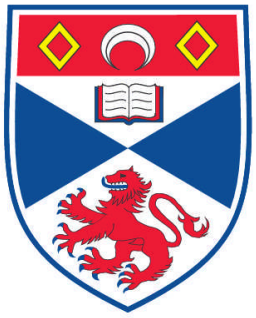
HEALTH, DISEASE, MORTALITY AND SURVIVAL IN WILD AND REHABILITATED HARBOR SEALS (PHOCA VITULINA) IN SAN FRANCISCO BAY AND ALONG THE CENTRAL CALIFORNIA COAST

Denise J. Greig

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



2011

Health, disease, mortality and survival in wild and rehabilitated harbor seals (*Phoca vitulina*) in San Francisco Bay and along the central California coast.

Denise J. Greig

A thesis submitted for the degree of Doctor of Philosophy

School of Biology, University of St Andrews

May 2011

Declarations

I, Denise Greig, hereby certify that this thesis, which is approximately 50,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 18 May 2011 Signature of candidate

I was admitted as a research student in September 2006 and as a candidate for the degree of Doctor of Philosophy in September 2006; the higher study for which this is a record was carried out in the University of St Andrews between 2006 and 2010.

Date 18 May 2011 Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date 18th May 2011 Signature of supervisor

In submitting this thesis to the University of St Andrews we understand that we are giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. We also understand that the title and the abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker, that my thesis will be electronically accessible for personal or research use unless exempt by award of an embargo as requested below, and that the library has the right to migrate my thesis into new electronic forms as required to ensure continued access to the thesis. We have obtained any third-party copyright permissions that may be required in order to allow such access and migration, or have requested the appropriate embargo below.

The following is an agreed request by candidate and supervisor regarding the electronic publication of this thesis: Access to printed copy and electronic publication of thesis through the University of St Andrews.

Date 18 May 2011 Signature of candidate

Signature of supervisor

Table of Contents

List of Tables……x

List of Figures……xiii

Abstract…..…….xvi

Acknowledgements…….....…xviii

Chapter 1: General Introduction…….…….….1

Harbor seals as sentinels of ocean health……1

Harbor seals in San Francisco Bay……2

Harbor seal survival and dispersal……..5

Disease in harbor seals: overview……..6

Disease in harbor seals in the San Francisco Bay Area……...10

Some methods to assess seal health...……..11

Contaminants……...13

Interactions among health, infectious diseases, and contaminants……..15

Thesis structure……..16

Chapter 2: Sampling overview: study sites and capture methods……...…19

Study sites……..19

Capture and sampling……23

Chapter 3: Hematology and serum chemistry in stranded and wild-caugh harbor seals in central California: reference intervals, predictors of survival, and parameters affecting blood variables…….27

Introduction……27

Methods…….29

Capture and sampling……..29

Sample analyses……...29

Data analyses……...30

Results……32

Predictors of survival……...32

Released pups compared to recently weaned wild pups……..33

Predictors of blood variables in wild harbor seals……...34

Discussion……..35

Chapter 4: Surveillance of zoonotic pathogens and pathogens previously reported in harbor seals (*Phoca vitulina*) from central California…….47

Introduction……..47

Methods…….48

Sampling……..50

Sample analysis……...50

Data analysis……55

Results……56

Fecal bacteria……...57

Giardia and *Cryptosporidium*……..61

Brucella……61

Avian influenza……62

Leptospira……63

Toxoplasma, Sarcocystis, Neospora……64

Morbillivirus……66

Discussion……..67

Fecal bacteria……...67

Giardia and *Cryptosporidium*……..68

Brucella……68

Avian influenza……69

Leptospira……69

Toxoplasma, Sarcocystis, Neospora……70

Morbillivirus……70

Chapter 5. Immune response in harbor seal pups during rehabilitation: relationships with growth and survival to release……73

Introduction……73

Methods……76

Sample collection……76

Clinical assessment……..76

Sample analyses……...77

Data analysis……78

Results……79

Clinical assessment of rehabilitating animals……..75

Comparison of immune measures between wild-caught and rehabilitating pups……..79

Sex, mass, and growth effects……..82

Comparison with longitudinal measurements of leukocytes and globulin……..83

Relationship between immune measures…….85

Discussion……..86

Chapter 6. Geography and stage of development affect persistent organic pollutants in stranded and wild-caught harbor seal pups from central California……89

Introduction……89

Methods…….90

Sampling……..90

Sample analysis……...91

Quality assurance and quality control……..92

Data analysis……92

Results……96

Pup morphology and blubber contaminants by group……..…….100

Summed contaminant compounds in the carcass group by location…….106

Individual blubber contaminant compounds by location……...110

Discussion……113

Chapter 7. Harbor seal dispersal and factors affecting the probability of survival…...119

Introduction…….119

Methods……...120

Samples analysis……121

Data analysis……..122

Results…….124

Distance travelled……..125

Morphology and blood variables……...128

Predictors of contaminant concentration……...129

Tag loss……..131

Correlates of survival……132

Discussion……138

Chapter 8. General Discussion: Health, disease, mortality and survival……...141

Thesis summary…….....141

Significance and Limitations..…….143

Future directions……..147

Literature Cited……150

List of Tables

Table 2.1. Maximum number of pups reported at Castro Rocks in San Francisco Bay and Tomales Bay from 2001-2006……..20

Table 2.2. Length and mass by age class for harbor seals (n=151) captured in May and June……..25

Table 3.1. Intervals for blood variables from dependent harbor seal pups at admission to The Marine Mammal Center 2007-2008…….38

Table 3.2. Reference intervals for blood variables from harbor seal pups at release from The Marine Mammal Center 2007-2008. Asterisks indicate significant difference from the blood variable thresholds in wild pups in Table 3.3……..39

Table 3.3. Reference intervals for blood variables from recently weaned, wild-caught harbor seal pups 2004, 2007, and 2008. Asterisks indicate significant difference from the blood variable thresholds in released pups in Table 3.2……40

Table 3.4. Model results for significant generalized linear models fitted to blood parameters from wild caught harbor seal pups……41

Table 3.5. Reference intervals for blood variables from wild-caught subadult and adult harbor seals, May and June 2004, 2007, and 2008……..43

Table 3.6. Model results for significant generalized linear models fitted to blood variables from wild-caught subadult and adult harbor seals……...……....44

Table 4.1. Summary of approach and samples used for each pathogen. Sources for sampling were wild-caught (wild) or stranded. “At admission” means live and dead stranded seals that were sampled on arrival to The Marine Mammal Center, while animals sampled during “necropsy” may have spent time in rehabilitation prior to sampling……...……...49

Table 4.2. Sample size and prevalence (percent positive) by pathogen tested……56

Table 4.3. Bacteria cultured from the feces of stranded (TMMC) and wild-caught harbor seals from San Francisco (SF) Bay and Tomales Bay…….58

Table 4.4. Odds ratios of culturing fecal pathogens from stranded (TMMC) versus wild-caught harbor seals from San Francisco (SF) Bay and Tomales Bay. Odds ratios significant at the p=0.05 level using the Fisher exact test are bolded. *Vibrio* spp is the combination of *V. cholerae*, *V. parahemolyticus*, and *V. alginolyticus*……..…….59

Table 4.5. Age and sex of the stranded harbor seals sampled for *Brucella* by PCR. Premature pups were those stranding underweight with full lanugo early in the season (1/31 to 4/9) while the fetus was full term, but found *in utero*……61

Table 4.6. Location and age class of harbor seals samples for avian influenza PCR…..62

Table 4.7. Number of harbor seals tested (n) and percent positive (%) with a titer to *Leptospira interrogans* Icterohemorrhagiae of 1:100 or greater by location and year……..63

Table 4.8a. *Toxoplasma*, *Sarcocystis* and *Neospora* titers in wild-caught harbor seals from San Francisco (n=75)……..65

Table 4.8b. *Toxoplasma*, *Sarcocystis* and *Neospora* titers in wild-caught harbor seals from Tomales Bay (n=97)……...65

Table 4.9. Location, year, and age class of samples tested for morbillivirus……..66

Table 6.1 Geometric mean contaminant concentrations and ranges in ng/g lipid weight for 177 harbor seal pups……..97

Table 6.2. Summary of the mass (kg), blubber depth (mm), percent lipid, and Julian day (as a proxy for age) sampled for 177 harbor seal pups. A lower case letter in common means no significant difference between groups in that column…….…….101

Table 6.3. Mean ± standard deviation (range) for mass (kg), blubber depth (mm), and lipid (%), plus range for the summed contaminant classes in ng/g wet weight (ww) and lipid weight (lw) for age classes other than pups (nd=none detected). All samples were from stranded animals that died in rehabilitation except for the yearlings denoted with an asterisk which were captured and released in the wild……102

Table 6.4. Component loadings of 34 compounds on the first two components which explained 85% of the variance in dataset for the neonate carcass (NC) group. Major contributors that do not overlap the two components are in bold (loadings<0.15 were considered minor contributors)…….111

Table 7.1. Mean and range for the morphology covariates by group (SF=San Francisco, TB=Tomales Bay and TMMC=The Marine Mammal Center)…….128

Table 7.2. Mean and range for the blood variables by group (SF=San Francisco, TB=Tomales Bay and TMMC=The Marine Mammal Center)…….128

Table 7.3. Geometric mean contaminant concentration by group in ng/g lipid weight……129

Table 7.4. Results for the significant general linear model of summed organohalogen contaminants (OH) controlling for group (SF=San Francisco, TB=Tomales Bay and TMMC=The Marine Mammal Center)…….129

Table 7.5. Model results from dataset 1: group and time……..132

Table 7.6. Model results from dataset 2: lipid weight contaminants…….133

Table 7.7. Model results from dataset 3: morphology……...134

Table 7.8. Model results from dataset 4: blood variables……..135

Table 7.9. Model results from dataset 5 incorporating covariates from previous models as well as a dispersal covariate…….136

Table 7.10. Model results for dataset 6: covariates specific to rehabilitating seal pups……137

List of Figures

Figure 1.1. Map of harbor seal locations including capture sites at Castro Rocks (San Francisco Bay) and Clam and Seal Islands (Tomales Bay)……3

Figure 2.1. Map of California with The Marine Mammal Center's response area shaded in gray……..……..22

Figure 2.2. Number of live stranded harbor seals admitted to The Marine Mammal Center from 1975 to 2009 by age class and year…….23

Figure 4.1. Map of stranding locations of harbor seals sampled for fecal pathogens in 2007 and 2008. Outfall locations are the California Environmental Protection Agency's National Pollutant Discharge Elimination System's (CA EPA NPDES) major outfalls and discharges. Wild-caught harbor seal capture locations are indicated with a star…..60

Figure 5.1a. Lymphocyte proliferation (mean and standard deviation) with no mitogen and two levels of stimulation with ConA in wild and rehabilitating harbor seal pups. Sample sizes are n=15 for rehabilitating pups and 9 for wild pups. The * indicates a statistically significant difference between the two groups of pups (t-test at $p<0.05$)…80

Figure 5.1b. Serum concentrations and white blood cell counts (1000/ul) in wild and rehabilitating harbor seal pups. Sample sizes are n=15 for rehabilitating pups and 9 for wild pups. The * indicates a statistically significant difference between the two groups of pups (t-test at $p<0.05$)……..81

Figure 5.2. The relationship between PHA response and the rate of mass gain from admission to the date of the PHA test (n=15, PHA response=3.2113 * (mass gain/number of days) + 0.3766, R^2=0.331, p=0.025)……..82

Figure 5.3. Total globulin concentrations (n=15) over the course of rehabilitation standardized to date of admission. After the first week, sample size varies as seals were not bled on a weekly basis (sample sizes are indicated below the data). Black circles indicate globulin values when the PHA test was performed. Error bars are standard error……..83

Figure 5.4. Leukocyte concentrations (n=15) over the course of rehabilitation standardized to date of admission. After the first week, sample size varies as seals were not bled on a weekly basis (sample sizes are indicated below the data). Black circles indicate leukocyte values when the PHA test was performed. Error bars are standard error……..84

Figure 5.5. The association between neutrophil count and SubConA induced lymphocyte response in the rehabilitating pups (n=15, neutrophils = -22.637*SubConA + 13.352, R^2=0.3196, p=0.028)……...85

Figure 6.1. Map of stranding locations for harbor seal pups in the carcass group (n=50). Black bars delineate the five geographical strata used in the statistical analyses (TBN, SFN, SFB, OCSSF, and MTY)……...95

Figure 6.2. Geometric mean summed PCBs and DDTs by group. Error bars are 95% confidence intervals. Lower case letters in common mean no significant difference between those groups……103

Figure 6.3. Geoometric mean summed BDEs and CHLDs by group. Error bars are 95% confidence intervals. Lower case letters in common mean no significant difference between those groups……104

Figure 6.4. Geometric mean summed HCHs by group. Error bars are 95% confidence intervals. Lower case letters in common mean no significant difference between those groups…….105

Figure 6.5. Log transformed SumPCBs (ng/g wet weight) by location. Locations are displayed from south to north. The plot shows the median value, the 25th and 75th percentiles, and maximum and minimum PCB values for each location……..107

Figure 6.6. Log transformed SumHCHs (ng/g lipid weight) by location. Locations are displayed from south to north. The plot shows the median value, the 25th and 75th percentiles, and maximum and minimum PCB values for each location. For SFB, the whiskers are 1.5 times the interquartile range of the data and two outliers are plotted individually……...108

Figure 6.7. SumPCBs:SumDDTs (ng/g wet weight) by location. Locations are displayed from south to north. The plot shows the median value, the 25th and 75th percentiles, and maximum and minimum PCB values for each location. For SFB, the whiskers are 1.5 times the interquartile range of the data and one outliers plotted individually.……109

Figure 6.8. The location of each sample (lipid weight) plotted by the first and second principal components (PC1 and PC2). 1=MTY, 2=OCSSF, 3=SFB, 4=SFN, 5=TBN…112

Figure 7.1. Proportion of tags still transmitting by week post-deployment. Tags that were permanently ashore were considered to be no longer transmitting……124

Figure 7.2. Map of the tracks taken by seals that travelled the furthest and the fastest from each location. The fastest TMMC seal was released from Fitzgerald Marine Reserve and the TMMC seal that travelled the furthest was released from Cypress Point, Monterey……126

Figure 7.3. Minimum average distance per day (km/day) travelled by satellite tagged seal pups over the course of their deployment……..…….127

Figure 7.4. Relationship between standard length and summed organohalogen contaminants (OH) for the three groups: Tomales Bay (R^2=0.6733), San Francisco Bay (R^2=0.2646), and The Marine Mammal Center (R^2=0.0003)...…….125

Figure 7.5. Survival based on satellite tag transmission data modeled with two exponential curves (solid lines) and tag survival based on a normal distribution for tag loss (grey line). The dashed lines display the effect of tag loss on the survival curve……..131

Abstract

Conventional methods for health assessment of wild-caught and stranded seals were used to describe the disease status of harbor seals in California. Clinical chemistry, infectious disease prevalence, immune function, and contaminant data were collected to evaluate harbor seal health with data collected from three groups of seals. Wild-caught seals of all ages were sampled at two locations: San Francisco Bay (a heavily urbanized estuary) and Tomales Bay (a less developed control site). Stranded seals entered rehabilitation from a more extensive portion of the California coast which included the locations where seals were caught.

Hematology reference intervals were generated to provide a baseline for health assessment among the seals. Individual variability in blood variables among seals was affected by age, sex, location, and girth. Disease surveillance focused on pathogens known to cause lesions in harbor seals, zoonoses, and those with terrestrial sources. Specific pathogens of interest were *E coli, Clostridium perfringens, Vibrio* spp, *Campylobacter* spp, *Salmonella, Giardia, Cryptosporidium,* avian influenza virus, *Brucella, Leptospira* spp., *Toxoplasma gondii, Sarcocystis neurona,* and *Neospora caninum, Leptospira* spp, and phocine and canine distemper virus. There was evidence of exposure to all pathogens except for phocine distemper virus.

Simple measures of immune response were used to evaluate the immune function of harbor seal pups in rehabilitation that had evidence of previous bacterial infection. The swelling response to a subcutaneous injection of phytohemagglutinin (PHA) was positively associated with growth rate, possibly illustrating the energetic trade-offs between growth and immunity.

Blubber contaminant concentrations (PCBs, DDTs, PBDEs, CHLDs, and HCHs) in harbor seal pups were grouped by extent of suckling and strand location. The ratio of PCB:DDT was increased in San Francisco Bay and decreased in Monterey Bay compared with other locations along the coast. Pups that weaned in the wild, lost weight and then stranded had the highest contaminant levels, equivalent to the concentrations detected in stranded adult harbor seals.

Dispersal and survival were monitored by satellite telemetry in harbor seal pups released from rehabilitation and recently weaned wild-caught pups to assess the effect of condition, health, and contaminant levels on survival probability. Increased contaminant levels and decreased thyroxine (T4) were associated with decreased survival probability. Increased mass, particularly among the rehabilitated pups, was associated with increased survival probability. This study demonstrates that health and survival of harbor seals pups along the central California coast are impacted by human activities such as contaminant disposal, pathogen pollution and boat traffic, although the variability in individual health measures requires carefully designed studies to detect these effects.

Acknowledgements

I would like to thank my two advisors, Ailsa Hall and Frances Gulland, for their endless support, and for being such amazing mentors and role models in the field of marine mammal disease ecology. And Jim Harvey and Sarah Allen for their support, encouragement and inspiration from my masters thesis through to the present.

I owe a debt of gratitude to Liz Wheeler, Tenaya Norris, Carlo Rios, Spencer Jang, Amber Makie, and Michelle Blascow for helping me to collect and process samples in multiple locations at once (field, hospital, necropsy, hematology lab and microbiology lab) for two extended field seasons.

I thank Marjorie Boor, Jim Harvey, Scott Hanson, John Hammond, Simon Moss, Charlotte Jones, and Suzanne Manugian for their time, generosity, and field skills.

I am thankful to many people who graciously allowed me into their laboratories and spent time teaching me: Ailsa Hall (PCB ELISA and complement protein); Pat Conrad, Andrea Packham, Ann Melli, Woutrina Miller, Melissa Miller and Stori Oates (protozoal serology, direct immunofluorescence assay and polymerase chain reaction); and Gina Ylitalo, Dar Boyd, Gladys Yanagida, and Jennie Bolton (blubber contaminants).

I am thankful to Ben Becker and Sarah Allen for a life saving quiet space to work in 2009. I thank Mike Lonergan and Bernie McConnell for data analysis assistance; and Lance Thompson, Alan Wallis and Matt Hoard for engineering assistance.

For years of support, quick answers to panicked questions, help with logistics, and assistance at all levels, I owe thanks to Emma Grigg, Debbie Green, Milton Levin, Sarah Jansson, Gill Braulik, Tracey Goldstein, Shawn Johnson, Robyn Stoddard, Cara Field, Daphne Carlson Bremer, JoAnn Yee, Kate Thomas, Chuck Morton, Emily Andrews, Sarah Codde, Sarah Lenz, Moe Flannary, and of course John, Willie and Solomon Memminger.

This work would not have been possible without funding from The Valentine Family Foundation and the John H Prescott Marine Mammal Rescue Assistance Grant Program.

Chapter 1: General Introduction

HARBOR SEALS AS SENTINELS OF OCEAN HEALTH

Harbor seals are distributed coastally throughout the northern hemisphere, and are relatively abundant throughout their range. Their habitat overlaps with humans on land, and they are impacted by human incursions into the marine environment including sewage, agricultural and surface runoff, chemical pollution, and vessel traffic. As carnivores feeding at the same trophic level as humans, they are sensitive to many of the same pathogens as humans, and accumulate toxins that biomagnify up the food web. They are thus good sentinels for changes in the ocean environment that might have effects on human health. Their accessibility and global distribution make them one of the best studied pinniped species. There is abundant literature on census methods, feeding behavior and reproductive strategies, however there is a relative paucity of data on factors impacting health of harbor seals and the importance of health changes on survival.

In the United States, with the passage of the Marine Mammal Protection Act in 1972, harbor seal numbers increased rapidly along the California coast until the 1990s (Carretta et al. 2007). Although recent counts have been less frequent than they were in the 1970s and 1980s, there is some evidence that the population has stabilized and may be close to carrying capacity (Carretta et al. 2007). A logistic growth curve of the harbor seal population at Double Point in Point Reyes National Seashore calculated growth of 13.7% per year from 1979 to 1983 and 2.2% per year from 1983 to 1997 (Sydeman and Allen 1999). Despite these increases along the coast, harbor seal numbers within San Francisco Bay from 1975-1995 were considered relatively stable at around 500 resident harbor seals (Grigg et al. 2004). Factors hypothesized to limit harbor seal numbers in San Francisco Bay have included oceanographic features and changes in prey availability, increased predation, limited haulout space (Sydeman and Allen 1999), and anthropogenic pollutants (Kopec and Harvey 1995; Neale et al. 2005b; Brookens et al. 2007). Limited studies on contaminant levels have not yet identified effects on harbor seal health, although pollutants have been detected blubber and blood. To date, the factors controlling population numbers within SF Bay remain poorly understood.

The harbor seal is a phocid seal with five recognized sub-species: *P.v.vitulina* in the eastern Atlantic, *P.v.concolor* in the western Atlantic, *P.v.richardsi* in the eastern Pacific, *P.v.stejnegeri* in the western Pacific and *P.v. mellonae* in the freshwater lakes of Quebec, Canada (Reidman 1990). The Pacific harbor seal is common throughout its range from Alaska to Mexico foraging in nearshore waters and coming ashore to rest and give birth (King 1983). Harbor seals are opportunistic feeders consuming a wide variety of fishes, crustaceans, and mollusks (Harvey et al. 1995) and can dive up to 480m (Eguchi 1998). Seals spend a greater proportion of time ashore during spring and summer when they breed and molt (Hanan 1996). After molt, however, more time is spent at sea, presumably foraging and rebuilding fat stores for the next pupping season (Allen et al. 1989). Females can live for 29 years, attain sexual maturity between 2 and 5 years of age, and give birth annually to a single pup (Bigg 1969). Annual survival rates for harbor seals older than 5 years are 71% for males and 85% for females (Bigg 1969).

Harbor seals in San Francisco Bay. Harbor seals are long term residents of San Francisco Bay (SF Bay) and have co-existed with humans for at least 4000 years (Risebrough et al. 1980). Sightings were reported in the 1800s (Scammon 1874) and they were apparently hunted along the California coast, although the harvest was never commercially viable like that of the northern elephant seal, *Mirounga angustirostris* (Bonnot 1928). The harbor seal population in SF Bay has been monitored by a number of researchers and agencies from the 1970s to present, and studies have focused on movements, foraging, disturbance and contaminant levels (Grigg 2003). A map of some of the locations used by seals in the SF Bay area and mentioned in the following text is shown in Figure 1.1.

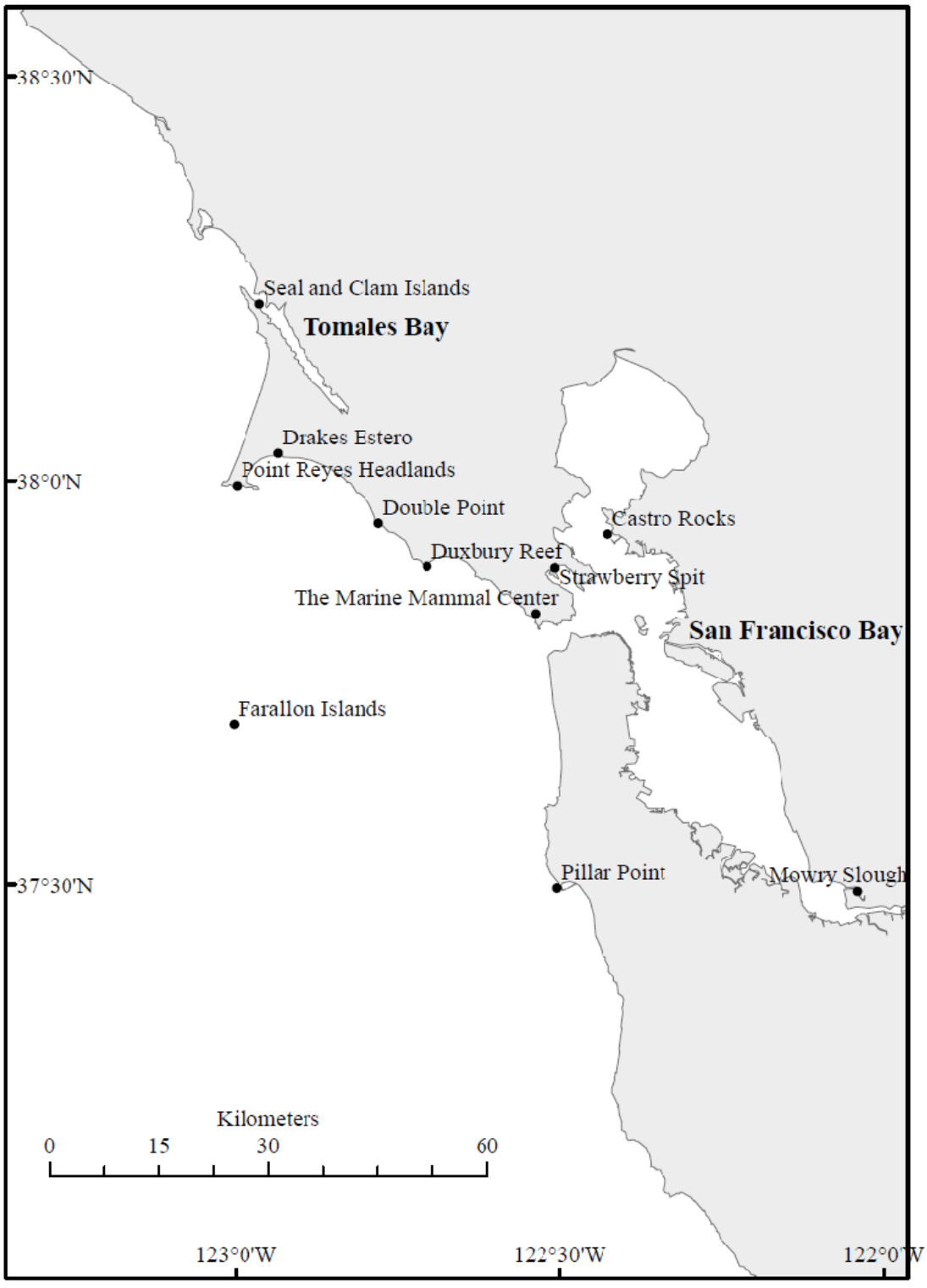


Figure 1.1. Map of harbor seal locations including capture sites at Castro Rocks (San Francisco Bay) and Clam and Seal Islands (Tomales Bay).

Harbor seals in California are genetically distinct from stocks in Oregon and Washington (Lamont et al. 1996), but genetic studies within California have been limited. Analysis of harbor seal microsatellite loci suggest that there is restricted gene flow between SF Bay, Point Reyes and Monterey sub populations (Scribner 2005). Studies of movements of radio tagged harbor seals confirm that there is movement between these groups of seals (Miller 1988; Greig 2002; Harvey and Goley 2005; Oates 2005).

Movement and habitat use of harbor seals in and around SF Bay have been studied extensively. From 2001 to 2005, 46 animals were tagged at Castro Rocks (27 VHF radio tags and 19 satellite tags): 12 of these animals regularly used areas outside SF Bay from Pillar Point to Point Reyes Headlands (Green et al. 2006). The at-sea movements of a subset of the radio tagged harbor seals were tracked to identify foraging areas. Primary foraging areas were located within SF Bay, just outside the bay, and at the Farallon Islands. Animals were located along at the coast at Duxbury Reef, Double Point, and Pillar Point, but foraged largely in SF Bay (Nickel 2003). Nickel (2003) hypothesized that animals were taking advantage of the fall run of Chinook salmon and the late winter/early spring spawning of anchovy and herring. These data were consistent with data from 1991-1992; radio tracked animals from Mowry Slough (n=59) spent the bulk of their time in SF Bay and very few offshore prey species were found in diet samples (Harvey and Torok 1994). Recent tracking data from animals captured in SF Bay and nearby Point Reyes showed similar movement patterns with some animals from SF Bay using coastal areas outside the Golden Gate and some animals from Tomales Bay traveling to other Point Reyes locations (Harvey and Goley 2005). While there is likely mixing at sites along the Point Reyes Peninsula, there is no evidence that the seals from SF Bay forage in Tomales Bay or that those from Tomales Bay come into SF Bay.

Maximum pup counts vary yearly but generally occur between the third week in April and the third week in May in Tomales Bay (Allen et al. 2004). In SF Bay, the first pups are seen in mid to late March and maximum numbers of pups observed at Castro Rocks have increased from 21 in 1999 to 50 in 2005 (Green et al. 2006). Harbor seal pups often disperse from their natal site and it is not known how far they go or when and where they recruit to a haulout area (Thompson et al. 1994; Lander et al. 2002; Oates

2005). This holds true for SF Bay where there is very little information available on pup movements: six weaned pups radio tagged at Castro Rocks in August dispersed in August and September while a seventh remained in the area. Five were located at sites along the coast, 1 of the 6 returned to Castro Rocks in December and another was found on the Farallon Islands in February (Green et al. 2006).

Despite the extensive human population around SF Bay, relatively little direct evidence of seal mortality exists. Carcasses have been detected during systematic directed searches and incidentally during other field work by biologists. In 1971 5 dead premature pups were found at Strawberry Spit, and in 1976 ground searches of Mowry Slough yielded 5 dead pups (Risebrough et al. 1980). In 1992, while collecting scats at Mowry Slough, 5 dead pups were recovered (Kopec and Harvey 1995). There was a dip in population numbers at Point Reyes during an El Nino event in 1998 (Sydeman and Allen 1999), and in 1997 and 2000 there were unusual mortality events at Point Reyes involving primarily adult animals (Allen et al. 2004). In addition, harbor seal pups "stranded" along the central California coast have been admitted each year to The Marine Mammal Center (TMMC), a hospital for sick and injured marine mammals. "Stranding" is a term used in the United States to describe seals that appear sick or injured (see full definition in Chapter 2). From 1997-2006, a mean of 12 pups per year were admitted from the SF Bay area or Point Reyes National Seashore (TMMC unpublished data). In most instances it is assumed that these pups would have died if they remained in the wild.

Harbor seal survival and dispersal. Among pinnipeds, adult survival and reproductive rates are usually relatively stable, while first year survival can be quite variable over time; thus first year, or juvenile, survival may be a primary determinant of population numbers (Gilmartin et al. 1993; York 1994). In phocids a number of maternal and pup characteristics have been linked to first year survival, such as maternal mass and age, pup sex, the mean squared distance between microsatellite alleles (d^2) which is a measure of individual genomic diversity, reproductive parity, birth mass, weaning mass and weaning condition (Coltman et al. 1998; Ellis et al. 2000; Bowen et al. 2001; Hall et al. 2001; Hall et al. 2002). Differential thermal stress on smaller versus larger harbor seal pups during their first winter has been suggested as a possible mechanism for

differential survival (Harding et al. 2005), as has immune response in grey seals (*Halichoerus grypus*, Hall et al. 2002) and climate change in Southern elephant seals (*Mirounga leonina,* McMahon and Burton 2005). Changes in maternal mass and pup mass, and therefore survival, have also been associated with prey availability prior to and during lactation, and with maternal foraging efficiency (Lunn et al. 1993).

In California, long distance movements have been recorded for newly weaned harbor seal pups, therefore ability to estimate survival depends on knowledge of pup dispersal. When Lander et al. (2002) compared the movements of wild-caught harbor seal pups with those of stranded pups after release from rehabilitation, they found that 48% of the rehabilitated pups and 24% of the wild pups dispersed (defined as one way movement from the natal site). The transmitter from one rehabilitated pup was found in Los Angeles, 480km south of the release site. Dive behavior and movements of rehabilitated and wild-caught pups were similar, and survival curves, estimated for 15 weeks post-release, were not statistically different between the two groups. After the conclusion of the study by Lander et al (2002), improvements were made to the rehabilitation facility and to the caloric value of the formulas fed to stranded pups, therefore, current post-release survival of rehabilitated harbor seal pups is not known.

Oates (2005) investigated dispersal and survival of wild-caught harbor seal pups from Monterey and Point Reyes National Seashore. Weaned pups tagged in Monterey Bay generally exhibited northward movement with some traveling to Point Reyes and one as far as Cape Mendocino (586 km north of the capture site). Some pups from Point Reyes also traveled northward, and others traveled as far south as Monterey (Oates 2005). Mean distance traveled for weaned pups was 162.7 km for male pups (min: 11 km, max: 587 km) and 79.1 km for female pups (min: 10 km, max: 170 km, Oates 2005). The 9 month survival of weaned harbor seal pups was estimated at 48% with the greatest mortality occurring within the first 6 months.

Disease in harbor seals: overview. Infectious disease outbreaks have caused major die-offs in harbor seal populations around the globe. In the North Sea, there have been two epidemics caused by phocine distemper virus (PDV). The first, in 1988, resulted in 23,000 dead seals and the second, in 2002, in 30,000 dead seals (Härkönen et al. 2006). Along the east coast of the United States and Canada, several smaller die-offs have been

attributed to influenza virus. In 1979-1980 an epidemic was caused by an influenza A virus with avian flu characteristics (Geraci et al. 1982). Another influenza A virus closely related to avian strains was isolated from western Atlantic harbor seals in 1982-1983 (Hinshaw et al. 1984) and two more viruses in 1991-1992 (Callan et al. 1995). More recently, influenza B, long considered a human pathogen, was detected in a harbor seal at a rehabilitation facility in the Netherlands (Osterhaus et al. 2000); and, based on serological evidence from Caspian seals, it appears that seals can acquire human strains of influenza A and B (Ohishi et al. 2002). Seals maintain titers to influenza A for longer than humans and harbor multiple strains suggesting that genetic reassortment of influenza strains adapted to mammalian hosts is a possibility in seals (Callan et al. 1995; Ohishi et al. 2002). Antibodies to PDV were detected in 2 of 160 harbor seals tested in Alaska (Zarnke et al. 2006), but to date there have not been any epidemics among marine mammals of the Pacific Ocean attributable to either morbilli or influenza viruses.

The bacterial genus *Brucella* is a major cause of reproductive failure in terrestrial mammals and livestock and serological evidence suggests that it is present in marine mammals worldwide (Foster et al. 2002). It was first isolated from marine mammals in Scotland (Ross et al. 1994), and based on molecular studies, it has been proposed that two distinct strains exist in pinnipeds and cetaceans., *B. pinnipediae* and *B. cetaceae*, and that they have been in the marine environment for a long time and are host adapted (Cloeckaert et al. 2001; Foster et al. 2002). Seroprevalence in harbor seals from Scottish waters was 49% (147/300 Foster et al. 2002) and from Alaska waters it was 46% (46/100 Zarnke et al. 2006). An unrecognized *Brucella* sp. isolated from a Pacific harbor seal was infectious to cattle and caused abortion (Rhyan et al. 2001) although it was considered less pathogenic to cattle than *Brucella abortus*. In a study of harbor seal carcasses examined in Scotland, *Brucella* was isolated from tissues but no *Brucella*-associated lesions were found (Foster et al. 2002). It is unknown whether harbor seal reproduction is impacted by this infection.

The spirochete *Leptospira* is shed in the urine of infected animals and can cause febrile illness in humans and abortion in terrestrial mammals (Williams and Barker 2001). Outbreaks of leptospirosis in humans are common in tropical regions and are associated

with heavy rainfall, flooding, poor sanitation and rodent populations (Victoriano et al. 2009). Cyclical outbreaks of leptospirosis in California sea lions (*Zalophus californianus*) have been caused by *Leptospira interrogans* serovar Pomona since the early 1970s (Smith et al. 1974; Lloyd-Smith et al. 2007; Zuerner and Alt 2009). A small number of leptospirosis cases have been reported based on histopathology and serology in stranded harbor seals (Stamper et al. 1998; Stevens et al. 1999) and Northern elephant seals (Colegrove et al. 2005), although no *Leptospira* organisms were cultured. *Leptospira* were cultured from harbor seal urine and kidney after a disease outbreak among captive harbor seals in the Netherlands; isolates were typed to serogroup Icterohaemorrhagiae (Kik et al. 2006).

Protozoal parasites have also been identified as disease agents in harbor seals. They have a global distribution, and most are impervious to destruction in the environment at some phase of their life cycle, allowing them to enter the marine environment from terrestrial sources. *Giardia* spp. are protozoan flagellates that are a common cause of diarrhea and intestinal malabsorption in humans. Several different species have been identified from amphibians, birds, reptiles and mammals. *Giardia lamblia* is found in humans, dogs, cats and a variety of wild mammals which have been implicated in waterborne outbreaks (Ortega and Adam 1997). Infections are generally transmitted through infected food or water: cysts can remain viable for months and are resistant to chlorination and UV light, but can be destroyed by ozone or halogens. In humans, up to 60% of the infected population can remain asymptomatic. *Giardia* cysts are shed intermittently and vary in numbers shed (Garcia et al. 1992).

Cryptosporidium parvum causes diarrheal illness in humans, calves and lambs and respiratory disease in poultry (Current and Garcia 1991). In humans, it can cause short term diarrhea in healthy people, but a long-term cholera-like illness in immune compromised individuals. It has also been associated with malnutrition in young children (Current and Garcia 1991). *Giardia* has been detected in harbor seals, ringed seals (*Phoca hispida*) and grey seals from eastern Canada (Olson et al. 1997; Measures and Olson 1999). *Giardia* and *Cryptosporidium* have been detected in the feces of California sea lions (Deng et al. 2000) and in harbor seals and river otters from Washington (Gaydos et al. 2007; Gaydos et al. 2008).

Toxoplasma gondii and *Sarcocystis neurona* are two additional protozoan parasites that have been widely reported in the marine environment and are known to cause disease in harbor seals (Lapointe et al. 1998; Miller et al. 2001). Felids are the definitive hosts for *T. gondii* and large numbers of oocysts can be shed by a single infected host (Fayer et al. 2004). Opossums are the definitive host for *Sarcocystis* while cats, skunks, raccoons and sea otters have been recognized as intermediate hosts (Dubey et al. 2001). *Toxoplasma gondii* oocysts can survive and sporulate in seawater (Lindsay et al. 2003) and have been detected in shellfish and sea otters along central California (Conrad et al. 2005). Freshwater runoff, as opposed to sewage outfall, posed a greater risk for sea otters for infection with *T. gondii* (Miller et al. 2002a). In Washington State, 7.6% (29 out of 380) of harbor seals tested positive for exposure to *T. gondii* (Lambourn et al. 2001).

Several enteric bacteria known to cause gastrointestinal illness in humans, such as *Vibrio* spp. *Salmonella* spp*, Clostridium perfringens* and *Campylobacter* spp., have been detected in marine mammals. *Vibrios* are common in seawater, and human infections are often associated with the consumption of raw or cooked shellfish: *V. cholerae* non-O1, *V. parahemolyticus* and *V alginolyticus* have all been associated with outbreaks of gastroenteritis (Blake et al. 1980).Although little is known about the general distribution of *Vibrios* in marine mammals (Dunn et al. 2001), *Vibrio cholerae* non-O1 has been isolated from a seal with diarrhea in the Netherlands (Visser et al. 1999).

Salmonellae have been detected in sewage, freshwater, sea, and surface water and their survival time in water varies by strain (Baudart et al. 2000). *Salmonellae* are common in apparently healthy wild seals and sea lions as well as sick individuals (Gilmartin et al. 1979; Baker et al. 1995; Thornton et al. 1998; Stoddard et al. 2005) and along with *Vibrio*, and *Clostridium perfringens* have been detected in shellfish used as sentinels of fecal pollution from the terrestrial environment (Miller et al. 2006). *Campylobacter,* does not survive for long in surface water, thus its presence in saltwater is usually interpreted as a sign of recent fecal contamination (Obiri-Danso et al. 2001; Stoddard et al. 2007). The odds of culturing *C. jejuni* from Northern elephant seals, *Mirounga angustirostris*, along the central California coast were higher from those seals at sites

with higher freshwater outflow, suggesting that, like shellfish, seals can be sentinels of fecal pollution from land (Stoddard et al. 2008).

Disease in harbor seals in the San Francisco Bay Area. Colegrove et al. (2005) documented the causes of stranding for harbor seals in the central California coastal area over ten years and found that the primary causes were malnutrition (52%), respiratory disease (10%), and trauma (8%). Their study also documented a number of meningoencephalitis cases caused by the protozoa *Sarcocystis sp.* (Colegrove et al. 2005). The most common infectious cause of mortality in stranded seal pups in rehabilitation in California was phocine herpesvirus (PhHV-1 Gulland et al. 1997). This virus caused adrenal failure in young pups, but older animals appeared more resistant, and antibodies were ubiquitous in adult seals along the west coast of the United States (Goldstein et al. 2003; Goldstein et al. 2005). Additional studies on stranded harbor seals have documented lesions associated with *Sarcocystis* and *Toxoplasma* (Lapointe et al. 1998; Miller et al. 2001), as well as an unidentified protozoan (Lapointe et al. 2003), phocine herpesvirus (Goldstein et al. 2005), the lungworm *Otostrongylus circumlitus* (Elson-Riggins et al. 2001; Elson-Riggins et al. 2004), leptospirosis (Stamper et al. 1998; Stevens et al. 1999), and various bacterial infections (Lapointe et al. 1999; Johnson et al. 2003). Congenital defects have also been documented in stranded harbor seals (Colegrove et al. 2005; McKnight et al. 2005; Buckles et al. 2006), and lead poisoning was recently implicated in the death of a harbor seal that had swallowed a lead sinker (Zabka et al. 2006b).

Although stranding data can provide insight into the types of diseases occurring in wild seals, they cannot be used to determine the prevalence or incidence of disease in the population, nor the effects of the diseases on host population dynamics (Gulland and Hall 2007). Furthermore, in California more than 90% of stranded seals are pups, so older age classes are poorly represented. Very little is currently known about the disease status of the wild seal population in California; however **t**here have been two harbor seal die-offs of unknown origin at Point Reyes National Seashore. One event occurred in 1997 and one in 2000. Histology reports from the 2000 outbreak (n=3) cite pneumonia as the cause of death and *Pseudomonas aeruginosa* was cultured from lung tissue for all three animals. This bacteria is usually a secondary infection and the

underlying factor causing the pneumonia remains unknown. Seals tested negative for morbillivirus antigen and antibody, but an unidentified virus was detected during viral isolation attempts in cell culture (Allen et al. 2004, Frances Gulland, pers. comm.). Recently, a novel coronavirus (HSCoV) was detected in archived lung tissue from the 2000 die-off (Nollens et al. 2010), but, like the *Pseudomonas*, limited fresh tissues were available for histology and its role in the die-off is not known.

Some methods to assess seal health. Health of harbor seals can be assessed by using measurements of condition, quantifying hematological and serum biochemical parameters, measuring circulating hormone levels and assessing immune function. Whether or not an animal is infected with a pathogen can be determined by culture techniques to isolate the infectious organism, visualization of the organism (microscopy, immunohistochemistry) and use of molecular techniques such as polymerase chain reaction (PCR) to detect the organism's DNA or RNA. Previous exposure to an organism can be detected by testing for presence of antibodies in the blood (serology), but does not provide information on the current infection status of the seal.

Body condition can be evaluated using morphological measurements (mass, length, girth, and blubber depth) and condition indices such as the mass/length ratio. Growth and nutrient use can be estimated using body composition measurements based on isotope dilution methods. For example, Muelbert et al (2003) used deuterium oxide to monitor changes in body composition in harbor seal pups during the post-weaning loss of mass and found that muscle growth occurred even while mass decreased. Body composition and morphological methods have been compared in recently weaned grey seal pups and total body fat was reliably estimated from girth measurements (Hall and McConnell 2007).

Complete blood counts (CBCs) and serum chemistries are routinely used in veterinary practice to evaluate health status. Results can indicate dehydration, nutritional status, inflammation, anemia, organ damage resulting in enzyme leakage, or failures of liver or kidney function based on comparison with reference intervals from healthy animals. In seals, few data are available from known healthy animals to establish age specific

normal ranges, and to determine changes associated with physiological states such as weaning and pregnancy (Bossart et al. 2001). In California, CBCs and serum chemistries have been compared among stranded harbor seals at admission to rehabilitation (presumed sick because they are stranding) and at release from rehabilitation (presumed healthy at good body mass and with no clinical signs of disease), and wild-caught harbor seal pups (Lander et al. 2003). Significant differences in a number of parameters among the three groups of seals indicate that certain parameters may be useful for detecting health changes associated with stranding. Compared with the rehabilitated pups at the time of admission, eosinophil counts and calcium levels were greater in the wild pups, while band neutrophils, aspartate aminotransferase, alanine aminotransferase, total bilirubin and chloride were greater among the newly admitted pups (Lander et al. 2003). These differences may be due to age (the pups in rehabilitation are generally younger than the weaned pups captured in the wild which may explain the differences in bilirubin), condition (malnourishment might explain the differences in electrolytes and liver enzymes) and disease status (greater bands occur in animals with active infections, Bossart et al. 2001; Lander et al. 2003).

Endocrine function can be evaluated by measuring circulating levels of hormones, their precursors and metabolites. In mammals, levels of hormones such as thyroxine and cortisol can vary with photoperiod, stress, age and physiological status (St. Aubin 2001). In grey seals, thyroxine values have been established in pups of various ages, and changes in levels have been associated with contaminant exposure (Hall et al. 1998b; Hall et al. 2003). Cortisol levels in rehabilitated harbor seals were higher in pups dying from PhHV-1 infection, suggesting stress was an important factor in the pathogenesis of this disease (Gulland et al. 1997).

Immune function can be evaluated by measuring serum levels of immunoglobulin, cytokines and intercellular transmitters, as well as by *in vitro* assessment of cellular immunity using T and B cell mitogen response tests (Aldridge et al. 2001). In grey seals, an association between immunoglobulin levels and survival has been

demonstrated (Hall et al. 2002), and *in vitro* mitogen responses have been shown to be influenced by exposure to organochlorines.

A variety of culture techniques are used to grow specific viruses, bacteria, fungi or protozoa of interest by inoculating different kinds of cells or culture media under varying conditions. Knowledge of the organism's growth requirements are needed for successful culture; and, fastidious organisms such as *Brucella* spp. may easily be missed. Once bacteria have grown, their sensitivity to antibiotics can be determined *in vitro*, and they can be stored at -80°C for molecular characterization (Zabka et al. 2006a). Aerobic bacteria from stranded seals in California have been surveyed (Thornton et al. 1998), antibiotic sensitivities determined (Johnson et al. 1998), and bacterial culture results also have been reported for stranded harbor seals from Washington State (Lockwood et al. 2006). A recent study of *Campylobacter* and *Escherichia coli* in elephant seals was the first to compare the prevalence of bacteria in stranded versus wild seals. Stranded seals had a higher prevalence of pathogenic bacteria and greater numbers of bacteria with resistance to greater numbers of antimicrobials (Stoddard et al. 2005).

Polymerase chain reaction (PCR) can be used to detect the presence of a specific disease agent by probing for a piece of its DNA or RNA with recognizable sequences. For example, PCR has been used to investigate the epidemiology and pathogenesis of phocine herpesvirus (PhHV-1), a virus common in seals along the coast California and a common cause of mortality of seal pups in rehabilitation (Goldstein et al. 2004).

Serology is used to test for exposure to infectious disease. Serologic tests detect the antibody response to virus, bacteria or parasites in the host serum (Zabka et al. 2006a). They thus indicate presence of previous exposure and immunity, but do not indicate whether infection is present (Hall et al. 2010).

Contaminants. Organohalogen contaminants such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and polybrominated diphenylethers (PBDEs) are pollutants that biomagnify in the environment and have been measured in

tissues of marine mammals world-wide (O'Shea 1999). These chemicals were designed and manufactured for a variety of reasons: PCB mixtures were used in electrical transformers, capacitors, hydraulic fluids, lubricating oils, and as additives in plastics, paints, printing inks, adhesives and sealants (Loganathan and Masunaga 2009); PBDEs are flame retardant chemicals used in plastic, upholstery, fabrics, carpets, and electronic devices (Kodavanti et al. 2008); while DDT, chlordanes (CHLDs), hexachlorocyclohexanes (HCHs) and other organochlorines were designed as pesticides. Because these contaminants are known to persist in the environment, bioaccumulate in the tissues of organisms, and are toxic at high concentrations, production of these compounds has been banned in most industrialized countries, although some countries still rely on organochlorine pesticides for control of malaria and other insect borne diseases. The manufacture and emission of most of these chemicals peaked in the 1970s and 1980s, however they continue to persist in the environment and in the tissues of marine organisms.

Associations between contaminant levels and disease have been reported: for example, polycyclic aromatic hydrocarbons in beluga whales (*Delphinapterus leucas*) and PCBs in California sea lions have been associated with cancer although causation has not been determined (Martineau et al. 2002; Ylitalo et al. 2005). In experimental exposure studies, harbor seals fed contaminated herring exhibited decreases in vitamin A and thyroid hormone concentrations (Brouwer et al. 1989), reductions in natural killer cell activity and T-cell responses (de Swart et al. 1996; Ross et al. 1996) and lowered reproductive success (Reijnders 1986).

Within SF Bay, PCBs, DDTs, and PBDEs have been measured in the blood and blubber of harbor seals (Risebrough et al. 1980; Kopec and Harvey 1995; Young et al. 1998; She et al. 2000; She et al. 2002; Neale et al. 2005b). Risebrough et al (1980) measured PCBs and DDTs in dead harbor seals; but, because mortality levels in Mowry Slough and the Double Point haulout at Point Reyes were similar, they concluded that contaminant levels were not a problem for SF Bay harbor seals. Despite this evaluation, contaminant levels in SF Bay harbor seals consistently have been measured at levels known to cause reproductive and immune effects in harbor seals (Neale et al. 2005b). In

wild-caught harbor seals from SF Bay, PCB congeners 153 and 180 averaged 79% of the ∑PCBs for the 10 congeners tested, and BDE 47 was the dominant PBDE congener (Neale et al. 2005b). When values from this study were compared with the previous results from SF Bay (Kopec and Harvey 1995; Young et al. 1998), PCB levels were lower than those measured 1991-1992 (18 ng/g versus 27ng/g wet weight), but congener pattern was the same: CB153 > CB180 > CB170 > CB128 (Neale et al. 2005b). PBDEs measured in blubber from dead stranded harbor seals ranged from 88-8325 ng/g lipid with BDE 47 comprising 40-84% of the ∑PBDEs and showed a dramatic increase over 10 year's time (She et al. 2002). PBDE levels in seal blubber were an order of magnitude greater than those measured in human breast adipose tissue from women living in the SF bay area and some of the highest levels reported for any mammal (She et al. 2002). These studies highlight the link between harbor seal and human health in the bay area and the need to further evaluate contaminant effects on SF Bay harbor seals.

Trace metal contaminants are another area of concern. Initial assessments of trace elements in harbor seals from SF Bay suggested that mercury and selenium might be of interest (Kopec and Harvey 1995), however recent work has shown that mercury levels within SF Bay are no greater than other locations in California (Brookens et al. 2007). Lead levels have been low in all harbor seals tested except for a single animal that died of acute lead poisoning (Kopec and Harvey 1995; Zabka et al. 2006b; Brookens et al. 2007). Mercury levels in fish were greater in SF and Tomales bays compared with other bays and estuaries, and both bays experience freshwater run-off from old mining operations (Brookens et al. 2007).

Interactions among health, infectious diseases, and contaminants. Contaminant effects on health have been investigated by looking at relationships between contaminant levels and hematology, blood chemistry, and endocrine markers such as thyroid hormone (Tabuchi et al. 2006) and vitamin A (Simms et al. 2000). A T lymphocyte delayed type hypersensitivity response to trace metals in harbor seals blood was measured using a lymphocyte transformation test: sensitizing metals were molybdenum, titanium, nickel, chromium, aluminum, lead, and tin (Kakuschke et al.

2005). In San Francisco Bay, harbor seal contaminant levels have been studied in conjunction with CBCs, serum chemistry and lymphocyte function (Kopec and Harvey 1995; Neale et al. 2005a; Neale et al. 2005b). Unlike a recent study in British Columbia which found high PCB levels associated with an increase in circulating retinol concentrations (Simms et al. 2000), Kopec and Harvey (1995) found no correlation between PCBs and retinol. And although CBCs and serum chemistries were not impacted in experimental studies of contaminated harbor seals (de Swart et al. 1995b), a positive association between PCB levels and leukocyte count was reported in SF Bay (Neale et al. 2005b).

Recent studies have taken an epidemiological approach to understanding the links between health, disease, immunity and contaminants (Hall et al. 2006; Mos et al. 2006). Mos et al. (2006) used groups of harbor seals in urban and non-urban settings to investigate contaminant effects on the immune system and found concurrent effects from terrestrial pathogens. In a case control study of harbor porpoise contaminants, risk of infection increased with increased PCB concentration in the blubber (Hall et al. 2006). Studies of stranded animals also are evolving from simply reporting on stranding events to understanding the pathogenesis of disease and its epidemiology (Buckles et al. 2007; Lloyd-Smith et al. 2007), the genetics of disease susceptibility (Acevedo-Whitehouse et al. 2003b), and the potential consequences of returning stranded animals to the wild (Stoddard et al. 2005).

THESIS STRUCTURE

The main goal of this thesis was to use current health assessment methods, contaminant analyses, and satellite telemetry to compare infectious disease prevalence between stranded and wild harbor seal pups, and to evaluate the effect of disease and contaminant exposure on first year survival. The pathogens and contaminants that were focused on are those that have been associated with lesions in stranded animals, are zoonoses, or are known to enter the marine ecosystem from anthropogenic sources.

The first objective was to characterize the health and disease status of the harbor seal population (Chapters 3 and 4). Chapter 3 analyzed the hematology data, generated reference ranges for the seals and evaluated the effects of age, sex, girth, body condition and geographic location on these blood values (Greig et al. 2010). To understand the role of the harbor seal as a sentinel of the coastal and nearshore marine environment and determine disease risks to seals and humans, a broad surveillance approach was used to assess pathogen exposure in stranded and wild-caught harbor seals (Chapter 4).

The second objective was to compare the immune function of stranded versus wild-caught harbor seals and assess the utility of simple immune parameters in assessing immune fitness in harbor seal pups (Chapter 5). The relationship between immune parameters, growth, and survival was explored in rehabilitating harbor seal pups.

The third objective was to understand the role of organohalogen pollution in the health of developing seal pups (Chapter 6). Differences in blubber contaminant levels in stranded and wild-caught pups were investigated while considering the effect of suckling and a rehabilitation diet on contaminant concentrations. Contaminant exposure differences based on stranding location were evaluated for newborn harbor seal pups.

The fourth objective was to assess the effects of health and contaminant levels on survival probability (Chapter 7). Satellite telemetry was used to track the dispersal and survival of recently weaned wild-caught pups and stranded harbor seals pups post-release. Mark-recapture methods were used to examine associations between the health and contaminant variables measured in the previous chapters on survival probability. Results and future directions were summarized in Chapter 8.

Chapter 2: Sampling overview: Study sites and capture methods

STUDY SITES

Harbor seals were sampled at three locations. Two were wild capture study sites, namely Castro Rocks in San Francisco Bay (37.9328°, -122.4176°) and Clam and Seal Islands in Tomales Bay (37.9328°, -122.4176°), and the third a rehabilitation and stranding response center at The Marine Mammal Center in Sausalito, California (Figure 1.1). The wild capture sites were selected based on their presumed differences in extent of anthropogenic habitat changes (see below). Blood and tissue samples for disease and contaminant analyses were collected from live and dead harbor seals that stranded along the California coast in 2007 and 2008 and from wild-caught harbor seals captured in May and June of 2007 and 2008. Archived serum samples from wild harbor seals captured at the San Francisco and Tomales Bay sites in 2004 as well as seals captured at the San Francisco Bay site in January and July 2001, January and August 2002, August 2003, January 2005, and August 2006 were also used for some analyses. Details of the numbers of animals sampled at each site and each year are given in the individual chapters.

San Francisco Bay (SF). San Francisco Bay, one of the largest estuaries in the United States, has been modified by human occupation and activity which increased dramatically after gold was discovered in the Sierra Nevada mountains in 1848 (Conomos et al. 1985). Most of the bay's saltwater and freshwater marshlands have been drained for farm, residential and industrial land; the diversion of fresh water from the major river systems for irrigation and human use has reduced freshwater inflows to the bay; and remaining inflows are polluted by agricultural, domestic and industrial waste (Nichols et al. 1986). Within the bay, Castro Rocks is one of the largest harbor seal haulouts (Green et al. 2006) consisting of a small island with rocky and sandy intertidal areas which are not available to seals at high tide. The island is located just south of the east span of the Richmond-San Rafael Bridge in close proximity to the Chevron oil refinery and pier. A high speed ferry running between San Francisco and Vallejo passes by several times a day. This site constituted a potentially 'contaminated' study site.

Tomales Bay (TB). Tomales Bay is 20 km by 1 km wide semi-enclosed estuary which receives little freshwater inflow during the summer (Kimbro et al. 2009). The harbor seals rest on sandbars (Clam and Seal Islands) located near the mouth of the bay which, like Castro Rocks, are inundated at high tide. The bay is relatively undeveloped and is bordered along its western coast by Point Reyes National Seashore. Disturbances to the seals occur primarily from small boat traffic and from recreational clammers which share the sandbars during weekend low tides (Flynn et al. 2009). This site constituted a cleaner, less disturbed site.

Harbor seals are present in SF and TB all year (Green et al. 2006, Sarah Allen pers. comm.). While effort and methodology have varied, maximum pup numbers have been reported from both sites (Table 2.1). Maximum harbor seal counts at haulout locations in Point Reyes National Seashore have varied between 2481 and 3506, and are thought to represent approximately 20% of the breeding population in mainland California (Allen et al. 2004).

Table 2.1. Maximum number of pups reported at Castro Rocks in San Francisco Bay and Tomales Bay from 2001-2006.

	Castro Rocks	Tomales Bay
2001	35*	179[1]
2002	44*	130[4]
2003	48*	144[4]
2004	56*	89[4]
2005	50*	188[2]
2006	37[3]	108[3]

*Green et al. 2006, [1]Allen et al. 2004, [2]Vanderhoof and Allen 2005, [3]Manna et al. 2006, [4]S. Allen, personal communication.

The Marine Mammal Center (TMMC). The Marine Mammal Protection Act (MMPA) was passed in 1972 prohibiting the harassment, hunting, capture, killing or collection of marine mammals in US waters. In response to public concern, a group of volunteers founded a non-profit organization in 1975, The Marine Mammal Center (TMMC), to assist sick and injured marine mammals with the goal of rehabilitating and returning them to the wild. In 1992, the MMPA was amended to form a national stranding network, the Marine Mammal Health and Stranding Response Program (MMHSRP). The Marine Mammal Center, as part of the national network, is now legally authorized by the MMHSRP to pick up live stranded pinnipeds and cetaceans. The term "stranding" is defined in the MMPA (section 420) as follows:

> "A. a marine mammal is dead and is--
>
> 1) on a beach or shore of the United States; or
>
> 2) in waters under the jurisdiction of the United States (including any navigable waters); or
>
> B. a marine mammal is alive and is—
>
> 1) on a beach or shore of the United States and unable to return to the water;
>
> 2) on beach or shore of the United States and, although able to return to the water, is in need of apparent medical attention; or
>
> 3) in the waters under the jurisdiction of the United States (including any navigable waters), but is unable to return to its natural habitat under its own power or without assistance."

The Marine Mammal Center responds to stranded animals from Mendocino (40.0000°, -124.0233°) to San Luis Obispo (35.0000°, -120.6402°, Figure 2.1). Harbor seals, primarily young of the year, are admitted to the hospital from the entire rescue range and numbers vary by year (Figure 2.2).

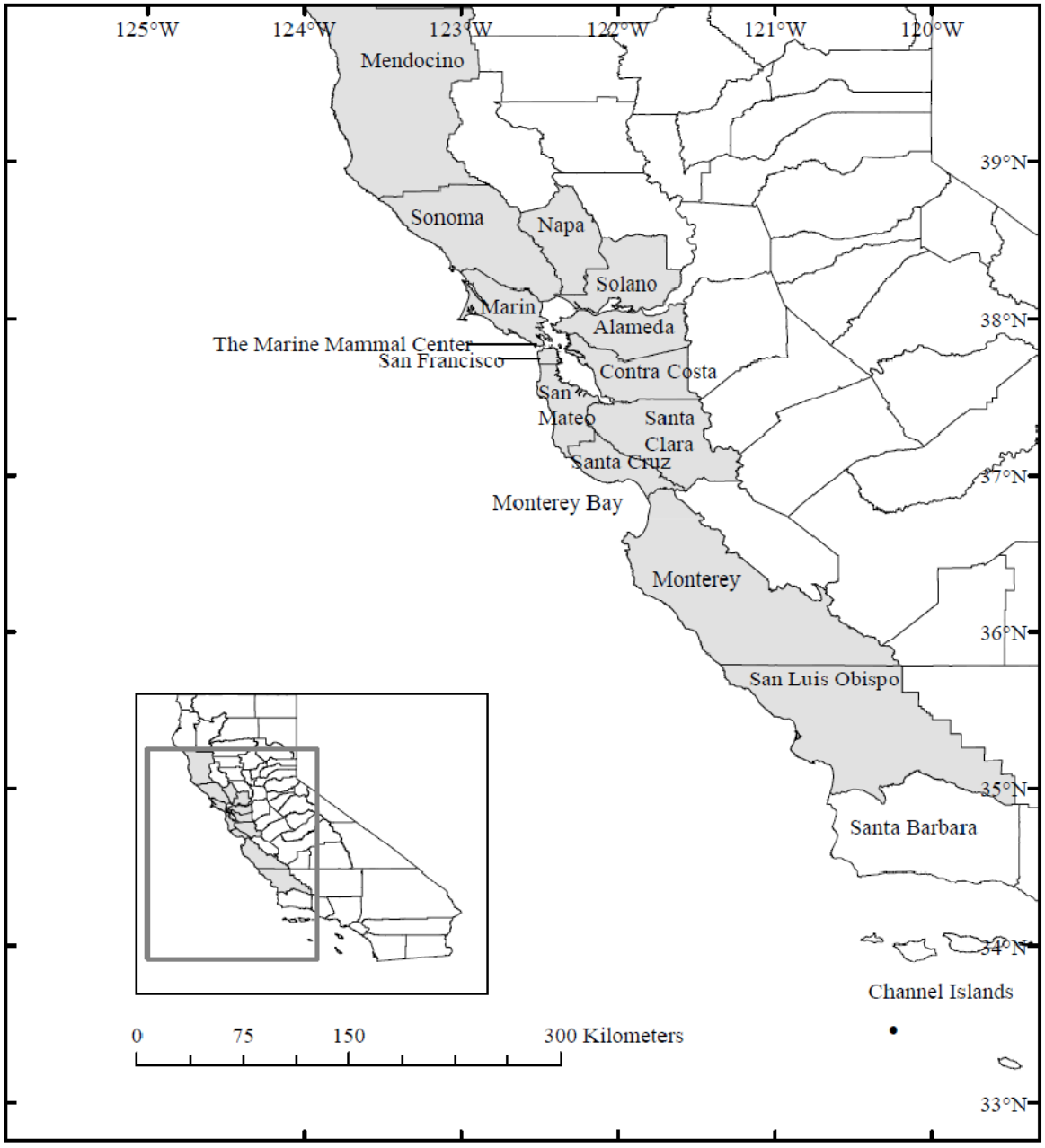


Figure 2.1. Map of California with The Marine Mammal Center's response area shaded in gray.

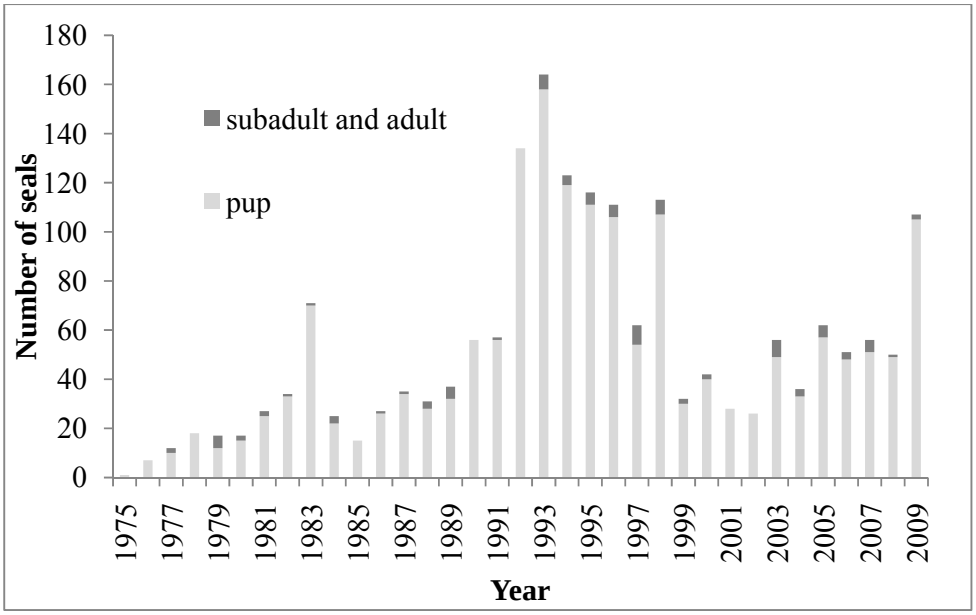


Figure 2.2. Number of live stranded harbor seals admitted to The Marine Mammal Center from 1975 to 2009 by age class and year.

CAPTURE AND SAMPLING

SF and TB. Harbor seals were captured using one of three techniques; hand held nets, monofilament netting that was suspended in the water column along seal haulouts, or seine nets deployed parallel to seal haulouts at high speed and then pulled ashore after Jeffries et al. (1993). Once captured, seals were contained in individual hoop nets and transported to nearby beaches for sampling. Seals were weighed with a hanging digital scale accurate to 0.5kg (Dyna-Link MSI-7200, Measurement Systems International, Seattle, Washington, USA), and physically and chemically restrained with 5mg/ml diazepam (Hospira, Inc. Lake Forest, Illinois, USA) at a dose of 0.25mg/kg. Length from nose to tail and girth at the axilla were measured to the nearest cm; and seals were flipper tagged and injected with a passive integrated transponder (PIT) tag for future identification if recaptured. Blood was drawn from the epidural venous sinus using the Vacutainer system (Becton Dickinson, Franklin Lakes, New Jersey, USA), blubber depth was measured with ultrasound (Hall and McConnell 2007), and a blubber sample was collected. Specific details of blubber sampling and analysis are given in Chapter 6.

Nasal and rectal swabs were collected for viral analyses, fecal swabs were collected for bacterial culture, and when possible feces were collected for protozoal identification. A uniquely numbered hat tag and radio frequency identification (RFID) tag (2007), or satellite tag (2008), was glued to the pelage of all recently weaned pups. Specific details of the marking and sampling methods are given in Chapter 7. Animals were grouped into age classes, based on mass and time of year (Bigg 1969; Greig 2002).

TMMC. Stranded harbor seals were sampled at two separate times: at admission to rehabilitation to determine disease and contaminant exposure prior to stranding, and at death or release to evaluate factors contributing to survival and mortality. At admission to rehabilitation, live harbor seals received a physical examination during which they were weighed and measured, flipper tagged, blood was drawn for complete blood counts (CBCs) and serum chemistries, and banked for serology, and feces was swabbed for culture of bacteria.

At release from rehabilitation, seals were weighed and measured, blood was drawn for CBCs and serum chemistries, blubber depth was measured with ultrasound, a blubber sample was collected, and a hat and RFID tag or satellite tag was glued to the pelage of the head.

Dead stranded harbor seals and seals that died during rehabilitation received post mortem examinations to determine cause of death: they were weighed and measured, blubber was collected for contaminant analysis, tissues were preserved in formalin for histology, and a suite of tissues were frozen at -80°C for follow up of histology or serology results.

Specific details of the sample collection, storage and analyses for the different health and disease variables are given in the appropriate chapters.

Harbor seal age classes.

Pre-weaned pups were less than 4 weeks old, weaned pups were 4 weeks old to 1 year, yearlings were 1 to 2 years old, subadults were 2-5 years old and adults were 5 years and older.

Wild captures occurred just after weaning (in May and June) and pups were easily distinguished from yearlings based on a combination of pelage, body condition and standard length. Mass was used as a general guideline to assign animals to subadult and adult age class after Bigg (1969) such than females over 45 kg and males over 60 kg were considered to be adult, but occasionally underweight animals were considered adults if they appeared to have nursed a pup (had a concave appearance to their abdominal region) or were the same length as the other adults captured at the same time.

Table 2.2. Length and mass by age class for harbor seals (n=151) captured in May and June.

	n	length (cm)	mass (kg)
weaned pup	43	88 (72 - 100)	20 (12.8 - 32)
yearling	14	107 (91 - 199)	30 (24 - 37)
subadult	30	118 (98 - 144)	41 (26 - 55)
adult female	41	138 (121 - 151)	63 (43 - 79)
adult male	23	146 (130 - 162)	74 (51 - 94)

At TMMC, pup age was further broken down based on size, date, and developmental characteristics. Pups estimated to be less than 5 days old based on mass, time of year, and the presence of an umbilical cord or lanugo coat were considered newborns. Any pup with an umbilical cord or lanugo coat was classified as a newborn, and pups under 10 kg (birth mass) stranding in March or April (before peak pup numbers in the wild) were considered newborn. Pups stranding in the first three weeks of May were considered pre-weaning, and pups stranding in the last week of May or later were considered confirmed weaners if they had parasites in their feces and probable weaners otherwise. In cases where specific age was important for the analysis (ex. for evaluating the route of contaminant exposure in Chapter 6), animals of unknown age were grouped accordingly and removed from analysis.

Chapter 3: Hematology and serum chemistry in stranded and wild-caught harbor seals in central California: reference intervals, predictors of survival, and parameters affecting blood variables

INTRODUCTION

Hematology and serum chemistry variables are used to help diagnose disease in marine mammals in rehabilitation and to assess the health of animals in wild populations. However, age, sex, season, reproductive status, captivity, diet, geographic location, and individual variability, as well as laboratory methodology have all been reported to affect results (Thompson et al. 1997; Morgan et al. 1998; Bossart et al. 2001; Trumble et al. 2006). When sampling the same individual repeatedly over time, changes in variables may reflect health trends; however, the results from a single blood sample can be difficult to interpret without baseline reference intervals. Age and sex specific reference intervals have been developed for humans, livestock and domestic animals, but few exist for wild mammals, especially marine mammals where sufficient samples are often scarce (e.g. Bossart et al. 2001).

Human medicine offers a growing body of literature on the choice of an appropriate reference population and the most appropriate methods to calculate reference intervals for blood values for those groups (Fraser 2004). Reference populations can vary by age, sex, or geographic location depending on the research question. For example, hematology and serum chemistry reference intervals from local populations were established for clinical trials in Africa because using reference intervals from Americans as inclusion criteria resulted in the rejection of clinically healthy Africans from the study (Karita et al. 2009). When calculating reference intervals, The International Federation of Clinical Chemistry (IFCC) recommends using nonparametric statistical techniques (Solberg 2004) which have recently been applied to terrestrial (riparian brush rabbit, *Sylvilagus bachmani riparius*; Black et al. 2009) and marine (bottlenose dolphin, *Tursiops truncatus*; Schwacke et al. 2009) wildlife. The resulting reference intervals have been used to evaluate differences in blood profiles among dolphins from different geographic locations as well as between sexes and among age classes (Hall et al. 2007; Schwacke et al. 2009). Such reference intervals for other wildlife species such

as seals, however, remain limited, yet are needed for health assessments of animals in veterinary care and in free ranging populations.

Harbor seal (*Phoca vitulina*) pups undergo a short, but intensive nursing period lasting 3 to 5 weeks. Each spring harbor seal pups that are still dependent on maternal milk are found alone on the beach and admitted to The Marine Mammal Center (TMMC) for rehabilitation, and release back to the wild: approximately 35 dependent pups are admitted each year. In the 10-yr period from 1992-2001, the most common health problems observed at admission among 940 harbor seals were malnutrition (52%), respiratory disease (10%), and trauma (8%); 78% of the harbor seals admitted were preweaned pups but it was unclear whether maternal separation occurred naturally or as a result of human disturbance (Colegrove et al. 2005).

On arrival at the hospital, each pup is examined and blood is drawn for hematology and serum chemistry tests. A treatment plan is devised based on blood results, age, and clinical signs. Previous work has shown that harbor seal pup blood values change during rehabilitation as they mature and their nutritional status improves (Lander et al. 2003). For example, bilirubin levels are often elevated in neonatal harbor seals, but decline to levels similar to those in weaned wild pups by the time of release (Dierauf et al. 1984; Lander et al. 2003). Because of differences in age and diet between newborns admitted to rehabilitation and their recently weaned wild conspecifics, reference intervals generated from the wild weaned population are not useful for assessing the individual health of dependent pups admitted to rehabilitation.

The objectives of this study were to

1) use blood variables from stranded harbor seals (at admission to and release from rehabilitation), recently weaned wild-caught harbor seals, and older wild-caught harbor seals to provide age specific ranges for evaluating harbor seal health in these four different groups of animals;

2) to evaluate the ability of blood variables measured at admission to predict survival during rehabilitation; and

3) to determine the effect of age class, sex, date (Julian day), location, size and body condition on blood variables from wild-caught harbor seals.

METHODS

Capture and sampling. Wild harbor seals of all age classes were sampled in San Francisco Bay, California (SF; 37.9328, -122.417) and Tomales Bay, Point Reyes National Seashore, California (TB; 38.2191, -122.9616) in May and June 2004, 2007, and 2008 after the pups were weaned to avoid disturbing nursing animals. Blood was drawn from the epidural venous sinus into blood collection tubes containing EDTA or serum separation gel (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey, USA) and stored at 4 C from within 5 min of collection until processing. Blood was centrifuged and smears made for manual differentials within 8 hours of collection and fully processed within 24 hours. In 2007 and 2008, stranded harbor seal pups along the California coast from Mendocino (40.0000, -124.0233) to San Luis Obispo (35.0000, -120.6402) counties were brought to TMMC for rehabilitation where they had blood drawn within 5 days of admission and within 48 hr of release. At admission, all pups were rehydrated for varying periods of 1-5 days with subcutaneous fluids and oral electrolytes and treated with oral amoxicillin based on clinical signs and bloodwork.

Sample analyses. A complete blood count (CBC) was obtained using an automated hematology analyzer (Vet ABC Heska, Loveland, Colorado, USA) and included leukocytes (WBC), erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), from which mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were derived, and platelets. White cell differential counts and cell morphology were read manually from a blood smear stained with Wright Giemsa stain (EMD Chemicals Inc., Gibbstown, New Jersey, USA). All differentials were read by the same person (Carlos Rios) knowing only the seal's identification number and species. The hematology analyzer, calibrated for canines, was recalibrated when needed using Minocal whole blood hematology calibrator (scil veterinary excellence, Viernheim, Germany). Three commercial controls (Minotrol 16 Vet whole blood hematology control, scil veterinary excellence) were tested every Monday, then alternated (one per day) Tuesday through Friday as recommended by the manufacturer.

Serum separator tubes were centrifuged and 0.5 ml of serum was used for chemistry analytes determined with an automated chemistry analyzer (Alfa Wasserman Vet Ace, West Caldwell, New Jersey, USA). Chemistry variables measured were iron, cholesterol, triglycerides, gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK), creatine kinase (CK), sorbitol dehydrogenase (SDH), bilirubin, glucose, blood urea nitrogen (BUN), creatinine, phosphorus, calcium, sodium, potassium, chloride, magnesium, total protein and albumin. Globulin was calculated as total protein minus albumin. The sample size for SDH was smaller than for the other variables because this enzyme was added to the chemistry panel midstudy. The sample size for serum chemistries from wild-caught seals was smaller than for hematology because only hematologic variables were determined in 2004. The chemistry analyzer was designed for human use and is calibrated once a month using a calibrator from the manufacturer. Two controls of lyophilized human serum from the manufacturer were run every day as were two separate controls for SDH (Genzyme Diagnostics, Framingham, Massachusetts, USA). If a control failed, it was repeated, and if it failed again the machine was recalibrated and reagents were checked. Monthly quality control data were sent to the Bio-Rad UNITY program, which compared results with peer groups using the same instrument. Quality control results remained within the range generated for these peer groups for all analytes tested.

Data analyses. Data were separated into four datasets based on when they sampled and regardless of why they were admitted to rehabilitation: pups at admission, pups at release, wild-caught pups, and wild-caught subadults and adults. Upper and lower thresholds consisting of 95% confidence intervals, with 90% confidence intervals for each threshold, were generated for each dataset using a nonparametric bootstrap analysis as recommended by the IFCC (Solberg 2004). Bootstrap analyses were performed using the R programming language (R Core Development Team 2009) after Schwacke et al. (2009). For sample sizes less than 40, the minimum and maximum values were reported as the upper and lower thresholds and 90% confidence intervals were not calculated. Three values were considered to be outliers because the difference between those values and the next lowest value was greater than one third of the range of all values for that variable (Schwacke et al. 2009): one band neutrophil count

(2,303/µl), a magnesium concentration (3.4 mg/dl), and a CK concentration (11,935 U/l) were deleted. One pup had a partial chemistry panel, but was retained in the dataset. Two values were deleted from the wild-caught pup dataset: an albumin concentration (0.1 g/dl) that was an outlier and the derived globulin. Three animals with obvious injuries were omitted from the wild-caught subadult and adult dataset as they had potential to affect the boundaries of the intervals.

Binary logistic regression models were used to test whether blood variables measured at admission were predictive of whether an animal would survive rehabilitation to release. Eight animals were omitted from this dataset because they were given antibiotics based on their blood values at admission and this treatment had the potential to affect their outcome. Date of stranding, mass at stranding, and sex were also tested as predictors of survival through rehabilitation. A chi-square test was used to determine whether prematurity (defined after Dierauf et al. 1986 as birth before 15 April and presence of a lanugo coat) was predictive of survival.

Differences in lower and upper thresholds for pups treated at TMMC just prior to release were compared to those of wild-caught recently weaned pups again using a bootstrap approach. Values for each group were estimated and the difference calculated 1000 times; when the 95% confidence intervals around the difference did not include zero, the differences were considered statistically significant (Schwacke et al. 2009).

Generalized linear models (GLMs) were used to evaluate the effects of different morphologic measurements (standard length, mass, axillary girth, and a body condition index), age (using capture date in Julian days as a proxy), sex, and location (SF versus TB) on the wild-caught harbor seal blood variables. The body condition index was the residuals from the linear relationship between mass (kg) and length (cm): mass=-26.47+0.53*length (R^2=0.57, P<0.005).

Length, mass and girth were significantly related to each other, so girth was chosen as the most reproducible of the measurements and a good indicator of body condition in phocid seal pups (Hall and McConnell 2007). Sex was not considered a relevant factor for the pup age class because no differences in physiology and behavior were expected until puberty (Hall 1998a). There was a temporal relationship between location and pup

age because in all three years pup capture effort began in SF and then moved to TB, thus, to investigate the effect of both location and age (Julian day), the starting model for wild-caught pups was additive and included location, age, and girth: blood variable~location+age+girth. Models were fitted to the data with a gamma error structure and logarithmic link function such that predicted Y=exp (intercept+ coefficient*x_1 +coefficient*x_2). Akaike's Information Criterion (AIC) was used to compare models and choose the most parsimonious for each blood variable. Residual analysis was used to assess goodness of fit. Blood variables with zeroes could not be fitted using the gamma error structure, so bands, monocytes, eosinophils, and basophils were fitted using a compound Poisson distribution (Tweedie model).

Because length, girth, and mass are significantly positively correlated with age, they were not used in the model for the older age classes. Instead, we chose location, sex, age class, and condition (which was not associated with age, R^2=0.024, P=0.20). Zero values were deleted from the dataset for monocytes (n=3), eosinophils (n=1), and basophils (n=1). Band neutrophil data were fitted with a compound Poisson distribution (Tweedie model). The fully parameterized model for the wild-caught subadult and adult dataset was blood variable~location+sex+age+ body condition. Parameters were considered significant at P=0.05, but nonsignificant parameters were retained in some models based on AIC.

RESULTS

Predictors of survival. Hematology and serum chemistry variables were measured in dependent pups (n=64) on admission to TMMC in 2007 and 2008 and intervals summarized (Table 3.1). Of the 64 seals, 42 were released back to the wild and 22 died during rehabilitation; one seal did not receive an admission chemistry and one seal did not receive an admission hematology. For the 22 pups that died, the mean number of days in rehabilitation was 20 (SD=19, range=1-70). Causes of death included septicemia and umbilical infection (n=13), malnutrition (n=2), encephalitis (n=2), enteritis, pyelonephritis, euthanasia because of blindness, herpesvirus, and congenital defect.

Two variables at the time of admission were significantly associated with survival through rehabilitation, but neither model would have been significant had a Bonferroni

correction for multiple tests approach been used, and both models were poor fits to the data. Decreased platelets were associated with decreased survival (disposition=platelets*0.005-1.353, P=0.03) and decreased levels of protein were associated with decreased survival (disposition=protein*1.3896-6.939, P=0.01). Neither date of stranding, nor sex affected survival through rehabilitation; however mass at stranding was predictive of survival (probability of survival=$\frac{e^{(\text{mass}*0.956-6.802)}}{1+e^{(\text{mass}*0.956-6.802)}}$, P=0.004) with all the pups that died during treatment stranding at under 10 kg. Prematurity was not predictive of survival during rehabilitation (X^2=1.675, df=1, P=0.20).

Dohle bodies, which are areas of dissolving rough endoplasmic reticulum within neutrophil cytoplasm (Prokocimer and Potasman 2008), were observed in 10 (16%) blood smears including samples with three of the four highest WBC concentrations. Large lymphocytes were observed in 18 (28%) blood smears. There was a wide range of hemoglobin and hematocrit values, which was likely a reflection of hydration status as MCHC was not as variable, but these variables were not associated with survival. Leukocytes also ranged dramatically with the highest and lowest WBC concentrations found in pups that died within 24 hr of admission. Because diseased individuals were included in the dataset, the intervals for this group do not represent true "reference" intervals for dependent harbor seal pups (Geffré et al. 2009).

Released pups compared to recently weaned wild pups. Harbor seal pups were released to the wild after an average of 84 days in rehabilitation (SD=20 days, range=30-143 days). Reference intervals for each blood variable were generated from 45 hematology and 43 serum chemistry samples collected just prior to release (Table 3.2) and from 40 recently weaned pups captured in May and June 2004, 2007, and 2008 (Table 3.3). The pups sampled at release are not directly paired with the pups sampled at admission as some of the pups in the release blood dataset were not sampled at admission (or their blood at admisstion or release was processed at an off-site laboratory and therefore not included in these analyses). Dohle bodies were present in 10 (22%) of the pre-release seals and large lymphocytes in four (9%). Among the wild-caught pups, no Dohle bodies were noted and large lymphocytes were observed in six (15%) seals. There were significant differences between the released pup reference intervals and the

wild pup reference intervals. Lower thresholds among the wild pups were less than the released pups for WBCs, neutrophils, cholesterol, ALT, glucose, phosphorus, sodium, potassium, total protein, albumin and globulin: lower thresholds among the released pups were less than among wild pups for HGB, HCT, MCV, chloride, and CK. Upper thresholds were greater among wild than released pups for HGB, HCT, and glucose, while upper thresholds were greater among released than wild pups for neutrophils, platelets, cholesterol, triglycerides, ALT, AST, SDH, bilirubin, phosphorus, potassium, total protein, and albumin (Tables 3.2 and 3.3).

Predictors of blood variables in wild harbor seals. Samples were obtained from 31 pups from SF and 9 pups from TB. The most consistent factor affecting wild-caught pup blood variables was age: monocytes, eosinophils, RBCs, HGB, HCT, MCH, MCHC, ALT, and creatinine all increased with age; while iron, ALK, CK, SDH, sodium, chloride, and magnesium decreased with age. Location was retained in several models: HCT and glucose were greater in SF than TB and concentrations of MCH, MCHC and SDH were greater in TB than SF. Eosinophils and GGT were negatively associated with girth while creatinine was positively associated with girth (Table 3.4). Increased numbers of monocytes (>200/ul) and eosinophils (>500/ul) appeared around Julian day 150, about 25 days after the pups had been weaned and toward the end of the sampling period.

Samples were obtained from 9 subadult and 10 adult harbor seals in SF and 24 subadult and 27 adult harbor seals in TB. Reference intervals for these animals are reported in Table 3.5. Age class was a significant variable for blood variables from subadult and adult harbor seals: WBCs, lymphocytes, eosinophils, basophils, RBCs, HGB, HCT and ALK were greater among the subadults, while MCH and BUN were greater among adults. Erythrocytes, HGB, and HCT were greater in TB, while monocytes, MCV, and MCH were greater in SF. Males had more neutrophils while females had greater concentrations of MCH. Eosinophils and GGT increased and SDH decreased with increased body condition (Table 3.6).

DISCUSSION

Although typically used to evaluate health and direct clinical management, the blood variables measured in this study at the time of admission were not predictive of whether seal pups would survive rehabilitation. Half of the seals that died during rehabilitation were septic, with the umbilicus as the suspected entry point of infection, although there were rarely signs of inflammation on the initial blood panel to guide treatment. This suggests that the immediate handling for hematologic assessment of neonates using the current routinely available tests may be less important than other features of the admission examination. While we support the use of hematology and serum chemistry to help diagnose illness and inform treatment during rehabilitation, the lack of prognostic changes in these data leads us to recommend that the initial treatment be based on mass and stage of development (e.g., presence of an umbilicus) of the seal. It should thus focus on minimizing the risk of umbilical infection and septicemia in neonates by cleaning the umbilical area regularly and using broad spectrum antibiotics.

The significant relationship between mass at stranding and the probability of survival through rehabilitation was consistent with the recognized association between mass and survival at various life stages in phocid seals: mass at weaning has been linked to first year survival in grey seals (*Halichoerus grypus;* Hall et al. 2002) and survival to two years old in monk seals (*Monachus schauinslandi*; Craig and Ragen 1999); and autumnal mass has been linked to over-winter survival in harbor seals (Harding et al. 2005). Low birth mass and prematurity are associated with decreased survival in humans (Goldenberg and Culhane 2007) and red deer (*Cervus elaphus*; Clutton-Brock et al. 1987). Interestingly, among free-ranging harbor seals on Sable Island, Nova Scotia, birth mass was not associated with survival to weaning (Coltman et al. 1998). Prematurity, rather than mass, has previously been associated with poor survival of seals in rehabilitation (Dierauf et al. 1986), but that was not the case in the current study.

Decreased HGB and HCT in the released pups compared with the wild pups may be a function of underdeveloped diving abilities. Pups in rehabilitation are housed in pools up to four feet deep, so have not experienced water depths to develop the dive capabilities that wild pups have. Increased oxygen stores in wild weaned pups compared with wild nursing pups described by (Clark et al. 2007) are consistent with the increases

in RBCs, HGB, HCT and MCHC with age observed in the recently weaned wild pups in this study.

Even though wild pups were captured during a relatively short time period (31 days), age was an important explanatory variable for several blood variables. These trends over time may indicate a switch from nursing to fasting, the development of increased dive capabilities, or growth and maturation in general. In an experimental study of recently weaned harp seal (*Phoca groenlandica*) pups, Worthy and Lavigne (1982) found that ALT, ALK, sodium, and CK were lower in fasting seals than feeding seals. In this study, ALK, sodium, and CK decreased over time suggesting a transition from nursing to fasting, but ALT increased over time. In domestic animals, the release of the enzyme ALT from tissues into the blood is associated with liver disease; however, in harbor seals ALT is also found in skeletal muscle and kidneys such that its presence in blood is not liver specific (Fauquier et al. 2008). ALT levels also may be related to specific food sources: In captive harbor seals, ALT was increased in those seals eating pollock as opposed to herring (Trumble et al. 2006). Although changes in hematologic variables with diet have been noted in harbor seals (Thompson et al. 1997), our study cannot directly address effects of diet changes in wild seals as prey consumption at the time of sampling was not known. Eosinophils, which have been associated with parasitism, and monocytes, which can indicate acute or chronic inflammation (Bossart et al. 2001; Piché et al. 2010), increased dramatically in the weaned harbor seal pups after 25 days. This may provide an initial estimate of the time from weaning to an inflammatory reaction to parasite acquisition from prey items.

The significance of the Dohle bodies noted in the neutrophils of harbor seal pups in rehabilitation is not known; Dohle bodies were not noted in the wild-caught weaned pups. In humans, the presence of Dohle bodies is an early indicator of infection and sepsis (Prokocimer and Potasman 2008), but their role in marine mammal neutrophils has not been documented. A greater percentage of harbor seals exhibited Dohle bodies on their blood smears at release than at admission and their presence was not associated with survival; thus we suspect that they are unlikely to be indicators of sepsis in newborn harbor seals. The meaning of the large lymphocytes in harbor seals at

admission is also unknown although in humans can result from bacterial, viral or helminthic infection (Prokocimer and Potasman 2008).

A number of hematologic variables varied with age, sex, condition and location among the wild-caught subadult and adult harbor seals. The slight hematologic variation with location may reflect exposure to different infectious agents with location as evidenced by the increased monocytes in SF. Chemistry variables were not affected by location and were fairly robust to differences in age, sex and condition with the exception of GGT, BUN and SDH. These variables, as well as the hematologic variables, should be interpreted with caution in the field and the clinical setting when only a single sample is available. In addition, these seals were sampled only during the breeding season when adult seals are in relatively poor body condition compared with winter months; this study did not test for the effect of season on hematology and serum chemistry values.

In summary, the intervals generated by this study can help clinicians evaluate blood variables from stranded harbor seals and the effects of age, sex, condition and location on wild harbor seal blood variables may inform study designs for future health assessment studies of these animals. However, this study indicates that, at the time of admission, the standard panel of variables used in veterinary practice today has little effect on treatment plan or rehabilitation outcome. Recently characterized markers of inflammation such as C-reactive proteins (Funke et al. 1997) and interleukins (Fonfara et al. 2008) may be more sensitive earlier in treatment and should be evaluated clinically. Furthermore, application of proteomics, genomics, and metabolomics (Abu-Asab et al. 2008) to marine mammal health evaluations is needed because of the limitations of using conventional hematology and serum chemistry panels alone to assess health status from a single sample.

Table 3.1. Intervals for blood variables from dependent harbor seal pups at admission to The Marine Mammal Center 2007-2008.

Blood variable	n	90% CI on lower threshold	Lower threshold	Median	Upper threshold	90% CI on upper threshold
WBC (/ul)	63	2000-4500	2800	8100	25000	13300-32900
neutrophils (mature)	63	1120-2173	1680	5440	22000	9842-26978
neutrophils (band)	62	0-0	0	56	864	570-990
lymphocytes	63	663-1127	720	2130	3888	3626-5088
monocytes	63	0-0	0	153	648	567-749
eosinophils	63	0-0	0	0	384	124-492
basophils	63	0-0	0	0	344	255-352
RBC (10^6/ul)	63	4.22-4.73	4.44	5.81	7.09	6.95-7.33
HGB (g/dl)	63	14.0-17.3	14.7	19.8	24.4	23.6-25.2
HCT (%)	63	39.9-49.2	42.5	57.6	68.8	68.1-73.4
MCV (fl)	63	90-93	92	102	113	110-114
MCH (pg)	63	29.7-31.8	30.8	35.2	40.8	38.5-40.8
MCHC (g/dl)	63	31.7-32.6	31.8	34.5	37.7	36.2-37.8
platelets (10^3/ul)	63	59-216	91	375	695	638-763
iron (ug/dl)	62	45-55	46	181	562	423-585
total cholesterol (mg/dl)	63	144-174	150	277	446	410-456
triglycerides (mg/dl)	63	31-66	47	112	276	239-328
GGT (U/l)	63	9-15	13	34	242	130-304
ALT (U/l)	63	19-26	20	71	325	220-340
AST (U/l)	63	15-29	24	61	462	154-621
ALK (U/l)	63	43-92	59	177	404	306-511
CK (U/l)	61	71-84	72	194	2569	1389-3140
SDH (U/l)	33		1.8	26.1	87.5	
total bilirubin (mg/dl)	63	0.3-0.7	0.6	2.4	24.1	13.6-24.8
glucose (mg/dl)	62	40-79	64	149	331	286-361
BUN (mg/dl)	63	7-13	11	26	59	47-70
creatinine (mg/dl)	63	0-0.1	0	0.4	0.6	0.6-0.6
phosphorus (mg/dl)	62	4.1-4.9	4.7	6.3	8.6	8.1-8.6
calcium (mg/dl)	62	7.2-8.0	7.5	9.0	10.2	9.9-10.6
sodium (mmol/l)	63	125-138	131	147	162	154-164
potassium (mmol/l)	63	3.1-3.9	3.3	4.7	5.8	5.4-6.5
chloride (mmol/l)	63	88-98	96	108	124	118-133
magnesium (mg/dl)	61	1.4-1.5	1.5	1.7	2.3	2.2-2.4
total protein (g/dl)	62	4.0-4.5	4.3	5.6	6.5	6.4-6.6
albumin (g/dl)	62	2.1-2.8	2.4	3.5	4.2	4.1-4.3
globulin (g/dl)	62	1.4-1.6	1.5	2.0	3.1	3.0-3.3

Table 3.2. Reference intervals for blood variables from harbor seal pups at release from The Marine Mammal Center 2007-2008. Asterisks indicate significant difference from the blood variable thresholds in wild pups in Table 3.3.

Blood variable	n	90% CI on lower threshold	Lower threshold	Median	Upper threshold	90% CI on upper threshold
WBC (/ul)	45	4900-7300	6200*	9700	15300	13600-16000
neutrophils (mature)	45	3080-3735	3348*	6077	11250*	8925-11520
neutrophils (band)	45	0-0	0	0	565	93-1224
lymphocytes	45	972-1617	1170	2790	4900	4150-5194
monocytes	45	0-0	0	74	900	480-918
eosinophils	45	0-0	0	136	1308	837-1332
basophils	45	0-0	0	238	666	558-721
RBC (10^6/ul)	45	4.23-4.80	4.54	5.33	6.03	5.74-6.70
HGB (g/dl)	45	15.9-17.2	15.9*	19	21.9*	21.3-22.1
HCT (%)	45	45.5-48.1	46.1*	54.4	62.0*	61.7-62.7
MCV (fl)	45	90-95	93*	103	112	109-115
MCH (pg)	45	33.0-33.9	33.2	36.4	39.1	38.6-40.1
MCHC (g/dl)	45	32.9-33.6	33.0	35.3	38.2	37.9-38.5
platelets (10^3/ul)	42	268-463	334	689	1130*	900-1490
iron (ug/dl)	43	65-111	95	199	494	417-566
total cholesterol (mg/dl)	43	243-257	248*	306	422*	370-535
triglycerides (mg/dl)	43	40-62	55	210	436*	392-550
GGT (U/l)	43	7-15	14	18	49	28-81
ALT (U/l)	43	22-41	28*	56	99*	78-176
AST (U/l)	43	30-45	32	75	191*	142-505
ALK (U/l)	43	43-83	62	136	307	265-434
CK (U/l)	43	72-103	79*	247	1986	1122-8472
SDH (U/l)	23		16.3	68.9	127.1*	
total bilirubin (mg/dl)	43	0.2-0.3	0.3	0.8	1.9*	1.7-2.3
glucose (mg/dl)	43	117-128	121*	145	176*	162-177
BUN (mg/dl)	43	25-35	29	48	75	61-77
creatinine (mg/dl)	43	0.3-0.4	0.3	0.5	0.8	0.7-1.0
phosphorus (mg/dl)	43	4.8-5.4	4.8*	7.3	10.1*	8.8-10.6
calcium (mg/dl)	43	8.8-9.1	8.9	9.9	10.4	10.3-10.4
sodium (mmol/l)	43	144-146	145*	150	160	152-166
potassium (mmol/l)	43	4.0-4.3	4.0*	4.9	5.8*	5.6-5.9
chloride (mmol/l)	43	99-103	100*	108	118	112-124
magnesium (mg/dl)	42	1.5-1.7	1.6	2.0	2.6	2.3-2.6
total protein (g/dl)	43	6.0-6.8	6.6*	7.5	8.9*	8.2-9.0
albumin (g/dl)	43	3.0-3.2	3.1*	3.5	4.0*	3.7-4.3
globulin (g/dl)	43	2.7-3.2	3.0*	4.0	5.2	4.7-5.4

Table 3.3. Reference intervals for blood variables from recently weaned, wild-caught harbor seal pups, California 2004, 2007, and 2008. Asterisks indicate significant difference from the blood variable thresholds in released pups in Table 3.2.

Blood variable	n	90% CI on lower threshold	Lower threshold	Median	Upper threshold	95% CI on upper threshold
WBC (/ul)	40	4300-4800	4300*	7550	13300	11300-13600
neutrophils (mature)	40	1968-2464	1968*	4193	8214*	6901-8296
neutrophils (band)	40	0-0	0	0	309	266-360
lymphocytes	40	1088-1364	1088	2354	4070	3294-4407
monocytes	40	0-0	0	142	812	486-960
eosinophils	40	0-0	0	236	1596	1224-2730
basophils	40	0-0	0	185	928	540-1088
RBC (10^6/ul)	40	4.70-4.82	4.70	5.76	6.43	6.07-6.70
HGB (g/dl)	40	17.3-17.9	17.3*	21.6	23.9*	22.9-24.5
HCT (%)	40	49.4-52.8	49.4*	60.6	68.7*	64.9-69.9
MCV (fl)	40	99-100	99*	106	113	111-113
MCH (pg)	40	33.6-34.2	33.6	36.9	40.7	40.1-42.2
MCHC (g/dl)	40	32.8-33.3	32.8	34.5	39.0	37.4-39.2
platelets (10^3/ul)	40	153-301	153	485	653*	622-795
iron (ug/dl)	35		68	184	646	
total cholesterol (mg/dl)	35		146*	245	361*	
triglycerides (mg/dl)	35		35	88	157*	
GGT (U/l)	35		5	17	81	
ALT (U/l)	35		19*	29	58*	
AST (U/l)	35		27	47	92*	
ALK (U/l)	35		37	126	540	
CK (U/l)	35		127*	309	1403	
SDH (U/l)	30		13.2	34.3	74.4*	
total bilirubin (mg/dl)	35		0.2	0.5	1.0*	
glucose (mg/dl)	35		99*	152	217*	
BUN (mg/dl)	35		25	36	62	
creatinine (mg/dl)	35		0.3	0.6	1	
phosphorus (mg/dl)	35		3.7*	4.8	6.5*	
calcium (mg/dl)	35		8.8	9.7	10.6	
sodium (mmol/l)	35		143*	149	157	
potassium (mmol/l)	35		3.8*	4.4	5.1*	
chloride (mmol/l)	35		105*	108	117	
magnesium (mg/dl)	35		1.6	2.1	2.8	
total protein (g/dl)	35		5.2*	5.8	7.7*	
albumin (g/dl)	34		2.3*	3.3	3.6*	
globulin (g/dl)	34		2.0*	2.5	5.4	

Table 3.4. Model results for significant generalized linear models fitted to blood parameters from wild caught harbor seal pups.

blood parameter	model parameter	estimate	SE	t value	p value
monocytes	intercept	-10.795	2.710	-3.98	0.00
	age_day	0.108	0.018	6.03	0.00
eosinophils	intercept	-2.457	3.227	-0.76	0.45
	location_TB	-0.825	0.468	-1.76	0.09
	age_day	0.091	0.020	4.48	0.00
	girth	-0.064	0.027	-2.42	0.02
erythrocytes (10^6/mm^3)	intercept	1.384	0.156		
	location_TB	-0.047	0.032	-1.43	0.16
	age_day	0.003	0.001	2.33	0.03
hemoglobin (g/dl)	intercept	2.438	0.123		
	age_day	0.004	0.001	5.05	0.00
hematocrit (%)	intercept	3.666	0.147		
	location_TB	-0.074	0.031	-2.41	0.02
	age_day	0.003	0.001	3.02	0.00
mean cell hemoglobin (pg)	intercept	3.361	0.106		
	location_TB	0.044	0.022	2.02	0.05
	age_day	0.002	0.001	2.28	0.03
mean cell hemoglobin conc. (g/dl)	intercept	3.353	0.064		
	location_TB	0.065	0.013	4.95	0.00
	age_day	0.001	0.000	2.94	0.01
iron (ug/dL)	intercept	8.168	0.951		
	age_day	-0.020	0.007	-2.93	0.01
GGT (U/L)	intercept	5.861	1.000		
	girth	-0.041	0.014	-2.97	0.01
ALT (U/L)	intercept	2.401	0.485		
	age_day	0.007	0.003	2.10	0.04
ALK (U/L)	intercept	10.632	1.258		
	location_TB	0.662	0.259	2.56	0.15
	age_day	-0.040	0.009	-4.40	0.00
CK (U/L)	intercept	9.873	1.235		
	age_day	-0.027	0.009	-3.13	0.00
SDH (U/L)	intercept	6.794	1.241		
	location_TB	0.793	0.244	3.25	0.00
	age_day	-0.025	0.009	-2.70	0.01
glucose (mg/dL)	intercept	5.083	0.031		
	location_TB	-0.256	0.064	-4.02	0.00
creatinine (mg/dL)	intercept	-2.558	0.532		
	age_day	0.010	0.003	3.70	0.00
	girth	0.010	0.005	2.11	0.04
sodium (mmol/L)	intercept	5.172	0.040		
	location_TB	-0.015	0.008	-1.86	0.07
	age_day	-0.001	0.000	-4.01	0.00

chloride (mmol/L)	intercept	4.797	0.050		
	age_day	-0.001	0.000	-4.33	0.00
	girth	0.001	0.000	1.50	0.14
magnesium (mg/dL)	intercept	1.154	0.358		
	location_TB	0.106	0.057	1.87	0.07
	age_day	-0.005	0.002	-2.63	0.01
	girth	0.004	0.003	1.54	0.13
albumin (g/dL)	intercept	0.995	0.097		
	location_TB	-0.038	0.022	-1.75	0.09
	girth	0.003	0.001	2.11	0.43

Table 3.5. Reference intervals for blood variables from wild-caught subadult and adult harbor seals, May and June 2004, 2007, and 2008.

Blood variable	n	90% CI on lower threshold	Lower threshold	Median	Upper threshold	90% CI on upper threshold
WBC (/ul)	70	5300-6800	5600	10900	18000	15500-19100
neutrophils (mature)	70	1276-2546	1520	5610	10325	9300-11050
neutrophils (band)	70	0-0	0	0	350	145-465
lymphocytes	70	440-1458	1140	3006.5	5940	4797-6000
monocytes	70	0-96	0	475.5	1562	1170-2397
eosinophils	70	0-464	408	1264.5	3441	2875-5157
basophils	70	0-109	91	397.5	900	764-1170
RBC (10^6/ul)	70	2.9-4.09	3.75	4.92	5.84	5.64-5.97
HGB (g/dl)	70	12.7-17	15.8	20.3	23.3	22.8-24.4
HCT (%)	70	34.7-48.5	43.3	56.65	63.2	62.4-65
MCV (fl)	70	97-107	100	115	126	122-131
MCH (pg)	70	35.9-37.6	36.3	41.4	46.7	43.9-47.7
MCHC (g/dl)	70	34-34.4	34.1	35.5	39.1	38.4-39.5
platelets (10^3/ul)	69	155-329	178	462	806	670-823
iron (ug/dl)	57	35-71	58	146	312	258-330
total cholesterol (mg/dl)	57	157-182	178	233	342	324-369
triglycerides (mg/dl)	57	23-46	38	86	245	174-487
GGT (U/l)	56	0-7	1	11	29	21-39
ALT (U/l)	57	20-32	22	54	119	101-159
AST (U/l)	57	53-66	59	113	326	251-510
ALK (U/l)	56	0-9	4	38	80	66-88
CK (U/l)	57	154-230	205	659	5125	2806-9462
SDH (U/l)	16	15.7-28.7	15.7	48.0	87.2	66.2-87.2
total bilirubin (mg/dl)	57	0.2-0.3	0.2	0.5	1.8	1.4-1.9
glucose (mg/dl)	57	86-95	87	120	161	145-172
BUN (mg/dl)	57	20-29	20	41	74	67-86
creatinine (mg/dl)	57	0.5-0.6	0.5	0.8	1.1	1-1.1
phosphorus (mg/dl)	57	1.9-2.7	2.5	4.8	7.2	6.8-11.3
calcium (mg/dl)	57	7.7-8.1	7.9	9.1	9.9	9.8-9.9
sodium (mmol/l)	57	145-147	146	149	154	153-162
potassium (mmol/l)	57	3.9-3.9	3.9	4.4	5.2	4.9-5.2
chloride (mmol/l)	57	104-106	106	109	117	113-120
magnesium (mg/dl)	57	1.4-1.5	1.5	1.8	2.2	2.1-2.4
total protein (g/dl)	57	7-7.3	7	7.9	9.5	9-9.9
albumin (g/dl)	57	2.5-2.5	2.5	3	3.4	3.3-3.5
globulin (g/dl)	57	4.1-4.4	4.3	5.0	6.7	6.1-6.7

Table 3.6. Model results for significant generalized linear models fitted to blood variables from wild-caught subadult and adult harbor seals.

Blood variable	Model parameter	Estimate	SE	*t* value	*P* value
WBC (/ul)	Intercept	9.241	0.043		
	age_sa	0.175	0.063	2.78	0.01
neutrophils (mature)	Intercept	8.573	0.054		
	sex_m	0.266	0.107	2.49	0.02
lymphocytes	Intercept	7.926	0.062		
	age_sa	0.201	0.090	2.23	0.03
monocytes	intercept	6.735	0.139		
	location_TB	-0.574	0.164	-3.49	<0.005
eosinophils	intercept	7.080	0.081		
	age_sa	0.412	0.118	3.51	<0.005
	condition	0.022	0.010	2.21	0.03
basophils	intercept	5.920	0.084		
	age_sa	0.310	-0.121	2.56	0.01
RBC (10^6/ul)	intercept	1.469	0.237		
	location_TB	0.107	0.025	4.30	<0.005
	age_sa	0.077	0.022	3.45	<0.005
HGB (g/dl)	intercept	2.925	0.023		
	location_TB	0.083	0.024	3.40	<0.005
	sex_m	-0.045	0.025	-1.81	0.07
	age_sa	0.054	0.022	2.50	0.01
HCT (%)	intercept	3.940	0.022		
	location_TB	0.078	0.023	3.41	<0.005
	age_sa	0.057	0.020	2.79	0.01
MCV (fl)	intercept	4.778	0.012		
	location_TB	-0.026	0.012	-2.11	0.04
	sex_m	-0.018	0.013	-1.46	0.15
	age_sa	-0.021	0.011	-1.95	0.06
MCH (pg)	intercept	3.758	0.013		
	location_TB	-0.027	0.013	-2.00	0.05
	sex_m	-0.030	0.014	-2.23	0.03
	age_sa	-0.023	0.012	-1.99	0.05
GGT (U/l)	intercept	2.390	0.084		
	age_sa	0.203	0.118	1.73	0.09
	condition	0.028	0.011	2.53	0.01
ALK (U/l)	intercept	3.472	0.093		
	age_sa	0.318	0.133	2.39	0.02
SDH (U/l)	intercept	3.832	0.090		
	condition	-0.314	0.015	-2.16	0.05

Table 3.6. continued. Model results for significant GLMs fitted to blood variables from wild-caught subadult and adult harbor seals.

Blood variable	Model parameter	Estimate	SE	*t* value	*P* value
BUN (mg/dl)	intercept	3.950	0.057		
	sex_m	-0.132	0.083	-1.59	0.12
	age_sa	-0.265	0.074	-3.56	<0.005

Chapter 4: Surveillance of zoonotic pathogens and pathogens previously reported in harbor seals (*Phoca vitulina*) from central California

INTRODUCTION

Infectious disease outbreaks have been reported in marine mammals worldwide (Gulland and Hall 2007) and some epizootics (such as those caused by phocine and canine distemper virus) have had serious impacts on marine mammal populations. For example, in 1988, approximately 18,000 harbor seals died from phocine distemper virus (PDV in the North Sea (Heide-Jorgensen et al. 1992) and thousands of Baikal seals (*Phoca sibirica*) died from canine distemper virus (CDV, Mamaev et al. 1996). In addition to the risk they pose to marine mammal populations, many diseases that affect marine mammals are zoonoses, raising concerns over coastal water pollution posing a risk to human health (Knap et al. 2002).

Zoonotic pathogens known to cause disease in harbor seals include influenza A (Geraci et al. 1982), *Brucella* (Garner et al. 1997), *Toxoplasma gondii* and *Sarcocystis neurona* (Lapointe et al. 1998; Miller et al. 2001), and *Leptospira interrogans* (Stamper et al. 1998; Stevens et al. 1999). In addition, surveys of apparently healthy animals in remote areas considered to be relatively pristine have demonstrated exposure to zoonotic bacteria such as *Brucella* sp. and protozoa such as *Giardia*, *Cryptosporidium*, *T. gondii*, and *S. neurona* (Dubey et al. 2003; Zarnke et al. 2006; Jensen et al. 2010), highlighting the role that marine mammals may play as sentinels of pollution in the marine environment or as reservoirs for pathogens that could affect humans (Ross 2000).

There are thus a variety of zoonotic organisms in the marine environment that can impact both marine mammal and human health. In coastal California, harbor seals share habitats with humans, with some habitats obviously impacted by anthropogenic effects such as sewage outfalls, and urban and agricultural developments (Grigg 2008).

The objectives of this study were

1) to assess the risks to harbor seals in the wild from pathogens known to cause disease in harbor seals; and

2) to assess the use of harbor seals in the wild as sentinels of pollution with zoonotic pathogens in the nearshore coastal environment.

Specific pathogens of interest were *E coli*, *Clostridium perfringens*, *Vibrio* spp, *Campylobacter* spp, *Salmonella*, *Giardia*, *Cryptosporidium*, *Brucella*, avian influenza virus, *Leptospira* spp, *Toxoplasma gondii*, *Sarcocystis neurona*, and *Neospora caninum*, and phocine and canine distemper virus.

METHODS

Disease exposure in mammalian populations can be measured using serology to detect the presence of serum antibodies to a specific pathogen, or using isolation or molecular techniques to detect the presence of an organism or its DNA/RNA. Identifying exposure or presence of an organism does not determine whether a pathogen is causing disease or mortality. To do so, the presence of the pathogen must be causally linked to a disease process or lesion, however the prevalence of exposure can be useful for understanding disease dynamics, for example, whether a pathogen is present in a population and the risk of encountering that pathogen (Hall et al. 2010).

We used two approaches to evaluate pathogen exposure in harbor seals: 1) the detection of pathogens from the seals including use of PCR to detect their nucleic acid and 2) the detection of antibodies in serum indicating previous exposure to specific pathogens. Samples for testing were acquired from two different sources: 1) wild-caught, clinically healthy, post-weaning seals of all age classes, and 2) primarily young of the year seals admitted to rehabilitation which lived to release, died during rehabilitation or stranded dead. These different approaches and sources (wild-caught and stranded, live and dead) involved a variety of laboratory techniques which are listed by pathogen in Table 4.1 and described in detail in the sample analysis section.

Table 4.1. Summary of approach and samples used for each pathogen. Sources for sampling were wild-caught (wild) or stranded. "At admission" means live and dead stranded seals that were sampled on arrival to The Marine Mammal Center, while animals sampled during "necropsy" may have spent time in rehabilitation prior to sampling.

Pathogen	**Detection method**	**Sample**	**Source**
Enteric bacteria - *E coli, Clostridium perfringens, Vibrio spp, Campylobacter spp, Salmonella*	culture	feces via swab	wild, stranded (at admission)
Giardia, Cryptosporidium	observation – DFA	feces	wild, stranded (at admission)
Giardia	molecular detection - PCR*	feces	wild, stranded (at admission)
Brucella	molecular detection - PCR	spleen, lymph node	stranded (necropsy)
Avian influenza	molecular detection - PCR	nasal/rectal swabs	wild, stranded (at admission)
Avian influenza	serology - ELISA	serum	wild, stranded (at admission)
Leptospira spp.	serology - MAT	serum	wild, stranded (at admission)
Toxoplasma gondii, *Sarcocystis neurona*, *Neospora caninum*	serology - IFAT	serum	wild, stranded (at admission)
Phocine and canine distemper virus	serology - SN	serum	wild, stranded (at admission)

DFA=direct immunofluorescent antibody test, PCR=polymerase chain reaction, ELISA=enzyme linked immunosorbent assay, MAT=microagglutination test, IFAT=indirect fluorescent antibody test, and SN=serum neutralization. *These samples were also used in a molecular study characterizing Giardia in marine vertebrates (Lasek-Nesselquist et al. 2010).

Sampling. In 2007 and 2008, exposure to infectious disease was determined in harbor seals brought to a seal rehabilitation hospital (The Marine Mammal Center- TMMC) and in harbor seals captured and released (Jeffries et al. 1993) at two locations: Castro Rocks in San Francisco Bay (SF, 37° 55′ 58″ N, 122° 25′ 3″ W) and Tomales Bay (TB, 38° 13′ 9″ N, 122° 57′ 42″ W, Figure 4.1). At the time of admission or capture, seals were weighed, measured (length and girth), and had a blood sample drawn. Seals were assigned to an age class based on mass, time of year, and stage of development (Bigg 1969; Dierauf et al. 1986; Greig 2002). Blood was centrifuged and serum archived at -80° C for serologic testing. Three rectal swabs were collected from each animal for the detection of fecal bacteria, placed into Cary Blair transport media (Becton Dickinson, Franklin Lakes, New Jersey, USA) and refrigerated at 4° C until processing. When available, a fecal sample was collected with a fecal loop and stored at 4° C for the detection of *Giardia* and *Cryptosporidium*. A nasal and rectal swab from each wild-caught harbor seal was placed into viral transport media for avian influenza detection and frozen at -80° C. To determine cause of death for stranded seals that died and to determine whether any disease processes were caused by the pathogens under investigation, a complete necropsy was performed at TMMC and histopathologic examination of tissues was performed by a pathologist. Spleen and tracheobronchial lymph node were frozen at -80° C for *Brucella* detection. Archived serum samples collected from wild-caught harbor seals in 2001, 2002, 2004, 2005 and 2006 were available for serology and additional swabs for fecal bacteria were collected in 2006.

Sample analysis.

Fecal bacteria. Rectal swabs were plated onto selective media, and bacteria that grew were identified using standard microbiological techniques at the UC Davis Veterinary Medical Teaching Hospital Microbiology Lab. Swab number one was plated onto MacConkey agar and incubated at 37º C for 24 hours to look for general enteric bacteria; the same swab was plated onto Xylose-Lysine-Tergitol 4 agar (XLT4) which is selective for non-*typhi Salmonella* and incubated for one day; and finally, swab number one was placed into selenite broth (for the selective enrichment of *Salmonella*) for 24 hours and then transferred to XLT4. Swab number two was plated onto Campy CVA agar which is selective for *Campylobacter spp* and contains three antimicrobials to

prevent the growth of normal fecal flora (Cefoperzone, Vancomycin, and Amphotericin B) and incubated at 37º C for >48 hours. Next, swab number two was placed into alkaline peptone (an enrichment media for *Vibrio spp*), incubated overnight and subcultured to thiosulfate citrate bile salts sucrose agar (TCBS) for the selective isolation of *Vibrio spp*. Swab number three was plated onto egg yolk agar (EYA) and incubated in an anaerobe chamber (Bactron IV) at 37º C for 48 hours to test for *Clostridium perfringens*. All media and reagents were purchased from Hardy Diagnostics (Santa Maria, California, USA) except for the EYA and selenite broth which were supplied by the UC Davis in-house Veterinary Medicine Biological Media Services.

Salmonella isolates were identified to serogroup (B1, C, D, E) by their agglutination to *Salmonella* anti sera (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and to strain with additional agglutination tests performed at the National Veterinary Services Lab (NVSL) in Ames, Iowa. Potential *Campylobacter* colonies that grew on the CVA agar were stained with BBL Gram stain reagents (Fisher Scientific) to check their morphology. Then the colony was subcultured to blood agar, and disks impregnated with nalidixic acid and cephalothin were placed on the inoculated blood agar plate and incubated for 48 hours. The sensitivity pattern of the isolate to the antibiotic disks was recorded, and further testing for confirmation included a catalase test (Hardy Diagnostics) and a hippurate disk test (Hardy Diagnostics). *Vibrio* colonies were identified to species using API 20E strips (bioMérieux Inc, Durham, North Carolina, USA). These strips consist of 21 biochemical tests which change color, some with the addition of reagents, depending on the metabolism of the bacteria cultured. *Clostridium perfringens* was identified by its characteristic reaction to EYA (lecithinase production), the Christie Atkins Munch-Petersen (CAMP) test, indole reation, aerotolerance and typical Gram stain morphology.

***Giardia* and *Cryptosporidium*.** Fecal samples were examined for *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts using a direct immunofluorescent antibody test (DFA, A100FLK Aqua-Glo G/C Direct Comprehensive Kit) from Waterborne, Inc (New Orleans, Louisiana, USA). Feces were washed with dilute dish washing detergent and strained through gauze into a 50 ml conical vial. Dilute detergent was added to 35

ml and conical vials spun for 10 minutes at 1000-1500g. The top layer of the pellet was then transferred to a DFA slide well using a 10ul fecal loop (Fisher Scientific). Slides were dried and kept up to two weeks before they were incubated with fluorescent antibody, and viewed under a microscope with a fluorescein filter at the Marine Wildlife Veterinary Care and Research Center (MWVCRC) in Santa Cruz.

Brucella. A survey to detect the presence of *Brucella* was conducted using tissues collected at necropsy from stranded harbor seals that were admitted dead or died during rehabilitation. Samples of spleen and tracheobronchial lymph node were archived at -80^{o}C and submitted to Mystic Aquarium and Institute for Exploration for PCR. DNA was extracted from tissues using a standard tissue extraction kit (DNeasy, Quiagen, Valencia, California, USA). Quantitative PCR (qPCR) was performed on samples using the technique described by Sidor et al. (2006). Briefly, a multiplex qPCR assay was developed using a Taqman probe-based assay which targets a 150 base pair amplicon from bcsp31; an outer membrane protein gene specific to the *Brucella* genus. The triplex assay includes two internal controls for DNA quality control and to detect endogenous inhibitors of PCR. Testing of this assay against a variety of common aquatic bacterial isolates demonstrated 100% specificity for *Brucella*, and assays of DNA extracted from pinniped and cetacean origin *Brucella* isolates demonstrate sensitivity at or below 3 bacteria. Real time PCR was then performed on samples using the Light Cycler 2.0 instrument (Roche Diagnostics, Alameda, California, USA), with an initial 15 minute denaturation step at 95^{o}C, followed by 45 cycles of 95^{o}C denaturation for 30 seconds and 63^{o}C annealing/extension for 60 seconds, with a final cooling step at 40^{o}C for 60 seconds. Each sample was run in triplicate and a reagent blank, negative tissue control, and positive bacterial dilutions were run in each batch of samples tested.

Avian influenza. Swabs in viral media were submitted to the National Wildlife Health Center for detection of all current subtypes of avian influenza using real-time RT-PCR (Ip 2008). After RNA extraction, a one-step RT-PCR kit (Qiagen, Valencia, California, USA) was used with a PCR primer set designed for a region conserved in all type A influenza virus Matrix genes (Spackman et al. 2002). Viral isolation in embryonating eggs was attempted from all 2008 samples. Allantoic fluids from each egg were tested

for the presence of hemagglutinating viruses using chicken and turkey red blood cells (Ip 2008).

Sera for the detection of antibodies to avian influenza were submitted to the Southeastern Cooperative Wildlife Disease Study at the University of Georgia in Athens, Georgia. Testing was performed using a commercially available blocking enzyme-linked immunosorbent assay (bELISA, FlockCheck AI MultiS-Screen antibody test kit; Idexx Laboratories, Westbrook, Maine, USA) which has been validated for use in avian and mammal species (Brown et al. 2009; Ciacci-Zanella et al. 2010). The test was performed and interpreted according to the manufacturer's instructions. Briefly, serum samples were diluted 1:10 in diluent provided by the manufacturer, 100 µl added to the antigen-coated test plate, and allowed to incubate for 60 min at room temperature. Each well was washed five times with test kit wash solution and 100 µl of anti-AI virus-horseradish peroxidase conjugate was added to each well, followed by 100 µl of 3,3',5,5'-tetramethylbenzidine as a color indicator. The reaction was stopped with 100 µl of stop solution and absorbance values were measured at a wavelength of 650nm. Positive and negative controls from the kit were included on each plate. The test was validated for mammals using sera from influenza infected ferrets as a mammalian control. Serum samples with a result-to-negative-control (S/N) absorbance ratio greater than or equal to 0.50 were considered negative for the presence of AI virus antibodies, and samples with an S/N values less than 0.50 were considered positive (Brown et al. 2009).

Leptospira. Serum samples were tested for antibodies against six serovars of *Leptospira* (*L. interrogans* serovar Bratislava, *L. interrogans* serovar Canicola, *L. kirshneri* serovar Grippotyphosa, *L interrogans* serovar Hardjo, *L. interrogans* serogroup Icterohemorrhagiae, and *L interrogans* serovar Pomona) by the California Animal Health and Food Safety Laboratory in Davis, California using the microagglutination test (Cole et al. 1973). Although it has not been validated in harbor seals, the *Leptospira* MAT has been validated for exposure in the California sea lion with titers of 1:100 and greater considered positive (Colagross-Schouten et al. 2002).

Toxoplasma, Sarcocystis, Neospora. An indirect fluorescent antibody test (IFAT) was used to test for antibodies to *Toxoplasma gondii*, *Sarcocystis neurona*, and *Neospora*

caninum after Miller et al (2001). Briefly, antigen slides were prepared by harvesting tachyzoites from cell culture, suspending them in sterile phosphate buffered saline (PBS) and applying 10 µl to each well on a 12 well slide. The slides were dried, fixed in formalin for 10 minutes, rinsed and stored at -70° C. Serum was serially diluted on a microtiter plate in doubling dilutions beginning with 1:40. Dilutions (10-15 µl) were applied to the 12 well antigen slide and incubated in a moist chamber at 37° C for 1 hour. The slides were washed 3 times, for 5 minutes each in PBS and blotted dry. Fluorescein isothiocyanate conjugated dog antibody at 1:100 dilution in PBS was added to each well (10 µl). Slides were incubated for 1 hour, washed and dried. Mounting media (.0715g $NaHCO_3$, .016g Na_2CO_3, 10 ml di H_2O, QS to 100ml w/ glycerol; pH 8.0-9.0) was added to each well and cover slipped. Slides were examined at 200X using a fluorescence microscope and the last well with distinctly fluorescent parasite outlines was the reported titer. The *Toxoplasma gondii* IFAT has not been validated for harbor seals, but has been validated for sea otters with active brain infections at a titer of 1:320 (Miller et al. 2002b).

Morbillivirus. Serum was submitted to the Athens Veterinary Diagnostic Lab at the University of Georgia for detection of phocine and canine distemper virus antibodies with a serum neutralization test (Saliki and Lehenbauer 2001; Saliki et al. 2002). Archived serum from 2001 and 2002 was sent to the Oklahoma Animal Disease and Diagnostic Laboratory at Oklahoma State University. At both laboratories, the test was conducted according to methods described in Saliki and Lehenbauer (2001). Briefly, serial twofold dilutions (in duplicate) of heat-inactivated sera were made in eight columns of 96-well plates using Eagle's minimum essential medium, starting at a 1:2 dilution. Next, 25 µl of the viruses containing approximately 100 50% tissue culture infective doses were added. The virus-serum mixtures were incubated at 37°C for 1 h in 5% CO2, and a Vero cell suspension (150 µl containing 10^4 cells/well) was added. The plates were incubated at 37°C in 5% CO2 for 4 days. Cell monolayers were examined under an inverted microscope for virus-specific cytopathic effects (CPE). Antibody titers were expressed as the reciprocals of the highest dilutions of sera that completely neutralized CPE in duplicate wells. All samples with a titer of 8 or greater were considered positive for morbillivirus antibody. For positive serum samples, the

homologous virus was considered to be the one against which the serum had the highest titer (Saliki and Lehenbauer 2001).

Data analysis. To evaluate whether pathogenic fecal bacteria were more likely to occur in stranded seals versus wild-caught seals, odds ratios were used to compare the probability of culturing fecal bacteria from seals in the three groups: TMMC, SF and TB. Odds are defined as (the proportion of cases)/(1-the proportion of cases) and are used to estimate the probability of becoming infected with a given pathogen based on proportion data, which in this instance was the number of cases divided by the number of controls (Rothman 2002). Chi square tests were used to compare antibody prevalence between locations. Odds ratio and chi square analyses were performed using the R programming language (R Core Development Team 2009).

Because the presence of *Campylobacter* is so ephemeral in the marine environment, strand date and location may be indicative of when and where the seal was exposed to the bacteria, although it is also possible that seals could be chronic carriers of the bacteria. SaTScan^{TM}v8.0 (Kulldorff 2009) was used to determine whether there were any patterns in space and time among the *Campylobacter* cases compared to the those that stranded without *Campylobacter*. Three likelihood ratios, the space-time scan statistic, the temporal scan statistic, and the spatial scan statistic were used with the Bernoulli model to test whether *Campylobacter* positives were randomly distributed in time and throughout the stranding response area (Kulldorff and Nagarwalla 1995). SatScan uses Monte Carlo simulations to obtain the probability distributions of scan windows of different sizes to find the most probable clusters of cases compared with controls (Kulldorff 1997). Binary logistic regression analysis was used to test whether *Campylobacter* positives were associated with distance to the California Environmental Protection Agency's National Pollutant Discharge Elimination System's (CA EPA NPDES) major outfalls and discharges.

RESULTS

The harbor seals admitted to TMMC from 2007 and 2008 were primarily young of the year seals, including pups likely separated or abandoned at birth (n=130) as well as weaned pups (n=28), yearlings (n=4), subadult (n=2), and adults (n=11). Wild-caught harbor seals captured from 2004 through 2008 (n=177) were a mixture of weaned pups (n=46), yearlings (n=20), subadults (n=38) and adults (n=73). Wild-caught seals were in good body condition and appeared healthy at the time of capture. Sample size and overall prevalence of exposure are displayed in Table 4.2 while sample age class and location are discussed by pathogen in the following sections.

Table 4.2. Sample size and prevalence (percent positive) by pathogen tested.

Pathogen	**wild-caught**	**stranded**
Isolation	**n(%)**	**n(%)**
Giardia-DFA	14 (7.1)	40 (0.0)
Cryptosporidium-DFA	14 (0.0)	40 (2.5)
*Giardia-PCR**	15 (6.7)	71 (5.6)
Brucella-PCR	0	58 (3.4)
avian influenza	96 (1.0)	11 (0.0)
Serology		
avian influenza	92 (6.5)	
Leptospira spp.	170 (61.2)	93 (29.0)
Toxoplasma gondii	172 (7.6)	111 (0.9)
Sarcocystis neurona	172 (9.3)	111 (0.9)
Neospora caninum	172 (7.0)	111 (0.9)
phocine distemper	217 (0.0)	109 (0)
canine distemper	217 (0.5)	109 (0)

* These samples form part of another student's dissertation; results of the genetic typing are presented in Lasek-Nesselquist et al. (2010).
Enteric bacteria results are presented separately in Table 4.3

Fecal bacteria. Fecal swabs were collected from 243 harbor seals (135 at admission to rehabilitation, 45 in SF and 61 in TB). *E. coli* was the most prevalent bacteria cultured in fecal samples from all three locations, followed by *V. parahemolyticus* in SF samples and *C. perfringens* in TMMC and TB samples (Table 4.3). *E. coli* was cultured from 75.6% of the stranded harbor seals with stranded animals 2.5 times more likely to have *E coli* cultured from their feces than wild-caught harbor seals from TB (Table 4.4). *Clostridium perfringens* was 2.9 times more likely in stranded harbor seals than the wild-caught seals from SF. Hemolytic *E coli* was present in all three groups of animals, but 6.2 times more likely in SF than TB or TMMC. *Campylobacter sp* were cultured from all three groups, but *Campylobacter jejuni* and *Campylobacter coli* were each cultured from a single stranded animal. The remaining eleven *Campylobacter* cultures could not be typed without molecular methods, but were determined not to be *C. jejuni* based on phenotypic characteristics. *Vibrio* spp were 12.0 times more likely to be cultured from seals in SF largely driven by the high prevalence of *V. parahemolyticus* in the SF samples. *V. alginolyticus* was more likely in SF and TB than TMMC seals and *V. cholerae* and *V. parahemolyticus* were not detected in any samples from TB. *Salmonella* was detected but at a very low prevalence with a single culture from the stranded seals and a single culture from the wild-caught seals. In addition to the targeted bacteria, several other enteric bacteria were detected including Beta-hemolytic *Streptococcus, Streptococcus bovis, Klebsiella pneumonia, Pleisomonas shigelloides, Edwardsiella tarda, Edwardsiella hoshinae,* and *Photobacterium damsela* (Table 4.3).

Among the fecal cultures from stranded animals, a spatial-temporal cluster of *Campylobacter* isolates with a radius of 72 km was found centered in Santa Cruz (36.948, -122.066) from 4/17/2008 to 6/11/2008 (p=0.010). Within this cluster, *Campylobacter* grew in 7 out of 12 fecal samples (Figure 4.1). In addition, a purely temporal cluster of *Campylobacter* was found in seals stranding between June 5-9, 2008 when *Campylobacter* was cultured from 4 out of 5 fecal samples (p=0.016): All four seals were weaned pups, two from Half Moon Bay harbor and two from Moss Landing. *Campylobacter* isolates were not associated with proximity to the major outfalls and discharges (p=0.423).

Table 4.3. Bacteria cultured from the feces of stranded (TMMC) and wild-caught harbor seals from San Francisco (SF) Bay and Tomales Bay.

	TMMC (n=135)		SF Bay (n=45)		Tomales (n=61)	
	# positive	%	# positive	%	# positive	%
E coli	102	75.6	24	51.1	44	72.1
hemolytic *E coli*	5	3.7	8	17.0	2	3.3
Campylobacter spp. (not jejuni)	11	8.1	5	10.6	4	6.6
Campylobacter jejuni	1	0.7				
Campylobacter coli	1	0.7				
Clostridium perfringens	78	57.8	16	34.0	29	47.5
Vibrio parahemolyticus	7	5.2	23	48.9		
V. alginolyticus	1	0.7	3	6.4	5	8.2
V. cholerae	9	6.7	1	2.1		
Salmonella enteritidis	1	0.7				
S. newport					1	1.6
Beta-hemolytic *Streptococcus*	1	0.7				
Streptococcus bovis	1	0.7				
Klebsiella pneumoniae	1	0.7				
Pleisomonas shigelloides	1	0.7	1	2.1	2	3.3
Edwardsiella tarda	1	0.7	1	2.1	2	3.3
Edwardsiella hoshinae	1	0.7				
Photobacterium damsela			1	2.1		

Table 4.4. Odds ratios of culturing fecal pathogens from stranded (TMMC) versus wild-caught harbor seals from San Francisco (SF) Bay and Tomales Bay. Odds ratios significant at the p=0.05 level using the Fisher exact test are bolded. *Vibrio* spp is the combination of *V. cholerae*, *V. parahemolyticus*, and *V. alginolyticus*.

			95% confidence intervals		
	Location	Odds ratio	lower	upper	p value
E coli	SF Bay	1.000			
	Tomales	2.242	0.997	5.135	0.065
	TMMC	**2.687**	**1.320**	**5.484**	**0.008**
hemolytic *E coli*	Tomales	1.000			
	TMMC	1.088	0.217	8.648	1.000
	SF Bay	**5.960**	**1.364**	**45.282**	**0.017**
Campylobacter spp.	Tomales	1.000			
	TMMC	1.478	0.491	5.603	0.591
	SF Bay	1.759	0.423	7.836	0.490
Clostridium perfringens	SF Bay	1.000			
	Tomales	1.631	0.741	3.666	0.239
	TMMC	**2.459**	**1.230**	**5.062**	**0.015**
Vibrio spp.	Tomales	1.000			
	TMMC	1.578	0.584	5.104	0.467
	SF Bay	**15.914**	**5.690**	**53.764**	**<0.005**
V. cholerae	SF Bay	1.000			
	TMMC	1.559	0.385	12.892	0.455
V. parahemolyticus	TMMC	1.000			
	SF Bay	**18.384**	**7.324**	**51.977**	**<0.005**
V. alginolyticus	TMMC	1.000			
	SF Bay	4.674	1.057	51.570	0.049
	Tomales	**5.877**	**1.397**	**54.518**	**0.012**

Note: *V. cholerae* and *V parahemolyticus* were not detected in any samples from Tomales Bay

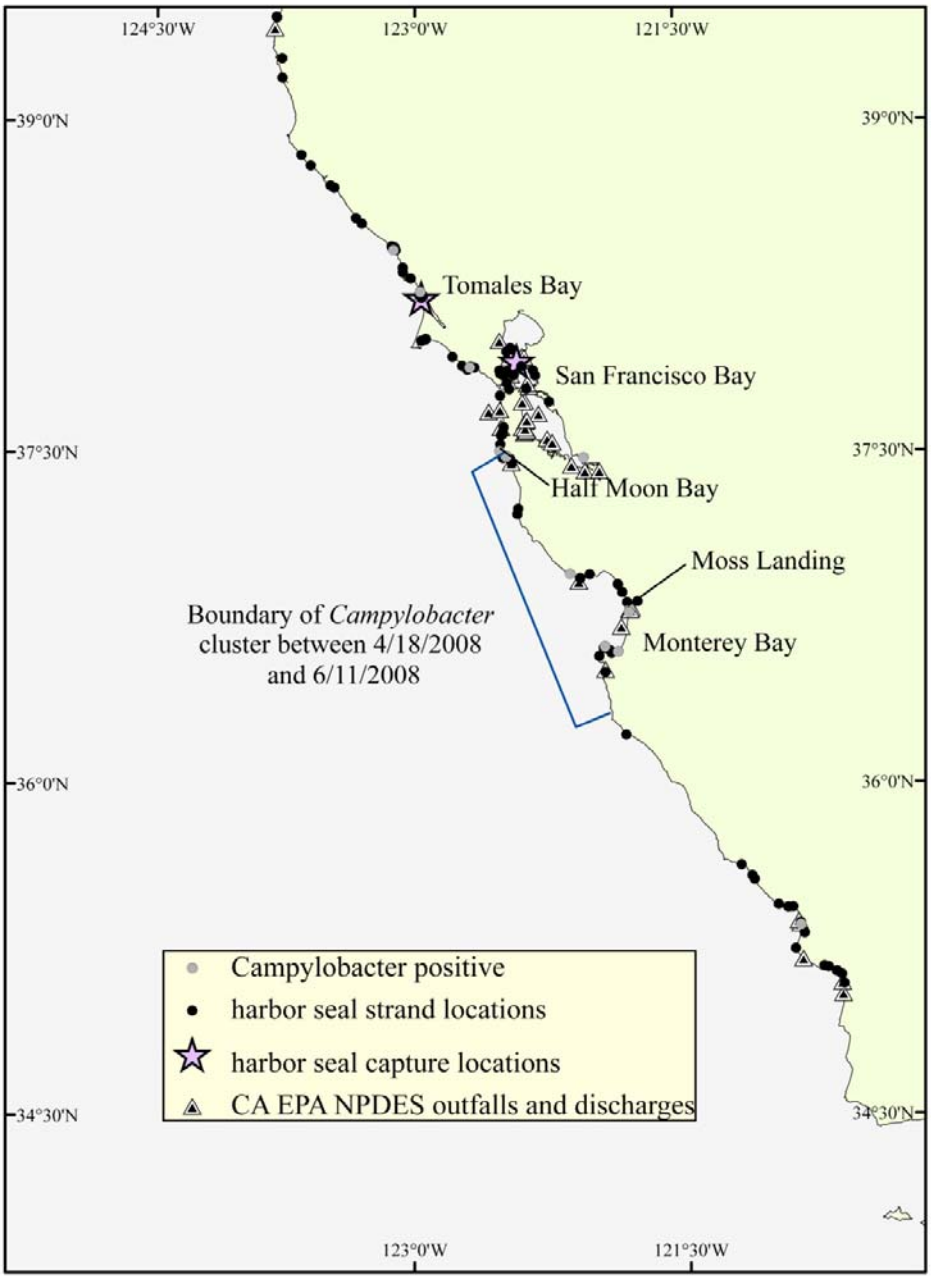


Figure 4.1. Map of stranding locations of harbor seals sampled for fecal pathogens in 2007 and 2008. Outfall locations are the California Environmental Protection Agency's National Pollutant Discharge Elimination System's (CA EPA NPDES) major outfalls and discharges. Wild-caught harbor seal capture locations are indicated with a star.

Giardia* and *Cryptosporidium. In 2007, fecal samples from 40 stranded seals (3 adults, 8 weaners, and 29 pups) yielded a single *Cryptosporidium* positive observed in the feces from a weaner that stranded alive at Linda Mar Beach south of San Francisco Bay (37.6030° N, 122.4990° W). Fecal samples from 14 wild-caught harbor seals (5 adults, 4 subadults, 2 yearlings, 3 weaners) yielded a single *Giardia* positive in a subadult from Tomales Bay. Because the number of detections was so much lower than reported in harbor seals to the north in Washington State (Gaydos et al. 2008), there was concern that the results were an artifact of methodology, and samples were sent to Wood's Hole Oceanographic Institute for *Giardia* PCR in 2008. In 71 stranded harbor seals tested by PCR from 2007 and 2008, there were 4 positives for *G. intestinalis* Assemblage B and in 15 wild-caught harbor seal samples, there was a single positive for *G. intestinalis* Assemblage B (Lasek-Nesselquist et al. 2010).

Brucella. Tissues from 58 stranded harbor seals from 2007 and 2008 were submitted for *Brucella* screening: samples included 54 tracheobronchial lymph nodes, 55 spleens, one placenta, and one lung with accompanying lungworms. Most samples were from pre-weaned pups (Table 4.5).

Table 4.5. Age and sex of the stranded harbor seals sampled for *Brucella* by PCR. Premature pups were those stranding underweight with full lanugo early in the season (1/31 to 4/9) while the fetus was full term, but found *in utero*.

Age class	female	male	Total
fetus	1		1
premature	3	12	15
pup	12	18	30
weaned pup	5	6	11
adult	1		1
Total	22	36	58

Two animals were *Brucella* positive: One weaned pup (HS 1754) had a brucella positive spleen and lymph node, while another (HS 1866) had positive spleen, but

negative lymph node, lung and lung worms. HS 1754 had a bronchopneumonia with *Otostrongylus* lungworms and liver lesions suggestive of sepsis, but no *Brucella*-associated pathology. HS 1866 had intranuclear inclusions in the lung and adrenal consistent with phocid herpesvirus-1 and a pneumonia with *Parafilaroides* lungworms, but no *Brucella*-associated pathology. Both positives were detected in weaned pups, for an overall prevalence of 3% (2 out of 58), or a prevalence of 17% (2 out of 12) among weaned pups and older.

Avian influenza. A total of 107 harbor seals were sampled for influenza virus by PCR (Table 4.6). Rectal and nasal swabs from 96 wild-caught harbor seals and 11 carcasses from the mouth of Drakes Estero, Point Reyes National Seashore were negative for avian influenza using the matrix gene RT-PCR test, however virus isolation yielded a culture from the rectal swab of a female subadult captured in Tomales Bay in June 2008. The viral isolate contained a mixed infection with undetermined hemagglutinin subtypes and N6 and N9 neuraminidase subtypes found by nucleotide sequencing.

Table 4.6. Location and age class of harbor seals samples for avian influenza PCR.

Location	Year	adult	subadult	yearling	weaner	pup	Total
San Francisco Bay	2007	4	3	2	7		16
	2008				19		19
Tomales Bay	2007	19	13	3	1		36
	2008	8	5	4	8		25
Drakes Estero	2008					11	11
Total		31	21	9	35	11	107

A subset of 92 serum samples from the seals tested by PCR were submitted for influenza serology (32 from SF and 60 from TB). Four of 36 (11%) TB seals in 2007 and two of 24 (8%) TB seals in 2008 had avian influenza antibodies. No positives were detected from seals sampled in SF yielding an overall prevalence of 6.5% (6 out of 92) for all samples tested. The PCR positive harbor seal was not seropositive.

Leptospira. Serum samples from 270 harbor seals were submitted for the *Leptospira* MAT. Seven TMMC samples were too contaminated for *Leptospira* testing and were omitted from analysis leaving 93 serum samples from stranded harbor seals and 170 samples from wild harbor seals. Among the stranded seals, antibodies to Icterohemorrhagiae were at the highest titer in all samples, and all titres were less than or equal to 1:800 except for one which had a titer >1:3200 to Icterohemorrhagiae and Grippotyphosa. This yearling female was found dead on the beach. At necropsy, several areas of alopecia were noted around the eyes, anus and ventral surface of the flippers; there was a large abscess on the left hip; and severe bronchopneumonia. No *Leptospira* were associated with lesions on histopathology. Among the wild-caught seals, Icterohemorrhagiae was the highest titer for all samples except for one adult male from TB that had a titer of 1:1600 to Grippotyphosa and 1:100 to Icterohemorrhagiae. All other titers were low (1:100, 1:200, 1:400 and one 1:800) and interpreted as evidence of exposure rather than infection. Prevalence of exposure varied by year with *Leptospira* antibodies detected in almost all wild-caught harbor seals in 2004 and 2005 (Table 4.7).

Table 4.7. Number of harbor seals tested (n) and percent positive (%) with a titer to *Leptospira interrogans* Icterohemorrhagiae of 1:100 or greater by location and year.

Location	Year	Adult n(%)	Subadult n(%)	Yearling n(%)	Weaner n(%)	Pup n(%)
San Francisco Bay						
	2004	8 (100)	4 (100)	1 (100)	5 (100)	
	2005	2 (100)	4 (100)	4 (100)	1 (100)	
	2006	4 (75)	3 (100)	1 (0)	0	
	2007	6 (50)	3 (100)	2 (0)	8 (25)	
	2008	0	0	0	19 (5)	
Tomales Bay						
	2004	24 (92)	5 (100)	4 (100)	3 (100)	
	2007	19 (42)	13 (46)	3 (67)	1 (100)	
	2008	7 (71)	5 (20)	3 (67)	8 (13)	
TMMC						
	2007	2 (0)	0	0	9 (78)	32 (66)
	2008	0	0	2 (100)	10 (40)	38 (5)

Toxoplasma, Sarcocystis, Neospora. Serum samples were tested for protozoal exposure from 111 harbor seals admitted to TMMC. All seals were pups except for two yearlings, one subadult, and four adults. One adult female had antibody titers to *Sarcocystis* (1:10240) and *Neospora caninum* (1:320): this animal had an extensive meningoencephalitis which is consistent with a *Sarcocystis* infection although no protozoa were evident on histologic examination. One subadult female had a titer to *Toxoplasma* (1:640), but cause of death was attributed to a severe bronchopneumonia. All stranded pups tested were negative for exposure to *Toxoplasma*, *Sarcocystis* and *Neospora* (n=104).

Titers to all three protozoa were detected in wild-caught seal serum from SF and TB (n=172, Table 4.8a and 4.8b). One adult female sampled in SF in August 2006 had a titer to *T. gondii* of 1:10240. She appeared healthy during capture, but her lymphocytes, triglycerides, alkaline phosphatase, glucose, phosphorus, magnesium, total protein, and globulin were all elevated and her creatine kinase was decreased compared with subadults and adults captured in May and June (Chapter 3). She was hat tagged and observed in SF with no visible symptoms through April 2007 when she likely shed her tag. There was no difference in the prevalences of *Toxoplasma* and *Sarcocystis* detected in SF and TB, but *N. caninum* was more prevalent in SF than TB (chi square, p=0.007).

Table 4.8a. *Toxoplasma, Sarcocystis* and *Neospora* titers in wild-caught harbor seals from San Francisco (n=75)

San Francisco	40	80	160	320	640	1280	2560	10240	%*
Toxoplasma				1	2		2	1	8
Sarcocystis	2		2	2	1	2			7
Neospora	11	5		4	3	1	1		12

* prevalence calculated using titers greater than 1:160

Table 4.8b. *Toxoplasma, Sarcocystis* and *Neospora* titers in wild-caught harbor seals from Tomales Bay (n=97)

Tomales Bay	40	80	160	320	640	1280	2560	10240	%*
Toxoplasma	6	4	3	3	2		2		7
Sarcocystis	6	2	5	4	2	3	2		11
Neospora	5	4	2	2			1		3

* prevalence calculated using titers greater than 1:160

Morbillivirus. A total of 326 serum samples were submitted for morbillivirus testing (109 from TMMC, 122 from SF, 95 from TB, Table 4.9). All samples were negative to CDV and PDV except for a single female weaned pup captured in Tomales Bay in 2004 which had titers to CDV (1:256) and PDV (1:128). This was considered to be a CDV positive with likely cross reaction to PDV (see methods).

Table 4.9. Location, year, and age class of samples tested for morbillivirus.

Location	Year	adult	subadult	yearling	weaner	pup	Total
San Francisco Bay	2001	5	6	4	6		21
	2002	8	7	4	6		25
	2004	8	4	1	5		18
	2005	2	4	4	1		11
	2006	4	3	2			9
	2007	6	3	2	8		19
	2008				19		19
Tomales Bay	2004	24	5	4	3		36
	2007	19	13	3	1		36
	2008	8	5	3	7		23
TMMC	2007	4	1		8	41	54
	2008			3	10	42	55
Total		88	51	30	74	83	326

DISCUSSION

Because serology cannot distinguish between current or previous exposure (Hall et al. 2010), serology and isolation detect pathogen presence at different time scales, and care must be taken when comparing prevalence data gathered by the two different approaches. For that reason, the results are discussed by pathogen followed by a discussion of the overall approach and conclusions.

Fecal bacteria. Although *E. coli* and *C. perfringens* were more likely to be cultured in stranded harbor seals than wild-caught seals, the high prevalence of these bacteria in all three locations in apparently healthy seals suggests that they are part of the normal gut flora of harbor seals. The prevalence of *Salmonella* in stranded harbor seals (1%) was dramatically lower than in stranded elephant seals (36.9%, Stoddard et al. 2005) although Newport, the most common serotype among the stranded northern elephant seals was detected in a harbor seal. Among stranded seals in the UK, grey seals also had a greater prevalence of *Salmonella* than harbor seals (Baker et al. 1995). Grey seals and northern elephant seals both remain on land from birth through their post weaning fast and may have increased exposure to fecal pollution from land compared with harbor seals which enter the water soon after birth. *Vibrios* in the coastal environment vary by season and location and their population dynamics are poorly understood (Thompson et al. 2005), thus patterns in seal exposure (i.e. increased prevalence in SF) may simply reflect differences in *Vibrio* distribution. The *Campylobacter* cultures provided evidence of recent fecal pollution with small boat harbors associated with seal exposure in this study. While the *Campylobacter* positive seals were not associated with known outfalls and sewage discharges, a more in depth analysis of the variable flow rates at the locations might be necessary to detect an association as was done with the northern elephant seal study (Stoddard et al. 2008).

While none of the bacteria were associated with illness in the seals, they have the potential to contribute to enteritis and wound infections. The prevalence of Salmonella in infected wounds and tissues (5%, Thornton et al. 1998) was greater than detected in the feces at admission to rehabilitation (0.7%, this study). *Plesiomonas shigelloides* was associated with gastroenteritis among harbor seals pups that had been in rehabitation 1-3 months (Koski and Vandenbroek 1986), but was cultured in this study from one harbor

seal admitted to rehabilitation and three clinically healthy wild harbor seal pups. The extent to which fecal bacteria are involved in the development of the septicemias and gastrointestinal disease observed in harbor seal pups in rehabilitation is unknown and beyond the scope of this study, but the fact that seals acquired these potentially pathogenic bacteria, whether through their prey or directly from the environment, is concerning for humans using the same resources. *Photobacterium damsela,* for example, has caused fatal infections in fishermen (Yamane et al. 2004).

Giardia* and *Cryptosporidium. The prevalence of *Giardia* previously reported in Washington State harbor seals (42%, Gaydos 2008) was much greater than the prevalence detected by this study (5% to 7%). This may be related to methodology: both this study and Gaydos et al (2008) used the same immunofluorescence antibody test although Gaydos et al (2008) used immunomagnetic separation to concentrate Giardia in the fecal samples prior to analysis. Lasek-Nesselquist et al (2010) detected a higher prevalence of Giardia in harbor seals on the east coast of the United States versus central California harbor seals using the same PCR primers for all samples so it is likely that the low number of detections in these samples using the DFA and PCR is accurate and may reflect stronger tidal flushing of haulout areas in central California compared with Washington State. Many of the Washington State locations were in small inlets, while most of the stranded seals in this study came from locations along the open coastline or areas within San Francisco Bay with strong tidal flushing (Conomos et al. 1985).

Brucella. *Brucella* has been shown to cause abortions in bottlenose dolphins (Miller et al.1999), but there is no evidence from this study to implicate *Brucella* as a causative agent of disease in the stillborn or premature harbor seals that we sampled. Excluding the negatives from the pre-weaned pups, 17% prevalence in the post weaning age classes is similar to the 11% reported in harbor seal tissue from the North Sea (Prenger-Berninghoff et al. 2008), but lower than the 46% reported inAlaska (Zarnke et al. 2006). This is worthy of consideration for humans in close contact with harbor seals whether in rehabilitation or the wild, and among people who consume pinnipeds for food (Higgins 2000) as *Brucella* species can be transmitted through mucosal membranes (Brew et al. 1999).

Avian influenza. The avian influenza isolate confirms that clinically healthy harbor seals might act as a reservoir for influenza viruses possibly allowing genetic reassortment and posing a potential risk to humans that work closely with these animals such as staff and volunteers working in the rehabilitation or other captive setting. The prevalence of non pathogenic strains of avian influenza in birds and seals in rehabilitation is low (0.1%), but occupational risk could increase dramatically if an animal stranded with a pathogenic strain (Siembieda et al. 2008). In addition, there is evidence that humans may pose a risk to seals (Ohishi et al. 2002). A directed study of influenza strains in seals and humans would be necessary to determine the rates of transmission between seals and humans and the risk they pose to each other. Transmission rates from birds to seals also are not known although avian influenza was recently detected in the Common Murre (*Uria algae*), a seabird with habitat that overlaps the harbor seal, in central California (Siembieda et al. 2010).

Leptospira. The significance of the low *Leptospira* titers in the wild and stranded harbor seals is unknown with only one stranded female yearling with a high titer suggesting active infection (>1:3200 to Grippotyphosa and Icterohemorrhagiae). This seal was dead when admitted to TMMC so it was not possible to submit paired titers for serology and there were no *Leptospira* observed on histology. The variation in low titer prevalence by year, however, is interesting. Over 60% of sea lions stranding at TMMC had titers to *Leptospira* in 2004 which was a peak year for cases of leptospirosis in stranded sea lions (Lloyd-Smith 2007). Almost 100% of the wild harbor seals tested in 2004 and 2005 exhibited signs of exposure to the bacteria. Additionally, in a search of the TMMC database from 2000 to 2010 (n=787), there were only three harbor seals that stranded with confirmed *Leptospira* infections, two in 2004 and one in 2005. The *Leptospira* have recently been reclassified and there is not agreement between serovar and genotype (Resch et al. 2007), such that without an isolate from a harbor seal it cannot be determined if the strain infecting harbor seals is the same or different from the strain isolated from sea lions. Furthermore, the confirmed infections, and the high titers in this study were to *L. kirshneri* serovar Gryppotophosa, but the greatest number of titers were low titers (less than or equal to 1:800) to *L. interrogans* serogroup Icterohemorrhagiae. These Icterohemorrhagiae titers suggestive of exposure to *Leptospira* in harbor seals may be modulated by some of the same environmental

drivers that affect cycles of leptospirosis in sea lions or reflect the cycles observed in the sea lion population.

Toxoplasma, Sarcocystis, Neospora. In Washington State, 7.6% (29 out of 380) of harbor seals tested positive for exposure to *T. gondii* (Lambourn et al. 2001) which is the same as observed in this study. Dubey et al (2003) reported a 3.5% prevalence of *N. caninum* in harbor seals from Alaska similar to the 3% we found in Tomales Bay. Harbor seals in SF, however, had greater levels of exposure to this pathogen (12%). The implications of the increased antibody titers in SF are unclear, and there are no documented cases of infection with this parasite in marine mammals (Dubey 2003). This is the first report of *S neurona* prevalence in harbor seals. The *S. neurona* infection that resulted in stranding and death and the high titer *T. gondii* case which resolved in the wild suggest that harbor seals may be better equipped to fight *Toxoplasma* than *Sarcocystis*, despite the similar exposure levels (7 to 11%). This is further supported by a search of the TMMC database from 2000 to 2010 which found 4 deaths caused by *S. neurona* and only 1 caused by *T. gondii*.

Transplacental infection with *T. gondii* has been reported in a sea otter and inconclusively in a harbor seal (Van Pelt and Dietrich 1973; Dubey et al. 2003; Miller et al. 2008), but our negative results from the young pups in rehabilitation provide no evidence for transplacental transmission of this protozoal pathogen or antibodies against it. However, serum was rarely obtainable from stillborn carcasses and these protozoa cannot be ruled out as a cause of reproductive failure in harbor seals.

Morbillivirus. Since their discovery in marine mammals, morbillivirus outbreaks have continued to have devastating consequences for seal populations; and in 2002, PDV was implicated in another 22,000 harbor seal deaths in the North Sea (Härkönen et al. 2006). Morbilliviruses have also been implicated in cetacean die-offs in the Mediterranean Sea, the Atlantic coast of North America, and the Gulf of Mexico (DiGuardo 2005). Reports of morbillivirus in the Pacific Ocean, however, have been rare. Reidarson et al (1998) detected titers to dolphin morbillivirus in six out of 18 common dolphins stranded in southern California, but characteristic morbilliviral lesions were not observed. Duignan et al (1995) found no serologic evidence of morbillivirus in 80 harbor seals from the Pacific coast of North America, nor did Zarnke et al (2006) in 286 harbor seals in

Alaska. Recently, however, viral nucleic acid identical to an isolate from the 2002 PDV outbreak in the North Sea was reported in sea otters in Alaska (Goldstein et al.2009). Phocine distemper virus remains undetected in harbor seals in California, but their naïve status puts them at risk. The canine distemper positive harbor seal pup detected in Tomales Bay could be from wild terrestrial canids as, in 2004, coyotes were observed preying on harbor seal pups at Point Reyes National Seashore which borders one side of Tomales Bay (Vanderhoof 2005).

There are pros and cons to a broad approach to pathogen surveillance involving different analytical methods and different sample sources. From a logistics perspective, it is difficult to sample for a number of pathogens at once when each pathogen requires a different sampling and analysis technique and level of expertise. In addition, not all tests are validated for use in harbor seals making interpretation of results difficult. New techniques for pathogen detection, such as metagenomics, are finding new pathogens from the tissues of sick animals (Ng et al. 2009; Victoria et al. 2009) and are ideal for investigating disease outbreaks of unknown etiology, but still require development before they can effectively screen the healthy population for evidence of disease exposure to multiple organisms. Microarrays can evaluate multiple nucleic acid sequences at once, but also require validation using conventional methods to link test results to a disease process.

Pathogen prevalence can vary with age class and because there was very little overlap in age class between the wild-caught seals and the stranded seals, prevalence rates could not be directly compared between these groups. The data can, however, provide insight into pathogen epidemiology. For example, the lack of exposure to *Toxoplasma*, *Sarcocystis* or *Neospora* in the young, stranded seals and their prevalence in the wild-caught seals suggests that these pathogens are acquired later in life. Another difficulty in comparing the data between stranded and wild-caught harbor seals occurs because the wild-caught seals are alive and usually clinically healthy. Exposure rate can be estimated, but it is not known how many seals were sickened or killed by the pathogen (i.e. only survivors are sampled).

In conclusion, while evidence of pathogen exposure was detected in seals of all ages, cases resulting in death were low and there is little evidence of risk to harbor seals from

infectious disease. One exception is infection with enteric bacteria which may not be a primary cause of disease, but can certainly compound the risk of mortality in debilitated seals. The second exception is phocine distemper virus which remains the greatest disease risk to the naïve wild harbor seal population unless harbor seals in the Pacific Ocean are less susceptible to morbilliviruses than other harbor seal populations. *Sarcocystis neurona,* continues to be implicated in stranding and death in harbor seals and future work to understand this disease process would be welcome.

With the exception of phocine distemper virus, all infectious desease agents tested for were detected in apparently clinically healthy wild-caught harbor seals. This highlights the role these animals may play as reservoir species for zoonotic disease and the extent to which pathogens with terrestrial sources have invaded the marine environment. Further work on other species in the area may help elucidate the role that seals play in the ecology of avian influenza. Harbor seals are useful sentinels and provide information about the risks to human health posed by these animals and the environment they share. There are likely low risks to humans working with seals as long as hygienic practices are observed, but humans living and fishing in central California certainly could be exposed to the same pathogens as the seals.

Chapter 5. Immune response in harbor seal pups during rehabilitation: relationships with growth and survival to release

INTRODUCTION

The vertebrate immune system responds to invading pathogens to kill and eliminate foreign proteins and parasites that may colonize the host causing disease and ultimately death. It has an innate component which is present prior to exposure to infection, while the more specific adaptive component gains "immune memory" over time through exposure to specific pathogens (Goldsby et al. 2003). In neonates, some adaptive immunity can also be acquired passively from maternal immunoglobulins passed through the placenta and colostrum (King et al. 1998). Innate and adaptive elements of the immune system interact with each other and include leukocytes (e.g. macrophages, neutrophils, basophils, eosinophils, and lymphocytes) and proteins (e.g. complement, immunoglobulins, and cytokines).

Lymphocytes are involved in the adaptive immune response and include B cells (which mature in the bone marrow) and T cells (which mature in the thymus). The B cells produce immunoglobulins of various types such as IgM which is produced by naïve lymphocytes and IgG, which is produced by lymphocytes that have been previously exposed to an antigen. When T cells encounter an antigen they recognize, they produce T helper cells which activate the B cells to produce antibodies (IgG) and T cytotoxic cells which can kill infected cells (Goldsby et al. 2003).

There are a number of ways to evaluate immunity including *in vitro* measures of innate (phagocytosis, natural killer cell activity) and adaptive (lymphocyte proliferation) immune function, and *in vivo* measures such as delayed type hypersensitivity response to determine whether an organism can respond to a specific antigen (e.g. the tuberculin skin test). Among *in vitro* methods, plant mitogens are used to elicit a general (i.e. not antigen specific) T lymphocyte response from a culture of peripheral blood mononuclear cells. It is not clear how these lectins stimulate growth, DNA synthesis and cell division, but one hypothesis is that they increase calcium levels in the cytoplasm triggering a cascade of activity that results in cell proliferation (Tsien et al. 1982). Lymphocyte responsiveness to the same plant mitogens can also be measured *in*

vivo. While traditionally associated with a T lymphocyte response, the swelling that results from the injection of the plant mitogen phytohemmaglutinin (PHA) under the skin involves components of the innate and adaptive immune system (Martin et al. 2006). Because of the number of immune system components involved in the response, it has been argued that the size of the response should not be directly associated with cell-mediated immunocompetence (Martin et al. 2006).

The PHA test has been used extensively in poultry and wild birds to evaluate immune function (Tella et al. 2008) where it has been used in conjunction with other measures of immunity, associated with life history traits such as body condition and survival, and used to detect the effects of immunosuppressive treatments on the immune system (Schrank et al. 1990; Møller and Saino 2004; Palacios et al. 2009). The subdermal injection of PHA has also been shown to generate a response in mammals including wildlife (grey seals, *Halichoerus grypus*, Hall et al. 1999; red deer, *Cervus elaphus*, Fernández-de-Mera et al. 2006; Brazilian free-tailed bats, *Tadarida brasiliensis*, Turmelle et al. 2010). The PHA response was age dependent in grey seal pups, with weaned pups exhibiting a stronger response than neonates (Hall et al. 1999). In house mice, *Mus musculus*, PHA response varied by sex, but was not associated with helminth resistence (Goüy de Bellocq et al. 2007).

In vitro immune function tests have been used extensively to understand the effects of disease, biotoxins, and contaminants on marine mammal immune response (e.g. De Guise et al. 1998; Levin et al. 2007; Schwacke et al. 2010). These *in vitro* mitogen induced T lymphocyte stimulation tests have been validated in harbor seals, *Phoca vitulina*, with concanavalin A (ConA) exhibiting a stronger stimulatory effect on lymphocytes than PHA (de Swart et al. 1993). In phocids, patterns of immune response generated from a number of immune functional and static measures have been linked to contaminant concentrations and disease outbreaks. Harbor seals fed herring from the contaminated Baltic Sea had higher blubber PCB concentrations and altered immune responses when compared with seals fed Atlantic herring. The more contaminated seals had decreased delayed-type hypersensitivity response to ovalbumin injection, decreased natural killer cell activity *in vitro*, and decreased response to the T cell mitogens PHA, ConA, and pokeweed mitogen (PWM) *in vitro* (Ross et al. 1995; de Swart et al. 1996;

Ross et al. 1996). A positive relationship between *in vitro* response to PHA and PCBs in blubber (Levin et al. 2005) and between leukocyte count and PCBs, DDTs, and PBDEs in blood has been reported in harbor seals (Neale et al. 2005b). Mos et al (2006) observed affects on similar immune function measurements that were explained by a combination of blubber contaminant concentration and other unknown exposures (possibly to enteric bacteria). Sormo et al (2009) measured decreased *in vitro* lymphocyte response to PHA stimulation in grey seal pups from the Baltic, compared with less PCB contaminated seals from the Atlantic. Interestingly, among the less contaminated seals from the Atlantic, they found that PWM and ConA (but not PHA) response were positively correlated with PCB levels.

Serum immunoglobulins (Ig) are antibodies produced as part of the acquired immune response, although they are also likely transferred to developing pups *in utero* and during lactation (King et al. 1994), and circulating levels are used as an indicator of immunity in marine mammals. In grey seals, approximately 90% of the immunoglobulin found in serum is gammaglobulin or IgG (Carter et al. 1990). Levels of IgG in grey and harbor seals increase from birth through to weaning and the low levels detected in newborn animals are hypothesized to be associated with neonatal susceptibility to infection and mortality (Carter et al. 1990; Ross et al. 1993). Conversely, elevated levels of IgG at the time of weaning are associated with decreased first year survival in grey seal pups (Hall et al. 2002). The mechanism behind the association between IgG and survival is not known and could reflect exposure to pathogens, genetics or immunosuppression (Hall et al. 2002).

Elevated total serum globulin concentrations can also indicate inflammatory disease, while decreased concentrations are associated with neonates and animals with acquired immune deficiency or exhaustion of the cellular immune system (Bossart et al. 2001). Total leukocyte count as well as differential white cell counts are also used to evaluate immune responses, and a persistent neutropenia or lymphopenia may be indicative of immune dysfunction (Aldridge et al. 2001). These blood variables can help rule out a differential diagnosis, but cannot clarify immune function on their own (Aldridge et al. 2001).

Despite the variety of *in vitro* and *in vivo* methods available to evaluate components of the immune response, the significance of a result from a single *in vitro* or *in vivo* test and its relationships with morbidity and mortality are unclear.

The objectives of this study were

1) to compare immune status between rehabilitating versus wild groups of pups; and

2) to investigate how specific immune function assays (particularly simple ones like the PHA skin response which is relatively easy to carry out compared to *in vitro* methods) relate to growth and rehabilitation outcome; and

3) to understand the context of the *in vivo* PHA test in the rehabilitating seals by evaluating additional simple immune status measures such as longitudinal total leukocyte and differential white cell counts and total globulin concentrations.

METHODS

Sample collection. In May and June 2008, blood was collected from wild-caught and rehabilitating harbor seal pups. At the time of blood collection, animals were weighed and length and girth were measured. Leukocytes were counted automatically and manually differentiated using a stained blood smear; and total serum globulin concentrations were calculated by subtracting albumin from total protein (see Chapter 3 for details). Fresh whole blood from the same animals was shipped to the University of Connecticut for *in vitro* lymphocyte proliferation tests. Serum was frozen at -80°C for the measurement of total IgG concentrations and shipped to the Sea Mammal Research Unit, St Andrews for analysis. In addition *in vivo* PHA stimulation tests were performed at The Marine Mammal Center on pups during rehabilitation but this was not possible for animals in the wild as the response needs to be measured 24 hours after injection.

Clinical assessment. Supportive care of the pups included subcutaneous fluids, vitamin and salt tablets, as well as pepto bismol, acidophilus, and metaclopramide to aid digestion. Several pups received antibiotics during treatment prior to the immune function tests, but only one was on antibiotics at the time of testing (as cover for lethargy and depression). Lymphocytes, neutrophils, albumin and globulin concentrations were compared with the released pup ranges from Chapter 3 to see if

there was any reason to suspect an immunological abnormality in any of the rehabilitating pups at the time of the immune response tests.

Sample analyses.

PHA swelling response. Methods for measuring the PHA response *in vivo* were adapted from Hall et al (1999). Five milligrams of lyophilized phytohemagglutinin (PHA, Sigma, St Louis, Missouri, USA,) were dissolved in 10 ml of sterile saline. Seals were briefly restrained and interdigital flipper thickness in each rear flipper was measured five times in the same location (Mitutoyo 547-500 digimatic thickness gauge, Kawasaki, Kanagawa, Japan) and the location marked with a permanent marker. Next, 100µl of sterile saline were injected in the left flipper and 100µl of PHA solution in the right flipper using a 0.5 ml tuberculin syringe with a 27 gauge, 1.27 cm needle (Terumo Medical Corporation, Elkton, Maryland, USA). Twenty four hours later, flipper thickness at the marked injection sites was again measured five times. The difference in mean flipper thickness before injection and 24 hours after injection was calculated as the PHA response.

IgG. A protein A enzyme linked immunosorbent assay (ELISA) was used to quantify total IgG after Ross et al (1993). Briefly, serum was diluted 1:800 in buffer (50mM NaHCO3, pH 9.5) and 100 µl transferred to three wells of a high binding, flat bottomed ELISA plate, incubated for 1 hour at 37° C. Plates were washed twice with Tris buffered saline and polysorbate (TBS-Tween). Next, 200 µl of gelatin solution (10ug/ml) were added to each well to block non-specific binding and incubated for 30 minutes at 37° C. Plates were washed twice in TBS-Tween and 100 µl of Protein A-horseradish peroxidase (1:2000 solution in TBS-Tween) was added to each well. Plates were incubated at room temperature for 30 min and then washed five times in TBS-Tween. Then 100 µl of a 50:50 solution of horseradish peroxidase substrate (2,2'-azino-di-[3-ethyl]-benzthiazoline-6-sulphonic acid and hydrogen peroxide) were added to each well and stopped after ten minutes with 100 µl of 2% oxalic acid. Plates were read at 405 nm using a microplate autoreader. Concentration in mg/ml was calculated from absorbance using a standard curve generated for each ELISA plate using dog reference serum with a known amount of IgG (Bethyl Laboratories, Montgomery, Texas, USA). Intra-assay coefficient of variation (CV) was <6.0%, inter-assay CV was 7.9%.

In vitro lymphocyte proliferation. Whole blood was centrifuged for 20 min at 220 G, and the buffy coat was collected and re-suspended into Dulbecco's modified eagle medium (DMEM, Gibco BRL, Grand Island, New York, USA) supplemented with 1 mM sodium pyruvate, 100 µM non-essential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, New York, USA), along with 10 % fetal bovine serum (Hyclone, Logan, Utah, USA), thereafter referred to as complete DMEM. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque plus (Amersham Biosciences, Uppsala Sweden) for 35 min at 990 g. The PBMCs were collected, washed once, and enumerated with their viability assessed using the exclusion dye trypan blue. Viability was typically greater than 90%.

PBMCs in complete DMEM were plated (2 x 105 cells/well) in 96 well flat bottom tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, New Jersey, USA), in triplicate. Cells were incubated at 37° C with 5% CO2 for a total of 66 hr alone (no_mitogen) or with the T cell mitogen, Con A (Sigma, St. Louis, Missouri, USA). Con A was used at an optimal concentration of 1.0 µg/ml (OptConA) and a sub-optimal concentration of 0.10 µg/ml (SubConA, Mori et al.2006). Lymphocyte proliferation was evaluated as the incorporation of 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, added for the last 18 hr of incubation, and further detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU - colorimetric, Roche Diagnostics GmbH, Mannheim Germany) as per manufacturer's instructions using an ELISA plate reader at 450 nm with a reference wavelength of 690 nm.

Data analysis. Results for total IgG; total leukocyte, neutrophil and lymphocyte counts; globulin; and lymphocyte proliferation tests (no_mitogen, OptConA, and SubConA) were compared between rehabilitating and wild harbor seals using t-tests after checking for normality and equal variances. When variances were unequal, Welch's two tailed t-test using unpooled variances was used.

Generalized linear models (GLMs) were used to evaluate the effects of sex, mass, and total leukocyte and neutrophil counts measured at the time of the PHA injection on PHA

response. The effect of mass gain (kg/day) from the time of admission until the PHA test was also evaluated.

Because differential white cell counts (leukocytes) are routinely used to assess health status of harbor seals during rehabilitation, we tested the relationship between these variables and the other immune measures in the rehabilitating and wild seals. General linear models were used and the starting model for the rehabilitating seals was white cell variable ~ IgG+SubConA+globulin+PHA and for the wild seals it was white cell variable ~ IgG+SubConA+globulin. White cell variables included total leukocyte, lymphocyte, and neutrophil counts. Residual plots were used to assess the model fit and Akaike's Information Criterion was used for model selection. All statistical analyses were performed using the R programming language (R Development Core Team, 2009).

RESULTS

The PHA response was tested in 15 pups during rehabilitation between 5/7/08 and 6/17/08. The mean PHA swelling response was 0.854 mm (SD=0.345, range 0.112 - 1.350). One pup died 11 days after the PHA test was administered (PHA swelling response was 0.400 mm), while the rest survived to release. Immune assays and blood parameters but not PHA swelling response, were also performed on nine recently weaned harbor seal pups captured in San Francisco Bay between 5/5/08 and 5/21/08.

Clinical assessment of rehabilitating animals. None of the rehabilitating pups exhibited a persistent lymphopenia or neutropenia. At the time of PHA testing, one pup had neutrophil numbers at the low end of the range for pups released from rehabilitation (3348 neutrophils/µl), but within the wild pup range. Another had a globulin concentration below the released pup range (2.6 g/dl), but again, was within range for the wild pups (see Chapter 3).

Comparison of immune measures between wild-caught and rehabilitating pups. Wild pups exhibited significantly greater spontaneous cell proliferation (no_mitogen, $t=-4.0009$, $df=22$, $p<0.005$) and OptConA mediated lymphocyte proliferation ($t=-3.9605$, $df=22$, $p= p<0.005$) than the pups in rehabilitation (Figure 5.1a). Rehabilitating pups had greater concentrations of total globulin ($t=17.6633$, $df=14.187$, $p<0.005$),

leukocytes (t=3.6015, df=22, p=0.002) and neutrophils (t=3.9762, df=22, p<0.005) than wild pups (Figure 5.1b)

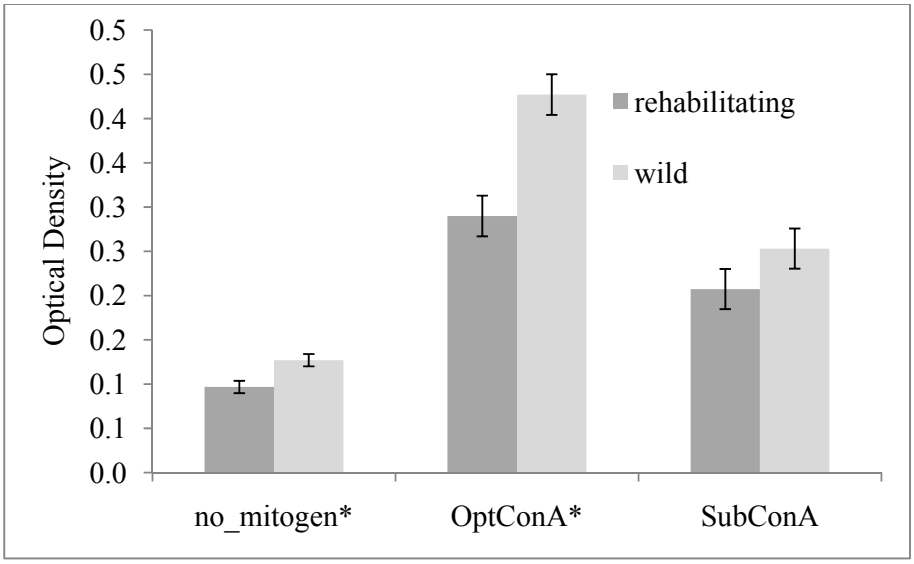


Figure 5.1a. Lymphocyte proliferation (mean and standard error) with no mitogen and two levels of stimulation with ConA in wild and rehabilitating harbor seal pups. Sample sizes are n=15 for rehabilitating pups and 9 for wild pups. The * indicates a statistically significant difference between the two groups of pups (t-test at p<0.005).

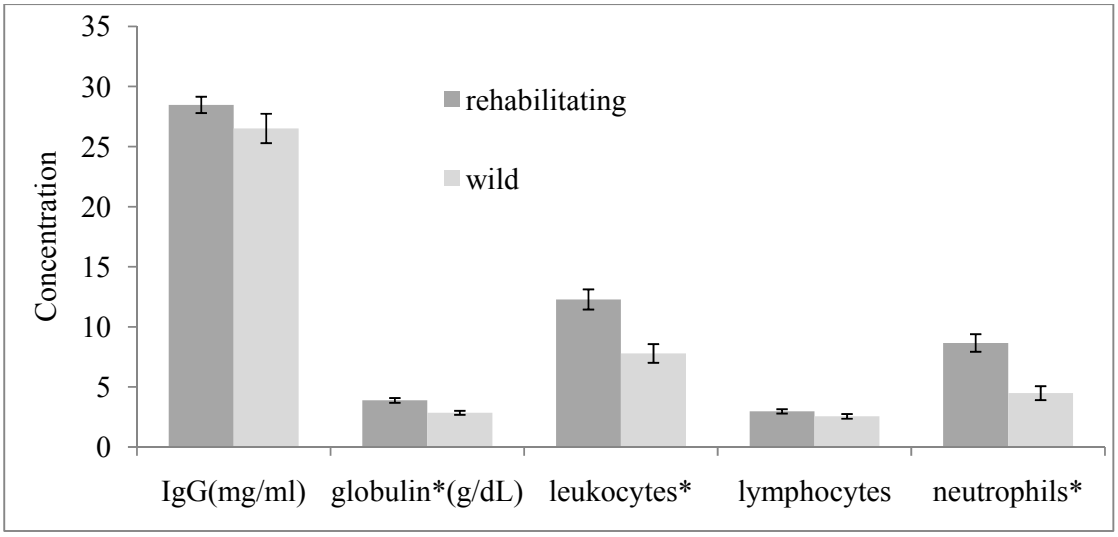


Figure 5.1b. Mean serum concentrations and white blood cell counts (1000/ul) in wild and rehabilitating harbor seal pups. Error bars are standard error. Sample sizes are n=15 for rehabilitating pups and 9 for wild pups. The * indicates a statistically significant difference between the two groups of pups (t-test at $p<0.005$).

Sex, mass and growth effects. The GLMs showed no effect of sex (male=6, females=12) or mass (range = 8.7 - 19.3 kg) on PHA response or leukocyte count among the rehabilitating seal pups. There was a significant positive association between PHA response and daily mass gain (R^2=0.331, p=0.025, Figure 5.2) and a negative association between leukocyte count and daily mass gain which was only significant at the 10% level (R^2=0.222, p=0.076). In the wild pups, there was no effect of sex (t=0.450, p=0.66) or mass at capture (t=-0.372, p=0.72) on leukocyte count.

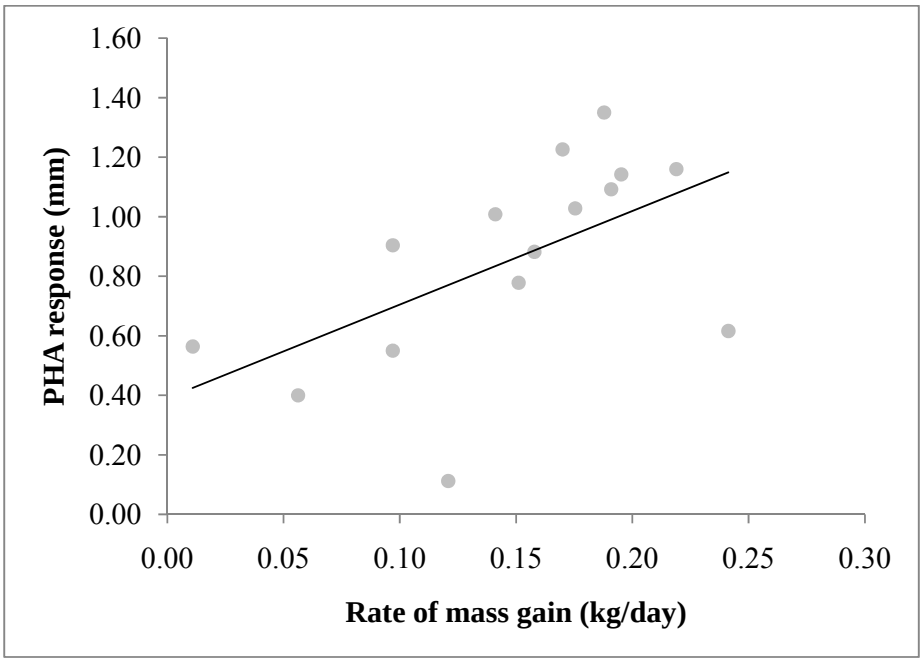


Figure 5.2. The relationship between PHA response and the rate of mass gain from admission to the date of the PHA test (n=15, PHA response=3.2113 * (mass gain/number of days) + 0.3766, R^2=0.331, p=0.025).

Comparison with longitudinal measurements of leukocytes and globulin. Among the rehabilitating animals, total globulin increased during rehabilitation (Figure 5.3). Leukocyte count varied throughout rehabilitation (Figure 5.4), but increased from admission values at some point during rehabilitation in all seals. There were two pups with elevated leukocyte counts at week 6 which did not have their PHA test until 4 weeks later. The two other pups sampled at week 6 had the elevated globulin levels. The pup that died with a low PHA response had the lowest globulin and highest leukocyte count of the animals that were tested.

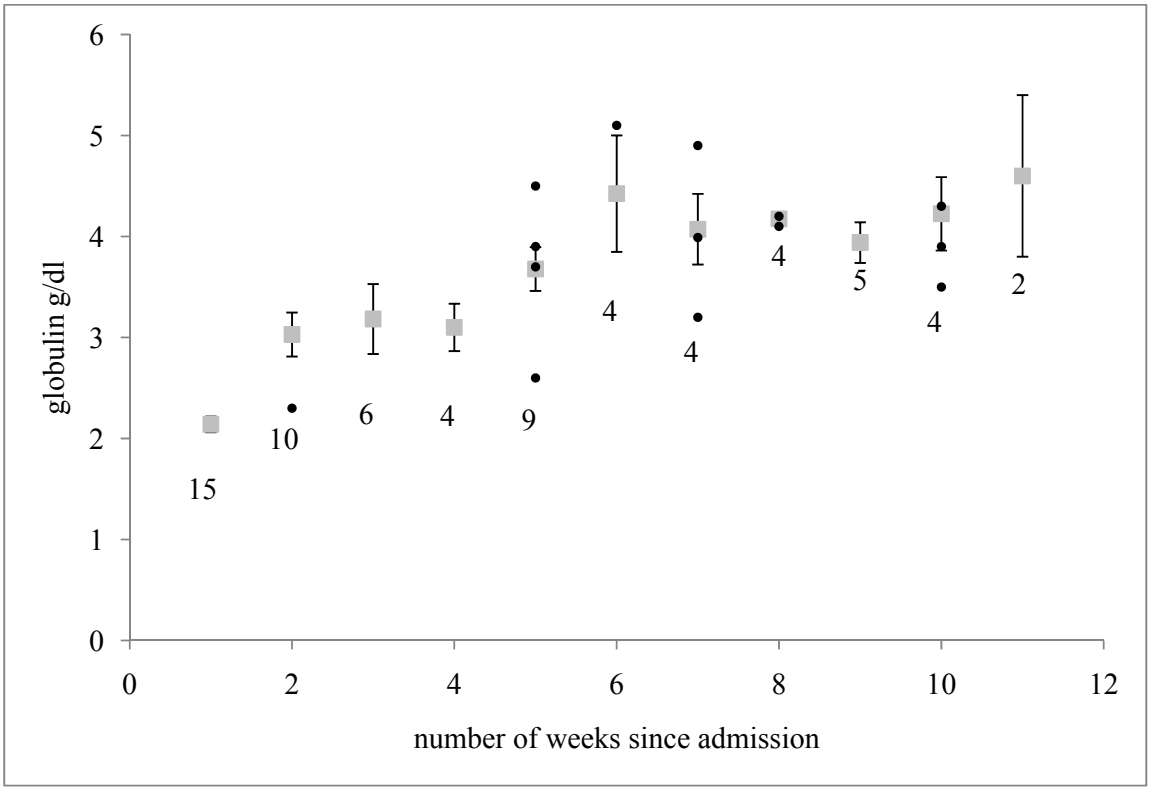


Figure 5.3. Total globulin concentrations (n=15) over the course of rehabilitation standardized to date of admission. After the first week, sample size varies as seals were not bled on a weekly basis (sample sizes are indicated below the data). Black circles indicate globulin values when the PHA test was performed. Error bars are standard error.

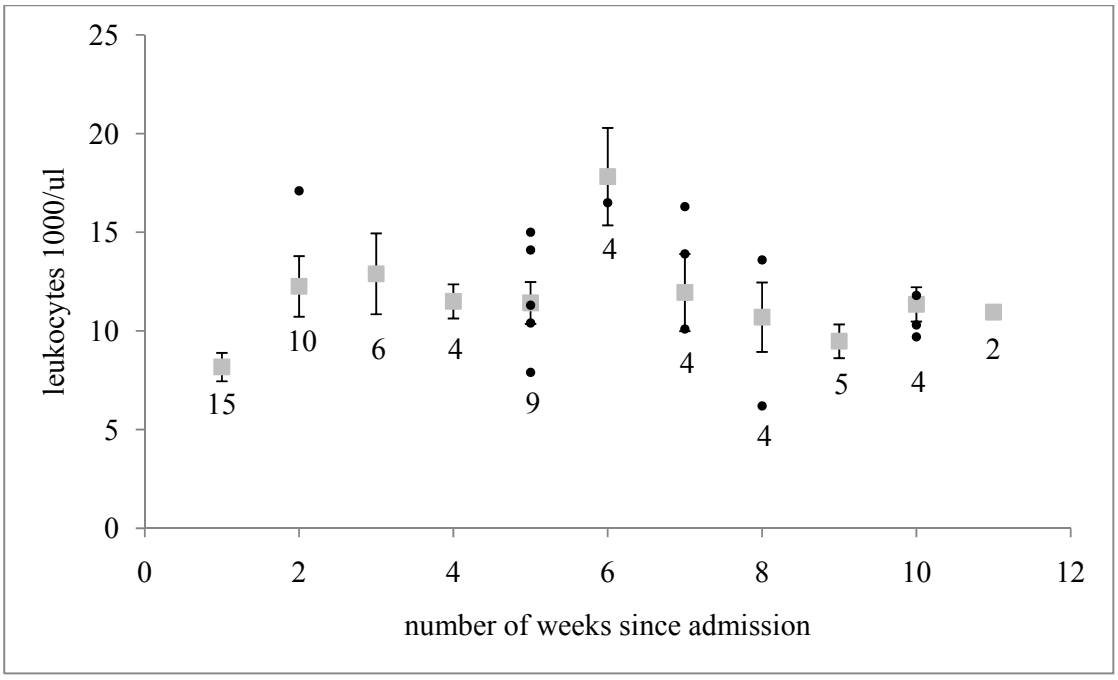


Figure 5.4. Leukocyte concentrations (n=15) over the course of rehabilitation standardized to date of admission. After the first week, sample size varies as seals were not bled on a weekly basis (sample sizes are indicated below the data). Black circles indicate leukocyte values when the PHA test was performed. Error bars are standard error.

Relationship between immune measures. There was a significant negative association between neutrophil count and SubConA induced lymphocyte response (Figure 5.5), but no association between the white cell variables and PHA response. When the highest leukocyte count measured for each pup during rehabilitation from admission to the day of the PHA test was used in the model (instead of leukocyte count on the day of the PHA test), leukocytes were negatively associated with PHA response (leukocytes=-6.463*PHA + 21.126, R^2=0.32, p=0.03). SubConA was a better fit to the white cell data than OptConA suggesting that harbor seal lymphocytes may be more sensitive to this mitogen dose. There was no direct relationship between the *in vitro* lymphocyte response to SubConA and the *in vivo* response to PHA, but both were negatively associated with white cell variables.

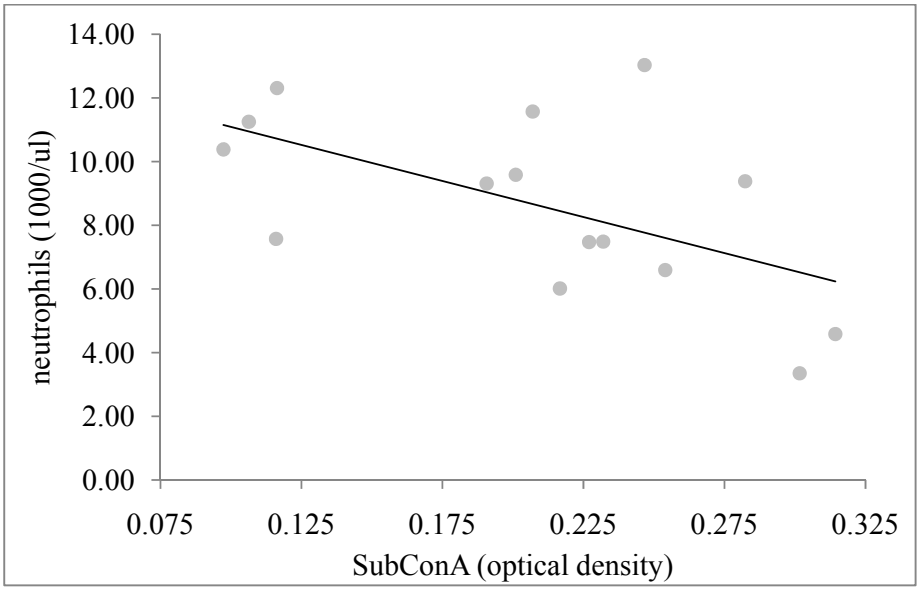


Figure 5.5. The association between neutrophil count and SubConA induced lymphocyte response in the rehabilitating pups (n=15, neutrophils = -22.637*SubConA + 13.352, R^2=0.3196, p=0.028).

DISCUSSION

Mounting an immune response (innate or adaptive) requires energy and can result in decreased growth and survival (Lochmiller and Deerenberg 2000). The anorexia and protein catabolism associated with the immune response can quickly cause severe mass loss (Lochmiller and Deerenberg 2000). In the rehabilitating harbor seal pups, an increase in neutrophil count was associated with decreased lymphocyte proliferation (when induced by SubConA) and decreased growth rate, suggesting that a trade-off occurs between the amount of energy engaged with fighting infection and that involved in growth. Bacterial infections leading to septicemia are a primary cause of death among rehabilitating harbor seals and the energetic cost of fighting these infections may determine which animals are likely to survive. This finding is also consistent with the decreased probability of survival with decreased mass at the time of admission reported in Chapter 3.

The association we found between white blood cells and lymphocyte proliferation is consistent with results from a longitudinal study of *in vitro* immune measures in rehabilitating harbor seals (De Guise et al. 2006). That study found high leukocyte counts were associated with increased odds of decreased PHA-induced T cell proliferation and low leukocyte counts were associated with greatly increased odds of increased T (PHA and ConA induced) and B (lipopolysaccharide induced) lymphocyte proliferation (De Guise unpublished data). We found no associations between leukocytes and the immune measures tested in the wild pups, and the relationships we observed between leukocytes, PHA response, and SubConA may only occur when pups are sick or diseased. There may also be a non linear response such that these relationships do not emerge until the leukocytes are above a certain threshold. Likewise, as PHA is a measure of immune system capacity, while a leukocyte count represents the clinical state of an animal at a single time point, there may be a PHA threshold under which the immune system is unable to respond at all.

Total serum globulin concentrations increased over time in rehabilitation in agreement with a previous study reporting low levels of globulin in neonatal harbor seals (Dierauf et al. 1986). The increase in globulins over several weeks in rehabilitation occurred in the absence of maternal transfer, but took longer than the 15 day increase observed in

harbor seals suckling in the wild (Ross et al. 1994). This is consistent with the production of large quantities of IgG and IgM observed in young northern elephant seals which also suggests synthesis of IgG by the growing pups, because Ig levels in the milk of nursing females were not high enough to account for the increase observed in the pups (King et al. 1998). As globulin appears to be highly variable with age in rehabilitation and the wild, it should be used with caution when evaluating the immune system or used diagnostically in young growing harbor seal pups.

PHA is known to cause a response among specific T cell populations (Byrd et al. 1973) but there are other immune elements involved in the response. Basophils, eosinophils, heterophils (avian neutrophils), lymphocytes, macrophages and thrombocytes have all been observed to infiltrate wing web tissues after PHA injection (Martin et al. 2006). And *in vivo* PHA response does not always correlate with *in vitro* PHA response (Bayyari et al. 1997). What this means for the overall success or strength of the immune response or how useful it is as a general immune fitness measure is difficult to say. The PHA skin response test is easier to carry out than traditional lymphocyte proliferations methods, and does not require large blood volumes, lab expertise, or extensive sample processing time (other than repeat access to the animal at between 12 and 24 hours). In terms of the treatment of rehabilitating animals, it may not enhance understanding of immune status beyond knowledge from white cell numbers and growth rate. However, there may be instances in the wild or captive setting where it is more practical to do a PHA test than a white cell count or differential such as where lab facilities are not available, or the ability to collect and store blood is limited.

In terms of the evolution of the immune response, it is unclear why mounting an immune response to sepsis should be so energetically demanding as to interfere with growth and survival (Lochmiller and Deerenberg 2000). In humans, where trauma (gunshot, vehicular accidents, penetrating wounds) has been associated with immunosuppression and the development of sepsis, it is thought that because trauma has only recently become a survivable event, the body has not evolved immune mechanisms for surviving it (Levy et al. 1984). Neonatal harbor seals develop immunocompetence as early as two weeks into their short nursing period. This precocious response may have evolved to quickly prepare them for a post weaning fast when they are exposed to new

sources of infection (Ross et al. 1994). However this strategy appears to be at odds with a severely underweight pup of pre-weaned age surviving a bacterial infection in rehabilitation at a time when mass gain is essential to survival.

This study has several limitations including a small sample size which means that patterns may not be discernable above individual variation. Working with animals in the rehabilitation setting has additional limitations because changes in lymphocyte proliferation can be affected by malnutrition, viral or bacterial infection, or stress (Aldridge et al. 2001). We did not observe the lymphopenia characteristic of harbor seals with phocine herpesvirus in California (Gulland et al. 1997); however, given the increases in white cells and the deaths of other pups attributed to septicemia, we suspect that the pups in this study likely began to mount an immune response to bacterial infection prior to the time of sampling. One animal that did not response to the PHA injection died. This is consistent with a grey seal pup (1 out of 26) which died soon after failing to respond to a PHA injection (Hall et al. 1999), but more data is needed to determine whether PHA response can be a useful prognostic tool. Measurement of the PHA response longitudinally throughout rehabilitation might be useful in concert with other variables such as white cell count and fecal cortisol levels to separate effects of age and immune system maturation from infection and stress of captivity.

Chapter 6. Geography and stage of development affect persistent organic pollutants in stranded and wild-caught harbor seal pups from central California

INTRODUCTION

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and polybrominated diphenylethers (PBDEs) are found in marine organisms throughout the world. These compounds are lipophilic and thus accumulate in the lipid rich blubber layers of marine mammals. Phocid seals are initially exposed to contaminants during gestation and lactation and then via the prey species they ingest. In addition, contaminants acquired during gestation and lactation are mobilized from the blubber into the circulatory system during the post-weaning fast (Debier et al. 2003a; Debier et al. 2003b). The greatest exposure occurs during gestation and lactation when these seals are developing and are potentially more vulnerable to the effects of these chemicals (Debier et al. 2006).

Harbor seals are born weighing 10 to 12 kg and are abruptly weaned three to five weeks later weighing 20-25 kg (Cottrell et al. 2002). In addition to the rapid mass gain and transfer of maternal antibodies, the greatest levels of contaminants are also acquired by the pups during suckling as lipid rich seal milk is at a higher trophic level and therefore typically more contaminated than the prey species the pups will consume after weaning (Wolkers et al. 2004; Thomas et al. 2005). Pups appear to store rather than metabolize these contaminants, and at weaning, the contaminant pattern in their blubber is similar to that of the milk they ingest (Wolkers et al. 2004). Little is known about the post-weaning fast in harbor seals because they do not remain ashore after weaning, but quickly disperse from their natal beaches, however the mobilization of contaminants from the blubber of grey seals (*Halichoerus grypus*) and elephant seals (*Mirounga angustirostris*) during the post-weaning fast is well described. It is expected that the blubber dynamics during fasting in harbor seals are similar to grey and elephant seals, with simultaneous movement of lipids and lipophilic contaminants into the serum and concentration of contaminants in the remaining blubber (Hall et al. 2003; Debier et al. 2006).

High levels of POPs have been implicated in disease and mortality in marine mammals. PCBs have been associated with mortality from infectious disease in harbor porpoises (Jepson et al. 2005) with increased concentrations associated with a greater risk of mortality (Hall et al. 2006). In California sea lions (*Zalophus californianus*), PCBs have been associated with cancer deaths (Ylitalo et al. 2005b). Harbor seals that died during a phocine distemper epidemic had higher levels of PCBs and DDTs than those that survived suggesting that contaminants may have affected the ability of the seals to respond to the virus (Hall et al. 1992). In experimental studies, PCBs in contaminated fish from the Wadden and Baltic Seas have been associated with reproductive failure and decreased immune system function in harbor seals (Reijnders 1986; Ross et al. 1995; de Swart et al. 1996; Ross et al. 1996), however, the effects of pervasive lower levels of environmental contaminants on marine mammal health are not well understood.

The potential role of contaminants in the births of premature harbor seal pups has long been a concern (Risebrough et al. 1980) and maternal separation is one of the primary reasons that harbor seal pups enter rehabilitation (Colegrove et al. 2005). To understand the role that contaminants might play in the health of developing seal pups in central California, differences in contaminant levels in stranded and wild-caught harbor seal pups were investigated. Primary objectives were to

1) consider the amount of time the pups suckled in the wild as well as the effects of growth during rehabilitation on contaminant levels; and

2) to evaluate the effect of stranding location on contaminant concentrations in newborn harbor seal pups.

METHODS

Sampling. Blubber was sampled in the same anatomical location from live and dead harbor seals: caudal to the left hip and lateral to the spine. For seals that stranded dead or died during rehabilitation, the depth of the blubber layer was measured and an area of blubber (~2cm x 2cm) extending from the skin to the muscle layer was excised with a scalpel, wrapped in solvent rinsed teflon sheeting (Port Plastics, San Jose, California, USA), and frozen at -80°C until analysis. Seals captured in the wild and rehabilitated

seals that were released were sedated with 5 mg/ml diazepam (Hospira, Inc. Lake Forest, Illinois, USA) administered intravenously at a dose of 0.25 mg/kg. The biopsy site was shaved, a 1ml dose of local anesthesia administered (2% lidocaine, Sparhawk Laboratories, Inc. Lenexa, Kansas, USA), and the blubber layer was visualized and its thickness measured using ultrasound (Sonovet 2000, BCF Technology, Livingston, United Kingdom). A sterile 8mm dermal biopsy punch (Miltex, Inc. York, Pennsylvania, USA) was used to obtain the blubber sample which was wrapped in solvent rinsed teflon and frozen at -80°C until analysis. At the time of sampling all animals were weighed, and measured (length and axillary girth).

Sample analysis. Persistent organic pollutants were extracted from tissue samples of harbor seals using a gas chromatography/mass spectrometry (GC/MS) method (Sloan et al. 2005). Briefly, each tissue sample was mixed with drying agents (sodium sulfate and magnesium sulfate), transferred to a 33-mL accelerated solvent extraction (ASE) cell and surrogate standards (PCB 103 and 4,4'-dibromooctafluorobiphenyl) were added to the top of each sample cell. Using the ASE, the POPs and lipids were sequentially extracted at 2000 psi and 100°C with two cell volumes using dichloromethane and the combined extract (~ 50 mL) collected in a 60-mL collection tube. The extract was thoroughly mixed using a Vortex mixer and a 1-2 mL aliquot of each sample extract was transferred to a GC vial for lipid quantitation by thin-layer chromatography/flame ionization detection (TLC/FID, Ylitalo et al. 2005a). The remaining non-lipid sample extract was filtered through a column of silica gel and alumina and concentrated for further cleanup to remove interfering lipid compounds. Size exclusion chromatography with high-performance liquid chromatography (HPLC) was used to collect the fraction containing the POPs (Sloan et al. 2005). The HPLC fraction was analyzed for POPs by capillary column GC/MS. The analytes were separated on a 60m DB-5 capillary column (25 um film thickness) and analyzed by GC/MS (Agilent 5973N Mass Selective Detector® Agilent Technologies) operated in the electron impact (EI) single ion monitoring (SIM) mode (Sloan et al. 2005). The instrument was calibrated using a series of multi-level calibrations standard solutions containing known amounts of several different POPs from which response factors relative to PCB 103 were computed.

Each lipid sample extract was spotted on a Chromarod (Type SIII) and developed in a chromatography tank containing 60:10:0.02 hexane:diethyl ether:formic acid (v/v/v). Various classes of lipids (e.g., wax esters/sterol esters, triglycerides, free fatty acids, cholesterol and polar lipids) were separated based on polarity, with the nonpolar compounds (e.g., wax esters/sterol esters) eluting first, followed by the more polar lipids (e.g., phospholipids). The lipid classes were measured by flame ionization using a Mark VI Iatroscan. Total lipid concentrations were calculated by adding the concentrations of the five lipid classes for each sample and were reported as percent lipid.

Quality assurance and quality control. The quality assurance criteria for analysis of POPs by gas chromatography/mass spectrometry followed the protocol described in Sloan et al. (2006). The laboratory has participated in the NIST National Marine Mammal Organic Contaminant Analytical Quality Assurance Program each year it has been conducted. A National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) was analyzed with each batch of samples to confirm accuracy of POP analysis. Each sample batch consisted of 10-12 field samples. For the NIST SRM analyzed with each batch of samples, the concentrations of $\geq$70% of the individual analytes were within 30% of either end of the 95% confidence interval range of the NIST certified values. Approximately 10% of the seal samples were analyzed in duplicate to measure precision of the analytical method. For duplicates, the relative percent difference was $\leq$30% for $\geq$90% of each of the analytes measured. A method blank was analyzed with each batch of field samples to monitor for laboratory contamination sources. Each method blank contained no more than five analytes that exceeded two times the lower limit of quantitation (LOQ). The recoveries of the surrogate standards ranged from 60 – 130%. For 0.25 – 0.5 g blubber samples, the LOQ for POPs ranged from approximately 1.0– 6.5 ng/g, wet weight.

Data analysis. In this study, PCBs is the sum of congeners 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101/90, 105, 110, 118, 128, 138/163/164, 149, 151, 153/132, 156, 158, 170, 171, 177, 180, 183, 187/159/182, 191, 194, 195, 199, 205, 206, 208, 209; DDTs is the sum of *o,p*'-DDD, *p,p'*-DDD, *o,p*'-DDE, *p,p*'-DDE, *o,p*'-DDT and *p,p*'-DDT; CHLDs is the sum of oxychlordane, γ-chlordane, heptachlor, heptachlor epoxide, nona-III-chlordane, α-chlordane, *trans*-nonachlor and *cis*-nonachlor;

hexachlorocyclohexanes (HCHs) are the sum of α-, β-, and γ-HCH isomers; and finally, PBDEs is the sum of congeners 28, 47, 49, 66, 85, 99, 100, 153, 154, and 183. Values for individual congeners that were below the LOQ were treated as zeroes when summing**.**

Young of the year animals (n=180 excluding two fetuses) were placed into seven groups based on age and potential for contaminant exposure during suckling:

1. Neonate carcasses (NC) included newborn pups found dead on the beach and newborn pups that stranded alive, but died in rehabilitation without gaining more than ten percent of their body mass at the time of admission (i.e. little or no suckling, no effect from a rehabilitation diet of artificial milk matrix (Zoologic, Hampshire, IL), salmon oil and frozen herring). Many of these pups were premature (i.e. stranded before viable pups were born in the wild), and this group also included 4 pups (near full term) that were found *in utero* after traumatic maternal death. We defined newborn as less than 5 days old based on mass, time of year, and the presence of an umbilical cord or patent umbilicus.

2. Neonate died (ND) were newborn pups that stranded alive and gained some mass in rehabilitation, but died before release (i.e. little or no suckling, some effect from rehabilitation diet).

3. Neonate released (NR) were newborn pups that survived rehabilitation (i.e. little or no suckling, strong rehabilitation effect as pups doubled or tripled their mass).

4. Suckled died (SD) were those that stranded in May or later, either as carcasses or died during rehabilitation, but were expected to have suckled, but not necessarily weaned, in the wild as maximum pup numbers in the San Francisco Bay area occur from the third week in April to the third week in May (Allen et al. 2004). The category also included those that stranded in May or later, gained more than 10% of their admit body mass in rehabilitation and then died (i.e. some suckling, some rehabilitation effect).

5. Weaned wild-caught (WW) were recently weaned pups that were captured and sampled in the field (i.e. full lactational input of contaminants, no rehabilitation effect).

6. Weaned died (WD) were those that weaned in the wild, then stranded, but died before re-gaining any mass (i.e. full lactational input of contaminants, followed by post-weaning mass loss, no rehabilitation effect).

7. Weaned released (WR) were those that weaned in the wild, stranded, and survived rehabilitation (i.e. full lactational input of contaminants, post-weaning mass loss, rehabilitation effect during mass gain).

Other age classes (fetuses, yearlings, subadults and adults) were evaluated separately.

Mass, blubber depth, percent lipid and sampling date (as a proxy for age) were summarized for each group. The geometric mean and 95% confidence intervals were calculated for each group eliminating values below the LOQ. Geometric mean contaminant concentrations were compared among groups using analysis of variance (ANOVA) with Tukey's Honest Significant Difference method (HSD) for multiple comparisons.

The effect of stranding location was evaluated for the neonate carcass group only (n=50) as the location of stranding for these newborns was likely to be close to their birth location and there was no effect from rehabilitation. This group was divided into five locations based on known harbor seal haulout locations: San Francisco Bay (SFB), North of San Francisco Bay (SFN), Tomales Bay north (TBN), South of San Francisco Bay (OCSSF), and Monterey south (MTY, Figure 6.1). Mean contaminants and the ratios of PCBs:DDTs and *p,p'*-DDE:*p,p'*-DDT were compared among locations using ANOVA with Tukey's HSD. A principal components analysis (PCA) was then used to compare the individual analytes by location. Only analytes for which 48 or more samples were above the LOQ were used in the PCA analysis: a value equal to one half of the LOQ was used for two PBDE99 samples, one PBDE100 sample, and one nona-III-chlordane sample. The PCA was run on log transformed concentrations using the R function "prcomp" which scales the variables to a unit variance.

Statistical analyses were performed using the R programming language (R Development Core Team, 2009).

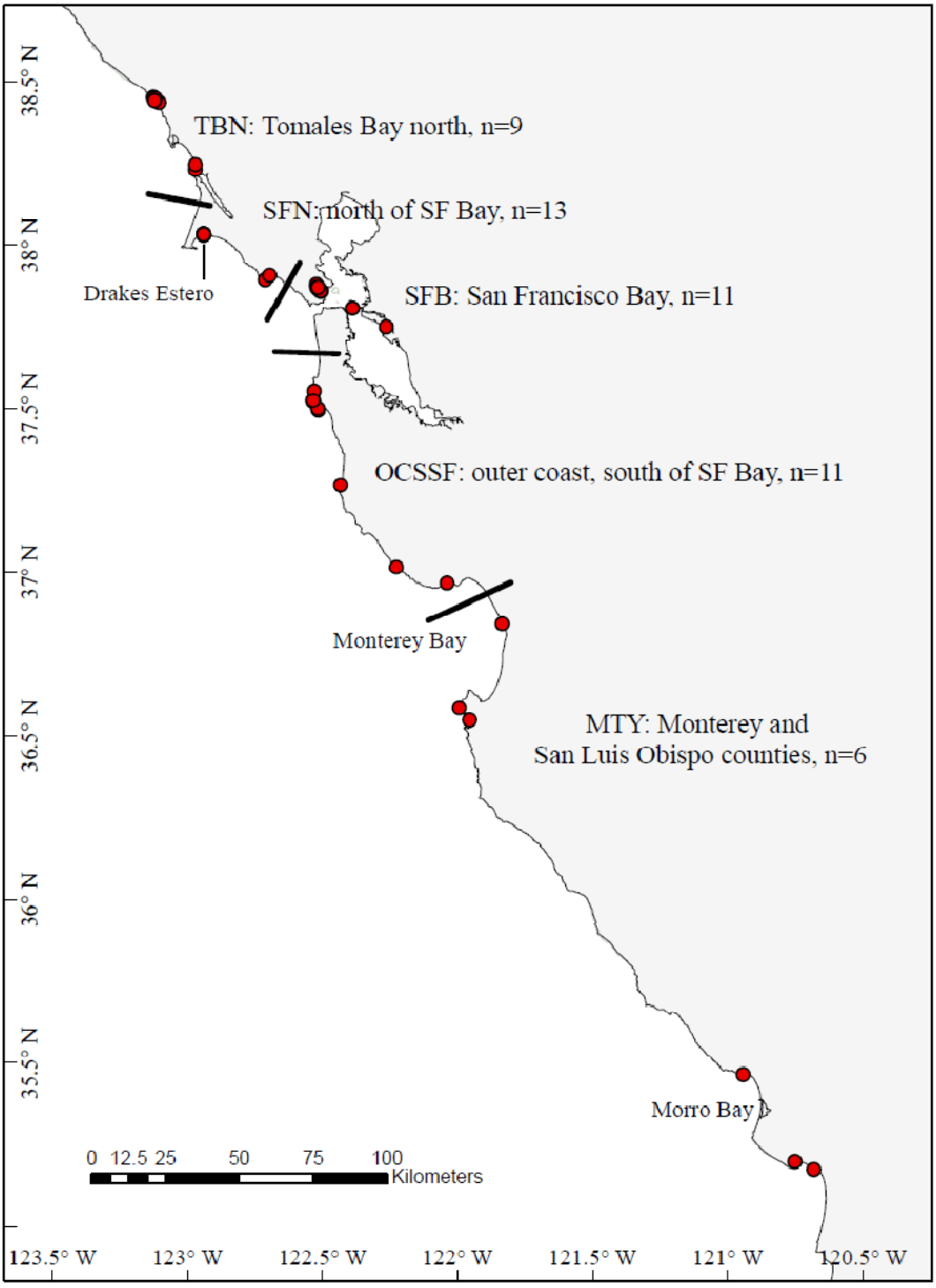


Figure 6.1. Map of stranding locations for harbor seal pups in the carcass group (n=50). Black bars delineate the five geographical strata used in the statistical analyses (TBN, SFN, SFB, OCSSF, and MTY).

RESULTS

Contaminants were measured in blubber samples from 205 animals, of which 180 were young of the year, 23 were older age classes and two were fetuses. Three pups with less than 1 percent lipid in the blubber were omitted from all analyses: all three were in the weaned died (WD) group.

Heptachlor, aldrin, and endosulfan and BDE183 were not detected in any of the blubber samples. Hexachlorobenzene, mirex and dieldrin were detected in most samples but at low levels. In all pup groups contaminant concentrations followed the same pattern: DDTs> PCBs> PBDEs> CHLDs> HCHs except for the WW group of pups which had more PCBs than other compounds (Table 6.1). PCB congener 153 was the biggest contributor to the sum PCBs (27%) followed by PCB 138 (19%), PCB 180 (10%) and PCB 187 (9% each), PCB99 (6%), PCBs 101, 170, 183, 149 (3%), and PCBs 177, 128, 199, 188 (2% each). Sum DDTs were primarily *p,p'*-DDE (97%) and *p,p'*-DDT (2%). Sum chlordanes were primarily *trans*-nonachlor (56%) and oxychlordane (29%). Sum PBDEs were primarily PBDE47 (83%), PBDE99 (8%), and PBDE100 (6%).The majority of the HCH detected was α (51%) and β (48%) isomers with *γ*-HCH (lindane) only detected in a subset of animals (39 out of 205). The highest prevalence of *γ*-HCH was detected in the WD group (33%). Among the other age classes, *γ*-HCH was only detected in one fetus, and 2 wild-caught yearlings from SFB.

Table 6.1. Geometric mean contaminant concentrations and ranges in ng/g lipid weight for 177 harbor seal pups.

	no suckling				suckling unknown				weaned					
compound	NC		ND		NR		SD		WW		WD		WR	
	n	mean (range)	n	mean (range)	n	mean (range)	n	mean (range)	n	mean (range)	n	mean (range)	n	mean (range)
HCB	37	6 (2-31)	11	16 (11-19)	45	11 (8-18)	12	7 (3-14)	33	6 (3-27)	9	5 (2-9)	11	11 (8-13)
α-HCH	48	10 (4-26)	12	8 (2-20)	45	11 (5-21)	12	14 (6-41)	35	15 (6-35)	12	57 (16-322)	11	15 (10-24)
β-HCH	45	10 (2-35)	12	10 (2-48)	42	11 (5-34)	9	21 (10-130)	34	14 (6-35)	11	77 (18-458)	10	16 (9-29)
γ-HCH	13	1 (1-3)	0	0 ()	11	2 (1-2)	1	2 (2-2)	7	2 (1-3)	4	3 (2-9)	1	2 (2-2)
α-chlordane	22	4 (1-21)	11	8 (4-14)	36	4 (2-8)	11	7 (2-21)	20	4 (2-34)	8	5 (2-18)	9	4 (3-8)
cis-nonachlor	47	9 (2-69)	12	9 (3-29)	42	6 (3-22)	12	29 (7-435)	35	15 (4-119)	12	63 (8-604)	11	9 (5-16)
γ-chlordane	2	1 (1-3)	2	2 (2-3)	0	0 ()	2	2 (2-3)	1	2 (2-2)	0	0 ()	0	0 ()
heptachlor	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()
heptachlor epoxide	47	11 (5-52)	11	10 (4-32)	42	6 (3-19)	12	22 (8-83)	35	18 (8-40)	12	80 (5-792)	11	16 (8-46)
nona-III-chlordane	49	14 (5-72)	12	14 (2-74)	42	6 (3-27)	12	38 (9-239)	35	19 (7-74)	12	176 (12-2500)	11	25 (11-42)
oxychlordane	50	63 (18-290)	12	49 (4-221)	45	21 (6-145)	12	157 (20-807)	35	104 (32-357)	12	553 (29-5833)	11	117 (31-828)
trans-nonachlor	50	121 (26-779)	12	104 (20-372)	45	53 (22-252)	12	394 (61-2826)	35	195 (68-1136)	12	1362 (116-19375)	11	224 (103-920)
aldrin	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()
diel	40	6 (2-26)	11	10 (5-19)	44	8 (5-23)	12	16 (3-89)	34	12 (4-61)	12	16 (3-75)	11	8 (5-16)
mirex	37	4 (2-26)	9	5 (2-13)	16	3 (1-7)	11	13 (4-83)	30	5 (2-30)	12	57 (3-958)	11	10 (2-32)
endosulfan	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()
PCB17	19	1 (0-2)	10	1 (0-2)	29	1 (0-2)	8	1 (0-1)	27	1 (0-2)	8	1 (0-7)	11	1 (0-3)
PCB18	14	2 (1-5)	3	2 (1-3)	10	2 (1-2)	4	4 (2-8)	8	3 (2-5)	3	3 (2-8)	5	3 (2-6)
PCB28	44	5 (1-13)	12	5 (2-11)	43	5 (3-9)	12	10 (4-42)	35	8 (3-26)	12	18 (4-71)	11	6 (4-10)
PCB31	35	2 (1-12)	11	4 (1-9)	41	3 (2-7)	10	5 (1-15)	35	3 (2-8)	11	5 (2-29)	11	4 (3-9)
PCB33	7	1 (1-4)	1	2 (2-2)	4	1 (1-2)	1	2 (2-2)	2	2 (1-2)	0	0 ()	2	3 (1-6)
PCB44	29	3 (1-12)	10	5 (3-9)	37	4 (3-6)	10	9 (3-30)	33	6 (3-18)	12	11 (2-63)	11	4 (3-7)
PCB49	50	16 (3-95)	12	11 (4-31)	44	8 (4-37)	12	48 (7-404)	35	40 (5-226)	12	121 (12-1417)	11	21 (10-73)
PCB52	50	46 (11-333)	12	34 (9-89)	45	23 (10-116)	12	140 (17-1754)	35	115 (14-677)	12	382 (34-4375)	11	77 (25-345)
PCB66	39	4 (1-15)	11	6 (3-10)	38	4 (3-6)	11	11 (3-50)	33	7 (4-24)	12	19 (3-67)	11	5 (3-8)
PCB70	37	4 (1-22)	11	7 (5-14)	44	6 (4-10)	10	10 (3-46)	34	6 (3-19)	12	13 (3-56)	11	5 (4-9)
PCB74	50	18 (4-78)	12	15 (4-47)	44	8 (4-33)	12	48 (9-266)	35	34 (6-179)	12	163 (15-1583)	11	22 (12-55)

	no suckling					suckling unknown					weaned			
compound		NC		ND		NR		SD		WW		WD		WR
PCB82	30	1 (0-2)	7	1 (1-2)	21	1 (1-2)	10	2 (0-10)	30	1 (1-4)	9	3 (0-18)	9	1 (1-2)
PCB87	50	16 (3-65)	12	13 (5-37)	44	9 (4-35)	12	48 (9-283)	35	30 (5-134)	12	141 (14-1271)	11	20 (12-57)
PCB95	50	17 (3-108)	12	14 (4-30)	45	13 (7-61)	12	59 (9-549)	35	43 (4-261)	12	149 (18-1604)	11	30 (14-113)
PCB99	50	183 (31-1255)	12	122 (10-357)	45	61 (14-625)	12	585 (54-5789)	35	413 (45-2793)	12	1946 (82-25000)	11	352 (87-2759)
PCB101	50	146 (26-736)	12	105 (16-298)	45	59 (20-404)	12	434 (63-2663)	35	313 (34-1862)	12	1341 (98-13542)	11	216 (74-1014)
PCB105	50	31 (5-113)	12	23 (4-58)	45	13 (5-57)	12	82 (13-565)	35	58 (10-207)	12	263 (25-2500)	11	38 (19-85)
PCB110	50	22 (4-89)	12	18 (6-38)	45	16 (8-48)	12	60 (12-478)	35	47 (5-166)	12	153 (18-979)	11	26 (18-43)
PCB118	50	87 (13-387)	12	66 (12-174)	45	37 (14-175)	12	245 (43-1957)	35	161 (26-615)	12	794 (70-7083)	11	104 (46-285)
PCB128	50	61 (7-476)	12	36 (2-89)	45	19 (4-257)	12	206 (17-2110)	35	132 (10-1006)	12	679 (34-9792)	11	122 (33-989)
PCB138/163/164	50	562 (84-4199)	12	358 (26-969)	45	184 (33-2390)	12	1755 (151-17544)	35	1233 (107-9125)	12	5839 (229-70833)	11	1120 (254-9885)
PCB149	50	85 (8-519)	12	52 (7-111)	45	39 (11-496)	12	294 (39-2743)	35	210 (14-1862)	12	835 (53-8333)	11	141 (58-1149)
PCB151	50	25 (2-165)	12	15 (2-33)	44	11 (3-129)	12	81 (7-652)	35	61 (4-503)	12	243 (16-1958)	11	36 (15-338)
PCB153/132	50	844 (126-6061)	12	542 (38-1395)	45	267 (46-3493)	12	2504 (203-24561)	35	1781 (156-13222)	12	7931 (310-89583)	11	1640 (381-14023)
PCB156	50	22 (3-156)	11	17 (4-36)	39	8 (2-97)	12	61 (4-557)	35	47 (3-298)	12	193 (10-2292)	11	34 (10-253)
PCB158	50	28 (3-216)	11	21 (6-47)	42	9 (3-123)	12	73 (4-842)	35	62 (4-540)	12	198 (10-2373)	11	41 (9-391)
PCB170	50	85 (10-649)	12	49 (3-128)	45	25 (3-423)	12	246 (18-3165)	35	193 (11-1583)	12	790 (32-7917)	11	172 (38-1762)
PCB171	50	18 (2-169)	11	12 (4-29)	29	8 (2-121)	11	71 (5-759)	33	51 (3-466)	12	162 (7-1610)	11	34 (8-391)
PCB177	50	51 (3-519)	11	32 (13-73)	43	16 (3-386)	12	174 (10-2743)	35	135 (4-1564)	12	524 (20-5208)	11	102 (27-1423)
PCB180	50	260 (35-1645)	12	147 (9-408)	45	76 (10-1268)	12	767 (56-8228)	35	579 (38-4655)	12	2548 (89-27083)	11	537 (131-5160)
PCB183	50	72 (9-563)	12	40 (2-111)	45	20 (3-404)	12	217 (14-2743)	35	176 (10-1639)	12	670 (26-7458)	11	138 (31-1726)
PCB187/159/182	50	232 (25-1645)	12	122 (6-321)	45	69 (8-1250)	12	718 (52-8228)	35	544 (31-5028)	12	2290 (94-22917)	11	473 (123-5160)
PCB191	31	6 (2-32)	7	3 (2-6)	9	4 (2-24)	9	21 (3-120)	29	12 (3-73)	9	61 (8-407)	8	8 (1-78)
PCB194	50	28 (2-167)	11	18 (8-32)	38	10 (3-143)	12	87 (6-1013)	35	60 (4-466)	12	332 (13-3750)	11	67 (15-712)
PCB195	44	9 (1-56)	10	5 (3-10)	19	5 (1-42)	9	48 (6-316)	31	25 (5-151)	11	77 (3-1051)	10	19 (3-205)
PCB199	50	40 (4-229)	12	21 (1-50)	44	12 (2-221)	12	126 (8-1456)	35	90 (5-764)	12	487 (20-5417)	11	97 (27-1103)
PCB205	17	3 (1-12)	2	3 (2-6)	3	4 (4-5)	4	20 (8-37)	20	4 (2-18)	8	25 (4-139)	3	14 (5-28)
PCB206	41	10 (1-43)	9	4 (2-8)	23	4 (1-37)	10	33 (4-370)	31	21 (5-132)	12	72 (4-1373)	10	21 (4-231)
PCB208	30	5 (1-16)	2	3 (2-3)	7	4 (2-16)	8	23 (3-143)	28	11 (3-60)	12	34 (2-508)	10	8 (2-98)
PCB209	23	4 (1-10)	1	3 (3-3)	5	3 (2-9)	8	15 (2-152)	27	6 (2-39)	9	36 (4-373)	7	8 (1-62)
o,p'-DDD	7	4 (1-19)	11	8 (4-16)	9	3 (2-6)	5	6 (1-15)	4	2 (1-6)	1	2 (2-2)	1	4 (4-4)

	no suckling				suckling unknown				weaned			
compound		NC		ND		NR		SD		WW		WD		WR
o,p′-DDE	17	2 (1-10)	5	3 (2-5)	1	2 (2-2)	5	4 (2-9)	5	4 (2-15)	7	9 (2-52)	0	0 ()
o,p′-DDT	7	2 (1-3)	0	0 ()	1	16 (16-16)	4	4 (3-8)	6	7 (2-16)	4	4 (3-7)	0	0 ()
p,p′-DDD	50	30 (4-126)	12	44 (5-97)	45	25 (14-53)	12	102 (18-1109)	35	64 (5-526)	12	145 (14-1458)	11	23 (15-38)
p,p′-DDE	50	4686 (750-19048)	12	3643 (579-13755)	45	1608 (293-9524)	12	9531 (1954-43584)	35	6208 (897-22556)	12	56455 (3859-1458333)	11	8534 (1996-25692)
p,p′-DDT	50	84 (15-381)	12	60 (7-260)	45	32 (10-532)	12	215 (27-2663)	35	161 (32-1635)	12	765 (39-10417)	11	148 (38-573)
PBDE28	6	3 (1-7)	0	0 ()	2	3 (3-4)	6	12 (4-37)	15	7 (4-15)	8	28 (12-80)	1	3 (3-3)
PBDE47	50	275 (38-1602)	12	181 (23-669)	45	130 (44-809)	12	872 (167-8261)	35	652 (40-3911)	12	3538 (235-54167)	11	538 (181-1779)
PBDE49	9	5 (2-9)	2	4 (4-4)	22	6 (4-12)	5	9 (6-18)	16	7 (4-32)	6	11 (6-17)	8	7 (4-10)
PBDE66	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	1	8 (8-8)	0	0 ()
PBDE85	1	6 (6-6)	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()
PBDE99	48	23 (4-134)	11	16 (8-63)	44	20 (8-50)	12	86 (18-802)	32	64 (10-297)	12	264 (16-4375)	11	51 (22-391)
PBDE100	49	24 (4-104)	11	19 (10-63)	44	15 (6-107)	12	78 (20-1065)	32	59 (10-372)	12	267 (23-3542)	11	36 (19-137)
PBDE153	22	9 (2-33)	4	5 (3-9)	7	8 (4-18)	9	30 (4-232)	27	21 (6-79)	10	112 (6-1250)	9	18 (5-126)
PBDE154	4	4 (2-7)	0	0 ()	1	4 (4-4)	5	12 (3-67)	6	5 (3-18)	7	17 (8-51)	1	5 (5-5)
PBDE183	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()	0	0 ()
Sum.PCBs.ww	50	1129 (240-10000)	12	823 (120-1700)	45	548 (180-7000)	12	3684 (380-45000)	35	3329 (260-27000)	12	7537 (1000-83000)	11	2450 (650-25000)
Sum.DDTs.ww	50	1761 (420-5800)	12	1587 (380-3800)	45	858 (250-4300)	12	3936 (1200-19000)	35	3167 (520-13000)	12	14253 (2300-70000)	11	3656 (1100-14000)
Sum.PBDEs.ww	50	120 (24-830)	12	87 (14-220)	45	87 (21-640)	12	425 (110-3000)	35	371 (21-2500)	12	1046 (160-6100)	11	275 (120-1200)
Sum.CHLDs.ww	50	81 (25-330)	12	82 (22-190)	45	48 (18-220)	12	264 (47-1400)	35	174 (48-900)	12	570 (110-1800)	11	172 (80-810)
Sum.HCHs.ww	50	7 (1-23)	12	7 (3-26)	45	11 (6-28)	12	10 (4-25)	35	14 (6-33)	12	30 (10-60)	11	12 (6-23)
Sum.PCBs	50	3098 (440-20779)	12	1982 (198-4651)	45	1071 (229-12868)	12	9318 (858-86498)	35	9318 (858-86498)	12	30196 (1348-333333)	11	5863 (1543-48276)
Sum.DDTs	50	4834 (808-19481)	12	3824 (628-14126)	45	1677 (318-10084)	12	9954 (2131-46005)	35	9954 (2131-46005)	12	57099 (3859-1458333)	11	8747 (1996-27668)
Sum.PBDEs	50	330 (46-1861)	12	209 (23-818)	45	171 (61-956)	12	1076 (213-10217)	35	1076 (213-10217)	12	4192 (268-62500)	11	658 (254-2135)
Sum.CHLDs	50	222 (57-1299)	12	198 (36-736)	45	94 (29-461)	12	668 (106-4130)	35	668 (106-4130)	12	2284 (175-29167)	11	411 (184-1862)
Sum.HCHs	50	18 (2-63)	12	18 (5-69)	45	22 (10-53)	12	26 (6-172)	35	26 (6-172)	12	121 (17-780)	11	29 (10-51)

Pup groups are neonate carcass (NC), neonate died (ND), neonate released (NR), suckled died (SD), weanedwild-caught (WW), weaned died (WD), and weaned released (WR). All values are in ng/g lipid weight unless indicated as wet weight (ww) in the compound name.

Pup morphology and blubber contaminants by group. Mass, length, girth, blubber depth, percent lipid and sampling date differed by group (Table 6.2). Among the pups that stranded without suckling (NC, ND, NR), mass, blubber depth, percent lipid, and sampling date increased between NC and NR (adjusted $p<0.0005$ for all four parameters). Likewise, among the pups that weaned (WW, WD, WR), the wild-caught pups (WW) had greater mass, blubber depth, and percent lipid than the weaned pups that stranded and died (WD). Among the pups sampled in the wild (WW) and the pups released from rehabilitation (NR and WR), there were no differences in mass, blubber depth, or percent lipid although there were differences in contaminant concentrations. The weaned pups that stranded and died (WD) were larger and sampled later in the year than the carcasses, but they had similar blubber depths and percent lipid (Table 2). Because of variability in percent lipid between groups, analyses of contaminants by group were based on lipid normalized values.

Among the three groups that stranded without receiving contaminants from milk, those that died soon after birth (NC) had significantly higher contaminant concentrations than those that increased their body mass in rehabilitation prior to release (NR, Table 6.3). This trend was significant for PCBs (adjusted $p<0.005$), DDTs (adjusted $p<0.005$), PBDEs (adjusted $p=0.01$) and CHLDs (adjusted $p<0.005$) but not for HCHs (Figures 6.2-6.4). Pups sampled in the wild soon after weaning (WW) had greater PCBs and PBDEs than the pup groups that did not suckle although DDTs were not significantly greater than detected in the carcass group. The weaned pups that stranded after losing mass (WD), with blubber depth and percent lipid equivalent to the newborn carcass group, had significantly greater contaminant concentrations than all pup groups in all contaminant classes (Figures 6.2-6.4). Some of the WD pups had values exceeding those detected in stranded adults (Table 6.3).

Table 6.2. Summary of the mass (kg), blubber depth (mm), percent lipid, and Julian day (as a proxy for age) sampled for 177 pups and weaners. A lower case letter in common means no significant difference between groups in that column.

		mass	blubber depth	percent lipid	day sampled
group	n	mean±sd (range)	mean±sd (range)	mean±sd(range)	mean±sd(range)
NC	50	7.4 ± 2.2^{a}(4.0 - 13.3)*	9 ± 4^{a}(3 - 23)*	39 ± 14^{a}(18 - 69)	89 ± 25^{a}(35 - 121)
ND	12	$9.2 \pm 2.5^{a,b}$(6.7 - 16.4)	$12 \pm 7^{a,b}$(4 - 29)	$44 \pm 13^{a,b}$(26 - 65)	114 ± 30^{b}(64 - 166)
NR	45	18.0 ± 2.4^{d}(12.9 - 25.0)	$17 \pm 2^{c,d}$(12 - 22)*	53 ± 12^{b}(26 - 79)	176 ± 22^{d}(122 - 206)
SD	12	$11.2 \pm 5.8^{a,b}$(7.9 - 27.0)*	$13 \pm 9^{a,b,c}$(1 - 30)	$46 \pm 18^{a,b}$(5 - 84)	144 ± 18^{c}(125 - 198)
WW	35	19.2 ± 3.8^{d}(12.8 - 26.8)	19 ± 3^{d}(14 - 25)*	50 ± 11^{b}(30 - 69)	143 ± 13^{c}(126 - 171)
WD	12	$13.2 \pm 4.0^{b,c}$(8.3 - 18.7)	$12 \pm 9^{a,b}$(3 - 30)	34 ± 23^{a}(5 - 74)	$183 \pm 28^{d,e}$(142 - 233)
WR	11	$17.7 \pm 4.1^{c,d}$(12.3 - 27.1)	$16 \pm 4^{b,c,d}$(11 - 25)	$43 \pm 11^{a,b}$(23 - 56)	210 ± 26^{e}(179 - 256)

* indicates a decreased sample size. for mass: NC n=38, SD n=10; for blubber depth: NC n=49, NR n=44, WW n=34

Table 6.3. Mean ± standard deviation (range) for mass (kg), blubber depth (mm), and lipid (%), plus range for the summed contaminant classes in ng/g wet weight (ww) and lipid weight (lw) for age classes other than pups (nd=none detected). All samples were from stranded animals that died in rehabilitation except for the yearlings denoted with an asterisk which were captured and released in the wild.

	fetus	yearling	yearling*	subadult	adult
n	2	3	9	2	9
mass (kg)	1.5-3.4	26.5±3.5 (24.0-29.0)	28.9±2.7 (26.0-34.0)	35.5-39.0	64.0±18.3 (32.7-88.5)
blubber depth	2-4	19±9 (11-29)	14±2 (10-17)	19-22	32±17 (5-60)
lipid (%)	17-31	65±9 (58-76)	49±14 (22-71)	55-60	61±13 (39-73)
PCBs, ww	1200-1700	3108 (1500-6900)	2964 (610-13000)	1900-6100	5241 (850-97000)
DDTs, ww	1000-2100	4564 (1800-12000)	4087 (2100-8400)	4400-10000	5352 (2000-28000)
PBDEs, ww	120-180	485 (240-720)	344 (93-1300)	190-620	578 (200-4300)
CHLDs, ww	52-120	186 (120-270)	159 (78-500)	90-280	221 (85-1900)
HCHs, ww	nd-5.7	13 (10-19)	9 (5-12)	11-12	8 (4-19)
PCBs, lw	3866-9942	4793 (1982-11897)	6263 (1543-29213)	3193-11051	8785 (1264-251948)
DDTs, lw	5848-6766	7038 (2378-19324)	8635 (4628-14237)	7395-18116	8971 (2928-72727)
PBDEs, lw	580-702	748 (317-1159)	726 (309-2203)	319-1123	970 (298-11169)
CHLDs, lw	304-387	288 (159-435)	336 (154-847)	151-507	371 (126-4935)
HCHs, lw	nd-18	20 (16-31)	19 (11-51)	18-22	14 (5-45)

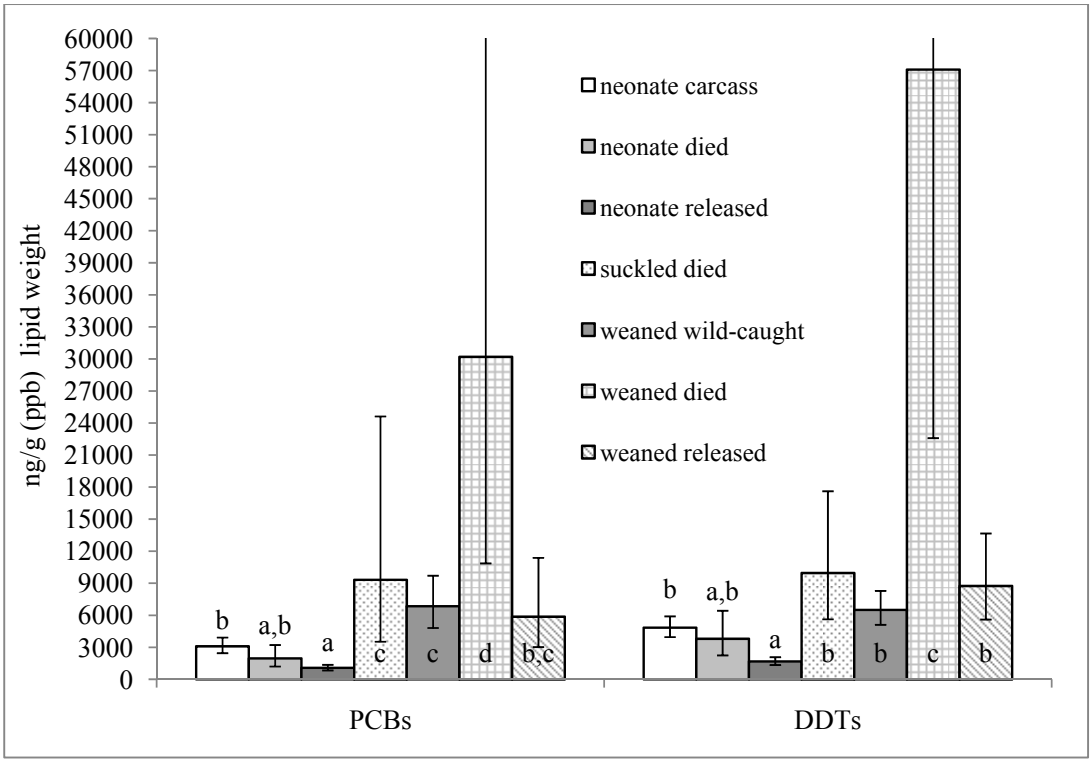


Figure 6.2. Geometric mean summed PCBs and DDTs by group. Error bars are 95% confidence intervals. Lower case letters in common mean no significant difference between those groups.

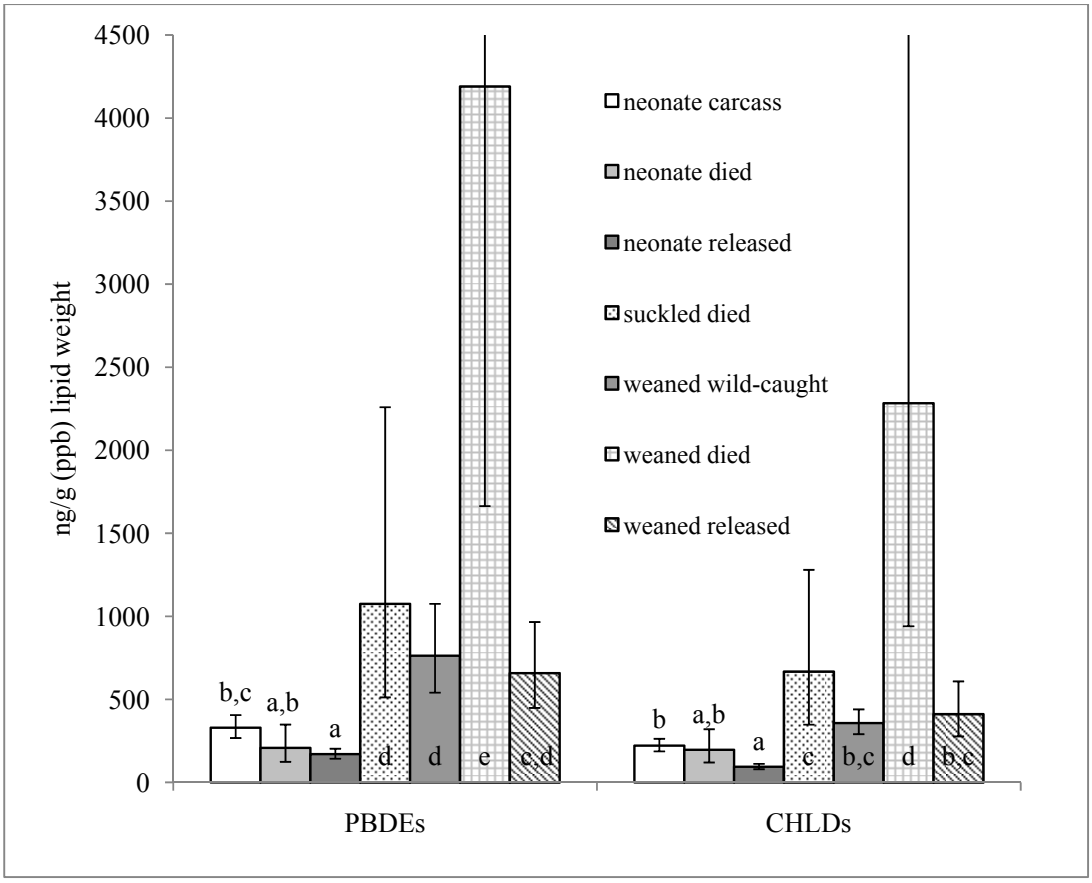


Figure 6.3. Geoometric mean summed BDEs and CHLDs by group. Error bars are 95% confidence intervals. Lower case letters in common mean no significant difference between those groups.

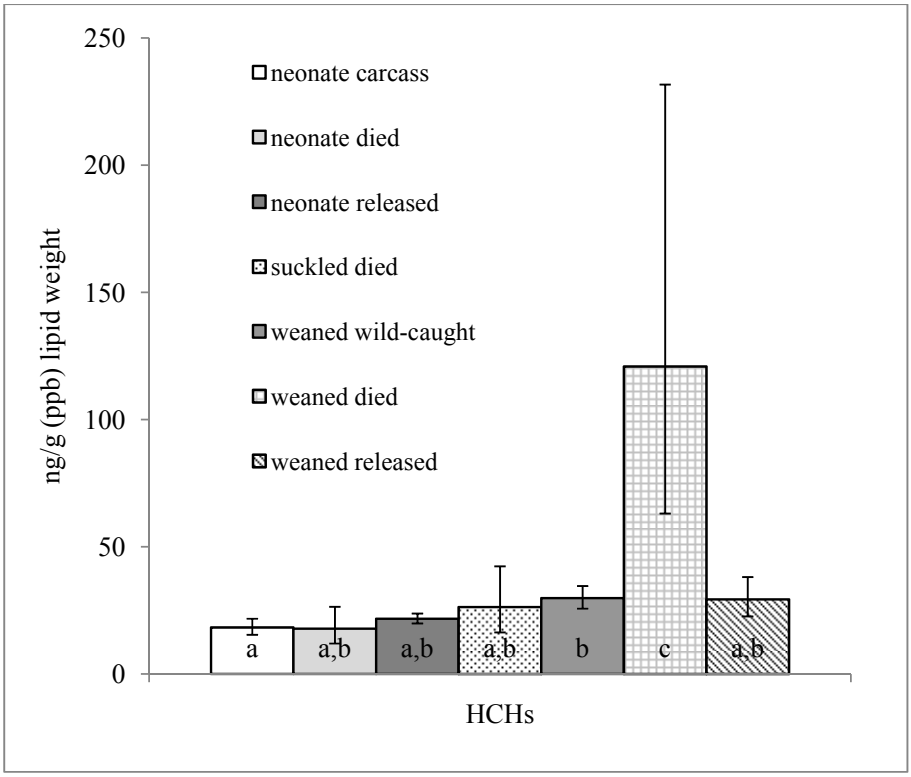


Figure 6.4. Geometric mean summed HCHs by group. Error bars are 95% confidence intervals. Lower case letters in common mean no significant difference between those groups.

Summed contaminant compounds in the carcass group by location. Concentrations of PCBs from SFB were significantly greater than those from MTY on a wet weight basis (adjusted p=0.035) and there were a wide range of PCB concentrations detected from SFN (Figure 6.5). There was no difference in the lipid normalized PCB values or wet weight or lipid normalized DDTs, CHLDs, or PBDEs between the five locations. Wet weight HCHs were significantly greater in pups from north of SFB (SFN, p=0.009 and TBN, p=0.026), but for lipid normalized HCHs, only TBN was greater than SFB (p=0.036, Figure 6.6). The ratio of PCBs to DDTs was significantly different between locations for wet weight and lipid weight concentrations (p<0.0005): MTY was significantly lower and SFB significantly higher than all other locations (Figure 6.7). There was no difference in PCB:DDT among TBN, SFN and OCSSF. There were no differences by location in the ratio of *p,p'*-DDE to *p,p'*-DDT.

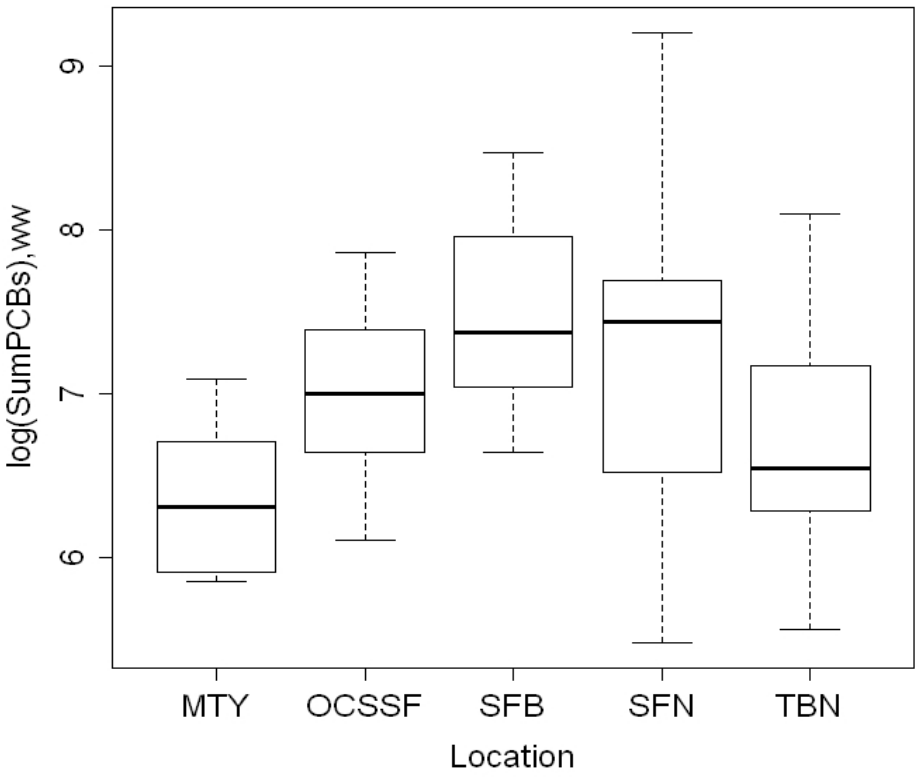


Figure 6.5. Log transformed SumPCBs (ng/g wet weight) by location. Locations are displayed from south to north. The plot shows the median value, the 25th and 75th percentiles, and maximum and minimum PCB values for each location.

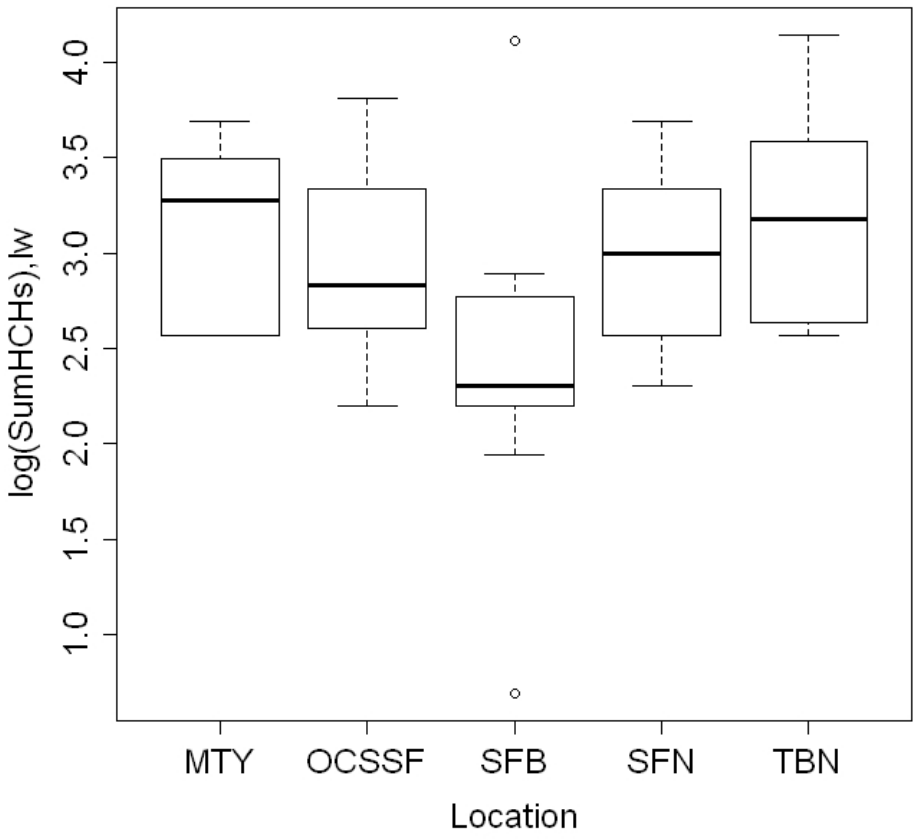


Figure 6.6. Log transformed SumHCHs (ng/g lipid weight) by location. Locations are displayed from south to north. The plot shows the median value, the 25th and 75th percentiles, and maximum and minimum PCB values for each location. For SFB, the whiskers are 1.5 times the interquartile range of the data and two outliers are plotted individually.

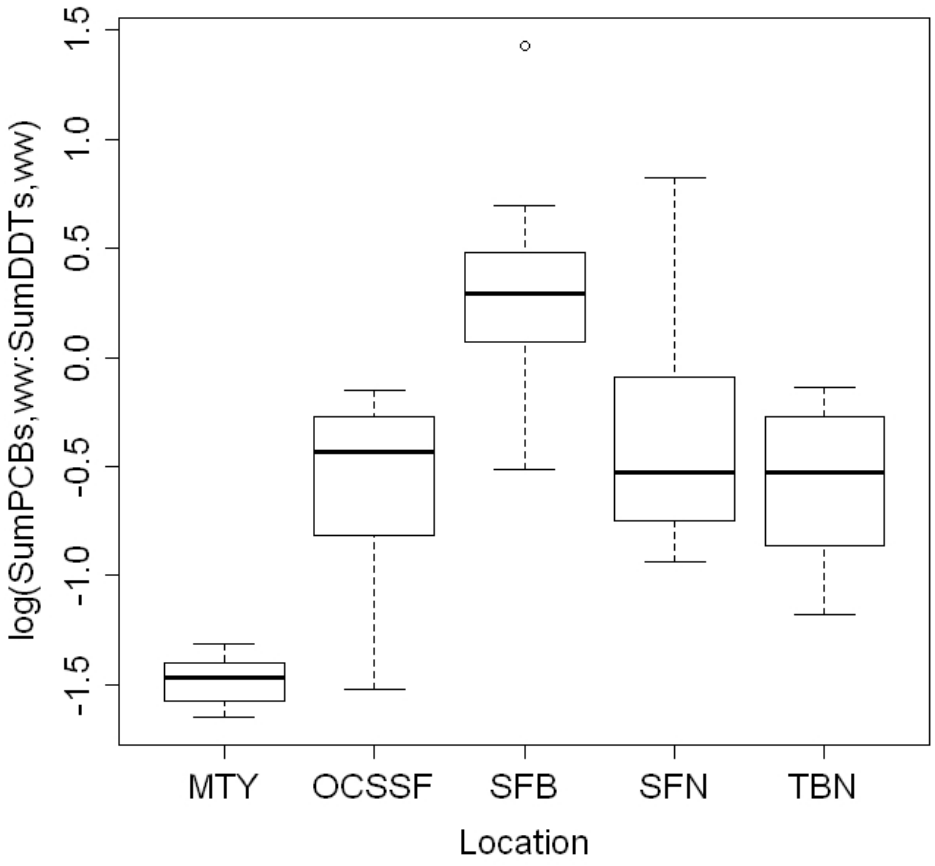


Figure 6.7. SumPCBs:SumDDTs (ng/g wet weight) by location. Locations are displayed from south to north. The plot shows the median value, the 25th and 75th percentiles, and maximum and minimum PCB values for each location. For SFB, the whiskers are 1.5 times the interquartile range of the data and one outliers plotted individually.

Individual blubber contaminant compounds by location. The first principal component explained 79% of the variability in the analytes and was negatively associated with all individual contaminants, most strongly with the PCB congeners. The second principal component explained an additional 7% of the variability in the dataset and was positively associated with most of the PCB congeners and negatively associated with the chlordanes, DDTs, PBDEs and some of the lower chlorinated PCB congeners (Table 6.4). These principal components were able to distinguish the animals from SFB and MTY (Figure 6.8). The variability in the SFN samples is also evident in Figure 6.8 indicating that animals just to the north of San Francisco Bay are likely made up of a combination of animals from the more PCB contaminated SFB as well as those from the more pesticide dominated areas to the north.

Table 6.4. Component loadings of 34 compounds on the first two components which explained 85% of the variance in dataset for the neonate carcass (NC) group. Major contributors that do not overlap the two components are in bold (loadings<0.15 were considered minor contributors).

Compound	PC1	PC2
PCB101	**-0.19**	0.01
PCB128	**-0.19**	0.05
PCB99	**-0.19**	0.02
PCB138	**-0.19**	0.09
PCB156	**-0.19**	0.09
PCB105	**-0.19**	-0.04
PCB158	**-0.19**	0.12
PCB153	**-0.19**	0.12
PCB151	**-0.19**	0.07
PCB149	**-0.18**	0.09
PCB187	-0.18	0.18
PCB118	**-0.18**	-0.05
PCB171	-0.18	0.18
PCB177	-0.18	0.17
PCB170	-0.18	0.17
PCB183	-0.18	0.19
PCB180	-0.18	0.19
PCB199	-0.18	0.21
PCB194	-0.18	0.22
PCB87	**-0.18**	-0.11
PCB52	**-0.17**	-0.07
PCB49	**-0.17**	-0.06
PCB74	-0.17	-0.21
PBDE47	-0.17	-0.17
PCB110	**-0.17**	-0.01
trans-nonachlor	-0.16	-0.26
PCB95	**-0.16**	-0.06
oxychlordane	-0.15	-0.21
PBDE100	-0.14	**-0.17**
p,p'-DDT	-0.14	**-0.29**
p,p'-DDE	-0.13	**-0.25**
p,p'-DDD	-0.11	**-0.25**
PBDE99	-0.11	**-0.22**
Nona-III-chlordane	-0.11	**-0.40**

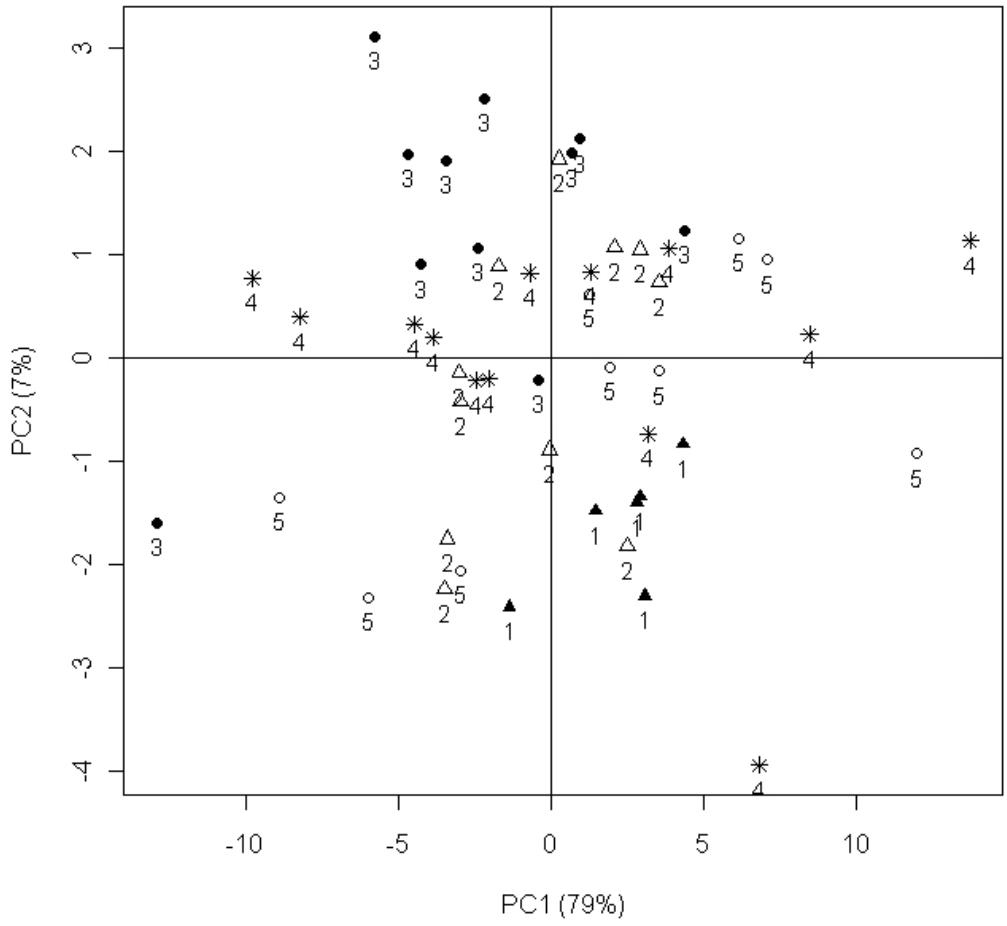


Figure 6.8. The location of each sample (lipid weight) plotted by the first and second principal components (PC1 and PC2). 1=MTY, 2=OCSSF, 3=SFB, 4=SFN, 5=TBN.

DISCUSSION

This is the first study to compare contaminant levels between wild and stranded harbor seal pups, categorizing them by extent of suckling which is an important route of exposure. Blubber contaminant concentrations in harbor seal pups varied dramatically across a two to three week time scale depending on whether the animal was nursing, fasting, or fed an artificial diet during that time. This has consequences for how contaminant concentrations are compared between studies and locations as well as for evaluating the health risks to these animals from persistent organic pollutants.

Based on the decreased levels of contaminants in the pups released from rehabilitation compared with the neonatal carcass group, it appears that there is a dilution effect on the contaminants acquired during gestation for the seals fed milk matrix, salmon oil, and frozen herring in rehabilitation. Alternatively, it is possible that the carcass group acquired greater concentrations of contaminants during gestation. Nevertheless, exposure during lactation exceeds exposure through prey sources (Wolkers et al. 2004; Thomas et al. 2005) and pups entering rehabilitation may lower their lifetime exposure to contaminants because they are not acquiring a large legacy through lactation. This point is illustrated by the 6 to 9 fold difference in blubber concentration between the pups released from rehabilitation and the weaned wild-caught pups with equivalent mass, blubber depth and percent lipid.

Although proportionally fewer contaminants are stored in the blubber during gestation than lactation, this does not rule out the possibility of exposure effects *in utero*. Compared with a study in the River Tees of contaminants associated with pups aged 2 to 5 days that failed to thrive, PCB concentrations in our NC pups were lower, but DDT concentrations were higher (Wilson 2001). The seal from the NC group with the highest PCBs, DDTs, PBDEs and CHLDs on a lipid weight (lw) basis was a pup from San Francisco Bay with a severe congenital defect although the cause of the defect is not known (Harris et al. 2011).

Among the pup groups, contaminant concentrations in the blubber were greatest in the weaned pups; and in the weaned pups that lost mass, stranded, and died, concentrations were comparable to levels measured in the blubber of stranded adults. In addition to concentrating to high levels in the blubber, contaminants are likely mobilized into the

blood during this period of mass loss with the potential to exert health effects on various systems just when these pups are learning to forage and fighting their first parasitic infections. While de Swart et al (1995a) did not observe any changes in immune function with fasting and mobilization of contaminants into the blood, the seals in their study were older (over 3 years) and only lost 16% of their body mass. In elephant seals, Debier et al (2006) found that greater concentrations of PCBs were mobilized into the serum of fasting females during late lactation. This increase in serum PCBs was more pronounced in leaner elephant seals suggesting that the retention capacity of the blubber was reduced more quickly in seals with lower lipid reserves (Debier et al. 2006). In this study, the harbor seal pups stranded near birth mass 3 to 8 weeks after weaning. Assuming a normal mass at weaning, these pups lost over 50% of their body mass potentially mobilizing a large portion of the lipophilic contaminants acquired during gestation and lactation into the serum where it has the potential to affect target organs.

Relatively few blubber contaminant concentrations have been reported for harbor seal pups in central California. Blubber contaminant concentrations were measured in eleven harbor seal pups in rehabilitation at TMMC in 1990 and 1991 similar to our ND and NR groups (Shaw 1998; Shaw 2001). Levels of PCBs ranged from 1400-5300 ng/g lw (this study 198-12862 ng/g lw) and DDE ranged from 700-13,400 ng/g lw (this study 522-9545 ng/g lw) suggesting a possible decrease in PCBs over the years among seals entering rehabilitation, but with little change in DDTs. Risebrough et al (1980) measured blubber PCBs and DDTs from 4 dead pups from south San Francisco Bay and one from Double Point, Point Reyes National Seashore in 1976 which can be compared to our NC pups from SFB and SFN. PCBs (approximating Aroclor 1260) ranged from 4,500 to 120,000 ng/g lw (this study 439-20,793 ng/g lw) and DDTs ranged from 7,200 to 21,000 ng/g lw (this study 952-19,494 ng/g lw). Also within SFB, PBDEs (sum of congeners 47, 99, 100, 153, and 154) for one full term fetus (collected 4/14/92) were 430 ng/g lw (66.9% lipid, She et al. 2002). This data point fits within our range of 49-1863 ng/g lw PBDEs. Recently PBDEs were reported in the blubber of harbor seal pups stranded in southern California (Meng et al. 2009). It is not clear which of the groups the pups would be most comparable to in terms of nutritional history, but levels were similar to our WD group. Based on comparison with the Risebrough data from the 1970s, it appears that PCBs have

declined in harbor seal pups from SFB, but DDTs are highly variable and in some cases remain at levels detected during the peak of pesticide use.

With the exception of the wild-caught group of pups, the pattern of contaminant classes we report is different than previously described from the livers of stranded harbor seals (PCBs>DDTs>CHLDs>HCHs, Kajiwara et al. 2001). In this study, DDTs were greater than PCBs as was reported in the livers of stranded sea lions and elephant seals from California (Kajiwara et al. 2001). The greater PCB concentrations in the wild-caught seals may be a location effect, as most of the WW pups were sampled in SFB. Among the PCB congeners, the pattern was similar to that reported around the world with 153 and 138 as the biggest contributors (Debier et al. 2003b). As seen in other studies, *trans*-nonachlor was the main contributor to sum CHLDs (Miranda Filho et al. 2009). We detected almost equal amounts of α- and β-HCH isomers in harbor seals in California which is an increase in the percent composition of β-HCH previously reported in harbor seal livers (Kajiwara et al. 2001). Kajiwara (2001) reported greater α-HCH in harbor seals compared with elephant seals and California sea lions, and α-HCH also is the predominant isomer in ringed seals in the Arctic (Kucklick et al. 2002; Addison et al. 2009). These species differences may reflect differences in HCH metabolism or differences in exposure level based on foraging locations. Exposure to the different isomers at different locations may also be a function of transport processes with atmospheric transport driving α-HCH levels and ocean current transport driving β-HCH levels (Addison et al. 2009).

These are the first data on CHLDs and HCHs in blubber from harbor seals in California. In southern elephant seals, HCHs (especially α-HCH) have a high transfer rate from maternal blubber to pup blubber during lactation (Miranda Filho et al. 2009). The seals that did not suckle (NC, ND, and NR) were therefore expected to have less HCH in their blubber than was measured in the recently weaned pups in the wild (WW), however no differences were observed. Overall, HCH levels were lower than the other contaminant classes. Lindane (γ-HCH), the most potent pesticide of the isomers, was substantially lower than levels found in UK harbor seals (Hall and Thomas 2007), but even low doses of γ-HCH are potent endocrine disruptors. They have been shown to cause chromosomal changes in human breast and prostate carcinoma cell lines such as inducing the formation

of micronuclei and increasing gene expression of the BCL-2 protein (Kalantzi et al. 2004).

Differences in blubber contaminant concentrations by location among the pups that likely stranded near where they were born (NC) presumably reflect maternal contaminant concentrations. This suggests that exposure levels among adults vary along the central California coast, with increased exposure to PCBs in SFB and increased exposure to DDTs to the south in MTY. These geographical patterns are consistent with those reported by the Mussel Watch program which uses shellfish to monitor coastal contaminant trends. They detected medium to high levels of CHLDs, DDTs and dieldrin in mussels collected at sites in Monterey Bay: medium levels of DDTs and PCBs in SFB; and medium levels of CHLDs, DDTs, and dieldrin at sites north of Tomales Bay (Kimbrough et al. 2008). Differences in harbor seal contaminants by location were also reported by Ross et al. (2004) for the Puget Sound area. Ross et al. (2004) separated samples taken from seals in industrialized regions from those collected in more remote areas based on the PCB homologue groups, with the higher chlorinated compounds (high log G and high K_{ow}) more prevalent in their industrialized location. We observed some of the lower chlorinated PCBs (74 and 95) grouping with the pesticides in less industrialized locations (i.e. away from SFB), however others were part of the industrial signal (PCB 99, 101, 105).

The high variability in contaminant concentrations detected from the SFN location is informative in terms of harbor seal movements and potential exposures. Most of the SFN pups (11 of 13) were recovered from a single beach at the mouth of Drakes Estero which is the largest nursery area for harbor seals in California with 300-500 pups born each year (Allen et al. 2004). The profiles of the pups from this location suggest that harbor seals from SFB as well as those from less PCB contaminated areas travel to Drakes Estero to give birth and nurse their pups emphasizing the importance of this nursery area for the harbor seal population especially those seals inhabiting SFB which have lost much of their nursery habitat (Allen 1991).

Differences in contaminant concentration by location were not as distinct in this study as differences reported among three areas in Denmark (Storr-Hansen and Spliid 1993). While there were differences in exposure that varied with location in this study, many

harbor seals in central California likely use a combination of differently contaminated locations and may show less site fidelity than the seals sampled in Denmark. This is consistent with data from harbor seals tagged in Drakes Estero which showed three separate movement patterns: resident, breeder, and transient (Allen et al. 1987). In another study, a pregnant female that was routinely observed in Elkhorn Slough (MTY) travelled to SFB and gave birth and nursed her pup before returning to MTY (Greig 2002).

In conclusion, contaminant concentrations in seal pup blubber vary dramatically based on extent of suckling and nutritional status, with the highest concentrations observed in harbor seal pups that suckled in the wild and then lost considerable mass during the post-weaning fast. This group is likely the most vulnerable to contaminant effects as these compounds also were likely circulating in high levels as the pups mobilized the fat stored in their blubber during fasting. However, contaminant effects on health and survival cannot be ruled out for any of the groups, and in the case of the neonatal carcass group, pups born in San Francisco Bay were exposed to higher concentrations *in utero* than pups born in other locations along the central California coast. High levels of DDTs were detected and it is unknown what effect these compounds may have on the health of developing harbor seals. Recent studies on rat models have shown synergistic effects between DDTs and biotoxins *in utero* (Tiedeken and Ramsdell 2009; Tiedeken and Ramsdell 2010) which has implications for marine mammals in central California that are routinely exposed to domoic acid (de la Riva et al. 2009). This study provides a comprehensive dataset of contaminant concentrations stratified by important life history characteristics which provides a useful baseline for future health and risk assessment work.

Chapter 7. Harbor seal dispersal and factors affecting the probability of survival

INTRODUCTION

Age specific survival rates are essential for understanding population dynamics and evaluating causes of population decline. For example, because most Hawaiian monk seals (*Monachus schauinslandi*) are tagged as pups and monitored yearly, it is well understood that the current decline in the population is result of low juvenile survival with few young seals surviving to reproduce (Antonelis et al. 2006; Baker and Thompson 2007). Investigations focused on the reasons for low juvenile survival are now guiding conservation and management of the species (Antonelis et al. 2006). Long term datasets of marked animals exist for other pinnipeds, for example Weddell seals (*Leptonychotes weddellii*, Hadley et al. 2007) and Australian fur seals (*Artocephalus pusillus doriferus*, Gibbens and Arnould 2009), however there are few such direct estimates of survival in harbor seals.

Harbor seal survival has primarily been estimated from mortality and life tables: annual adult survival has been estimated at aproximately 80% (Bigg 1969; Boulva and McLaren 1979), while juvenile survival is highly variable with pre-weaning survival ranging from 69% to 83% (Boulva and McLaren 1979; Steiger et al. 1989) and post-weaning first year survival ranging from 35% to 80% (Bigg 1969; Reijnders 1978). In addition to having good estimates of survival, it is particularly important to understand the factors influencing juvenile survival to be able to understand potential risks to population stability and growth.

In pinnipeds, juvenile survival has been variably linked to genetics (Coltman et al. 1998), protein reserves (Bennett et al. 2007), climate perturbations such as El Nino (Ono et al. 1987), thermal stress (Harding et al. 2005), sex, immunity (Hall et al. 2002), contaminant concentrations (Hall et al. 2009) and mass at weaning (Hall et al. 2001) which has in turn been linked to maternal mass, maternal experience and prey availability during lactation (Lunn et al. 1994; Bowen et al. 2001; McMahon and Burton 2005). In addition, the effect of some factors, like mass at weaning, may vary annually and become even more important for post-weaning survival when there are changes in prey availability or climate (McMahon and Burton 2005). Many studies of juvenile

survival have focused on the effect of environmental covariates on maternal and pup body condition and physiology, but fewer have assessed the possible effects of contaminants and disease.

Experimental studies have demonstrated contaminant effects on immunity and reproduction in the harbor seal (Reijnders 1980; Ross et al. 1995; de Swart et al. 1996). In addition, *in vitro* studies have shown contaminant effects on immune function (Neale et al. 2002; Levin et al. 2005). As endocrine disruptors, organochlorine contaminants may also affect growth and metabolism through alterations in circulating thyroid hormones (Hall et al. 1998). In grey seals, PBDE concentration was positively associated with total serum levels of thyroxine (T4) and triiodothyronine (T3) when days post weaning and body condition were taken into account (Hall et al. 2003). It has also been suggested that contaminant exposure increases the likelihood of disease and mortality in sea lions and cetaceans (De Guise et al. 1995; Jepson et al. 2005; Ylitalo et al. 2005b): and in harbor seals, PCBs, PBDEs, and DDE were associated with an increase in leukocyte count (Neale et al.2005b). Most studies of contaminant exposure have focused on effects on immunity and reproduction and indirectly survival, and only recently have direct associations with survival probability been considered (Hall et al. 2009).

In harbor seals, it is difficult to estimate juvenile survival because the seals disperse soon after weaning and it is not known when or if they return to their natal beaches. For this reason, radio tags or other marks that require re-locating the animal to determine survival are of limited utility (Lander et al. 2002). The objective of this study was to use satellite telemetry to determine whether health measures and contaminant concentrations were associated with survival in wild-caught and rehabilitated harbor seal pups. In addition, the effects of dispersal on survival, and the effects of factors specific to rehabilitation on post-release survival (such as date of admission, mass gain and days spent in rehabilitation) were considered.

METHODS

In 2008, satellite tags were deployed on three groups of seals: recently weaned, wild-caught, pups from San Francisco Bay (SF) and Tomales Bay (TB), and rehabilitated

pups from The Marine Mammal Center (TMMC). At the time of deployment, pups were weighed, length and girth were recorded and blubber depth was measured by ultrasound. Blood was drawn to measure leukocyte count (WBC), immunoglobulin (IgG), thyroxine (T4) and triiodothyronine (T3) and a blubber sample was collected for contaminant analysis. Satellite linked transmitters were glued to the pelage of the head using loctite 422 (Loctite Corp., Connecticut, USA).

Satellite tags (Spot5, Wildlife Computers, Redmond, WA, USA) weighed 49 grams, measured 48 x 42 x 14 mm, and had temperature and wet/dry sensors. Each tag was estimated to have 30,000 lifetime transmissions: the tags were programmed to transmit a maximum of 100 transmissions every other day to extend the battery life beyond the following year's molt. The tags transmitted data on location, temperature, and percent time the tag was dry (as a proxy for time spent ashore). The Argos system of orbiting satellites used satellite location and the time lag between messages received (doppler effect) to calculate the location of the tagged seal (CLS 2008). Accuracy was estimated for each location and a location class assigned with the estimated error varying from <250m (location class 3) to >1500m (location class 0) and no accuracy assigned to location class A or B (CLS 2008).

Sample analysis. Blood samples were analyzed for WBC and IgG as described in Chapters 3 and 5. Blubber samples were analyzed for organohalogen pollutants (OH) as described in Chapter 6 and the summed contaminant classes used as individual covariates: contaminant classes were polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane and its metabolites (DDT), polybrominated diphenylethers (PBDE), chlordanes (CHLD), and hexachlorocyclohexanes (HCH).

Thyroid hormones. Blood samples were analyzed for total thyroxine (T4) and total triiodothyronine (T3) by enzyme-linked immunosorbent assay (ELISA, Fortress Diagnostics, Belfast, UK). Briefly, 25ul of serum and 100ul of a buffered solution of T4 (or T3)-horseradish peroxidase were added to microplate wells coated with T4 (or T3) antibody and allowed to incubate for 60 minutes. The contents were decanted and rinsed with a wash buffer. Substrate solution was added to each well, incubated for 15 minutes and stopped with hydrochloric acid before the absorbance in each well was read at 450 nm. All samples were assayed in duplicate with controls and standards included in all

assay runs. Total T4 intra-assay coefficient of variation (*CV*) was 10.4%. Total T3 intra-assay *CV*s were 7.6% (Hall et al.2007).

Data analysis. Morphology covariates and blood variables were analyzed by group using analysis of variance (ANOVA) and Tukey's Honest Significant Difference method. The body condition index was the residuals from the linear relationship between mass (kg) and length (cm): mass=-21.90+0.47*length (R^2=0.52, P<0.005). Generalized linear models were used to evaluate whether contaminant concentrations were predictive of thyroid levels. The contaminant classes were summed for each seal and generalized linear models were used to determine whether morphology or blood variables were associated with overall organohalogen contamination (OH). Statistical analyses were performed using the R programming language (R Development Core Team, 2009).

The tags were neutrally buoyant (i.e. would not float if detached from an animal) and could only communicate successfully with a satellite when the tag antenna was out of the water. Thus, when transmissions were no longer received, this was interpreted as death of the animal or loss of the tag. Loss of the tag could result from either mechanical failure of the tag or detachment of the tag from the animal. While it is not possible to estimate the probability of mechanical failure of the tag, all tags were from the same batch and there was no reason to expect differential deployment of bad tags on one group of seals over another. Therefore, mechanical failure was not expected to impact comparisons between rehabilitated and wild-caught seals. Tag loss from detachment was estimated assuming that detachment would be normally distributed with a high mean (Thompson and Lonergan 2009). Tags that continued to transmit, but were dry 100% of the time (i.e. on shore) and never returned to the water, were assumed to have come ashore with an animal that was dead or dying.

To estimate the minimum distance each seal travelled during tag deployment, distances were first calculated between the highest quality location data from each transmission day to the next (omitting location class B). The spherical law of cosines was used to calculate the distance between latitude and longitude:

Distance (km) = arccosine(sin(lat1)*sin(lat2)+cos(lat1)*cos(lat2)*cos(lon2-lon1))*R

where R is the mean radius of the earth (6371 km) and latitude and longitude are in radians. The distances were then summed and divided by the length of time the tag transmitted to calculate an average minimum distance per day (speed). This was a minimum estimate of distance travelled because 1) tracks occasionally crossed land instead of going around points or peninsulas, 2) there was a maximum of one location every other day and the path the seal took between those two points was not known, and 3) a high quality transmission was not always received every other day. This speed covariate provided an estimate of dispersal because if travel was unidirectional, the total distance travelled was greater as was the distance per day. Speed was assumed to be constant throughout deployment, but if seals travelled faster initially, the covariate would be biased toward faster speeds among the seals that stopped transmitting earlier. Distance from capture/release directly to the last location received was also calculated as a minimum distance travelled from haulout, but was also biased as not all travel was directed away from the haulout.

Program MARK (1999) was used to model the effect of individual covariates on the probability of survival using a Cormack Jolly Seber live resighting framework (Hall et al. 2009). Each location received was considered to be a recapture, and binary encounter data were summarized for each animal by week as still transmitting (1) or no transmissions received (0). Several datasets with different individual covariates were examined each containing the same weekly encounter data and group covariate based on location (SF, TB, or TMMC). MARK used maximum likelihood to estimate a survival probability (Phi) and recapture probability (p) that best fit the encounter data. Akaike's information criterion, corrected for small sample size (AICc), was calculated: this criterion balanced the fit of the model with the precision lost when additional parameters were estimated (Cooch and White 2009) and was used to compare models within a set of candidate models. Models with a delta AICc < 2 when compared to the model with the minimum AICc were considered strongly supported by the data (Hadley et al. 2007; Hall et al. 2009). Models with a delta AICc between two and four are also considered to be supported by the data (Burnham and Anderson 2002). Median c-hat was used to test for goodness of fit which could only be evaluated in datasets without individual covariates.

RESULTS

Transmissions from satellite tags deployed on seals from San Francisco Bay (n=19), Tomales Bay (n=7) and The Marine Mammal Center (n=21) were received for eight months and survival appeared to be greater among the pups from Tomales Bay (Figure 7.1). One pup from Tomales Bay transmitted only on the day it was captured and was omitted from the study. One pup was recovered dead within San Francisco Bay five days after deployment with a fractured the skull and humerus. The injury was consistent with collision with a high speed vessel and the animal was otherwise healthy. The pup had a thick blubber layer (34mm) and its buoyancy and inexperience compared with older animals may have increased its risk to boat traffic. Three tags presumed to have come ashore with dead or dying seals transmitted for 388, 403, and 417 days confirming that the tags were capable of transmitting for over a year.

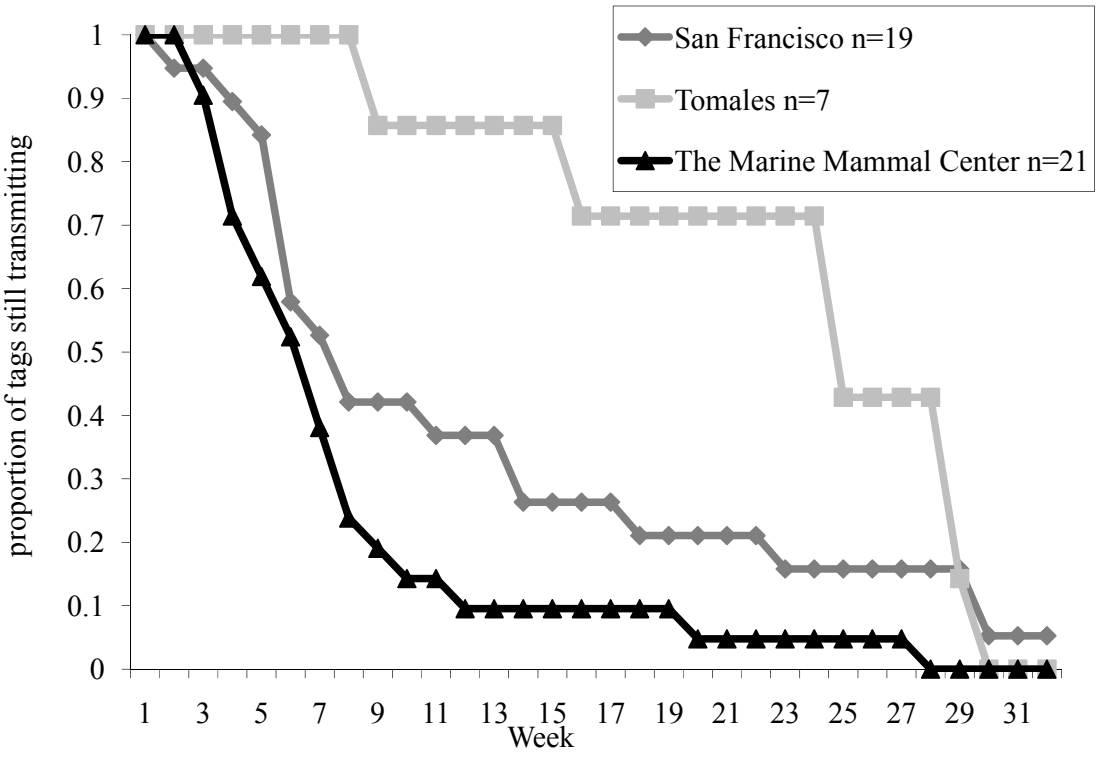


Figure 7.1. Proportion of tags still transmitting by week post-deployment. Tags that were permanently ashore were considered to be no longer transmitting.

Distance travelled. Distance of final location from capture/release site ranged from 5 km to 802 km with the furthest location reached by a pup from SF that stopped transmitting just south of Playas de Rosarito, Mexico (32.138°, -117.084°). Minimum distance travelled ranged from 5 to 2,530 km with the shortest distance covered by the boat strike pup from SF which transmitted for 4 days and with the greatest minimum distance covered by an SF pup which transmitted for 232 days and stopped transmitting offshore of Coos Bay, Oregon (43.336°, -124.450°) 637 km from the capture site (Figure 7.2). Minimum distance travelled per day varied from 0.5 to 49 km/day. Of those animals that transmitted for less than 6 weeks, 17% travelled over 20 km/day (24% of TMMC pups and 16% of SF Bay pups, Figure 7.3). It is possible that these relatively higher speeds were indicative of travel with very little time spent foraging in one place leading to a quick decrease in energy stores.

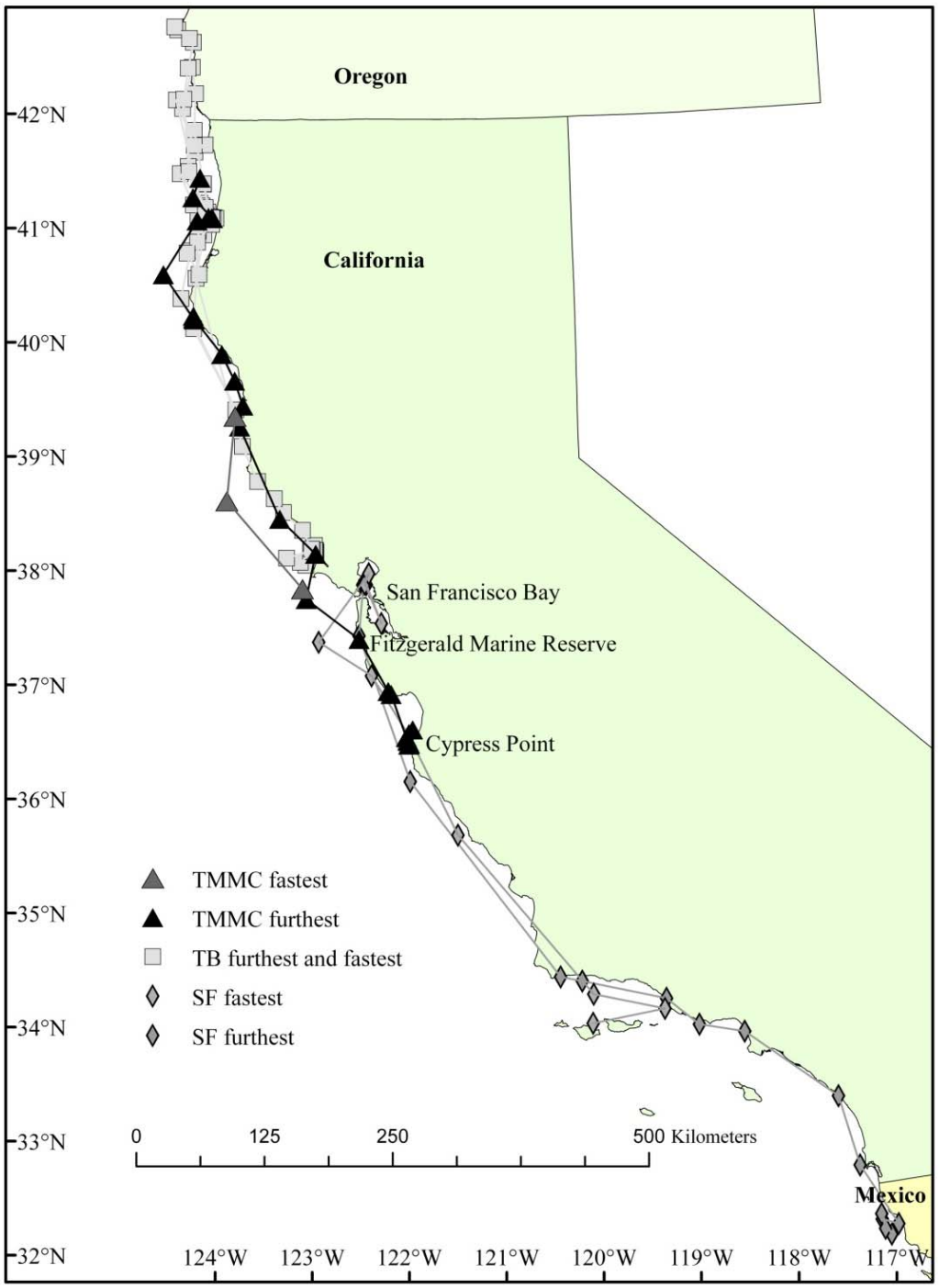


Figure 7.2. Map of the tracks taken by seals that travelled the furthest and the fastest from each location. The fastest TMMC seal was released from Fitzgerald Marine Reserve and the TMMC seal that travelled the furthest was released from Cypress Point, Monterey.

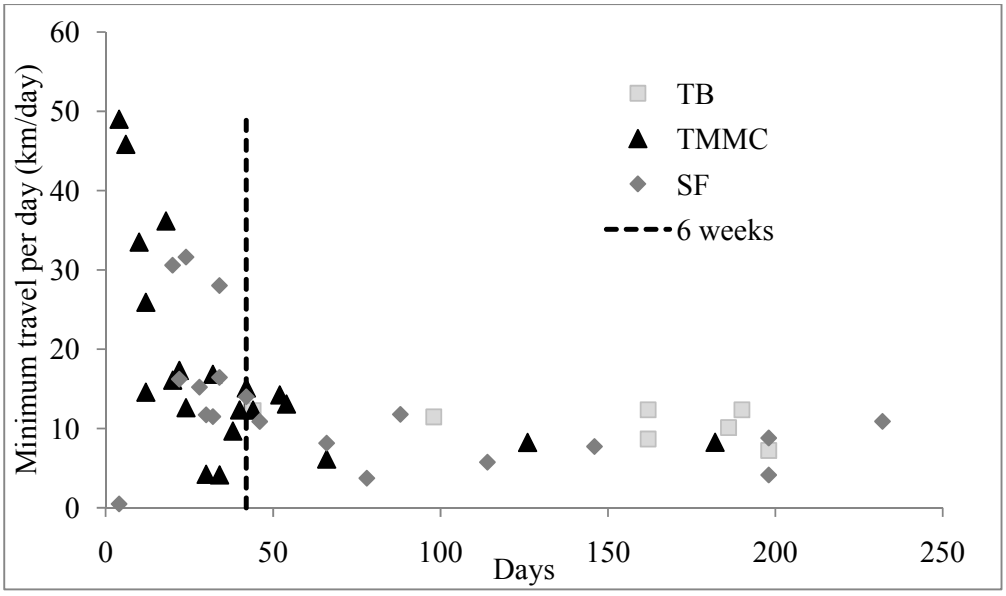


Figure 7.3. Minimum average distance per day (speed) travelled by satellite tagged seal pups over the course of their deployment.

Morphology and blood variables. There were no differences among the seal groups in any of the morphology covariates except for body condition. The TMMC pups had significantly poorer body condition compared to the SF pups (adjusted p=0.036, Table 7.1). There were differences by group for the blood variables (Table 7.2). Immunoglobulins were significantly increased in TMMC pups compared with SF (adjusted p<0.005) and TB (adjusted p=0.039) pups. Leukocyte count was significantly higher in TMMC pups than SF pups (adjusted p=0.004). Thyroxine levels varied by group with T4 levels significantly higher in TB than SF (adjusted p=0.008) and TMMC (adjusted p<0.005) pups. There were no differences in T3 between group (anova, p=0.113, df=2). Contaminant concentrations were not predictive of T4 or T3 for any of the three groups (p>0.05).

Table 7.1. Mean and range for the morphology covariates by group (SF=San Francisco, TB=Tomales Bay and TMMC=The Marine Mammal Center).

group	n	mass (kg)	length (cm)	girth (cm)	bd (mm)	condition
SF	19	19 (13-27)	85 (72-100)	72 (61-86)	19 (14-25)	1.0 (-3.0-7.5)
TB	7	20 (16 -25)	88 (83-95)	74 (65-83)	19 (14-23)	0.1 (-1.9-1.9)
TMMC	21	18 (13-25)	86 (79-96)	69 (58-93)	18 (13-22)	-1.0 (-5.4-2.8)[a]

bd=blubber depth, n=20 for blubber depth from TMMC pups, superscript letters in common represent no significant difference

Table 7.2. Mean and range for the blood variables by group (SF=San Francisco, TB=Tomales Bay and TMMC=The Marine Mammal Center).

group	sex (m,f)	IgG	WBC	T4	T3
SF	9,10	24 (20-33)	7.4 (4.3-11.1)[b]	28 (10-58)	0.86 (0.53-2.32)
TB	5,2	26 (21-29)	8.3 (4.9-13.6)[a,b]	46 (18-62)	0.61 (0.39-0.94)
TMMC	9,12	29 (26-32)[a]	10.1 (6.2-15.0)[a]	19 (7-40)[a]	0.88 (0.52-1.46)

superscript letters in common represent no significant difference

Predictors of contaminant concentration. Contaminant concentrations were significantly lower in TMMC pups (adjusted $p<0.005$) and TB pups (adjusted $p=0.025$) than the pups from SF (Table 7.3). When the groups were controlled for, length was the only other significant predictor of contaminant concentration with shorter animals associated with higher OH levels (Table 7.4, Figure 7.4).

Table 7.3. Geometric mean contaminant concentration by group in ng/g lipid weight

	n	PCB	DDT	PBDE	CHLD	HCH	OH
SF	19	9777	7179	1053	373	27	18601
TB	7	1794	3897	192	252	43	6302
TMMC	21	1148	1616	154	99	24	3160

Table 7.4. Results for the significant generalized linear model of summed organohalogen contaminants (OH) controlling for group (SF=San Francisco, TB=Tomales Bay and TMMC=The Marine Mammal Center).

Dependent variable	model parameter	estimate	SE	t value	p value
OH	intercept	13.853	1.626		
	location_TB	-0.929	0.319	-2.91	0.006
	location_TMMC	-1.706	0.226	-7.554	<0.005
	length	-0.047	0.019	-2.486	0.017

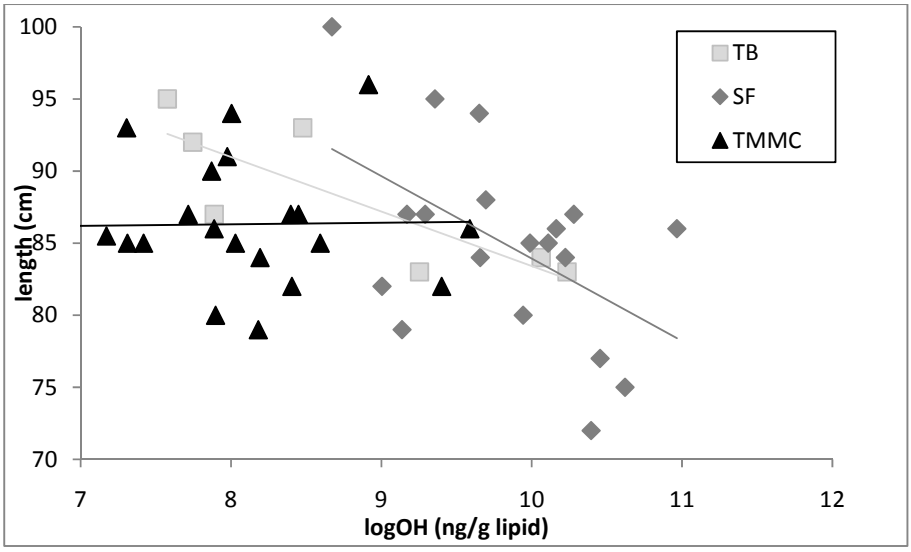


Figure 7.4. Relationship between standard length and summed organohalogen contaminants (OH) for the three groups: Tomales Bay (R^2=0.6733), San Francisco Bay (R^2=0.2646), and The Marine Mammal Center (R^2=0.0003).

Tag loss. Using the TMMC and a combined SF + TB dataset to estimate tag loss, two exponential curves fit the data best and yielded a normal tag loss curve with a mean of 201 days and sd=20 days (Figure 7.5). Subtracting two standard deviations from the mean resulted in 161 days or 23 weeks of the satellite data before effects from tag loss might have affected survival estimates, therefore 23 weeks of satellite data were used to model the individual covariate effects on survival. The effect of tag loss is more evident on the survival curve for the wild-caught seals because so few of the TMMC animals survived long enough for tag loss to be an issue (Figure 7.5).

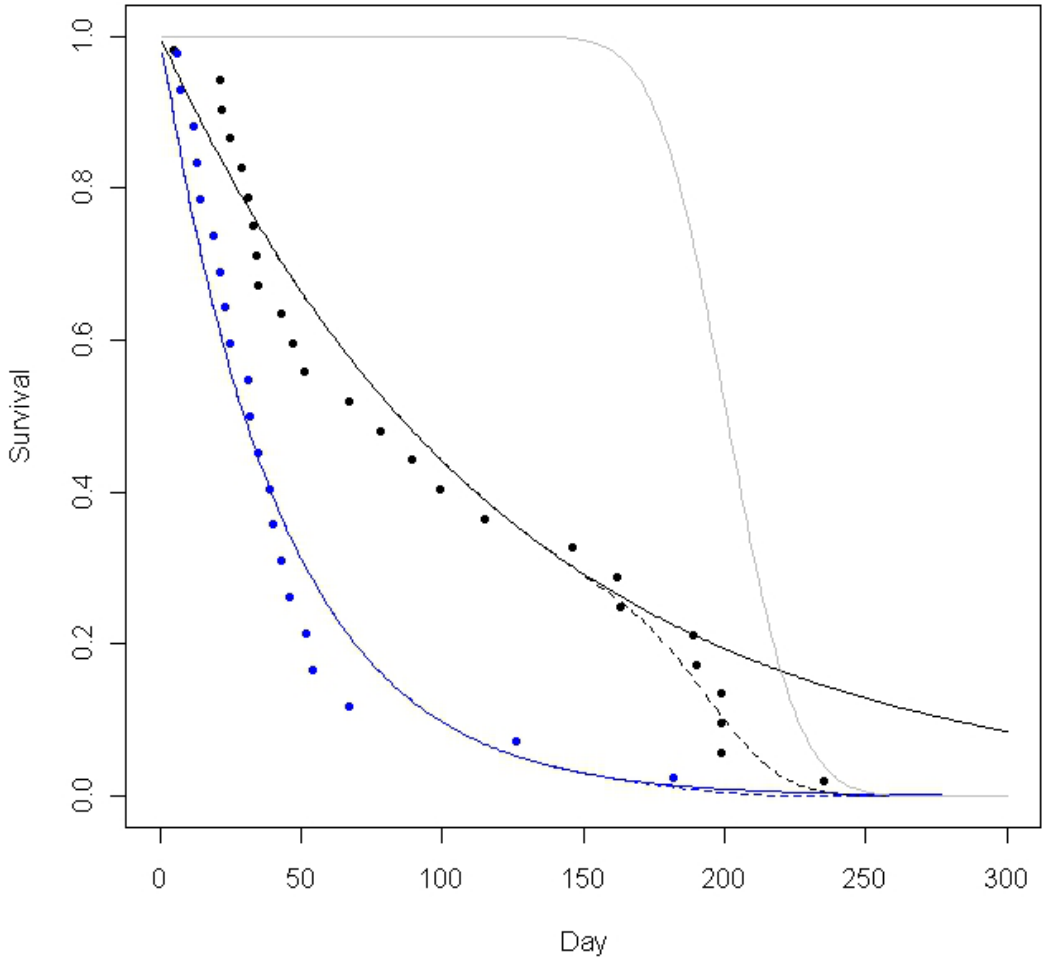


Figure 7.5. Survival based on satellite tag transmission data modeled with two exponential curves (solid lines) and tag survival based on a normal distribution for tag loss (grey line). The dashed lines display the effect of tag loss on the survival curve.

Correlates of survival.

Dataset 1 (group and time). The first dataset contained location (SF, TMMC, TB) as a group covariate. The best model included the group covariate with all three locations (Phi(g), Table 7.5). Recapture probability (p) was estimated to be 1, and was set to 1 in subsequent models because the satellite tags provided 100% recapture rates until they stopped transmitting. This reduced by one the number of parameters to be estimated and effectively modeled the data using a known fate framework. The Phi (group) model was a good fit to the data with a median c-hat of 1.15 (indicating very little over dispersion of the data). This c-hat was used in subsequent models to convert the AICc to QAICc for evaluating the best models in each dataset. There was no support for a model with time dependent survival. There was support for grouping SF and TMMC together, but this model would not be appropriate for datasets with contaminant concentration as an individual covariate because of the difference in contaminant concentration between those two groups.

Table 7.5. Model results from dataset 1: group and time.

Model	QAICc	delta QAICc	QAICc weights	model likelihood	no. parameters	deviance
Phi(g)	222.45	0.000	0.538	1.000	3	49.658
Phi(SF and TMMC v TB)	222.95	0.501	0.419	0.779	2	52.184
Phi(SF and TB v TMMC)	227.55	5.102	0.042	0.078	2	56.786
Phi(.)	233.43	10.983	0.002	0.004	1	64.684
Phi(.) p(.)	235.45	13.000	0.001	0.002	2	64.684
Phi(t)	254.10	31.655	0.000	0.000	22	41.095
Phi(g*t)	320.74	98.291	0.000	0.000	66	0.000

t=time, g=group (SF=San Francisco, TB=Tomales Bay, and TMMC=The Marine Mammal Center), Phi=survival, p=recapture and was set to 1 unless indicated, the dashed line delineates models with delta QAICc<2.

Dataset 2 (contaminants). This dataset contained the location grouping and the lipid weight summed contaminants for the various contaminant classes (PCB, DDT, PBDE, CHLD, and HCH) as individual covariates. There was support for all the models with contaminant covariates (delta QAICc < 2) although group was still the best fit to the data (Table 7.6).

Table 7.6. Model results from dataset 2: lipid weight contaminants

model	QAICc	delta QAICc	QAICc weights	model likelihood	no. parameters	deviance
Phi(g)	220.57	0.000	0.226	1.000	3	214.517
Phi(g+logPBDE)	221.04	0.473	0.179	0.789	4	212.955
Phi(g+logHCH)	221.11	0.544	0.172	0.762	4	213.026
Phi(g+logCHLD)	221.67	1.104	0.130	0.576	4	213.586
Phi(g+logPCB)	222.06	1.491	0.107	0.475	4	213.973
Phi(g*logHCH)	222.25	1.683	0.098	0.431	6	210.070
Phi(g+logDDT)	222.46	1.894	0.088	0.388	4	214.376

Dataset 3 (morphology). Group was again retained and mass, length, girth, body condition, blubber depth and sex were added as individual covariates. The best models included length, mass, and sex (Table 7.7) with increased length, increased mass, and female pups each associated with increased survival probability.

Table 7.7. Model results from dataset 3: morphology.

model	QAICc	delta QAICc	QAICc weights	model likelihood	no. parameters	deviance
Phi(g+length)	219.90	0.000	0.191	1.000	4	211.810
Phi(g+mass)	220.44	0.544	0.145	0.762	4	212.354
Phi(g+sex+length)	220.46	0.567	0.144	0.753	5	210.334
Phi(g)	220.57	0.672	0.136	0.715	3	214.517
Phi(g+sex)	220.83	0.935	0.120	0.627	4	212.745
Phi(g+sex+mass)	221.16	1.267	0.101	0.531	5	211.034
Phi(g+bd)	222.23	2.330	0.060	0.312	4	214.140
Phi(g+cond)	222.45	2.557	0.053	0.278	4	214.368
Phi(g+girth)	222.56	2.662	0.050	0.264	4	214.472

Dataset 4 (blood variables). This dataset contained the group covariate as well as T4, T3, WBC, and IgG as individual covariates. The best model was Phi(group +T4) with decreased T4 associated with decreased probability of survival (Table 7.8).

Table 7.8. Model results from dataset 4: blood variables

model	QAICc	delta QAICc	QAICc weights	model likelihood	no. parameters	deviance
Phi(g+T4)	217.45	0.000	0.481	1.000	4	209.366
Phi(g+T4+T3)	219.49	2.033	0.174	0.362	5	209.356
Phi(g+T3)	220.04	2.587	0.132	0.274	4	211.953
Phi(g)	220.57	3.116	0.101	0.211	3	214.517
Phi(g+wbc)	221.49	4.040	0.064	0.133	4	213.406
Phi(g+IgG)	222.04	4.589	0.048	0.101	4	213.955

Dataset 5 (previous models plus dispersal covariate). Based on the results from datasets 1 through 4, this dataset contained the group covariate and OH, mass, T4 and minimum average distance travelled per day (speed) as individual covariates. Total OH was used for the contaminant covariate because all the contaminant classes were similarly supported, and mass was used instead of length to avoid confounding because length was associated with contaminant concentration. The best model was Phi(g+speed+T4), but there was also strong support for Phi(group+speed+T4+logOH) and moderate support for Phi(group+speed+T4+mass). Increased speed, increased OH and decreased T4 were associated with decreased survival probability (Table 7.9).

Table 7.9. Model results from dataset 5 incorporating covariates from previous models as well as a dispersal covariate.

model	QAICc	delta QAICc	QAICc weights	model likelihood	no. parameters	deviance
Phi(g+speed+T4)	210.75	0.000	0.334	1.000	5	200.624
Phi(g+speed)	212.04	1.282	0.176	0.527	4	203.950
Phi(g+speed+T4+logOH)	212.40	1.645	0.147	0.439	6	200.218
Phi(g+speed+T4+mass)	212.80	2.050	0.120	0.359	6	200.622
Phi(g+speed+logOH)	213.70	2.946	0.077	0.229	5	203.570
Phi(g+speed+mass)	213.73	2.974	0.076	0.226	5	203.599
Phi(g+speed+T4+logOH+mass)	214.44	3.686	0.053	0.158	7	200.197
Phi(g+T4)	217.45	6.698	0.012	0.035	4	209.366
Phi(g+mass)	220.44	9.687	0.003	0.008	4	212.354
Phi(g)	220.57	9.814	0.002	0.007	3	214.517
Phi(g+log(OH))	222.19	11.437	0.001	0.003	4	214.105

Dataset 6 (TMMC – rehabilitation covariates). This dataset contained only the encounter data from TMMC pups and incorporated individual covariates that were specific to the animals in rehabilitation, such as date of admission (in Julian days), number of days in rehabilitation, change in mass from admission to release, and mass gain per day, as well as the individual covariates from dataset 5. Phi(mass) was the best model with increased mass at the time of release associated with increased survival, and there was still support for a positive association with T4 (Table 7.10). There was also support for the rehabilitation specific covariates: greater total mass gain, a shorter time in rehabilitation, and admission to rehabilitation earlier in the season were all associated with an increase in survival probability.

Table 7.10. Model results for dataset 6: covariates specific to rehabilitating seal pups

model	QAICc	delta QAICc	QAICc weights	model likelihood	no. parameters	deviance
Phi(mass)	101.79	0.000	0.220	1.000	2	97.704
Phi(T4)	102.08	0.287	0.190	0.866	2	97.991
Phi(.)	102.42	0.625	0.161	0.732	1	100.387
Phi(change in mass)	102.80	1.013	0.133	0.603	2	98.717
Phi(days in rehab)	103.47	1.679	0.095	0.432	2	99.383
Phi(admit)	103.73	1.942	0.083	0.379	2	99.646
Phi(logOC)	104.38	2.593	0.060	0.273	2	100.297
Phi(mass per day)	104.46	2.666	0.058	0.264	2	100.370

DISCUSSION

The survival probability of the pups from Tomales Bay was greater than from San Francisco Bay or The Marine Mammal Center. After controlling for these groups, an increase in OH concentration and a decrease in T4 were associated with decreased survival. Dispersal speed was also associated with decreased survival and it appears that factors associated with growth (T4), behavior (speed) and morphology (mass) were more important than those associated with infection (WBC and IgG). This may simply confirm that the animals were clinically healthy at the time of sampling, and had any of them been ill, these factors might have been more strongly associated with survival probability. Even though WBC and IgG levels were increased in the TMMC pups, there was only moderate support for their association with the survival data and it is possible that there is a threshold effect for these variables as was noted by Neale et al. (2005b). Neale et al. (2005b) reported a significant positive relationship between WBC and PCB which was driven by four data points with a WBC greater than 17 which is higher than found in the seals in this study. In harbor seal pups in Washington and British Columbia, Tabuchi et al (2006) found decreased circulating T4 and an increase in thyroid receptors in the blubber among harbor seal pups with increased PCB concentrations in the blubber. This study extends that link between decreased T4 and increased contaminant levels to survival probability.

In contrast to Hall and Thomas (2007), there were no associations detected between contaminant classes and circulating levels of T3. Their study, however, was composed primarily of adult harbor seals so there could be differences in the way contaminants interact with the endocrine system in adults versus pups. There are also differences in the contaminant profiles of harbor seals in the UK compared the harbor seals sampled in this study (Chapter 6; Hall and Thomas 2007).

Although blubber PCBs were not directly associated with circulating T4 levels, we were not able to control for days post-weaning as was done in a previous study by Hall et al (2003). In harbor seal pups, T4 levels decline from birth to weaning (Haulena et al. 1998). Likewise levels in pre-weaned grey seals were greater than post-weaned grey seals Hall (1998). Without knowing the exact age of the pups, or the thyroid dynamics in rehabilitating seals, it was not possible to separate contaminant effects on thyroid

level from the effects of growth on this hormone. However, the association between length and contaminant concentration does provide support for a possible contaminant effect on growth.

While the survival curve of the pups released from rehabilitation was similar to that of the wild-caught pups from San Francisco, survival probability was associated with different factors. Mass at the time of release was the most important correlate of survival in the rehabilitated pups. Harbor seal pups on Sable Island took 4-6 weeks before they were able to forage effectively and during this time lost approximately half their fat stores, but only about 20% of their total body mass (Muelbert et al. 2003). Two harbor seal pups that re-stranded after release from rehabilitation in 2007 lost 21% of their body mass in 16 days and 32% in 37 days suggesting that compared with the wild pups in Muelbert et at (2003), they were depleting more of their fat stores and/or failing to add lean muscle. Lander et al (2002) reported two similar instances of mass loss in rehabilitated seals post-release. Pups raised in captivity may take longer than wild pups to forage effectively and an extra reserve of body fat may be essential for carrying them past the period of adjusting to a new environment and learning to forage.

Survival among rehabilitated and wild-caught pups was decreased compared with Lander et al (2002). That may represent annual variation or be a function of satellite versus radio telemetry or annual variation. It may also be related to the release masss of the harbor seals as the rehabilitated pups in Lander et al (2002) were not released until they were 20kg (i.e. heavier than some of the pups in this study). Lander et al (2002) additionally suggested that difference in pelage quality between rehabilitated and wild-caught seals may have resulted in differential tag loss. This study does not address this directly, but one rehabilitated seal retained its tag as long as any of the wild-caught seals, and the rest stopped transmitting well before tag loss was expected to occur.

Harbor seal pups dispersed extensively, travelling as far as Oregon and Mexico, well beyond what is generally assumed based on genetics (Lamont et al. 1996). Whether these pups return to their natal beaches before recruiting to the population as reproductive adults is not known. Lander et al (2002) had one tag return 480 km from the release site, but this study is the first to show the full extent of harbor seal pup dispersal along the west coast of the United States and Mexico. Although the harbor

seal population in California is presently considered to be stable (Carretta et al. 2007), these dispersal patterns have management implication should juvenile mortality become a concern for this population as these pups travel well outside of current marine protected areas.

In summary, growth (T4 and mass), behavior (dispersal speed), and contaminant factors were all associated with survival probability; and harbor seal pups that gained the most mass in rehabilitation had an increased probability of surviving their first year. In terms of conservation and management, the most important anthropogenic factor affecting survival, organohalogen contamination, has been addressed in the United States with bans on production and use of PCBs, DDT, CHLDs, and HCHs since the 1970s (Goldberg 1991) and more recently on most PBDEs in the 2000s (Betts 2008). However, these lipophilic contaminants are still present and exerting effects in the marine environment and it will be important to monitor the effects of these as well as new, replacement compounds. The effect of other factors that were not part of the study, such as boat strikes, on the survival of harbor seal pups in San Francisco Bay merit further investigation. Among stranded pups in rehabilitation, the best strategy to increase first year survival appears to be increasing pup mass prior to release. Survival correlates like growth and behavior may be related to the ecosystem and prey availability, and further study on their variability as well of patterns of dispersal will be useful for understanding harbor seal population numbers in California.

Chapter 8. General Discussion: Health, disease, mortality, and survival

THESIS SUMMARY

Conventional health assessment methods were used to describe the health status of harbor seals in California. Hematology and serum chemistry, surveillance for the presence of infectious disease agents, a test of immune function, and contaminant data were all used to evaluate harbor seal health in three groups of seals. Wild-caught seals of all ages were sampled at two locations: San Francisco Bay (SF, a heavily urbanized estuary) and Tomales Bay (TB, a less developed control site). Stranded seals entered rehabilitation (TMMC) from a more extensive portion of the California coast which included the locations where seals were caught. Satellite telemetry data was used to test whether health and contaminants were associated with survival probability.

Previous investigations of harbor seal health have relied primarily on data collected from stranded animals which by definition are not healthy (ex. Colegrove et al. 2005) or surveys of wild caught animals which are generally presumed healthy based on clinical signs (for example, Kopec and Harvey 1995; Zarnke et al. 2006). This project sampled both sources of seals in an attempt to provide an integrated assessment of health and disease. The first step was to describe the blood parameters of stranded and wild-caught harbor seals. Hematology and serum chemistry intervals were generated and provide a baseline for evaluating the health status of wild-caught and stranded harbor seals of different age classes. Analysis of this dataset also provided a guide for researchers to decide whether to consider life history characteristics like age class and sex in their analyses of these blood parameters.

The disease status of harbor seals was evaluated through broad surveillance for the presence of disease causing organisms in stranded and wild-caught animals. Among the pathogens previously reported to cause disease in harbor seals (*Leptospira spp*, *Toxoplasma gondii*, *Sarcocystis neurona,* influenza virus, phocine and canine distemper virus), *Leptospira*, *Toxoplasma*, *Sarcocystis* and avian influenza virus were detected in the wild-caught harbor seals (post-weaning age classes). The stranded, pre-weaned harbor seals also exhibited antibody titers to *Leptospira*, but not to *Toxoplasma* or *Sarcocystis* (and a small number tested negative for exposure to avian influenza virus).

No wild-caught harbor seals were exposed to phocine distemper virus (n=217) although a single low antibody titer to canine distemper virus was measured in a year when coyotes were observed interacting with harbor seals. No stranded harbor seals tested positive for phocine or canine distemper.

Based on necropsy and histology data, there was no evidence for infectious causes of reproductive failure in the dead newborn pups. In particular, there was no evidence of the pathogens implicated in reproductive failure in humans and other animals (*Brucella*, *Toxoplasma,* Foster et al. 2002, Cenci-Goga et al. 2011).

Among the pathogens known to infect humans (*E. coli*, *Clostridium perfringens*, *Vibrio* spp, *Campylobacter* spp, *Salmonella*, *Giardia*, *Cryptosporidium*, avian influenza, *Brucella*, and *Leptospira* spp), all were detected in harbor seals except for *E coli* serotype 0157. The prevalence of Brucella (17%) was similar to that reported in harbor seals in the North Sea (11%, Prenger-Berninghoff et al. 2008), but lower than that reported in Alaska (46%, Zarnke et al 2006). The prevalence of *Giardia* and *Cryptosporidium* were substantially lower than has been reported on the east coast of the United States (Lasek-Nesselquist et al. 2010).

The immune system responds to invading pathogens to kill and eliminate foreign proteins and parasites that may colonize the host causing disease and ultimately death. A PHA skin test was used to assess the immune capacity of rehabilitating neonatal harbor seals. As with previous work on harbor seal immunity (Ross et al. 1994; King et al. 1994), the results of the PHA test indicated that even at a young age, they are capable of mounting an immune response. Doing so, however, requires energy (Lochmiller and Deerenberg 2000). The association between decreased PHA response and slow growth indicated that there may be a cost to developing that immune capacity.

Environmental pollutants have also been associated with marine mammal health and disease (for example, Martineau et al. 2002). Effects from these chemicals have been documented in cell cultures and laboratory animals, but few studies have evaluated the effects of exposure on animals in the wild. Experimental studies on harbor seals have documented associations between PCB contamination and decreased immune response and reproduction (Reijnders 1986, de Swart et al. 1996, Ross et al. 1996). This thesis

investigated whether naturally acquired quantities and mixtures of contaminants were correlated with a life history endpoint, survival.

First, blubber concentrations of PCBs, PBDEs, DDT and other organochlorine pesticides were evaluated in terms of route of exposure. The highest concentrations were measured in seal pups that were exposed to contaminants through milk and subsequently lost substantial amounts of mass after weaning. Patterns of contamination also varied with stranding location with greater concentrations of PCBs in seals from a more urbanized location (SF) and greater concentrations of pesticides in more agricultural location (Monterey Bay).

Next, contaminant concentrations as well as blood parameters were used as correlates in a mark-recapture model fitted to satellite telemetry data. Seal group (SF, TB and TMMC), dispersal speed, serum thyroxine, and contaminant concentration were all associated with survival. When the TMMC group of seals was considered separately, mass, thyroxine, and contaminant concentration were still important, but the amount of mass gained in rehabilitation, the number of days spent in rehabilitation, admission date (Julian day), and the amount of mass gained per day were equally important. In conclusion, harbor seals in central California are exposed to a number of infectious disease agents, growth and mass are associated with immune status and survival probability in rehabilitation, and growth (thyroxine and mass), behavior (dispersal speed) and contaminants were all related to first year survival probability.

SIGNIFICANCE and LIMITATIONS

In terms of the health of harbor seals in central California, clinical chemistry references intervals and data on disease and contaminant exposure will be useful for monitoring the health of these seals and their environment over time. The results could also be useful in the development of regional conservation and management plans for these harbor seals; for example, if the population went into decline, it might be wise to focus protection on harbor seal nursery habitat in less urbanized areas.

Human health. The prevalence of zoonotic pathogens in harbor seals is important from a veterinary public health standpoint for understanding the risks to humans working with these animals in a wild or captive setting. In a study of people working with marine

mammals, the most commonly reported health problems were bites, wounds and skin reactions, although respiratory illness and prolonged illness were also reported (Hunt et al. 2008). The presence of zoonotic pathogens like *Salmonella*, *Campylobacter*, and *Brucella* in these animals should dictate standards of hygiene and personal protection when working with diseased and healthy seals. Immune suppressed people should also be aware that these animals have the potential to be a reservoir for zoonotic pathogens like influenza viruses. Seal exposure to zoonotic pathogens associated with abortion in humans such as *Leptospira* spp and *Toxoplasma gondii* indicate that, for example, pregnant women should be especially careful when working with seals, ingesting the food that seals eat, or engaging in recreational activities near seal haulout areas.

There are already advisories in place for SF Bay anglers, particularly pregnant women and children, to limit the number of sport fish they ingest, primarily white sturgeon (*Acipenser transmontanus*) and striped bass (*Morone saxatilis*), to limit exposure to mercury. These advisories are supported by the contaminant concentrations detected in the harbor seals in this thesis as well as the effect of these contaminants on juvenile survival.

Disease. Among the infectious disease agents surveyed in this thesis, phocine distemper virus poses the greatest risk to the harbor seal population. Morbilliviruses are highly infectious and can have devastating consequences on naïve or unvaccinated populations (Di Guardo et al. 2005). In Europe where multiple PDV epizootics have been observed, mortality in harbor seals has been much greater than in grey seals (Harwood et al. 1989, Härkönen et al. 2006). One hypothesis to explain this differential susceptibility to morbilliviruses has been a differential impact of contaminants on the immune system (Hammond et al. 2005). Hammond et al (2005) found that phagocytosis, respiratory burst, and cytotoxic activity of harbor seal leukocytes incubated with PCBs were decreased compared with similarly treated grey seal leukocytes providing support to this idea of contaminant mediated immunity in harbor seals but not grey seals. In this thesis, no evidence of exposure to phocine distemper virus was detected in eight years of testing suggesting that these contaminated harbor seals would be particularly vulnerable if they encountered the virus.

Infection with *Sarcocystis neurona* has been noted previously as a cause of brain lesions in harbor seals (Miller et al. 2001; Colegrove et al. 2005) and continues to be notable as a cause of death among stranded non-pup harbor seals: of 60 seals greater than one year old stranding between 2000 and 2010, *S. neurona* was detected in 10% and was a cause of death in 7% (TMMC, unpublished data). How this relates to the 9% prevalence reported among wild-caught harbor seals in this thesis is not clear.

The role of disease in two harbor seal die-offs in central California in 1997 and 2000 (Allen et al 2004) is still unresolved and harbor seals are likely susceptible to toxins or pathogens that have not yet been detected or described. For example, three previously unknown protozoal parasites have recently been described in California sea lions with at least one of them capable of infecting and causing disease in harbor seals (Colegrove et al. 2011).

Stranded harbor seals remain the most useful group of animals for disease detection, but are not ideal for estimating disease prevalence because of the low number of older age classes that strand. Live stranded animals present challenges because, in a hospital setting, variables cannot always be controlled for in the same way they can in an experimental setting.

Mortality and survival. While this thesis provides information on mortality among newborn harbor seals admitted to rehabilitation, there is very little information on estimates and causes of pre-weaning mortality in the wild and this thesis was not able to evaluate whether contaminant levels were associated with death in the newborn pups. To compare the contaminant concentrations of seals that stranded dead to those that survived rehabilitation to release, live stranded pups would need to be sampled at the time of admission rather than release or wild-caught pups would need to be sampled soon after birth. But, given the results from Chapter 3, these underweight pups would likely not benefit from the additional procedure with current methods. Nor is it practical to sample a comparison group of healthy newborn harbor seal pups in the wild because of concerns that the bond between mother and pup could be disrupted. Linking contaminants and mortality remains difficult unless direct associations between contaminant exposure and lesions observed at necropsy can be drawn. And even when severe developmental abnormalities are observed in dead seals, it is not always possible

to draw conclusions because the timing of contaminant exposure is not known (Harris et al. 2011).

In terms of post-weaning mortality, the single carcass recovered from the satellite telemetry study points to human activity (boat strike) as a cause of mortality. Two rehabilitated animals that re-stranded post-release suggest that malnutrition and parasitism are likely additional significant contributors to mortality. Causes of morbidity and mortality in adult harbor seals remain unknown as so few strand. This may be because death occurs quickly or far from shore (such as shark predation or fishing interactions) or may be a function of behavior such that sick seals do not come ashore in populated or accessible locations like California sea lions do. For example, domoic acid toxicity has been diagnosed in harbor seals (TMMC, unpublished data), but large numbers of them do not come ashore with neurological symptoms as occurs with California sea lions (Goldstein et al. 2008). Harbor seals may forage in different locations than sea lions limiting their exposure to domoic acid, they may succumb to domoic acid toxicity before they can get to shore, or they simply may not come ashore when affected.

As has been found in studies of grey seals (Hall et al. 2001; Hall et al. 2002), increased growth and body size was associated with higher post-weaning survival probabilities. And as with grey seals, there was an association between blubber contaminant concentration and survival probability (Hall et al. 2009). Contaminants are likely to be of ongoing concern for the harbor seal population as concentrations of some of the legacy contaminants such as DDT were as elevated in this study's harbor seals as they were in the 1970s when DDT was manufactured and used in the United States (Risebrough et al. 1980).

FUTURE DIRECTIONS

This thesis provides a baseline from which to develop future studies of factors affecting harbor seal mortality and survival. The broad survey of known infectious agents used in this thesis is useful for ruling out known pathogens as causes of mortality, but it is not optimal for determining the cause of a harbor seal die off as samples have to be sent to multiple laboratories for multiple tests. With ongoing development of tools to detect a wide range of pathogens at once, such as oligonucleotide microarrays and multiplex PCR (Wang et al. 2008; Liang et al. 2010), it may become easier to investigate the causes of die-offs with limited samples, like the two that occurred at Point Reyes National Seashore in 1997 and 2000.

Recent reports of morbillivirus exposure in sea otters in Alaska (Goldstein et al. 2009) and Washington (Brancato et al. 2009) are concerning. The harbor seals tested in this thesis did not exhibit exposure to morbilliviruses which implies that harbor seals in California are not immune and are at risk for infection. If morbilliviruses are circulating in the Pacific, this presents a management and conservation challenge for harbor seals and for the endangered Hawaiian monk seal which has also tested negative for morbillivirus antibodies (Littnan et al. 2007). To evaluate the risk to these species, however, more work needs to be done to evaluate this family of viruses in the marine environment and to validate the tests used to detect morbillivirus antibodies for different species. For example, in the serum neutralization test, it is possible that some compound in sea otter serum other than antibodies to PDV and CDV is cross reacting with the PDV and CDV antigens. It is also possible that exposure to an undescribed morbillivirus circulating in the Pacific protects harbor seals and monk seals against infection with PDV or CDV.

Another pathogen which may be interacting with multiple marine mammal species is *Leptospira* spp. for which extensive work has been conducted in Californa sea lions (Gulland et al. 1996, Acevedo-Whitehouse et al. 2003a; Lloyd-Smith et al. 2007). The high prevalences reported in harbor seals in this thesis coincide with an outbreak year among the sea lions. The disease dynamics and reservoir hosts of *Leptospira* spp in the marine environment may be quite different than the terrestrial environment where rodents typically act as a reservoir and disease vector (Ko et al. 2009). Culturing and

identifying *Leptospira* spp strains from marine mammals that are sympatric with California sea lions would be the first step to determining whether different species are involved in *Leptospira* spp transmission dynamics.

There are still basic questions about recruitment age and survival to reproductive age that are not known for harbor seals in California. The dispersal patterns observed in this thesis differ from populations in Alaska (Small et al. 2005) and Scotland (Thompson et al. 1994). Seal pups in California travelled further and showed no tendency to return to their natal beaches within the eight months that their tags transmitted. This is a conservation issue in terms of marine protection areas, but also in terms of infection risk. Harbor seals in Oregon are considered a different stock based on genetics (Lamont et al. 1996), but the movements of these pups from California suggest that they could exposed to infected seals in Oregon even if they do not breed with them.

Enhancing the survival of neonatal harbor seals in the rehabilitation setting continues to present challenges to clinicians and rehabilitators. Many pups that ultimately die in treatment fail to gain mass during rehabilitation. The PHA response documented in rehabilitating seals highlights the interaction between the immune response and growth. Other processes that may interfere with growth include stress and genetics. The stress of being in a captive setting may initially suppress the immune response and ultimately lead to adrenal exhaustion and insufficiency (Dierauf et al. 1986). Genetic measures of inbreeding have also been associated with neonatal survival in harbor seals (Coltman et al. 1998), and it would be useful to devise a way to calculate a probability of survival that incorporates all these factors early in the rehabilitation process.

A larger sample size would have improved the telemetry study in this thesis, but in addition to more reliable capture techniques, there are other challenges such as how to mark these animals for longer term study as the annual molt currently limits the duration of tags that are glued to the fur. In addition, monitoring the survival of known age individuals to reproduction will require the development of a technique that is more economical than satellite telemetry because it is a big investment to put tags on a large number of pups when few of them survive their first year. Ultimately, a simple, inexpensive, non-invasive, long lasting marking technique is needed. Possibilities include brands or tags implanted under the skin or blubber, but current size and

deployment methods for these tags may not be appropriate for recently weaned pups about to enter the vulnerable post-weaning stage of life.

In future studies of the factors affecting post-weaning survival, it would be interesting to investigate inter annual variability in survival and to monitor whether thyroxine (T4) levels increase as concentrations of legacy contaminants in the population decrease. Meanwhile, the effect of emerging contaminants (for example perfluorinated chemicals and non brominated flame retardants) on survival probability should also be investigated. Future studies might also combine telemetry and disease investigation to model epidemiological parameters such as encounter rates between individuals and the possible spread of infection as is being attempted with avian species (Gaidet et al. 2010).

In addition, more information is needed for harbor seals in California on direct human impacts that have the potential to affect harbor seal survival such as disturbance, ocean noise, boat traffic, and fishing interactions as well as other correlates of survival that were not investigated as part of this thesis like El Ninos, and climate change. Premature harbor seal carcasses should continue to receive full necropsies to investigate causes of reproductive failure in harbor seals. The relationship between newborn pups that strand and enter rehabilitation and natural mortality is not clear, and future studies should investigate levels and causes of pre-weaning mortality in the wild.

LITERATURE CITED

Abu-Asab, M., Chaouchi, M. and Amri, H. (2008). Evolutionary medicine: A meaningful connection between omics, disease, and treatment. PROTEOMICS - Clinical Applications 2(2): 122-134.

Acevedo-Whitehouse, K., de la Cueva, H., Gulland, F.M., Aurioles-Gamboa, D., Arellano-Carbajal, F. and Suarez-Guemes, F. (2003a). Evidence of *Leptospira interrogans* infection in California sea lion pups from the Gulf of California. Journal of Wildlife Diseases 39(1): 145-151.

Acevedo-Whitehouse, K., Gulland, F., Greig, D. and Amos, W. (2003b). Inbreeding: Disease susceptibility in California sea lions. Nature 422(6927): 35.

Addison, R.F., Muir, D.C., Ikonomou, M.G., Harwood, L. and Smith, T.G. (2009). Hexachlorocyclohexanes (HCH) in ringed seal (*Phoca hispida*) from Ulukhaktok (Holman), NT: trends from 1978 to 2006. Science of the Total Environment 407(18): 5139-46.

Aldridge, B.M., King, D.P., Kennedy-Stoskopf, S. and Stott, J.L. (2001). Immunology. CRC Handbook of marine mammal medicine. L. A. Dierauf and F. M. D. Gulland. Boca Raton, FL, CRC Press**:** 237-252.

Allen, S.G., Ainley, D.G., Fancher, L.E. and Shuford, D. (1987). Movement and activity patterns of harbor seals (*Phoca vitulina*) from the Drakes Estero population, California, 1985-86. Gulf of the Farallones Natinal Marine Sanctuary. Final report to Sanctuary programs office, NOAA NOAA Technical Memorandum NOS MEMD 6: 36pp.

Allen, S.G., Huber, H.R., Ribic, C.A. and Ainley, D.G. (1989). Population dynamics of harbor seals in the Gulf of the Farallones, California. California Fish and Game 75(4): 224-232.

Allen, S.G. (1991). Harbor Seal Habitat Restoration at Strawberry Spit, San Francisco Bay. Marine Mammal Commission.

Allen, S., Waber, S., Holter, W. and Press, D. (2004). Long-term monitoring of harbor seals at Point Reyes, California, 5-year annual report 1997-2001: 1-66.

Antonelis, G.A., Baker, J.D., Johanos, T.C., Braun, R.C. and Harting, A.L. (2006). Hawaiian monk seal (*Monachus schauinslandi*): status and conservation issues. Atoll Research Bulletin 543: 75-101.

Baker, J.D. and Thompson, P.M. (2007). Temporal and spatial variation in age-specific survival rates of a long-lived mammal, the Hawaiian monk seal. Proceedings of the Royal Society B: Biological Sciences 274(1608): 407-415.

Baker, J.R., Hall, A., Hiby, L., Munro, R., Robinson, I., Ross, H.M. and Watkins, J.F. (1995). Isolation of salmonellae from seals from UK waters. Veterinary Record 136: 471-472.

Baudart, J., Lemarchand, K., Brisabois, A. and Lebaron, P. (2000). Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer regions. Applied and Environmental Microbiology 66(4): 1544-52.

Bayyari, G.R., Huff, W.E., Rath, N.C., Balog, J.M., Newberry, L.A., Villines, J.D., Skeeles, J.K., Anthony, N.B. and Nestor, K.E. (1997). Effect of the genetic selection of turkeys for increased body weight and egg production on immune and physiological responses. Poultry Science 76(2): 289-296.

Bennett, K.A., Speakman, J.R., Moss, S.E., Pomeroy, P. and Fedak, M.A. (2007). Effects of mass and body composition on fasting fuel utilisation in grey seal pups (*Halichoerus grypus* Fabricius): an experimental study using supplementary feeding. Journal of Experimental Biology 210(Pt 17): 3043-53.

Betts, K.S. (2008). New thinking on flame retardants. Environmental Health Perspectives 116(5): A210-A213.

Bigg, M.A. (1969). The harbor seal in British Columbia. Bulletin of the Fisheries Research Board of Canada 172: 33.

Black, D.M., Gilardi, K.V.K., Hamilton, L.P., Williams, E., Williams, D.F., Kelly, P.A. and Gardner, I. (2009). Hematologic and biochemistry reference values for the endangered riparian brush rabbit (*Sylvilagus bachmani riparius*). Journal of Wildlife Diseases 45(2): 491-496.

Blake, P.A., Weaver, R.E. and Hollis, D.G. (1980). Diseases of humans (other than cholera) caused by vibrios. Annual Review of Microbiology 34(1): 341-367.

Bonnot, P. (1928). Report on the seals and sea lions in California. California Fish and Game, Fisheries Bulletin 14: 61 pp.

Bossart, G.D., Reidarson, T.H., Dierauf, L.A. and Duffield, D.A. (2001). Clinical pathology. CRC Handbook of Marine Mammal Medicine. L. A. Dierauf and F. M. D. Gulland. Boca Raton, FL, CRC Press**:** 383-436.

Boulva, J. and McLaren, I.A. (1979). Biology of the harbor seal, *Phoca vitulina*, in Eastern Canada. Bulletin of the Fisheries Research Board of Canada 200: 1-24.

Bowen, W.D., Iverson, S.J., Boness, D.J. and Oftedal, O.T. (2001). Foraging effort, food intake and lactation performance depend on maternal mass in a small phocid seal. Functional Ecology 15(3): 325-334.

Brancato, M.S., Milonas, L., Bowlby, C.E., Jameson, R., Davis, J.W. (2009). Chemical contaminants, pathogen exposure and general health status of live and beach-cast Washington sea otters (*Enhydra lutris kenyoni*): NOAA Marine Sanctuaries Conservation Series ONMS-09-01; 189pp.

Brew, S.D., Perrett, L.L., Stack, J.A., MacMillan, A.P. and Staunton, N.J. (1999). Human exposure to *Brucella* recovered from a sea mammal. Veterinary Record 144(17): 483.

Brookens, T., Harvey, J. and O'Hara, T. (2007). Trace element concentrations in the Pacific harbor seal (*Phoca vitulina richardii*) in central and northern California. Science of the Total Environment 372: 676-692.

Brouwer, A., Reijnders, P.J.H. and Koeman, J.H. (1989). Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). Aquatic Toxicology 15(1): 99-105.

Brown, J.D., Stallknecht, D.E., Berghaus, R.D., Luttrell, M.P., Velek, K., Kistler, W., Costa, T., Yabsley, M.J. and Swayne, D. (2009). Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. Clinical and Vaccine Immunology 16(6): 824-9.

Buckles, E.L., Gulland, F.M., Aldridge, B.M., Gelatt, T.S., Ross, P.S., Haulena, M. and Lowenstine, L.J. (2006). Fetus in fetu in a harbor seal (*Phoca vitulina richardi*): histopathologic, genetic, and toxicologic analysis. Veterinary Pathology 43(4): 541-4.

Buckles, E.L., Lowenstine, L.J., Delong, R.L., Melin, S.R., Vittore, R.K., Wong, H.N., Ross, G.L., St Leger, J.A., Greig, D.J., Duerr, R.S., Gulland, F.M. and Stott, J.L. (2007). Age-prevalence of Otarine Herpesvirus-1, a tumor-associated virus, and possibility of its sexual transmission in California sea lions. Veterinary Microbiology 120(1-2): 1-8.

Burnham, K.P. and Anderson, D.R. (2002). Model selection and multimodel inference: a practical information-theoretic approach. 2nd edition. New York, NY, Springer: 488 pp.

Byrd, W.J., Von Boehmer, H. and Rouse, B.T. (1973). The role of the thymus in maturational development of phytohemagglutinin and pokeweed mitogen responsiveness. Cellular Immunology 6(1): 12-24.

Callan, R.J., Early, G., Kida, H. and Hinshaw, V.S. (1995). The appearance of H3 influenza viruses in seals. Journal of General Virology 76(1): 199-203.

Carretta, J.V., Forney, K.A., Lowry, M.S., Barlow, J., Baker, J., Hanson, B. and Muto, M.M. (2007). U.S. Pacific marine mammal stock assessments: 2007: 316 pp.

Carter, S.D., Hughes, D.E. and Baker, J.R. (1990). Characterization and measurement of immunoglobulins in the grey seal (*Halichoerus grypus*). Journal of Comparative Pathology 102(1): 13-23.

Cenci-Goga, B.T., Rossitto, P.V., Sechi, P., McCrindle, C.M. and Cullor, J.S. (2011). Toxoplasma in animals, food, and humans: an old parasite of new concern. Foodborne Pathogens and Disease 8(7): 1-12.

Ciacci-Zanella, J.R., Vincent, A.L., Prickett, J.R., Zimmerman, S.M. and Zimmerman, J.J. (2010). Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. Journal of Veterinary Diagnostic Investigation 22(1): 3-9.

Clark, C.A., Burns, J.M., Schreer, J.F. and Hammill, M.O. (2007). A longitudinal and cross-sectional analysis of total body oxygen store development in nursing harbor seals (*Phoca vitulina*). Journal of Comparative Physiology B 177: 217-227.

Cloeckaert, A., Verger, J.-M., Grayon, M., Paquet, J.-Y., Garin-Bastuji, B., Foster, G. and Godfroid, J. (2001). Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. Microbes and Infection 3(9): 729-738.

CLS. (2008). "Argos User's Manual." from http://www.argos-system.org/manual/.

Clutton-Brock, T.H., Major, M., Albon, S.D. and Guinness, F.E. (1987). Early development and population dynamics in red deer. I. Density-dependent effects on juvenile survival. Journal of Animal Ecology 56: 53-67.

Colagross-Schouten, A.M., Mazet, J.A., Gulland, F.M., Miller, M.A. and Hietala, S. (2002). Diagnosis and seroprevalence of leptospirosis in California sea lions from coastal California. Journal of Wildlife Diseases 38(1): 7-17.

Cole Jr, J.R., Sulzer, C.R. and Pursell, A.R. (1973). Improved microtechnique for the leptospiral microscopic agglutination test. Applied Microbiology 25(6): 976-980.

Colegrove, K.M., Greig, D.J. and Gulland, F.M.D. (2005). Causes of Live Strandings of Northern Elephant Seals (*Mirounga angustirostris*) and Pacific Harbor Seals (*Phoca vitulina*) Along the Central California Coast, 1992-2001. Aquatic Mammals 31(1): 1-10.

Colegrove, K., Grigg, M.E., Carlson-Bremer, D., Miller, R.H., Gulland, F.M., Ferguson, D.J., Rejmanek, D., Barr, B.C., Nordhausen, R., Melli, A.C. and Conrad, P.A. (2011). Discovery of Three Novel Coccidian Parasites Infecting California Sea Lions (*Zalophus Californianus*) with Evidence of Sexual Replication and Interspecies Pathogenicity. Journal of Parasitology.

Coltman, D.W., Bowen, W.D. and Wright, J.M. (1998). Birth weight and neonatal survival of harbour seal pups are positively correlated with genetic variation measured by microsatellites. Proceedings of the Royal Society B: 265(1398): 803-9.

Conomos, T.J., Smith, R.E. and Gartner, J.W. (1985). Environmental setting of San Francisco Bay. Hydrobiologia 129(1): 1-12.

Conrad, P.A., Miller, M.A., Kreuder, C., James, E.R., Mazet, J., Dabritz, H., Jessup, D.A., Gulland, F. and Grigg, M.E. (2005). Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. International Journal for Parasitology 35(11-12): 1155-68.

Cooch, E. and White, G. (2009). Program MARK "A Gentle Introduction", http://www.phidot.org/software/mark/docs/book/.

Cottrell, P.E., Jeffries, S., Beck, B. and Ross, P.S. (2002). Growth and development in free-ranging harbour seal (*Phoca vitulina*) pups from southern British Columbia, Canada. Marine Mammal Science 18(3): 721-733.

Craig, M.P. and Ragen, T.J. (1999). Body size, survival, and decline of juvenile Hawaiian monk seals, *Monachus schauinslandi*. Marine Mammal Science 15(3): 786-809.

Current, W.L. and Garcia, L.S. (1991). Cryptosporidiosis. Clinical Microbiology Reviews 4(3): 325-58.

De Guise, S., Martineau, D., Beland, P. and Fournier, M. (1995). Possible mechanisms of action of environmental contaminants on St. Lawrence beluga whales (*Delphinapterus leucas*). Environmental Health Perspectives 103 Suppl 4: 73-7.

De Guise, S., Martineau, D., Béland, P. and Fournier, M. (1998). Effects of in vitro exposure of beluga whale leukocytes to selected organochlorines. Journal of Toxicology and Environmental Health A 55(7): 479-93.

De Guise, S., Mori, C., Levin, M., Trailsmith, M., Morsey, B., Leibrecht, H. and Dunn, L.J. (2006). Diagnostic and prognostic tools in stranded marine mammal. IAAAM 37th Annual Conference Proceedings, Nassau, Bahamas.

de la Riva, G.T., Johnson, C.K., Gulland, F.M., Langlois, G.W., Heyning, J.E., Rowles, T.K. and Mazet, J.A. (2009). Association of an unusual marine mammal mortality event with *Pseudo-nitzschia* spp. Blooms along the southern California coastline. Journal of Wildlife Diseases 45(1): 109-21.

de Swart, R.L., Kluten, R.M.G., Huizing, C.J., Vedder, L.J., Reijnders, P.J.H., Visser, I.K.G., UytdeHaag, F.G.C.M. and Osterhaus, A.D.M.E. (1993). Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (*Phoca vitulina*). Veterinary Immunology and Immunopathology 37(3-4): 217-230.

de Swart, R.L., Ross, P.S., Timmerman, H.H., Hijman, W.C., de Ruiter, E.M., Liem, A.K.D., Brouwer, A., van Loveren, H., Reijnders, P.J.H., Vos, J.G. and Osterhaus, A.D.M.E. (1995a). Short term fasting does not aggravate immunosuppression in harbour seals (*Phoca vitulina*) with high body burdens of organochlorines. Chemosphere 31(10): 4289-4306.

de Swart, R.L., Ross, P.S., Vedder, L.J., Boink, F.B.T.J., Reijnders, P.J.H., Mulder, P.G.H. and Osterhaus, A.D.M.E. (1995b). Haematology and clinical chemistry values for harbour seals (*Phoca vitulina*) fed environmentally contaminated herring remain within normal ranges. Canadian Journal of Zoology 73(11): 2035-2043.

de Swart, R.L., Ross, P.S., Vos, J.G. and Osterhaus, A.D. (1996). Impaired immunity in harbour seals (*Phoca vitulina*) exposed to bioaccumulated environmental contaminants: review of a long-term feeding study. Environmental Health Perspectives 104(Supplement 4): 823-8.

Debier, C., Pomeroy, P.P., Dupont, C., Joiris, C., Comblin, V., Le Boulengé, E., Larondelle, Y. and Thomé, J.-P. (2003a). Dynamics of PCB transfer from mother to pup during lactation in UK grey seals *Halichoerus grypus*: differences in PCB profile between compartments of transfer and changes during the lactation period. Marine Ecology Progress Series 247: 249-256.

Debier, C., Pomeroy, P.P., Dupont, C., Joiris, C., Comblin, V., Le Boulengé, E., Larondelle, Y. and Thomé, J.-P. (2003b). Quantitative dynamics of PCB transfer from mother to pup during lactation in UK grey seals *Halichoerus grypus*. Marine Ecology Progress Series 247: 237-248.

Debier, C., Chalon, C., Le Boeuf, B.J., de Tillesse, T., Larondelle, Y. and Thomé, J.P. (2006). Mobilization of PCBs from blubber to blood in northern elephant seals (*Mirounga angustirostris*) during the post-weaning fast. Aquatic Toxicology 80(2): 149-57.

Deng, M.-Q., Peterson, R.P. and Cliver, D.O. (2000). First Findings of *Cryptosporidium* and *Giardia* in California Sea Lions (*Zalophus californianus*). The Journal of Parasitology 86(3): 490-494.

Dierauf, L.A., Dougherty, S.A. and Baker, B. (1984). Neonatal hyperbilirubinemia in harbor seals (*Phoca vitulina ricardsi*). Journal of Zoo Animal Medicine 15: 55-59.

Dierauf, L.A., Dougherty, S.A. and Lowenstine, L.J. (1986). Survival versus nonsurvival determinants for neonatal harbor seals. Journal of the American Veterinary Medical Association 189(9): 1024-8.

Di Guardo, G., Marruchella, G., Agrimi, U. and Kennedy, S. (2005). Morbillivirus infections in aquatic mammals: a brief overview. Journal of Veterinary Medicine A 52(2): 88-93.

Dubey, J.P., Rosypal, A.C., Rosenthal, B.M., Thomas, N.J., Lindsay, D.S., Stanek, J.F., Reed, S.M. and Saville, W.J.A. (2001). *Sarcocystis neurona* Infections in Sea Otter (*Enhydra lutris*): Evidence for Natural Infections with Sarcocysts and Transmission of Infection to Opossums (*Didelphis virginiana*). The Journal of Parasitology 87(6): 1387-1393.

Dubey, J.P., Zarnke, R., Thomas, N.J., Wong, S.K., Bonn, W.V., Briggs, M., Davis, J.W., Ewing, R., Mense, M., Kwok, O.C.H., Romand, S. and Thulliez, P. (2003). *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. Veterinary Parasitology 116(4): 275-296.

Duignan, P., Saliki, J., StAubin, D., Early, G., Sadove, S., House, J., Kovacs, K. and Geraci, J.R. (1995). Epizootiology of morbillivirus infection in North American harbor seals (*Phoca vitulina*) and gray seals (*Halichoerus grypus*). Journal of Wildlife Diseases 31(4): 491-501.

Dunn, L.J., Buck, J.D. and Robeck, T.R. (2001). Bacterial diseases of cetaceans and pinnipeds. CRC Handbook of marine mammal medicine. L. A. Dierauf and F. M. D. Gulland. Boca Raton, FL, CRC Press**:** 309-335.

Eguchi, T. (1998). Morphology of the pacific harbor seal (*Phoca vitulina richardsi*) using Elkhorn Slough, CA, and their movements and diving behavior in the Monterey Bay area. Marine Sciences, California State University, Fresno. MS: 1-105.

Ellis, S., Bowen, W., Boness, D. and Iverson, S. (2000). Maternal effects on offspring mass and stage of development at birth in the harbor seal, *Phoca vitulina*. Journal of Mammalogy 81(4): 1143-1156.

Elson-Riggins, J.G., Al-Banna, L., Platzer, E.G. and Kaloshian, I. (2001). Characterization of *Otostrongylus circumlitus* from Pacific Harbor and Northern Elephant Seals. The Journal of Parasitology 87(1): 73-78.

Elson-Riggins, J.G., Riggins, S.A., Gulland, F.M. and Platzer, E.G. (2004). Immunoglobulin responses of northern elephant and Pacific harbor seals naturally infected with *Otostrongylus circumlitus*. Journal of Wildlife Diseases 40(3): 466-75.

Fauquier, D.A., Mazet, J.A.K., Gulland, F.M.D., Spraker, T.R. and Christopher, M.M. (2008). Distribution of tissue enzymes in three species of pinnipeds. Journal of Zoo and Wildlife Medicine 39(1): 1-5.

Fayer, R., Dubey, J.P. and Lindsay, D.S. (2004). Zoonotic protozoa: from land to sea. Trends in Parasitology 20(11): 531-6.

Fernández-de-Mera, I.G., Höfle, U., Vicente, J., Garcia, A., Rodriguez, O. and Gortázar, C. (2006). Optimal dose and timing in phytohaemagglutinin skin-testing of deer. New Zealand Veterinary Journal 54(6): 357-9.

Flynn, E., Press, D., Codde, S., Roberts, D. and Allen, S. (2009). Pacific Harbor Seal (*Phoca vitulina richardsi*) monitoring at Point Reyes National Seashore and Golden Gate National Recreation Area: 2008 Annual Report, NPS/SFAN/NRTR-2009/267: 1-26.

Fonfara, S., Kakuschke, A., Rosenberger, T., Siebert, U. and Prange, A. (2008). Cytokine and acute phase protein expression in blood samples of harbour seal pups. Marine Biology 155(3): 337-345.

Foster, G., MacMillan, A.P., Godfroid, J., Howie, F., Ross, H.M., Cloeckaert, A., Reid, R.J., Brew, S. and Patterson, I.A. (2002). A review of *Brucella* sp. infection of sea mammals with particular emphasis on isolates from Scotland. Veterinary Microbiology 90(1-4): 563-80.

Fraser, C.G. (2004). Inherent biological variation and reference values. Clinical Chemistry and Laboratory Medicine 42(7): 758-764.

Funke, C., King, D.P., Brotheridge, R.M., Adelung, D. and Stott, J.L. (1997). Harbor seal (*Phoca vitulina*) C-reactive protein (C-RP): purification, characterization of specific monoclonal antibodies and development of an immuno-assay to measure serum C-RP concentrations. Veterinary Immunology and Immunopathology 59(1-2): 151-162.

Gaidet, N., Cappelle, J., Takekawa, J.Y., Prosser, D.J., Iverson, S.A., Douglas, D.C., Perry, W.M., Mundkur, T. and Newman, S.H. (2010). Potential spread of highly pathogenic avian influenza H5N1 by wildfowl: dispersal ranges and rates

determined from large-scale satellite telemetry. Journal of Applied Ecology 47: 1147-1157.

Garcia, L.S., Shum, A.C. and Bruckner, D.A. (1992). Evaluation of a new monoclonal antibody combination reagent for direct fluorescence detection of *Giardia* cysts and *Cryptosporidium* oocysts in human fecal specimens. Journal of Clinical Microbiology 30(12): 3255-7.

Garner, M.M., Lambourn, D.M., Jeffries, S.J., Hall, P.B., Rhyan, J.C., Ewalt, D.R., Polzin, L.M. and Cheville, N.F. (1997). Evidence of *Brucella* infection in Parafilaroides lungworms in a Pacific harbor seal (*Phoca vitulina richardsi*). Journal of Veterinary Diagnostic Investigation 9(3): 298-303.

Gaydos, J.K., Miller, W.A., Gilardi, K.V., Melli, A., Schwantje, H., Engelstoft, C., Fritz, H. and Conrad, P.A. (2007). *Cryptosporidium* and *Giardia* in marine-foraging river otters (*Lontra canadensis*) from the Puget Sound Georgia Basin ecosystem. Journal of Parasitology 93(1): 198-202.

Gaydos, J.K., Miller, W.A., Johnson, C., Zornetzer, H., Melli, A., Packham, A., Jeffries, S.J., Lance, M.M. and Conrad, P.A. (2008). Novel and Canine Genotypes of *Giardia duodenalis* in Harbor Seals (*Phoca vitulina richardsi*). Journal of Parasitology 94(6): 1264-1268.

Geffré, A., Friedrichs, K., Harr, K., Concordet, D., Trumel, C. and Braun, J.P. (2009). Reference values: a review. Veterinary Clinical Pathology 38(3): 288-98.

Geraci, J.R., Aubin, D.J.S., Barker, I.K., Webster, R.G., Hinshaw, V.S., Bean, W.J., Ruhnke, H.L., Prescott, J.H., Early, G., Baker, A.S., Madoff, S. and Schooley, R.T. (1982). Mass Mortality of Harbor Seals: Pneumonia Associated with Influenza A Virus. Science 215(4536): 1129-1131.

Gibbens, J. and Arnould, J.P.Y. (2009). Interannual variation in pup production and the timing of breeding in benthic foraging Australian fur seals. Marine Mammal Science 25(3): 573-587.

Gilmartin, W.G., Vainik, P.M. and Neill, V.M. (1979). Salmonellae in feral pinnipeds off the Southern California coast. Journal of Wildlife Diseases 15(4): 511-514.

Gilmartin, W.G., Johanos, T.C. and Eberhardt, L.L. (1993). Survival rates for the Hawaiian monk seal (*Monachus schauinslandi*). Marine Mammal Science 9(4): 407-420.

Goldberg, E.D. (1991). Halogenated hydrocarbons: Past, present and near-future problems. The Science of The Total Environment 100: 17-28.

Goldenberg, R.L. and Culhane, J.F. (2007). Low birth weight in the United States. American Journal of Clinical Nutrition 85(2): 584S-590S.

Goldsby, R.A., Kindt, T.J., Osborne, B.A. and Kuby, J. (2003). Immunology. New York, W.H. Freeman and Company.

Goldstein, T., Gulland, F.M., Aldridge, B.M., Harvey, J.T., Rowles, T., Lambourn, D.M., Jeffries, S.J., Measures, L., Yochem, P.K., Stewart, B.S., Small, R.J., King, D.P., Stott, J.L. and Mazet, J.A. (2003). Antibodies to phocine herpesvirus-1 are common in North American harbor seals (*Phoca vitulina*). Journal of Wildlife Diseases 39(3): 487-94.

Goldstein, T., Mazet, J.A., Gulland, F.M., Rowles, T., Harvey, J.T., Allen, S.G., King, D.P., Aldridge, B.M. and Stott, J.L. (2004). The transmission of phocine herpesvirus-1 in rehabilitating and free-ranging Pacific harbor seals (*Phoca vitulina*) in California. Veterinary Microbiology 103(3-4): 131-41.

Goldstein, T., Mazet, J.A., Lowenstine, L.J., Gulland, F.M., Rowles, T.K., King, D.P., Aldridge, B.M. and Stott, J.L. (2005). Tissue distribution of phocine herpesvirus-1 (PhHV-1) in infected harbour seals (*Phoca vitulina*) from the central Californian coast and a comparison of diagnostic methods. Journal of Comparative Pathology 133(2-3): 175-83.

Goldstein, T., Mazet, J.A.K., Gill, V.A., Doroff, A.M., Burek, K.A. and Hammond, J.A. (2009). Phocine distemper virus in northern sea otters in the Pacific Ocean, Alaska, USA. Emerging Infectious Diseases 15(6): 925-927.

Goüy de Bellocq, J., Porcherie, A., Moulia, C. and Morand, S. (2007). Immunocompetence does not correlate with resistance to helminth parasites in house mouse subspecies and their hybrids. Parasitology Research 100(2): 321-328.

Green, D.E., Grigg, E., Allen, S. and Markowitz, H. (2006). Monitoring the potential impact of the seismic retrofit construction activities at the Richmond San Rafael Bridge on harbor seals (*Phoca vitulina*): May 1, 1998-September 15, 2005, Richmond Bridge Harbor Seal Survey: 99 pp.

Greig, D.J. (2002). Pregnancy and parturition rates of harbor seals in Monterey Bay, CA. Biological Sciences, Moss Landing Marine Laboratories and San Jose State University. MS: 1-68.

Greig, D.J., Gulland, F.M.D., Rios, C.A. and Hall, A.J. (2010). Hematology and serum chemistry in stranded and wild-caught harbor seals in central California: reference intervals, predictors of survival, and parameters affecting blood variables. Journal of Wildlife Diseases 46(4): 1172-1184.

Grigg, E.K. (2003). Pacific harbor seals (*Phoca vitulina richardii*) in San Francisco Bay, California: a review of the literature, San Francisco Estuary Institute: 1-24.

Grigg, E.K., Allen, S.G., Green, D.E. and Markowitz, H. (2004). Harbor seal, *Phoca vitulina richardii*, population trends in the San Francisco Bay estuary, 1970-2002. California Fish and Game 90(2): 51-70.

Grigg, E.K. (2008). Environmental predictors of habitat use patterns of Pacific harbor seals (*Phoca vitulina richardii*) in an urbanized estuary. University of California, Davis. PhD: 1-112.

Gulland, F.M.D., Koski, M., Lowenstine, L.J., Colagross, A., Morgan, L. and Spraker, T. (1996). Leptospirosis in California sea lions (*Zalophus californianus*) stranded along the central California coast, 1981-1994. Journal of Wildlife Diseases 32(4): 572-580.

Gulland, F.M., Lowenstine, L.J., Lapointe, J.M., Spraker, T. and King, D.P. (1997). Herpesvirus infection in stranded Pacific harbor seals of coastal California. Journal of Wildlife Diseases 33(3): 450-8.

Gulland, F.M.D. and Hall, A.J. (2007). Is marine mammal health deteriorating? Trends in the global reporting of marine mammal disease. EcoHealth 4: 135-150.

Hadley, G.L., Rotella, J.J. and Garrott, R.A. (2007). Influence of maternal characteristics and oceanographic conditions on survival and recruitment probabilities of Weddell seals. Oikos 116: 601-613.

Hall, A.J., Law, R.J., Wells, D.E., Harwood, J., Ross, H.M., Kennedy, S., Allchin, C.R., Campbell, L.A. and Pomeroy, P.P. (1992). Organochlorine levels in common seals (*Phoca vitulina*) which were victims and survivors of the 1988 phocine distemper epizootic. Science of the Total Environment 115(1-2): 145-162.

Hall, A.J. (1998a). Blood chemistry and hematology of gray seal (*Halichoerus grypus*) pups from birth to postweaning. Journal of Zoo and Wildlife Medicine 29(4): 401-407.

Hall, A.J., Green, N.J.L., Jones, K.C., Pomeroy, P.P. and Harwood, J. (1998b). Thyroid hormones as biomarkers in grey seals. Marine Pollution Bulletin 36(6): 424-428.

Hall, A.J., Licence, S.T. and Pomeroy, P.P. (1999). The response of grey seal pups to intradermal phytohaemagglutinin injection. Aquatic Mammals 25.1: 25-30.

Hall, A.J., McConnell, B.J. and Barker, R.J. (2001). Factors affecting first-year survival in grey seals and their implications for life history strategy. Journal of Animal Ecology 70: 138-149.

Hall, A.J., McConnell, B.J. and Barker, R.J. (2002). The effect of total immunoglobulin levels, mass and condition on the first-year survival of grey seal pups. Functional Ecology 16: 462-474.

Hall, A.J., Kalantzi, O.I. and Thomas, G.O. (2003). Polybrominated diphenyl ethers (PBDEs) in grey seals during their first year of life--are they thyroid hormone endocrine disrupters? Environmental Pollution 126(1): 29-37.

Hall, A.J., Hugunin, K., Deaville, R., Law, R.J., Allchin, C.R. and Jepson, P.D. (2006). The risk of infections from polychlorinated biphenyl exposure in the harbor porpoise (*Phocoena phocoena*): A case-control approach. Environmental Health Perspectives 114(5): 704-711.

Hall, A.J. and McConnell, B.J. (2007). Measuring changes in juvenile gray seal body composition. Marine Mammal Science 23(3): 650-665.

Hall, A.J. and Thomas, G.O. (2007). Polychlorinated biphenyls, DDT, polybrominated diphenyl ethers, and organic pesticides in United Kingdom harbor seals (*Phoca vitulina*)--mixed exposures and thyroid homeostasis. Environmental Toxicology and Chemistry 26(5): 851-61.

Hall, A.J., Wells, R.S., Sweeney, J.C., Townsend, F.I., Balmer, B.C., Hohn, A.A. and Rhinehart, H.L. (2007). Annual, seasonal and individual variation in hematology and clinical blood chemistry profiles in bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, Florida. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 148(2): 266-277.

Hall, A.J., Thomas, G.O. and McConnell, B.J. (2009). Exposure to Persistent Organic Pollutants and First-Year Survival Probability in Gray Seal Pups. Environmental Science & Technology 43(16): 6364-6369.

Hall, A.J., Gulland, F.M.D., Hammond, J.A. and Schwacke, L. (2010). Epidemiology, disease, and health assessment. Marine Mammal Ecology and Conservation. I. L. Boyd, W. D. Bowen and S. J. Iverson. New York, Oxford University Press**:** 144-164.

Hammond, J.A., Hall, A.J. and Dyrynda, E.A. (2005). Comparison of polychlorinated biphenyl (PCB) induced effects on innate immune functions in harbour and grey seals. Aquatic Toxicology 74(2): 126-38.

Hanan, D.A. (1996). Dynamics of abundance and distribution for Pacific harbor seal, *Phoca vitulina*, on the Coast of California. Biology. Los Angeles, UCLA: 158.

Harding, K.C., Fujiwara, M., Axberg, Y. and Harrkonen, T. (2005). Mass-dependent energetics and survival in harbour seal pups. Functional Ecology 19: 129-135.

Härkönen, T., Dietz, R., Reijnders, P., Teilmann, J., Harding, K., Hall, A., Brasseur, S., Siebert, U., Goodman, S.J., Jepson, P.D., Dau Rasmussen, T. and Thompson, P. (2006). The 1988 and 2002 phocine distemper virus epidemics in European harbour seals. Diseases of Aquatic Organisms 68(2): 115-30.

Harris, H.S., Facemire, P., Greig, D.J., Colegrove, K.M., Ylitalo, G.M., Yanagida, G.K., Nutter, F.B., Fleetwood, M. and Gulland, F.M.D. 2011. Congenital neuroglial heterotopia in a neonatal harbor seal (*Phoca vitulinla richardsi*) with evidence of recent exposure to polycyclic aromatic hydrocarbons. Journal of Wildlife Diseases 47(1): 246-254.

Harvey, J. and Torok, M. (1994). Movements, dive behaviors, and food habits of harbor seals (*Phoca vitulina richardsi*) in San Francisco Bay, CA, Department of Water Resources: 89 pp.

Harvey, J.T., Helm, R.C. and Morejohn, G.V. (1995). Food habits of harbor seals inhabiting Elkhorn Slough, California. California Fish and Game 81(1): 1-9.

Harvey, J.T. and Goley, D. (2005). Determining a correction factor for aerial surveys of harbor seals in California, A Final Report to the NMFS and PSFMC: 35.

Harwood, J., Carter, S.D., Hughes, D.E., Bell, S.C., Baker, J.R. and Cornwell, H.J.C. (1989). Seal disease predictions. Nature 339: 670.

Haulena, M., St Aubin, D.J. and Duignan, P.J. (1998). Thyroid hormone dynamics during the nursing period in harbour seals, *Phoca vitulina*. Canadian Journal of Zoology 76: 48-55.

Heide-Jorgensen, M.P., Härkönen, T., Dietz, R. and Thompson, P.M. (1992). Retrospective of the 1988 European seal epizootic. Diseases of Aquatic Organisms 13: 37-62.

Higgins, R. (2000). Bacteria and fungi of marine mammals: a review. Can Vet J 41(2): 105-16.

Hinshaw, V.S., Bean, W.J., Webster, R.G., Rehg, J.E., Fiorelli, P., Early, G., Geraci, J.R. and St Aubin, D.J. (1984). Are seals frequently infected with avian influenza viruses? J Virol 51(3): 863-5.

Hunt, T.D., Ziccardi, M.H., Gulland, F.M., Yochem, P.K., Hird, D.W., Rowles, T. and Mazet, J.A. (2008). Health risks for marine mammal workers. Diseases of Aquatic Organisms 81(1): 81-92.

Jeffries, S.J., Brown, B.F. and Harvey, J.T. (1993). Techniques for capturing, handling and marking harbour seals. Aquatic Mammals 19.1: 21-25.

Jensen, S., Aars, J., Lydersen, C., Kovacs, K. and Åsbakk, K. (2010). The prevalence of *Toxoplasma gondii* in polar bears and their marine mammal prey: evidence for a marine transmission pathway? Polar Biology 33(5): 599-606.

Jepson, P.D., Bennett, P.M., Deaville, R., Allchin, C.R., Baker, J.R. and Law, R.J. (2005). Relationships between polychlorinated biphenyls and health status in harbor porpoises (*Phocoena phocoena*) stranded in the United Kingdom. Environmental Toxicology and Chemistry 24(1): 238-48.

Johnson, S.P., Nolan, S. and Gulland, F.M. (1998). Antimicrobial susceptibility of bacteria isolated from pinnipeds stranded in central and northern California. Journal of Zoo Wildlife Medicine 29(3): 288-94.

Johnson, S.P., Jang, S., Gulland, F.M., Miller, M.A., Casper, D.R., Lawrence, J. and Herrera, J. (2003). Characterization and clinical manifestations of *Arcanobacterium phocae* infections in marine mammals stranded along the central California coast. Journal of Wildlife Diseases 39(1): 136-44.

Kajiwara, N., Kannan, K., Muraoka, M., Watanabe, M., Takahashi, S., Gulland, F., Olsen, H., Blankenship, A.L., Jones, P.D., Tanabe, S. and Giesy, J.P. (2001). Organochlorine pesticides, polychlorinated biphenyls, and butyltin compounds in blubber and livers of stranded California sea lions, elephant seals, and harbor seals from coastal California, USA. Archives of Environmental Contamination and Toxicology 41(1): 90-9.

Kakuschke, A., Valentine-Thon, E., Griesel, S., Fonfara, S., Siebert, U. and Prange, A. (2005). Immunological impact of metals in harbor seals (*Phoca vitulina*) of the North Sea. Environmental Science and Technology 39(19): 7568-75.

Kalantzi, O.I., Hewitt, R., Ford, K.J., Cooper, L., Alcock, R.E., Thomas, G.O., Morris, J.A., McMillan, T.J., Jones, K.C. and Martin, F.L. (2004). Low dose induction of micronuclei by lindane. Carcinogenesis 25(4): 613-22.

Karita, E., Ketter, N., Price, M.A., Kayitenkore, K., Kaleebu, P., Nanvubya, A., Anzala, O., Jaoko, W., Mutua, G., Ruzagira, E., Mulenga, J., Sanders, E.J., Mwangome, M., Allen, S., Bwanika, A., Bahemuka, U., Awuondo, K., Omosa, G., Farah, B., Amornkul, P., Birungi, J., Yates, S., Stoll-Johnson, L., Gilmour, J., Stevens, G., Shutes, E., Manigart, O., Hughes, P., Dally, L., Scott, J., Stevens, W., Fast, P. and Kamali, A. (2009). CLSI-derived hematology and biochemistry reference intervals for healthy adults in eastern and southern Africa. PLoS One 4(2): e4401.

Kik, N.J.L., Goris, M.G., Bos, J.H., Hartskeerl, R.A. and Dorrestein, G.M. (2006). An outbreak of leptospirosis in seals (*Phoca vitulina*) in captivity. Veterinary Quarterly 28(1): 33-39.

Kimbro, D.K., Largier, J. and Grosholz, E.D. (2009). Coastal oceanographic processes influence the growth and size of a key estuarine species, the Olympia oyster. Limnology and Oceanography 54(5): 1425-1437.

Kimbrough, K.L., Johnson, W.E., Lauenstein, G.G., Christensen, J.D. and Apeti, D.A. (2008). An assessment of two decades of contaminant monitoring in the nation's coastal zone. NOAA Technical Memorandum NOS NCCOS 74: 105.

King, D.P., Lowe, K.A., Hay, A.W.M. and Evans, S.W. (1994). Identification, characterisation, and measurement of immunoglobulin concentrations in grey (*Haliocherus grypus*) and common (*Phoca vitulina*) seals. Developmental & Comparative Immunology 18(5): 433-442.

King, D.P., Sanders, J.L., Nomura, C.T., Stoddard, R.A., Ortiz, C.L. and Evans, S.W. (1998). Ontogeny of humoral immunity in northern elephant seal (*Mirounga angustirostris*) neonates. Comparative Biochemistry and Physiology Part B 121: 363-368.

King, J.E. (1983). Seals of the World. Ithaca, NY, Cornell University Press.

Knap, A., Dewailly, E., Furgal, C., Galvin, J., Baden, D., Bowen, R.E., Depledge, M., Duguay, L., Fleming, L.E., Ford, T., Moser, F., Owen, R., Suk, W.A. and Unluata, U. (2002). Indicators of ocean health and human health: developing a research and monitoring framework. Environmental Health Perspectives 110(9): 839-45.

Kodavanti, P.R.S., Senthil Kumar, K. and Loganathan, B.G. (2008). Organohalogen Pollutants and Human Health. International Encyclopedia of Public Health. H. K. Heggenhougen and S. R. Quah. Oxford, Academic Press. **4:** 686-693.

Kopec, A.D. and Harvey, J.T. (1995). Toxic pollutants, health indices, and population dynamics of harbor seals in San Francisco Bay, 1989-1992. Moss Landing, CA, Moss Landing Marine Laboratories: 138.

Kucklick, J.R., Struntz, W.D., Becker, P.R., York, G.W., O'Hara, T.M. and Bohonowych, J.E. (2002). Persistent organochlorine pollutants in ringed seals and polar bears collected from northern Alaska. Science of the Total Environment 287(1-2): 45-59.

Kulldorff, M. and Nagarwalla, N. (1995). Spatial disease clusters: detection and inference. Statistics in Medicine 14: 799-810.

Kulldorff, M. (1997). A spatial scan statistic. Communications in Statistics: Theory and Methods 26: 1481-1496.

Kulldorff, M.and Information Management Services, Inc. (2009) SaTScan™ v8.0: Software for the spatial and space-time scan statistics. http://www.satscan.org/.

Lambourn, D.M., Jeffries, S.J. and Dubey, J.P. (2001). Seroprevalence of *Toxoplasma gondii* in Harbor Seals (*Phoca vitulina*) in Southern Puget Sound, Washington. The Journal of Parasitology 87(5): 1196-1197.

Lamont, M.M., Vida, J.T., Harvey, J.T., Jeffries, S., Brown, R., Huber, H.H., DeLong, R. and Thomas, W.K. (1996). Genetic substructure of the Pacific harbor seal (*Phoca vitulina richardsi*) off Washington, Oregon, and California. Marine Mammal Science 12(3): 402-413.

Lander, M., Harvey, J. and Gulland, F. (2003). Hematology and serum chemistry comparisons between free-ranging and rehabilitated harbor seal (*Phoca vitulina richardsi*) pups. Journal of Wildlife Diseases 39(3): 600-609.

Lander, M.E., Harvey, J.T., Hanni, K.D. and Morgan, L.E. (2002). Behavior, movements, and apparent survival of rehabilitated and free-ranging harbor seal pups. Journal of Wildlife Diseases 66(1): 19-28.

Lapointe, J.-M., Duignan, P.J., Marsh, A.E., Gulland, F.M., Barr, B.C., Naydan, D.K., King, D.P., Farman, C.A., Huntingdon, K.A.B. and Lowenstine, L.J. (1998). Meningoencephalitis Due to a *Sarcocystis Neurona*-like Protozoan in Pacific Harbor Seals (*Phoca vitulina richardsi*). The Journal of Parasitology 84(6): 1184-1189.

Lapointe, J.M., Gulland, F.M., Haines, D.M., Barr, B.C. and Duignan, P.J. (1999). Placentitis due to *Coxiella burnetii* in a Pacific harbor seal (*Phoca vitulina richardsi*). Journal of Veterinary Diagnostic Investigation 11(6): 541-3.

Lapointe, J.M., Duignan, P.J., Barr, B.C., Petrich, A.K., MacPherson, D.W., Gulland, F.M. and Dubey, J.P. (2003). Meningoencephalitis associated with an unidentified apicomplexan protozoan in a Pacific harbor seal. Journal of Parasitology 89(4): 859-62.

Lasek-Nesselquist, E., Welch, D.M. and Sogin, M.L. (2010). The identification of a new Giardia duodenalis assemblage in marine vertebrates and a preliminary analysis of G. duodenalis population biology in marine systems. International Journal for Parasitology 40(9): 1063-1074.

Levin, M., De Guise, S. and Ross, P.S. (2005). Association between lymphocyte proliferation and polychlorinated biphenyls in free-ranging harbor seal (*Phoca vitulina*) pups from British Columbia, Canada. Environmental Toxicology and Chemistry 24(5): 1247-52.

Levin, M., Leibrecht, H., Mori, C., Jessup, D. and De Guise, S. (2007). Immunomodulatory effects of organochlorine mixtures upon in vitro exposure of peripheral blood leukocytes differ between free-ranging and captive southern sea otters (*Enhydra lutris*). Veterinary Immunology and Immunopathology 119(3-4): 269-77.

Levy, E.M., Alharbi, S.A., Grindlinger, G. and Black, P.H. (1984). Changes in mitogen responsiveness lymphocyte subsets after traumatic injury: relation to development of sepsis. Clinical Immunology and Immunopathology 32(2): 224-33.

Liang, T., Ji, H., Lian, L., Wu, T., Gu, W. and Wang, W. (2010). A rapid assay for simultaneous detection of *Spiroplasma eriocheiris* and white spot syndrome virus in *Procambarus clarkii* by multiplex PCR. Letters in Applied Microbiology doi: 10.1111/j.1472-765X.2010.02927.x.

Lindsay, D., Collins, M., Mitchell, S., Cole, R., Flick, G., Wetch, C., Lindquist, A. and Dubey, J. (2003). Sporulation and survival of *Toxoplasma gondii* oocysts in seawater. Journal of Eukaryotic Microbiology 50: 687-688.

Littnan, C.L., Stewart, B.S., Yochem, P.K. and Braun, R. (2007). Survey for selected pathogens and evaluation of disease risk factors for endangered Hawaiian monk seals in the main Hawaiian Islands. Eco Health 3: 232-244.

Lloyd-Smith, J.O., Greig, D.J., Hietala, S., Ghneim, G.S., Palmer, L., St Leger, J., Grenfell, B.T. and Gulland, F.M.D. (2007). Cyclical changes in seroprevalence of leptospirosis in California sea lions: endemic and epidemic disease in one host species? BMC Infectious Diseases 7:125.

Lochmiller, R.L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88(1): 87-98.

Lockwood, S., Chovan, J. and Gaydos, J. (2006). Aerobic bacterial isolations from harbor seals (*Phoca vitulina*) stranded in Washington: 1992-2003. Journal of Zoo and Wildlife Medicine 37(3): 281-291.

Loganathan, B.G. and Masunaga, S. (2009). PCBs, dioxins, and furans: human exposure and health effects. Handbook of Toxicology of Chemical Warfare Agents. R. C. Gupta**:** 245-253.

Lunn, N., Boyd, I., Barton, T. and Croxall, J. (1993). Factors Affecting the Growth Rate and Mass at Weaning of Antarctic Fur Seals at Bird Island, South Georgia. Journal of Mammalogy 74(4): 908-919.

Lunn, N.J., Boyd, I.L. and Croxall, J.P. (1994). Reproductive Performance of Female Antarctic Fur Seals: The Influence of Age, Breeding Experience, Environmental Variation and Individual Quality. Journal of Animal Ecology 63(4): 827-840.

Mamaev, L.V., Visser, I.K., Belikov, S.I., Denikina, N.N., Harder, T., Goatley, L., Rima, B., Edginton, B., Osterhaus, A.D. and Barrett, T. (1996). Canine distemper virus in Lake Baikal seals (*Phoca sibirica*). The Veterinary Record 138(18): 437-9.

Manna, J., Roberts, D., Press, D. and Allen, S. (2006). Harbor Seal Monitoring San Francisco Bay Area. Annual Report, National Park Service: 22 pp.

Martin, L.B., Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C. and Wikelski, M. (2006). Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Functional Ecology 20(2): 290-299.

Martineau, D., Lemberger, K., Dallaire, A., Labelle, P., Lipscomb, T.P., Michel, P. and Mikaelian, I. (2002). Cancer in wildlife, a case study: beluga from the St. Lawrence estuary, Quebec, Canada. Environmental Health Perspectives 110(3): 285-92.

McKnight, C.A., Reynolds, T.L., Haulena, M., deLahunta, A. and Gulland, F.M. (2005). Congenital hemicerebral anomaly in a stranded Pacific harbor seal (*Phoca vitulina richardsi*). Journal of Wildlife Diseases 41(3): 654-8.

McMahon, C.R. and Burton, H.R. (2005). Climate change and seal survival: evidence for environmentally mediated changes in elephant seal, *Mirounga leonina*, pup survival. Proceedings of the Royal Society B 272: 923-928.

Measures, L.N. and Olson, M. (1999). Giardiasis in pinnipeds from eastern Canada. J Wildl Dis 35(4): 779-82.

Meng, X.Z., Blasius, M.E., Gossett, R.W. and Maruya, K.A. (2009). Polybrominated diphenyl ethers in pinnipeds stranded along the southern California coast. Environmental Pollution 157(10): 2731-6.

Miller, M., Conrad, P., James, E.R., Packham, A., Toy-Choutka, S., Murray, M.J., Jessup, D. and Grigg, M. (2008). Transplacental toxoplasmosis in a wild southern sea otter (*Enhydra lutris nereis*). Veterinary Parasitology 153(1-2): 12-8.

Miller, M.A., Sverlow, K., Crosbie, P.R., Barr, B.C., Lowenstine, L.J., Gulland, F.M., Packham, A. and Conrad, P.A. (2001). Isolation and Characterization of Two Parasitic Protozoa from a Pacific Harbor Seal (*Phoca vitulina richardsi*) with Meningoencephalomyelitis. The Journal of Parasitology 87(4): 816-822.

Miller, M.A., Gardner, I.A., Kreuder, C., Paradies, D.M., Worcester, K.R., Jessup, D.A., Dodd, E., Harris, M.D., Ames, J.A., Packham, A.E. and Conrad, P.A. (2002a). Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*). International Journal for Parasitology 32(8): 997-1006.

Miller, M.A., Gardner, I.A., Packham, A., Mazet, J.K., Hanni, K.D., Jessup, D., Estes, J., Jameson, R., Dodd, E., Barr, B.C., Lowenstine, L.J., Gulland, F.M. and Conrad, P.A. (2002b). Evaluation of an Indirect Fluorescent Antibody Test (IFAT) for demonstration of antibodies to *Toxoplasma gondii* in the Sea Otter (*Enhydra lutris*). The Journal of Parasitology 88(3): 594-599.

Miller, S.A. (1988). Movement and activity patterns of harbor seals at the Point Reyes Peninsula, California. Wildland Resource Science. Berkeley, University of California. MS: 70.

Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Byrne, B.A., Jang, S., Harris, M., Ames, J., Jessup, D., Paradies, D., Worcester, K., Melli, A. and Conrad, P.A. (2006). *Salmonella* spp., *Vibrio* spp., *Clostridium perfringens*, and *Plesiomonas shigelloides* in marine and freshwater invertebrates from coastal California ecosystems. Microbial Ecology 52(2): 198-206.

Miller, W.G., Adams, L.G., Ficht, T.A., Cheville, N.F., Payeur, J.P., Harley, D.R., House, C. and Ridgway, S.H. (1999). Brucella-induced abortions and infection in bottlenose dolphins (*Tursiops truncatus*). Journal of Zoo and Wildlife Medicine 30(1): 100-10.

Miranda Filho, K.C., Metcalfe, C.D., Metcalfe, T.L., Muelbert, M.M., Robaldo, R.B., Martinez, P.E., Colares, E.P. and Bianchini, A. (2009). Lactational transfer of PCBs and chlorinated pesticides in pups of southern elephant seals (*Mirounga leonina*) from Antarctica. Chemosphere 75(5): 610-6.

Møller, A.P. and Saino, N. (2004). Immune response and survival. Oikos 104(2): 299-304.

Morgan, L., Kumaresan, S., Thomas, C. and MacWilliams, P. (1998). Hematology and chemistry reference values for free-ranging harbor seals (*Phoca vitulina*) and the effects of hemolysis on chemistry values of captive harbor seals. Journal of Zoo and Wildlife Medicine 29(4): 394-400.

Mos, L., Morsey, B., Jeffries, S.J., Yunker, M.B., Raverty, S., De Guise, S. and Ross, P.S. (2006). Chemical and biological pollution contribute to the immunological profiles of free-ranging harbor seals. Environmental Toxicology and Chemistry 25(12): 3110-7.

Muelbert, M.M., Bowen, W.D. and Iverson, S.J. (2003). Weaning mass affects changes in body composition and food intake in harbour seal pups during the first month of independence. Physiological and Biochemical Zoology 76(3): 418-27.

Neale, J.C., van de Water, J.A., Harvey, J.T., Tjeerdema, R.S. and Gershwin, M.E. (2002). Proliferative responses of harbor seal (*Phoca vitulina*) T lymphocytes to model marine pollutants. Developmental Immunology 9(4): 215-21.

Neale, J.C., Kenny, T.P., Tjeerdema, R.S. and Gershwin, M.E. (2005a). PAH- and PCB-induced alterations of protein tyrosine kinase and cytokine gene transcription in harbor seal (*Phoca vitulina*) PBMC. Clinical and Developmental Immunology 12(2): 91-7.

Neale, J.C.C., Gulland, F.M.D., Schmelzer, K.R., Harvey, J.T., Berg, E.A., Allen, S.G., Greig, D.J., Grigg, E.K. and Tjeerdema, R.S. (2005b). Contaminant loads and hematological correlates in the harbor seal (*Phoca vitulina*) of San Francisco Bay, California. Journal of Toxicology and Environmental Health, Part A 68: 617-633.

Ng, T.F., Suedmeyer, W.K., Wheeler, E., Gulland, F. and Breitbart, M. (2009). Novel anellovirus discovered from a mortality event of captive California sea lions. Journal of General Virology 90(Pt 5): 1256-61.

Nichols, F.H., Cloern, J.E., Luoma, S.N. and Peterson, D.H. (1986). The Modification of an Estuary. Science 231(4738): 567-573.

Nickel, B.A. (2003). Movement and habitat use patterns of harbor seals in the San Francisco Bay Estuary, California. Biology. San Francisco, San Francisco State University. MS: 121.

Nollens, H.H., Wellehan, J.F.X., Archer, L., Lowenstine, L.J. and Gulland, F.M.D. (2010). Detection of a respiratory coronavirus from tissues archived during a pneumonia epizootic in free-ranging Pacific harbor seals *Phoca vitulina richardsii*. Diseases of Aquatic Organisms 90(2): 113-120.

O'Shea, T.J. (1999). Environmental contaminants and marine mammals. Biology of marine mammals. J. E. Reynolds III and S. A. Rommel. Washington, DC, Smithsonian Institution Press**:** 485-563.

Oates, S. (2005). Survival, movements, and diet of juvenile harbor seals along central California. Marine Science, Moss Landing Marine Laboratories and San Jose State University. MS: 228 pp.

Obiri-Danso, K., Paul, N. and Jones, K. (2001). The effects of UVB and temperature on the survival of natural populations and pure cultures of *Campylobacter jejuni*,

Camp. coli, *Camp. lari* and urease-positive thermophilic campylobacters (UPTC) in surface waters. Journal of Applied Microbiology 90(2): 256-267.

Ohishi, K., Ninomiya, A., Kida, H., Park, C.H., Maruyama, T., Arai, T., Katsumata, E., Tobayama, T., Boltunov, A.N., Khuraskin, L.S. and Miyazaki, N. (2002). Serological evidence of transmission of human influenza A and B viruses to Caspian seals (*Phoca caspica*). Microbiology and Immunology 46(9): 639-44.

Olson, M.E., Roach, P.D., Stabler, M. and Chan, W. (1997). Giardiasis in ringed seals from the western arctic. Journal of Wildlife Diseases 33(3): 646-8.

Ono, K.A., Boness, D.J. and Oftedal, O.T. (1987). The effect of a natural environmental disturbance on maternal investment and pup behavior in the California sea lion. Behavioral Ecology and Sociobiology 21: 109-118.

Ortega, Y.R. and Adam, R.D. (1997). *Giardia*: overview and update. Clin Infect Dis 25(3): 545-9; quiz 550.

Osterhaus, A.D.M.E., Rimmelzwaan, G.F., Martina, B.E.E., Bestebroer, T.M. and Fouchier, R.A.M. (2000). Influenza B Virus in Seals. Science 288(5468): 1051-1053.

Palacios, M.G., Cunnick, J.E., Vleck, D. and Vleck, C.M. (2009). Ontogeny of innate and adaptive immune defense components in free-living tree swallows, *Tachycineta bicolor*. Developmental & Comparative Immunology 33(4): 456-463.

Piché, C., Measures, L., Bédard, C. and Lair, S. (2010). Bronchoalveolar lavage and pulmonary histopathology in harp seals (*Phoca groenlandica*) experimentally infected with *Otostrongylus circumlitus*. Journal of Wildlife Diseases 46(2): 409-421.

Prenger-Berninghoff, E., Siebert, U., Stede, M., Konig, A., Weiss, R. and Baljer, G. (2008). Incidence of Brucella species in marine mammals of the German North Sea. Diseases of Aquatic Organisms 81(1): 65-71.

Prokocimer, M. and Potasman, I. (2008). The added value of peripheral blood cell morphology in the diagnosis and management of infectious diseases--part 1: basic concepts. Postgraduate Medical Journal 84(997): 579-85.

R Development Core Team (2009). R: A Language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing. http://www.R-project.org

Reidarson, T.H., McBain, J., House, C., King, D.P., Stott, J.L., Krafft, A., Taubenberger, J.K., Heyning, J. and Lipscomb, T.P. (1998). Morbillivirus

infection in stranded common dolphins from the Pacific Ocean. Journal of Wildlife Diseases 34(4): 771-6.

Reidman, M. (1990). The pinnipeds: seals, sea lions, and walruses. Berkeley, CA, University of California Press.

Reijnders, P.J.H. (1978). Recruitment in the harbour seal (*Phoca vitulina*) population in the Dutch Wadden Sea. Netherlands Journal of Sea Research 12(2): 164-179.

Reijnders, P.J.H. (1980). Organochlorine and heavy metal residues in harbour seals from the Wadden Sea and their possible effects on reproduction. Netherlands Journal of Sea Research 14(1): 30-65.

Reijnders, P.J.H. (1986). Reproductive Failure in Common Seals Feeding on Fish from Polluted Coastal Waters. Nature (London) 324(4): 456-457.

Resch, G., Awad-Masalmeh, M., Bakoss, P. and Jarekova, J. (2007). Utility of phylogenetic studies in the identification of *Leptospira* strains. Epidemiology and Infection 135(8): 1266-73.

Rhyan, J.C., Gidlewski, T., Ewalt, D.R., Hennager, S.G., Lambourne, D.M. and Olsen, S.C. (2001). Seroconversion and abortion in cattle experimentally infected with *Brucella* sp. isolated from a Pacific harbor seal (*Phoca vitulina richardsi*). Journal of Veterinary Diagnostic Investigation 13(5): 379-82.

Risebrough, R.W., Alcorn, D., Allen, S.G., Anderlini, V.C., Booren, L., DeLong, R.L., Fancher, L.E., Jones, R.E., McGinnis, S.M. and T.T.Schmidt (1980). Population Biology of Harbor Seals in San Francisco Bay, California, Bodega Bay Institute of Pollution Ecology. US Department of Commerce, National Technial Information Service Report No. MMC-76/19: 67 pg.

Ross, H.M., Foster, G., Reid, R.J., Jahans, K.L. and MacMillan, A.P. (1994). *Brucella* species infection in sea-mammals. Veterinary Record 134(14): 359.

Ross, P., Pohajdak, B., Bowen, W. and Addison, R. (1993). Immune function in free-ranging harbor seal (*Phoca vitulina*) mothers and their pups during lactation. Journal of Wildlife Diseases 29(1): 21-29.

Ross, P.S., De Swart, R.L., Visser, I.K.G., Vedder, L.J., Murk, W., Bowen, W.D. and Osterhaus, A.D.M.E. (1994). Relative immunocompetence of the newborn harbour seal, *Phoca vitulina*. Veterinary Immunology and Immunopathology 42: 331-348.

Ross, P.S., De Swart, R.L., Reijnders, P.J.H., Loveren, H.V., Vos, J.G. and Osterhaus, A.D.M.E. (1995). Contaminant-Related Suppression of Delayed-Type

Hypersensitivity and Antibody Responses in Harbor Seals Fed Herring from the Baltic Sea. Environmental Health Perspectives 103(2): 162-167.

Ross, P., De Swart, R., Timmerman, H., Reijnders, P., Vos, J., Van Loveren, H. and Osterhaus, A. (1996). Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. Aquatic Toxicology 34(1): 71-84.

Ross, P.S. (2000). Marine Mammals as Sentinels in Ecological Risk Assessment. Human and Ecological Risk Assessment: An International Journal 6(1): 29-46.

Ross, P.S., Jeffries, S.J., Yunker, M.B., Addison, R.F., Ikonomou, M.G. and Calambokidis, J.C. (2004). Harbor seals (*Phoca vitulina*) in British Columbia, Canada, and Washington State, USA, reveal a combination of local and global polychlorinated biphenyl, dioxin, and furan signals. Environmental Toxicology and Chemistry 23(1): 157-65.

Rothman, K.J. (2002). Epidimiology-An Introduction. New York, Oxford University Press: 223 pp.

Saliki, J.T. and Lehenbauer, T.W. (2001). Monoclonal Antibody-Based Competitive Enzyme-Linked Immunosorbent Assay for Detection of Morbillivirus Antibody in Marine Mammal Sera. Journal of Clinical Microbiology 39(5): 1877-1881.

Saliki, J.T., Cooper, E.J. and Gustavson, J.P. (2002). Emerging Morbillivirus Infections of Marine Mammals. Annals of the New York Academy of Sciences 969: 51-59.

Scammon, C.M. (1874). The marine mammals of the northwest coast of North America, and American whale fishing. San Francisco, John H. Carmany and Co. (republished 1968 by Dover Publications, New York).

Schrank, C.S., Cook, M.E. and Hansen, W.R. (1990). Immune response of mallard ducks treated with immunosuppressive agents: antibody response to erythrocytes and in vivo response to phytohemagglutinin-P. Journal of Wildlife Diseases 26(3): 307-315.

Schwacke, L.H., Hall, A.J., Townsend, F.I., Wells, R.S., Hansen, L.J., Hohn, A.A., Bossart, G.D., Fair, P.A. and Rowles, T.R. (2009). Hematology and clinical blood chemistry reference intervals for free-ranging common bottlenose dolphins (*Tursiops truncatus*) and variation related to geographic sampling site. American Journal of Veterinary Research 70(8): 973-985.

Schwacke, L.H., Twiner, M.J., De Guise, S., Balmer, B.C., Wells, R.S., Townsend, F.I., Rotstein, D.C., Varela, R.A., Hansen, L.J., Zolman, E.S., Spradlin, T.R., Levin, M., Leibrecht, H., Wang, Z. and Rowles, T.K. (2010). Eosinophilia and biotoxin exposure in bottlenose dolphins (*Tursiops truncatus*) from a coastal area

impacted by repeated mortality events. Environmental Research 110(6): 548-555.

Scribner, J.A. (2005). Mitochondrial and microsatellite DNA analysis of population subdivision in the Pacific harbor seal (*Phoca vitulina richardii*) in California. Biology. San Francisco State University. MS: 110 pp.

Shaw, S.D. (1998). Organochlorines and biomarkers of immune and endocrine effects in Pacific harbor seal and norther elephant seal pups, Columbia University. PhD: 359 pp.

Shaw, S.D. (2001). Perinatal Exposure to Persistent Organic Pollutants: Parallels in Seals and Humans. Organohalogen Compounds 53: 21-26.

She, J., Petreas, M., Winkler, J., Visita, P., McKinney, M., Jones, R. and Kopec, A.D. (2000). Harbor seals as indicators of halogenated contaminants in San Francisco Bay. Organohalogen Compounds 49: 422-425.

She, J., Petreas, M., Winkler, J., Visita, P., McKinney, M. and Kopec, D. (2002). PBDEs in the San Francisco Bay Area: measurements in harbor seal blubber and human breast adipose tissue. Chemosphere 46: 697-707.

Sidor, I.F., Dunn, L.J., Romano, T.R., Meegan, J., Carlson, J. and Frasca, S. (2006). Use of real-time PCR for detection of *Brucella* spp. in marine mammal tissues. Proceedings of the American Association for Zoo Veterinarians, Tampa, Florida.

Siembieda, J., Johnson, C.K., Boyce, W., Sandrock, C. and Cardona, C. (2008). Risk for avian influenza virus exposure at human-wildlife interface. Emeging Infectious Diseases 14(7): 1151-3.

Siembieda, J.L., Johnson, C.K., Cardona, C., Anchell, N., Dao, N., Reisen, W. and Boyce, W. (2010). Influenza A Viruses in Wild Birds of the Pacific Flyway, 2005-2008. Vector-Borne and Zoonotic Diseases.

Simms, W., Jeffries, S., Ikonomou, M. and Ross, P. (2000). Contaminant-related disruption of vitamin A dynamics in free-ranging harbor seal (*Phoca vitulina*) pups from British Columbia, Canada, and Washington State, USA. Environmental Toxicology and Chemistry 19(11): 2844-2849.

Sloan, C.A., Brown, D.W., Pearce, R.W., Boyer, R.H., Bolton, J.L., Burrows, D.G., Herman, D.P. and Krahn, M.M. (2005). Determining aromatic hydrocarbons and chlorinated hydrocarbons in sediments and tissues using accelerated solvent extraction and gas chromatography/mass spectrometry. Techniques in Aquatic Toxicology - Volume 2. G. K. Ostrander. Boca Raton, FL, CRC Press**:** 631-651.

Sloan, C.A., Brown, D.W., Ylitalo, G.M., Buzitis, J., Herman, D.P., Burrows, D.G., Yanagida, G.K. and Krahn, M.M. (2006). Quality assurance plan for analyses of environmental samples for polycyclic aromatic compounds, persistent organic pollutants, fatty acids, stable isotope ratios, lipid classes, and metabolites of polycylic aromatic compounds, NMFS-NWFSC-77: 30.

Small, R.J., Lowry, L.F., ver Hoef, J.M., Frost, K.J., Delong, R.A. and Rehberg, M.J. (2005). Differential movements by harbor seal pups in contrasting Alaska environments. Marine Mammal Science 21(4): 671-694.

Smith, A.W., Brown, R.J., Skilling, D.E. and DeLong, R.L. (1974). *Leptospira pomona* and reproductive failure in California sea lions. Journal of the American Veterinary Medical Association 165(11): 996-8.

Solberg, H.E. (2004). The IFCC recommendation of estimation of reference intervals. The RefVal Program. Clinical Chemistry and Laboratory Medicine 42(7): 710-714.

Sormo, E.G., Larsen, H.J., Johansen, G.M., Skaare, J.U. and Jenssen, B.M. (2009). Immunotoxicity of polychlorinated biphenyls (PCB) in free-ranging gray seal pups with special emphasis on dioxin-like congeners. Journal of Toxicology and Environmental Health A 72(3-4): 266-76.

Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T. and Suarez, D.L. (2002). Development of a Real-Time Reverse Transcriptase PCR Assay for Type A Influenza Virus and the Avian H5 and H7 Hemagglutinin Subtypes. Journal of Clinical Microbiology 40(9): 3256-3260.

St. Aubin, D.J. (2001). Endocrinology. CRC Handbook for Marine Mammal Medicine. L. A. Dierauf and F. M. D. Gulland. Boca Raton, FL, CRC Press**:** 165-192.

Stamper, M.A., Gulland, F.M. and Spraker, T. (1998). Leptospirosis in rehabilitated Pacific harbor seals from California. Journal of Wildlife Diseases 34(2): 407-10.

Steiger, G.H., Calambokidis, J., Cubbage, J.C., Skilling, D.E., Smith, A.W. and Gribble, D.H. (1989). Mortality of harbor seal pups at different sites in the inland waters of Washington. Journal of Wildlife Diseases 25(3): 319-28.

Stevens, E., Lipscomb, T.P. and Gulland, F.M. (1999). An additional case of leptospirosis in a harbor seal. Journal of Wildlife Diseases 35(1): 150.

Stoddard, R.A., Gulland, M.D., Atwill, E.R., Lawrence, J., Jang, S. and Conrad, P.A. (2005). *Salmonella* and *Campylobacter* spp. in northern elephant seals, California. Emeging Infectious Diseases 11(12): 1967-9.

Stoddard, R.A., Miller, W.G., Foley, J.E., Lawrence, J., Gulland, F.M., Conrad, P.A. and Byrne, B.A. (2007). *Campylobacter insulaenigrae* Isolates from Northern Elephant Seals (*Mirounga angustirostris*) in California. Applied and Environmental Microbiology 73(6): 1729-35.

Stoddard, R.A., Atwill, E.R., Gulland, F.M., Miller, M.A., Dabritz, H.A., Paradies, D.M., Worcester, K.R., Jang, S., Lawrence, J., Byrne, B.A. and Conrad, P.A. (2008). Risk factors for infection with pathogenic and antimicrobial-resistant fecal bacteria in northern elephant seals in California. Public Health Reports 123(3): 360-70.

Storr-Hansen, E. and Spliid, H. (1993). Coplanar polychlorinated biphenyl congener levels and patterns and the identification of separate populations of harbor seals (*Phoca vitulina*) in Denmark. Archives of Environmental Contamination and Toxicology 24(1): 44-58.

Sydeman, W.J. and Allen, S.G. (1999). Pinniped population dynamics in Central California: Correlations with sea surface temperature and upwelling indices. Marine Mammal Science 15(2): 446-461.

Tabuchi, M., Veldhoen, N., Dangerfield, N., Jeffries, S., Helbing, C.C. and Ross, P.S. (2006). PCB-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging harbor seals (*Phoca vitulina*). Environmental Health Perspectives 114(7): 1024-31.

Tella, J.L., Lemus, J.s.A., Carrete, M. and Blanco, G. (2008). The PHA Test Reflects Acquired T-Cell Mediated Immunocompetence in Birds. PLoS ONE 3(9): e3295.

Thomas, G.O., Moss, S.E., Asplund, L. and Hall, A.J. (2005). Absorption of decabromodiphenyl ether and other organohalogen chemicals by grey seals (*Halichoerus grypus*). Environmental Pollution 133(3): 581-6.

Thompson, D. and Lonergan, M. (2009). Harbour seal pups survival determined by satellite telemetry. SCOS-BP 09/8: 1-3.

Thompson, J.R., Pacocha, S., Pharino, C., Klepac-Ceraj, V., Hunt, D.E., Benoit, J., Sarma-Rupavtarm, R., Distel, D.L. and Polz, M.F. (2005). Genotypic diversity within a natural coastal bacterioplankton population. Science 307(5713): 1311-3.

Thompson, P.M., Kovacs, K.M. and McConnell, B.J. (1994). Natal dispersal of harbour seals (*Phoca vitulina*) from breeding sites in Orkney, Scotland. Journal of Zoology (London) 234(4): 668-673.

Thompson, P.M., Tollit, D.J., Corpe, H.M., Reid, R.J. and Ross, H.M. (1997). Changes in haematological parameters in relation to prey switching in a wild population of harbour seals. Functional Ecology 11(6): 743-750.

Thornton, S.M., Nolan, S. and Gulland, F.M. (1998). Bacterial isolates from California sea lions (*Zalophus californianus*), harbor seals (*Phoca vitulina*), and northern elephant seals (*Mirounga angustirostris*) admitted to a rehabilitation center along the central California coast, 1994-1995. Journal of Zoo and Wildlife Medicine 29(2): 171-6.

Tiedeken, J.A. and Ramsdell, J.S. (2009). DDT Exposure of Zebrafish Embryos Enhances Seizure Susceptibility: Relationship to Fetal *p,p′*-DDE Burden and Domoic Acid Exposure of California Sea Lions. Environmental Health Perspectives 117(1).

Tiedeken, J.A. and Ramsdell, J.S. (2010). Zebrafish seizure model identifies *p,p′* -DDE as the dominant contaminant of fetal California sea lions that accounts for synergistic activity with domoic acid. Environmental Health Perspectives 118(4): 545-51.

Trumble, S.J., Castellini, M.A., Mau, T.L. and Castellini, J.M. (2006). Dietary and seasonal influences on blood chemistry and hematology in captive harbor seals. Marine Mammal Science 22(1): 104-123.

Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982). T-cell mitogens cause early changes in cytoplasmic free Ca2+ and membrane potential in lymphocytes. Nature 295(5844): 68-71.

Turmelle, A.S., Ellison, J.A., Mendonca, M.T. and McCracken, G.F. (2010). Histological assessment of cellular immune response to the phytohemagglutinin skin test in Brazilian free-tailed bats (*Tadarida brasiliensis*). Journal of Comparative Physiology B.

Van Pelt, R.W. and Dietrich, R.A. (1973). Staphylococcal infection and toxoplasmosis in a young harbor seal. Journal of Wildlife Diseases 9(3): 258-61.

Vanderhoof, M. and Allen, S. (2005). Harbor seal monitoring at Point Reyes National Seashore and Golden Gate National Recreation Area: 19 pp.

Victoria, J.G., Kapoor, A., Li, L., Blinkova, O., Slikas, B., Wang, C., Naeem, A., Zaidi, S. and Delwart, E. (2009). Metagenomic analyses of viruses in stool samples from children with acute flaccid paralysis. Journal of Virology 83(9): 4642-51.

Victoriano, A.F., Smythe, L.D., Gloriani-Barzaga, N., Cavinta, L.L., Kasai, T., Limpakarnjanarat, K., Ong, B.L., Gongal, G., Hall, J., Coulombe, C.A.,

Yanagihara, Y., Yoshida, S. and Adler, B. (2009). Leptospirosis in the Asia Pacific region. BMC Infectious Diseases 9: 147.

Visser, I.J., Vellema, P., van Dokkum, H. and Shimada, T. (1999). Isolation of *Vibrio cholerae* from diseased farm animals and surface water in The Netherlands. Veterinary Record 144(16): 451-2.

Wang, L.C., Pan, C.H., Severinghaus, L.L., Liu, L.Y., Chen, C.T., Pu, C.E., Huang, D., Lir, J.T., Chin, S.C., Cheng, M.C., Lee, S.H. and Wang, C.H. (2008). Simultaneous detection and differentiation of Newcastle disease and avian influenza viruses using oligonucleotide microarrays. Veterinary Microbiology 127(3-4): 217-26.

Williams, E.S. and Barker, I.K. (2001). Infectious diseases of wild mammals, Iowa State University Press.

Wilson, S.C. (2001). Population growth, reproductive rate and neo-natal morbidity in a re-establishing harbour seal colony. Mammalia 65(3): 319-334.

Wolkers, H., Lydersen, C. and Kovacs, K.M. (2004). Accumulation and lactational transfer of PCBs and pesticides in harbor seals (*Phoca vitulina*) from Svalbard, Norway. Science of the Total Environment 319(1-3): 137-46.

Worthy, G.A.J. and Lavigne, D.M. (1982). Changes in blood properties of fasting and feeding harp seal pups, *Phoca groenlandica*, after weaning. Canadian Journal of Zoology 60: 586-592.

Yamane, K., Asato, J., Kawade, N., Takahashi, H., Kimura, B. and Arakawa, Y. (2004). Two cases of fatal necrotizing fasciitis caused by *Photobacterium damsela* in Japan. Journal of Clinical Microbiology 42(3): 1370-2.

Ylitalo, G.M., Hufnagle, L. and Krahn, M.M. (2005a). Determination of lipid classes and lipid content in tissues of aquatic organisms using a think layer chromatography/flame ionization detection (TLC/FID) microlipid method. Techniques in Aquatic Toxicology - Volume 2. G. K. Ostrander. Boca Raton, FL, CRC Press**:** 227-237.

Ylitalo, G.M., Stein, J.E., Hom, T., Johnson, L.L., Tilbury, K.L., Hall, A.J., Rowles, T., Greig, D., Lowenstine, L.J. and Gulland, F.M. (2005b). The role of organochlorines in cancer-associated mortality in California sea lions (*Zalophus californianus*). Marine Pollution Bulletin 50(1): 30-9.

York, A.E. (1994). The population dynamics of northern sea lions, 1975-1985. Marine Mammal Science 10(1): 38-51.

Young, D., Becerra, M., Kopec, D. and Echols, S. (1998). GC/MS analysis of PCB congeners in blood of the harbor seal *Phoca vitulina* from San Francisco Bay. Chemosphere 37(4): 711-733.

Zabka, T.S., Goldstein, T. and Nollens, H.H. (2006a). "Diagnostic Techniques in Aquatic Animal Medicine." from http://www.iaaam.org/.

Zabka, T.S., Haulena, M., Puschner, B., Gulland, F.M., Conrad, P.A. and Lowenstine, L.J. (2006b). Acute lead toxicosis in a harbor seal (*Phoca vitulina richardsi*) consequent to ingestion of a lead fishing sinker. Journal of Wildlife Diseases 42(3): 651-7.

Zarnke, R.L., Saliki, J.T., Macmillan, A.P., Brew, S.D., Dawson, C.E., Ver Hoef, J.M., Frost, K.J. and Small, R.J. (2006). Serologic survey for *Brucella* spp., phocid herpesvirus-1, phocid herpesvirus-2, and phocine distemper virus in harbor seals from Alaska, 1976-1999. Journal of Wildlife Diseases 42(2): 290-300.

Zuerner, R.L. and Alt, D.P. (2009). Variable nucleotide tandem-repeat analysis revealing a unique group of *Leptospira interrogans* serovar Pomona isolates associated with California sea lions. Journal of Clinical Microbiology 47(4): 1202-5.