ORGANOCHLORINES IN STELLER SEA LIONS (*Eumetopias jubatus*)

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Abstract

Existing populations of Steller sea lions (*Eumetopias jubatus*) have declined precipitously over the last half-century. Investigations into the cause of this downward trend have focused on many different possible factors. Toxicity caused by the accumulation of organochlorines (OCs), such as polychlorinated biphenyls (PCBs) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane or dichlorodiphenyltrichloroethane (DDT), has been demonstrated in marine mammals and was considered here as one possible factor that may have contributed to the decline of Steller sea lions or their failure to recover. The focus of this project was to investigate the relationship of contaminant loads to hormone levels, specifically thyroid hormones and cortisol in Steller sea lions. Two approaches were taken to this study. Firstly, baseline hormone concentrations were identified for the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), and cortisol. This involves comparison and extrapolation. Secondly, possible risk effects were examined by comparing levels of OCs in captive and free-ranging Steller sea lions to known effects in related species with known physiological thresholds. Serum concentrations of total T_4 were highest in Steller sea lions followed by total T_3 concentrations. Concentrations of free T_4 and free T_3 were three to four orders of magnitude lower. Concentrations for all four thyroid hormone measurements tended to a lower level as animals matured beyond the neonatal stage. When thyroid hormones from captive sea lions were evaluated across seasons, all thyroid hormones were highest in the July to September period. Cortisol concentrations were similar in male and female pups. Cortisol varied with age but when considered in regards to time of year when sampled, followed a seasonal pattern. Cortisol was elevated in fall months in captive sea lions (non-pups), which is similar to what is seen in other marine mammals and is likely associated with the annual molt. Male pups from Alaska had lower levels of $\sum$PCBs and $\sum$DDT when compared to male pups from Russia. Female pups from Alaska were significantly lower in $\sum$PCBs than Russian female pups as were female pups for $\sum$DDT levels between areas. Anywhere from 12 to 64% (depending on rookery) of Steller sea lion pups investigated for contaminants had concentrations of $\sum$PCBs that are high

enough to cause physiological problems. Concentrations in blood taken monthly for 2 years in three captive Steller sea lions were similar at any given sampling time and followed a seasonal pattern with levels significantly higher in the summer months of July to September and lower in the three month winter period January to March. Concentrations of OCs in blubber samples collected quarterly for the captive females followed an analogous pattern to blood samples but the captive male sea lion was considerably lower and declined over the study period. A significant relationship between blubber contaminants and lipids was noted in the three captive Steller sea lions. Even though OC contamination has not been hypothesized to be the primary factor that precipitated the population decline, there is a potential for these chemicals to have a negative effect on the health of free-ranging Steller sea lions. These data suggest that concentrations of OCs in Steller sea lions may be high enough to cause endocrine or reproductive dysfunction and could potentially impact fertility or fecundity. Therefore, OC contaminants can not be dismissed as a contributing source to either the decline or the failure to recover of the Steller sea lion population.

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Preface

The work contained in this dissertation is that of Matt Myers. In a tragic situation, Matt died in a scuba diving incident in September 2007. Fortunately he had defended his Ph.D. dissertation successfully in May 2007, although the written document needed some work. As such, all of the chapters of this dissertation were originally written by Matt, however there are substantial changes to what Matt originally wrote and what is presented in the ensuing pages of this dissertation. The chapters had the following status at the time of Matt's death:

Chaper 1 – Published in Comparative Biochemistry and Physiology, a peer-reiviewed international journal in 2006.

Chapter 2 – Unpublished but I recently submitted it with the assistance of Ms. Angie Steeves, and it has been conditionally accepted pending revision.

Chapter 3 – Submitted and accepted for publication pending revision. Ms. Gina Ylitalo, Dr. Peggy Krahn and I completed the revisions and this chapter was published in April 2008.

Chapter 4 – Unpublished but I plan to submit it this spring.

These and Matt's other scientific papers and abstracts are listed at the end of this preface.

Although Matt's acknowledgements were not completed, I am sure he would want to thank his committee for their support and encouragement throughout his tenure as a graduate student at the University of Alaska Fairbanks.

1. Dr. Lorrie Rea was the first committee member that Matt approached for guidance. She has co-authored Matt's first peer-reviewed manuscript and co-authored grants to help fund his work.
2. Dr. Peggy Krahn opened her laboratory for Matt to learn the assays for organochlorines. Her staff, especially Ms. Gina Ylitalo, provided the guidance to help Matt fulfill his dreams to study the impacts of chemical contaminanats in our environment.

3. Dr. Michael Castellini provided mentoring for Matt when he spent a semester in Fairbanks and was always available for guidance.
4. Dr. Alexander Burdin provided samples that were unique in nature and created an excitement in Matt's scientific direction that Matt loved.
5. Dr. Jo-Ann Mellish gave valuable guidance especially in the area of writing. Her efforts were needed and valued.

In addition to Matt's committee, much of his work was focused in my endocrinology laboratory. The guidance and cammaraderie of Kendall Mashburn, Jacqueline Mitchell, Lisa Petrasuskas, Daniel O'Neil, Beate Litz, Mandy Keogh and numerous other students and researchers in my lab was a focal point for Matt. A multitude of other students and staff at Alaska SeaLife Center (ASLC) were always there for Matt and he thrived on their friendship, including Jason Waite, John Maniscalco, Monica Bando, Daniela Maldini, Peter Nilsson, Chuck Adams, Susan Inglis, Kathrin Huelck, Caroline Oki, and many others.

Matt's project would not have been possible without the support of Mr. Don Calkins, Dr. Tom Loughlin, Dr. Ken Pitcher, Dr. Pam Tuomi, Dr. Qing Li, Dr. Lisa Mazzaro, Dr. Vladimir Burkanov, Dr. Dan Hennen, and the husbandry, veterinary and research departments staff at ASLC, and the adminsistrative staff at Seward Marine Center.

Without a doubt this dissertation could not have been completed without the support of Ms. Angie Steeves. Her time spent in editing changes from various committee members and co-authors, as well as formatting to UAF standards was irreplaceable. I greatly appreciate her help and I know Matt would be indebted to her for her efforts. In addition, the awarding of this degree posthumously would not have been possible without the support of Drs. Denis Wiesenburg and Mike Castellini and the UAF administration. Their support is greatly appreciated.

Matt's work was funded by the Pollock Conservation Cooperative Research Center (PCCRC), the Alaska SeaLife Center's Steller Sea Lion Research Program with funds from National Marine Fisheries Service, and U.S. Fish and Wildlife Service

Pacific Islands Office. In addition, PCCRC awarded a grant to establish the Matt Myers Memmoral Graduate Student Travel Fund, to assist two graduate students in Marine Biology and Fisheries, per year to attend a scientific conference for the next three years. For his memory, we are all thankful for PCCRCs generosity.

Most importantly I know Matt would want to thank his devoted wife, Michelle, and his two sons, Jacob and Cody. Without their love and support, along with that from his much larger extended family, Matt could not have fulfilled his vision of a life he felt was close to perfection.

Shannon Atkinson, Ph.D.
Professor of Marine Sciences
Committtee Chair for Ph.D. Candidate Matt Myers
June 2008

A full list of Matt's scientific work is presented on the following pages.

Scientific Peer-Reviewed Publications that have been authored or co-authored by Matt Myers during his tenure as a graduate student.

1. **Myers, M.J.**, Rea, LD. and Atkinson, S. 2006. The effects of age, season and geographic region on thyroid hormones in Steller sea lions (*Eumetopias jubatus*) Comp. Biochem. Phys. A 145,90-98.
2. Ylitalo, G.M., **Myers, M.**, Stewart, B.S., Yochem, P.K., Braun, R., Kashinsky, L., Boyd, D., Antonelis, G.A., Atkinson, S., Aguirre, A.A. and Krahn, M.M. 2008. Organochlorine contaminants in endangered Hawaiian monk seals from four subpopulations in the Northwestern Hawaiian Islands. Mar. Pollut. Bull. 56,231-244.
3. **Myers, M.J.**, Ylitalo, G.M., Krahn, M.M., Boyd, D., Calkins, D., Burkanov, V. and Atkinson, S. 2008. Organochlorine contaminants in endangered Steller sea lion pups (*Eumetopias jubatus*) from Western Alaska and the Russian far east. Sci. Total Environ. 396(1),60-69.
4. **Myers, M.J.** and Atkinson, S. In Review. The effects of age, season and geographic region on circulating cortisol concentrations as a biomarker of stress in threatened and endangered Steller sea lions (*Eumetopias jubatus*). Gen. Comp. Endocr.
5. **Myers, M.J.** and Shannon Atkinson In Prep. Temporal variability in organochlorine contamination in both blood and blubber in captive Steller sea lions (*Eumetopias jubatus*)

Scientific abstracts presented either orally or via poster by Matt Myers during his tenure as a graduate student.

1. Atkinson, S., **Myers, M.** and Rea, L. 2001. Thyroid and cortisol hormone concentrations in Steller sea lions (*Eumetopias jubatus*). 14th Biennial Conference on the Biology of Marine Mammals. (Poster presentation) VanCouver, BC. Canada

2. **Myers, M.,** Atkinson, S. and Rea, L. 2001. Development of an index for metabolic condition in Steller sea lions *(Eumetopias jubatus)* utilizing leptin hormone concentrations. 14th Biennial Conference of the Biology of Marine Mammals. (Poster presentation), Vancouver, BC. Canada
3. **Myers, M.** and Atkinson, S. 2002. Thyroid hormones and the effects of contaminants in Steller sea lions (*Eumetopias jubatus*). e.Hormone Conference. (Poster presentation), New Orleans, LA.
4. **Myers, M**. Rea L.D., Mashburn, K.L. and Atkinson. S. 2003. Thyroid and cortisol hormones as an indication of metabolic function and well-being in Steller sea lions (*Eumetopias jubatus*). Marine Science in the Northeast Pacific: Science for Resource Dependent Communities, (Oral presentation), Anchorage, AK.
5. Atkinson, S., Hong, S.M., Campbell, S., **Myers, M.,** Springer, A. and Li, Q. 2003. Organochlorine contaminants in tissues from two subarctic pinnipeds. 15th Biennial Conference on the Biology of Marine Mammals. (Oral presentation), Greensboro, NC.
6. **Myers, M**. and Atkinson, S 2003. The effect of contaminants on hormone function in Steller sea lions. 5th Biennial Conference on the Biology of Marine Mammals, (Oral presentation), Greensboro, NC.
7. **Myers, M.** and Atkinson, S. 2004. Chemical contamination levels in Steller sea lion pups from Southwest Alaska and Russian far east. 22nd Wakefield Fisheries Symposium. Sea Lions of the World: Conservation and Research in the 21st Century, (Poster presentation), Anchorage, AK.
8. **Myers, M.** and Atkinson, S. 2004. Chemical contamination levels in Steller's sea lion pups from southwest Alaska and the Russian far-east. 22nd Wakefield Fisheries Symposium. Sea Lions of the World: Conservation and Research in the 21st Century, (Oral presentation), Anchorage, AK.
9. Atkinson, S., **Myers, M.** and Ylitalo, G. 2004. Contaminants in North Pacific Basin Marine Mammals. 3rd International Conference of Marine Mammals of the HolArctic (Oral presentation), Ukraine, Crimea, Koktebel.

10. **Myers, M.** and Atkinson, S. 2004. Organochlorine contamination in Steller sea lion pups from four Russian rookeries. 3rd International Conference of Marine Mammals of the HolArctic, (Oral presentation), Ukraine, Crimea, Koktobel.
11. Atkinson, S., **Myers, M.** and Ylitalo, G. 2005. Contaminants in North pacific basin marine mammals. Marine Science of Alaska Symposium, (Oral presentation), Anchorage AK.
12. Atkinson, S., **Myers M.** and Ylitalo, G. 2005. Organochlorine contaminants in pinnipeds and cetaceans from the North Pacific. 36th Annual IAAAM Conference (Oral presentation), Seward AK.
13. **Myers, M.** and Atkinson, S. 2005. Contaminants in Steller sea lions (*Eumetopias jubatus*). 36th Annual IAAAM Conference (Oral presentation), Seward AK.
14. **Myers, M**. and Atkinson, S. 2005. Contaminants in Steller sea lions (*Eumetopias jubatus*) and other marine mammals from the North Pacific. Marine Mammal Conference, (Poster presentation), San Diego, CA.

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General Introduction

Background and relevance

In the North Pacific Ocean, the western Steller sea lion (*Eumetopias jubatus*) population has been decreasing over the past several years. Over the past 40 years, the numbers of Steller sea lions in the United States have declined by about 50% (Braham et al., 1980, Calkins et al., 1999). This trend has led to the species being listed as endangered under the Endangered Species Act (specifically, the western stock of Steller sea lions is listed as endangered and the eastern stock as threatened) and the subsequent development of specific recovery plans for the species. As part of the directives in the plan, requirements include investigation into pollution as a proximate cause for the decline or the failure of the population to recover.

The ecological consequence of manufacturing industrial and agricultural chemicals on a massive scale has lead to large amounts of these compounds being released into the environment. Organochlorines (OCs) are a diverse group of compounds synthesized for various purposes including pesticides and lubricants in machinery. Many of the OCs are highly fat soluble (lipophilic) but have low water solubility (hydrophobic) and differentially accumulate in the lipids of animals. Designed for chemical stability, some of the OCs are extremely persistent in the environment and resistant to metabolic degradation, thereby increasing in concentrations as the compounds move up food webs (O'Shea, 1999). Due to the fact that the oceans of the world act as a transport for many of these compounds and airborne chemicals are deposited in cold air sinks (Wania and Mackay, 1993), many of these chemicals find their way into the habitat of North Pacific pinnipeds (Iwata et al., 1993).

Synthetic chemicals, such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), have potent biological effects including immunosuppression, carcinogenicity, reproductive toxicity, neurotoxicities, and disturbances in energy metabolism. The mechanism by which these compounds are thought to cause these effects is by disrupting normal hormonal pathways in biota. Endocrine disruptors act by either enhancing or interfering with the actions of natural

hormones. Because hormones are especially important in regulating immune function, reproduction, and development, the effects of endocrine disruptors may be the greatest on these processes (USEPA, 1997). Endocrine disrupting compounds can express adverse effects as especially persistent parent compounds and as metabolized congeners. Similarly, several PCB metabolites are so structurally similar to thyroxine they can compete for thyroxine binding sites on the transport protein, transthyretin (TTR) (Lans et al., 1993). This competitive binding by OCs can cause reduction in plasma tetraiodothyroxine (T_4) levels in rodents (Lans et al., 1993, Brouwer et al., 1998). Hydroxylated metabolites of PCBs have been shown *in vitro* to have binding affinities 10 times greater for TTR than for T_4 (Lans et al., 1993). Bergman et al. (1994) found that this preferential binding resulted in the persistence of these metabolites in blood of both seals and humans exposed to PCBs in the environment. Competitive binding by PCB metabolites with T_4 for TTR or other endogenous proteins could cause toxic effects such as thyroid dysfunction resulting from the imbalance in the synthesis and regulation of thyroid hormones (Klassen-Wehler et al., 1998). A recent study on harbor seals (*Phoca vitulina*) indicated that PCBs can cause immunosuppression (deSwart et al., 1996). The seals were fed fish that was originally destined for human consumption. The reference group received relatively uncontaminated fish, and the experimental group received fish containing PCBs. After a two-month exposure period, lymphocytes in the experimental group were severely reduced. PCBs have also been found to cause thyroid hormone deficiency in the harbor seal (Brouwer et al., 1998). Chlorinated pesticides have been linked to premature births in pinnipeds (DeLong et al., 1973). California sea lions (*Zalophus californianus californianus*) in California's Channel Islands experiencing premature births were found to have two to eight times more organochlorine pesticides and PCBs than populations with normal births.

The present investigation helped to establish baseline data that indicate normal hormonal levels. The physiological effects of thyroid hormones are protean, accounting for the myriad of symptoms seen in thyroid disorders. These effects include protein synthesis, cell growth and differentiation, critical effects on the maturation of the central

nervous system and skeleton, maintenance of the oxidative metabolism and heat production, and maintenance of the cardiovascular function. The circulating levels of thyroid hormone affect muscle tone, deep tendon reflexes and maturation of the epidermis. Thyroid hormones occur in either a free or bound state *in vivo.* In humans, 80% to 90% of secretory output product of the thyroid gland is T_4 and 10% is T_3. T_3 is three to four times as potent as T_4 and 75% of T_3 is synthesized from T_4. Secreted T_4 and T_3 circulate in the bloodstream almost entirely bound to proteins. Normally, only 0.03% of the total plasma T_4 and 0.3% of the total plasma T_3 are in the free-state. About 70% to 80% of the circulating T_4 and T_3 are bound to TBG (thyroxine-binding globulin); the remainder is bound to albumin and to a thyroid-binding prealbumin (TBPA). T_4 stimulates mitochondrial respiration and oxidative phosphorylation. T_3 and T_4 increase both the number of mitochondria and the number of their cristae. Mitochondrial protein synthesis is increased, while degradation of their proteins is decreased. Thyroid hormones also influence body growth and the development of the nervous system during fetal life (Genuth, 1988).

Cortisol is a glucocorticoid that is essential to life by virtue of its effects on carbohydrate and protein metabolism. In addition, cortisol is released in response to stressful stimuli. The most important overall action of cortisol is to facilitate the conversion of protein to glycogen. In addition, cortisol is involved in facilitating fat metabolism, supporting vascular responsiveness and modulating central nervous system function. Cortisol also affects skeletal turnover, muscle function, immune responses and renal function. In addition, cortisol shows a pronounced circadian rhythm. Peak daily cortisol concentrations usually occur just prior to or immediately after awakening and coincide with the onset of locomotor activity. This programmed elevation of cortisol concentrations increases blood pressure and cardiac output prior to the active phase of the day and is not controlled by an increase in activity levels. In regards to stress, studies have shown that, in addition to epinephrine and norepinephrine, an entire assortment of hormones are known to be involved in the mediation of stress and change in concentrations over the course of the stress response. Initially, stress causes the release

of epinephrine from the adrenal medulla and of norepinephrine from the sympathetic nervous system. Within minutes of the onset of the stressor, the adrenal cortex begins to secrete cortisol. Epinephrine and cortisol are commonly known as the stress hormones, despite the fact that their major endocrine functions involve metabolism. Levels of epinephrine and norepinephrine return to normal quickly after removal of the stressor. However, cortisol levels remain high for longer periods (Genuth, 1988).

In marine mammals, such as pinnipeds, the physiological role of fat is slightly different than in other, terrestrial mammals. Marine mammals are dependent on fat for thermoregulation against their relatively cool water environment, for buoyancy, and energy storage. Many pinnipeds undergo seasonal fasting periods during summer breeding events. For example, female Steller sea lions may fast for one to two weeks while nursing a new pup. Males defending a territory may fast four to five months while maintaining extremely active energy expenditures, fighting to control territory and access to females. Also, newborn pups may fast for days at a time waiting for 'mom' to return from a foraging trip with sustenance. During any of these phases, animals will lose body fat. Unlike other mammals, this seasonal or developmental metabolization of blubber energy stores is a product of the evolution of these species to a mostly-aquatic lifestyle. Adipose tissue is known to maintain the largest energy reserves in the animal body. In addition to this role, recent research suggests that adipocytes may function as a center in the regulation of energy management. Adipose tissue is responsive to a number of endocrine signals (Hwang et al., 1997). An adipocyte-specific gene, the obese gene, was recently identified and found to encode a hormone that plays a major role in the regulation of energy intake and expenditure. Leptin, commonly referred to as the ob or obesity protein, is a peptide hormone secreted by adipocytes (Campfield et al., 1996). Leptin, and the recent elucidation of the fundamentals of its regulatory physiology, has been identified as a plausible candidate for a humoral signal with the requisite endocrinology and neurobiology that may act to integrate somatic energy stores, energy expenditure and fertility (Rosenbaum and Leibel, 1998). The specific molecular and biochemical pathways of action associated with leptin are still being intensively studied.

However, it is thought that leptin acts as a negative feedback signal to satiety centers in the hypothalamus to regulate body energy stores. High levels of leptin, found in association with abundant adipose reserves, are secreted and signal the brain to regulate energy balance or in this case, decrease food intake. Research on mice, lemmings, humans and non-human primates has shown that leptin is a hormone secreted from the adipose tissue that acts on the central nervous system centers to influence food intake and energy balance. The role of leptin in comparative animal systems has received much less attention to date, although significant advances have been made in the study of hibernating mammals (Ormseth et al., 1996, Boyer et al., 1997). The gene that codes for the production of leptin is expressed only in adipose tissue and leptin is produced and secreted in proportion to the size and number of fat cells present. In several species studied to date, it has been demonstrated that a close correlation exists between the blood leptin concentration and total body fat mass (Frederich et al., 1995, Nagy et al., 1998).

In an important study done in northern fur seals, Beckmen et al., (1999) found correlations between contaminants with thyroid hormones and retinol, a vitamin associated with growth. Retinol levels in northern fur seal neonates were negatively correlated to PCBs. Serum retinol levels in neonates were also negatively correlated to the Toxic Equvalents Scheme (TEQs) in the perinatal period. Total T_4 was also negatively correlated with contaminants (Beckmen et al., 1999).

The investigation into the cause of the decline of the western Steller sea lion population will not produce a reputable result unless there is a reliable and quantitative procedure to assess the physiological and metabolic condition of specific animals and groups. At the present time, most procedures to measure body condition or analysis of body fat are too invasive and involved to use in the field on a large scale. It is one objective of this study to develop and investigate the suitability of using only a blood sample and the subsequent analysis using a combination of tests based upon circulating hormone levels that will provide usable indications of fitness in free-ranging animals. In addition, any detrimental effect of organic pollutants must be investigated to either substantiate current research trends or direct new investigations.

Many marine mammals go through remarkable periods of fasting followed by periods of fat amassing. As the fat is utilized for energy during fasting, OC concentrations in the remaining fat may increase, changing the balance *in vivo*. Physiological effects can be measured at different levels of biological organization, from the ecosystem level down to the molecular. There is a significant problem in that biomarkers measurable at a molecular level respond early but are not readily interpreted ecologically. There are often considerable species differences in sensitivity to specific OCs as well as differences in response. It is, therefore, often difficult to generalize results found in one species to another species. Other factors such as fat dynamics, delayed implantation, differences in physiology, and toxicokinetics may make animals more or less susceptible to the effects of OCs. A wide range of effects are currently being used as biological markers for OC exposure. These include, among other things, effects on reproduction, development, cytochrome P450 enzymes, the immune system, the adrenals, the thyroid gland, thyroid hormone levels and vitamin A levels (Kannan et al., 2000).

Two approaches have generally been taken in identifying and estimating the risk for possible effects (de Wit et al., 2004). The first involves comparison and extrapolation by studying the risk of possible effects when comparing levels of OCs in a particular species to known physiological thresholds. The difficulties in extrapolation relate generally to differences in sensitivity, (i.e. fish vs. birds vs. mammals) (Kim and Hahn, 2002). Comparison and extrapolation have some intrinsic weaknesses, however. It is difficult to extrapolate the toxic effects seen at high acute doses to possible adverse effects at lower but chronic exposures. Another approach studies physiological effects by probing subtle indicators of biological responses or biomarkers, to contaminants. "Examination of the animals for responses known to be associated with the contaminants found in their tissues is perhaps the only way to make a convincing case either for or against the hypothesis that trace contaminants are acting biologically on the animals" (de Wit et al., 2004). Changes at the molecular level are the most common type of biomarker (McCarthy and Shugart, 1990, Huggett et al., 1992, Peakall, 1992). Biomarkers are

typically measures within an accepted norm that arbitrarily measure abnormal values as a result of exposure to some contaminant, and elevated levels of organochlorines were measured in Steller sea lions in the 1970's (Lee et al., 1996). Based on these different types of studies, a weight-of-evidence argument can be established. For example, it was concluded that PCBs provoked the 1988 morbillivirus-related epizootic in harbour seals in northwestern Europe (Ross et al., 1996, Vos et al., 2003).

Objectives

The primary goal of this study was to investigate any relationship between the body burden of organic pollutants and variation in homeostasis as interpreted by hormone levels. A secondary objective was to develop an index or tool for the assessment of metabolic condition in free-ranging Steller sea lions based upon circulating hormone levels. These goals were accomplished through the successful completion of the following objectives:

1. Determine baseline thyroid and cortisol hormone levels in Steller sea lions with components for temporal, seasonal and cohort variation.
2. Survey PCBs and congeners, DDT, DDE and DDD in a select sub-sample of the population and consider the level and effect of organochlorines on circulating hormones.

Methodology

Hormone and OC Assays:

Thyroid hormones T_4, T_3 and cortisol concentrations were measured in both serum and plasma blood samples by direct assay using solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). In these assays, ^{125}I-labeled hormone competes for a fixed time with hormone in the animal sample for binding sites on a highly specific antibody that is coated onto the test tube. After a specified time, the supernatant is decanted causing immobilization of the antibody, termination of competition and isolation of the antibody-bound fraction of the radiolabeled hormone. A gamma counter yields counts per minute for each sample, which are converted to a concentration via a calibration curve. A standard curve for each assay is log-logit

transformed, enabling extrapolation of sample concentration. Nonspecific binding and the sensitivity of the assay used are calculated. Nonspecific binding of other proteins from the sample are considered minimal only if the antibody is highly specific. Sensitivity is indicated by the detection limit or minimal detectable dose defined as the apparent concentration at 95% maximum binding/nonspecific binding to specify that samples fell into the range.

The analysis techniques for cortisol and thyroid hormones were validated for Steller sea lions in this project. Tests of parallelism and linearity under dilution were performed to verify that the assays used were applicable to Steller sea lions. Samples were analyzed in batches to reduce inter-assay variation. Controls of a known hormone concentration, pooled plasma and serum sample and a specific calibration curve were run with each assay to calculate intra and inter-assay variation.

Organochlorines were measured in blood and blubber samples. All samples were analyzed for PCBs and congeners, plus DDT, DDE and DDD. All samples were analyzed at the NMFS Northwest Fisheries Science Center in Seattle, Washington. All methods followed those outlined in Hyvarinen and Sipila (1984), Krahn et al. (1993), Menchero et al. (1994). The procedure in Seattle was a HPLC/PDA (high-performance liquid chromatography with photodiode array detection) method that was developed to rapidly screen for toxic "dioxin-like" CBs and congeners.

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CHAPTER 1.

The effects of age, season and geographic region on thyroid hormones in Steller sea lions (*Eumetopias jubatus*)*

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Abstract

The purpose of this study was to investigate thyroid hormone concentrations, thyroxine (T_4) and triiodothyronine (T_3), in order to determine basic levels in Steller sea lions of different ages and over seasons. Serum concentrations of total T_4 were highest in Steller sea lions followed by total T_3 concentrations. Concentrations of free T_4 and free T_3 were three to four orders of magnitude lower. Concentrations for all four thyroid hormone measurements tended to a lower level as animals matured beyond the neonatal stage. When thyroid hormones from captive sea lions were evaluated across seasons, all thyroid hormones were highest in the July to September period. When compared across the geographic range, animals in southeast Alaska tended to have lower thyroid hormone levels, while the Steller sea lions west of Prince William Sound and animals from the Russian Far East had significantly higher concentrations. Significant inter-annual differences in concentrations were also observed across the geographic range. With an understanding of the basic changes in thyroid hormone concentrations, changes in plane of nutrition or life history states (i.e. fasting, pregnancy or lactation) can now be evaluated for their effect on the overall health of this endangered species.

Introduction

Steller sea lions (*Eumetopias jubatus*) range from the California Channel Islands along the North Pacific Rim to northern Japan. In the 1970's, the greatest concentration of animals occurred in the western Gulf of Alaska and along the eastern Aleutian Islands. This portion of the population has experienced a dramatic decline over the last 30 years. Overall, Steller sea lion population numbers decreased more than 80% since 1976 (Sease et al., 2001) and about 70% since 1985 (Calkins et al., 1999). This decline led to the species being listed as threatened under the U.S. Endangered Species Act in 1990 (55 U.S. Federal Register 49204). In 1997, genetic information and population trends indicated that distinct stocks existed (Bickham et al., 1996, Loughlin, 1997). Subsequently, the western stock, which occurs from 144° West longitude (just east of Prince William Sound, Alaska) to Japan, was listed as endangered (62 U.S. Federal Register 24345). The eastern stock in southeast Alaska to California remains listed as threatened.

Nutritional stress has been one of the leading hypotheses related to the decline of the Steller sea lion population. If animals are perishing as a result of either low food resources or poor food quality, then this should be reflected in the circulating thyroid hormone concentrations of the animal. In mammals, thyroid hormones relate directly with energy intake; the responses are rapid and indicate a continuous ongoing regulation of metabolism in relation to caloric supply (Eales, 1988). Many actions of thyroid hormones are mediated through the effects of these hormones on stimulation of cellular protein synthesis (Hadley, 2000). Thyroid hormones effect mammalian metabolism by a calorigenic or thermogenic action and specific effects are related to carbohydrate, lipid and protein metabolism (Norris, 1997). T_4 stimulates mitochondrial respiration and oxidative phosphorylation, which in turn, increases heat production (thermogenesis) and increases the animal's metabolic rate (Hadley, 2000). Thyroid hormones are also indispensable for normal growth and development and altered thyroid hormone concentrations at critical developmental periods may be a special concern, especially during early development (Porterfield, 1994, Porterfield and Stein, 1994).

The structure and function of the thyroid gland is similar in most mammals (Hadley, 2000, Norris, 1997). In the blood, thyroid hormones occur in either a free or bound state *in vivo.* Secreted T_4 and T_3 circulate in the bloodstream almost entirely bound to proteins. T_4, which is considered the less biologically active form of the hormone, is produced in much greater quantity than T_3. T_4 is converted to T_3 by monodeiodination and this conversion of T_4 is a dominant source of T_3 in the circulation. T_3 is the major physiologically active thyroid hormone regulating cellular activity in many species, although T_4 is still an active hormone (Hadley, 2000).

In several marine mammals, thyroid hormone concentrations decrease with postnatal development and maturation (harbor seals *Phoca vitulina,* Haulena et al., 1997, gray seals *Halichoerus grypus*, Hall et al., 1998, Woldstad and Jenssen ,1998; harp seals *Pagophilus groenlandicus*, Engelhardt and Ferguson ,1980, Leatherland and Ronald, 1979; southern elephant seals *Mirounga leonina*, Little, 1991). St. Aubin et al., (1996) also found that free T_4 declined with maturity in wild dolphin (*Tursiops truncatus*) populations. Thyroid hormones have also been shown to vary with season and are associated with the annual molt of some marine mammals. Although there is some inconsistency in the literature, the majority of reports indicate that thyroid hormones are very important regulators in the molt. Boily, (1996) showed an increase in thyroid hormones in association to the molt in captive gray seals. Harbor seals also exhibited higher thyroid hormone concentrations in summer months (Renouf and Noseworthy, 1991), which were associated with the conclusion of the molt (Riviere et al., 1977). Ashwell-Erickson et al., (1986) also looked at thyroid concentrations associated to molt in the harbor seal and in the spotted seal (*Phoca largha*). Thyroid levels increased to a seasonal high in the late phases of the molt for both species. Harp seals were also shown to increase thyroid concentrations during the molt phase (John et al., 1987). In most marine mammals studied, thyroid hormone concentrations were consistently higher in the late summer, mainly associated with the late phase of the molt. Additionally, some of the lowest yearly concentrations are reported at the onset of molt.

The present study investigated thyroid hormone concentrations in free-ranging and captive Steller sea lions of various ages in order to determine basal concentrations and trends through development and over seasons. The intent was to clarify thyroid concentrations of different age and sex classes of Steller sea lions under the hypothesis that thyroid hormones in Steller sea lions would decrease with age, vary with season and not with sex. In addition, a comparison between different geographic areas, within the Steller sea lions range, was investigated to compare the discrete genetic populations.

Materials and Methods

Animal selection and sample collection

In this study, 1385 separate blood samples were collected from 1165 free-ranging and captive Steller sea lions (free-ranging n=1156, captive n=9). The majority of samples (n=1091) were collected in collaboration with the Alaska Department of Fish and Game (ADF&G) and National Marine Fisheries Service (NMFS) during the field seasons of 2000, 2001 and 2002. Of the 1091 blood samples collected in 2000, 2001 and 2002, all known aged animals were pups (n=1006) or juvenile animals (age 1 to 3 years, n=85). Although most of the free-ranging samples were from recent years, one sub-group of archived adult samples (age 4 to 15 years) was included and consisted of samples from harvest and subsistence taken as early as 1976 (n=65, collected 1976 to 1997). Captive samples were collected from nine animals (5 males and 4 females) at the Vancouver Aquarium and later from three of the same animals that were housed at the Alaska SeaLife Center in Seward, Alaska (n=229).

Free-ranging samples came from as far west as the Kuril Islands in Russia across the Aleutian Islands and the Gulf of Alaska to southeast Alaska, the majority of the full geographical range of Steller sea lions. Blood samples were taken by venipuncture from a rear flipper or the caudal gluteal vein. Samples were taken throughout the year and the specific time of year is reflected in the animal's age as the cohort matured. All samples from one month old pups were taken at approximately the same time each year and the age of one month was based on a median birthday for that cohort. All blood samples were taken between 9 am and 4 pm for free-ranging animals but the specific time within

that period varied and may account for some variation in thyroid hormone concentrations. However, as the time to process all animals at a given site was significant, the variation would be more likely within a specific site as the average time of sampling between sites was similar. Whole blood was kept cool on ice packs until centrifugation and was typically processed within 4 to 6 hours of collection. Serum or plasma was separated and kept frozen at -80°C until analysis. Animals were weighed and length measured in the field. In order to investigate seasonal variability of these hormone concentrations, monthly samples were collected from a group of captive Steller sea lions housed outdoors in natural seawater and under natural photoperiod. Longitudinal seasonal samples from these sea lions were evaluated in four seasons, January to March, April to June, July to September and October to December.

Hormone measurements

Four forms of thyroid hormones, total T_4, total T_3, free T_4 and free T_3, were measured in both serum and plasma samples using solid phase radioimmunoassay (RIA). All kits were manufactured by Diagnostic Products Corporation, Los Angeles, CA (Total T_4 catalog number TKT41 or 45, Total T_3, catalog number TKT31 or 35. Free T_4, catalog number TKF41 or 45, Free T_3, catalog number TKF31 or 35). Mean nonspecific binding (NSB), which is the percentage of radioactive hormone that binds in the absence of antibody divided by the total counts, for each hormone was total T_4=0.88%, total T_3=0.87%, free T_4=0.71% and free T_3=0.76% (n=10 assays per hormone). The lower limit of sensitivity of assays for each hormone was total T_4=0.51 ng/ml, total T_3=0.09 ng/ml, free T_4=3.5 pg/ml and free T_3=0.1 pg/ml (n=10 assays per hormone).

The analysis technique for each of the four hormones measured was validated for Steller sea lions. Tests of parallelism and linearity were performed on each hormone to verify that the assay used was applicable to Steller sea lions. Parallelism was tested by combining 50% of each standard concentration calibrator with 50% of a pooled sample of Steller sea lion serum. Next, regression of pure standard concentration calibrators was compared with the mixed calibrators. Standard curves were parallel and slope values varied less than 10%. The slope of total T_4 with standard calibrators was -0.95 and the

slope of calibrators mixed with pooled serum was -1.03. The slope of total T_3 with standard calibrators was -1.10 and the slope of calibrators mixed with pool was -1.05. The slope of free T_4 with standard calibrators was -0.58 and the slope of calibrators mixed with pool was -0.59. The slope of free T_3 with standard calibrators was -0.79 and the slope of calibrators mixed with pool was -0.74. In addition, linearity tests utilizing various amounts of a pooled sample of Steller sea lion sera (1/2, 1, 2 and 4 times the recommended amount) were assayed and regression applied to verify that as the amount of the sample changes, the relationship of hormone concentration in the pool sample to the standard curve is maintained. For total T_4 r^2=0.99; total T_3 r^2=0.99; free T_4 r^2=0.96; and free T_3 r^2=0.97. Controls of a known hormone concentration and pooled plasma and serum samples were run with each assay to calculate intra and inter-assay variation. Intra-assay coefficients of variation were <5% for all assays. Samples were analyzed in batches to reduce inter-assay variation. Inter-assay coefficients of variation utilizing the median control were total T_4=11.8%, total T_3=7.9%, free T_4=10.8% and free T_3=8.2%. Regression of plasma versus serum was correlated for each of four assays (n=30, total T_4 r^2=0.72 P<0.001, total T_3 r^2=0.74 P<0.001, free T_4 r^2=0.81 P<0.001 and free T_3 r^2=0.71 P<0.001) and justified using the two interchangeably.

Data Analysis

All hormone concentrations were determined after a log-logit transformation of the standard curve (Rodbard, 1974). For hormone concentration plots versus age group, animals that were less than one year were considered pups. Animals one year to three years old were considered juveniles and animals older than four years as adults.

Descriptive statistics were employed to illustrate ranges and mean concentrations. Linear regression was utilized to characterize the relationships between the different forms of thyroid hormones. One-way ANOVAs (for groups of three or more) and t-tests (for two groups) were used for comparisons of group means. For all tests completed, a P-value of less than 0.05 was chosen as the level of significance, however, where multiple comparisons were performed Bonferroni adjustments were applied by multiplying the resulting p value by the number of pair-wise comparisons.

Results

Overall, there were no differences between males and females. Therefore, sex was disregarded in all further statistical analyses. Utilizing the entire data set for general analysis, we discovered that almost 97% of the circulating thyroid hormones in Steller sea lions is thyroxine (T_4) and just a little over 3% is triiodothyronine (T_3). Our work also shows that in Steller sea lions 0.02% of T_4 and 0.01% of T_3 is in the free-state. Table 1.1 shows a correlation matrix for all four forms of thyroid hormone. Total T_4 and free T_4 were the most highly related to each other (0.77). Total T_3 was similar to total T_4 and free T_4 more that 50% of the time (total T_3 versus total T_4 = 0.53, total T_3 versus free T_4 = 0.55). Free T_3 was the least associated to any of the other forms of thyroid hormones (Table 1.1).

In order to consider the effect of growth and development on thyroid levels, thyroid hormone concentrations were evaluated for the entire free-ranging data set (Fig 1.1, Table 1a). In all four thyroid hormones measurements, pups had the highest concentrations measured in this study and adults had the lowest concentrations.

In an area comparison between Steller sea lion pups from southeast Alaska, southwest Alaska and Russia for both 2001 and 2002, southeast animals had the lowest concentrations for all four hormone measurements in both years. In 2001, Russian animals had the highest concentrations for total T_4 but southwest animals were higher for total T_3 and free T_4 and T_3. In 2002, Russian animals had the highest concentrations for all four hormone measurements (Figure 1.2, Tables 1b and 1c). In 2002 for free T_3, a one-way ANOVA between groups showed significant differences between two of the three areas (Russia vs. southeast Alaska, P=<0.001; Russia vs. southwest Alaska, P=0.029). There was not a difference between southwest Alaska and southeast for free T_3 in 2002 (P=0.056). Trends for all hormones by area were similar between years except for free T_3. In 2001 for free T_3, all areas were far less than the same area in 2002.

In a seasonal comparison of thyroid hormones for captive animals, all four hormone measurements were highest in late summer (July to September, Figure 1.3, Table 1d), when grouped as three-month seasons. For total T_4, total T_3 and free T_4 the

season with the highest concentrations measured was the July to September, which was significantly different than all other seasons. For free T_3, a one-way ANOVA between groups showed the only significant difference for the April to June season compared to the July to September season (April to June vs. July to September, P=0.001). In addition, the October to December period showed the lowest concentrations of the year for all hormones measured except free T_3 (where April to June was the lowest of the year).

Table 1.2 is included as a reference to show ranges, means and comparative concentrations between groups. Groups include free-ranging pups, juveniles and adults and captive juveniles and adults.

Discussion

This study established background information for four forms of thyroid hormone measured in Steller sea lions of various ages. There was no literature encountered that indicated any of these hormones had been previously investigated for Steller sea lions. Thyroid hormones are one component of a large complex network of responses to a number of environmental and physiological factors, many of which also influence growth, development, and metabolism. Thyroid hormones are involved in the regulation of energy management in mammals, functioning primarily to help control basal metabolic rate by regulating lipid metabolism (Hadley, 2000, Norris, 1997).

Free-ranging animals were grouped as pups, juveniles and adults to compare animals of varying maturity (Fig 1.1, Table 1a). Changes in thyroid hormones with increasing maturity were similar for total and free T_4 and all differences were significant. Total and free T_3 levels were also similar and followed the same pattern as total and free T_4 but pups and juveniles were not significantly different. Still, for all four hormones measured in this study, pups were significantly higher than adults.

Without knowing what environmental perturbations may be acting on Steller sea lion populations, it is conceivable that pups in southeast Alaska, where overall populations are increasing, could be considered hormonally stable. This is consistent with their hormone concentrations being lower than sea lions whose environment may stimulate enhancement of thyroid concentrations (Fig 1.2, Tables 1b and 1c). Although

large sample sizes may allow for slight differences to be significant, this does not necessarily mean that the differences are biologically or physiologically relevant. However, the fact that the trends between the three areas are essentially consistent between the two years investigated, suggests that the differences are notable. Following this logic, it would seem that sea lion pups in southwest Alaska and especially Russia, where populations have declined, would have thyroid hormone concentrations that are elevated in an effort to enhance metabolism and subsequently heat production in order to maintain homeostasis. However, in a preliminary study, Hoopes et al., (2004) found no difference in metabolic rates for pups or juveniles when comparing southwest sea lions from Prince Williams Sound to southeast sea lions. In this study, higher thyroid hormone concentrations in southwest animals may have been due to differences in age and ambient temperatures. Still, this suggests that the difference in hormone concentrations is not because of differences in metabolic rates. Another reason for lower concentrations in southeast animals may be associated with recent information that southeast pups have slower growth rates and face longer fasts due to shorter maternal attendance patterns (Fadely et al., 2004). Southeast pups are also slightly smaller than their southwestern counterparts (Davis et al., 2004). Due to the fact that fasting may cause a depression in thyroid concentrations, this could account for the lower thyroid concentrations in southeast animals.

The changes in hormones over time show how these hormones vary in relation to seasons or possibly life history stages (Fig 1.3, Table 1d). An increase in thyroid hormone concentrations during the warmer summer months is likely associated with social, reproductive and molting behavior. In a natural state, this time correlates to when the animals are more often out of the water for breeding and social purposes and for the annual molt of the pelage. Enhancement of thyroid hormone concentrations at this time is similar to what was found in other pinnipeds (Ashwell-Erickson et al., 1986, Boily, 1996, John et al., 1987, Renouf and Noseworthy, 1991, Riviere et al., 1977).

This study demonstrated that concentrations of T_4 in Steller sea lions tend to be highest in pups. This is also similar to what has been seen in other pinnipeds

(Leatherland and Ronald, 1979, Engelhardt and Ferguson, 1980, Little, 1991, Haulena et al., 1997, Hall et al., 1998, Woldstad and Jenssen, 1998). This trend suggests that the newborn Steller sea lion may initiate a high metabolic rate in response to the thermoregulatory challenge of life outside the womb. Otariids, including Steller sea lions, are typically born with a minimal layer of subcutaneous fat (Jonker and Trites, 2000). Without this important insulation and energy reserve, it may be imperative that a newborn Steller sea lion is able to maintain body heat production through increased metabolism. As the neonate grows and develops, pups maintain relatively high concentrations of thyroid hormones but are supplementing energy reserves by adding blubber stores which help insulate core temperatures. Juveniles and especially adults are better able to regulate homeostasis while pups have to rely on other physiological mechanisms, evidence of the important role of these hormones in tissue synthesis, growth and thermoregulation (Renouf and Noseworthy, 1991, Woldstad and Jenssen, 1998).

Almost 97% of the circulating thyroid hormone in Steller sea lions is thyroxine (T_4) and just a little over 3% is triiodothyronine (T_3). Our work shows that in Steller sea lions, 0.02% of T_4 and 0.01% of T_3 is in the free-state. Measures of total and free concentrations for each hormone indicate what portion of the molecule is in the bound state (total minus free concentration is equivalent to how much of the molecule is bound to transport proteins). As most T_3 is produced by the deiodination of T_4, and because the concentrations are controlled collectively by a negative feed-back loop (Hadley, 2000), both total and free T_3 and T_4 are maintained in the system in balance. This indicated that the concentrations should be related to each other not accounting for any amount of hormone that is bound intracellular at an active site. A correlation between total and free T_4 indicated that they are related 77% of the time. Total and free T_4 are also related to total T_3 more than 50% of the time. Interestingly, free T_3 is the least correlated to the other hormones suggesting that either free T_3 concentrations in the blood are regulated independently of the other forms of thyroid hormone or it may be that the binding affinity for free T_3 at the cellular binding sites is significantly higher than for the other forms of thyroid hormones, resulting in a constant removal of free T_3 from circulation. Williams

et al., (2001) demonstrated that the basal metabolic rate, as measured by oxygen consumption, is higher in otariids than in humans. An enhanced metabolic state may account for the actions of free T_3 in Steller sea lions.

This study was designed to enable basal concentrations of thyroid hormones to be determined in Steller sea lions, utilizing assays which have been validated for this species. In addition, thyroid hormone concentration changes in relation to age, season and sex have also been elucidated. The relationships between these hormones and food deprivation, reproductive state, other circulating hormones, immunoglobulins and contaminants can now be identified by further analysis.

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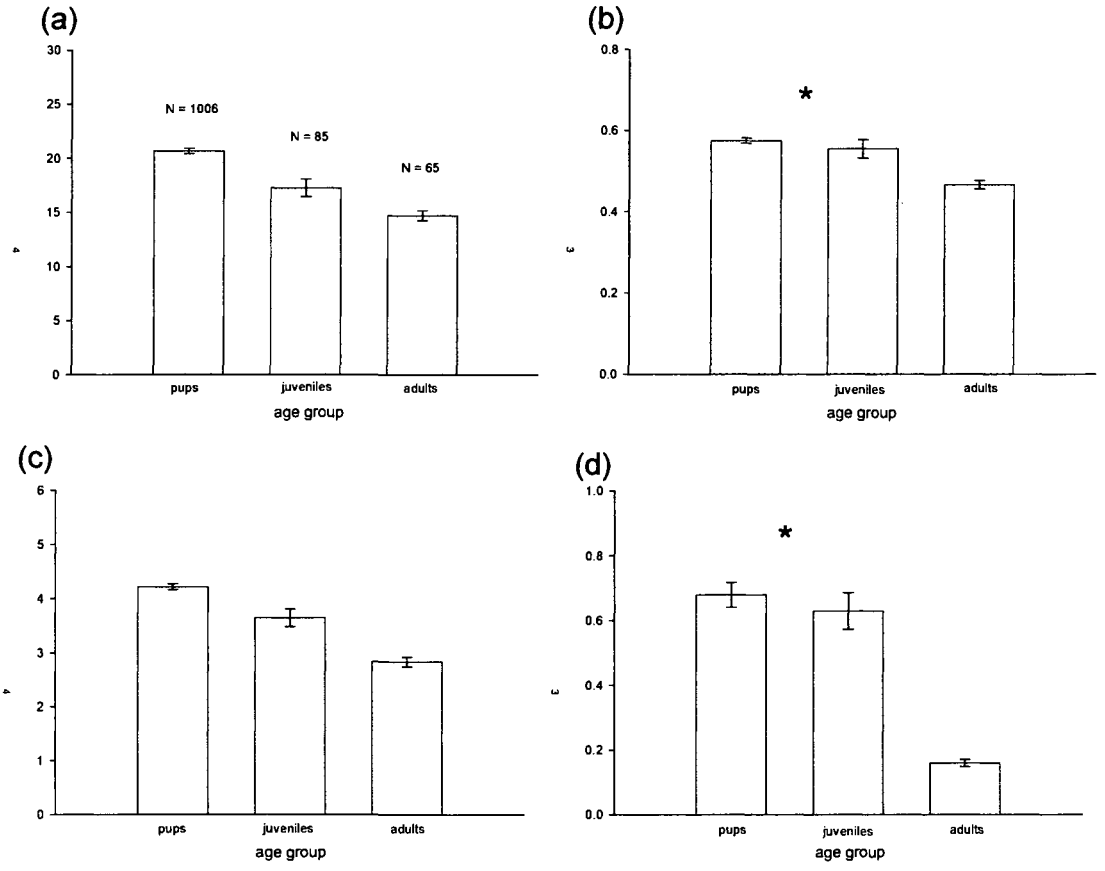

Figure 1.1. Mean (±se) thyroid hormone concentration, (a) total T_4, (b) total T_3, (c) free T_4 and (d) free T_3 over age group (pups < one year, juveniles 1 year to < 4 years, adults 4 years and older) in free-ranging Steller sea lions. Sample size is shown on graph (a) and is approximately the same for (b), (c) and (d). An * shows where groups were not significantly different. Statistics for individual pair-wise comparisons are presented in Table 1.a.

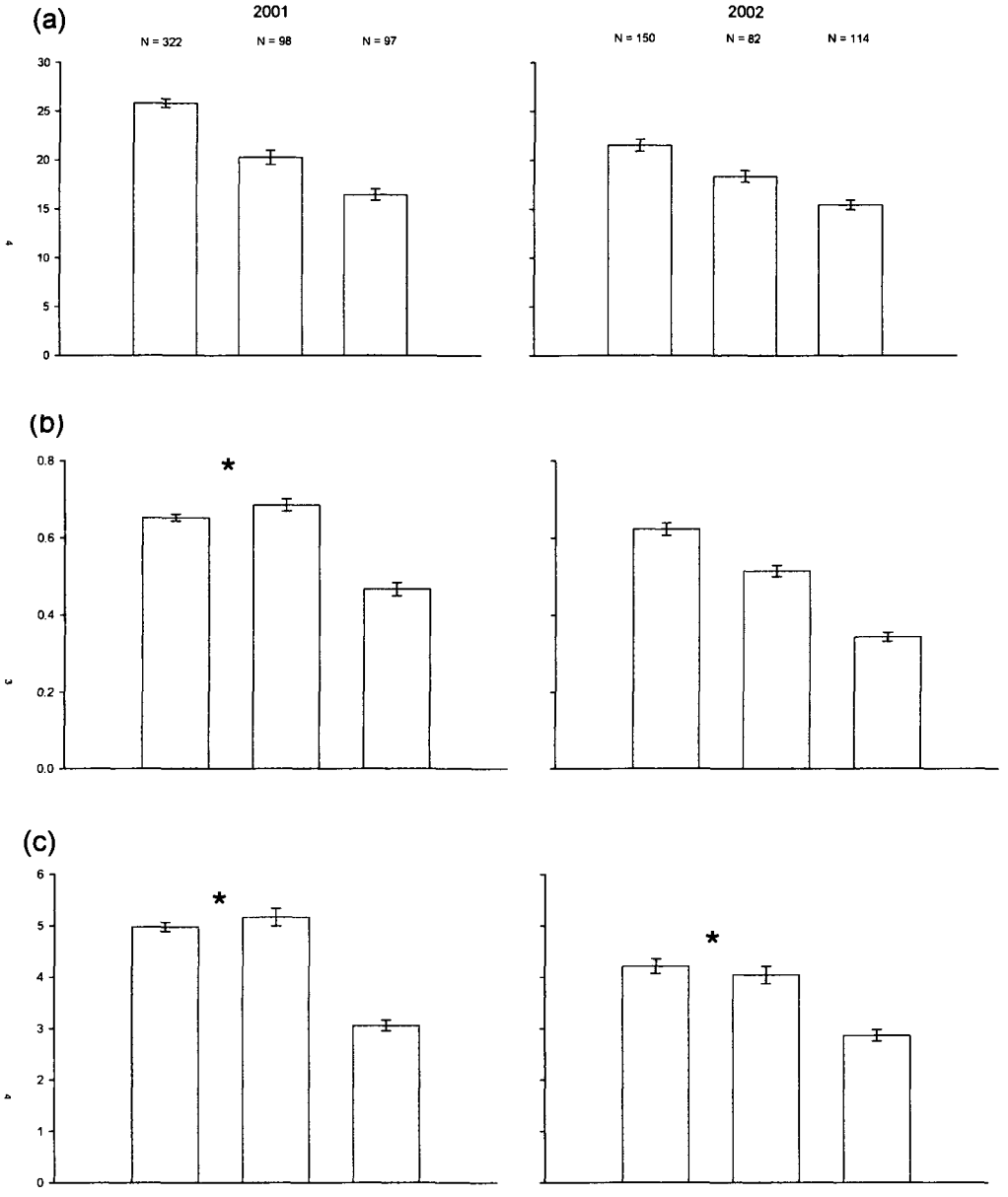

Figure 1.2. Mean (±se) thyroid hormone concentration for free-ranging Steller sea lion pups (all animals were approximately 1 month of age), (a) total T_4, (b) total T_3, (c) free T_4 and (d) free T_3 over areas with 2001 shown to the left and 2002 to the right. Sample size is shown on graph (a) and is approximately the same for (b), (c) and (d). An * shows were groups were not significantly different. Statistics for individual pair-wise comparisons are presented in Tables 1.b and 1.c.

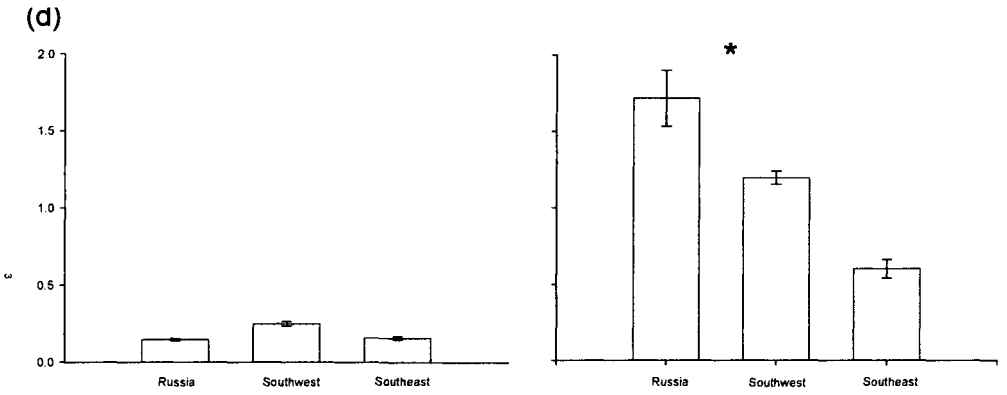

Figure 1.2 continued.

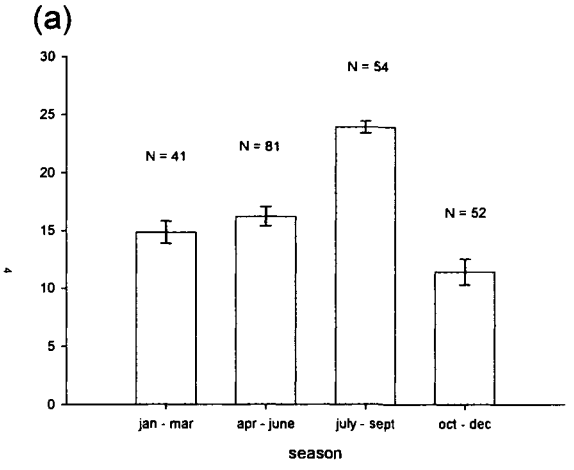

Figure 1.3. Seasonal changes in mean (±se) thyroid hormone concentrations. Season of the year (January to March, April to June, July to September and October to December) versus average (a) total T_4, (b) total T_3, (c) free T_4 and (d) free T_3 in captive Steller sea lions (n=9). Sample size is shown on graph (a) and is approximately the same for (b), (c) and (d). Statistics for individual pair-wise comparisons are presented in Table 1.d.

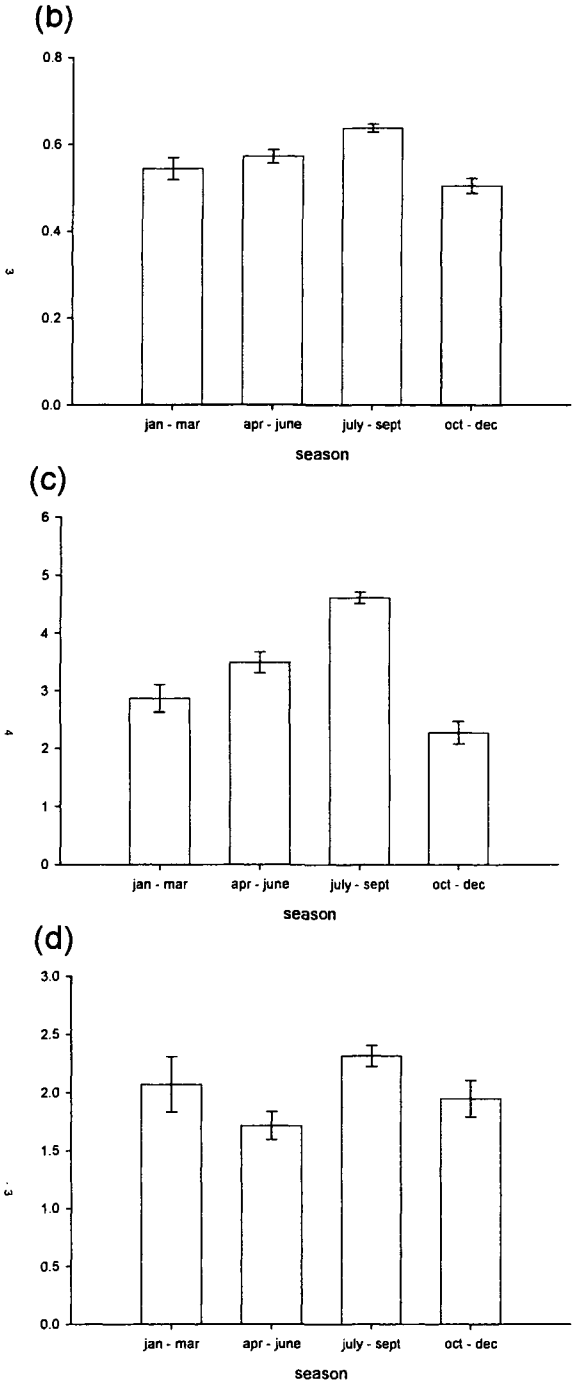

Figure 1.3 continued.

Table 1.1. Correlation matrix comparing each thyroid hormone (total T_4, total T_3, free T_4 and free T_3) to itself (correlation factor =1) and each of the other hormones for free-ranging Steller sea lions n=1132). The matrix was produced utilizing regression between various hormones, which resulted in the r values reported.

	total T_4	**total T_3**	**free T_4**	**free T_3**
total T_4	1	0.528 (p=<0.001)	0.770 (p=<0.001)	0.0115 (p=0.724)
total T_3	0.528 (p=<0.001)	1	0.552 (p=<0.001)	0.0669 (p=0.537)
free T_4	0.770 (p=<0.001)	0.552 (p=<0.001)	1	0.0351 (p=0.281)
free T_3	0.012 (p=<0.724)	0.067 (p=<0.537)	0.035 (p=0.281)	1

Table 1.2. Range and mean (± standard error) for all four thyroid hormones (total T_4, total T_3, free T_4 and free T_3) for the entire sample set, broken into age groups for both free-ranging and captive Steller sea lions (pups < one year, juveniles 1-3 yrs, adults >3yrs). und.= undetectable. One-way ANOVA between groups for all values of total T_4, total T_3, free T_4 and free T_3 ($P < 0.001$).

Population Data N = 1156		
Hormone	**Range**	**Mean ± se**
Total T_4	und. to 58.50 (ng/ml)	20.20±0.24 (ng/ml)
Total T_3	und. to 1.38 (ng/ml)	0.57±0.01 (ng/ml)
Free T_4	und. to 13.4 (pg/ml)	4.11±0.05 (pg/ml)
Free T_3	und. to 6.70 (pg/ml)	0.69±0.04 (pg/ml)
Pups (free-ranging) N = 1006		
Hormone	**Range**	**Mean ± se**
Total T_4	und. to 58.50 (ng/ml)	20.68±0.25 (ng/ml)
Total T_3	und. to 1.38 (ng/ml)	0.58±0.01 (ng/ml)
Free T_4	und. to 13.4 (pg/ml)	4.21±0.05 (pg/ml)
Free T_3	und. to 6.70 (pg/ml)	0.68±0.04 (pg/ml)
Juveniles (free-ranging) N = 85		
Hormone	**Range**	**Mean ± se**
Total T_4	und. to 44.30 (ng/ml)	17.28±0.82 (ng/ml)
Total T_3	und. to 1.34 (ng/ml)	0.56±0.02 (ng/ml)
Free T_4	und. to 7.21 (pg/ml)	3.64±0.16 (pg/ml)
Free T_3	und. to 4.42 (pg/ml)	0.95±0.09 (pg/ml)
Adults (free-ranging) N= 48		
Hormone	**Range**	**Mean ± se**
Total T_4	2.15 to 29.59 (ng/ml)	15.35±0.94 (ng/ml)
Total T_3	und. to 0.68 (ng/ml)	0.41±0.02 (ng/ml)
Free T_4	und. to 7.63 (pg/ml)	3.05±0.24 (pg/ml)
Free T_3	und. to 0.70 (pg/ml)	0.23±0.03 (pg/ml)
Juveniles (captives) N = 69		
Hormone	**Range**	**Mean ± se**
Total T_4	und. to 16.49 (ng/ml)	6.99±0.55 (ng/ml)
Total T_3	und. to 1.07 (ng/ml)	0.63±0.02 (ng/ml)
Free T_4	und. to 7.90 (pg/ml)	2.36±0.16 (pg/ml)
Free T_3	und. to 0.60 (pg/ml)	0.21±0.02 (pg/ml)
Adults (captive) N =160		
Hormone	**Range**	**Mean ± se**
Total T_4	1.51 to 34.61 (ng/ml)	14.42±0.54 (ng/ml)
Total T_3	und. to 0.90 (ng/ml)	0.49±0.01 (ng/ml)
Free T_4	und. to 7.10 (pg/ml)	2.76±0.10 (pg/ml)
Free T_3	und. to 0.50 (pg/ml)	0.14±0.01 (pg/ml)

Statistical Analysis

Table 1a. Statistical data (group, difference of means and *P* value) comparing mean hormone concentration to age group in free-ranging Steller sea lions (related to Figure 1.1).

Groups	**Difference of means**	***P value***
total T_4		
pups vs. adults	5.95	*P*=<0.001
pups vs. juveniles	3.40	*P*=<0.001
juveniles vs. adults	2.55	*P*=<0.001
total T_3		
pups vs. adults	0.11	*P*=<0.001
pups and juveniles	0.02	not significantly different
juveniles vs. adults	0.09	*P*=0.001
free T_4		
pups vs. adults	1.38	*P*=<0.001
pups vs. juveniles	0.57	*P*=0.005
juveniles vs. adults	0.81	*P*=<0.001
free T_3		
pups vs. adults	0.52	*P*=<0.001
pups and juveniles	0.05	not significantly different
juveniles vs. adults	0.47	*P*=<0.001

Table 1b. Statistical data (group, difference of means and *P* value) comparing mean hormone concentrations to area for free-ranging Steller sea lion pups in 2001 (related to Figure 1.2).

Areas and year	**Difference of means**	***P value***
total T_4 2001		
Russia vs. southwest	5.54	*P*=<0.001
Russia vs. southeast	9.35	*P*=<0.001
southwest vs. southeast	3.81	*P*=0.001
total T_3 2001		
Russia vs. southwest	0.03	not significantly different
Russia vs. southeast	0.19	*P*=<0.001
southwest vs. southeast	0.22	*P*=<0.001
free T_4 2001		
Russia vs. southwest	0.19	not significantly different
Russia vs. southeast	1.92	*P*=<0.001
southwest vs. southeast	2.12	*P*=<0.001
free T_3 2001		
Russia vs. southwest	0.04	*P*=0.046
Russia vs. southeast	0.05	*P*=0.009
southwest vs. southeast	0.09	*P*=0.001

Table 1c. Statistical data (group, difference of means and *P* value) comparing mean hormone concentrations to area for free-ranging Steller sea lion pups in 2002 (related to Figure 1.2).

Areas and year	**Difference of means**	***P value***
total T_4 2002		
Russia vs. southwest	3.15	*P*=0.001
Russia vs. southeast	6.08	*P*=<0.001
southwest vs. southeast	2.94	*P*=0.005
total T_3 2002		
Russia vs. southwest	0.11	*P*=<0.001
Russia vs. southeast	0.28	*P*=<0.001
southwest vs. southeast	0.17	*P*=<0.001
free T_4 2002		
Russia vs. southwest	0.17	not significantly different
Russia vs. southeast	1.35	*P*=<0.001
southwest vs. southeast	1.18	*P*=<0.001
free T_3 2002		
Russia vs. southwest	0.56	*P*=0.029
Russia vs. southeast	1.10	*P*=<0.001
southwest vs. southeast	0.54	not significantly different *P*=0.056

Table 1d. Statistical data (group, difference of means and *P* value) comparing mean seasonal changes in thyroid hormone concentrations in captive Steller sea lions (related to Figure 1.3).

Season	**Difference of means**	***P value***
total T_4		
Jan. to March vs. July to Sept.	9.09	*P*=<0.001
Jan. to March vs. April to June	1.38	not significantly different
Jan. to March vs. Oct. to Dec.	3.43	not significantly different
April to June vs. July to Sept.	7.72	*P*=<0.001
April to June vs. Oct. to Dec.	4.80	*P*=0.009
July to Sept. vs. Oct. to Dec.	12.5	*P*=<0.001
total T_3		
Jan. to March vs. July to Sept.	0.09	*P*=0.004
Jan. to March vs. April to June	0.03	not significantly different
Jan. to March vs. Oct. to Dec.	0.04	not significantly different
April to June vs. July to Sept.	0.07	*P*=<0.001
April to June vs. Oct. to Dec.	0.07	not significantly different
July to Sept. vs. Oct. to Dec.	0.13	*P*=<0.001
free T_4		
Jan. to March vs. July to Sept.	1.75	*P*=<0.001
Jan. to March vs. April to June	0.62	not significantly different
Jan. to March vs. Oct. to Dec.	0.59	not significantly different
April to June vs. July to Sept.	1.13	*P*=<0.001
April to June vs. Oct. to Dec.	1.21	*P*=<0.001
July to Sept. vs. Oct. to Dec.	2.34	*P*=<0.001
free T_3		
Jan. to March vs. July to Sept.	0.25	not significantly different
Jan. to March vs. April to June	0.35	not significantly different
Jan. to March vs. Oct. to Dec.	0.12	not significantly different
April to June vs. July to Sept.	0.60	*P*=0.001
April to June vs. Oct. to Dec.	0.23	not significantly different
July to Sept. vs. Oct. to Dec.	0.37	not significantly different

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CHAPTER 2

The effects of age, season and geographic region on circulating cortisol concentrations as a biomarker of stress in threatened and endangered Steller sea lions (*Eumetopias jubatus*)*

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Abstract

As the northern most of the otariids, Steller sea lions (SSLs) exhibit unique adaptations and physiological coping strategies that allow them to successfully inhabit the sub-arctic ecosystem. Cortisol can be elevated in association with acute or chronic changes in an animal's environment and was validated here as a potential barometer of stress in sub-populations, specifically the threatened southeast Alaska population, the endangered western Alaska population and SSLs from the Russian Far East. Cortisol concentrations were similar in male and female pups. Cortisol varied with age but when considered in regards to time of year when sampled, followed a seasonal pattern. Cortisol was elevated in fall months in captive sea lions (non-pups), which is similar to what is seen in other marine mammals and is likely associated with the annual molt. Cortisol concentrations from free-ranging juvenile sea lions were significantly elevated (145.1±6.6 ng/ml) above captive sea lions (87.4±4.8 ng/ml, $p < 0.001$), however there were no differences in captive and free-ranging adult sea lions. This may suggest environmental influences on the well-being of free-ranging juvenile sea lions. A similar trend occurred in both regions of Alaska with cortisol concentration in 2000 significantly lower than in 2001, and 2002 levels the highest of the three years. In 2001 and 2002, an additional sampling protocol provided samples from Russian pups that were consistently higher than the western Alaska pup concentrations and also tended to increase in the year sampled. Our results suggest that cortisol concentrations in SSLs are a useful diagnostic tool to compare the physiology between groups of sea lions.

Introduction

Steller sea lions (*Eumetopias jubatus*) range from the California Channel Islands along the North Pacific Rim to northern Japan. In the 1970's, the greatest concentration of animals occurred in the western Gulf of Alaska and along the eastern Aleutian Islands. This portion of the population has been experiencing a dramatic decline over the last 30 years. Overall, Steller sea lion population numbers decreased more than 80% since 1976 and about 70% since 1985 (Calkins et al., 1999, Sease et al., 2001, Atkinson et al., 2008a). This decline led to the species being listed as threatened under the U.S. Endangered Species Act in 1990 (55 U.S. Federal Register 49204). In 1997, genetic information and population trends indicated that distinct stocks existed (Bickham et al., 1996, Loughlin, 1997). Subsequently, the western stock, which occurs from 144° West longitude (just east of Prince William Sound, Alaska) to Japan, was listed as endangered (62 U.S. Federal Register 24345). The eastern stock in Southeast Alaska to California remains listed as threatened.

Nutritional stress has been one of the leading hypotheses related to the decline of the western stock of Steller sea lions. If animals are perishing as a result of either low food resources or poor food quality, then this should be reflected in the overall stress response of the population (Atkinson et al., 2008b). In mammals, cortisol is one of the hormones secreted by the adrenal glands in response to a stressor (Ortiz and Worthy, 2000, St. Aubin, 2001, Oki and Atkinson, 2004). Cortisol is a glucocorticoid which is secreted by the adrenal cortex in response to stimulation by adrenocorticotropic hormone (ACTH) (Hadley 1992, Mashburn and Atkinson, 2004, 2007, Petrauskas and Atkinson, 2006, Petrauskas et al., 2006). All organisms must be able to cope with changes or short-term stressors in their environment in order to survive and reproduce (Dobson and Smith, 2000) and the high concentrations of serum glucocorticoids serve as primary indicators of a successful stress response. Under optimal environmental conditions, stressors are occasional events and corticoid actions on the body are acute and short-lived. Sub-optimal or severe conditions, such as long term food deprivation, can cause chronic release of corticoids and a subsequent chronic stimulation of corticoid-sensitive organs.

Mean initial cortisol levels were similar in both Pacific harbor seals (*Phoca vitulina richardii*) that survived and died in rehabilitation (Gulland et al., 1999, O'Neil, 2005). However, survivors saw a decrease in cortisol levels over time while those that succumbed exhibited increasing cortisol levels. Petrauskas et al., (2006) showed that fecal corticosterone concentrations increased in association with specific stressors in a Steller sea lion pup in rehabilitation. In yearling Hawaiian monk seals (*Monachus schauinslandi*), emaciated seals had higher cortisol concentrations compared to healthy animals (Atkinson and Oki, unpublished data).

Cortisol has also been shown to vary with season and is associated with the annual molt of some marine mammals. Harbor seals exhibited higher cortisol concentrations in association to cessation of hair growth and loss of pelage (Riviere et al., 1977). Ashwell-Erickson et al., (1986) showed that both Alaskan harbor seals and spotted seals (*Phoca largha*) had maximum cortisol levels just before or during the main shedding of hair. Oki and Atkinson, (2004) reported that the diurnal pattern of circulating cortisol concentrations was abandoned in the summer months in harbor seals resident at 60° north, which may be due to the additional requirements of cortisol associated with the molt.

Scientific handling can cause an acute increase in cortisol levels and must be considered in relation to interpretation of stress effects (St Aubin and Geraci, 1979, Thomson and Geraci, 1986, St Aubin and Dierauf, 2001). Engelhard et al., (2002) showed that both lactating mother and pup southern elephant seals (*Mirounga leonina*) had increased levels of cortisol in association to handling but that these animals tolerated moderate degrees of handling disturbance. In Atlantic bottlenose dolphins (*Tursiops truncatus*) circulating levels of cortisol were higher in wild animals encircled by capture nets versus semidomesticated animals that were trained to voluntarily present tails for blood sampling (St. Aubin et al., 1996). Mashburn and Atkinson, (2004, 2007) stimulated an acute stress response in Steller sea lions by injecting ACTH in both summer and winter seasons. They found that animals responded immediately with

increased mean cortisol concentrations in the sera and the response was abated within two days as cortisol was metabolized and excreted through the feces as corticosterone.

The present study investigated serum cortisol concentrations in Steller sea lions of various ages and over temporal and spatial scales that reflect the majority of the geographical range of the population. The intent was to clarify normal cortisol concentrations of different age and sex classes of Steller sea lions in order to evaluate basic levels and trends over seasons. The hypothesis was that cortisol in Steller sea lions would vary with season and not vary with sex. In addition, pre-defined Steller sea lion pup populations, which differ genetically and by geographic area, were compared to investigate any differences which may be indicated by mean population cortisol concentrations. In order to evaluate any effect of handling on cortisol concentrations in Steller sea lions, an additional objective considered that there would be no difference in cortisol concentrations as handling time increased.

Materials and Methods

Animal selection and sample collection

In this study, 1368 separate blood samples were collected from free-ranging and captive Steller sea lions (free-ranging n=1144, captive n=10). The majority of samples (n=1079) were collected in collaboration with the Alaska Department of Fish and Game (ADFG), National Marine Fisheries Service (NMFS) and Alaska SeaLife Center cruises during the field seasons of 2000, 2001 and 2002. Of the 1079 blood samples collected in 2000, 2001 and 2002, all known aged animals were pups (n=995) or juvenile animals (age 1 to 3 years, n=84). Although most of the free-ranging samples were from the last few years, one sub-group of archived adult samples (age 4 to 15 years) was included and consisted of samples from harvest and subsistence taken as early as 1976 (n=65, collected 1976 to 1997). Captive samples were also provided from archive, which was collected from resident animals at the Alaska SeaLife Center and the Vancouver Aquarium (224 samples, age 3 to 9 years, collected 1995 to 2001, n=9 sea lions).

Free-ranging samples came from as far west as the Kuril Islands in the Russian far east across the Aleutian Islands and the Gulf of Alaska to southeast Alaska, the majority

of the full geographical range of Steller sea lions. Blood samples were taken by venipuncture from a rear flipper vein or the caudal gluteal vein. Samples were taken throughout the year and the specific time of year is reflected in the animal's age as the cohort matured. All samples from one month old pups were taken at approximately the same time each year and the age of one month was based on a median birthday for that cohort. All blood samples were taken between 9 am and 4 pm for free-ranging animals and the specific time within that period was recorded. Whole blood was kept cool on ice packs until centrifugation and was typically processed within 4 to 6 hours of collection. Serum or plasma was separated and kept frozen at -80°C until analysis. Animals were weighed and length measured in the field.

In order to investigate seasonal variability of cortisol hormone concentrations, monthly samples were collected from a group of captive Steller sea lions housed outdoors in natural seawater and under natural photoperiod. Longitudinal seasonal samples from these sea lions were evaluated in four seasons, January to March, April to June, July to September and October to December.

Hormone measurements and statistical analysis

Cortisol concentrations were measured in both serum and plasma samples by direct assay using a solid phase radioimmunoassay (RIA). All kits were manufactured by Diagnostic Products Corporation, Los Angeles, CA (catalog number TKCO1). Regression of plasma versus serum was correlated for cortisol ($n=30$, $r^2=0.92$) and justified using the two interchangeably. Mean nonspecific binding (NSB), which is the percentage of radioactive hormone that binds in the absence of antibody divided by the total counts, was 1.02% (n=10 assays). Mean sensitivity or the lower limit of sensitivity for cortisol was 17.0 ng/tube (n=10 assays).

The analysis technique for cortisol was validated for Steller sea lions. Tests of parallelism and linearity were performed to verify that the assay used is applicable to a given species. Parallelism tests involved combining 50% of each standard concentration calibrator with 50% of a pooled sample of Steller sea lion serum. Next, regression of pure standard concentration calibrators was compared with the mixed calibrators.

Standard curves were parallel and slope values varied less than 10%. Slope of cortisol with standard calibrators =-0.77; slope of calibrators mixed with pool =-0.85. In addition, linearity tests utilizing various amounts of pool (1/2, 1, 2 and 4 times the recommended amount) were performed and regression applied to verify that as the amount of the sample changes, the hormone concentration was maintained (r^2=0.99, $p<0.001$). Controls of a known hormone concentration were run with each assay to calculate intra and inter-assay variation. Intra-assay coefficients of variation were <5%. Samples were analyzed in batches to reduce inter-assay variation. Inter-assay coefficient of variation utilizing the median control were 10.49%.

Data Analysis

All hormone concentrations were determined after a log-logit transformation of the standard curve (Rodbard, 1974). For plots of hormone concentration versus age, all animals that were less than one year or were considered pups. Animals one year to three years old were considered juveniles and animals older than four years as adults.

Descriptive statistics were employed to illustrate ranges and mean concentrations. One-way ANOVAs (for groups of three or more) and t-tests (for two groups) were used for comparisons of group means. For all tests completed, a P-value of less than 0.05 was chosen as the level of significance.

Results

Overall, there were no differences in cortisol concentration between males and females. Further comparison of 24 rookeries with at least 10 individual samples and a normal distribution of males and females, indicated that only four had significant sex differences. Therefore, sex was disregarded in all further statistical analyses. Cortisol concentrations in Steller sea lions varied over a wide range for all samples analyzed. The range of concentrations for the entire population sampled was the same as for free-ranging pups and accounted for the lowest and highest concentrations measured (Table 2.1). Captive juveniles had significantly lower mean concentrations (mean 87.4±4.8 ng/ml) than free-ranging juveniles (mean 145.1±6.6 ng/ml, $P<0.001$). There was no significant difference in cortisol concentrations between captive adults (mean 77.6±2.4

ng/ml) and free-ranging adults (mean 74.2±5.4 ng/ml). However, there were no recent adult samples from free-ranging animals and the comparison was based on archived adult samples from 1976 to 1997. Breaking down the archived samples and comparing them to recent adult captives, there was still no difference between adults taken in the 1970s, the 1980s, the 1990s and captive adults sampled recently.

The sampling protocol dictated that all samples were taken during the same portion of the day for free-ranging animals but the specific time within that period varied on a per animal processed basis and may account for some variation in cortisol hormone concentrations. To investigate any effect of handling on cortisol concentration, a regression of cortisol concentration by sampling order or time progression was conducted and determined no relationship at any of the 24 field sampling sites. As researchers landed on the rookery and handled pups throughout the sampling period, cortisol concentrations did not vary consistently over time as animals were processed, indicating that cortisol concentration was not associated with increased handling time.

In a temporal comparison between Steller sea lion pups from all regions (i.e. southeast Alaska, western Alaska and Russia) for 2000, 2001 and 2002, cortisol concentrations increased significantly (one-way ANOVA $P<0.001$) in each of the three years investigated (Figure 2.1). Individual regions also exhibited temporal increases during the years we sampled (Figure 2.2, one-way ANOVA, Tukey Test $P<0.001$). The spatial comparison using the same regions revealed that southeast Alaska cortisol concentrations were significantly higher ($p=0.045$) than in western Alaska in 2000. In 2001, southeast and Russia had similar cortisol concentrations and were significantly higher ($p<0.001$) than in western Alaska (Figure 2.2). In 2002, all three areas had similar concentrations ($p=0.75$).

There were significant ($P<0.001$) inter-island differences in cortisol concentrations from Steller sea lions in specific areas within each year (Table 2.2). The overall highest mean concentration was recorded at Koslova Cape in Russia in 2002 and the next highest spot was Amak Island in western Alaska in 2002 (Table 2.2). The lowest concentrations were at Sugarloaf Island in western Alaska in 2000 followed by Marmot

Island near Kodiak Island in western Alaska in 2000 and Ugamak Bay in western Alaska in 2001 (Table 2.2). The highest cortisol concentrations measured in this study were from Koslova Cape in 2002 (see Table 2.2) and suggest that at the time of sampling pups from Koslova Cape may have experienced a significant stress factor or factors. Many sites did follow increasing yearly trends, for example for Marmot and Sugarloaf in southwest Alaska, cortisol concentrations went up significantly from 2000 to 2002 but neither site was visited in 2001 (Marmont and Sugarloaf, $p<0.001$). Cortisol concentrations also went up between 2001 and 2002 for Lowrie Island in southeast Alaska ($p<0.005$). Inconsistent with the typical and overall trends, Iony Island in Russia went down between 2001 and 2002 ($p<0.001$).

A seasonal comparison of cortisol concentrations in captive Steller sea lions showed significant differences between seasons ($p<0.001$) with the highest concentrations in late summer to early fall period from July to September, which coincides with the period when animals molt their pelage (Figure 2.3). The lowest levels were in the winter period of January to March (Figure 2.3).

Discussion

This study established baseline for serum cortisol concentrations in Steller sea lions of various ages, different seasons, and of different genetic and geographic populations. Free-ranging pups showed the greatest range of concentrations which may be indicative of the trials and tribulations that very young animals face in a sometimes harsh sub-artic environment. Fortunately the animal handling protocol for this study did not influence the cortisol results in contrast to what Engelhard et al., (2002) reported. Captive juvenile Steller sea lions had lower cortisol concentrations than free-ranging animals and this may further indicate that free-ranging animals face more physiological challenges compared to animals held under stable environmental conditions with good nutrition and veterinary care (St. Aubin et al., 1996, Gardiner and Hall, 1997, Gulland et al., 1999).

Inter-annual differences in circulating cortisol concentrations may be related to any number of environmental changes but may suggest that the coping mechanisms of

sea lions have increased at least over the period of our sampling (Figure 2.1). It would take a multi-year study to properly address this in detail. However, there is a pattern in that all three areas increased in all years investigated (Figure 2.1). It is interesting that in our third year of investigation, 2002, all areas had their highest concentrations and the differences between areas were not significant (Figure 2.2), possibility indicating that environmental stress factors had manifest equally throughout the Steller sea lion range during our sampling time. As cortisol is the dominant circulating glucocorticoid in marine mammals and functions to maintain homeostasis against environmental pressures (St. Aubin et al., 1996, Ortiz and Worthy, 2000), it can be inferred that those environmental pressures have increased on Steller sea lion pups over this three year period. As cortisol is a non-specific stress indicator, factors stimulating its production and synthesis could not be identified specifically by this study.

Comparing areas (Figure 2.2), it is surprising that cortisol concentrations from pups in western Alaska were lower than those from southeast Alaska for both 2000 and 2001. Cortisol concentrations in animals from the Russian Far East were only measured for two years but in the years that were measured, Russian animals had higher concentrations compared to their closest relatives in western Alaska in 2001 but Russia was similar to all areas in 2002. It is difficult to infer the progression of an unknown stressor geographically but our data specify that cortisol concentrations during this study were higher on the periphery of the endangered population and increased over the time investigated into the central portion of the population (western Alaska population). The western Alaska portion of the Steller sea lion range historically housed the bulk of the population but also has experienced the greatest declines (Calkins et al., 1999, Sease et al., 2001). It is likely that stressors that acted on the population during the substantial decline have changed (Atkinson et al., 2008a).

The specific sites sampled varied within areas and between years and makes it difficult to interpret the data from the perspective of grouped areas over years investigated. A few islands were sampled between years and trends from limited and/or disconnected data sets may be misleading (Table 2.2).

In captive Steller sea lions, cortisol concentrations were highest in the July to September season (Figure 2.3). This would be expected as cortisol has been associated with the molt in other marine mammals such as harbor seals and spotted seals (Riviere et al., 1977, Ashwell-Erickson et al., 1986). Gardiner and Hall, (1997) also showed higher cortisol concentration in harbor seals during the season they referred to as the breeding/molt versus a post season. Engelhardt and Ferguson, (1980) found higher cortisol concentrations in harp (*Phoca groenlandica*) and gray (*Halichoerus grypus*) seal pups that peaked three weeks after birth and was associated with the molt and weaning.

This study was designed to enable a baseline index of cortisol concentrations to be determined in Stellers sea lions, utilizing assays which have been validated for this species. The relationships between cortisol and other circulating hormones, reproductive state, immunoglobulins and contaminants can now be identified by further analysis. Cortisol concentrations in Steller sea lions indicate that seasonal variation is occurring and shows that free-ranging Steller sea lions have elevated concentrations, which is likely associated with environmental pressures. Cortisol concentrations also allow comparisons between groups of animals to be made indicating groups that are exhibiting more of a physiological response which may reflect environmental stressor.

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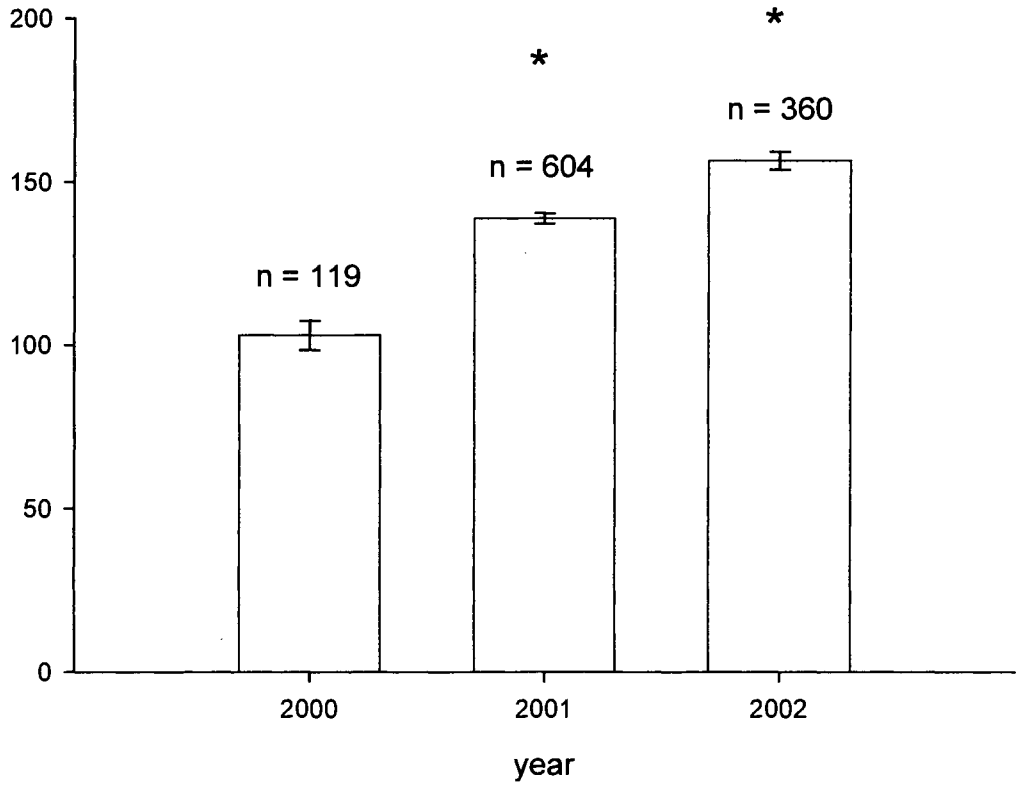

Figure 2.1. Mean (±se) cortisol concentration (in ng/ml) by year (2000, 2001 and 2002) in free-ranging Steller sea lion pups (less than one month of age) from southeast Alaska, western Alaska and the Russian Far East combined. Asterisk denotes significant ($P<0.001$) differnce between years.

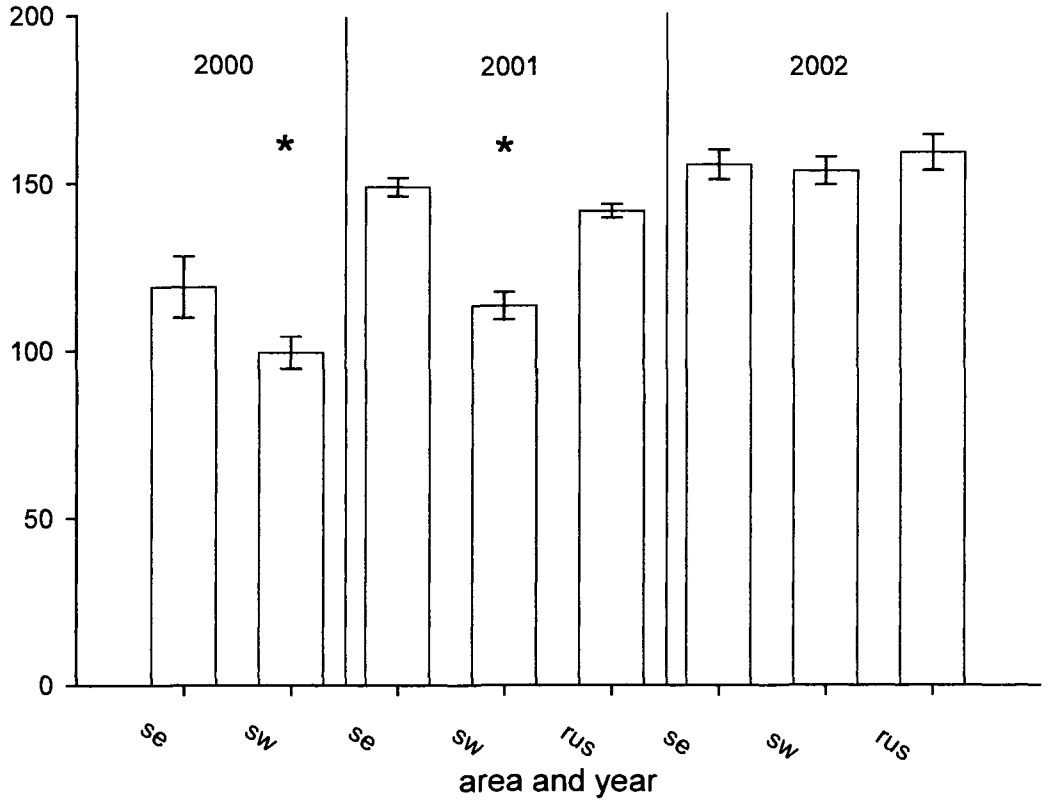

Figure 2.2. Mean (±se) cortisol concentration (in ng/ml) by year (2000, 2001 and 2002) and area (Southeast Alaska [se], Western Alaska [sw] and Russia [rus]) in free-ranging Steller sea lion pups (less than one month of age). Asterisk denotes significant ($P<0.05$) change between areas within each year.

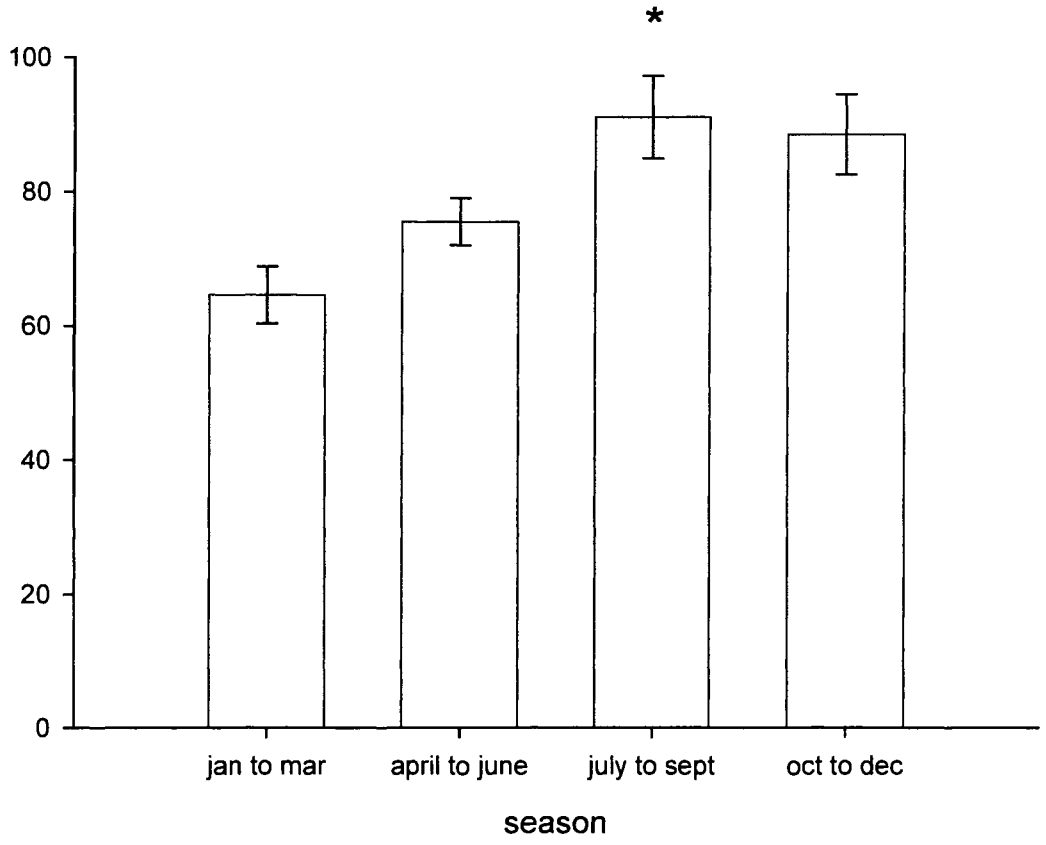

Figure 2.3. Seasonal changes in mean (±se) cortisol concentration (in ng/ml). Season of the year binned into three month blocks (January to March [jan to mar], April to June [april to june], July to September [july to sept] and October to December [oct to dec]) versus mean cortisol concentration in captive Steller sea lions (n=9). Asterisk denotes significantly ($P<0.001$) increased cortisol concentration in July – September above January – March.

Table 2.1. Range and mean (±se) for serum cortisol concentrations (in ng/ml) for the total population, broken into age groups (pups ≤ one year, juveniles 1 to 3 years, adults > 3 years) and for captive Steller sea lions.

Population Data	**Range**	**Mean**	**±se**	**N**
	3.1 to 442.2	141.2	1.5	1079
Pups (free-ranging) less than one year	**Range**	**Mean**	**± se**	**N**
	3.1 to 442.2	140.8	1.5	995
Juveniles (free-ranging) one to three years	**Range**	**Mean**	**±se**	**N**
	38.9 to 283.2	145.1	6.6	84
Adults (free-ranging) over three years[1]	**Range**	**Mean**	**±se**	**N**
	9.6 to 119.0	74.2	5.4	29
Captives (juveniles)[2]	**Range**	**Mean**	**±se**	**N**
	30.3 to 206.4	87.4	4.8	68[2]
Captives (adults)[3]	**Range**	**Mean**	**±se**	**N**
	23.1 to 193.8	77.6	2.4	155[2]
	[1]actual age range is 4 to 16+ years [2]from eight individuals aged 1 year to 3 years [3]from nine individuals (5 males and 4 females) ages 5-12 years.			

Table 2.2. Mean (±se) cortisol concentration (ng/ml) in serum from free-ranging Steller sea lion pups less than one month of age by area and island sampled in 2000, 2001 and 2002.

AREA	ISLAND	AVERAGE	SE	N
SW 2000	Marmot Island	94.5	6.8	20
	Sugarloaf Island	70.0	5.4	32
	Chiswell Island	116.4	7.7	4
SE 2000	Sail Island	108.9	18.5	8
	SW Brothers	131.0	31.5	4
SW 2001	Seguam/Saddleridge	117.6	8.6	10
	Akutan/Cape Morgan	108.8	7.6	11
	Ugamak/North	112.6	4.6	20
	Ugamak/Ugamak Bay	92.2	10.2	22
	Chowiet Island	142.0	9.3	8
	Wooded (Fish)	99.8	21.2	9
	Seal Rocks	105.2	8.5	18
SE 2001	Lowrie Island	137.6	3.7	55
	Grassy Island	158.1	7.8	21
	Hazy Island	129.8	5.8	21
R 2001	Antsiferov	138.0	7.5	40
	Lovushki	148.3	4.5	59
	Raikoke	113.4	3.4	51
	Sregnego	138.2	4.0	51
	B. Chirpoev	139.0	4.3	44
	Iony Island	164.1	4.2	73
SW 2002	Amak Island	179.1	13.1	10
	Pinnacle Rock	145.8	19.7	10
	Atkins Island	146.6	10.1	10
	Chirikof Island	133.4	11.4	10
	Marmot Island	144.2	7.2	20
	Sugarloaf Island	161.5	8.6	22
SE 2002	Lowrie Island	159.4	5.6	49
	White Sisters	154.0	9.0	42
	Graves Rock	150.2	8.0	20
R 2002	Iony Island	124.3	8.4	31
	Yamskie	121.6	10.4	34
	Medny Island	155.9	8.5	42
	Koslova Cape	218.8	6.6	42
AVERAGE		**139.9**	**0.1**	**913**

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CHAPTER 3.

Organochlorine contaminants in endangered Steller sea lion pups (*Eumetopias jubatus*) from western Alaska and the Russian Far East*

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Abstract:

Investigations into the cause of the Steller sea lion population decline have focused on numerous factors, including exposure to organochlorines (OCs). OCs such as polychlorinated biphenyls (PCBs), 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, or dichlorodiphenyltrichloroethane (DDT), are associated with deleterious biological effects in marine mammals. We measured these compounds in whole blood of free-ranging Steller sea lion pups to characterize the magnitude of contamination across their geographical range. Whole bloodflow of 212 pups from western Alaska was analyzed for OCs. ∑PCB concentrations of pups from Alaska ranged 0.21 to 13 ng/g wet weight (ww) (mean 2.1±0.27 ng/g ww). The Russian animals, ∑PCBs in pups were 0.33 to 36 ng/g ww (mean of 4.3±0.44 ng/g ww). ∑DDT concentrations in pups from western Alaska ranged 0.18 to 11 ng/g ww (mean 1.6±0.23 ng/g ww). In Russia, ∑DDT in pups ranged from undetectable to 26 ng/g wet weight (mean 3.3±0.36 ng/g ww). OC concentrations were higher in Russian pups compared to Alaskan pups (PCBs and DDTs, $p<0.001$) and in both locales females had higher concentrations than males. Male pups from Alaska had lower levels of ∑PCBs and ∑DDT when compared to male pups from Russia (PCBs and DDTs $p<0.001$). Female pups from Alaska were significantly lower in ∑PCBs than Russian female pups (PCBs $p=0.009$) as were female pups for ∑DDT levels between areas (DDTs $p=0.026$). These data indicate that SSL pups have measurable concentrations of OCs. The physiological role these chemicals may have in either the decline or failure of the endangered SSL population to recover needs further investigation. This research indicates specific geographic areas and animals that may be most at risk.

Introduction:

Steller sea lions (*Eumetopias jubatus*) range from the California Channel Islands along the North Pacific Rim to northern Japan. In the 1970s, the greatest concentration of animals occurred in the western Gulf of Alaska and along the eastern Aleutian Islands. This portion of the population has experienced a dramatic decline over the last 30 years. Overall, Steller sea lion population numbers have decreased more than 80% since 1976 (Sease et al., 2001) and about 70% since 1985 (Calkins et al., 1999). This decline led to the species being listed in 1990 as threatened under the U.S. Endangered Species Act (55 U.S. Federal Register 49204). In 1997, genetic information and population trends indicated that distinct stocks existed (Bickham et al., 1996, Loughlin, 1997). Subsequently, the Western stock, which occurs from 144° West longitude (just east of Prince William Sound, Alaska) to Japan, was listed as endangered (62 U.S. Federal Register 24345) whereas the eastern stock from southeast Alaska to California remains listed as threatened. Recently, Baker et al., (2005) suggested that the Russian Far East stock may be genetically distinct from the two Alaskan stocks.

The subsequent development of specific recovery plans for Steller sea lions incorporated requirements to include investigation into pollution as a proximate cause for the decline or the failure of the Steller sea lion population to recover (National Marine Fisheries Service (NMFS) 2006). As one form of pollution, organochlorines are a diverse group of compounds synthesized for various purposes including use as pesticides and as lubricants in machinery and electrical equipment. OCs are highly fat soluble (lipophilic), have low water solubility (hydrophobic) and differentially accumulate in the lipids of animals. Designed for chemical stability, most OCs are persistent in the environment and are resistant to metabolic degradation. OCs generally biomagnify or increase in concentration as the compounds move up through food webs (O'Shea, 1999). Due to this biomagnification, the highest concentrations typically are found in top-level predators (de Wit et al., 2004). The world's atmosphere and oceans transport many of these compounds to northern latitudes (Iwata et al., 1993, Wania and Mackay 1993, Arctic Monitoring and Assessment Program (AMAP), 2004). Airborne chemicals

precipitate out in cold air sinks resulting in deposition of these chemicals in pinniped habitats of the North Pacific (Li et al., 2002).

Exposure to OCs, such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), has been linked to a host of potent biological effects including immunosuppression, carcinogenicity, reproductive toxicity, neurotoxicity and endocrine disruption in mammals and may potentially impact the health or reproductive success of Steller sea lion populations. For example, OC exposure has been associated to premature births and cancer in California sea lions (DeLong et al., 1973, Ylitalo et al., 2005c) as well as immune dysfunction in harbor seals (Ross et al., 1995, de Swart et al., 1996, Hammond et al., 2005) and northern fur seals (Beckmen et al., 2003). Holmes and York, (2003) suggested that Steller sea lion population declines in the 1990s were associated with disproportionately low fecundity rates that may be related to overt toxicity or endocrine disruption caused by exposure to high levels of environmental contaminants.

Surveys have shown that Steller sea lions have relatively high levels of organic pollutants in their systems, specifically OCs, such as PCBs and DDT. Sea lion blubber had PCB concentrations as high as 41 μg/g (lipid weight) and DDT concentrations as high as 17 μg/g (Lee et al., 1996) in samples collected during the height of the population decline. Even though OC contamination has not been hypothesized to be the primary factor that precipitated the population decline, there is a potential for these chemicals to contribute to the decline as one of a number of stressors (e.g., reduced quality or quantity of prey) that act together. Furthermore, no other single threat has been shown to be the cause of the decline (NMFS 2006).

The intent of the current study was to measure OCs in whole blood of Steller sea lion pups in order to determine the extent and magnitude of contamination across the geographic range of the population. The null hypotheses included no difference in OC concentrations would exist between males and females, no differences would exist among rookeries in the U.S and Russia. In order to test these hypotheses, 212 whole blood samples were collected in 2002 from free-ranging Steller sea lion pups in western Alaska

and the Russian Far East. Blood samples were analyzed for selected OCs, including dioxin-like and other PCB congeners, as well as DDT and its metabolites.

Materials and Methods

Sample Collection

In the current study, whole blood samples were collected for contaminant analysis from 212 free-ranging one month old Steller sea lion pups (Figure 3.1 shows a map of the area sampled with the specific rookeries indicated). All samples were collected in collaboration with the NMFS and North Pacific Wildlife Consulting during the 2002 field season. Of the 212 samples collected, 76 pups (36 females and 40 males) were from western Alaska and the other 136 (63 females and 73 males) were from the Russian Far East. Samples for western Alaska animals came from six rookeries including Amak (females n=5, males n=5), Pinnacle Rock (females n=3, males n=6), Atkins Island (females n=5, males n=4), Chirikof Island (females n=5, males n=5), Marmot Island (females n=9, males n=9) and Sugarloaf Island (females n=9, males n=11). Samples from the Russian Far East came from four rookeries including Iony Island (females n=12, males n=14), Yamskie (females n=13, males n=20), Koslova Cape (females n=17, males n=21) and Medney Island (females n=21, males n=18).

Blood samples were taken by venipuncture from a rear flipper or the caudal-gluteal vein. Whole blood was collected into tubes with EDTA additive and kept cool on ice packs until freezing, which was typically within 4 to 6 hr of collection. Animals were weighed and length measured in the field. The samples were shipped on dry ice to the analytical laboratory and stored at -80°C until chemical analyses.

OC and lipid analyses

All samples were analyzed at the NMFS Northwest Fisheries Science Center in Seattle, WA. Blood samples from Steller sea lion pups were analyzed for dioxin-like PCBs and other selected OCs by a high-performance liquid chromatography/photodiode array (HPLC/PDA) method (Krahn et al., 1994). Blood (3.0 - 8.0 g), hexane/pentane (1:1 v/v), sodium sulfate (10 g for whole blood) and a surrogate standard were homogenized and separated from interfering compounds (e.g., lipids, aromatic compounds) on a gravity

flow cleanup column that contained neutral, basic and acidic silica gels eluted with hexane/methylene chloride (1:1 v/v). Prior to the cleanup step, a 1-mL aliquot of each sample extract was removed for lipid quantiflcation by thin layer chromatography with flame ionization detection (TLC/FID) (Krahn et. al., 2001, Ylitalo et. al., 2005b). Eight dioxin-like PCB congeners (PCBs 77, 105, 118, 126, 156, 157, 169, 189) were resolved from other frequently measured PCBs (PCBs 101, 128, 138, 153, 170/194, 180) and six chlorinated pesticides [o,p'-DDD, p,p'-DDD, p,p'-DDE, o,p'-DDT, p,p'-DDT, hexachlorobenzene (HCB)] were also determined by HPLC on two Cosmosil PYE analytical columns, connected in series and cooled to 16°C. The congeners were measured by an ultraviolet (UV) photodiode array detector and were identifled by comparing their UV spectra (200-310 nm) and retention times to those of reference standards in a library. The lower limits of quantitation (LLOQs) for the PCB congeners ranged from <0.03 to <0.3 ng/g, ww. The LLOQ for DDT and DDT metabolites ranged from 0.1 to <1.4 ng/g, ww.

Sum PCBs (∑PCBs) were calculated using the following formula: ∑PCBs= ∑concentrations of 15 PCBs listed above (based on individual response factor)+ ∑concentrations of other PCB congeners (calculated by summing areas of peaks identified as PCBs and using an average PCB response factor). Sum DDT (∑DDTs) concentrations were calculated by summing the concentrations of five DDTs (o,p'-DDD, p,p'-DDD, p,p'-DDE, o,p'-DDT, p,p'-DDT). Using the concentrations of the individual dioxin-like PCBs, toxic equivalent (TEQ) values were calculated. For this calculation, the molar concentration of each dioxin-like PCB congener was multiplied by the appropriate toxic equivalency factor (TEF), recommended recently by World Health Organization for human and wildlife health (van den Berg, et. al., 1998). The following TEF values were used for calculating the PCB TEQ values: PCB77 (0.0001), PCB105 (0.0001), PCB118 (0.0001), PCB126 (0.1), PCB156 (0.0005), PCB157 (0.0005), PCB169 (0.01) and PCB189 (0.0001).

Concentrations of lipid classes and total lipid of the Steller sea lion pup whole blood samples were measured by thin-layer chromatography with flame ionization

detection (TLC/FID) using an Iatroscan Mark 6 (Iatron Laboratories, Tokyo, Japan) as described by Krahn et al., (2001) and Ylitalo et al., (2005a). Various classes of lipids (i.e., wax esters/sterol esters, triglycerides, free fatty acids, cholesterol and polar lipids) were measured for each sample, and total lipid concentrations were calculated by adding the concentrations of the five lipid classes and were reported as percent total lipid.

Quality Assurance

A sample set for OC analysis contained 10–14 field samples, a method blank and a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM 1945) blubber sample. Laboratory quality assurance criteria were met for all quality control procedures (Sloan et al., 2006). For example, concentrations of ≥70% of individual analytes (see OC list above) that were measured in the NIST SRM 1945 were within 35% of either end of the 95% confidence interval range of the published NIST certified concentration of the OC (Wise et al., 1993). Method blanks contained no more than four analytes that exceeded four times the LLOQ, unless the analyte was not detected in the associated samples of the set.

Statistical analyses

Student t-tests were used to compare mean concentrations of OCs between areas or stocks, as well as between male and female Steller sea lions pups at each rookery. Analysis of variance (ANOVA) and the Tukey-Kramer HSD test were used to determine differences in mean concentrations of OCs among the different rookeries. The level of significance used for all statistical tests was $p \leq 0.05$. The correlation between percent lipid and summed OC concentrations of the blood samples was assessed by simple correlation analyses (Zar 1999).

Results

Concentrations of ∑PCBs, ∑PCB TEQs and ∑DDTs and ranges for each across the entire population, and broken down into pups from western Alaska and pups from the Russian Far East, are shown in Table 3.1 (Table 3.1a is ww and Table 3.1b is lipid adjusted) and graphically in Figure 3.2. Concentrations of ∑PCBs, ∑PCB TEQs and ∑DDTs and ranges for the western Alaska rookeries are shown in Table 3.2 (Table 3.2a

is ww and Table 3.2b is lipid adjusted). Concentrations of ∑PCBs, ∑PCB TEQs and ∑DDTs and ranges for the Russian Far East rookeries are shown in Table 3.3 (Table 3.3a is ww and Table 3.3b is lipid adjusted). Because no significant correlations were found between percent lipid and ∑PCBs (r^2 = -0.00404, p = 0.69), ∑DDTs (r^2 = -0.002, p = 0.475) and ∑PCB TEQs (r^2 = -0.0040, p = 0.59) in the sea lion blood samples, we presented the OC concentration data on a ww basis. We also reported the lipid-adjusted OC levels in the Steller sea lion blood samples in order to compare the contaminant levels in these animals with those previously described in the blood of other species of marine mammals.

Overall, whole blood levels of ∑PCBs were higher than ∑DDTs in sea lion pups from both regions (for PCBs versus DDTs ww and lipid weight in western Alaska p=0.001, in the Russian Far East for ∑PCBs versus ∑DDTs ww p<0.001 and lipid weight p=0.007) (Figure 3.2). Whether or not the OC concentrations were normalized to lipid, this was consistent when OCs were compared by rookery with the exception of two sites. The only notable difference was that Koslova Cape in the Russian Far East had higher ∑PCBs than all other areas (one way ANOVA for ∑PCBs ww by site p<0.001) but for ∑DDTs, Iony Island was higher than Koslova Cape but the difference between these two sites was not significant (Figure 3.6).

Regardless of geographic region, the trend was for female pups to have higher concentrations of both ∑PCBs and ∑DDTs when compared to males but the differences were not significant (p>0.05) (Figure 3.3). However, we found significant differences (p<0.001) in mean body masses between males and females from each region (Figure 3.4). Males from western Alaska averaged 35.1±0.7 kg whereas the mean mass of female pups was 27.8±0.7 kg. In pups from the Russian Far East, males averaged 33.5±0.5 kg and the average for females was 27.9±0.5 kg. There were no significant differences (p>0.05) in body mass between western Alaska and the Russian Far East when males and females were considered separately.

Average contaminant concentrations compared on a ww basis were significantly higher in Russian animals compared to western Alaska (for PCBs and DDTs p<0.001;

Figure 3.2). Male pups from western Alaska had significantly lower levels of ∑PCBs and ∑DDT when compared to male pups from Russia (for PCBs and DDTs $p<0.001$; Figure 3.2). Female pups from western Alaska were significantly lower in ∑PCBs than Russian female pups (for PCBs $p=0.009$) as were female pups for ∑DDT levels between areas (for DDTs $p=0.026$; Figure 3.2). The average lipid concentration for western Alaska was 0.04 ± 0.00 ng/g. The average lipid concentration for the Russian Far East was 0.09±0.02 ng/g. There was no significant difference in lipids concentrations between areas (Table 3.1).

Based on rookery, we found significant differences in mean concentrations of ∑PCBs and ∑DDTs between locations (one way ANOVA for PCBs and DDTs in both western Alaska and the Russian Far East, $p<0.001$; Tables 3.2 and 3.3; Figures 3.5 and 3.6). ∑PCB TEQs were also significantly different. For pups from western Alaska average ∑TEQs were 0.03±0.00 ng/g (ww; Tables 3.1, 3.2). Animals from the Russian Far East ∑TEQs averaged 0.08±0.01 (ww) (for TEQs $p<0.001$; Tables 3.1, 3.3).

Discussion

Steller sea lion pups from the Russian Far East contained higher contaminant concentrations than western Alaska pups (Figure 3.2) and these levels may be of significant concern. In light of information that animals from the Russian Far East are a unique stock (Baker et. al., 2005), significant differences in contaminant concentrations in sea lions from the two regions suggest that Russian animals may be exposed to considerably more point source pollution or may be in a more direct line for long-range atmospheric or oceanic transport. Similarly, indigenous people from the Chukotka Peninsula in the Russian Far East had higher maternal and cord blood levels of HCB, DDTs and PCBs compared to people from other regions of the Russian North and Western Alaska (AMAP, 2004). These contaminants were most likely obtained from consuming contaminated fish, marine mammals and other biota collected from this area. Another explanation for differences in contaminant levels in sea lions from the two regions, though less likely, may be that there are genetic differences in the way animals store or metabolize these compounds.

In order to compare results found here to other studies and species it is important to take into consideration some of the fallacies that coincide with such an attempt. It is difficult to compare concentrations between studies and species as sampling protocols, sample medium, laboratory procedures and reporting formats fluctuate and therefore exclude most direct comparisons. However, there are some other recent reports on OCs in Steller sea lions. Barron et al., (2003) reported that PCB and DDT concentrations measured in the 1980s were the highest recorded for any Alaskan pinniped. In order to gain perspective between the threatened southeastern Alaska population of Steller sea lions and the endangered western population, the AMAP reported on unpublished data collected by Beckmen between 1998 and 2000 (de Wit et al., 2004). The AMAP report based on these data concluded that Steller sea lions from the eastern Aleutian Islands had higher levels of PCBs and DDTs in feces compared to southeast Alaska animals. Hoshino et al., (2006) looked at contaminants in Steller sea lions from areas in the western North Pacific and concluded that some animals may have levels high enough to cause physiological problems and that these levels were also higher than animals from the eastern North Pacific.

In an effort to compare our results on OCs in Steller sea lions to other North Pacific marine mammals, only studies that report concentrations in blood are suitable for evaluation. Unfortunately, there are only a few of these types of studies. Compared to the current study, Beckmen et al., (1997) found much higher OC levels in blood of northern fur seal (*Callorhinus ursinus*) neonates and pups ($\sum$PCBs means from 16.2 to 22.8 ng/g ww; $\sum$DDTs means from 2.94 to 13.2 ng/g ww) but only 11 PCB congeners and three DDT metabolites were measured. In our study, we resolved 14 PCB congeners and six DDT metabolites. In addition, Beckmen et al., (1999) had lipids (means from 0.22 to 0.30 ng/g) that were much higher than those reported here (mean for all pups 0.08 ng/g). There is some work that has been done on OCs in blood for polar bears (*Ursus maritimus*) from around the North Pacific. Andersen et al., (2001) and Lie et al., (2003) also reported higher blood levels of PCBs but lower DDT levels in animals from the eastern Siberian Sea and the Chukchi Sea compared to our study ($\sum$PCBs means from

18.4 to 26.7 ng/g ww; ∑DDTs means from 0.14 to 0.28 ng/g ww) but only measured 6 congeners for ∑PCBs and one metabolite for ∑DDTs and these studies had even higher lipids (from 0.55 to 0.75 ng/g). Again, due to differences in study design, it is difficult to deduce much from these types of comparisons.

Concentrations of PCBs were higher than DDTs in sea lions from both Russia and western Alaska. One finding of interest in the current study was that female sea lion pups had higher OC concentrations compared to males (Figure 3.3). Previous studies have found that circulating levels of OCs in male and female pups of other pinniped species were not significantly different (Beckmen et al., 1999, Debier et al., 2003). The differences in OC levels found between the sexes in the current study may be due to differences in sizes (body masses) that were found between female and male pups (Figure 3.4). Costa et al., (1998) reported that the blood volumes of female New Zealand sea lions (*Phocarctos hookeri*) increased with increasing body masses. If we assume that the total OC burdens of one-month old sea lion pups (both males and females) are similar, it is possible for larger pups (males), due to their greater volume of blood, to have lower (diluted) circulating levels of OCs than smaller animals (females). However, without information on the total OC body burdens of the Steller sea lion pups, it is difficult to determine if body mass influences the circulating OC levels of these animals. To further illustrate the significance of the differences between areas and sexes, it is important to note that male pups were similar in size between Russia and western Alaska, as were females, further indicating that the higher levels in Russia were not due to a sampling bias by sex.

∑PCB and ∑PCB TEQ concentrations in blood of Steller sea lion pups were compared to levels associated with biological and physiological effects in blood of harbor seals (*Phoca vitulina*) (Kannan et al., 2000). The levels in harbor seals were based on the findings of various studies that measured a range of toxicological endpoints (e.g., natural killer cell activity, thyroid hormone concentrations and levels of vitamin A) and levels of PCBs. For PCB effects, Kannan et al., (2000) recommended a PCB threshold concentration of 11,000 ng/g lipid weight. In the current study, the population mean of

∑PCBs based on lipid weight is below the threshold (∑PCBs in all pups was 9,000 ng/g lipid weight). When broken down into areas, ∑PCBs for western Alaskan pups averaged only 5,200 ng/g lipid weight, which is well below threshold levels. However, in western Alaskan pups, 9 out of 76 pups (or 12% of our sample population) exceeded the threshold concentration. Russian pups had an average ∑PCBs of 11,000 ng/g lipid weight, which is right at threshold levels. In Russian pups, 39 of the 136 pups sampled (or 29%) exceeded the threshold concentration.

When comparing OC concentrations based on rookery, differences in mean contaminant concentration emerged. Koslova Cape in the Russian Far East had the highest ∑PCB concentrations in this study (Figure 3.6). The average level of ∑PCBs (lipid adjusted) in blood of pups at Koslova Cape was 18,000 ng/g, nearing twice that of the 11,000 ng/g lipid weight identified by Kannan et al., (2000) as critical limits. Eighteen of 38 or 47% of the Koslova Cape animals exceeded this threshold, suggesting that certain individuals had high PCB concentration (one individual had ∑PCB concentration of 120000 ng/g lipid weight). Iony Island in Russia had the highest ∑DDT concentrations in this study (Figure 3.6) and these values were similar to those of pups at Koslova Cape. Medney Island, at the end of the Aleutian chain but part of the Russian Far East, had much lower levels of OCs compared to the other Russian sites and was similar to Sugarloaf and Marmot Islands in western Alaska. The highest levels of OCs in western Alaska were found at Amak Island (Figure 3.5). The lowest concentrations of OCs were at Atkins Island in western Alaska (Figure 3.5).

In a captive harbor seal study, immunosuppressive effects were measured in seals that had been fed Baltic Sea herring that contained high levels of PCBs (de Swart et al., 1994, 1996, Ross et al., 1995). The mean concentration of TEQs measured in the blood of the immune-compromised harbor seals was 72 pg/g lipid weight. In the present study, our Steller sea lion pup population average TEQ was 158 pg/g lipid weight or more than twice the threshold found in harbor seals. Western Alaskan pups TEQ averaged 71 pg/g lipid weight, which is near the threshold and 30 of the 76 pups or approximately 40% exceeded this threshold. For Russian pups the TEQ average was 206 pg/g lipid weight or

almost three times the threshold value and 87 of the 136 Russian pups or 64% of the animals exceeded the threshold TEQ value. The TEQ values determined using the HPLC/PDA method are conservative because only selected PCBs were quantified and the PDA limits of detection (LOD) were higher than the LODs of high-resolution gas chromatography/mass spectrometry. Still, they provide an estimate of the relative toxic potency present in the Steller sea lion pup blood samples.

Any comparison of OC contamination is incomplete without consideration of life history parameters of individuals. In most mammal studies, OC concentrations increase or bioaccumulate as animals mature (an exception is that females may transfer contaminants to their offspring via gestation and lactation) (de Wit et al., 2004). As all of the animals in this study are one month old pups that have only been exposed via gestation and lactation, the concentrations reported here should be considered the minimum for this species and will most likely be expected to increase over time. Considering females off-load the majority of their contaminant load to their first born (Beckmen et al., 1999, Debier et al., 2003, Wolkers et al., 2004), it may be pups from the primiparous births have the highest concentrations encountered in this study but there is no way to verify this. All the threshold effect levels used to make comparisons were based on studies of older animals and are therefore already conservative when compared to one month old pups, as in this study.

The concentrations of OC contaminants in Steller sea lion pups suggest that levels may be capable of causing physiological problems such as immunosuppression in individuals, as well as affecting other biological factors, and thus have potential effects to the sea lion population. Therefore, OC contaminants can not be dismissed as a contributing source to either the decline of the Western Steller sea lions or the failure of the population to recover. These data suggest that concentrations of OCs in certain Steller sea lion pups may be high enough to potentially cause reproductive dysfunction and impact fecundity. It is possible that OC contaminants were a contributing source to lower fecundity in the 1990s (Holmes and York, 2003) but as there are no samples available from that time period, this postulate is not verifiable. Census data from 2000,

2002 and 2004 showed that the Steller sea lion population may be beginning to recover (NMFS, 2006). As world levels of OCs are tending to decrease (de Wit et al., 2004), it is probable that concentrations were higher in the past and the concentrations represented here may be low enough in some areas to allow for population growth. However, other problematic chemicals (e.g., polybrominated diphenyl ethers) are rapidly increasing in concentrations in North America and these pollutants (and others) may replace PCBs as chemicals of concern (de Wit et al., 2004). Future work should include investigations into how contaminants change over time in captive Steller sea lions and if there are relationships between OC contaminants and physiological biomarkers.

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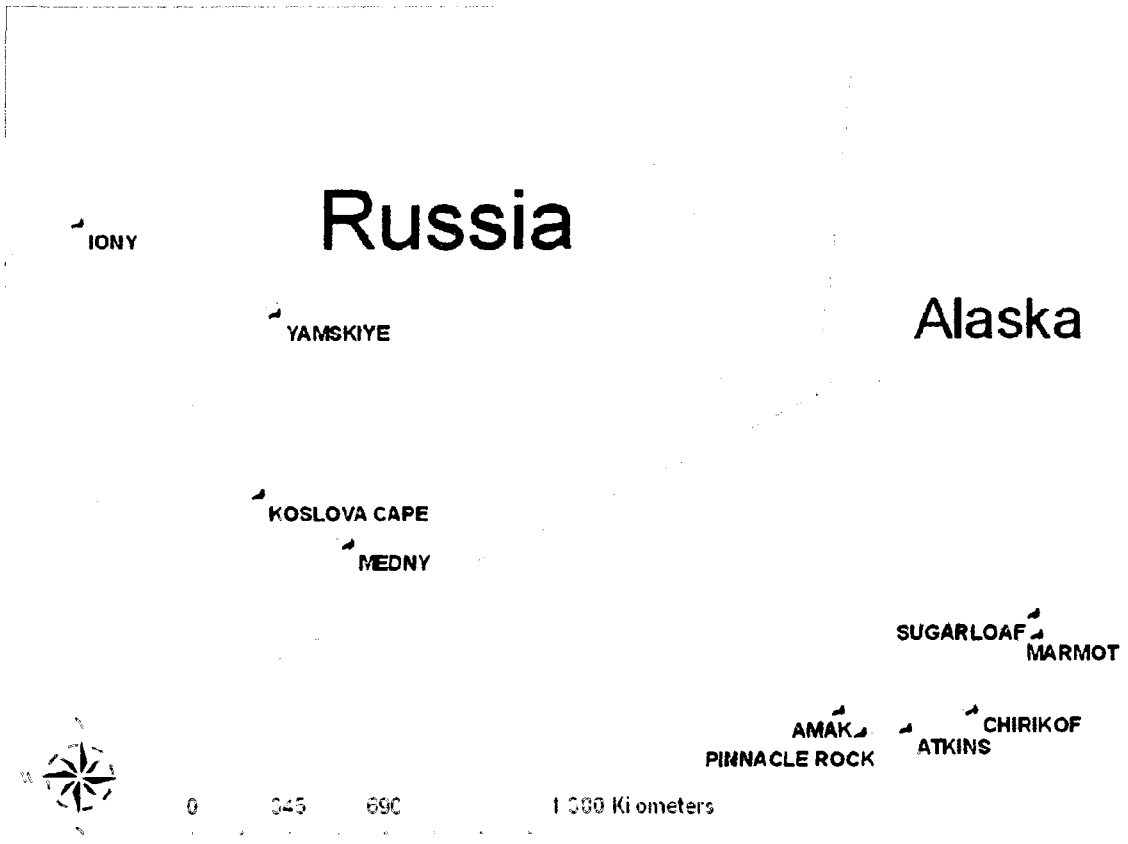

Figure 3.1. Map of the study area and locations of rookeries sampled.

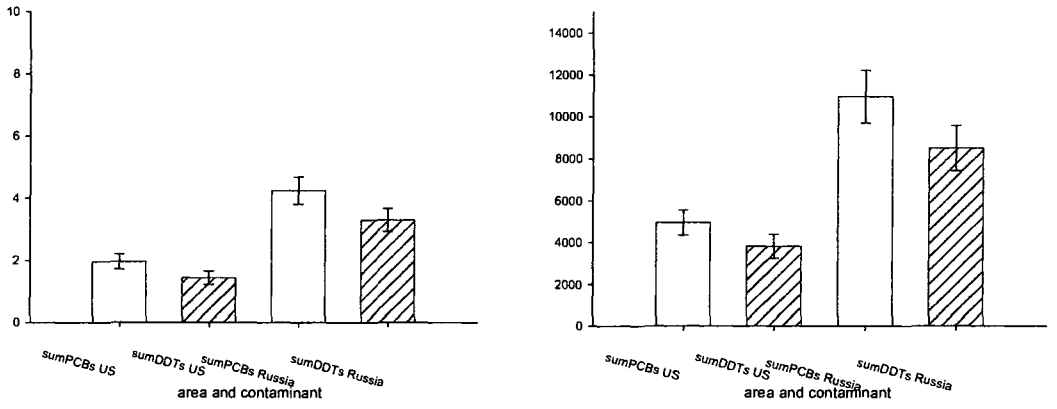

Figure 3.2. Mean (± se) organochlorine concentrations in blood (ng/g, ww on left, lipid weight on right) by area in free-ranging Steller sea lion pups (< 1 month of age). Solid bars are ∑PCBs and striped bars are ∑DDTs. ∑PCBs are higher than ∑DDTs in both regions ($P \leq 0.00$) and for both ww and lipid weight concentration ($P < 0.01$). Russian sea lion pup had significantly higher concentrations than Alaskan pups ($P < 0.001$).

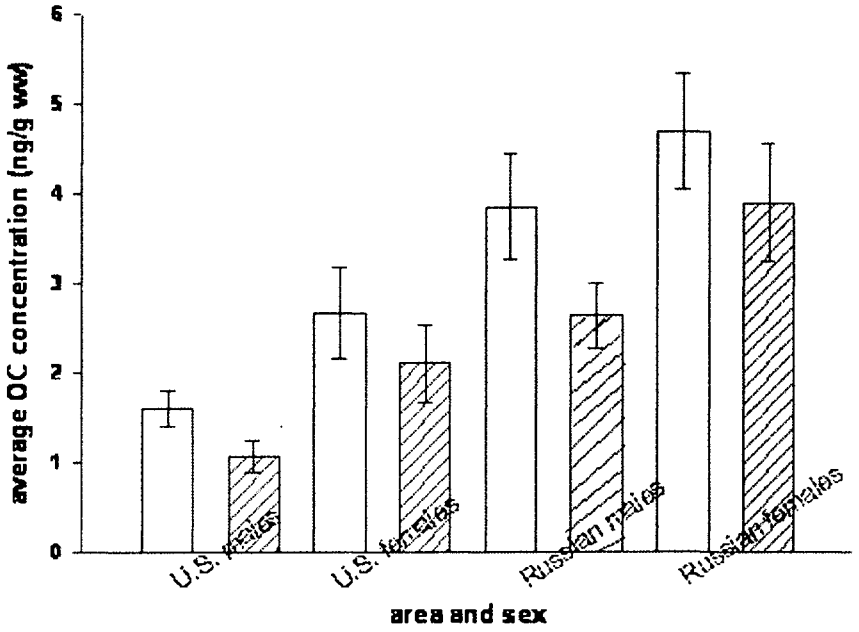

Figure 3.3. Mean (± se) organochlorine concentrations in blood (ng/g, ww) by area and sex in free-ranging Steller sea lion pups (< 1 month of age). Western Alaska is shown in white and Russia in gray. Striped bars are ∑DDTs and solid bars are ∑PCBs.

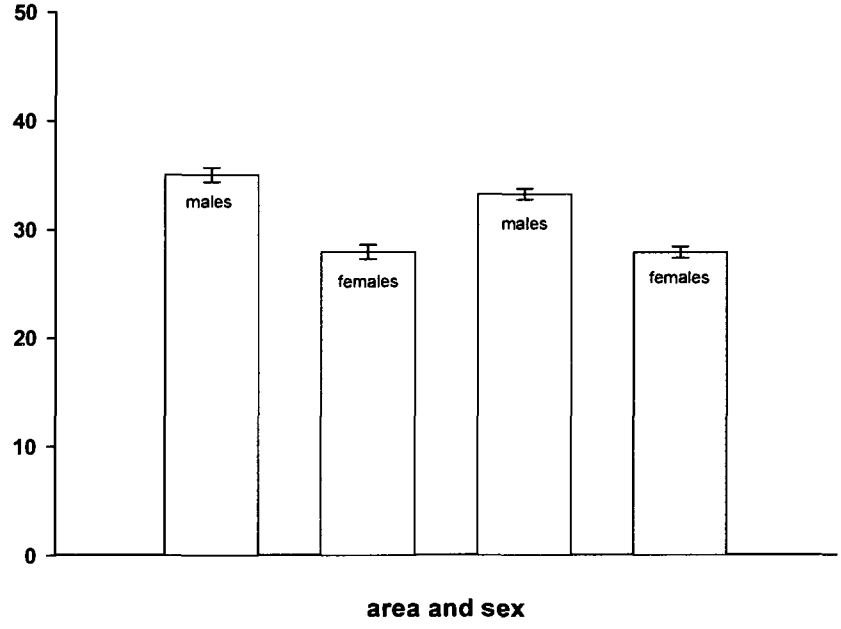

Figure 3.4. Mean (± se) body mass (kg) by area and sex in free-ranging Steller sea lion pups (< 1 month of age). Western Alaska is shown in white and Russia in gray. Male sea lion pups were significantly heavier than female pups ($P < 0.001$).

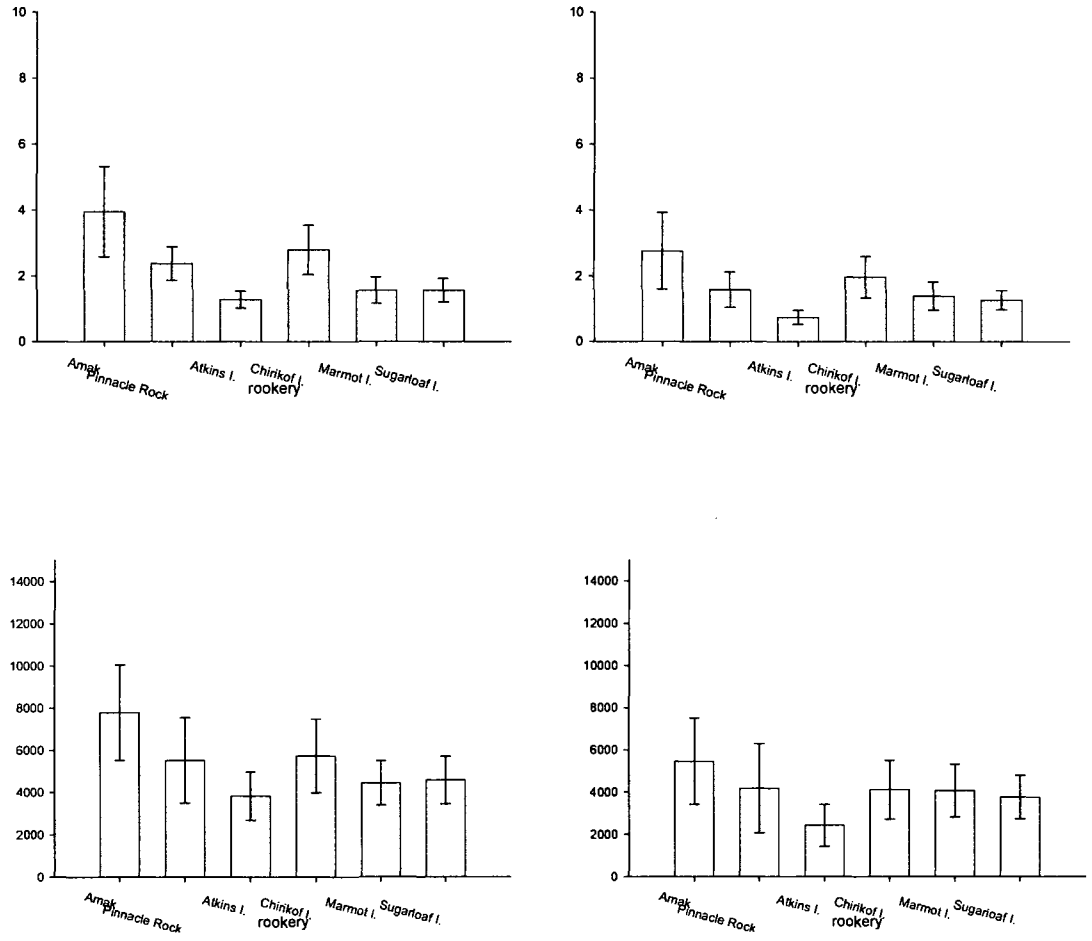

Figure 3.5. Mean (± se) organochlorine concentrations in blood (ng/g) by rookery in free-ranging Steller sea lion pups (< 1 month of age) from western Alaska. ∑PCBs are shown in the left panels and ∑DDTs are to the right. Top panels are ww and lower panels are lipid adjusted.

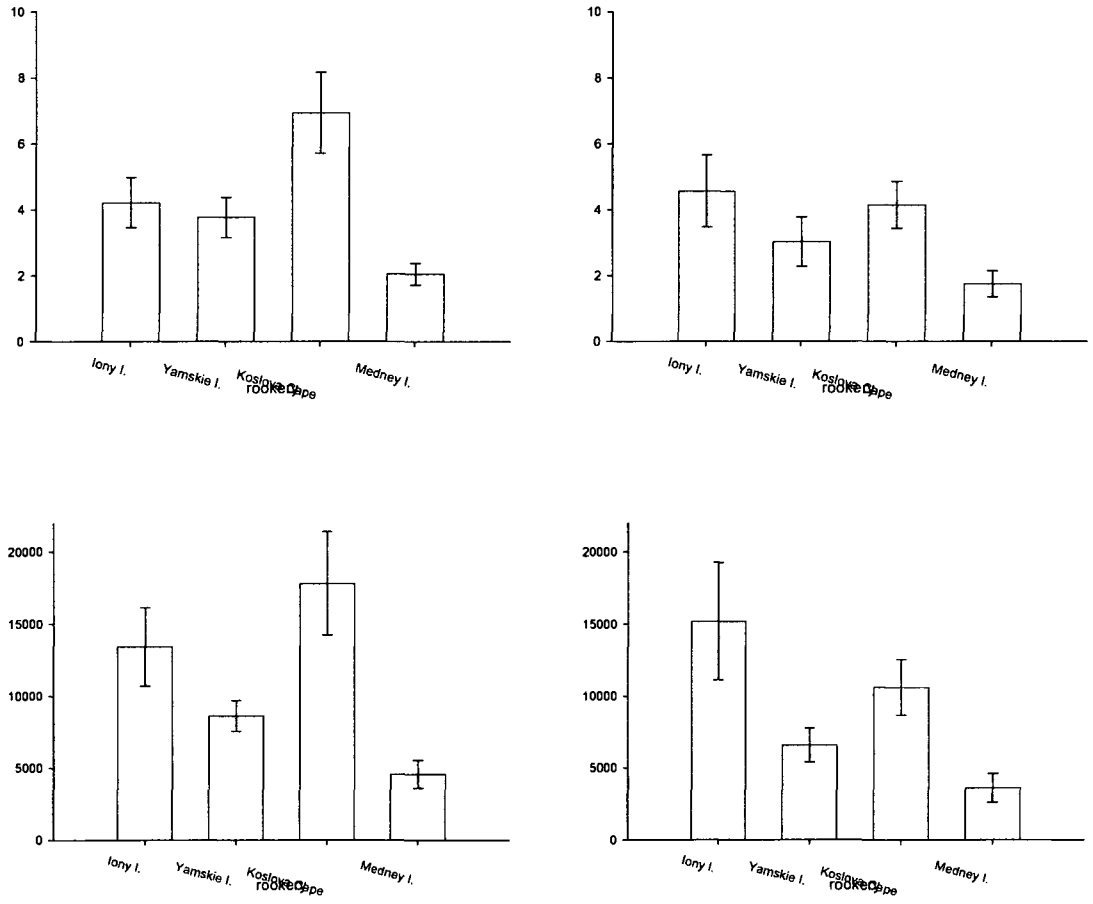

Figure 3.6. Mean (± se) organochlorine concentrations in blood (ng/g) by rookery in free-ranging Steller sea lion pups (< 1 month of age) from the Russian Far East. ∑PCBs are shown in the left panels and ∑DDTs are to the right. Top panels are ww and lower panels are lipid adjusted.

Table 1. Mean (±SE) concentrations of sum PCBs (∑PCBs), sum PCB TEQs (∑PCB TEQs) and sum DDTs (∑DDTs) in blood samples from free-ranging Steller sea lion pups.

N	Site or group	Percent Lipid*	∑PCBs ng/g, wet wt.	∑PCB TEQs pg/g, wet wt.	∑DDTs ng/g, wet wt.	∑PCBs ng/g, lipid wt.	∑PCB TEQs pg/g, lipid wt.	∑DDTs ng/g, lipid wt.
212	All pups	0.08 ± 0.01	3.49 ± 0.31	0.06 ± 0.01	2.67 ± 0.26	8896.7 ± 862.8	157.5 ± 20.5	6885.9 ± 737.1
113	Males	0.07 ± 0.02	3.08 ± 0.41	0.06 ± 0.01	2.08 ± 0.25	8478.6 ± 1296.4	159.7 ± 33.3	6078.3 ± 846.6
99	Females	0.09 ± 0.03	3.96 ± 0.46	0.07 ± 0.01	3.36 ± 0.47	9369.6 ± 1114.4	154.9 ± 22.0	7807.6 ± 1246.5
76	Western Alaska pups	0.04 ± 0.00	2.12 ± 0.27	0.03 ± 0.00	1.56 ± 0.23	5155.1 ± 610.2	71.3 ± 8.2	3994.5 ± 574.6
40	Males	0.05 ± 0.00	1.61 ± 0.20	0.03 ± 0.00	1.07 ± 0.18	3978.5 ± 622.8	61.4 ± 7.4	3016.2 ± 676.7
36	Females	0.04 ± 0.00	2.67 ± 0.50	0.04 ± 0.01	2.10 ± 0.43	6429.7 ± 1046.2	82.3 ± 15.1	5081.6 ± 928.7
136	Russia pups	0.09 ± 0.02	4.25 ± 0.44	0.08 ± 1.01	3.30 ± 0.36	10960.0 ± 1263.0	205.6 ± 30.9	8501.7 ± 1080.4
73	Males	0.08 ± 0.03	3.86 ± 0.60	0.07 ± 0.02	2.63 ± 0.36	10882.7 ± 1906.6	213.5 ± 50.5	7756.3 ± 1216.3
63	Females	0.12 ± 0.04	4.70 ± 0.65	0.08 ± 0.01	4.11 ± 0.69	11049.6 ± 1614.4	196.4 ± 32.4	9365.4 ± 1863.8

Table 2. Mean (±SE) concentrations of sum PCBs (∑PCBs), sum PCB TEQs (∑PCB TEQs) and sum DDTs (∑DDTs) in blood samples from free-ranging western Alaska Steller sea lion pups by rookery.

N	Site or group	Percent Lipid†	∑PCBs ng/g, wet wt.	∑PCB TEQs pg/g, wet wt.	∑DDTs ng/g, wet wt.	∑PCBs ng/g, lipid wt.	∑PCB TEQs pg/g, lipid wt.	∑DDTs ng/g, lipid wt.
10	Amak Island	0.05 ± 0.01	3.95 ± 1.37	0.07 ± 0.02	2.76 ± 1.16	7797.2 ± 2268.2	126.6 ± 37.5	5463.76 ± 2050.3
5	Male	0.05 ± 0.01	2.00 ± 0.27	0.04 ± 0.01	0.87 ± 0.11	4324.6 ± 499.1	86.1 ± 9.6	2007.0 ± 433.5
5	Females	0.04 ± 0.01	5.90 ± 2.55	0.09 ± 0.04	4.65 ± 2.07	11269.8 ± 4107.5	167.1 ± 73.5	8920.4 ± 3571.3
9	Pinnacle Rock	0.06 ± 0.01	2.38 ± 0.51	0.03 ± 0.00	1.58 ± 0.54	5532.0 ± 2025.7	69.2 ± 14.9	4178.0 ± 2114.3
6	Males	0.06 ± 0.01	1.91 ± 0.45	0.03 ± 0.00	1.10 ± 0.37	3783.6 ± 1214.7	53.6 ± 9.4	2377.4 ± 1125.6
3	Females	0.05 ± 0.01	3.33 ± 1.19	0.04 ± 0.01	2.55 ± 1.47	9028.9 ± 5717.8	100.3 ± 38.4	7781.1 ± 6129.9
9	Atkins Island	0.04 ± 0.00	1.30 ± 0.30	0.02 ± 0.01	0.74 ± 0.22	3692.2 ± 1295.4	54.3 ± 18.1	2427.1 ± 994.7
4	Male	0.05 ± 0.01	1.54 ± 0.36	0.03 ± 0.00	0.73 ± 0.16	2780.2 ± 453.7	69.4 ± 13.0	1831.3 ± 635.3
5	Females	0.03 ± 0.00	1.15 ± 0.44	0.01 ± 0.01	0.75 ± 0.39	4239.4 ± 2106.5	42.2 ± 31.6	2903.8 ± 1786.6
10	Chirikof Island	0.06 ± 0.01	2.80 ± 0.75	0.05 ± 0.01	1.97 ± 0.63	5739.6 ± 1755.8	91.8 ± 19.3	4116.5 ± 1401.3
5	Male	0.06 ± 0.02	1.29 ± 1.18	0.03 ± 0.00	0.60 ± 0.06	2603.5 ± 503.3	61.8 ± 13.1	1362.5 ± 344.8
5	Females	0.05 ± 0.01	4.30 ± 1.17	0.06 ± 0.01	3.35 ± 0.92	8875.6 ± 2950.0	121.7 ± 32.4	6870.5 ± 2219.2
18	Marmot Island	0.04 ± 0.00	1.57 ± 0.40	0.02 ± 0.01	1.39 ± 0.44	4449.8 ± 1056.4	52.6 ± 14.4	4071.6 ± 1256.0
9	Male	0.04 ± 0.00	1.28 ± 0.32	0.02 ± 0.00	1.15 ± 0.45	4402.0 ± 1490.2	56.7 ± 19.8	4253.4 ± 1986.0
9	Females	0.04 ± 0.01	1.86 ± 0.74	0.02 ± 0.01	1.63 ± 0.77	4497.7 ± 1588.0	48.5 ± 21.9	3889.9 ± 1656.5
20	Sugarloaf Island	0.04 ± 0.00	1.57 ± 0.36	0.02 ± 0.00	1.27 ± 0.30	4592.1 ± 1133.3	58.9 ± 13.8	3752.0 ± 1033.2
11	Male	0.04 ± 0.00	1.70 ± 0.59	0.02 ± 0.01	1.42 ± 0.49	4533.0 ± 1757.5	55.3 ± 19.7	3993.6 ± 1731.4
9	Females	0.03 ± 0.00	1.41 ± 0.37	0.02 ± 0.01	1.08 ± 0.29	4664.5 ± 1434.7	63.3 ± 20.1	3456.7 ± 1016.9

Table 3. Mean (±SE) concentrations of sum PCBs (∑PCBs), sum PCB TEQs (∑PCB TEQs) and sum DDTs (∑DDTs) in blood samples from free-ranging Russian Far East Steller sea lion pups by rookery.

N	Site or group	Percent Lipid†	∑PCBs ng/g, wet wt.	∑PCB TEQs pg/g, wet wt.	∑DDTs ng/g, wet wt.	∑PCBs ng/g, lipid wt.	∑PCB TEQs pg/g, lipid wt.	∑DDTs ng/g, lipid wt.
26	Iony Island	0.15 ± 0.08	4.22 ± 0.77	0.06 ± 0.01	4.56 ± 1.10	13429.6 ± 2727.8	189.9 ± 42.6	15199.8 ± 4077.8
14	Male	0.04 ± 0.00	3.59 ± 0.54	0.05 ± 0.01	3.67 ± 0.84	11816.8 ± 2264.3	171.1 ± 32.9	12271.1 ± 3198.3
12	Females	0.28 ± 0.17	4.95 ± 1.55	0.07 ± 0.03	5.60 ± 2.19	15311.2 ± 5382.9	211.9 ± 85.7	18616.7 ± 8112.2
33	Yamskie	0.05 ± 0.00	3.77 ± 0.62	0.07 ± 0.01	3.02 ± 0.75	8627.3 ± 1083.3	165.6 ± 20.6	6575.6 ± 1184.9
20	Male	0.05 ± 0.00	3.52 ± 0.53	0.07 ± 0.01	2.52 ± 0.47	8170.3 ± 1295.7	161.3 ± 27.1	5843.1 ± 1085.2
13	Females	0.04 ± 0.00	4.15 ± 1.37	0.07 ± 0.02	3.80 ± 1.79	9330.6 ± 1945.9	172.1 ± 32.8	7702.7 ± 2541.9
39	Medny Island	0.09 ± 0.04	2.06 ± 0.33	0.03 ± 0.00	1.75 ± 0.40	4592.9 ± 971.3	75.3 ± 22.5	3613.7 ± 1002.7
18	Male	0.05 ± 0.00	1.53 ± 0.32	0 02 ± 0.01	0.94 ± 0.24	5001.1 ± 1779.1	92.9 ± 45.9	3683.2 ± 1784.3
21	Females	0.13 ± 0.07	2.51 ± 0.53	0.03 ± 0.01	2.56 ± 0.72	4243.0 ± 1004.9	60.2 ± 15.2	3554.0 ± 1108.0
38	Koslova Cape	0.11 ± 0.05	6.94 ± 1.23	0.15 ± 0.03	4.15 ± 0.72	17830.6 ± 3588.0	384.7 ± 163.5	10608.0 ± 1934.2
21	Male	0.15 ± 0.09	6.36 ± 1.87	0.14 ± 0.05	3.49 ± 0.96	17884.6 ± 5927.0	394.9 ± 163.5	10059.7 ± 2977.4
17	Females	0.05 ± 0.00	7.67 ± 1.55	0.16 ± 0.04	4.95 ± 1.08	17764.0 ± 3507.0	372.1 ± 84.6	11285.3 ± 2366.0

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CHAPTER 4.

Temporal variability in organochlorine contamination in both blood and blubber in captive Stellers sea lions *(Eumetopias jubatus).* *

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Abstract

Three adult captive Steller sea lions (two females and one male) housed at the Alaska SeaLife Center in Seward, Alaska were repeatedly sampled for organochlorine contaminants (OCs) over a two year period (March 2001 to March 2003). Samples were analyzed in both blood (n=69) and blubber (n=19). Concentrations in blood were similar between sea lions at any given sampling time and followed a seasonal pattern with levels significantly higher in summer months of July to September and lower in the three month winter period January to March. Concentrations of OCs in blubber for the females followed an analogous pattern to blood samples but the male was considerably lower and declined over the study period. A significant relationship between blubber contaminants and lipids was noted in the three captive Steller sea lions. Paired blood and blubber samples (n=18) indicated that there was a positive correlation in organochlorine contamination between the two media and justified using the two interchangeably. The way in which concentrations of organochlorine contaminants change over time in Steller sea lions indicates that while blubber levels may vary between sexes, blood levels are consistent and change with season which is likely associated with seasonal metabolism of blubber stores for various physiological needs.

Introduction

Steller sea lions (*Eumetopias jubatus*) range from the California Channel Islands along the North Pacific Rim to northern Japan. In the 1970s, the greatest concentration of animals occurred in the western Gulf of Alaska and along the eastern Aleutian Islands. This portion of the population experienced a dramatic decline over the last 30 years. Overall, Steller sea lion population numbers decreased more than 80% since 1976 (Sease et al., 2001) and about 70% since 1985 (Calkins et al., 1999). This decline led to the species being listed as threatened under the U.S. Endangered Species Act in 1990 (55 U.S. Federal Register 49204). In 1997, genetic information and population trends indicated that distinct stocks existed (Bickham et al., 1996, Loughlin, 1997). Subsequently, the Western stock, which occurs from 144° West longitude (just east of Prince William Sound, Alaska) to Japan, was listed as endangered (62 U.S. Federal Register 24345) whereas the eastern stock from southeast Alaska to California remains listed as threatened. The subsequent development of a recovery plan for this species incorporated recovery tasks that include investigation into pollution as a proximate cause for the decline or the failure of the Steller sea lion population to recover (National Marine Fisheries Service (NMFS), 2008).

Organochlorines (OCs) are a diverse group of compounds synthesized for various purposes including pesticides and lubricants in machinery and electrical equipment. OCs are highly fat soluble (lipophilic), have low water solubility (hydrophobic) and differentially accumulate in the lipids of animals. Designed for chemical stability, most OCs are persistent in the environment and are resistant to metabolic degradation. OCs generally biomagnify or increase in concentrations as the compounds move up through food webs (O'Shea, 1999). Due to this biomagnification, the highest concentrations typically are found in top-level predators (de Wit et al., 2004). In addition to point or local sources of pollution, the world's atmosphere and oceans transport many of these compounds to northern latitudes (Iwata et al., 1993, Wania and Mackay, 1993). Airborne chemicals precipitate out in cold air sinks resulting in deposition of these chemicals in pinniped habitats of the North Pacific (Li et al., 2002).

Exposure to OCs, such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) has been linked to a host of potent biological effects including immunosuppression, carcinogenicity, reproductive toxicity, neurotoxicity and endocrine disruption in mammals and may potentially impact the health or reproductive success of Steller sea lion populations. For example, OC exposure has been associated to premature births and cancer in California sea lions (DeLong et al., 1973, Ylitalo et al., 2005) as well as immune dysfunction in harbor seals (Ross et al., 1995; de Swart et al., 1996, Hammond et al., 2005) and northern fur seals (Beckmen et al., 2003). Holmes and York, (2003) suggested that Steller sea lion population declines in the 1990s were associated with disproportionately low fecundity rates that may be related to overt toxicity or endocrine disruption caused by exposure to high levels of environmental contaminants.

Surveys have shown that Steller sea lions have relatively high levels of organic pollutants in their systems, specifically OCs, such as PCBs and DDT. Sea lion blubber had PCB concentrations as high as 41 μg/g (lipid weight) and DDT concentrations as high as 17 μg/g (Lee et al., 1996) in samples collected during the height of the population decline. Barron et al., (2003) reported that PCB and DDT concentrations measured in the 1980s were the highest recorded for any Alaskan pinniped. Hoshino et al., (2006) looked at contaminants in Steller sea lions from areas in the western North Pacific and concluded that some animals may have levels high enough to cause physiological problems. Myers et al., (2008) surveyed Steller sea lion pups throughout their range and found that a significant portion of animals have OC concentrations that are high enough to potentially cause physiological problems. Even though OC contamination has not been hypothesized to be the primary factor that precipitated the population decline (Atkinson et al., 2008), there is a potential for these chemicals to have a negative effect on the health of these animals. Furthermore, no other single threat has been shown to be the cause of the decline (Atkinson et al., 2008, NMFS, 2008). Very little information is available on the way OCs change in marine mammals or on temporal analysis of contaminants in Steller sea lions.

The intent of the current study was to measure OCs in whole blood and blubber of captive Steller sea lions in order to investigate the way OC concentrations change over time within individual animals. The null hypotheses were no difference in OC concentrations would exist between males and females and no difference would exist between seasons in both blood and blubber. In order to test these hypotheses, 69 whole blood samples and 19 blubber samples were collected from three captive Steller sea lions over approximately a two year period (March 2001 to March 2003). Blood and blubber samples were analyzed for selected OCs, including dioxin-like and other PCB congeners, as well as DDT and its metabolites. OCs in whole blood and blubber from paired samples (n=18) were evaluated in order to make a blood versus blubber assessment.

Materials and Methods

Sample Collection

Whole blood and blubber samples were collected for contaminant analysis from three captive Steller sea lions (n=88) kept at the Alaska Sealife Center in Seward, Alaska. Blood samples were taken by venipuncture from a rear flipper or the caudal-gluteal vein. Whole blood was collected into tubes with EDTA additive and immediately frozen. A blubber biopsy sample (3–4 cm in length) was collected from the pelvic region using a 6-mm diameter biopsy punch. After collection, the blood samples were placed in solvent-rinsed glass vials; the blubber samples were wrapped in Teflon paper then placed in aluminum foil and stored at –20°C or colder until shipment to the analytical laboratory. Animals were weighed and length measured at the time of sampling. The samples were shipped on dry ice to the analytical laboratory and stored at -80°C until chemical analyses.

OC and lipid analyses

All samples were analyzed at the NMFS Northwest Fisheries Science Center in Seattle, WA. Blood and blubber samples from Steller sea lions were analyzed for dioxin-like PCBs and other selected OCs by a high-performance liquid chromatography/photodiode array (HPLC/PDA) method (Krahn et al., 1994, Myers et al., 2008). Blubber (0.20–0.35 g) or blood (3.0-8.0 g), hexane/pentane (1:1 v/v), sodium

sulfate and a surrogate standard were homogenized and separated from interfering compounds (e.g., lipids, aromatic compounds) on a gravity flow cleanup column that contained neutral, basic and acidic silica gels eluted with hexane/methylene chloride (1:1 v/v). Prior to the cleanup step, a 1-mL aliquot of each sample extract was removed for lipid quantification by thin layer chromatography with flame ionization detection (TLC/FID) (Krahn et al., 2001, Ylitalo et al., 2005b). Eight dioxin-like PCB congeners (PCBs 77, 105, 118, 126, 156, 157, 169, 189) were resolved from other frequently measured PCBs (PCBs 101, 128, 138, 153, 170/194, 180) and six chlorinated pesticides [o,p'-DDD, p,p'-DDD, p,p'-DDE, o,p'-DDT, p,p'-DDT, hexachlorobenzene (HCB)] were determined by HPLC on two Cosmosil PYE analytical columns, connected in series and cooled to 16°C. The congeners were measured by an ultraviolet (UV) photodiode array detector and were identified by comparing their UV spectra (200-310 nm) and retention times to those of reference standards in a library (Krahn et al., 1993). The lower limits of quantitation (LLOQs) for the PCB congeners ranged from <0.03 to <0.3 ng/g, wet weight (ww). The LLOQ for DDT and DDT metabolites ranged from 0.1 to < 1.4 ng/g, ww.

Sum PCBs (∑PCBs) were calculated using the following formula: ∑PCBs=∑ concentrations of 15 PCBs listed above (based on individual response factor) + ∑ concentrations of other PCB congeners (calculated by summing areas of peaks identified as PCBs and using an average PCB response factor). Sum DDT (∑DDTs) concentrations were calculated by summing the concentrations of five DDTs (o,p'-DDD, p,p'-DDD, p,p'-DDE, o,p'-DDT, p,p'-DDT). Using the concentrations of the individual dioxin-like PCBs, toxic equivalent (TEQ) values were calculated. For this calculation, the molar concentration of each dioxin-like PCB congener was multiplied by the appropriate toxic equivalency factor (TEF), recommended recently by World Health Organization for human and wildlife health (van den Berg et al., 1998). The following TEF values were used for calculating the PCB TEQ values: PCB77 (0.0001), PCB105 (0.0001), PCB118 (0.0001), PCB126 (0.1), PCB156 (0.0005), PCB157 (0.0005), PCB169 (0.01) and PCB189 (0.0001).

Concentrations of lipid classes and total lipid of the Steller sea lion whole blood and blubber samples were measured by thin-layer chromatography with flame ionization detection (TLC/FID) using an Iatroscan Mark 6 (Iatron Laboratories, Tokyo, Japan) as previously described (Krahn et al., 2001, Ylitalo et al., 2005a, Myers et al., 2008). Various classes of lipids (i.e., wax esters/sterol esters, triglycerides, free fatty acids, cholesterol and polar lipids) were measured for each sample, and total lipid concentrations were calculated by adding the concentrations of the five lipid classes and were reported as percent total lipid.

Quality Assurance

A sample set for OC analysis contained 10–14 field samples, a method blank and a National Institute of Standards and Technology (NIST) Standard Reference Material (SRM 1945) blubber sample. Laboratory quality assurance criteria were met for all quality control procedures (Sloan et al., 2006). For example, concentrations of ≥70% of individual analytes (see OC list above) that were measured in the NIST SRM 1945 were within 35% of either end of the 95% confidence interval range of the published NIST certified concentration of the OC (Wise et al., 1993). Method blanks contained no more than four analytes that exceeded four times the LLOQ, unless the analytes was not detected in the associated samples of the set.

Statistical analyses

Student t-tests were used to compare mean concentrations of OCs between animals or seasons, as well as between male and female Steller sea lions. Analysis of variance (ANOVA) and the Tukey-Kramer HSD test were used to determine differences in mean concentrations of OCs when groups of animals numbered more than two. The level of significance used for all statistical tests was $p \leq 0.05$.

Results

The mean values and ranges for ww OC contaminants in the captive animals combined and each individual animal are shown in Table 4.1. The lipid quantity, the mean lipid adjusted OC values and ranges for the same animals are shown in Table 4.2. For blood, the male was significantly lower than the females in all measurements except

for lipid adjusted PCB TEQs (Tables 4.1 and 4.2, Kruskal-Wallis One Way Analysis of Variance on Ranks for lipids $p = 0.045$; for PCBs ww $p = 0.002$; for PCB TEQs ww $p=0.010$; for DDTs both wet and lipid weight $p<0.001$; One Way ANOVA for PCBs lipid weight $p=0.021$). However, when the females were compared to each other, the only value that was significantly different was the % lipid (Table 4.2, Mann-Whitney Rank Sum Test $p=0.019$). When comparing mean ∑PCBs to ∑DDTs in each animal, values and profiles were similar. Only the male had significant differences between blood values for PCBs compared to DDTs when lipid adjusted (Table 4.2, $p=0.033$). For blubber OCs, the male was again lower than the females when compared on a ww basis (Table 4.1, One Way ANOVA for lipids $p=0.018$; for PCBs, TEQs and DDTs $p\leq0.001$). Conversely, there were no differences between animals when compared on a lipid adjusted basis (Table 4.2).

In order to make a blood versus blubber comparison, paired samples from the same individuals were compared. A significant relationship was revealed for PCBs $r=0.67$, $r^2=0.45$, $p=0.002$ [PCB ww blubber =-467.089+(385.586 * PCB ww blood)] and for DDTs $r=0.77$, $r^2=0.59$, $p<0.001$ [DDT ww blubber=-399.795+(517.676 * DDT ww blood)] when OCs were measured by ww (Figure 4.1).

In blood, there was considerable variability in all OCs measured in each animal over time. Still, each animal followed a similar trend over the course of the study (Figure 4.2). The changes for DDTs over time are exceptionally similar to the PCBs. Wet weight measurements of OCs measured in blubber also varied considerably over the time line of the study. The females showed a similar pattern in blubber to that which was seen in blood (Figure 4.3) but the concentrations of OCs in the male were significantly lower compared to the females and although there was some variance in the male, the trend was at a consistently lower level.

In order to quantify seasonal changes, all three animals were combined and binned into three month seasons (Figure 4.4, January to March, April to June, July to September and October to December). The July to September period was the highest for both ∑PCBs and ∑DDTs (Kruskal-Wallis One Way Analysis of Variance on Ranks

$\sum$PCBs ww p=0.001; for $\sum$DDTs ww p=0.003) and the winter season of January to March was the lowest. For blubber, the July to September period was again the highest for both $\sum$PCBs and $\sum$DDTs but the differences were not significant (Figure 4.5).

A comparison between % lipids to blubber OC concentrations was considered. A significant positive relationship was uncovered for both $\sum$PCBs and $\sum$DDTs ww compared to % lipid (Figure 4.6). For $\sum$PCBs ww compared to % lipids the equation for the line was $y=4.43+(0.02 * x)$, $r^2=0.73$ and $p<0.001$. For $\sum$DDTs ww compared to % lipids the equation for the line was $y=3.28+(0.02 * x)$, $r^2=0.75$ and $p<0.001$.

Blood $\sum$PCB and $\sum$PCB TEQ concentrations of Steller sea lion pups were compared to blood levels associated with biological and physiological effects in harbor seals (*Phoca vitulina*) (Kannan et al., 2000). The levels in harbor seals were based on the findings of various studies that measured a range of toxicological endpoints (e.g., natural killer cell activity, thyroid hormone concentrations and levels of vitamin A) and levels of PCBs. For PCB effects, Kannan et al., (2000) recommended a PCB threshold concentration of 11,000 ng/g lipid weight. In this study for $\sum$PCBs the mean for captive animals measured 10,378 ng/g lipid weight. For SSL-1, 12 measurements out of 24 (or 50%) were above the threshold level. For SSL-2, 7 out of 22 (or 32%) of measurements were above the threshold. Lastly, for SSL-3 the male, only 3 of 23 (or 13%) were above the threshold.

In a captive harbor seal study, immunosuppressive effects were measured in seals that had been fed Baltic Sea herring that contained high levels of PCBs (de Swart et al., 1994, 1996, Ross et al., 1995). The mean concentration of TEQs measured in the blood of the immune-compromised harbor seals was 72 pg/g lipid weight. In this study for $\sum$PCB TEQs the mean for captive animals measured 177 pg/g lipid weight. For SSL-1, 24 measurements out of 24 (or 100%) were above the threshold level. For SSL-2, 20 out of 22 (or 91%) of measurements were above the threshold. Finally, for SSL-3, only 19 of 23 (or 83%) were above the threshold.

Discussion

Comparing the three captive animals, it is not surprising that the male has lower overall OC concentrations. Myers et al., (2008) found that in Steller sea lion pups, females were consistently smaller than males but had higher OC concentrations. In addition, as the captive females in the study are nulliiparous, and have not had a pup and potentially off-loaded a portion of their contaminant load to their first born (Beckmen et al., 1999, Debier, 2003, Wolkers et al., 2004), the levels in the females are likely higher than compared to free-ranging females in general.

The relationship elucidated in the paired blood and blubber samples indicates that in Steller sea lions, blood will work well as a medium to measure OC contaminants on a ww basis. This is in contrast to what Lydersen et al., (2001) found in harp seals, in samples taken at day 1, 14 and 28 of a fasting experiment, there was no change in blubber OCs but that blood OCs increased. However, seals in the study were losing body mass. Blubber OCs may change over a longer time period, as in the current study blubber samples were taken once per quarter. In combination with the small sample size (n=5) may have affected the results.

The extreme variability in both blood and blubber samples over time (Figures 4.2 and 4.3) indicates that any sampling regime that surveys Steller sea lions, and marine mammals in general, needs to take into account that a single time point sample may have substantial errors with it, and may not be an accurate indication of that animal's overall OC burden. There are hundreds of studies in the literature that do just that and need to have the error associated with those values considered. Most studies on marine mammals encountered in the literature that considered a temporal scale, looked at changes over years and not in the same individuals. However, given that there is a seasonal trend (Figures 4.4 and 4.5) in captive animals, if time of year is taken into consideration, the data can be measured in relation to the changes that are expected over the annual cycle. In addition, it may be beneficial to future studies to consider the time of year for the sampling protocol in order to assess either the time when levels are likely to be highest or lowest. It would be ideal to sample in both the late summer and winter to determine an

indication of range in individual animals if specific animals could be identified and re-sampled over time.

In the current study, all three animals had similar profiles in OC contaminants measured in ww in the blood (Figure 4.2) and the females were also similar in the blubber (Figure 4.3). Although it is hypothesized that the changes over time are due to a seasonal cycle, it may be only indicated in captive animals that are semi-controlled for nutritional needs, veterinary care and held in a more constant environment and therefore not dynamically challenged by coping with an open environment. The much lower OC concentrations measured in ww in the blubber of the male and the lack of a seasonal signal were associated with samples that also had lower % lipids indicative of an older animal with fiberous tissue on his blubber. Due to the fact that the male was similar to the females in the OCs measured in ww in blood, it is surprising that there is not even a seasonal signal in the blubber. When the male was compared to the females and OCs were lipid adjusted, there is no difference between the sexes (Table 4.2). This may indicate that the male is similar in OC blood profiles because he has the same seasonal metabolic needs and has metabolized OCs correspondingly from blubber to blood.

The indication that OC contaminants were highest in the late summer months (Figures 4.4 and 4.5) is interesting. Myers et al., (2006) and Myers and Atkinson, (*Chapter 2, in press*) found that the same season was highest for serum concentrations of thyroid hormones and cortisol in the same captive Steller sea lions. The July to September period of the year was associated with the time when free-ranging animals may be fasting and are out of the water for the molt and is similar for many marine mammals (Ashwell-Erickson et al., 1986, Renouf and Noseworthy, 1991, Boily, 1996, Riviere et al., 1997,). It may be that molting animals have an increased metabolic demand at a time when they are ingesting fewer calories. This could lead to increased metabolism of blubber stores which leads to the increase in OC concentrations in the blood.

The purpose of lipid adjusting OC samples is to make them comparable between tissues (de Wit et al., 2004, Willcox et al., 2004). It is interesting that when OCs in

blubber were lipid adjusted and compared between the captive female sea lions there was no difference between animals or over time. Therefore, the relationship between percent lipids and OCs measured in ww is not surprising (Figure 4.6). Again, it may be only indicated in captive animals that are in a semi-controlled and more constant environment. It is well understood that OCs are highly fat soluble (lipophilic), have low water solubility (hydrophobic) and differentially accumulate in the lipids of animals (O'Shea, 1999). Still, a similar relationship between percent lipids and OC contaminants in marine mammals was not seen in the literature and may be first reported here. Krahn et al., (2001) reported that lipid levels could be used to estimate the quality of a blubber sample in a gray whale and found a relationship between animal length and lipids.

The comparison of blood ∑PCB and ∑PCB TEQ concentrations for captive Steller sea lions compared to blood levels associated with biological and physiological effects in harbor seals (de Swart et al., 1994, 1996, Ross et al., 1995 Kannan et al., 2000), indicates that an individual animal may have OC concentrations that vary above and below a threshold (Myers et al., 2008). This makes interpretation of a static threshold figure less exacting. However, when an individual animal is temporally compared to a given threshold it may provide more insight. For example, when comparing captive animals to the ∑PCB threshold indicated by Kannan et al., (2000), SSL-1 was above the threshold in 50% of samples, while SSL-2 was only above threshold in 32% of samples. This may suggest that SSL-1 is more liable to experience the negative repercussions due to her contaminant load. SSL-3, with only 13% of samples above the threshold may be less likely to have problems. As there is a portion of time that each animal is above and below the threshold, it indicates that it may be at a specific time of the year that an animal will be susceptible to any potential impacts their contaminant load.

The indication for physiological effects based on the ∑PCB TEQs is more concerning. The captive population is more than two times the recommended threshold that was determined by dosing harbor seals (de Swart et al., 1994, 1996, Ross et al., 1995). SSL-1 was always over this threshold and may experience problems due to her OC load. SSL-2 was over the threshold 91% of the time and also may have tribulations

due to her contaminant load. In addition, SSL-3 was over this threshold 83% of the time. It may be prudent, although somewhat arbitrary, to consider the range of time an individual animal was over the two thresholds of ∑PCBs and ∑PCB TEQs combined. In this way, SSL-1 falls somewhere between 50 – 100% likely to have problems associated with her contaminant load. For SSL-2 it was possible that 32 – 91% of the time she may have been susceptible and SSL-3, the male, was anywhere from 13 – 83% liable to experience problems. The range is considerable for any animal, but may be more realistic than using a single threshold. Indeed, the ranges for these captive animals, the single point threshold for free-ranging pups, indicating that bioaccumulations was occurring (Myers et al., 2008). However, as the females in this study are nulliiparous, and a significant portion of their load can be transferred to their first born (Beckmen et al., 1999, Debier, 2003, Wolkers et al., 2004), there was at least an opportunity for these animals to see some reduction in their OC load. The free-ranging juveniles (which were not sampled temporally, and therefore may still show some variation in OC loads over time) had only 5% of ∑PCB samples above threshold and 18% above the ∑PCB TEQ threshold.

The way in which concentrations of organochlorine contaminants change over time in captive Steller sea lions indicates that any single sampling regime may not be indicative of the dynamic nature of these stable chemicals. The variability over time in individual animals must be taken into consideration, especially when body burden of OLS is compared to physiological effects. In addition, this study has confirmed that while blubber levels may vary between sexes, blood levels are more consistent and both blood and blood OC concentrations change with season which is likely associated to metabolism of blubber stores for various physiological needs.

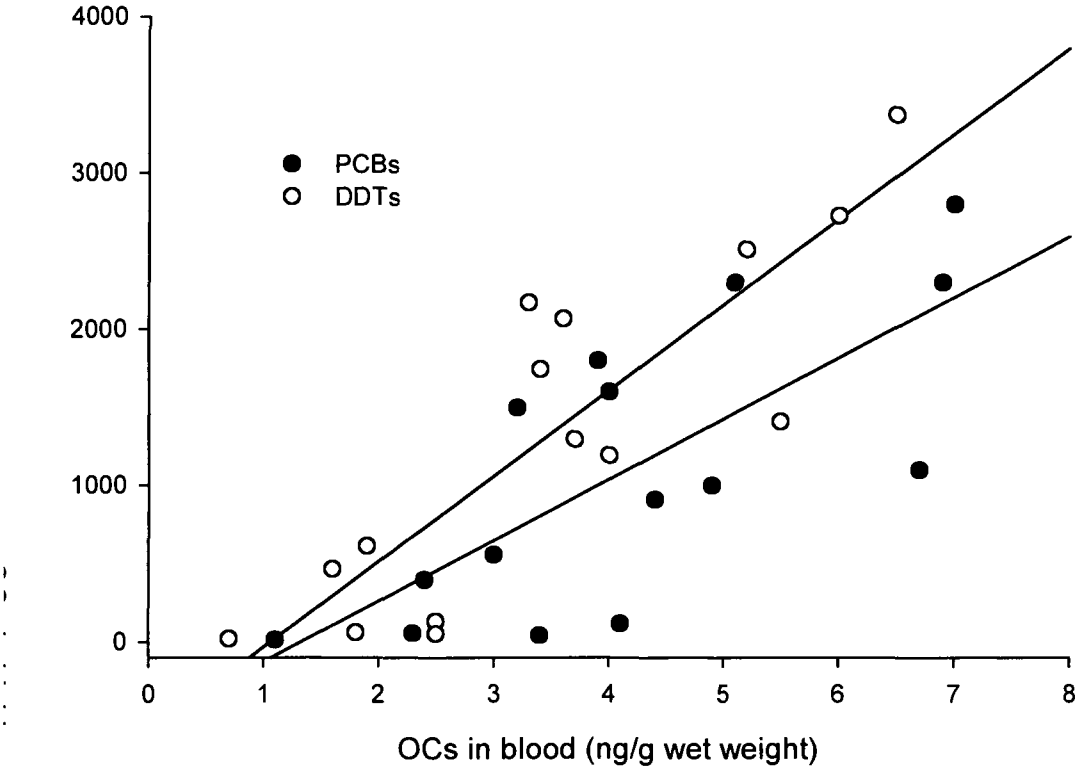

Figure 4.1. Concentrations of organochlorine contaminants (PCBs and DDTs ng/g ww) in blubber versus blood in captive Steller sea lions (n=18). For PCBs, r=0.67, r^2=0.45, p=0.002, y=-467.089+(385.586 * x) and for DDTs, r=0.77, r^2=0.59, p<0.001, y=-399.795 +(517.676 * x).

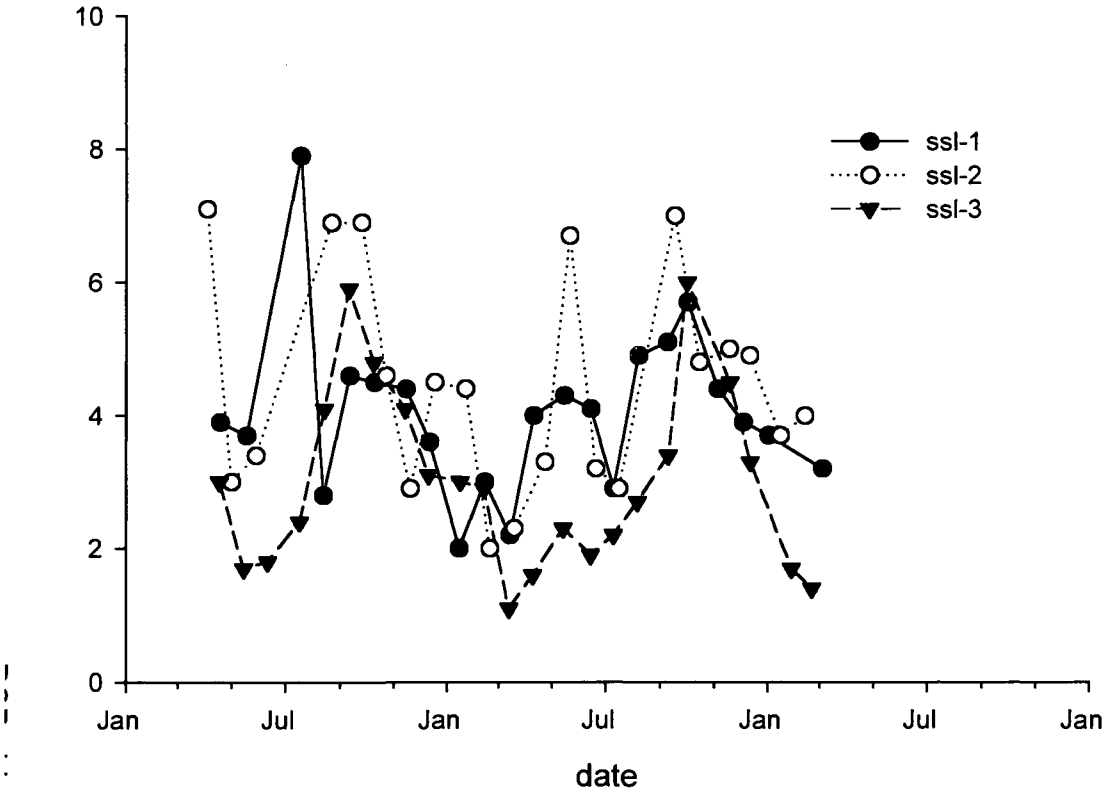

Figure 4.2. PCBs (ng/g ww) in blood (n=69) from three captive Steller sea lions (two females and one male) over a two year period (March 2001 to March 2003).

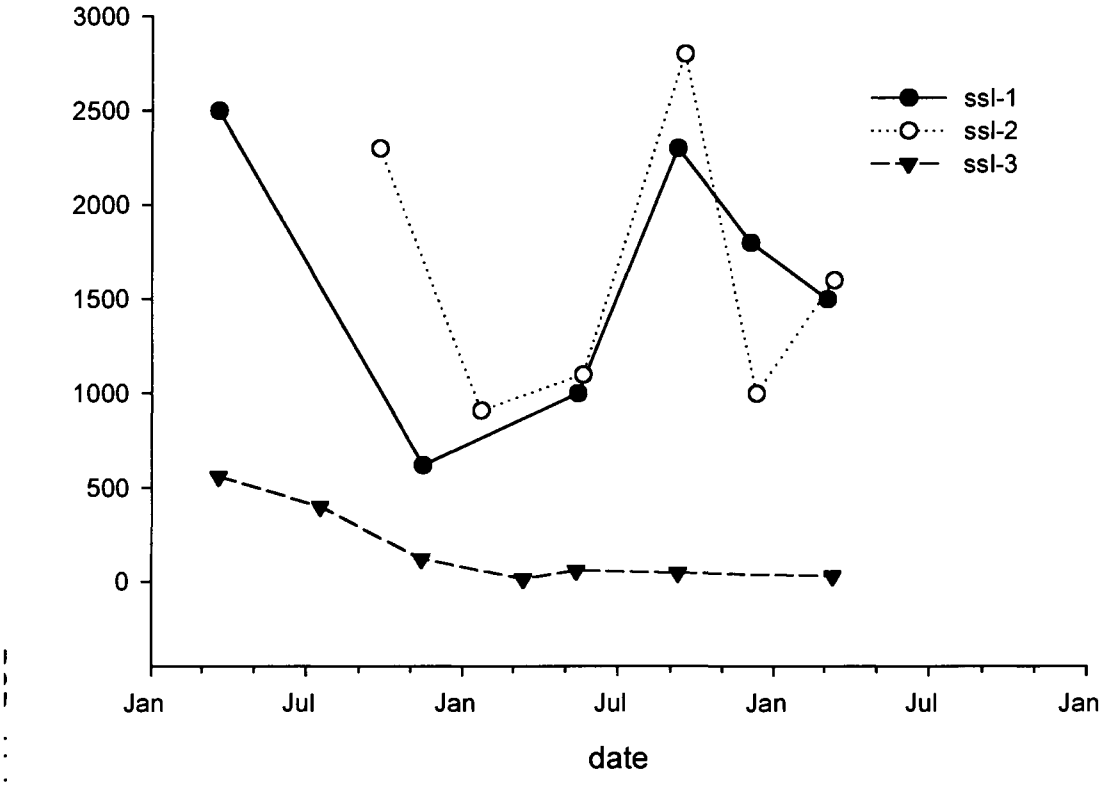

Figure 4.3. PCBs (ng/g ww) in blubber (n=19) from three captive Steller sea lions (two females and one male) over a two-year period (March 2001 to March 2003).

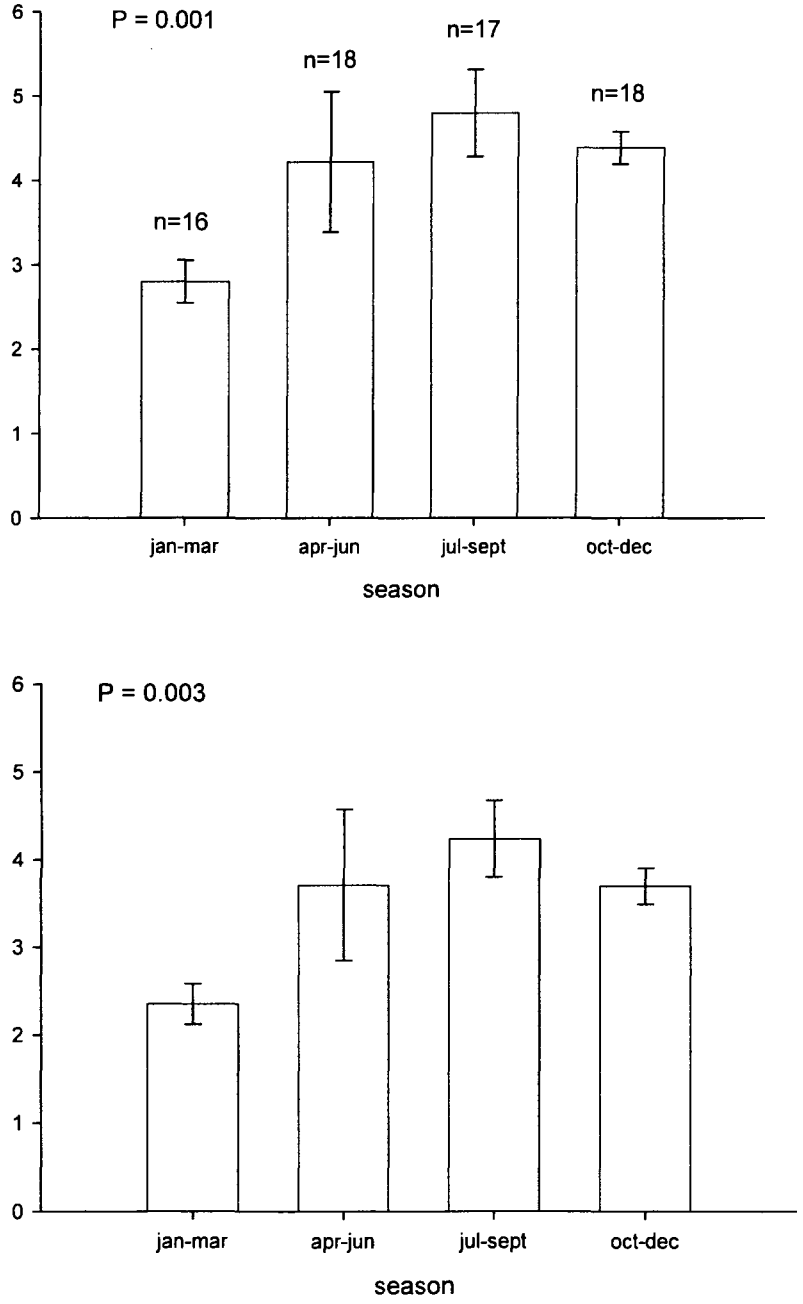

Figure 4.4. Mean (±se) concentrations of organochlorine contaminants (∑PCBs to the left and ∑DDTs to the right, ng/g ww) in blood (n=69) from three captive Steller sea lions (two females and one male) by seasons (January to March, April to June, July to September and October to December).

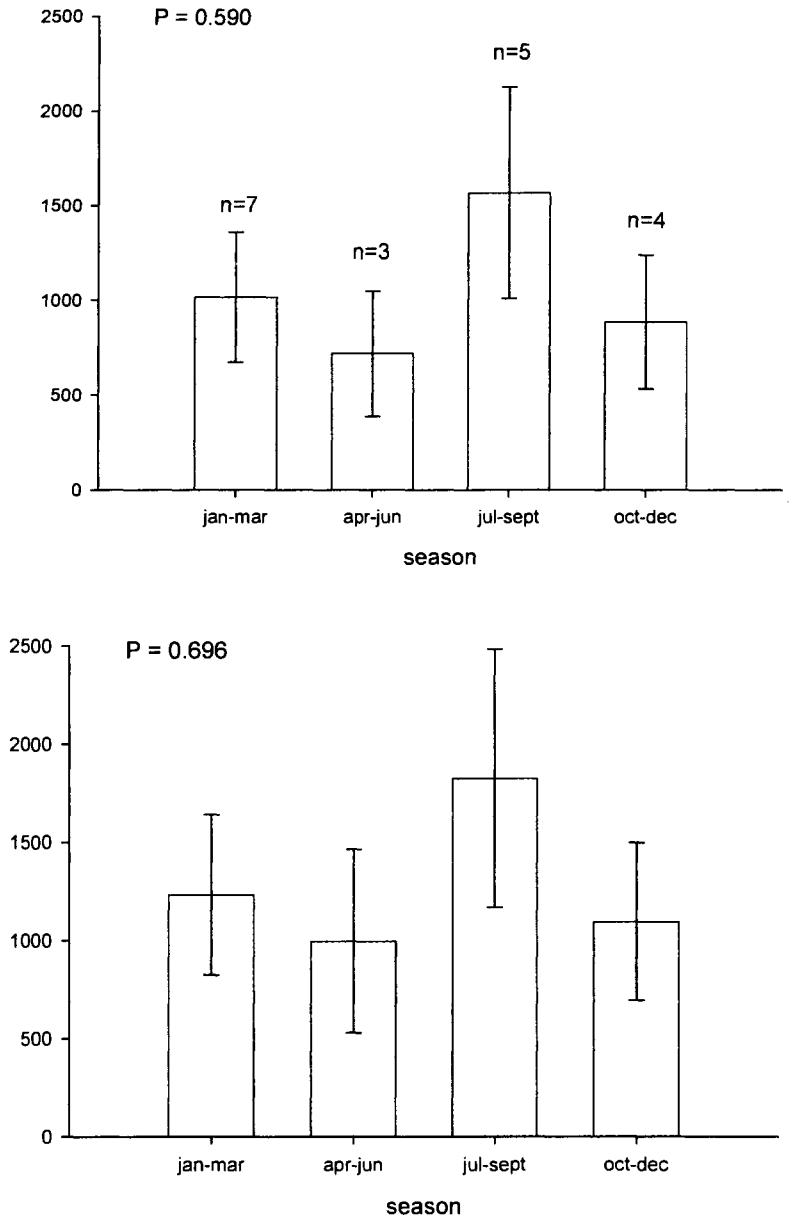

Figure 4.5. Mean (±se) concentrations of organochlorine contaminants (∑PCBs to the left and ∑DDTs to the right, ng/g ww) in blubber (n=19) from three captive Steller sea lions (two females and one male) by seasons (January to March, April to June, July to September and October to December).

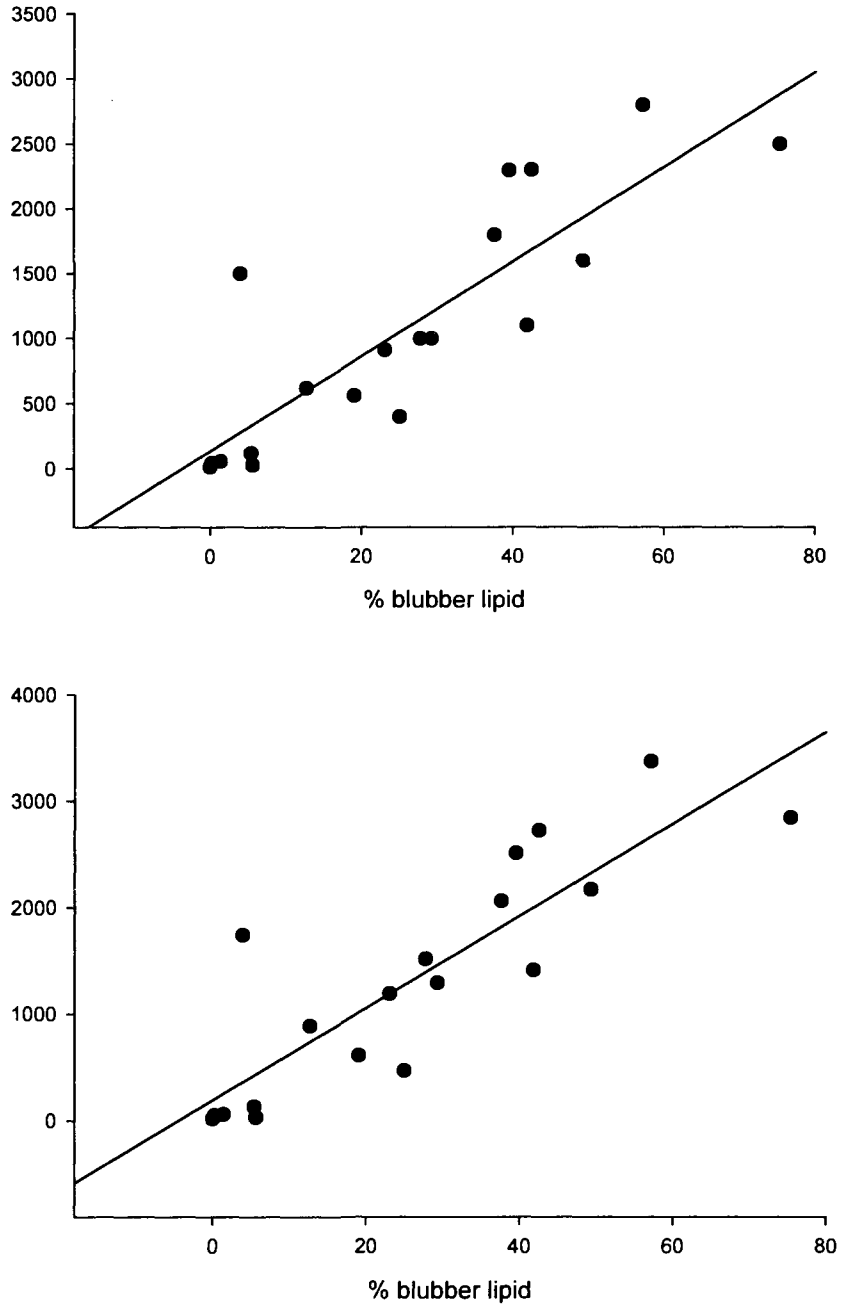

Figure 4.6. Concentrations of organochlorine contaminants (PCBs to the left and DDTs to the right, ng/g ww) in blubber versus % lipid (n=19) from three captive Steller sea lions (two females and one male). For ∑PCBs, r=0.85, r^2=0.73 and p<0.001, y=4.43+(0.02 * x). For ∑DDTs, r=0.86, r^2=0.75 and p<0.001, y=3.28+(0.02 * x).

Table 1. Mean (±SE) concentrations of sum PCBs (∑PCBs), sum PCB TEQs (∑PCB TEQs) and sum DDTs (∑DDTs) wet weight in blubber and blood samples of captive Steller sea lions. SSL 1 & 2 are females and SSL 3 is male.

Tissue		∑PCBs		∑PCB TEQs		∑DDTs	
Type	animal	ng/g, wet wt.	range	pg/g, wet wt.	range	ng/g, wet wt.	range
Blood	all captives (n = 69)	4.1 ± 0.3	1.1 - 17.0	0.07 ± 0.00	0.02 - 0.20	3.5 ± 0.3	0.7 - 16.9
	SSL-1 (n = 24)	4.6 ± 0.6	2.0 - 17.0	0.07 ± 0.01	0.03 - 0.20	4.2 ± 0.6	2.0 - 16.9
	SSL-2 (n = 22)	4.7 ± 0.4	2.0 - 9.0	0.09 ± 0.01	0.02 - 0.17	4.2 ± 0.3	2.4 - 7.3
	SSL-3 (n = 23)	3.0 ± 0.3	1.1 - 6.0	0.05 ± 0.01	0.02 - 0.12	2.2 ± 0.3	0.7 - 5.5
Blubber	all captives (n = 19)	1087 ± 211	14 - 2800	8.6 ± 1.7	* - 23.5	1323 ± 247	22 - 3371
	SSL-1 (n = 6)	1620 ± 298	620 - 2500	12.3 ± 2.2	5.6 - 20.4	1931 ± 288	893 - 2848
	SSL-2 (n = 6)	1618 ± 317	910 - 2800	13.5 ± 2.8	7.5 - 23.5	2029 ± 362	1194 - 3371
	SSL-3 (n = 7)	175 ± 82	14 - 560	1.3 ± 0.5	0.2 - 3.3	198 ± 91	22 - 611

* = < LLOQ

Table 2. Mean (±SE) concentrations of sum PCBs (∑PCBs), sum PCB TEQs (∑PCB TEQs) and sum DDTs (∑DDTs) lipid adjusted in blubber and blood samples of captive Steller sea lions. SSL 1 & 2 are females and SSL 3 is male.

Tissue Type	Site	Percent Lipid*	∑PCBs ng/g, lipid wt.	range	∑PCB TEQs pg/g, lipid wt.	range	∑DDTs ng/g, lipid wt.	range
Blood	all captives (n = 69)	0.09 ± 0.05	10378 ± 835	216 - 53125	177 ± 13	5 - 610	8844 ± 802	187 - 52844
	Kiska (n = 24)	0.04 ± 0.00	13342 ± 1903	6250 - 53125	205 ± 23	106 - 610	12037 ± 1895	6563 - 52844
	Sugar (n = 22)	0.19 ± 0.14	9738 ± 1117	216 - 20909	178 ± 27	5 - 478	8714 ± 875	187 - 18182
	Woody (n = 23)	0.04 ± 0.00	7897 ± 817	2698 - 18750	145 ± 17	21 - 357	5637 ± 624	2000 - 11935
Blubber	all captives (n = 19)	26.12 ± 4.93	6379 ± 2101	518 - 38660	49± 15	4 - 279	7645 ± 2435	625 - 44949
	Kiska (n = 6)	32.79 ± 10.24	10180 ± 5708	3318 - 38660	76 ± 41	27 - 279	12184 ± 6568	3781 - 44949
	Sugar (n = 6)	40.5 ± 5.13	3927 ± 430	2634 - 5414	33 ± 4	22 - 47	4953 ± 453	3377 - 6417
	Woody (n = 7)	8.09 ± 3.75	5030 ± 2789	518 - 18750	38 ± 18	4 - 115	5797 ± 3300	625 - 22083

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General Conclusions

Any factor that may play a potential role in the Steller sea lion (SSL) population decline, or affect the ability of populations to recover, can only be justifiably eliminated from the list of possible factors if it has been clearly demonstrated to have no effect on the population structure. This is clearly not the case for pollution. To date there is little other reported information on organochlorines such as PCBs and DDTs and some other heavy metals and virtually no data on other know pollutants (not to mention, the myriad of other chemicals that are constantly being leached into the environment). It is inconceivable that any group of scientists would consider eliminating a potential factor without a nominal investigation into specific concentrations and the prospective for any negative physiological effect. The extensive body of published literature over the last 35 years is overwhelming in regards to contaminants and problems associated in amphibians, reptiles and terrestrial mammals. In addition, recent understanding of the way these chemicals are transferred around the planet and the enhanced deposition in high latitude areas indicates that Steller sea lion habitat could be in jeopardy.

Presently, there has been only minimal investigation into the area of contaminants in regards to Steller sea lions. Historically, there is only one published manuscript that addresses contaminants such as PCBs and DDTs in Steller sea lions. Surveys from samples collected during the height of the population decline have shown that Steller sea lions had relatively high levels of organic pollutants. Sea lion blubber had PCB concentrations as high as 41 μg/g (lipid weight) and DDT concentrations as high as 17 μg/g (Lee et al., 1996). As far as recent work, there is still only a paucity of materials available to consider. Barron et al., (2003) reported that there are many synthetic chemicals that are measurable in Steller sea lion tissues. In addition, PCB and DDT concentrations measured in the 1980s were the highest recorded for any Alaskan pinniped. Lastly, Barron et al., (2003) concluded that, "there are insufficient data to reject the hypothesis that contaminants play a role in the continued decline of SSL". In order to gain perspective between the threatened southeastern Alaska population of Steller sea lions and the endangered western population, the AMAP reported on

unpublished data collected by Beckman between 1998 and 2000 (de Wit et al., 2004). The AMAP report concluded that Steller sea lions from the eastern Aleutian Islands had higher levels of PCBs and DDTs in feces compared to southeast Alaska animals. Hoshino et al., (2006) looked at contaminants in Steller sea lions from areas in the western North Pacific and concluded that some animals may have levels high enough to cause physiological problems and that these levels are also higher than animals from the eastern North Pacific.

In this dissertation, I have reported that anywhere from 12 to 64% (depending on rookery) of pups investigated for contaminants have concentrations of ∑PCBs that are high enough to cause physiological problems. Blood ∑PCB and ∑PCB TEQ concentrations of Steller sea lion pups were compared to blood levels associated with biological and physiological effects in harbor seals (Kannan et al., 2000). The levels in harbor seals were based on the findings of various studies that measured a range of toxicological endpoints (e.g., natural killer cell activity, thyroid hormone concentrations and levels of vitamin A) and levels of PCBs. All the threshold effect levels used to make comparisons were based on studies of older animals and are therefore already conservative when compared to one month old pups, as in our study.

The way in which contaminants change overtime in individual animals shows the importance of additional factors that must be taken into consideration when establishing a sampling protocol or interpreting contaminants data, such as season. The seasonal variability in contaminants shows that concentrations in different tissue change over time as OCs are metabolized or are in flux between blood and blubber tissues. Not only is the time a sample is taken important but additionally, any comparison of OC contamination is incomplete without consideration of life history parameters of individuals. In most mammal studies, OC concentrations increase or bioaccumulate as animals mature (an exception is that females may transfer contaminants to their offspring via gestation and lactation) (de Wit et al., 2004). Considering females off-load the majority of their contaminant load to their first born, it may be the primiparous births that have the highest potential for problems. From an ecological prospective, the fitness of an individual is not

determined until the offspring of an animal successfully reproduces. This type of data would take a minimum of three to four years (females) and up to 10 to 15 years (males) to collect. Therefore, the nuances associated with Steller sea lion sampling techniques and the time to complete an in-depth study, indicate that actually being able to conclusively determine the role of contaminants may continue to elude researchers. In addition, many scientists will not accept studies of association and would require a dosing study done in the specific species of interest. In an endangered species such as Steller sea lions and given the permit problems presently, it is very unlikely that this type of data will ever be available. However, the concentrations of OC contaminants in Steller sea lion pups suggest that levels may be capable of causing physiological problems such as immunosuppression in individuals and to the population. Therefore, OC contaminants can not be dismissed as a contributing source to either the decline or the failure of the population to recover. These data suggest that concentrations of OCs in Steller sea lions may be high enough to cause reproductive dysfunction and could potentially impact fecundity. Even though OC contamination has not been hypothesized to be the primary factor that precipitated the population decline, there is a potential for these chemicals to have a negative effect on the health of these animals. Furthermore, no other single threat has been shown to be the cause of the decline (Atkinson et. al., 2008).

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