Exxon Valdez Oil Spill
Restoration Project Final Report

Synthesis of Nearshore Recovery following the 1989 *Exxon Valdez* Oil Spill: Sea Otter Liver Pathology and Survival in Western Prince William Sound, 2001 – 2008

Restoration Projects 070808 and 070808A
Final Report

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February 2014

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Study History:

The sea otter population in western Prince William Sound (WPWS) was injured as a result of the 1989 *Exxon Valdez* oil spill (EVOS). The U.S. Geological Survey, Alaska Science Center has conducted a range of studies from 1989-2013 to assess the post-spill status of sea otters in WPWS and evaluate factors constraining population recovery. In this report, we present two components of Restoration Project –0808: (1) an evaluation of liver pathology and histopathology of sea otters sampled at sites with varying oiling histories from 2001-2008 (component of Project 070808A), and (2) an analysis of correlates of survival of sea otters captured, radio-tagged and monitored in oiled areas of WPWS from 2002-2008 (component of Project 070808). Work presented herein builds on previous Restoration Studies 01534, 02585, 030620, 040774, and 0407758.

Abstract:

We examined livers and liver biopsies collected from captured sea otters in WPWS, 2001–2008, to determine whether indicators of liver health correlated with history of oil contamination from the 1989 *Exxon Valdez* oil spill. Sea otters captured in oiled areas had a significantly higher proportion of livers with gross pathological change, based on visual inspection at the time of capture, than those from unoiled areas. Of the 10 histopathology variables scored on liver biopsies, only two (vacuolar change and pigment) differed between animals from oiled and unoiled areas, and neither correlated with gross pathology scores. Vacuolar change indicates physiological disturbance, which is consistent with potential effects from oil exposure but also could be influenced by a number of other factors. We concluded that, as of 2008, some differences in liver health were evident between sea otters from oiled and unoiled areas; these differences were consistent with, but not specific to, effects that might be expected with sub-lethal exposure to lingering *Exxon Valdez* oil. We also quantified variation in survival of radio-marked sea otters within oiled areas of WPWS in relation to age, sex, body condition, selected blood serum chemistry variables, and histological scores indicative of liver health. Of the variables considered, only the serum enzyme aspartate aminotransferase (AST) and the ratio of serum proteins albumin and globulin (A/G) were correlated with survival, with higher levels of AST and lower levels of A/G associated with increased likelihood of mortality. High AST and low A/G both may be indicative of liver disease. Taken together, results reported here suggest that liver health of sea otters in oiled areas was slightly poorer than those from unoiled areas and,

further, that this may have translated to poorer survival through 2008, nearly 2 decades after the spill. More recently collected information indicated that mortality patterns and abundance had returned to pre-spill conditions between 2010 and 2013, suggesting that the effects that we detected through 2008 may have represented the end of effects related to exposure to lingering oil.

Key Words:

Sea otters, liver, pathology, histopathology, survival, Prince William Sound, oil, *Exxon Valdez*

Project Data:

Data will be kept in digital format (csv files with metadata) at the Alaska Science Center, U.S. Geological Survey, Anchorage, Alaska. Data custodian – Daniel Esler, Research Wildlife Biologist and Project Leader, Nearshore Marine Ecosystem Research Program, Alaska Science Center, U.S. Geological Survey, Anchorage, Alaska.

Citation:

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EXECUTIVE SUMMARY

Since the 1989 *Exxon Valdez* oil spill in Prince William Sound, Alaska, the U.S. Geological Survey has conducted a series of studies to evaluate effects of the spill on sea otters (*Enhydra lutris*) and assess status of population recovery. This report includes two chapters with findings from studies of liver condition and survival of sea otters in western Prince William Sound (WPWS) through 2008.

In the first chapter, we present results of visual examinations of livers at the time of capture and histopathological examination of liver biopsies collected from captured sea otters in oiled and unoiled areas of WPWS, 2001–2008. Based on data collected during the late 1990s, we concluded that continuing exposure to oil was limiting sea otter population recovery (Bodkin and others, 2002; Peterson and Holland-Bartels, 2002). Sea otter survival was depressed in oiled areas (Monson and others, 2000; Ballachey and others, 2003), and we speculated that liver damage resulting from chronic exposure to residual *Exxon Valdez* oil was influencing survival (Bodkin and others, 2002, 2012; Ballachey and others, 2003). To evaluate the hypothesis that chronic oil exposure affected sea otter liver health, we compared liver gross pathology and histopathology scores between sea otters from oiled and unoiled areas of WPWS. We found that a significantly higher proportion of sea otters in oiled areas had livers with gross pathology than those in unoiled areas through 2008. Of 10 histopathology variables scored, two (vacuolar change and pigment) differed between animals from oiled and unoiled areas, and we found no correlation between either of these variables and the gross pathology scores. Differences in pigment are not thought to be a pathological finding. However, differences in vacuolar change between otters from oiled and unoiled areas may be indicative of physiological disturbance in some animals from oiled areas. Vacuolar change could be related to toxicological effects of oil exposure, but also may vary with a number of other factors, including otter condition, reproductive state (i.e., pregnancy or lactation in females), and nutritional status. Our finding of area differences in liver health through 2008 is consistent with other research results from the same time period that suggested continued exposure to lingering oil (Bodkin and others, 2012; Miles and others, 2012), poor survival (Monson, 2009; Monson and others, 2000, 2011), and reduced numbers of sea otters in heavily oiled areas of WPWS (Bodkin and others, 2011).

In the second chapter, we present results of analyses of correlations between sea otter survival and age, sex, body condition, selected blood serum chemistry variables, and histological scores indicative of liver health. This work was based on tracking of radio-tagged otters in oiled areas of WPWS. We hypothesized that elevated mortality potentially related to chronic oil exposure would be reflected in correlations between survival and health attributes (blood chemistry and histopathology). Of the variables examined, only the serum enzyme aspartate aminotransferase (AST) and the ratio of serum proteins albumin and globulin (A/G) were related to survival probability, with higher levels of AST and lower levels of A/G associated with increased likelihood of mortality. High levels of AST and low levels of A/G both may be indicative of liver disease, which we interpret as suggestive that chronic oil exposure and associated liver pathologies may have been compromising sea otter survival through 2008.

When considered together, the results from the 2 chapters in this report suggest that liver health of sea otters in oiled areas was somewhat poorer than in otters from unoiled areas and that this may have affected survival through 2008, nearly 2 decades after the spill. These results are consistent with other findings from that period describing potential chronic effects of lingering oil (e.g., Miles and others, 2012) and lack of full population recovery (Bodkin and others, 2011, 2012). However, these results are best interpreted in light of more recently collected information (Ballachey and others, 2014) which indicated that mortality patterns and abundance returned to pre-spill conditions between 2010 and 2013, suggesting that subtle effects that we detected through 2008 may have represented the end of effects related to exposure to lingering oil.

CHAPTER 1. Examination of Livers from Sea Otters in Western Prince William Sound, Alaska, 2001–2008, for Gross Pathology and Histopathological Change

By B.E. Ballachey, D. Monson, F.C. Mohr, T.P. Lipscomb, M.J. Murray, and S. Howlin

Introduction

Acute effects of the *Exxon Valdez* oil spill (EVOS) included the documented mortality of nearly 1,000 sea otters, with total acute deaths estimated to be as high as several thousand (Ballachey and others, 1994). Acute oil exposure in sea otters resulted in lung, liver, and kidney damage (Lipscomb and others, 1993, 1994). Changes in serum enzymes associated with liver damage were documented in wild sea otters in 1992, and again, although to a lesser extent, in 1996–1998 (Ballachey and others, 2002, 2003), suggesting that sea otters in the wild may have experienced pathologies similar to those seen in animals dying shortly after the spill. As documented by Short and others (2004, 2006) and Li and Boufadel (2010), oil has persisted on shorelines and presented a continuing risk of exposure to animals utilizing nearshore areas (Bodkin and others, 2012) for at least two decades.

In 2001, we initiated studies that included capture of sea otters and collection of liver biopsies. Here we present results of liver examinations, including gross and histological findings, for sea otters sampled from 2001 through 2008 in oiled and unoiled areas of western Prince William Sound (WPWS).

Methods

In summers 2001–2005 and 2008, and late winter 2006, we captured sea otters in WPWS, in the area of northern Knight Island (heavily oiled area) and at Montague Island (unoiled reference area). In 2008, sea otters also were captured at Prince of Wales Passage (moderately oiled area). Capture and handling methods have been described by Bodkin and others (2002, 2012). Briefly, sea otters were sedated and body measurements taken, including mass and total length. A tooth was collected to determine age (Bodkin and others, 1997), and if a tooth was not available, age was estimated based on tooth wear, morphometrics, and pelage coloration. Blood was taken by jugular venipuncture for hematology, clinical chemistry, and biomarker analyses. Sea otters were tagged on the rear flippers with numbered, colored plastic tags (Temple Tag®, Temple, Tex.). Following reversal from anesthesia, otters were released in the same vicinity as captured.

From sedated adults, juveniles and large pups (>20 lb), a liver biopsy weighing approximately 0.1 g was laparoscopically collected for histological examination. At the time of biopsy, the attending veterinarian (MJM) made notes on the visual appearance of the liver, including whether or not the liver appeared “normal.” Notes were made on only a few otters in 2001 (those with readily observed abnormalities), and in subsequent years, notes were made for all otters sampled. Based on these visual observations, livers were assigned a gross pathology score ranging from 0 to 2, with 0 indicating normal, 1 indicating mild to moderate abnormality, and 2 indicating more severe abnormality. Liver biopsies were collected from sea otters captured at Knight Island in all years and at Montague Island in all years except 2003 and 2005, and at

Prince of Wales Passage only in 2008. The biopsy was immediately fixed in 10 percent neutral buffered formalin for subsequent histopathology examination.

The liver tissues in formalin were processed routinely for histopathology, and hematoxylin and eosin stained sections were examined microscopically by two board-certified veterinary pathologists (TPL, FCM) who worked independently and were not provided any specimen collection locations or information about the sea otters, although they conferred to ensure the variables assessed and the scoring system were consistent. Ten variables were semi-quantitatively scored—(1) parenchymal inflammation, (2) portal inflammatory cells, (3) intra-sinusoidal inflammatory cells, (4) composite score for inflammation, (5) vacuolar change, (6) necrosis, (7) apoptosis, (8) eosinophilic foci, (9) pigment, and (10) capsular thickness. For all metrics, we used the following scale—none (0), minimal (1), slight (2), moderate (3), and marked or severe (4). A description of the variables and the scoring system, and a summary of findings was provided by the initial reader (FCM) and is presented in the Appendix.

Data Analysis

For data analyses, sea otters from Prince of Wales Passage were combined with those from Knight Island as both were considered "oiled" areas. Histopathological scores from readings of the two pathologists were averaged for basic statistical descriptions and analyses.

We used models to investigate gross visual appearance and 10 histological response variables, and the influence of four main effects—sex (male, female), age (juvenile, 0,1 years of age; prime-age, 2–8 years of age, and old-age, 9+ years of age at capture), area (oiled, unoiled), and time (years, 2001–2008). We hypothesized two-way interactions might be present, including (1) area by age interaction, as effect of area could vary with the age of the otter at capture, (2) area by year interaction, as the effect of area could vary with the year of the otter's capture, (3) age by year interaction, as the effect of age could vary with the year of the otter's capture, and (4) age by sex interaction, as the effect of age could vary by sex.

We initially evaluated significance of several two-way interactions to guide subsequent construction of models incorporating main effects. Main effects were considered when the two-way interactions were not supported by the data. Models using the multinomial distribution and incorporating repeated measures did not converge, thus further modeling was based on the binomial distribution. The binomial response was equal to "0" for average histology scores equal to 0 or 1 and "1" for average scores 2, 3, or 4. The binomial model is a generalized linear model with logit link function and is fit with maximum likelihood methods. For binomial models, the four main effects were considered; model construction proceeded in a forward selection manner, with $p < 0.10$ for retention.

We also conducted Spearman correlations (PROC CORR, SAS Institute©, Inc., Cary, N.C.) on gross scores (1) with histological variables that assessed inflammation (parenchymal inflammation, portal inflammation, intra-sinusoidal inflammation, and composite score for inflammation) because visual assessments of gross pathology often related to swollen lobes; (2) with vacuolar change scores because they have the most direct connection to physiological stress; and (3) with the pigmentation scores because visual assessment frequently indicated livers were pale in color. In addition, we included three serum enzymes potentially indicative of liver function in the correlation analysis: gamma glutamyl transferase (GGT), alkaline phosphatase (AP), and alanine aminotransferase (ALT).

Results

Over the 8-year period of sampling, 206 biopsy samples were taken (table 1.1); this included multiple samples on a subset of 29 sea otters captured at least twice over the time period (table 1.2). Proportions of livers that were scored as grossly normal (0), mildly/moderately abnormal (1) or severely abnormal (2) by year for oiled and unoiled areas are presented in table 1.3.

We found no significant interactions for any of the histological response variables or the gross pathology response variable (figs. 1.1–1.11). Then, based on models with main effects of gender, area, age class and year, five histological response variables and the gross pathology response variable were significantly related to gender, area, or age class; these results are presented in table 1.4. We found a difference between sea otters from oiled and unoiled areas for the gross appearance score, as well as for vacuolar change, and pigment (table 1.4).

Based on the visual assessment of gross pathology, individuals living in oiled areas were significantly more likely to have livers described as "abnormal" to some degree, as were aged adults (table 1.4; fig. 1.1). In the unoiled area, 86 percent (42 of 49) of livers were classified as "normal," while only 49 percent (65 of 133) of livers received a "normal" classification at the oiled area (table 1.3). Of note, 35 percent (9 of 26) of recaptured animals at the oiled area were assessed with at least moderate abnormalities at least one time and another 50 percent (13 of 26) were assessed as severely abnormal at least one time, while only 16 percent (4 of 26) had "normal" assessments at all captures.

One prime-age animal (5 years of age) with a severely abnormal liver assessment died about 2 months after capture in the oiled area, during or soon after a severe autumn storm. Nine individuals continued to live at least a year or more after having been assessed with a severely abnormal liver, with four individuals receiving the same gross pathology score (2) upon recapture and the others showing improved visual liver assessments.

Abnormal liver scores at visual assessment did not translate into consistently poor scores when those liver tissues were examined histologically. Gross pathology score did not correlate with any inflammation score (Spearman CC $p > 0.1$ for all CC), with pigmentation score (Spearman CC = -0.3, $p = 0.65$), or vacuolar change (Spearman CC = -0.03, $p = 0.67$). However, gross pathology scores were positively correlated with two serum enzymes related to liver function (GGT, Spearman CC = 0.28, $p < 0.01$; AP, Spearman CC = 0.19, $p = 0.05$); these relationships were in the direction expected, but no correlation was seen with the third serum enzyme tested, ALT (Spearman CC = -0.87, $p = 0.36$).

Histological scores for pigment varied by area and age (table 1.4), with otters at the unoiled area having higher values than those from the oiled area (fig. 1.10). Vacuolar change also varied significantly by area (table 1.4) with otters in the oiled area having higher scores than those from the unoiled area (fig. 1.6), as anticipated if oil exposure was a concern. Eosinophilic foci scores varied by sex (higher in males; table 1.4), and tended to be higher in older otters, but area did not appear to be a factor (fig. 1.9). Sinusoidal and composite inflammation scores also varied by sex (higher for females; table 1.4; figs. 1.4 and 1.5). Other histological scores (parenchymal inflammation, portal inflammation, necrosis, apoptosis and capsular thickness) did not vary by any of the main effects.

Table 1.1. Number of sea otters sampled for liver biopsies each year in oiled and unoiled areas of western Prince William Sound.

[Age classes are juveniles (Juv, 0–1 years), prime age adults (Prime, 2–8 years) and old adults (Old, 9 years and older)]

	Unoiled area						Oiled area					
	Female			Male			Female			Male		
Year	Juv	Prime	Old	Juv	Prime	Old	Juv	Prime	Old	Juv	Prime	Old
2001	3	6	3	0	2	1	0	8	3	0	3	1
2002	1	4	1	0	4	0	4	12	2	0	5	4
2003	0	0	0	0	0	0	1	13	2	0	2	4
2004	1	11	4	0	0	0	1	11	3	1	2	2
2005	0	0	0	0	0	0	0	9	2	0	5	4
2006	0	3	2	0	2	0	1	5	3	0	1	0
2008[1]	5	3	4	0	3	0	0	20	4	1	2	2
Subtotal:	10	27	14	0	11	1	7	78	19	2	20	17
Total by area: Unoiled 51 females, 12 males; Oiled 104 females, 39 males												
Grand total, all years, both areas: 206												

[1]In 2008, the oiled area includes both northern Knight Island and Prince of Wales Passage.

Table 1.2. Sea otters sampled for liver biopsies in more than 1 year, 2001–2008.

Otter No		2001	2002	2003	2004	2005	2006	2008
01-01		x			x			
01-22		x		x	x			
01-24		x	x					
01-27		x	x	x				
01-28		x	x			x		x
02-04			x	x				
02-07			x		x	x		
02-27			x				x	
02-28			x	x				
02-31			x	x				
02-32			x	x	x			
03-06				x	x			
03-07				x	x			
03-09				x		x		
03-10				x	x			
03-13				x	x			
03-15				x		x		
04-02					x	x		
04-04					x	x		
04-05					x			x
04-07					x	x		
96-02		x		x		x		
97-21			x	x				
97-28			x	x	x			
97-43						x		x
98-29		x	x			x		
98-38			x	x	x			
08-06[1]	?							x
08-08[1]	?							x

[1]Otters 08-06 and 08-08 were caught in 2008 and had holes in their flippers from previous tags and biopsy scars on their livers seen by endoscopy, but as the tags had been lost, it was not possible to definitively identify them with their previous otter ID number.

Table 1.3. Gross pathology observation scores of livers from sea otters in oiled and unoiled areas of Prince William Sound, 2002–2008, based on observations at the time of endoscopy and biopsy sampling.

[Scores range from 0 to 2, with 0 being normal, 1 mild/moderately abnormal, and 2 more severely abnormal]

			Gross Liver Pathology Score			
Year	**Area**		**0**	**1**	**2**	**Total # scored**
2002	Oiled	#	10	5	11	26
		%	*38%*	*19%*	*42%*	
	Non-oiled	#	10	0	1	11
		%	91%	0%	9%	
2003	Oiled	#	11	7	4	22
		%	*50%*	*32%*	*18%*	
2004	Oiled	#	10	3	7	20
		%	*50%*	*15%*	*35%*	
	Non-oiled	#	13	1	2	16
		%	*81%*	*6%*	*13%*	
2005	Oiled	#	12	3	5	20
		%	*60%*	*15%*	*25%*	
2006	Oiled	#	5	4	2	11
		%	*45%*	*36%*	*18%*	
	Non-oiled	#	5	1	0	6
		%	*83%*	*17%*	*0%*	
2008	Oiled	#	15	9	4	28
		%	*54%*	*32%*	*14%*	
	Non-oiled	#	13	1	1	15
		%	*87%*	*7%*	*7%*	
2002–2008 Total	**Oiled**	**#**	**63**	**31**	**33**	**127**
		%	***49.6%***	***24.4%***	***26%***	
	Non-oiled	**#**	**41**	**3**	**4**	**48**
		%	***87%***	***7%***	***7%***	

[1]In 2001, notes on visual observations of liver were made for only 7 otters, of 30 sampled, and those data are not presented in this table.

Table 1.4. Results of binomial models to evaluate effects of area, age, gender, and year on histopathological response variables scored in liver biopsies, and on gross pathology score.

Histopathology variable		Estimate	SD	Z	*p*-value	
Sinusoidal inflammation	Intercept	0.05	0.16	0.32	0.7472	
	Gender - M	-0.75	0.34	-2.20	0.0275	
	Comment	Females had a significantly higher probability of more severe inflammation values				
Composite inflammation	Intercept	1.64	0.22	7.51	0.0001	
	Gender - M	-0.77	0.38	-2.03	0.0424	
	Comment	Females had a significantly higher probability of more severe inflammation values				
Vacuolar change	Intercept	1.79	0.36	4.98	0.0001	
	Area - Oil	1.17	0.53	2.21	0.0273	
	Comment	Sea otters in the oiled area had a significantly higher probability of more severe vacuolar change values				
Eosinophilic foci	Intercept	-3.92	0.58	-6.72	0.0001	
	Gender - M	2.08	0.71	2.93	0.0034	
	Comment	Males had a significantly higher probability of more severe eosinophilic foci values.				
Pigment	Intercept	0.62	0.31	2.02	0.0436	
	Area – Oil	-2.01	0.36	-5.61	0.0001	
	Age Class – Old adult	-0.10	0.39	-0.26	0.7965	
	Age class - Juvenile	-2.95	1.08	-2.74	0.0062	
	Comment	Otters in the unoiled area had a significantly higher probability of elevated pigment values, and adult otters had a significantly higher probability of elevated pigment values than juvenile otters				
Gross score variable						
Gross score	Intercept	-3.25	0.58	-5.62	0.0001	
	Area – Oil	1.59	0.57	2.81	0.0050	
	Age Class – Old adult	1.33	0.41	3.25	0.0011	
	Age class - Juvenile	0.61	0.71	0.85	0.3939	
	Comment	Otters in the oiled areas had a significantly higher probability of abnormal liver values. Aged adult otters had a significantly higher probability of abnormal liver values than adult otters				

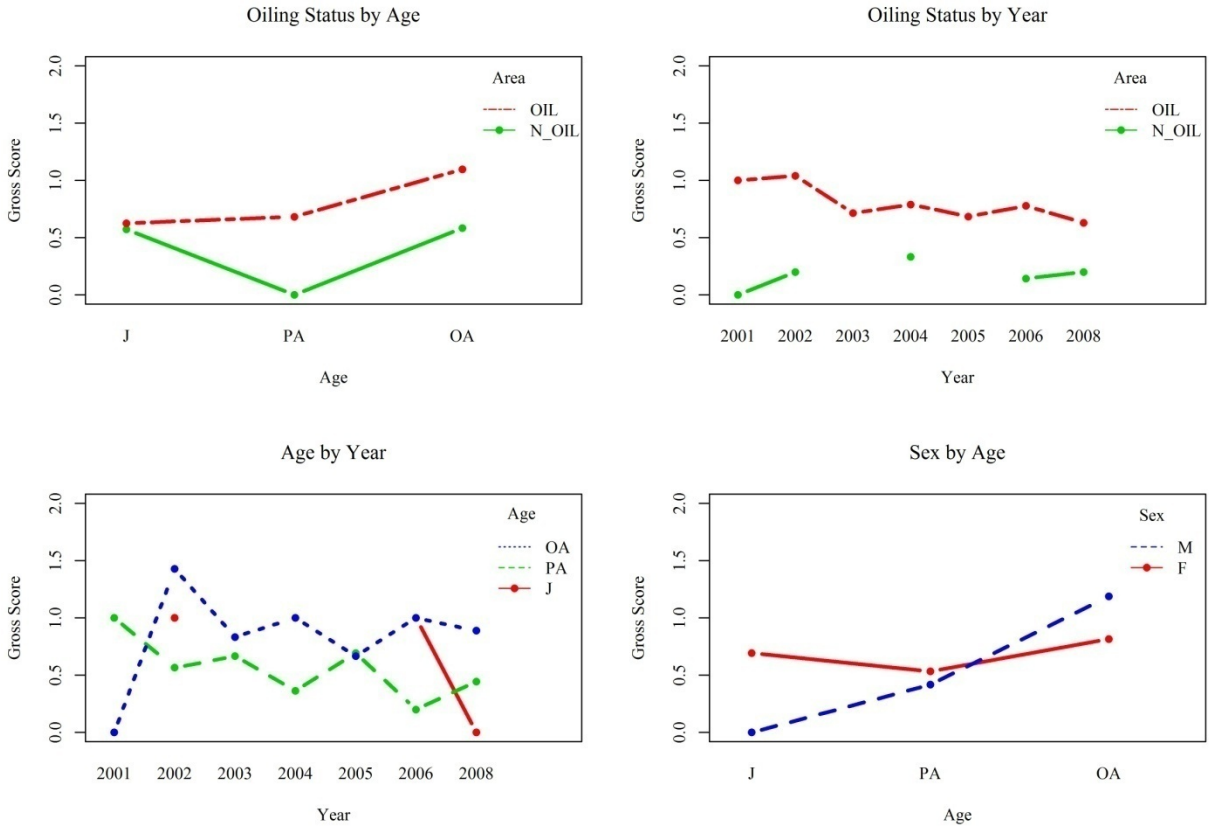

Figure 1.1. Interactions between gross visual appearance scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

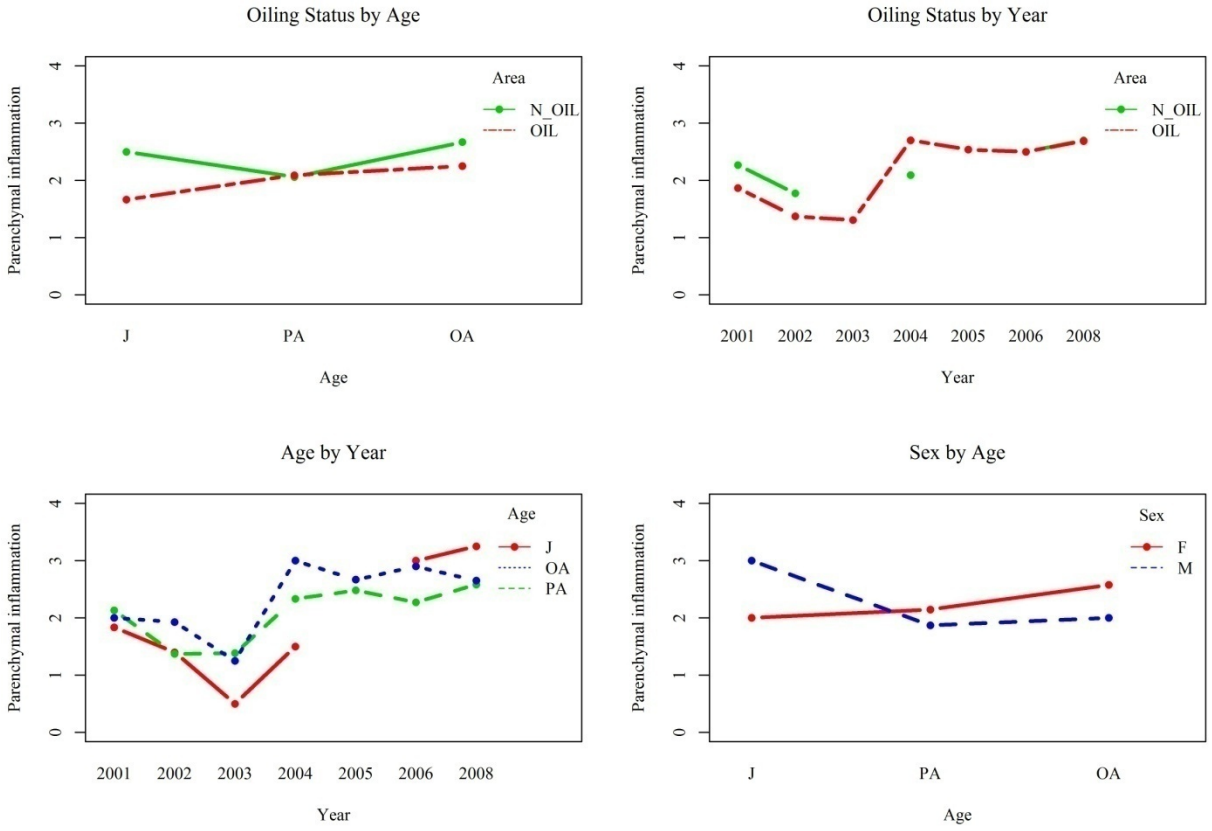

Figure 1.2. Interactions between parenchymal inflammation scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

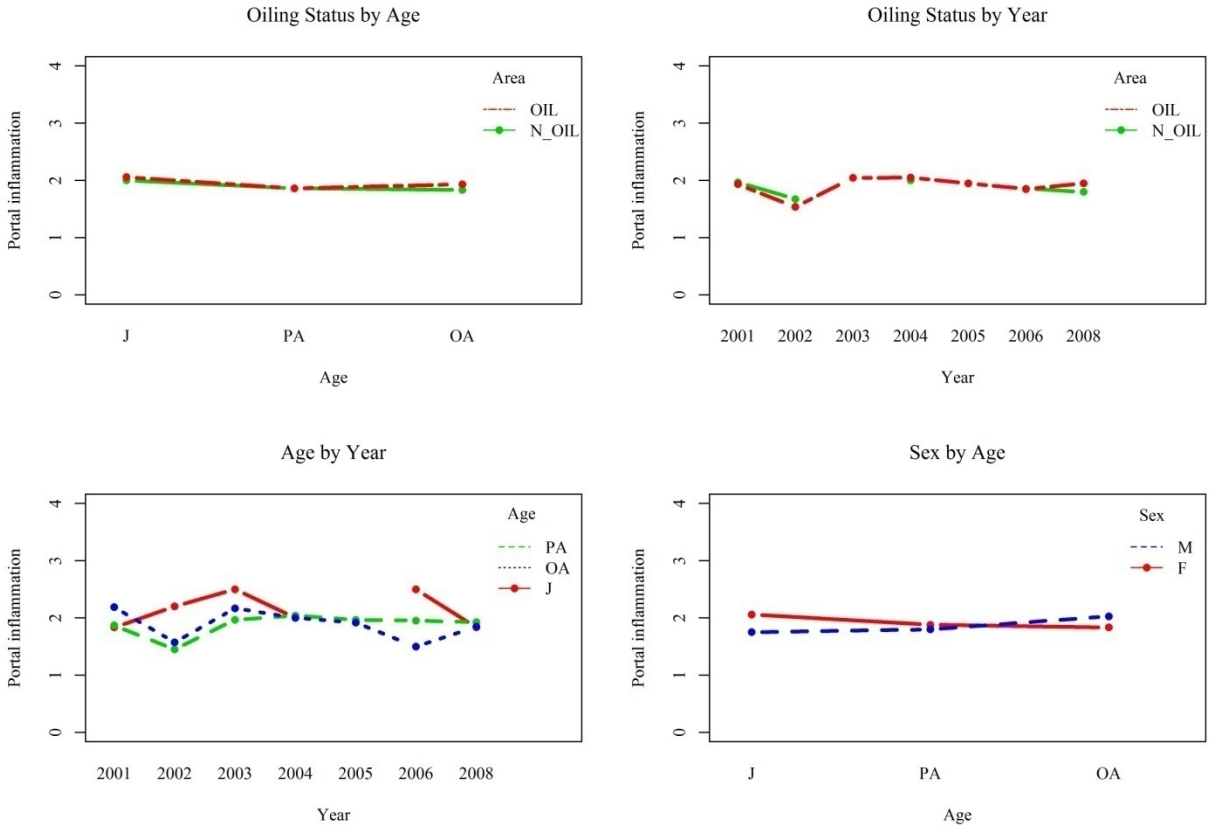

Figure 1.3. Interactions between portal inflammation scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

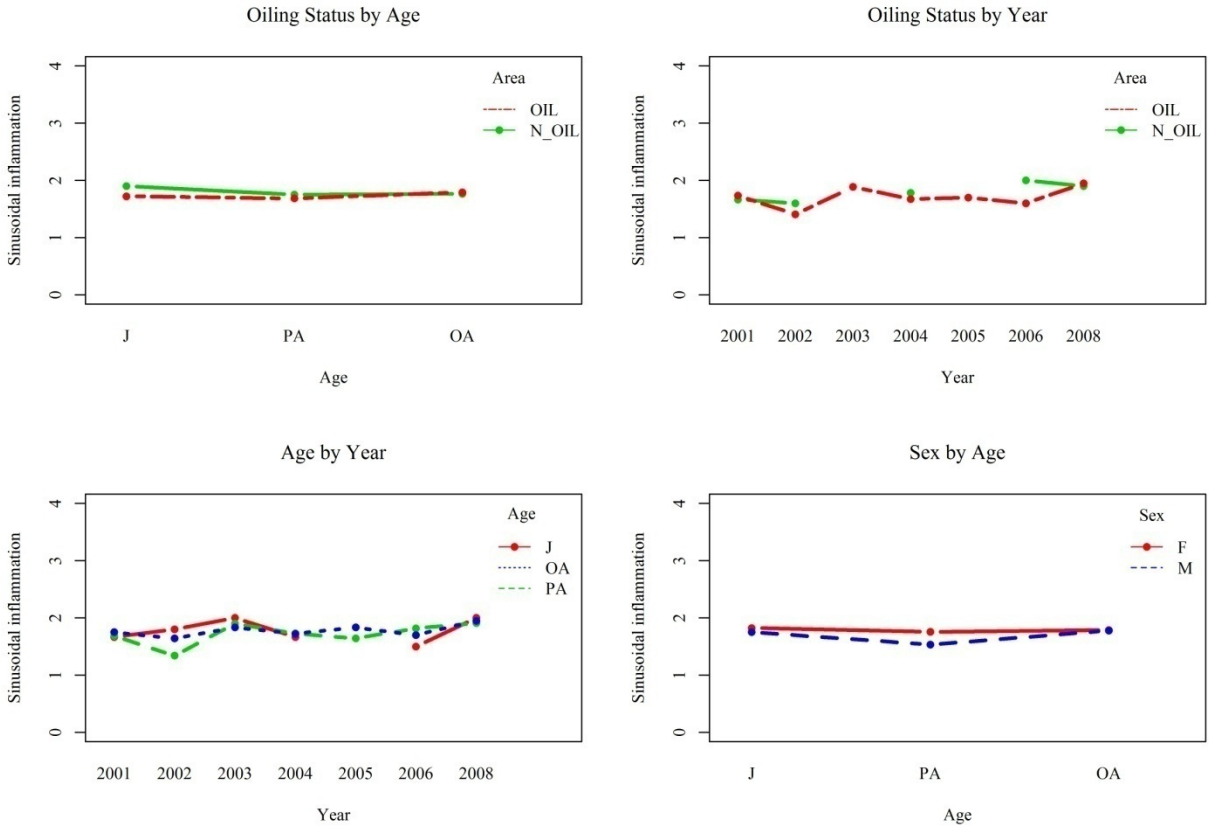

Figure 1.4. Interactions between intra-sinusoidal inflammation scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

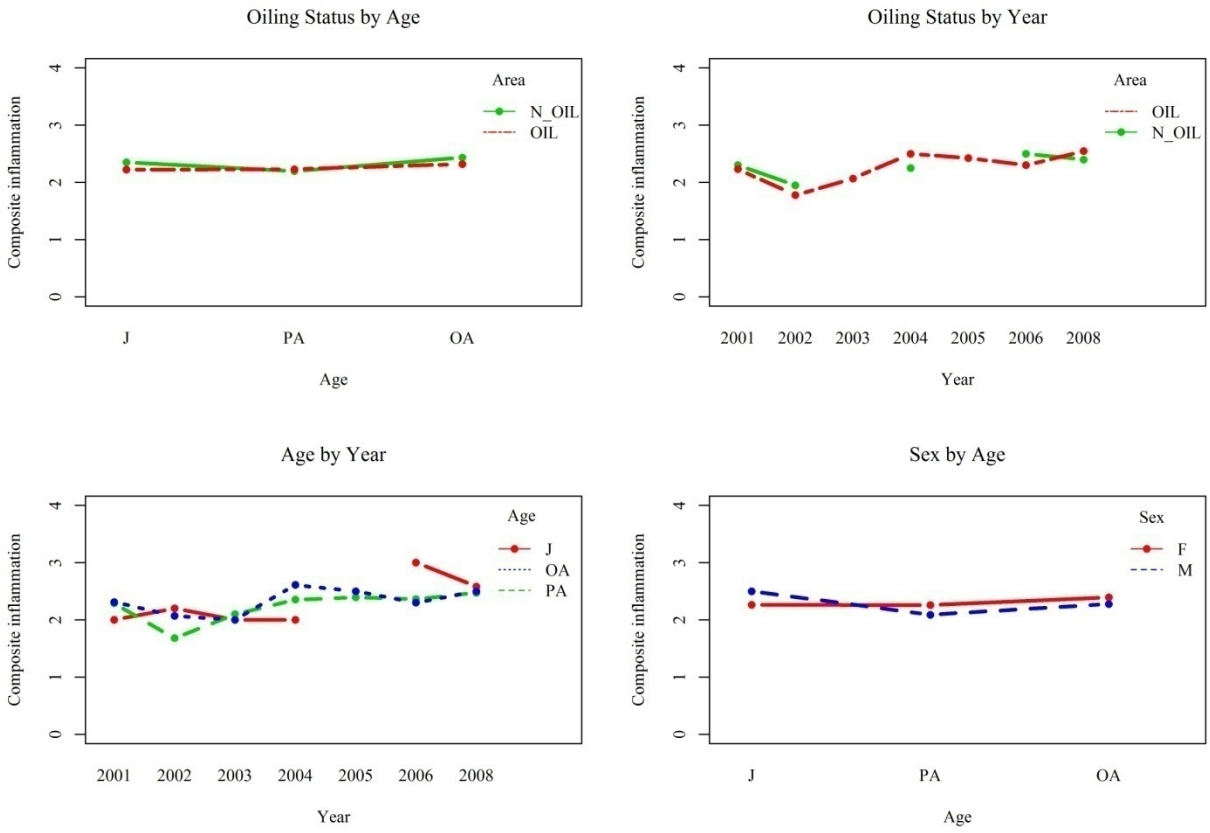

Figure 1.5. Interactions between composite inflammation scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

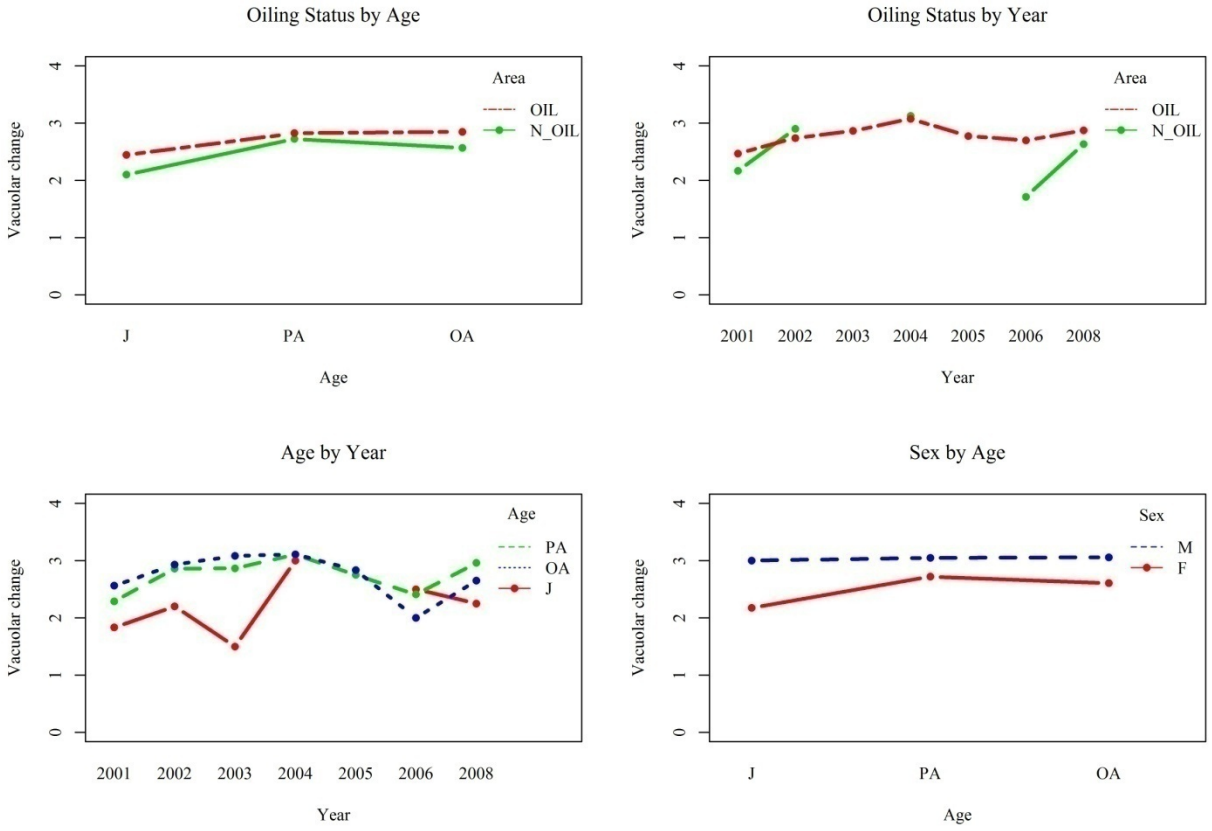

Figure 1.6. Interactions between vacuolar change scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

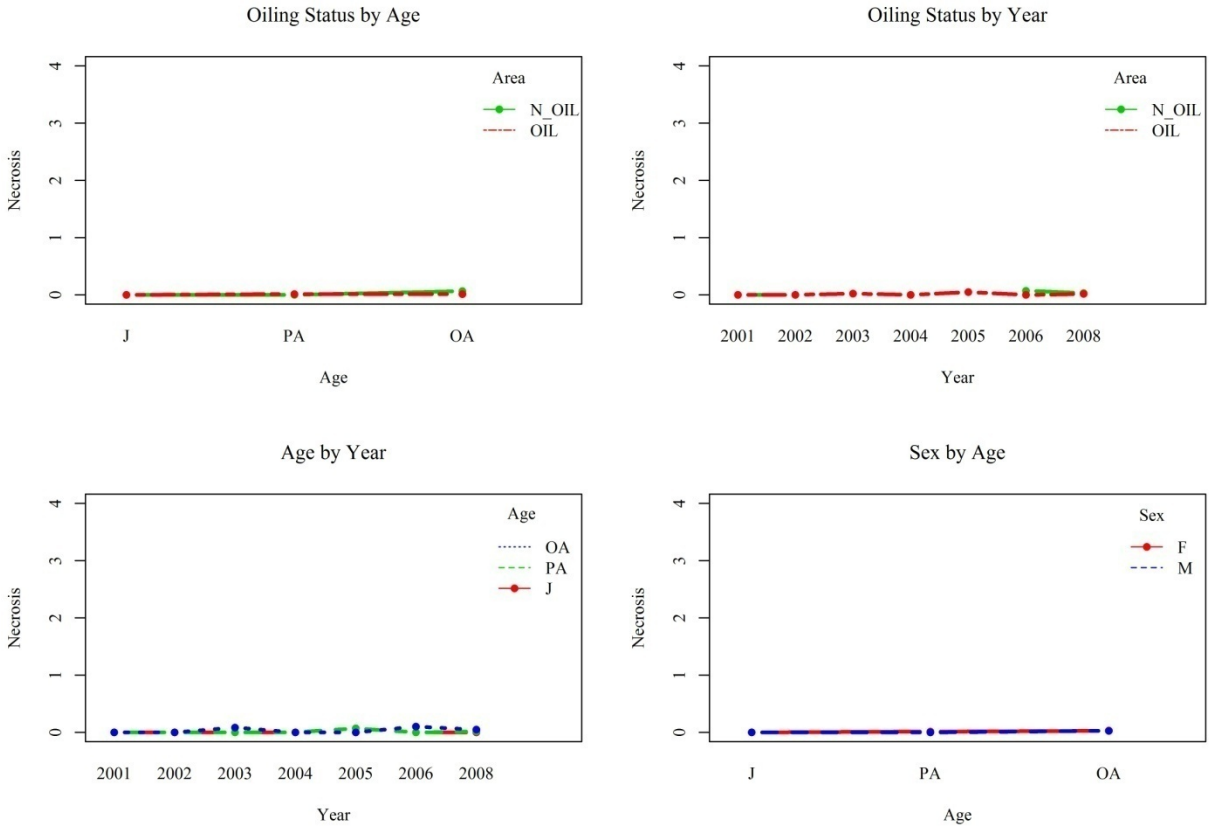

Figure 1.7. Interactions between necrosis scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

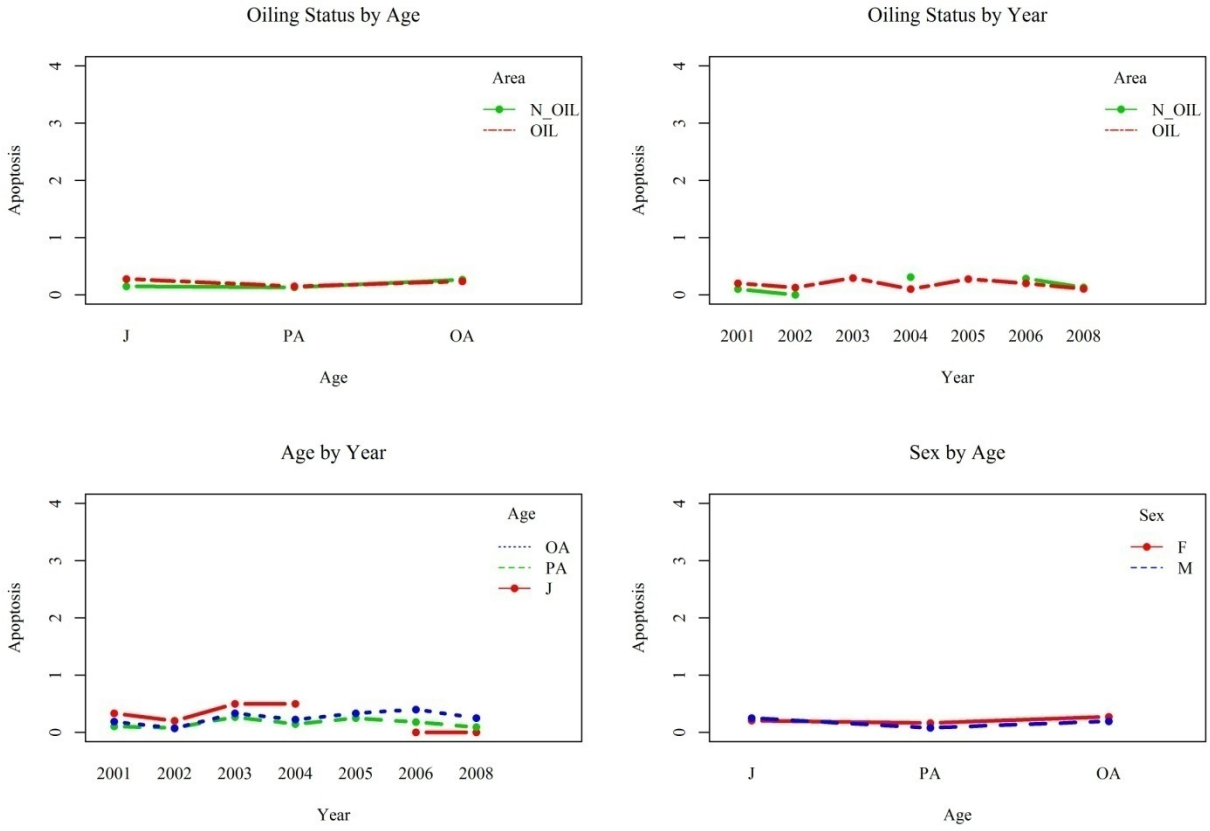

Figure 1.8. Interactions between apoptosis scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

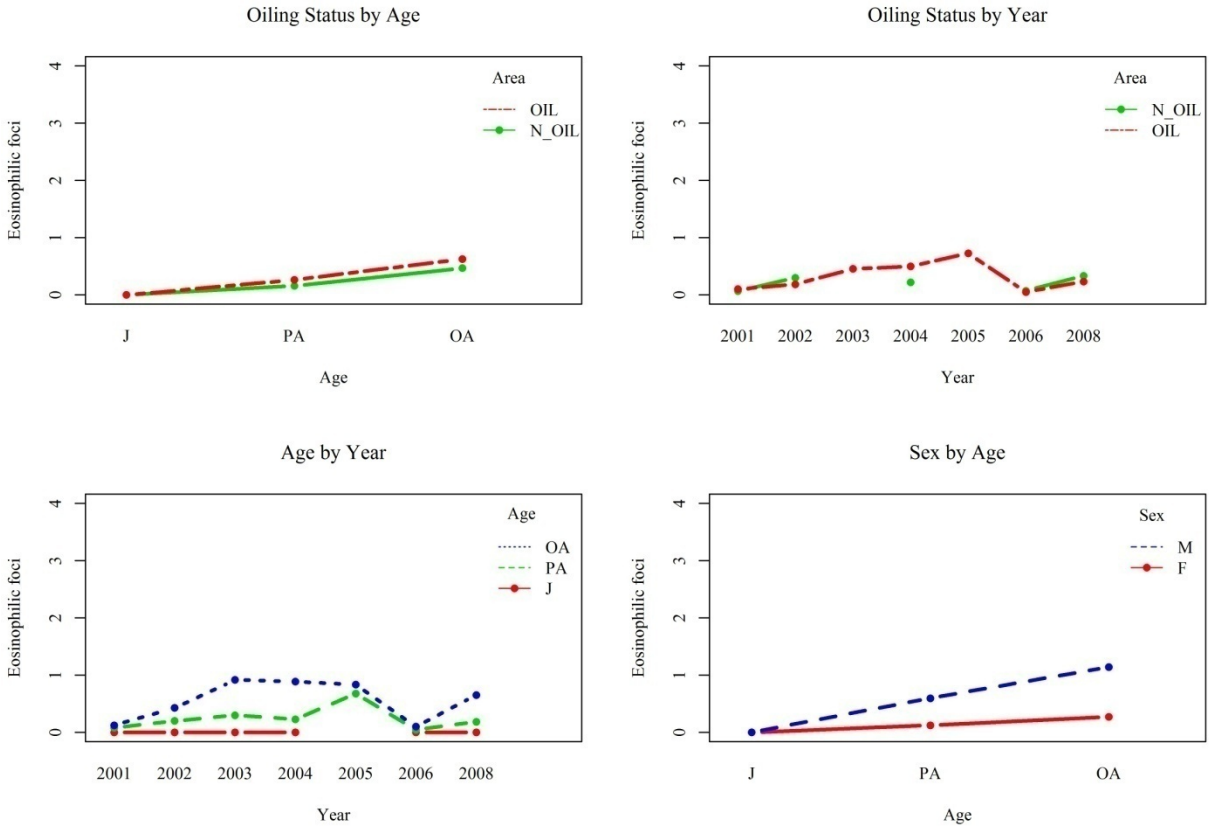

Figure 1.9. Interactions between eosinophilic foci scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

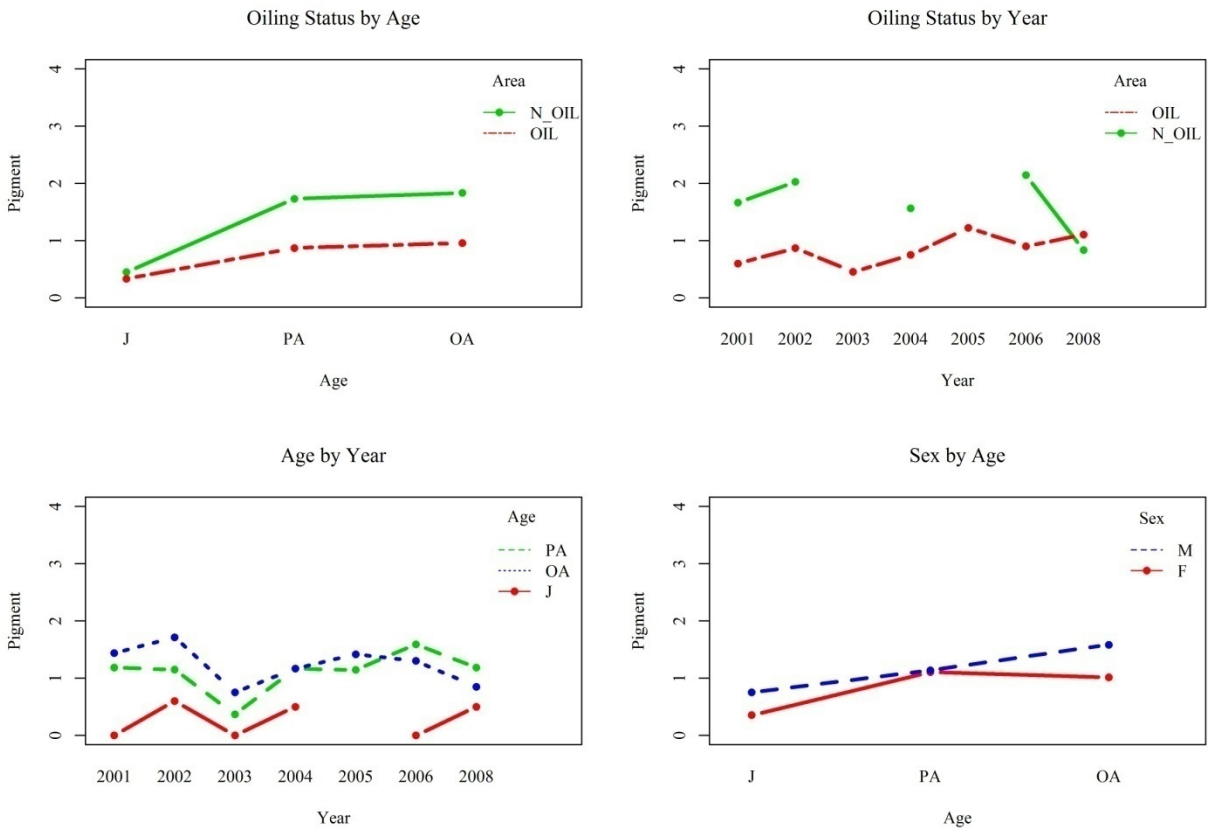

Figure 1.10. Interactions between pigment scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

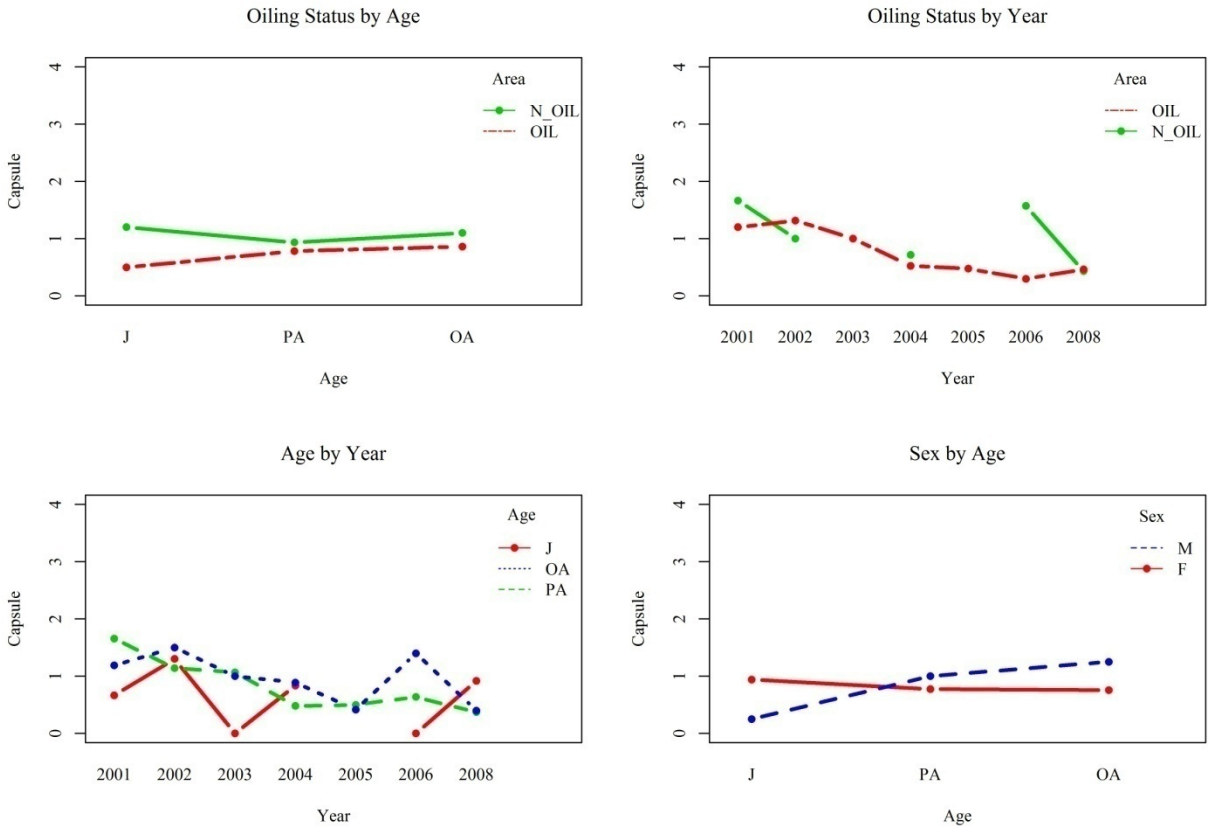

Figure 1.11. Interactions between capsular thickness scores, sex, age, and year of capture. Age classes were J – juvenile (0–1), A – prime age adult (2–8), and OA – old adult, >9 years of age.

Discussion

Differences in gross visual assessments of livers from sea otters residing in oiled and unoiled areas were noted, consistent with our hypothesis that sea otters residing in oiled areas may have had hepatic abnormalities associated with persistent low-grade exposure to residual oil. We are not aware of other differences in physiological status between sea otters in oiled and unoiled areas that could have explained these observations. We acknowledge that gross scores were subjective, and that area was known at time of scoring. We further recognize that our scale, 0–2, for gross abnormalities did not provide for a great deal of resolution in discriminating among different pathologies and, as such, may have reduced our ability to detect area differences.

Gross visual assessments correlated positively with two of three serum enzymes potentially related to liver function (correlations with GGT and AP, but not with ALT); higher levels of these enzymes can indicate problems with liver function. In addition, visual scores indicated poorer liver condition in older otters, which is consistent with a longer time period for exposure as well as potentially higher levels of exposure for animals that were born closer to the time of the oil spill.

Gross visual assessment scores, however, did not correlate with histological readings. As acknowledged above, our gross scoring system (values from 0 to 2, with 2 indicating more severe abnormality, and only about 20 percent of samples overall categorized as 2) did not differentiate among different pathologies, diminishing our ability to detect relationships with other variables. It also may be that the regenerative ability of the liver generally kept up with the low-grade toxicological challenges and thus much of the liver tissue was essentially "normal" most of the time (and in particular during summer, when otters were captured and when other environmental stressors may be at a minimum). This is consistent with the finding that some animals with poor gross liver assessment scores in one year had improved gross liver appearance in a later year. Also, a biopsy collects only a small piece of tissue (less than 0.1 g wet weight) and so abnormalities evident in the visual inspection may be missed in the biopsy sample.

The only histological variables that differed between sea otters from oiled and unoiled areas were vacuolar change (elevated in oiled area) and pigment (lower in oiled area). The difference in vacuolar change score is potentially indicative of physiological disturbance and reflects an adaptive response of the hepatocytes. However, there can be multiple causes of vacuolar change, including toxicological effects, reproductive status (that is, pregnancy or lactation for females) and nutritional status. Pigment scores are related to hemosiderin content, which would have to be present in very large concentrations to change observed liver color (and in fact, "dark" livers were rarely observed in our sample). The bulk of hepatic hemosiderin comes from red blood cell breakdown. Hemosiderin also accumulates in the liver with aging, which is consistent with the tendency for higher values in older otters, and also can accumulate by an increase in iron delivery to the liver. Overall, the difference in pigment scores may not have any relation to liver function and otter health, as liver color generally was not observed to be dark but rather to be normal, or slightly pale, and pigment was not related to vacuolar change, inflammation scores, or blood variables examined in this study. Three histological scores (sinusoidal and composite inflammation, and eosinophilic foci) varied by sex; the biological significance of these findings is not clear.

Gross pathology assessments we observed in otters sampled from 2001-2008 may be indicative of a low level toxicological challenge, and the lack of histological results may simply reflect the rapid regenerative ability of the liver. If so, then low-grade exposure may not present a significant physiological problem, particularly at times when other environmental challenges are low. However, in combination with other environmental stressors faced by wild animals, there could be synergistic effects that present a significant physiological challenge. For example, a 5-year old female sea otter caught in 2003 had an abnormal liver upon gross examination, and high ALT and GGT levels. She also was caring for a large pup, imposing an extra physiological burden (Monson, 2009). Approximately 2 months after capture, this animal died during or following a major storm event. We knew the timing of her death as the radio implant provided a mortality signal that was detected during aerial surveys, but because of logistical problems, we were not able to recover the carcass, so we have no data on the ultimate cause of death. However, we speculate that the cumulative effects of low-grade oil exposure, caring for a large pup, and challenging environmental conditions all may have contributed to the demise of this prime-age animal. This scenario is consistent with reduced life expectancy in the oiled area indicated by mortality models based on age-at-death data (Monson and others, 2000, 2011). In general, liver pathologies may have been a factor in reduced survival of sea otters noted in related studies examining survival from 1993 to 2009 (Ballachey and others, 2003; Monson and others, 2000, 2011).

Overall, area differences in gross liver pathology and vacuolar change that were noted through 2008 suggest concerns for health and survival of sea otters in oiled areas, at least through two decades post-spill. These observations coincided temporally and spatially with results of related studies suggesting some level of exposure to lingering oil (Bodkin and others, 2012; Miles and others, 2012), poor survival rates (Monson, 2009; Monson and others, 2011) and lack of increase in abundance (Bodkin and others, 2011). Liver biopsies from sea otters have not been sampled since 2008, and thus more recent data are not available, but most recent findings on sea otter numbers and ages at death (Ballachey and others, 2014) suggest any negative effects of exposure to lingering oil on health of sea otters have abated since 2008.

CHAPTER 2. Correlates of Survival of Sea Otters in Oiled Areas of Western Prince William Sound, 2002–2008

By B. Ballachey, S. Howlin, and G.G. Esslinger

Introduction

Following the 1989 *Exxon Valdez* oil spill, depressed survival was recognized as a concern for recovery of sea otters in oiled areas of western Prince William Sound (WPWS). Reduced survival of otters in oiled areas of WPWS was identified from radio-telemetry studies of sea otters in oiled and unoiled areas of WPWS in the early 1990s (Monnett and Rotterman, 1995; Ballachey and others, 2003), resighting rates of tagged otters in oiled areas from 1996 to 2005 (Monson, 2009), and ages-at-death, based on pre- and post-spill carcass recoveries through 2008 (Monson and others, 2000, 2011). Although poor survival was identified as a constraint to population recovery, little information was available on factors contributing to mortality, in part because fresh carcasses of sea otters are rarely recovered.

In 2002, we initiated a radio-telemetry study of sea otters in the area of northern Knight Island, where shorelines were heavily oiled following the spill; one objective of this effort was to monitor factors influencing survival of the instrumented sea otters. Here, we present results of an analysis of data from that study. We used a Cox proportional hazards model to discern effects of otter age, sex, condition, liver histopathology, and selected blood values on survival, following the analytical methods described in Ballachey and others (2003). Cox proportional hazards models frequently are used in survival analyses as they provide an approach to assess the importance of various explanatory variables (covariates) in the survival times of individuals, through the hazard function.

Methods

In summers 2002–2004, sea otters were captured in WPWS, in the area of northern Knight Island (heavily oiled study area), and were implanted with VHF radios. Radio-implants have been widely used in sea otter studies since the mid-1980's (Ralls and others, 1989; Lander and others, 2001). Capture and handling methods for this study have been described by Bodkin and others (2012) and Esslinger and others (in press). Sea otters were sedated and body measurements taken including mass and total length. A vestigial premolar tooth was taken for age determination (Bodkin and others, 1997), and if tooth age was not available, we estimated age at capture based on tooth wear, morphometrics, and pelage coloration. Blood was taken by jugular venipuncture for hematology and clinical chemistry. Otters were marked on the rear flippers with numbered colored plastic tags (Temple Tag®, Temple, Tex.). Following reversal from anesthesia, sea otters were released in the same vicinity as captured.

As presented in Chapter 1, liver biopsies were collected from these captured sea otters for histological examination using laparoscopic procedures. Scoring of histopathology variables is described in Chapter 1 and in the Appendix.

Sea otters with radio transmitters were relocated weekly by aircraft or boat for at least 1 year following capture, and observations of radioed animals (based on radio frequency signals or, after radios no longer functioned, on flipper tags) were made during the course of related studies through summer 2008. Although radio-implants were only done in 2002–2004, there

were additional captures in 2005, 2006, and 2008 for related studies, and sea otters were frequently captured more than one time over this 7-year period of the study.

Proportional-Hazards Model of Survival

Survival was modeled for 47 sea otters from the time VHF radio transmitters were implanted. All otters that were fit with a VHF radio and had covariate values for blood serum, histology, and capture were in the analysis. Eight of these otters were confirmed to have died during the study and all other otters were censored at the time of last detection (fig. 2.1). The model incorporates time-dependent covariates, that is, covariates with values that change during the study. Capture covariates of age, condition, pup presence, and blood serum and histology parameters were updated with current values obtained when otters were recaptured. The only covariate that did not change with time in this analysis was gender.

Survival was estimated using a Cox proportional hazards model in which the hazard function is nonparametrically modeled based on the distribution of the survival times of individuals. The model allows for incorporation of continuous covariates through a linear function of the log of the hazard ratio. All covariates were measured when the sea otters first entered the study and their effects on survival were modeled as multiplicative influences on the hazard function (Collett, 1994). The parameter estimates from the models can be interpreted as changes in the hazard rate as a result of changes in the covariate(s) in the model while holding values of the other covariates constant.

The Cox model parameterizes the hazard of death ("instantaneous probability of death") for an individual as the product of a baseline hazard and an exponentiated linear function on a set of covariates (Allison, 1995). Covariates were included in the linear part of the model to assess their effect on the hazard function and appear in the model with a corresponding β coefficient as in:

$$h_i(t) = \exp\left(\beta_1 X_{1i} + \beta_2 X_{2i} + \cdots + \beta_p X_{pi}\right) h_0(t)$$

where

$h_i(t)$ is the hazard function for individual i at time t,
β is the vector of coefficients of the explanatory variables x1, x2,…, xp, and
h0(t) is the baseline hazard function at time t.

Unknown parameters and their standard errors were estimated using maximum likelihood. Approximate confidence intervals were calculated and hypothesis tests for the null hypothesis that β=0 were conducted with a Wald test using SAS® 9.2 (SAS Institute©, Cary, N.C.).

Time-dependent covariates are those that change in value throughout the course of the study. To utilize time dependent covariates, the model required the value of each time-dependent covariate associated with each sea otter at risk at each time period. Because the time-dependent variables included here were internal variables, that is, measured on individual otters, we do not present the estimation of survival curves (Allison, 1995) but focus on the relationship between the covariates and the hazard ratio.

Cox models with time-dependent covariates are not strictly proportional hazards models, thereby allowing us to relax the assumption of proportional hazards (Zhang, 2005). Allison (1995) asserts a mis-specified Cox model is analogous to not including the interaction between a covariate and time, but if there is a significant interaction with time, the Cox model incorporates the non-proportionality. Evaluations of Shoenfeld residuals allowed a determination of correct model specification (Collett, 1994; Zhang, 2005).

There were 39 variables evaluated for relationships with survival (table 2.1). Initially, univariate models were run for each covariate individually, and those with a *P* value < 0.20 were retained for further analysis in multivariate models. The group of explanatory variables contained enough missing values to result in large variation in the sample size of fitted models. Univariate comparisons were unaffected by this, but multivariate models could not be compared with the AIC statistic. Therefore multivariate model selection was conducted based on p-values (using $p > 0.10$ as the rejection criterion for discarding variables from the model) of the parameter estimates, and proceeded in a forward manner. Univariate results are reported here to highlight consistencies with multivariate models.

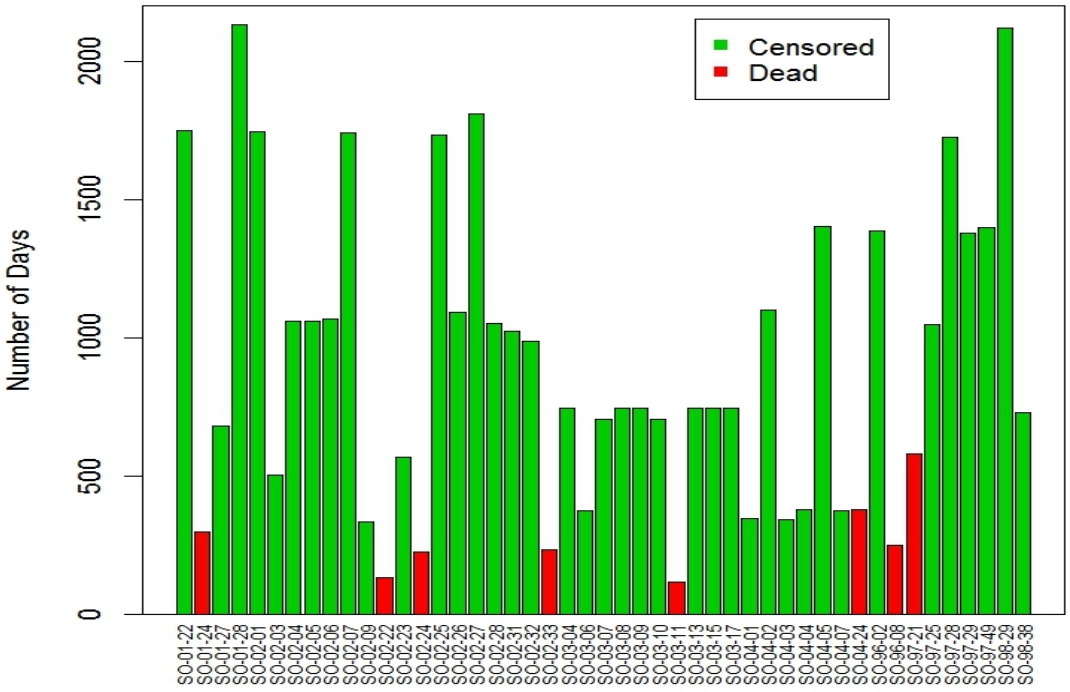

Figure 2.1. Number of days each sea otter was monitored for the survival analysis. Animals that are "censored" were alive at the time of the last observation.

Table 2.1. Variables in the survival analysis of sea otters.

Name	Description
Capture Covariates	
Age Class	Age of otter at capture
Sex	Gender of otter
Pup	Presence of pup with female
Wt/Ln	Weight/Length, a measure of body condition
Histology Covariates	(see appendix 2 for further detail)
I_parenchymal	Parenchymal inflammation
I_triads	Portal inflammation
ISI_Cells	Sinusoidal inflammation
CSI	Composite inflammation
VacuolarChg	Vacuolar change
Necrosis	Necrosis
Apoptosis	Apoptosis
Efoci	Eosinophilic foci
Pigment	Pigment
Capsule	Capsular thickness
Blood Serum Covariates	
Gluc	Glucose, mg/dL
Bun	Blood urea nitrogen, mg/dL
Creat	Creatinine, mg/dL
Bun/Cr	Ratio of BUN to CREAT
UricA	Uric acid, mg/dL
Na	Sodium, mEq/L
K	Potassium, mEq/L
Cl	Chloride, mEq/L
Ca	Calcium, mg/dL
P	Phosphorus, mg/dL
TotProt	Total protein, g/dL
Alb	Albumin, g/dL
Glob	Globulin, g/dL
A/G	Albumin/globulin ratio
Trig	Triglycerides, mg/dL
Chol	Cholesterol, mg/dL
HDL	High-density lipoproteins, mg/dL
VLDL	Very low-density lipoproteins, mg/dL
LDL	Low density lipoproteins, mg/dL
TotBili	Total bilirubin, mg/dL
DirBili	Direct bilirubin, mg/dL
GGT	Gamma glutamyl transferase, IU/L
AP	Alkaline phosphatase, IU/L
AST	Aspartate aminotransferase, IU/L
ALT	Alanine aminotransferase, IU/L

Results

Univariate Survival Results

Nine blood serum parameters were significantly associated with sea otter survival at the p=0.20 level of significance. ALB, HDL, A/G, CHOL, and GLUC were positively correlated with survival, and AST, P, K, and GLOB were negatively correlated with survival (see table 2.1 for description of variables). None of the capture or histology parameters showed a significant association with survival at the univariate stage of analysis.

Multivariate Survival Results

A multivariate model of survival was constructed for the set of all 47 otters. The final model contained effects of AST and A/G (table 2.2). Increased levels of AST were associated with decreased survival. Values of AST ranged from 72 to 683 (mean = 161.7) for censored otters while the range for known dead otters was 93 to 2,237 (mean = 383.6). Increased levels of A/G were associated with increased survival. Values of A/G ranged from 0.5 to 0.9 for censored otters and known dead otters. The mean for censored otters was 0.71 while the mean for known dead otters was 0.66. Interpretation of parameter estimates from the multivariate model was entirely consistent with the univariate analysis.

The parameter estimates from the models can be interpreted as changes in the hazard ratio due to changes in the covariate(s) in the model while holding values of the other covariates constant. For a 1 unit increase in AST, the change in the hazard ratio is 1.003 (90-percent confidence interval [% CI]: 1.0004, 1.005). To compare the minimum to maximum values in the dataset, the hazard of death for an otter with 2,237 IU/L was 229 (90% CI: 224234) times the rate for an otter with 72 IU/L. For a 1 unit increase in A/G, the change in the log-hazard ratio is 0.0003 (90% CI: 0, 0.213). To compare the minimum to maximum values in the dataset, the hazard of death for an otter with A/G of 0.50 was 25.22 (90% CI: 22.61–27.83) times the rate for an otter with a A/G of 0.90.

Table 2.2. Coefficients in multivariate model of survival.

Parameter	DF	Parameter estimate	Standard error	Chi-square	P-value	Hazard ratio
AST	1	0.0025	0.0013	3.63	0.0567	1.0025
A/G	1	-8.0690	3.9783	4.11	0.0425	0.0003

Discussion

This survival model was used to assess whether there was evidence of significant effects of age, sex, body condition, selected serum chemistry variables, and liver histology scores on survival of a group of sea otters that were instrumented with VHF radio transmitters and monitored closely for a 2-year period, and at a much lesser intensity for up to 6 years. Only two blood serum variables, AST and A/G, were correlated with survival, with lower levels of AST and higher A/G ratios associated with increased likelihood of survival. Both these variables can be indicative of liver disease (high AST, low A/G), although neither is necessarily specific to liver and can reflect status of other organs or disease processes as well. We note that one of the sea otters that died (SO-03-11, a 5-year old female with a large pup) had the highest AST value (2,237) and a low A/G value (0.60), as well as high levels of other serum enzymes ALT, AP, and GGT, and gross pathological abnormalities in her liver at capture. This individual was found dead several months after capture, days after a major storm event, and it seems possible that liver disease contributed to her death, perhaps combined with stresses associated with pup-rearing and experiencing a period of foul weather.

In this analysis, we did not estimate survival rates but rather evaluated correlates of survival. Monson (2009) conducted a survival analysis which included many of the same animals from this study, using resight data on otters captured and tagged (color-coded flipper tags enabling individual identification) in WPWS between 1996 and 2005. That analysis provided an estimated annual survival rate of 0.77 (95% CI = 0.73–0.81) for prime age adult otters (ages 2–8 years), which is low relative to survival rates estimated for the WPWS otters just prior to the spill (Udevitz and Ballachey, 1998). Further, this rate is likely insufficient to sustain a stable or growing population (Bodkin and Ballachey, 2010). This relatively low survival rate (Monson 2009) also is consistent with models demonstrating relatively low survival limiting population growth based on ages at death from sea otters carcass recoveries in WPWS during the same time period (Monson and others, 2011).

In general, findings of this analysis do not suggest any effects of age, gender, condition, or liver histopathology variables on survival, nor is there an apparent relation between most serum chemistry variables and survival, with the exception of AST and A/G. The dataset was not large, with 47 sea otters and only 8 confirmed deaths over the period of study, and observations were made 13 to 19 years post-spill. For individual sea otters, however, as exemplified by sea otter SO-03-11, we suggest that chronic oil exposure and liver pathologies may have been a factor contributing to mortality. At a population level, alterations in liver health may have contributed to the depressed survival rates observed for sea otters in oiled areas of WPWS through the mid-2000s (Monson, 2009; Monson and others, 2000, 2011).

SYNTHESIS AND CONCLUSIONS

In this report, we present findings from two components of studies conducted over the past two decades to assess the status of recovery of sea otters in western Prince William Sound, Alaska, following the 1989 *Exxon Valdez* oil spill. In the first chapter, we present results of examinations of livers and liver biopsies collected from captured sea otters in WPWS, 2001–2008, to determine whether indicators of liver health correlated with history of oil contamination from the 1989 *Exxon Valdez* oil spill. Sea otters captured in oiled areas had significantly higher proportion of livers with gross pathological change, based on visual inspection at the time of capture, than sea otters captured from unoiled areas. Of the 10 histopathology variables scored on liver biopsies, only two (vacuolar change and pigment) differed between animals from oiled and unoiled areas, and neither correlated with the gross pathology scores. The area difference in vacuolar change is of concern as this indicates physiological disturbance, which is consistent with potential effects from oil exposure but also could be influenced by a number of other factors. We conclude that, as of 2008, differences in liver health were evident between oiled and unoiled areas; these were consistent with, but not necessarily specific to, effects that might be expected with sublethal exposure to lingering *Exxon Valdez* oil.

In the second chapter, we present results of a model used to quantify variation in survival of radio-tagged sea otters within oiled areas of WPWS in relation to age, sex, body condition, selected blood serum chemistry variables, and histological scores indicative of liver health. Of the variables considered, only the serum enzyme aspartate aminotransferase (AST) and the ratio of serum proteins albumin and globulin (A/G) correlated with survival, with lower levels of AST and higher levels of A/G associated with increased likelihood of survival. High AST and low A/G both may be indicative of liver disease.

Taken together, results reported here suggest that liver health of sea otters in oiled areas was slightly poorer than in sea otters from unoiled areas through 2008, and that this may have translated to poorer survival during that time frame. In general, we consider that a range of toxicological, energetic and behavioral effects of chronic exposure cumulatively may have contributed to diminished survival for sea otters experiencing long-term low level exposure to residual oil (Ballachey and others, 2013), and that a combination of factors may have limited population growth in WPWS over the two decades post-spill. However, these conclusions are best interpreted in light of more recently collected information (Ballachey and others, 2014) indicating that between 2010 and 2013, mortality patterns and abundance of sea otters in WPWS returned to pre-spill conditions. We suggest that differences we detected in health and survival of sea otters through 2008, presented in this report, likely represented the last effects related to exposure to lingering oil.

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APPENDIX: Histological Descriptions and Scoring System for Sea Otter Liver Biopsies

By F.C. Mohr

General: Samples were small, fragmented, and some had compression artifact. All tissue sections were stained with hematoxylin and eosin (H&E). All elements of particular category were considered when assigning grading. For example, in grading the extent of inflammation in the portal triads, if one triad contained a very high number of inflammatory cells but all other triads had few cells, a grade of slight would be given because the overall appearance of the triads was one of a slight change. If a section of liver has three granulomas in the parenchyma with one granuloma being very large in relation to the others, the grade would still be minimal because of the number of granulomas present.

The cytoplasm of hepatocytes was uniformly eosinophilic unless there was vacuolar (presumed fatty change) and/or whispy cytoplasm with irregular clear spaces (presumed glycogen accumulation). Mild variation in nuclear size was common.

Parenchymal inflammation: This was classified based on the number of aggregates of inflammatory cells throughout the section. This number was dependent on the size of the examined tissue. Aggregates were of two types. One was composed of aggregates of epithelioid macrophages with variable neutrophils. These aggregates resembled granulomas and were mainly present in the hepatic parenchyma but were also occasionally observed either adjacent or within portal triads. Within the granulomas, necrotic debris, necrotic cells (rare), or hemosiderin was variably present. On a few occasions isolated hepatocytes were embedded with the granuloma stroma. Except for one sample with a parasite egg embedded within a granuloma (Box 03, 04:128 SO-03-06 7/_/04), no organisms were observed in the granulomas. Size of the granulomas was variable from just a few inflammatory cells to very large aggregates of cells.

The other cellular aggregates that were counted did not have the morphological characteristics of a granuloma. Instead they were small clusters of inflammatory cells randomly dispersed throughout the hepatic parenchyma. Cell types were mostly mononuclear (lymphocytes) with some aggregates also including neutrophils. Sometimes the cells comprising these aggregates were not formed into small clusters but, instead, were dispersed within a focal area of hepatic sinusoids. While not well formed compared to the granulomas described above, all of these cellular aggregates were still considered and counted as focal lesions.

When different tissue sections were present (not replicate sections), cellular aggregates (both types) were counted and recorded for each section.

None (0): No aggregates observed
Minimal (1): 1-3 aggregates observed
Slight (2): 4-6 aggregates observed
Moderate (3): 7-9 aggregates observed
Severe (4): 10 or greater aggregates observed

Inflammation- portal triads: This was defined as the presence of inflammatory cells within the portal triads. The most common cells observed were lymphocytes followed by neutrophils and occasionally plasma cells. Eosinophils were rarely observed. Inflammatory cells were found either scatted throughout the portal triad or in small clusters that were randomly distributed or surrounded bile ducts (suggestive of a cholangitis). Rarely portal inflammatory cells breeched the limiting plate (border between the portal triad and hepatic parenchyma), which was consistent with interface hepatitis. Occasionally granulomas were observed either impinging upon a portal triad or possibly originating within the triad; although, this was difficult to determine. Therefore granulomas were considered to be part of the parenchyma and counted under the category of Parenchymal Inflammation for grading purposes. The number of portal triads observed per slide was variable.

Normal (0): no inflammatory cells observed
Minimal (1): only a few inflammatory cells were present and randomly dispersed throughout the triad, portal triads without inflammatory cells were also observed
Slight (2): an increase in numbers of inflammatory cells throughout the portal triad either randomly dispersed or in clusters, portal triads with a lesser level of inflammatory cell involvement were present
Moderate (3): large numbers of inflammatory cells randomly dispersed or in clusters (some may be large), most portal triads showed similar changes
Severe (4): more numerous inflammatory cells *filling* the triads or organized into clusters of cells (some large), most portal triads had a similar level of involvement

Sinusoidal inflammatory cells: There were subjective differences in the overall number of circulating inflammatory cells (mononuclear and neutrophils) present within the hepatic sinusoids. This could be reflective of inflammatory cell recruitment into the hepatic parenchyma or of an increase in circulating inflammatory cells in the sinusoids because of non-hepatic inflammation (attributed to leukocytosis). It was sometimes difficult to distinguish between the Slight and Moderate and the Moderate and Severe classifications.

Normal (0): no inflammatory cells observed throughout the sinusoids
Minimal (1): few sinusoidal inflammatory cells observed, distribution not uniform over the entire section
Slight (2): increase in sinusoidal inflammatory cells with uniform distribution
Moderate (3): large numbers of sinusoidal inflammatory cells with uniform distribution
Severe (4): more numerous sinusoidal inflammatory cells, distribution was uniform over the entire section

Composite score for inflammation: Scores for parenchymal inflammation, inflammation-triads and sinusoidal inflammatory cells were summed and graded according to the scheme below. Double scores were recorded when two, non-replicate, sections were present.

Normal (0):
Minimal (1): Score of 1-3
Slight (2): Score of 4-6
Moderate (3): Score of 7-9
Severe (4): Score of 10 or greater

Vacuolar change: This was a very common cytoplasmic change. There were two patterns with often both present within the same cell. The first appearance was of small round cytoplasmic clear vacuoles of variable number. These vacuoles caused the nucleus to be displaced peripherally in some but not all instances. The vacuoles were most consistent with microvesicular fatty change. The other pattern was of irregular clear spaces throughout the cytoplasm or in conjunction with microvesiculation described above. This pattern was consistent with glycogen accumulation. Both of these accumulations had either no effect on cell size or caused enlargement. The latter change was considered to be a significant accumulation of either lipid or glycogen within the cytoplasm. Vacuolar change was either random or diffuse throughout the section. A zonal distribution to the vacuolization and clear spaces was not common.

None (0): no cells with intracytoplasmic vacuoles or clear spaces were observed
Minimal (1): random distribution involving only a few cells with intracytoplasmic vacuoles and/or clear spaces throughout the parenchyma
Slight (2): random distribution of cells with intracytoplasmic vacuoles and/or clear spaces, a moderate number of cells were devoid of vacuoles and clear spaces
Moderate (3): diffuse distribution of cells with intracytoplasmic vacuoles and/or clear spaces with most cells involved, cells were normal in size, a minority of cells were devoid of vacuoles and/or clear spaces
Severe (4): diffuse distribution of cells with intracytoplasmic vacuoles and/or clear spaces involving all cells, a significant number of cells were enlarged, some containing only one large vacuole

Necrosis: Coagulation necrosis was a rare finding. The highest level of grading was minimal.

None (0): none observed
Minimal (1): 1-2 areas of necrosis

Apoptosis: This refers to single cell death with the morphological pattern of necrosis. It was not a common finding and was considered minimal and most likely normal.

None (0): none observed
Minimal (1): 1-3 cells

Eosinophilic foci: These foci were characterized as an irregular grouping of hepatocytes that were larger and had a deeper eosinophilic cytoplasm when compared to non-involved (neighboring) cells. The cytoplasm of the cells within an eosinophilic focus blended together. A clear, noticeable separation between these foci and the normal parenchyma was obvious. Some foci had inflammatory cells intercalated with the cells. Fine vacuolization of cells in these foci was rarely observed.

None (0): 0 foci
Minimal (1): 1-2 foci
Slight (2): 3-4 foci
Moderate (3): 5-6 foci
Severe (4): > 6 foci

Pigment: Retractile golden-brown intracellular pigment, considered to be hemosiderin, showed a pattern ranging from pinpoint stippling to course aggregates. The vast majority of hemosiderin was found in Kupffer's cells. Only when the accumulation became severe did hepatocytes also accumulate the pigment.

None (0): no pigment observed
Minimal (1): only a few Kupffer's cells contained minor stippling of pigment, not observed at every high power field (HPF) magnification (400x)
Slight (2): Minor stippling of pigment observed in every HPF in multiple Kupffer's cells
Moderate (3): Pronounced fine stippling to course aggregates within Kupffer's cells and occasionally hepatocytes in every HPF
Severe (4): Course aggregates of pigment were present in Kupffer's cells and in many hepatocytes, often pigmented cells aggregated into small clusters and were present throughout the parenchyma

Capsule: The normal capsule was barely visible as a thin lining of mesothelial cells that surrounds the hepatic parenchyma. The capsule was subjectively graded as either normal, mildly, or moderately thickened with fibrous connective tissue. The capsular thickening was either uniform along the tissue surface or only focally present. It was likely a response of the liver to an injury originating either outside the liver, *e.g.*, peritonitis or inside *e.g.*, parenchymal inflammation. Occasionally associated with a thickened capsule was hypertrophy of the lining mesothelial cells, which might be an indication of increased fluid in the peritoneal cavity. Capsular thickening observed near an obvious ligament was not counted and subcapsular hemorrhage was considered an artifact of procurement.

Normal (0): normal, capsule barely noticeable
Minimal (1): mildly thickened capsule, focal in distribution
Slight (1): mildly thickened capsule, uniformly present on the liver surface
Moderate (3): moderately thickened capsule, focal in distribution
Severe (4): moderately thickened capsule, uniformly present on the liver surface