 ± 6.0	ns
% Diploid	17.9 ± 7.2	15.6 ± 6.1	ns
% Tetraploid	16.6 ± 7.6 (n = 10)	10.5 ± 2.6 (n = 4)	ns
Age ^d	5.5 ± 4.6 (n = 10)	6.1 ± 1.9 (n = 14)	ns

^a 27 otters were captured but because not all samples were successfully collected or processed from each otter, n values for different measurements vary.

^b From a t-test to test differences between area means. ns = not significantly different.

^c The α_t values from the Sperm Chromatin Structure Assay.

^d Estimated from premolar section.

Appendix A. Genotoxic effects of exposure to the *Exxon Valdez* oil spill in sea otters (*Enhydra lutris*) assayed by flow cytometry. Authors: J. W. Bickham and M. J. Smolen. LGL Ecological Genetics Inc.

Exposure to genotoxic pollutants has the potential to result in DNA damage to somatic and germinal tissues. The effects on germinal cells results in reduced fertility as well as heritable mutations. Exposure of somatic tissues results in various effects including physiomorphological (growth rate alterations, respiration patterns, blood chemistry, enzyme levels, histological and general morphological parameters) effects, ecological effects such as changes in community structure, genetic effects such as tumor formation, behavioral effects, and bioaccumulation. The use of natural populations of mammals as sentinels to detect such responses was recently reviewed by McBee and Bickham (1990). Although sea otters have not been used to detect the toxic effects of petrochemicals in the environment, both American and European river otters have been investigated as to the bioaccumulation of heavy metals (Anderson-Bledsoe and Scanlon 1983, Mason et al. 1986). Potentially, such marine and fresh water mammals are valuable indicators of environmental damage because of their position at the top of the food chain. Not only direct exposure of the otters to chemicals in the water, but also chronic exposure through food items in which chemicals might become concentrated, can cause specific physiological effects. This study investigated potential DNA damage in the white blood cells of sea otters that were exposed to the Exxon Valdez oil spill in Prince William Sound using flow cytometric measurements of DNA content from non-exposed control, and exposed experimental, wild-caught animals.

Blood samples were obtained from adult male sea otters collected between 11 September and 4 October 1990. A total of 25 specimens were examined: 11 from a population that was not exposed to the oil spill (controls) and 14 from a population exposed to the spill (experimentals). Whole blood was drawn into heparinized vacutainer tubes and centrifuged to separate the cellular components. The white blood cells, contained in the buffy coat just above the red blood cells, were removed and placed into cryotubes containing 1 ml of Ham's F10 cell culture media with fetal calf serum (18%) and glycerine (10%). The cells were refrigerated a few hours before being stored in liquid nitrogen. The frozen cells were shipped to Texas A&M University for cytometric analysis.

Cells were thawed, diluted to a total volume of 2 ml with Hanks' balanced saline solution, filtered through 35 μ m nylon mesh filter cloth, and centrifuged. Media and Hanks' solution were discarded and the pelleted cell button was resuspended in 0.5 ml Hanks' solution with RNAase (10 mg/100 ml Hanks' solution). Cells were incubated at room temperature for 10 minutes and then 10 drops of Hanks' solution with propidium iodide (41 mg/100 ml Hanks' solution) was added. The cells were allowed to stain for 10 minutes before being analyzed on a Coulter Profile II flow cytometer.

Intensity of red fluorescence was measured from approximately 400-10,000 cells per individual. Fluorescence was measured both from gated and ungated 2C cell populations. Cells were gated on forward-angle light scatter to minimize the contribution of nuclei with

cytoplasmic debris attached. Fluorescent microspheres (beads) were analyzed periodically to check the alignment and focus of the cytometer. The machine was highly stable throughout the study.

The differences in group means and coefficients of variation (half-peak or HPCV) were not statistically significant. This means that mean genome size and dispersion of DNA values around the means were not significantly influenced by exposure to the oil spill. However, variance was significantly increased in the experimental population relative to the control population ($P=0.03$) for the gated HPCV. It thus appears that exposure to the oil spill has resulted in increased variability for gated HPCV in blood cells, which is an anticipated response to exposure to genotoxins, particularly in uncontrolled field situations. To underscore this observation, there are four (out of 14) animals from the experimental population that exceeded the 95% confidence limits of the control population. Three of the four also exceed the 99% confidence limits and two of these animals had the highest CVs found in the study. It can be concluded that these four animals have experienced a significant effect from mutagen exposure.

In summary, the data presented in this report are interpreted to mean that a significant effect was observed in the variation of HPCV in the experimental population likely as a result of exposure to environmental mutagens resulting from the *Exxon Valdez* oil spill. Although these effects are significant, they appear to be less severe than those observed in some other studies of vertebrates exposed to petrochemical (McBee and Bickham 1988) and nuclear (Bickham et al. 1988, Lamb et al., in press) environmental contaminants. In those studies, the mean coefficients of variation were increased in the exposed populations. The fact that mean HPCVs were not increased in the sea otters could be due to the fact that the population is in the process of recovering from the insult. If that is so, then enough of the individuals in the population have recovered to bring the mean values close to that of the control necessitating larger sample sizes to detect any significant difference. However, some animals have not yet recovered to control levels. This is possibly the result of these individuals being more sensitive to DNA alterations or else variation in the degree of exposure (or both).

It thus appears that additional studies on the mutagenic effects of oil exposure in sea otters is warranted. Larger sample sizes from more populations are needed to better define the effects and response of the animals. The monitoring of these populations in the years to come would be highly desirable.

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Appendix B. Analytical methods for sperm and testicular samples.

The Sperm Chromatin Structure Assay (SCSA)

The SCSA is a technique that uses acridine orange (AO) staining and flow cytometry to evaluate the structural stability of sperm nuclear chromatin. The utility of AO is based on its metachromatic emission of fluorescence upon laser excitation. AO intercalated into double-stranded nucleic acids fluoresces green, whereas when bound to single-stranded nucleic acids, it fluoresces red. Mature sperm cells are essentially devoid of RNA and so the red and green fluorescence intensities of AO-stained sperm are indicative of levels of single and double stranded DNA, respectively.

Alpha-t (α_t), defined as the ratio of red to total (red + green) fluorescence, is computed for each sperm cell by computer protocols, and the distribution of the α_t values computed for each sample. Although the theoretical distribution of α_t values is continuous from 0 to 1, the measurements are made over 1000 channels (levels) of fluorescence, and thus values obtained and analyzed are expressed on a scale of 1 to 1000.

Based on the distribution of α_t values, the following statistics are computed for each sample: 1) the mean of the alpha-t distribution ($X\alpha_t$), 2) the standard deviation of the alpha-t distribution ($SD\alpha_t$), and 3) proportion of "cells outside the main peak" of alpha-t ($COMP\alpha_t$). The last value, $COMP\alpha_t$, is essentially equal to the percentage of cells that have increased susceptibility to denaturation, relative to the "normal" cells in the sample. All three values are useful to quantify differences in susceptibility of the DNA in chromatin to denaturation. Higher values are associated with increased levels of chromatin heterogeneity and single-stranded DNA, and a "poorer" quality semen sample.

Flow Cytometry Measurements

Measurement of DNA in sperm cells and testicular biopsies was done by Dr. Donald Evenson of Biomedical Diagnostics, located in Station Biochemistry, South Dakota State University, Brookings. This flow cytometry laboratory is the only one in North America which is routinely measuring sperm cells by the SCSA. Measurements were made with a Cytofluorograf II (Ortho Diagnostic Systems, Westwood, MA) interfaced to an Ortho Diagnostics 2150 data handling system. Five thousand cells were measured per sample wherever possible. Some sperm samples were very low in concentration and fewer cells were measured.

For flow cytometry of sperm cells, samples were prepared as described for the SCSA by Ballachey et al. (1988). Samples were thawed and the concentration of sperm was quickly estimated with a hemocytometer count. Depending on the concentration, the sample was diluted as needed with TNE buffer to achieve a concentration of $1-2 \times 10^6$ sperm/ml. A 0.2 ml aliquot of the diluted sample was mixed with 0.4 ml of a solution containing 0.1% (v/v) Triton X-100, 0.08 N HCl, and 0.15 M NaCl (pH 1.2). This low pH, non-ionic solution

improves dye uptake and potentially induces partial denaturation of the DNA in chromatin. After 30 seconds, 1.2 ml of staining solution (0.2 M Na₂HPO₄, 1mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, pH6.0, with 0.006 mg acridine orange stain added per ml) was added and samples were measured 3 minutes later by flow cytometry. Relative levels of double-stranded and single-stranded DNA in the nucleus of each cell were recorded; the ratio of single-stranded to total DNA provides an index, α_1 , of chromatin stability.

For flow cytometry of the testicular cells, the samples were spun down and the ethanol fixative solution was pipetted off. Pellets were resuspended in 4 ml of PIPES buffer (0.1 M PIPES and 0.002 M MgCl₂) and allowed to equilibrate for 10 minutes. Samples were respun and resuspended in 1 ml of PIPES buffer with 0.1% Triton-X added (pH 6.4). Five λ of RNAase was added and the sample incubated at room temperature for 30 minutes, then placed on ice. For FCM measurement, an aliquot of sample was admixed with 0.4 ml of Triton-X staining solution and 1.2 ml of propidium iodide staining solution was added. FCM measurements were made 3.5 min. after staining. The relative DNA content of 5000 cells per sample was measured by flow cytometry, providing a DNA profile. Proportions of tetraploid (4c), diploid (2c) and haploid (1c) cells were computed for each sample using software programs for this purpose.

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