DETERMINING THE IMMUNE STATUS OF STELLER SEA LIONS (*EUMETOPIAS JUBATUS*): AN ENVIRONMENTAL AGENTS OF DISEASE PERSPECTIVE

By

Stephanie Nichole Kennedy, B.Sc., M.Sc.

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Stephanie N. Kennedy

APPROVED:

Dr. Todd O'Hara, Committee Co-Chair

Dr. Andrea Ferrante, Committee Co-Chair

Dr. Thomas Kuhn, Committee Member

Dr. Thomas Trainor, Committee Member

Dr. Lorrie Rea, Committee Member

Dr. Tom Green, Chair *Department of Chemistry*

Dr. Leah Berman, Dean *College of Natural Science and Mathematics*

Dr. Michael Castellini,
Dean of the Graduate School

Abstract

The integrity of the immune system is paramount for preserving overall health for many organisms. Investigating environmental and physiological factors that may be associated with alterations of the immune status in non-traditional sentinel species, like the Steller sea lion (SSL), is a prominent undertaking in eco-immunology research. Changes to immune homeostasis likely impacts the health and survival of SSLs. Recent studies have reported that mercury concentrations in hair in 24 to 36% of newborn SSLs of the Western Aleutian Islands (WAI) exceed thresholds (>30 ppm) for potential adverse effects. Many of these individuals were from WAI rookeries that have historically experienced significant population declines with some slow to recover. Retrospective, and ongoing, analyses of mercury in lanugo coats (natal hair) from young pups of the WAI demonstrate *in utero* exposure to relatively high levels of mercury during late gestation. Therefore, this dissertation focuses on the notion that dietary acquired mercury could potentially alter immune response in SSLs, especially young pups, and may contribute to the lack of recovery from population declines. In order to gain an understanding of the potential for mercury to adversely affect the immune response of SSLs, selected aspects of immunity were measured (blood cell counts, haptoglobin, immunoglobulins, and cytokines) and investigated within the context of body condition, age, mercury exposure and regional population dynamics. In Chapter Two, the acute phase response protein, haptoglobin, was found to vary significantly with age and region. Individual SSL pups with greater concentrations of mercury had lower predicted concentrations of haptoglobin. In Chapter Three, a colorimetric protein A enzyme-linked immunosorbent assay was modified for enhancing accurate measurement of immunoglobulin concentrations in SSL serum. This improved methodology was then used in Chapter Four for comparing immunoglobulins in young developing SSL pups and dams as a measure of maternal investment of immunity among different rookeries. Lastly, Luminex multiplex technologies were employed for quantifying cell-signaling proteins (cytokines) in SSL serum to compare associations among rookery pups in Chapter Five. Although mercury concentrations in some individuals exceed adverse effects thresholds that are defined for other mammals, no statistically significant associations were found between immunoglobulins and cytokines

relative to mercury concentrations in young developing pups. These thesis chapters provide a powerful baseline and improved methods for ongoing and future assessments of haptoglobin, immunoglobulins, and cytokines (combined with traditional hematologic measures) observed in young developing SSL pups in regions experiencing population decline when compared with rookeries with stable or increasing pup production. Some of these findings, especially for haptoglobin, are indicative of alterations in immune status in young SSL pups born to dams from different natal rookeries with higher mercury exposure. Understanding the cause of the differences in the immune status of young SSLs will require additional assessments of the maternal-fetal interface of immunity and other factors like nutrition, metabolic status, and infectious disease that may shape neonatal immunity leading to the regional differences observed.

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Lastly, this dissertation is dedicated to Captain Brian Corcoran Hughes (August 1, 1984 – July 29, 2018). I discovered my love of marine life when Brian and the Hughes family shared their island home of Hilo, Hawaii in the summer of 2003. This was, and forever will be, the fuel that sparked a fiery

passion within me to contribute to the understanding of the health of marine ecosystems for conservation. This dissertation exemplifies that mission. Cheers, Brian.

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Chapter 1 - **Introduction**[1]

[1]Stephanie Kennedy

1.1 Overview

The mammalian immune system is dynamic and can respond to multiple perturbations and insults (i.e. disease agents, antigens, nutritional and/or physiological stress, and toxicants). Monitoring changes to the immune status of a threatened or endangered species such as the Steller Sea Lions (SSL) may serve as a model for Oceans and Human Health (Bossart, 2011). Considering there is little information regarding the immune response in SSLs, it is critical to establish methods and baselines of immune markers for identifying specific epidemiological, physiological, and environmental factors that may have the greatest effect on their immune status. Of these factors, toxicants in particular can lead to varying immune dysfunction (Das et al., 2008; Desforges et al., 2016; Dupont et al., 2016) and this may impact survival (Hall et al., 2009; Van Loveren et al., 2000) especially in young pups (Hall et al., 2002). Chemical-associated immunotoxicity and reduced immune function following contaminant exposure have been reported in other pinnipeds and cetaceans (Beckmen et al., 2003; De Swart et al., 1996; Frouin et al., 2012; Mori et al., 2006; Mos et al., 2006). Effects of immunotoxicity may increase susceptibility of populations to mass mortality events (Van Loveren et al., 2000) and this might also be relevant for SSL population dynamics. Adverse effects following toxicant exposure may be of primary concern in developing young (Van Hoomissen et al., 2015), especially in species such as SSLs that are exposed to levels exceeding safety thresholds (Beckmen et al., 2002; Castellini et al., 2012; Rea et al., 2013). Recent studies reported that 24 to 36% of SSL pups sampled in the western Aleutian Islands (WAI) and the Gulf of Alaska, regions that have not yet recovered from significant population decline (Fritz et al., 2013, 2014; Loughlin and York, 2000; Pendleton et al., 2006), had concentrations of mercury exceeding adverse effects thresholds (>30 ppm) that were defined for other mammals (Basu et al., 2007; Castellini et al., 2012; Crawford et al., 2019; Rea et al., 2013). This lends support to the hypothesis that mercury may affect the immune status of SSLs.

1.2 Immune status and mercury

Understanding factors that influence the immune status in SSLs (i.e. mercury exposure) will provide context for assessing proposed causes for the lack of recovery of SSLs in the (WAI) and for addressing the overall health status of SSLs (Bowen et al., 2006; Burek et al., 2005; Jemison et al., 2013; Pendleton et al., 2006; Rea et al., 1998; Trites and Donnelly, 2003). In the same vein, this thesis serves to provide information regarding the immune status of SSLs by measuring various immune proteins and comparing levels with cell counts, body condition, and demographic data with emphasis on *in utero* exposure to mercury in developing young. An innate inflammatory protein, haptoglobin (Hp), was found to be a useful biomarker in SSLs (Thomton and Mellish, 2007; Zenteno-Savin et al., 1997), and was measured in Chapter Two to investigate acute phase response within the context of mercury exposure (Kennedy et al., 2019). To further investigate immune status in SSLs within the context of regional variations in *in utero* mercury exposure, maternal antibody investment (Chapters Three and Four) and cell signaling proteins (Chapter Five) were measured and compared in young SSL pups from different regions, including the WAI.

1.3 Expanding the eco-immunology toolkit

There is a need to enhance the toolkit for the field of eco-immunology with regards to SSL research. This thesis provides improved methodology (Chapter Three) for measuring total immunoglobulin G concentrations in non-traditional wildlife species using SSL serum (Kennedy et al., 2018). I used current advanced methodology available (Kennedy et al., 2018; Levin et al., 2014; Thomton and Mellish, 2007) for measuring immune proteins in SSLs to identify associations of immune regulation proteins (haptoglobin and cytokines) and maternal immune investment (total immunoglobulin G) with mercury during a critical and sensitive developmental period in young pups. The samples collected for mercury analysis also represent appropriate time periods of interest and account for important life history stages of SSLs (Peterson et al., 2015). The results presented in this

dissertation constitute a holistic approach towards understanding immune status and potential effects of mercury in SSLs, particularly young pups.

1.4 Steller sea lions as a sentinel species

SSLs can be considered a sentinel species within the One Health paradigm given their mammalian physiology and their importance to the marine ecosystem in the Pacific northwest (Castellini et al., 2012). Therefore, this dissertation establishes a baseline understanding of interactions of mercury and immune status in young developing SSLs and these findings may also be of relevance to other piscivorous mammalian species, including humans.

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Chapter 2 - **Regional and age-related variations in haptoglobin concentrations in Steller sea lions (*Eumetopias jubatus*) from Alaska**[2]

[2]Kennedy, S.N., Castellini, J.M., Hayden, A.B., Fadely, B.S., Burkanov, V.N., Dajles, A., O'Hara, T.M., Rea. L.D. 2019. Regional and Age-Related Variations in Haptoglobin Concentrations in Steller Sea Lions (*Eumetopias jubatus*) from Alaska. Journal of Wildlife Diseases. 55(1): 91-104.

2.1 Abstract

Varying concentrations of the highly conserved acute phase response protein, haptoglobin, can indicate changes to the health and disease status of mammals, including Steller sea lions (*Eumetopias jubatus,* SSL). To better understand factors relating to acute phase response in SSL, circulating haptoglobin concentrations (Hp) were quantified in plasma collected from 1,272 individuals sampled near rookeries and haulouts off the coast of Alaska. We compared Hp in SSL between sexes and among different age classes (young pups, young-of-the-year, yearlings, subadults, and adults) sampled within distinct regions in Alaska (Aleutian Islands, Gulf of Alaska, Southeast Alaska). Regional and age-related differences were observed, particularly in younger SSL. No sex-related differences were detected. We identified weakly significant relationships between Hp and hematology measurements including white blood cell counts (WBC) and hematocrit (Hct) in young pups from the Aleutian Islands and Southeast Alaska. No relationship between Hp and body condition was found. Lastly, a non-linear relationship of plasma Hp and whole blood total mercury concentrations (THg) was observed in SSL from the endangered western distinct population segment in Alaska. These results demonstrated that regional variation in Hp, especially in younger SSL, may reflect regional differences in health and circulating THg.

2.2 Introduction

Haptoglobin, a protein biomarker of inflammation in mammals (Murata et al. 2004), is upregulated during the primary inflammatory response and concentrations vary with physical and environmental stressors (Petersen et al. 2004). More specifically, haptoglobin binds free reactive hemoglobin (Hb) released from damaged red blood cells and prevents damaging redox reactions, minimizing oxidative stress (Alayash 2011; Bertaggia et al. 2014). This function is likely conserved considering the genetic and structural homology of haptoglobin among mammals (Polticelli et al. 2008; Andersen et al. 2012). The health of some species of free-ranging wildlife, including the Steller sea lion (*Eumetopias jubatus*; SSL), may be assessed using haptoglobin (Zenteno-Savin et al. 1997; Bertelsen et al. 2009).

Unlike the recently delisted eastern distinct population segment (eDPS) of SSLs, the endangered western distinct population segment (wDPS) has been slow to recover from population decline (Loughlin and York 2000; Fritz et al. 2013; 2014). Mean Hp in SSLs from the wDPS were reported to be significantly greater than from the eDPS (Zenteno-Savin et al. 1997). Thomton and Mellish (2007) reported that Hp in SSL increased with inflammation, infection, and trauma.

Contaminants may adversely impact the health of the wDPS SSL (Rea et al. 2013; Beckmen et al. 2016). Concentrations of mercury (Hg) are significantly greater and more variable in young SSL from the declining wDPS than from the eDPS (Castellini et al. 2012; Rea et al. 2013). Subclinical effects of Hg in pinnipeds may occur (Das et al. 2008; Van Hoomissen et al. 2015). Greater than 20% of young SSL pups from the wDPS had THg in lanugo above benchmarks for neurological or reproductive effects for fish-eating mammals (Health Canada 2007; Basu et al. 2009; Rea et al. 2013). The majority (8/15) of the SSL pups sampled at Agattu Island, Alaska in 2011 (Rea et al. 2013) had blood THg greater than concentrations that stimulate the pro-inflammatory response and alter the blood proteome inflammatory pathway in humans (Gardner et al. 2009; Birdsall et al. 2010). We aimed to determine whether high THg are associated with increased haptoglobin concentrations (Hp) in SSLs.

We measured Hp in plasma collected over 21 yr from SSL in the eDPS and wDPS of Alaska. We tested for differences in Hp among SSL from the Aleutian Islands (AI), Gulf of Alaska (GOA), and Southeast Alaska (SEA), and metapopulations within these regions (defined as western AI, central AI, eastern AI, western GOA, central GOA, and eastern GOA, northern SEA and southern SEA; York et al. 1996) based on sex, and age class. A reference range of Hp was determined to be used as a baseline for comparing Hp in SSLs. We also identified relationships between Hp and physiological measurements including white blood cell counts (WBC), hematocrit (Hct), and body condition index (BCI). Finally, we explored the relationship of Hp with whole blood THg in young pups from regions that had sufficient data for statistical analysis.

2.3 Methods

2.3.1 Sample collection and hematology

From 1992 to 2013, SSL young pups (<1.5 mo), young-of-the-year (>1.5-12 mo), yearlings (>12-24 mo), subadults (>24-44 mo), and adult females of similar reproductive status were captured and sampled on, or near rookery or haulout sites (Figure 2.1) in the AI (n=452), GOA (n=377), and in SEA (n=443). Age classification followed other SSL studies (King et al. 2007; Rea et al. 2016). Routine capture, restraint, and sampling methodologies were employed to collect whole blood samples (Heath et al. 1997; Raum-Suryan et al. 2004). Blood and plasma aliquots were frozen (-10 C while shipboard), shipped on dry ice, and then frozen (-80 C) until analysis. Data such as sex, age class, metapopulations (as defined above) and regions of capture (AI, GOA, SEA), and morphometrics (mass, axillary girth and dorsal standard length) were recorded. Body condition index (BCI) was calculated as axillary girth/standard length x 100 using morphometric data. White blood cells were manually counted aboard the research vessel, and Hct was measured using manual or automated methods. Geographic, morphometric, BCI, and hematological data collected in Alaska from 1992-1996 and 1998-2011 were previously reported (Rea et al. 1998; Lander et al. 2013).

2.3.2 Haptoglobin analysis

We measured Hp in plasma using the Tridelta Phase Haptoglobin Assay (Tridelta Development, Maynooth, Ireland). Samples and standard control Hp dilutions were prepared for each 96-well plate (Immulon Microtiter Plate, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Absorbance values at a wavelength of 630 nm at 37 C were detected using a UV spectrophotometer (Spectramax, 340PC, Molecular Devices, San Jose, California, USA) and transcribed (SoftMax Pro 4.8, San Jose, California, USA). Measurements were taken following a 5 and a 10 min incubation, and readings corresponding with the best fit calibration curve were used. Mean was calculated from triplicate measurements. Samples were re-analyzed if the coefficient of variance was greater than 10%. If Hp were higher than the upper range of the standard curve (linear range: 0.005 to 1.75 mg/mL), the out-of-range samples were diluted and re-analyzed.

2.3.3 Total mercury concentration (THg) determination

Whole blood THg was measured in duplicate using a DMA-80 direct mercury analyzer (Milestone Inc., Shelton, Connecticut, USA; US Environmental Protection Agency Method 7473) at the Wildlife Toxicology Laboratory at the University of Alaska Fairbanks, (Rea et al. 2013; Peterson et al. 2016). Certified reference materials (DORM-3, National Research Council, Ottawa, Ontario, Canada; Seronorm, Westbury, New York, USA), calibration verifications, and system and method blanks were included in each run for quality assurance. Recoveries for certified reference materials and liquid standards were previously reported for samples analyzed from fifteen pups from Agattu Island in 2011 (90-106%; Rea et al. 2013), and recoveries for samples analyzed from rookery pups in SEA, AI, and GOA in 2000, 2012 and 2013 reported in this study were 103.24 ± 0.02% (DORM-3), 102.00 ± 0.01% (Seronorm), and 96.00 ± 0.01 % (1 mg/kg mercury chloride).

2.3.4 Statistical analyses

Central tendency, summary statistics, statistical analyses, and graphics were computed using the statistical program R version 3.1.2 (lme4, MuMIn, MASS, referenceIntervals, rpart, and ggplot2 packages; R Development Core Team 2006) to compare the variation of Hp (mg/mL) in SSL with sex, age class, metapopulation, region, and hematological measurements (WBC, Hct), BCI, and whole blood THg. To meet the assumptions of normality and homogeneity of variances required for parametric statistical testing, Hp were logarithmically transformed prior to further analyses. Mean Hp are reported with standard error, and statistical differences were considered significant with an alpha value of <0.05.

A generalized linear mixed model (GLMM) was used to identify descriptive factors affecting the variability in Hp for SSLs. To account for temporal variability, capture year was incorporated into the GLMM as a random factor. The appropriate geographical scale used for analysis was determined by comparing mean Hp among metapopulations for each age class using ANOVAs. Age classes where sample sizes were not adequately represented across metapopulations ($n<10$), were excluded from analyses. When no differences were detected among metapopulations, data were pooled. Criteria for subsequent analyses for SSLs were determined from the most parsimonious model based on the change in Akaike Information Criterion (AIC). For important factors, two sample t-tests, ANOVAs, and multiple comparisons (Tukey's test) were used to identify groups contributing to the differences observed. We also reported the proportion of pups with Hp at least double or greater the regional mean concentration, criteria indicating acute phase response in other mammalian species (Petersen et al. 2004). Outliers were detected and removed using methods described by Horn et al. (2001), and reference ranges were computed using a nonparametric 95% reference limit criterion, and 90% confidence intervals.

Simple linear regression models were used to determine if Hct, WBC, or BCI, explained the variability in Hp for a subset of young pups. The relationship of Hp with THg was assessed for young pups in AI ($n=186$, years 2011-2013) and GOA ($n=25$, years 2000, and 2010) with matched plasma Hp

and whole blood THg data. Classification and regression tree analysis (Loh 2011) was employed to estimate mean Hp of groups based on the variability in THg for data with sufficient sample sizes.

2.4 Results

2.4.1 Comparison of region and age class-specific Hp

The range of Hp in the plasma of SSL off the coast of Alaska (n=1,272) was 0.01-11.03 mg/mL (Table 2.1; Figure 2.1). Adult female and subadult SSLs were excluded from the GLMM because sample sizes did not meet our criteria. Considering there were no significant differences in mean Hp among metapopulations ($P \geq 0.050$) for young pups, young-of-the-year, and yearlings, region was considered the appropriate geographical scale to include for the GLMM. Therefore, the full GLMM for SSLs included region, age class, sex, and their interactions with capture year included as a random effect on the variability of Hp for young pups, young-of-the-year, and yearlings. The most parsimonious model included region, age class, and their interaction. The inclusion of sex and its interactive effects were negligible when comparing the change in AICs and r-squared values among models. Further, the model including sex as a main factor did not statistically differ (ANOVA, $F_{11, 1201}$=2.77, P=0.249) from the model without sex. Therefore, Hp for males and females were pooled for each age class within a region. Given these findings, subsequent analyses were conducted to compare the differences in mean Hp among all age classes within each region, and regional differences for each age class separately.

Significant differences in mean Hp were detected among age classes when comparing within each region: AI (t-test, t_{80}=-8.22, P<0.001), GOA (ANOVA, $F_{3,367}$=3.00, P=0.031), and SEA (ANOVA, $F_{3,428}$ =21.92, P<0.001). Mean Hp in SSL from AI were similar among most age classes, with the exception that young-of-the-year had a significantly lower mean Hp concentration (0.49±0.06, n=69) compared with mean Hp in young pups (1.33±0.07, n=373; Table 2.1). Multiple comparisons tests showed that for SSL in the GOA, mean Hp in young pups (1.57±0.12, n=155), young-of-the-year (1.35±0.24, n=124), and yearlings (1.13±0.08, n=72) were not different from one another, and all were significantly less than mean Hp (2.04±0.24, n=20) observed for adult females. Adult females from SEA had similar mean Hp

(2.22±0.16, n=25) to young pups (2.93±0.15, n=210) and young-of-the-year (1.52±0.09, n=99) in this region, and all were significantly greater than mean Hp (1.30±0.09, n=98) in yearlings in SEA (Table 2.1). Subadults were excluded from analyses due to insufficient sample sizes.

When comparing mean Hp among regions for each age class of SSL, significant regional differences were observed for young pups (ANOVA, $F_{2, 735}$=57.38, P<0.001), and young-of-the-year (ANOVA, $F_{2, 289}$=49.60, P<0.001), whereas no significant regional differences were detected for yearlings (t_{165}=0.40, P=0.691), or adult females (t-test, t_{43}= -0.25, P=0.800; Figure 2.2). We did not have sufficient sample sizes of subadult SSL for statistical comparisons. Multiple comparisons tests revealed that mean Hp measured for young pups in the AI (1.33±0.07, n=373) were similar (1.57±0.12, n=155) to GOA, however, both were significantly lower (2.93±0.15, n=210; P<0.001) than SEA (Figure 2.2A). For young-of-the-year, the difference was largely driven by significantly lower mean Hp (0.49±0.06, n=69; P<0.001) measured for AI compared with (1.35±0.10, n=124) GOA and (1.52±0.09, n=99) SEA (Figure 2.2B).

Following the detection and removal of 15 outliers, the lower and upper reference thresholds of Hp for SSLs were calculated as 0.13 mg/mL and 5.06 mg/mL respectively (n=1,272). The percentage of individuals falling outside this range in each region was low (2-5%). However, the greatest percentage of SSLs with Hp less than the lower reference threshold were from AI and GOA. The percentage of SSLs with Hp greater than the upper threshold of Hp in SEA were more than double that of GOA and AI (Table 2.2). Lastly, a greater proportion of young pups from GOA (13.5%) had Hp that were at least two times the regional mean compared with young pups from AI (9.6%), and SEA (9.7%).

2.4.2 Relationships of Hp with WBC, Hct, and BCI

Considering the regional differences observed, subsequent analyses to investigate relationships of Hp with WBC, Hct, and BCI were performed on young pups for each region separately. The mean, median, and range for WBC, Hct, and BCI, were determined for young pups sampled from the AI, GOA, and SEA over various years between 1992 and 2013 (Table 2.3). A statistically significant positive relationship of

Hp with WBC was observed in young pups from the AI (r^2=0.05, P<0.009; Figure 2.3A) and SEA (r^2=0.36, P<0.014; Figure 2.3B). There was a significant negative relationship (r^2=0.09, P<0.001) between Hp and Hct in young pups from the GOA (Figure 2.3C). Haptoglobin concentrations in young pups from other regions were not related to corresponding WBC or Hct measurements (P>0.050). Lastly, BCI was not related to Hp for young pups, and this finding was consistent across all regions (P>0.050). Two individuals with the lowest Hct (11.75% and 17.5%) from SEA were both underweight for their age (mass of 26kg and 32kg for ~2months old), although both were within normal ranges for total protein (6.2gd/L, and 5.5gd/L; Lander et al. 2013). Young pups from SEA had lower mean Hct than young pups from other regions, however, no relationship of Hp with Hct in SEA animals was observed, regardless of the removal of the two individuals.

2.4.3 Variations in Hp with whole blood THg

Whole blood THg ranged from 0.01-0.35 mg/kg in a subset of young pups with matched Hp in SEA, the AI and GOA and mean THg for these regions were 0.01±0.01 mg/kg, 0.06±0.01 mg/kg, and 0.04±0.01 mg/kg, respectively (Table 2.3). No statistical analysis could be performed for young SEA pups. No linear relationship between Hp and THg was identified for GOA young pups (years 2000 & 2010), and regression tree analysis was not performed on this subset of young pups from GOA given the relatively small sample size. Haptoglobin concentrations in young pups from AI (years 2011-2013) varied significantly with THg; however, the relationship did not fit a linear model (Figure 2.4A). Regression tree analysis computationally assigned a node at a THg of 0.11mg/kg (Figure 2.4B). The resulting regression tree model indicated that young pups from the AI with whole blood THg below 0.11mg/kg had an average Hp of 1.54mg/mL. Conversely, young pups from AI with whole blood THg equal to or exceeding 0.11mg/kg had a lower predicted average Hp of 0.95mg/mL (Figure 2.4B). On average, Hp were predicted to be 62% greater in pups below 0.11 mg/kg THg.

2.5 Discussion

Regardless of possible individual and regional variability, haptoglobin may provide insight to general physiological changes when taken into consideration with key descriptive factors and other important physiological indicators (Kakuschke et al. 2010). From an ecological perspective, using baseline Hp as an index of health has the strength of repeatability, and of being predictive of inducible changes to acute phase response (Matson et al. 2012).

A greater range of Hp were observed in SSL than in most marine mammal species (Beckmen et al. 2003; Krafft et al. 2006; Frouin et al. 2013), and mean Hp were greatest in SSLs from SEA. In domestic animals, an acute phase response occurs when peak Hp are 1.5-10 times greater than baseline, indicating changes to general health status (Petersen et al. 2002, 2004; Cray and Belgrave 2014). In hospitalized dogs, the range and median of Hp were greater compared with healthy individuals, except dogs with liver disease had significantly lower median Hp compared to dogs with other illnesses (Crawford et al. 2013). Humans and other species with hemolytic disease or liver cirrhosis also had lower Hp (<0.3mg mL-1) indicating a compromised acute phase response (Marchand et al. 1980; Dobryszycka 1997; Körmöczi et al. 2006). For SSLs with confirmed infection, or trauma-induced acute phase response, Hp were greater than 5 mg/mL (Thomton and Mellish 2007), similar to our upper reference threshold value (5.06 mg/mL). In our study, a small proportion of young SSL pups from each region (9-13%) had Hp that were at least two times the regional mean or greater. A greater proportion of young-of-the-year SSLs in the AI were below the lower limit of the normal range for other mammalian species, yet the number of SSLs with Hp greater than the upper reference threshold was more than doubled in SEA. Abnormally low Hp or abnormally high Hp may indicate a compromised or active acute phase response.

Elevated Hp in harbor seals (*Phoca vitulina*) was attributed to the phocine distemper virus epidemic but no age nor regional differences in Hp were found (Kakuschke et al. 2010). Unlike harbor seals, the variation in Hp in young SSL pups and young-of-the-year depended on region and age. In general, Hp in younger SSLs from the wDPS were lower compared with the eDPS, which was the opposite of findings

previously reported for a smaller number of SSLs (Zenteno-Savin et al. 1997). Furthermore, we found no regional differences in mean Hp for yearlings and adults.

Poor agreement among commercialized Hp assays is not uncommon (Czopowicz et al. 2017) and newer commercial assays for measuring Hp in marine mammals (Thomton and Mellish 2007) may be responsible for differing results among studies on SSLs over the past decades. Plasma samples previously assayed using gel electrophoresis (Zenteno-Savin et al. 1997) were re-analyzed using the colorimetric assay and higher Hp were measured for some SEA animals than previously reported, suggesting potential inconsistency of the gel assay (Supplemental Figure 2.1). These presumed gel issues likely resulted in the discrepancy between the previously reported low Hp in SEA and our current regional pattern of Hp. Several findings in this study are in agreement to those previously reported for SSL using colorimetric assays. For example, no differences in mean Hp were found between sexes of all pre-reproductive age classes, and Hp in juveniles (1.33±0.17mg/mL) were similar (1.13±0.08mg/mL) in similarly aged individuals we sampled (Thomton and Mellish 2007). Prolonged freezing also may contribute to significant changes in measures of protein biomarkers when using archived samples, however, this is unlikely for the SSL archive Hp plasma samples. Martins et al. (2017) found that Hp remained stable over time regardless of freezing duration or freeze thaw cycles.

Given that Hp increases following pregnancy, parturition, and lactation in other mammals (Berkova et al. 2001, Hiss et al. 2009), the greater mean Hp for adult females sampled a few months after the breeding season may be a result of reproductive or metabolic status. Furthermore, Hp compared among AI, GOA, and SEA were not statistically different for yearlings and adult females. Therefore, the range of Hp observed for older SSL in this study are likely representative of normal variability for those age classes.

The relationship of Hp with other physiological parameters involved with the acute phase response gives insight into the potential physiological status of each animal (Hanthorn et al. 2014). White blood cell counts are an important indicator of changes to immune response and survival (Shuert et al. 2015).

The majority of hematological measurements, including WBC, in young pups from the AI and GOA were within reference ranges (Lander et al. 2013), and mean Hp was not significantly different among these regions.

The relationship of Hp with hemoglobin was assessed using Hct as a proxy for hemoglobin (O'Brien et al. 2014; Quintó et al. 2006). The Hct values for the majority of young pups were within normal ranges, and similar to the subset of these animals previously reported (Rea et al. 1998; Lander et al. 2013). A weak, negative relationship of Hp with increasing Hct was observed for young pups in GOA. A similar relationship was previously reported for juveniles from the wDPS (Thomton and Mellish 2007). The mean Hct for SEA young pups was lower on average than in GOA and AI, although no relationship of Hp with Hct in SEA animals was observed. Finally, body condition had no effect on the variability of Hp and this finding was consistent among regions. Therefore, young pups with poor body condition have the same variability in Hp as young pups with good body condition, supporting that other extrinsic factors may be involved with changes to Hp. We caution that Hp should not be used as an indicator of nutritional stress, and that BCI estimates may not accurately represent total body fat content (Rea et al. 2016), especially in young pups (Rea et al. 1998).

A greater proportion of SSL in the wDPS have THg in blood and hair (Rea et al. 2013) that exceed established threshold levels for adverse effects in humans and some wildlife (Clarkson and Magos 2006; AMAP 2011; Dietz et al. 2013), although it is uncertain if exposure to Hg adversely effects SSL. In harbor seals, subclinical effects of Hg may occur above these threshold levels (Das et al. 2008; Van Hoomissen et al. 2015). It is possible that Hg above thresholds for adverse effects in young SSLs may contribute to the lack of recovery of SSL in the AI and GOA (Holmes et al. 2008, Castellini et al. 2012; Rea et al. 2013). These findings warrant investigation of physiological factors that may be influenced by Hg exposure, including protective proteins like haptoglobin. Considering contaminants can influence acute phase proteins (Yiangou et al. 1991), we investigated the relationship of whole blood THg with Hp in a subset of young wDPS pups and found that individuals with greater concentrations of THg have

lower Hp compared with young pups with lower THg. The cut-off node (THg of 0.11 mg/kg) statistically assigned from the regression tree analysis of Hp and THg concentrations from AI young pups is similar to the critical level determined for risk of adverse effects of Hg exposure in humans (0.1mg/kg; Health Canada 2007). The mean Hp for the group of individuals with THg greater than 0.11mg/kg THg is almost half of that of Hp in pups with lower concentrations of THg. These individuals with greater THg were also well above the lower limit (58μg/L) benchmark in maternal cord blood for adverse effects in humans (National Research Council 2006). Exposure to Hg exceeding critical, at-risk thresholds may have indirect effects on the Hp pathway leading to decreased Hp. However, the biological significance of decreased Hp of this magnitude in SSL is unknown. We are cautious to make any conclusions about the adverse effects of Hg on Hp in SSL, given the distribution of the data (*i.e.,* fewer young pups with THg>0.11mg/kg than <0.11mg/kg) and lack of clinically validated reference ranges of Hp for SSLs.

Acute phase response can be modified by contaminant exposure via fish consumption (Gump et al. 2012), and Hp may be altered if oxidative stress from contaminant exposure damages hepatocytes where the majority of Hp is produced. Controlled feeding studies with sled dogs (Sonne et al. 2007) and river otters (Ben-David et al. 2001) showed that exposure to naturally accumulated contaminants such as PCBs and polycyclic aromatic hydrocarbons resulted in markedly decreased levels of expression or Hp compared with the control group and was suggested to be the result of damaged hepatocytes incurred from toxicant-related oxidative stress. The greatest THg in young SSL is observed in liver tissue compared to other tissues (Correa et al. 2014) and individuals that experience high levels of exposure *in utero* may be most vulnerable to toxic levels during development (Rea et al. 2013; Oliveira et al. 2015). Therefore, it is possible that contaminants like Hg that accumulate in hepatocytes where is manufactured and regulated (Andersen et al. 1966), also could influence Hp in SSLs.

Regional differences in Hp observed in young pups and young of the year may indicate differences in acute phase response among the regions in the wDPS and eDPS. Given the Hp pathway depends on the interaction of the Hp and hemoglobin complex with macrophages (Thomsen et al. 2013), and that WBCs

and Hp share a positive relationship during acute phase response (Thomton and Mellish 2007), it is not surprising that a positive relationship of WBC and Hp was observed in some cases. However, the lack of this relationship in young pups from GOA may indicate a difference in acute phase response, or lack thereof, in this group. Although the health status relevance of the noted statistically significant relationship of THg and Hp is unclear, these results lend support that there may be an underlying biological mechanism worthy of further exploration. Determining Hp in endangered SSL can be useful for inferring changes to acute phase response that may correlate with changes to general health in SSL groups among different geographical regions, especially when considered in conjunction with other physiological measurements.

2.6 Acknowledgments

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2.7 Figures

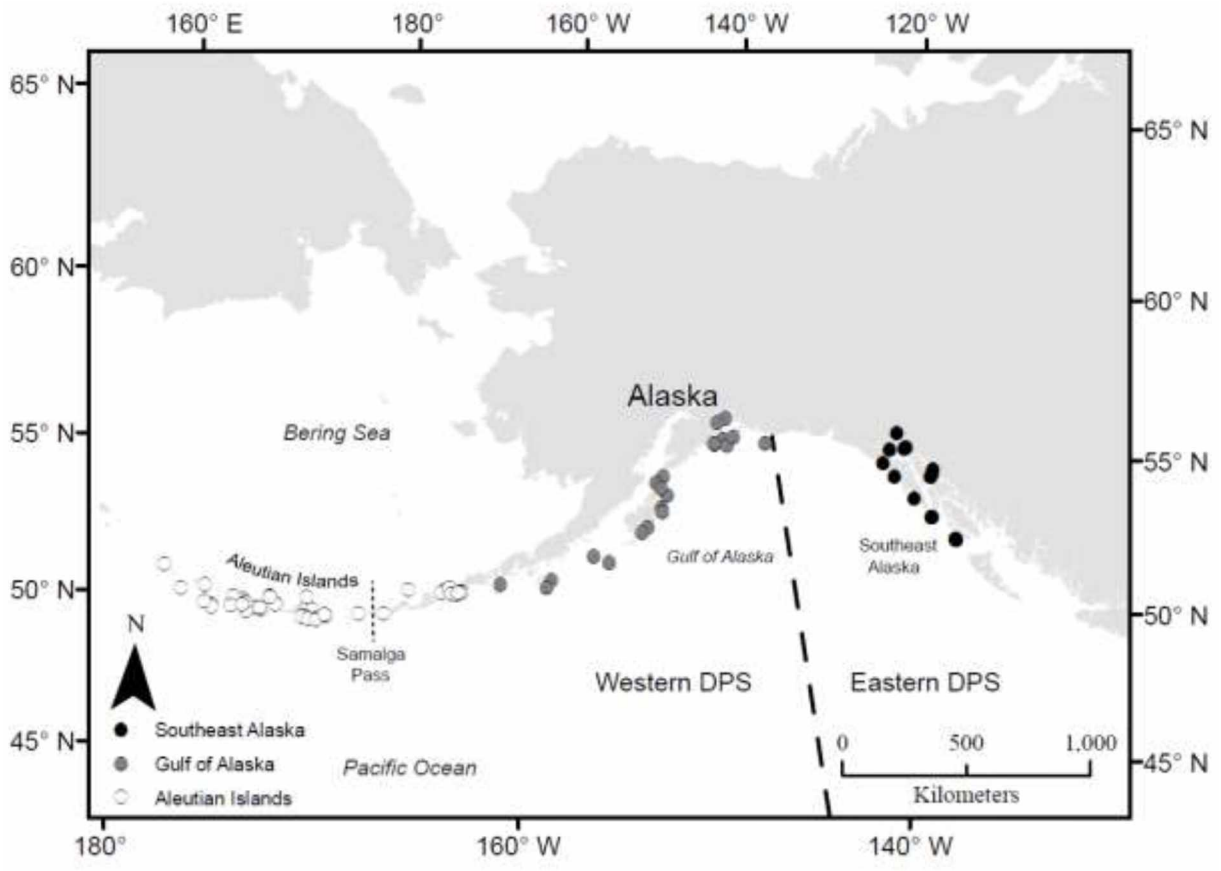


Figure 2.1 - Steller sea lion (*Eumetopias jubatus*) sampling locations for haptoglobin within rookeries across the coastal Alaska. The eastern and western Distinct Population Segments (DPS) for Alaskan SSLs are designated by longitude 144ºW.

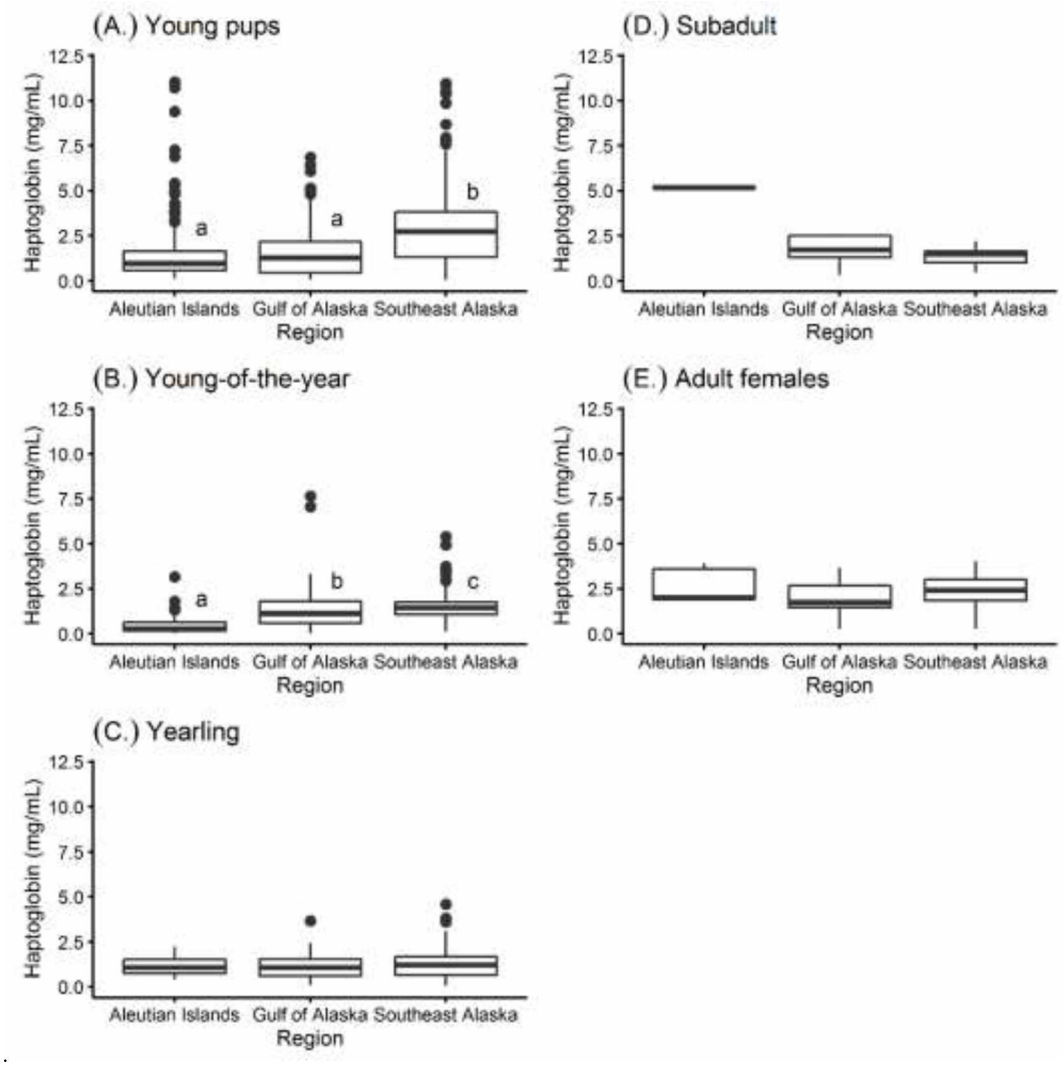


Figure 2.2 - A comparison of haptoglobin concentrations (mg/mL) among regions in Alaska for each age class; A) young pups, B) young-of the year, C) yearlings, D) subadults, and E) adult females of Steller sea lion (*Eumetopias jubatus*). Lower case letters designate significant differences ($P < 0.05$) and groupings from multiple comparisons tests (a-c). Sample sizes of subadults in the Aleutian Islands, Gulf of Alaska, and Southeast Alaska did not meet the criterion ($n < 10$) for comparison.

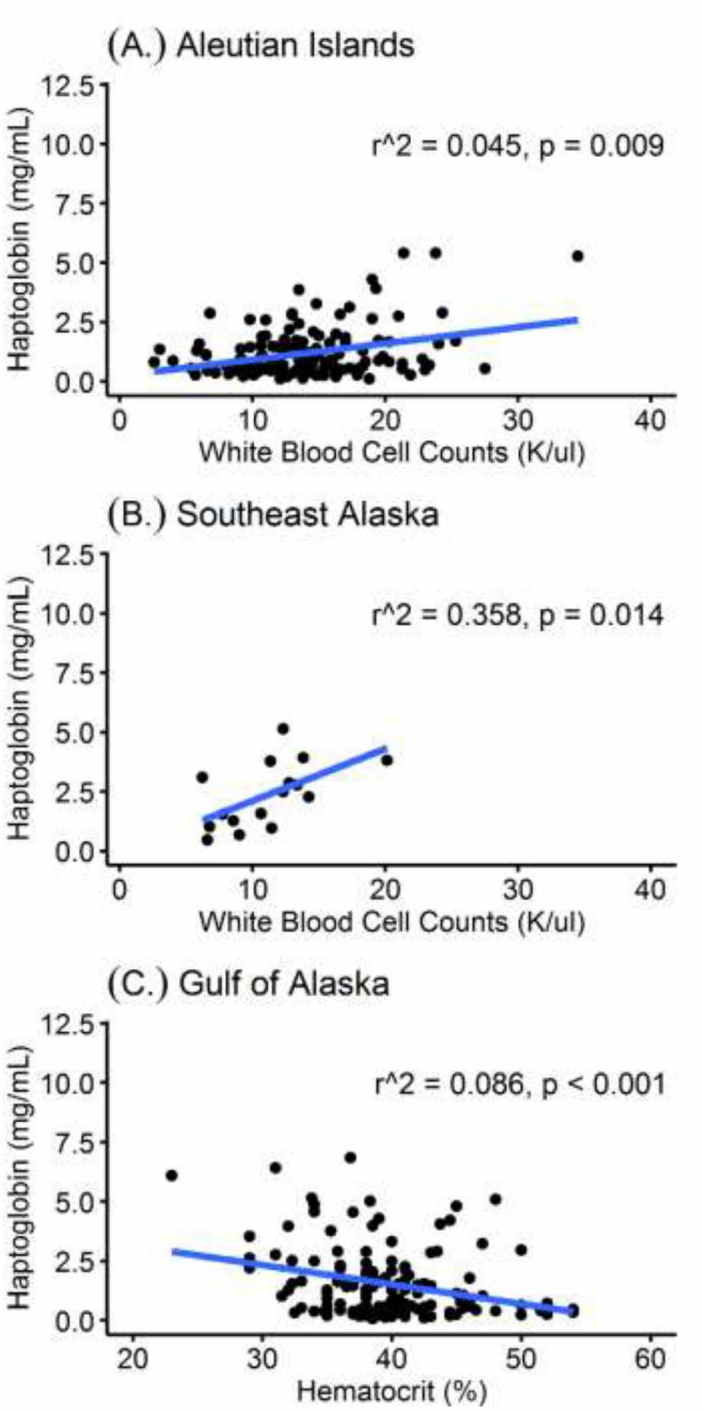


Figure 2.3 - The relationships of plasma haptoglobin concentrations with white blood cell counts (total cells x 10^3) in A) young pups of Steller sea lion (*Eumetopias jubatus*) from the Aleutian Islands, and B) Southeast Alaska, and relationships of Hp with hematocrit (%) in young pups from C) Gulf of Alaska.

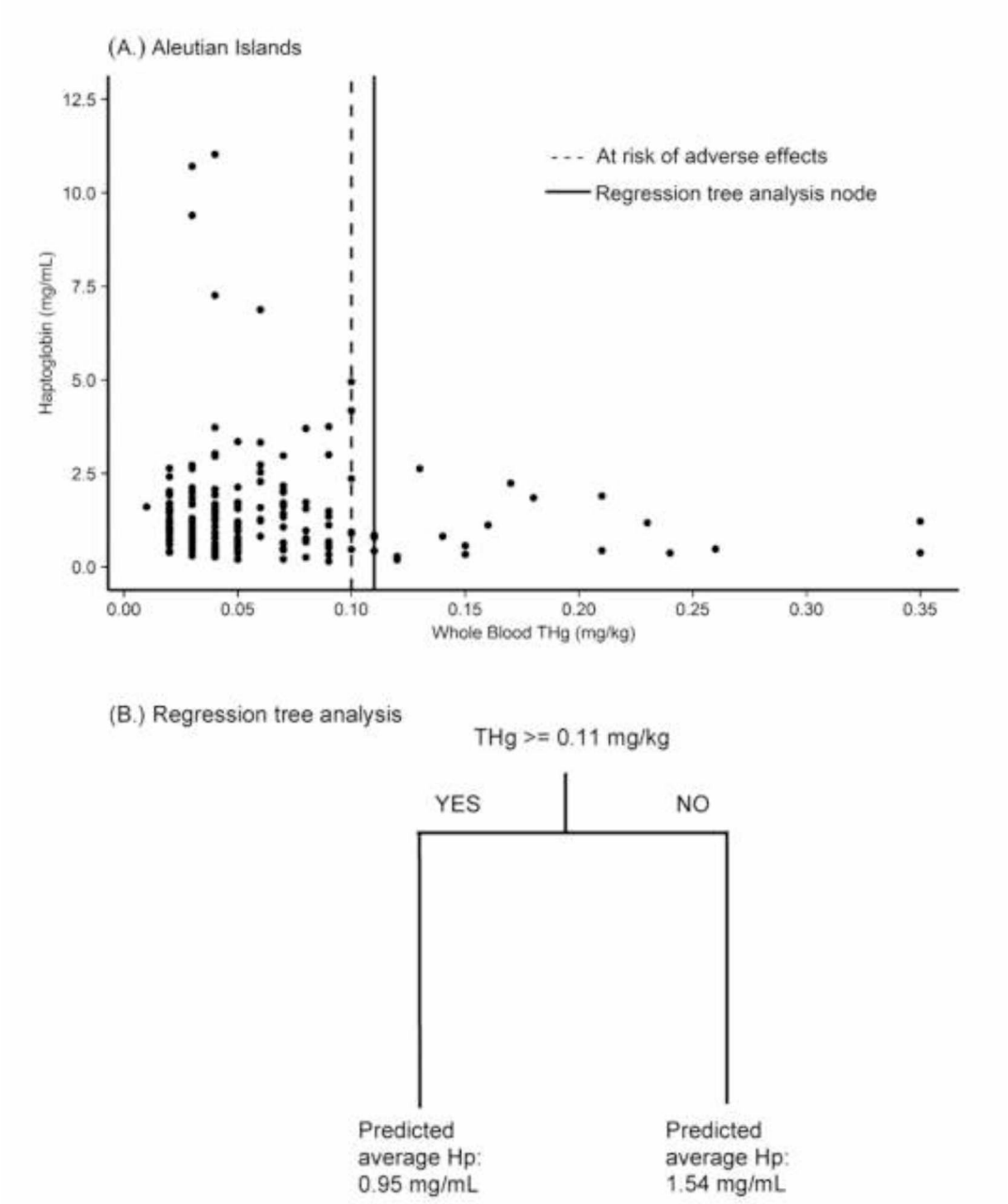


Figure 2.4 - The distribution of plasma haptoglobin concentrations with matched whole blood total mercury concentrations (mg/kg). A) Young pups of Steller sea lions (*Eumetopias jubatus*) from the Aleutian Islands and B) A regression tree demonstrating a statistically derived bifurcation of the data. The "at risk" adverse effects threshold for mammals (Health Canada 2007) and the regression tree analysis node are also denoted as dashed lines in A).

2.8 Tables

Table 2.1 - Mean (± SE) and sample size of haptoglobin concentrations (Hp, mg/mL) for Steller sea lions (*Eumetopias jubatus*) of different age classes that were sampled from each three regions in Alaska (1992 to 2013). Bold indicates significantly (**P*=0.031 , ***P*<0.001) lower Hp when comparing among age classes within a region. Statistical comparisons were made when $n > 10$.

Region	Age classes[a]									
	n	Young pups	*n*	Young-of-the-year	*n*	Yearlings	*n*	Subadults	*n*	Adult Females
Aleutian Islands	373	1.33 ± 0.07	69	**0.49 ± 0.06****	4	1.20 ± 0.39	1	5.18	5	2.66 ± 0.45
Gulf of Alaska	155	**1.57 ± 0.12***	124	**1.35 ± 0.10***	72	**1.13 ± 0.08***	5	1.68 ± 0.28	20	2.04 ± 0.24
Southeast	210	2.93 ± 0.15	99	1.52 ± 0.09	98	**1.30 ± 0.09****	10	1.34 ± 0.24	25	2.22 ± 0.16

[a] Young pups (<1.5 mos.), Young-of-the-year (>1.5-12 mos.), Yearlings (>12-24 mos.), Subadults (>24-44 mos), Adult Females (>44 mos.)

Table 2.2 - Sample size and the number of Steller sea lions (*Eumetopias jubatus*) with Hp outside the upper and lower thresholds of the reference interval for haptoglobin in plasma by region in Alaska. The calculated reference range for Hp for Steller sea lions (n=1, 272) was 0.13–5.06 mg/mL following the removal of fifteen outliers (Horn et al. 2001).

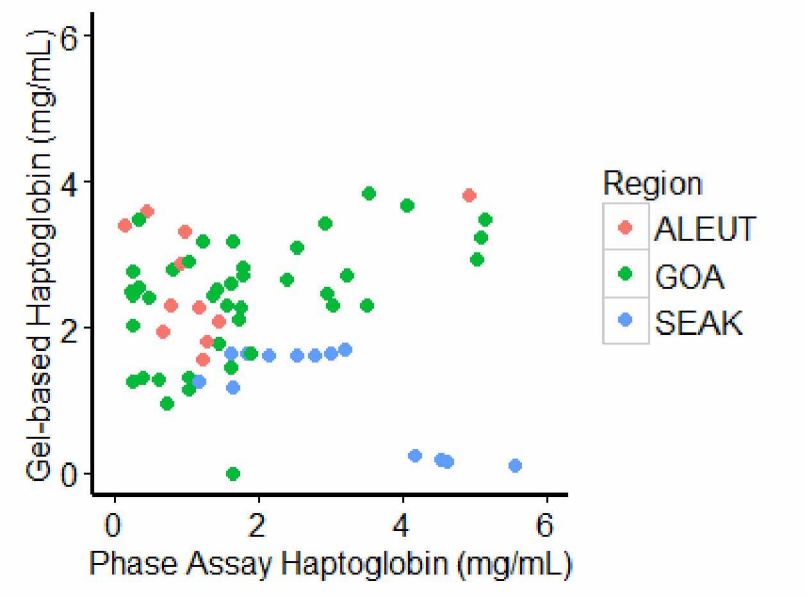
Region	Total *n*	Haptoglobin levels outside of reference interval			
		≤0.13 mg/mL		≥5.06 mg/mL	
		n	%	*n*	%
Aleutian Islands	452	13	2.9	9	2.0
Gulf of Alaska	377	12	3.2	7	1.9
Southeast Alaska	443	9	2.0	24	5.4

Table 2.3 - Mean (±SE) and sample size for the parameters, white blood cell count (WBC), hematocrit (Hct), body condition (BCI), and whole blood total mercury (THg) in Steller sea lions (*Eumetopias jubatus*) young pups from three regions in Alaska. Significant relationships between haptoglobin concentrations are indicated in bold, and the level of significance is denoted as **P*=0.009, ***P*<0.001, and ****P*=0.014. Whole blood THg represent a subset of data previously reported by Rea et al. (2013), Peterson et al. (2016).

Region	Total	*n*	Parameters						
			WBC	n	Hct	n	BCI	n	THg
Aleutian Islands	373	150	**13,890±255***	167	40.16 ±0.22	362	70.15 ± 0.21	186	0.06 ± 0.01
Gulf of Alaska	155	119	12,510±513	143	**39.64 ± 0.43****	146	68.43 ±0.27	25	0.04 ± 0.01
Southeast Alaska	210	15	**11,083±249*****	108	33.17 ± 0.32	205	70.37 ± 0.22	3	0.01 ± 0.01

WBC=white blood cell count; Hct=hematocrit; BCI=body condition index; THg=whole blood total mercury.

2.9 Appendices



Supplementary Figure 2.1- Haptoglobin concentrations (mg/mL) in SSL sampled in AI, GOA, and SEA from 1992-1994 (n = 66) using the gel electrophoresis methodology (previously reported in Zenteno-Savin et al.1997) are compared with Hp concentrations (mg/dl) measured for the same individuals using the Phase Tridelta Hp assay in this study. The correlation of the two values was assessed using Pearson's correlation coefficients and associated P-values that were computed using R statistical software. Haptoglobin measurements using the two different methodologies were positively correlated for some of the samples compared (r_{30} = 0.380, P = 0.034)). However, Hp values compared for the two methodologies in individuals from the SEA were not in agreement (P > 0.05). From this comparison, it is likely that the gel-based assay may have failed for these samples from SEA. Regardless, the Phase Tridelta assay used in the current study is reliable, and commonly used for comparing Hp in domestic and wildlife species.

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Chapter 3 - **Enhanced quantification of serum immunoglobulin G from a non-model wildlife species, the Steller sea lion (*Eumetopias jubatus*), using a protein A ELISA**[3]

[3]Kennedy, S.N., Wilhite, B., Castellini, J.M., Rea, L.D., Kuhn, T., Ferrante, A., O'Hara, T. 2018. Enhanced quantification of serum immunoglobulin G from a non-model wildlife species, the Steller sea lion (*Eumetopias jubatus*), using a protein A ELISA. Journal of Immunological Methods. 462: 42-47.

3.1 Abstract

Immunoglobulins (Ig) are proteins that preserve immune homeostasis and are quantified to infer changes to the acquired humoral immune response in mammals. Measuring Ig in non-model wildlife for immune surveillance often requires ingenuity, and rigorous standardization of methodologies to provide reliable results especially when lacking species-specific reagents. We modified and optimized existing ELISA methodology utilizing the binding properties of *Staphylococcus*-derived Protein A (PrtA) to immunoglobulin G (IgG). We enhanced the assay for quantifying IgG in Steller sea lion (SSL) serum using critical quality control measures including dilution linearity, spike and percent recoveries, and internal controls. Of the modifications made, heat treatment of SSL serum enhanced accuracy and precision of IgG measurements by improving linearity and percent recovery in parallel dilutions and serum spikes. Purified canine IgG standard was not affected by heat inactivation. These results support that confounding serum proteins interfere with binding of Prt A with IgG demonstrating the need for heat treatment of serum to optimize IgG quantification using the PrtA-ELISA. Further, essential validation measures ensure proper assay performance. Consequently, the improved PrtA-ELISA provides species-independent IgG detection with validation criteria to enhance accuracy and precision for addressing future immunological questions in non-model wildlife in clinical, ecological, and conservation contexts.

3.2 Introduction

The immunoglobulins (Ig) are important protective proteins of the acquired humoral immune response and variations in total Ig concentrations may be indicative of an exposure to an immunogen or immune dysfunction in mammals. Commercialized species-specific reagents are available for measuring Ig concentrations in humans, research animals (rat, mouse, dog, rabbit), and some wildlife species. However, for non-model wildlife species, specialized methodology for measuring Ig are limited given the lack of species-specific reagents available (Boughton et al., 2011; Garnier et al., 2017; Taylor et al., 2002). With respect to marine mammals, few diagnostic assays exist to adequately measure pathogen-specific immune response due to the lack of diagnostic tools to adequately isolate and identify novel pathogens specific to the marine environment (Fahsbender et al., 2015; Visser et al., 1989). For this reason, measurements of total Ig are commonly used for assessing pinniped (seals, sea lions, and walrus) immune status (Brock et al., 2013; Fahsbender et al., 2015; Frouin et al., 2013).

Changes in circulating total Ig can be driven by environmental exposure to contaminants (McMahon et al., 2014), or can result from a heritable immunological disease (Lim et al., 2013). Of the five isotypes of Ig, IgG occurs in the highest concentration in circulation in most taxa, therefore total IgG is often used for assessing changes to immune response in marine mammals (Frouin et al., 2013; Ross et al., 1994). Importantly, adequate quantities of protective maternal IgG transferred to neonates are essential for their survival since the humoral response requires time to develop after birth (Cabrera et al., 2012; Ross et al., 1994). The IgG concentrations below adequate concentrations may indicate failure of passive transfer of immunity (Palmeira et al., 2012; Weaver et al., 2000). Therefore, detecting changes to total IgG concentrations in non-model wildlife using precise and reliable species-independent methodology presents an approach to assessing individual and population-level immune status, especially for marine mammals.

Few reliable methodologies exist for measuring total IgG in non-model species that do not require species-specific reagents. The radial diffusion technique is one example (Castinel et al., 2008, Taylor et

al., 2002), while other species-independent methods exploit the binding properties of IgG with modified recombinant bacterial proteins derived from *Staphylococcus aureus*, protein A (PrtA) (Boyle and Reis, 1987; Goding 1978; Langone 1982). Although differences in the accuracy between techniques have been debated (Gelsinger et al., 2015), the advantages of the PrtA- ELISA supersede the radial diffusion technique (Ruiz et al., 2009).

Additional bacterial protein derivatives and mixtures other than PrtA can be used for Ig capture assays (e.g. Protein G for human IgG, and protein L for total Ig), yet, PrtA is superior for dogs and related species, including pinnipeds (Arnason et al., 2006; Choe et al., 2016; Nymo et al., 2013). More recently, conjugated protein L was used as a detection method for quantifying total virus-specific Ig of all isotypes in the California sea lion (*Zalophus californianus*) (Fahsbender et al., 2015). To assess the immune competence of developing harbor seal pups, Ross et al. (1993) developed an indirect PrtA-ELISA for measuring IgG using a conjugated anti-PrtA detector antibody. Although the PrtA-ELISA methodology was developed for harbor seals decades ago (Ross et al., 1993), it has been used since for other non-model pinniped species to address rehabilitation success (Frouin et al., 2013) and ecological immunology concerns. For example, this methodology was used to determine serum IgG from grey seals as an assessment of their immune response in relation to survival (Hall et al., 2002), and in Galapagos sea lions to infer changes to immune status in human-impacted colonies (Brock et al., 2013).

Notably, previous studies relying on the species-independent PrtA-ELISA for measuring IgG in pinnipeds did not consider the potential effect of interfering serum proteins on quantification of total IgG. Further, these studies did not include assay validation results, or spike control, and recovery or QA/QC (Brock et al., 2013; Frouin et al., 2013; Hall et al., 2002; Ross et al., 1993, 1994). Rigorous controls and validation methodologies are necessary to ensure assay performance (Andreasson et al., 2015) and to address the potential for serum protein interference. Matrix spike control and recovery is an important validation technique adopted from analytical chemistry analysis (Pang and Cowen, 2017). This validation technique is not routine in marine mammal immune assays but may reveal potential confounding serum

matrix-specific effects interfering with analyte detection. Previous studies using the PrtA-ELISA also do not report use of a heat inactivation step, an important step for minimizing interference effects of heat labile serum complement proteins (Gelsinger et al., 2015; Tate and Ward, 2004). Complement proteins are known to bind to bacterial proteins directly, or bind to the Fc region of Ig (Choe et al., 2016; Langley et al., 2005) which could potentially disrupt binding of the IgG with PrtA in the PrtA-ELISA. Methodology for measuring immune protein analytes in pinnipeds also should include validation control measures for each species prior to use to avoid erroneous results (Richmond and Zinn, 2009).

We adopted the PrtA-ELISA assay following Ross et al. (1993) and Frouin et al. (2013) and modified the assay by incorporating standardized QA/QC validation techniques for enhancing the precision and accuracy of the species-independent indirect PrtA-ELISA methodology for quantifying IgG in serum from Steller sea lions (SSL, *Eumetopias jubatus*). Validation measures included dilution linearity, standard controls, serum matrix spike and recovery, and intra- and inter-assay precision. Specifically, we addressed the issue of potential confounding effects of innate immune (heat labile) serum protein interference by comparing IgG measurements following heat inactivation treatment of the serum.

3.3 Methods

Methods were adopted from Ross et al. (1993) and Frouin et al. (2013) and modified accordingly for SSL serum. High binding, flat bottom 96-well plates (Spectra Plate-96 HB, PerkinElmer, Hopkinton, MA USA) were coated with 100µl per well of PrtA 1 µg/mL (Pierce, Thermo Scientific, Ward Hill, MA, USA) in a 0.5 M carbonate buffer (Alfa Aesar, Tewksbury, MA, USA) and incubated at 37°C for 2 hrs. Next, plates were washed 3 times using 300 µl per well of wash buffer (0.1 M PBS and 0.05 % Tween-20) and blocked with 300 µl per well of blocking buffer containing 1 % Bovine Serum Albumin (BSA, Lot# 1357C442, VWR, Amnesco Life Sciences, Radnor, PA, USA), 5 % NaCl, (Lot#2127CC500, VWR, BDH Chemicals, Radnor, PA) 0.05 % Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) in 0.01 M PBS.

Once blocking buffer was added, plates were incubated at 37 ° C for 1 hr, and washed 4 times. Standard, controls, and sample dilutions were prepared during incubations.

An 11-point standard curve (ranging from 1.8 μg/mL to 32 μg/mL) and three internal controls of known concentrations (5.06 μg/mL, 6.75 μg/mL, 9 μg/mL) were prepared by serially diluting 3:1 purified unlabeled canine IgG (#0129-01 Southern Biotech, Birmingham, AL, USA) in ELISA buffer (1 % BSA, 0.1 % Tween-20, 0.1 % Triton, 5 % NaCl in 1X PBS). Similarly, serum from a juvenile SSL (animal ID SSL 101PWS00, MMPA Permit # 358-1564 issued to Alaska Department of Fish and Game) was serially diluted 3:1 using a starting dilution of 1:300 across five tubes. To test the effects of immune modulating proteins in the SSL serum, such as complement, we treated SSL serum at 56 ° C for 30 mins (water bath). Following heat inactivation of heat labile proteins, the serum and canine IgG were centrifuged at 16,000 g_{max} (eppendorf centrifuge, Spectrafuge, Edison, NJ, USA) for approximately 5 secs. to pellet the denatured heat labile serum proteins and/or precipitates. Serial dilutions of heat inactivated SSL serum followed that of the control SSL serial dilutions (3:1). Dilution linearity was assessed for serum with and without heat treatment and linearity was considered adequate if the relationship of the observed with the expected IgG concentrations had an *r*-squared value > 0.8, and the slopes of the lines were between 0.8 and 1.2. Additionally, we assessed the effect of heat treatment on the standard, the purified canine IgG, using the same heat treatment protocol and prepared the same dilutions as the standard curve. Percent recovery for each heat-treated standard dilution was calculated.

Spike and percent recovery was assessed using purified canine IgG and the SSL serum matrix. Heat inactivated SSL serum was also prepared for comparing the effects of heat on spike and recovery. Purified canine IgG standard was diluted to a concentration of 3 μg/mL and the SSL serum and heat inactivated SSL serum were diluted to 1:500 in ELISA buffer. Five hundred microliters of each dilution were pipetted into their respective 1.5ml centrifuge tubes for the spike and the control. Each matrix received an additional spike of 100 μl of purified canine IgG (3 μg/100 μl) standard. Measurements of IgG in the control were used to determine the amount of IgG recovered in the spiked sample matrices.

Standards, internal controls, and spikes were loaded into 96 well plates (triplicates, 100 µl per well), incubated for 1 hr at 37ºC, and then washed 5 times. A combination of ELISA buffer and a polyclonal goat anti-Protein-A antibody conjugated with horseradish peroxidase (10 mg/mL, GeneTex, Irvine, CA USA) antibody was added (1µg/mL, 100 µl per well), incubated at 37 º C for 1 hr, and then washed 7 times. Tetramethylbenzidine (TMB, Thermo Scientific, Rockford, IL, USA) was added at 100 µl per well to the plate and developed at room temperature for 2-4 mins or until a clear change in coloration was visible. The reaction was stopped with 4 N sulfuric acid (50 µl per well). Absorbance was measured immediately at 450nm in a spectrophotometer (PerkinElmer 2030 Victor X5). Concentrations of IgG were calculated for the standard curve and sample treatments from the raw absorbance values processed by the associated program software (PerkinElmer 2030 Program Manager), and using those values in an online four parameter logistic curve analysis template (https://www.myassays.com, MyAssays, Ltd 2011). Percent recoveries for internal controls, dilution linearity, and spiked samples were calculated, and QA/QC were acceptable if the recoveries ranged within 80 to 120 %.

Mean (± SE) IgG concentrations were compared for SSL serum treated with (n = 12) and without heat inactivation (n = 12) for serum spike controls using two-tailed paired sample t-test. Multiple regression was used to test the effect of heat treatment on the relationship of predicted and observed concentrations of the 5 IgG serial parallel dilutions (n = 12 per dilution). If the absorbance value for a dilution was outside the detection limit of the standard curve, it was excluded from analysis. Statistics were computed using R (R Core Team, 2014), and r-squared values and slopes for parallel dilutions were also reported. Differences were considered significant at an alpha < 0.05.

The modified IgG PrtA-ELISA was also repeated (n = 3) on serum pooled from four individual young SSL pups (animal IDs SSL2016 E254, SSL2016 E258, SSL2016 62V-SE, SSL2016 17V-SE; Alaska Department of Fish and Game Permit # 18537, Alaska Sea Life Center Permit # 18438-00, and National Park Service Permit # GLBA-2016_SCI-0011, Animal Care and Use Committee Permit # 2015-38B).

3.4 Results

Assay controls for each experiment were within the acceptable QA/QC range with average percent recoveries within range from 80 to 120%, and coefficients of determination were < 15 %. Inter-assay variability was 8.71 % and intra-assay variability was 8.02 %, indicating adequate precision and repeatability among plates, and precision among all sample triplicates. Internal control recoveries for three known concentrations of purified canine IgG standard (5.06 μg/mL, 6.75 μg/mL, 9 μg/mL) and were 103 ± 0.15 %, 87 ± 0.08 %, and 90 ± 0.04 %, respectively. Mean percent recovery for SSL serum that was spiked with purified canine IgG standard without heat inactivation was 103 ± 0.06 % and 118 ± 0.07 % once heat inactivated.

Immunoglobulin G was detected in every dilution, and the sensitivity of the assay for IgG concentrations was determined by assessing the linear range of the standard curves, and is approximately 3 μg/mL to 14 μg/mL (Figure 3.1). The mean percent recovery for the heat treated purified canine IgG standard dilutions was 114 ± 5.82 % and is within acceptable range (Table 3.1). Mean IgG concentrations observed for the heat inactivated SSL serum spike control (X = 7,002.25 ± 581 μg/mL) were greater than that of the SSL serum spike control without heat inactivation (X = 4,961 ± 240 μg/mL), and the difference was statistically significant (t-value = 5.02, $P < 0.01$).

The reliability of measurements as indicated by the percent recoveries for the dilution linearity measurements (Table 3.2) depended on heat treatment of serum. Percent recoveries for more concentrated dilutions of SSL IgG with and without heat treatment were ideal when they were within the linear portion of the standard curve (Table 3.2). Percent recoveries for IgG in more dilute SSL serum that did not receive heat treatment did not meet the QA/QC criteria, and total IgG concentrations increased with greater dilutions demonstrating poorer dilution linearity (Table 3.2). For heat inactivated SSL serum, dilution linearity improved, and average percent recoveries in all dilutions falling within the acceptable QA/QC criteria. Further, IgG concentrations were significantly greater (X = 8,090.70 ± 274 μg/mL) in heat inactivated SSL serum compared with serum that did not receive heat treatment (X = 5,030.75 ± 437

μg/mL; *t*-value= 5.98, $P = 0.002$) for all serial dilutions. For the SSL serum IgG parallel dilutions, the average *r*-squared values were 0.953 and 0.946 for serum without (Figure 3.2A) and with heat inactivation (Figure 3.2B), respectively. The mean of the slopes of the fitted lines among serum dilutions with heat treatment is closer to 1 ($X = 0.906$) compared with the slopes without heat treatment ($X = 1.353$; Figure 3.2). Further, the heat treatment significantly affected the relationship of predicted and observed concentrations of IgG in parallel dilutions, and the interaction of treatment with the observed IgG concentrations was significant (multiple $r^2 = 0.862$, $F_{2,35} = 109.30$, $P = <0.001$).

Similarly, the influence of the heat treatment significantly affected the relationship of predicted and observed concentrations of IgG in parallel dilutions for the pooled serum (multiple $r^2 = 0.907$, $F_{2,6} = 29.08$, $P = 0.001$). Dilution linearity of pooled pup serum was adequate without heat treatment with slopes and *r*-squared values relatively close to within our criteria (slope = 1.23 and $r^2 = 0.77$) although dilution linearity was improved (slope = 0.83 and $r^2 = 0.92$) with heat treatment (Figure 3.3). The average percent recoveries among serial dilutions with and without heat treatment were within acceptable range (89 % and 87 % respectively). The average percent recovery of the SSL serum matrix spike and recovery control was 105.03 %. Mean IgG detected in pooled serum without heat treatment ($X = 1{,}775.06 \pm 172$ μg/mL) was 23 % less than pooled serum that received heat treatment ($X = 2{,}311.45 \pm 190$ μg/mL), however, this difference was not statistically significant (*t*-value = -2.41; $P = 0.054$).

Given that turbidity in samples are a known contributing factor for bioassay interference (King and Florkowski, 2010) it is important to note that the serum samples from the juvenile SSL (animal ID SSL 101PWS00) exhibited some turbidity whereas the majority of serum samples from four young SSL pups (3/4) were relatively clear.

3.5 Discussion

The modified PrtA-ELISA successfully measured IgG in SSL serum. The methodology presented incorporates technical steps and validation control measures that ensure assay repeatability and reliability. Considering the species-independent design of the ELISA, the reliability of the assay depends on successful binding of the Fc portion of IgG with the *Staphylococcus* PrtA. To decrease suspected interference of some serum proteins (*e.g.* complement) that may bind with the Fc region of IgG, a heat treatment step was incorporated to inactivate heat labile proteins to enhance successful binding of IgG to PrtA (Miller and Nussenzweig, 1975).

Protein A is preferred to capture the majority of subclasses of IgG as a measure of total IgG in dogs (Choe et al., 2016) and related species including pinnipeds (Frouin et al., 2013; Ross et al., 1993). Recombinant antigens and alternative protein mixtures can be used for measuring IgG, although they are typically used for detecting and/or quantifying virus-specific Ig concentrations in marine mammals. For example, Burek et al. (2005) reported Ig concentrations for calicivirus in SSLs using a group-specific ELISA methodology that used recombinant calicivirus antigen as the capture protein and PrtA alkaline phosphatase as the detector. Similarly, a serological assay was developed for detecting novel anellovirus in California sea lions, (*Zalophus californianus*) that used PrtA and Protein L (PrtL) for detection (Fahsbender et al., 2015). Protein L binds the light chain region of all Ig isotypes making it useful for measuring primary virus-specific immune response (Fahsbender et al., 2015). However, the use of (PrtL) for capturing IgG may not give a reliable signal due to variable steric hindrance from other Ig isotypes with greater surface area that bind PrtL (i.e. IgM pentamers). The binding capabilities of Fc regions of IgG with bacterial capture proteins may vary among subclasses (Kronvall et al., 1969; Lewis et al., 2008), although we assumed measurements of SSL IgG bound with PrtA are adequately quantifying total IgG (predominant IgG subclasses). Protein A has the greatest affinity for capturing all subclasses of IgG to measure total IgG (Choe et al., 2016), and was selected for measuring IgG in SSLs. Indeed, SSL serum IgG were detected and measured using the modified PrtA-ELISA protocol. The modifications we made to

the original assay enhanced precision and accuracy as demonstrated from the validation measures of dilution linearity, mean percent recovery of standards, mean percent recovery of spikes, and sensitivity (Plikaytis et al., 1994). Most notable was the effect of heat treatment on the measurement of SSL serum IgG as this step was a key factor in reducing the variability in dilution linearity and spikes, improving percent recoveries, and increasing IgG signal.

The consistent percent recoveries observed for the heat treated purified standard canine IgG supports that heat treatment did not influence the ability to quantify the amount of added purified IgG to spike the serum for this QA/QC measure. Considering a centrifugation step was added directly following heat inactivation of the SSL serum to pellet denatured heat labile proteins, the increased signal is likely due to enhanced binding of free IgG with PrtA. The results support that heat inactivation enhances the detection of IgG by decreasing potential interactions with proteins that may interfere, or compete with the Fc binding region of SSL IgG with PrtA.

IgG was detected in every dilution of SSL serum measured regardless of heat treatment, and the *r*-squared values demonstrate a strong relationship of the predicted values with the observed values in the dilution series. In addition, heat treatment of the purified canine IgG standard dilutions did not impact the percent recoveries within this linear range of the assay. Considering IgG is stable at 56°C (Ishizaka et al., 1967) and the canine IgG standard is purified and does not contain interfering serum proteins, the observation that heat treatment had no effect on the purified canine IgG standard is reassuring.

It is evident from our results that individual SSLs likely differ in their serum protein composition, leading to the difference in dilution linearity for pooled SSL serum from young pups using the PrtA-ELISA compared with the juvenile SSL serum. Interfering proteins can give rise to spurious bioassay results (King and Florkowski, 2010), and that likely alters measurement of IgG. Body weight and caloric intake can influence serum complement protein components in humans (Pomeroy et al., 1997). The composition of interfering proteins may differ depending on the health status, age, and condition of the

individual. Therefore, differences in body condition, diet, and immune competence between the juvenile SSL and young SSL pups may explain our results with regards to the heat treatment and improvements to dilution linearity. It should be noted that the young SSL pups were sampled less than one month after birth and dependent on milk. Therefore, their circulating IgG are likely of maternal origin.

Dilution linearity was adequate for the pooled SSL serum from young pups, and the slopes of the lines were close to within the adequate range regardless of heat treatment. The treatment of heat likely improves dilution linearity for serum samples that may contain greater concentrations of interfering proteins. Regardless of parallel dilutions, the signal of IgG measurement increased following heat treatment for the juvenile and pooled young pup SSL serum. This increase in signal was not statistically significant for the pooled SSL serum, however, this increase in signal was consistent with the juvenile results nonetheless. Considering heat treatment improved dilution linearity for the juvenile SSL serum sample, and a consistent increase in IgG signal was observed following heat treatment in all experiments, it remains an important step to minimize erroneous error associated with interfering heat labile proteins when measuring total IgG.

Presumably, there is a density dependent effect of innate serum proteins in dilutions that contain greater amounts of IgG (Yang et al., 2017). Dependent young typically have lesser concentrations of IgG than older individuals, and the juvenile SSL serum contained greater concentrations of IgG. Solubilization of antibody-antigen complexes is known to occur with increased aggregation of IgG, and this phenomenon may relate to the observed variability among IgG concentrations in the juvenile SSL serum for dilutions that did not receive heat treatment (Yang et al., 2017). Heat inactivation of the juvenile SSL serum improved the precision in quantifying IgG, and the linearity, and percent recoveries among serum parallel dilutions. These improvements are most likely due to inactivating the heat labile proteins (*e.g.* C1q) responsible for potential interference effects that could interact with antibody binding with PrtA (Yang et al., 2017).

Pathogen-specific Ig detection methods are useful for monitoring diseases in marine mammals (Fahsbender et al., 2015; Visser et al., 1989), yet few methods are available for marine mammal species. Detection and isolation of a known pathogen is necessary for developing pathogen-specific immune assays and identification and isolation of pathogens in marine mammals often eludes most standard techniques. Molecular techniques have advanced our knowledge of the microbiota of marine mammals (Bik et al., 2016), and mining for potential pathogens among commensals using next generation sequencing will likely enhance our abilities for pathogen identification and disease surveillance. Once marine mammal pathogens are identified, it will be possible to develop serological assays for assessing pathogen-specific Ig response in marine mammals. Until these methods are further developed, measurements of total IgG using the modified PrtA-ELISA offers a species-independent, simple assay design for detecting general changes to humoral response at an individual and/or population level.

3.6 Conclusion

The results from this study demonstrate that the addition of heat treatment of serum for the PrA-ELISA methodology is recommended to avoid confounding interference from complement and other serum proteins that may influence the accuracy of total IgG measurements in serum samples (Correia, 2010). Heat inactivation is straightforward, rapid, and cost effective in comparison with other purification methods (*e.g.* Hall et al., 2002) for reducing unwanted interference of serum proteins. The improved and validated PrtA-ELISA provides a species-independent approach with validation criteria to enhance accuracy, precision, and repeatability for measuring total IgG. The modified PrtA-ELISA methodology and QA/QC validation techniques can be applied to address future immunological questions concerning the total IgG humoral response of SSLs, or other non-model wildlife species, in a clinical, ecological, and conservation context.

3.7 Acknowledgements

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3.8 Figures

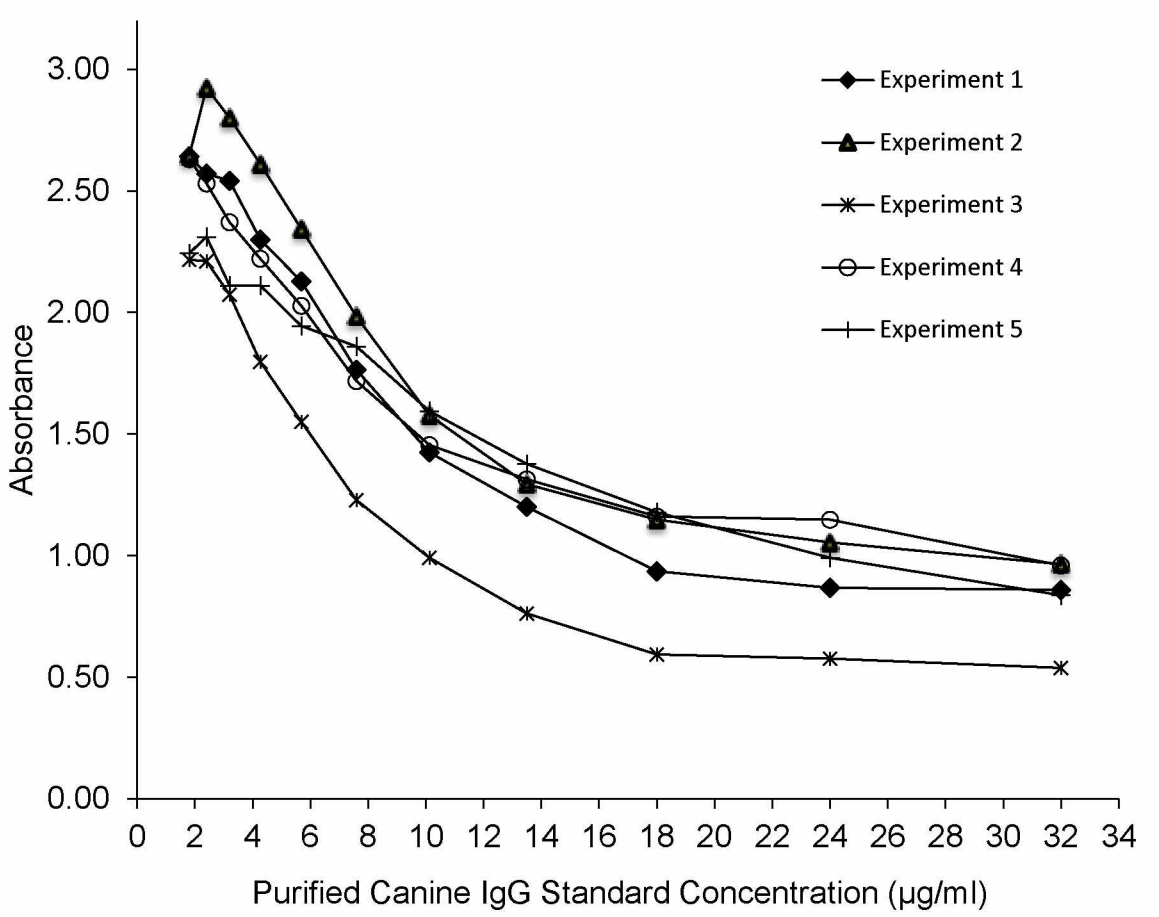
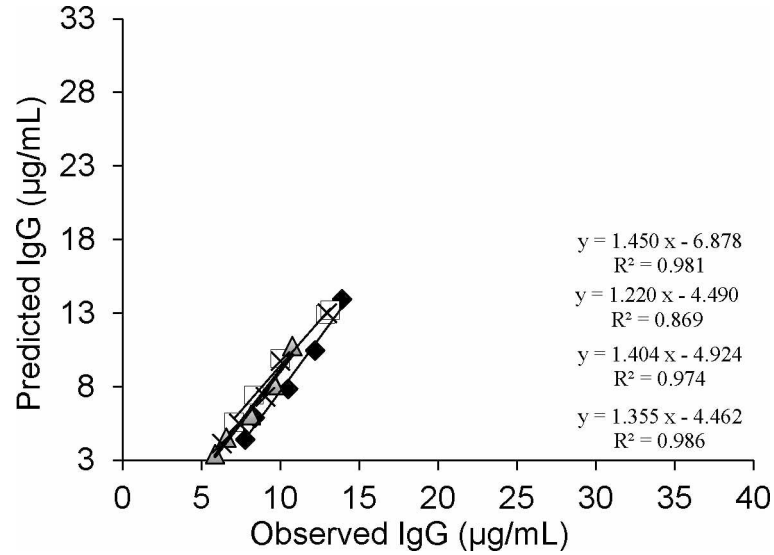


Figure 3.1 - Comparison of mean raw absorbance values for the 11-point dilution series of purified canine IgG standard for each protein A ELISA experiment ran between 21Sep17 and 23Mar18. The linear range and assay sensitivity of 2 µg/mL to 14 µg/mL is consistent among assay runs regardless of variability in raw absorbance values.

A.



B.

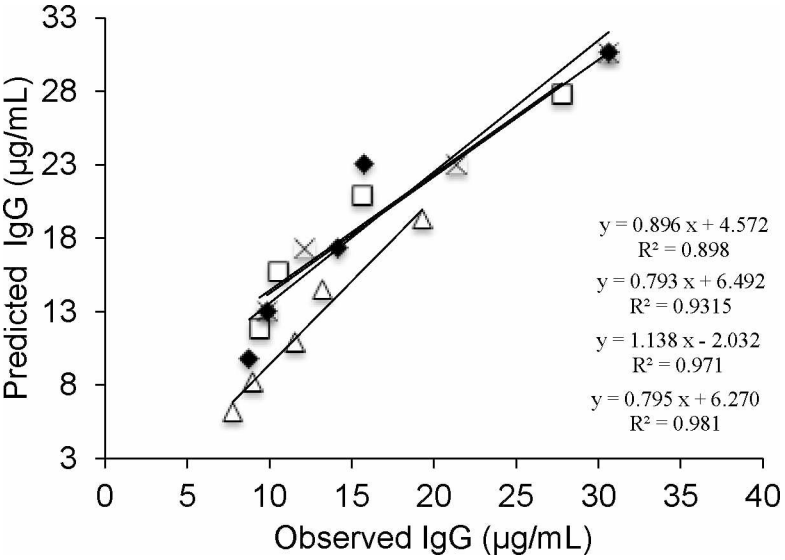


Figure 3.2 - Dilution linearity demonstrated for Steller sea lion serum from a juvenile (SSL 101 PWS00) for 3:1 serial dilution series starting at 1:300 for four experiments run between 21Sep17 and 21Nov17 without heat inactivation (A.), and with heat inactivation (B.). The unique symbols represent the means of the observed and expected IgG concentrations for the serial dilutions for each individual experiment. The linear models and *r*-squared values for serial dilutions for individual experiments are also provided. Note: the axes are the same range for (A.) and (B.) to accurately compare the differences observed following heat treatment.

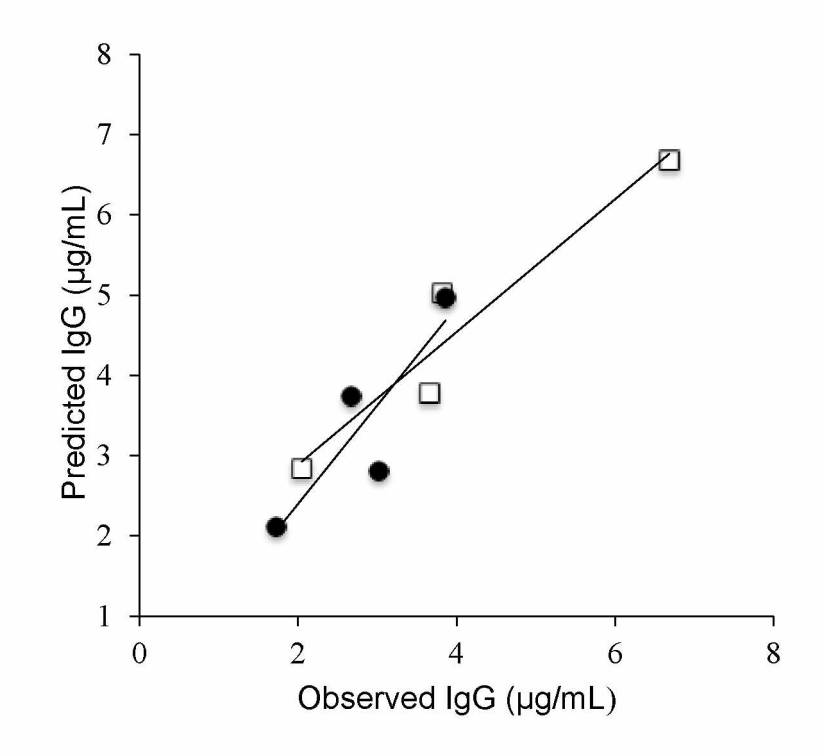


Figure 3.3 - Dilution linearity demonstrated for pooled serum from four young Steller sea lion pups (SSL2016 E254, SSL2016 E258, SSL2016 62V-SE, SSL2016 17V-SE) for 3:1 serial dilution series starting at 1:400 without heat inactivation (closed circles), and with heat inactivation (open squares). The fitted linear models (*r*-squared) values for without heat and with heat were y = 1.229 x - 0.063 (r^2 = 0.770) and y = 0.828 x + 1.229 (r^2 = 0.923) respectively.

3.9 Tables

Table 3.1 - Percent recoveries of purified canine IgG standard curve dilutions following heat inactivation.

Expected concentration (µg/mL)	Observed heat inactivated (µg/mL)[a]	% Recovery
32.00	>curve	>curve
24.00	>curve	>curve
18.00	>curve	>curve
13.50	16.1	119.259
10.13	9.865	97.384
7.59	8.031	105.810
5.69	6.914	121.511
4.27	5.013	117.401
3.20	4.047	126.469
2.40	3.328	138.667
1.80	1.577	87.611

[a]mean inter-assay %CV = 13.04
>curve = absorbance values greater than the linear portion of the standard curve where concentration could not be computed by the four parameter curve.

Table 3.2 - Dilution linearity was assessed for 5 dilutions (3 replicates each) made with serum without and with heat inactivation of confounding heat labile serum proteins. The IgG concentrations reported are the mean (±SE) of the four assays, and the percent recoveries from what is to be expected amount based on the dilution factor (predicted value).

	Dilution	IgG Observed (µg/mL)	IgG Predicted (µg/mL)	Percent Recovery	IgG Conc. (Observed X DF, µg/mL)
Without	(1:300)	12.64	12.64	100.00	3791.25
	(1:400)	11.27	9.50	118.60	4507.50
	(1:533)	9.42	7.13	132.08	5021.66
	(1:710)	7.67	5.36	143.12	5444.99
	(1:946)	6.75	4.03	167.76	6388.34
					5030.75 ± 437
With Heat	(1:300)	24.98	24.98	100.00	7494.00
	(1:400)	21.85	24.00	91.05	8740.00
	(1:533)	15.70	18.04	87.00	8366.77
	(1:710)	10.40	13.57	76.62	7380.63
	(1:946)	8.96	10.20	87.80	8472.14
				Average	8090.71 ± 274

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Chapter 4 - **Temporal and regional variation in maternal investment of immunity and *in utero* mercury exposure in Steller sea lions (*Eumatopias jubatus*) from Alaska**[4]

[4]Kennedy, S.N., Keogh, M., Levin, M., Maniscalco, J., Castellini, J.M., Lian, M., Fadely, B., Ferrante, A., Rea, L.D., O'Hara, T.M. (*In Prep*). Temporal and regional variation in maternal investment of immunity and *in utero* mercury exposure in Steller sea lions (*Eumatopias jubatus*) from Alaska. Developmental and Comparative Immunology.

4.1 Abstract

Like most mammals, neonatal pinnipeds rely on passive maternal immunity for protection against antigenic challenge until they develop functional humoral and cellular responses. Measurements of immunoglobulins in young pups 1) reflect maternal immune investment and 2) represent a critical component of assessing neonatal immune status. Maternal antibody investment has not been previously studied in the Steller sea lion (SSL) and in utero exposure to mercury is of concern for pups in regions lacking recovery from historical population decline. Therefore, total immunoglobulin G concentrations (IgG) were measured in serum from free-ranging adult females and young pups (<1.5 months old) from natal rookeries in Alaska to compare IgG among years sampled and between sexes, and identified relationships of IgG with age, body condition, lymphocyte counts, and whole blood total mercury concentrations ([THg]) among regions. Male free-ranging SSL pups had greater IgG than females from Agattu Island, and IgG differed significantly among rookeries. Regional differences were driven by lower average IgG in young pups from Agattu Island and Ugamak Island compared with greater IgG in Chiswell Island pups. No significant differences or associations were found among the other factors tested. To understand variations in maternal antibody investment shortly after birth and through the post weaning period, IgG measurements were also collected from a captive dam and her two pups from the Alaska Sea Life Center. Temporal variation in maternal antibody investment corresponded with gestation, lactation, and weaning in captive SSLs. IgG declined in the captive dam following the birth of the second pup possibly indicating costs associated with maternal antibody investment in consecutive births, although this cost did not affect the IgG in the second born pup because IgG were consistent between the captive pups. Inadequate investment of maternal humoral immunity in developing young may compound the effects of other environmental stressors that can challenge neonatal immunity, although further investigation is needed to better understand the temporal trends and regional differences we observed in adult females and young pups.

4.2 Introduction

Passive transfer of maternal antibodies to developing young is an important aspect of postnatal humoral immunity in pinnipeds. Details regarding the ontogeny of postnatal immunity in young pinnipeds have yet to be elucidated, although measurements of immunoglobulins in early stages of life have been used to assess postnatal immune status and survival outcomes in pinnipeds (Hall et al., 2002). Previous studies have addressed immuno-competence in harbor seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*), and elephant seals (*Mirounga angustirostris*) (Cavagnolo and Vedros, 1979; Ross et al., 1994) supporting that passive transfer of maternal immunity to pups likely occurs in utero via passage of immunoglobulins though the endotheliochorial placenta and through suckling (dam's colostrum). Until the immune system is able to mount antigen-specific responses, neonatal pups must rely on the passive transfer of maternal antibodies, or immunoglobulins, for protection against antigenic challenge and infectious agents (Chucri et al., 2010; Prabhudas et al., 2015). Without sufficient protective maternal antibodies, developing young that are not yet immunocompetent may succumb to infections thereby potentially impacting their survival (Hall et al., 2002). Although knowledge regarding the specific mechanisms of passive transfer of immunity from the dam in pinnipeds is limited, assessments of immunoglobulins in young pups are an important component of neonatal immune status and general health and can serve as a proxy of maternal immune investment in young developing pups (Ross et al., 1994).

Inadequate transfer of maternal humoral immunity in developing young may compound the effects of other environmental factors that can adversely affect their health. Steller sea lions (SSL) are sentinels for the environmental health of the North Pacific (Castellini et al., 2012; Phillips et al., 2011) and pup production in regions west of Samalga Pass have not recovered from population decline (Fritz et al., 2014). Young SSL pups also have varying concentrations of mercury that coincidently correspond to geographically distinct population dynamics (Castellini et al., 2012; Rea et al., 2013a). Mercury concentrations have been on the rise in circumpolar north (Dietz et al., 2013), and 20%-35% of SSL pups

sampled from various rookeries within the western and central Aleutian Islands (AI) had total mercury concentrations ([THg]) that were above critical adverse effects thresholds defined for other fish eating mammalian species (Castellini et al., 2012; Rea et al., 2013a). Mercury can negatively impact immune response in marine mammals (Desforges et al., 2016) and developing SSL pups from rookeries within the AI had greater average mercury concentrations and decreased average concentrations of the acute phase protein, haptoglobin (Kennedy et al., 2019). This prompted the current investigation of [THg] associations with other aspects of immunity, like passive maternal antibody investment, in developing young SSL pups.

Regardless of [THg], maternal antibody investment is an important component of postnatal immune status and health of young animals and we introduce the use of this aspect of immunity in our health assessments of SSL pups. Serum IgG levels have been used as a proxy for maternal antibody investment and immune status in other eared seals like the California and New Zealand sea lions (Espinosa-de Aquino et al. 2017), although previous serological studies in young SSLs were focused on infectious agent exposure (Burek et al., 2005). Therefore, we used an enhanced protein A ELISA (Kennedy et al. 2018) to measure total immunoglobulin G ([IgG]) concentrations and compared maternal investment of passive humoral immunity in young SSL pups within the context of regional population dynamics and mercury concentrations. To infer maternal antibody investment as part of investigating the immune status of free-ranging SSLs, immunoglobulin G concentrations were measured in free-ranging adult females and young pups (<1.5 months old) sampled along different rookeries from Alaska where pup production (Fritz et al., 2013, 2014) and [THg] varies regionally (Castellini et al., 2012; Rea et al., 2013). To identify important factors that might relate to changes in maternal antibody investment in young free-ranging SSL pups (within the context of in utero exposure to mercury) we compared the variation in immunoglobulin concentrations among years sampled and between sexes, and explored relationships of IgG with age, body condition, lymphocyte counts, and whole blood [THg] among regions. We also addressed the temporal link of immunoglobulin G concentrations between a captive dam

and her two pups to understand variations in maternal antibody investment shortly after birth and through the post weaning period.

4.3 Methods

4.3.1 Sample collection

Free-ranging SSL pups were sampled shortly after birth (< 1.5 mo.) at natal rookeries from Agattu Island (n = 21) in 2013, 2015 and 2017, Ulak Island (n = 12) in 2013 and 2017, Ugamak Island (n = 23) in 2017, and Chiswell Island (n = 16) and Graves Rock (n = 12) in 2016. Estimated age (days) was determined based on video recordings of births from the Alaska Sea Life Center monitoring program for most of the Chiswell Island animals born on the rookery from dams that had natural, identifiable markings or were previously branded (Maniscalco et al., 2010). Age for all other free-ranging young pups were estimated based on date of capture and size. Free-ranging adult females (n = 5) were sampled at Kiska Island, Adak Island, Kanaga Island, and Ulak Island of the Aleutian Islands using previously reported methodology (Haulena, 2014; Rea et al., 2013). Routine capture, restraint, and sampling methodology was used to collect whole blood from as previously described (Castellini et al., 2012; Lander et al., 2013; Raum-Suryan et al., 2004). Demographic data such as sex, age class, and rookery were recorded for each pup (Figure 3.1). We also investigated temporal variation in maternal antibody investment in SSLs by collecting paired serum samples from a captive dam and her female and male pups that were born in captivity and cared for by the personnel at the Alaska SeaLife Center (ASLC) in Seward, Alaska. Serum samples were collected periodically during routine examinations (Keogh et al., 2010) starting August 2013 through December 2015.

3.3.2 Immunoglobulin measurements

Total immunoglobulin G concentrations ([IgG]) were quantified from SSL serum using an enhanced colorimetric species-independent Protein A ELISA with spike and recovery controls from previously described methodology (Frouin et al., 2013; Kennedy et al., 2018; Ross et al., 1994). Specific

information regarding reagents, buffer mixtures, sample handling, ELISA setup and analysis for SSL serum was previously reported in Kennedy et al. (2018). Briefly, serum samples and positive control serum from a juvenile SSL (animal ID SSL 101PWS00, MMPA Permit #358-1564 issued to Alaska Department of Fish and Game) were heat inactivated in 56 °C for 30 min (water bath) and centrifuged at 16,000gmax (Eppendorf centrifuge, Spectrafuge, Edison, NJ, USA) prior to diluting. Adult female serum samples were diluted 1:400, whereas pup serum samples were diluted to 1:200. High binding 96 well plates (Spectra Plate-96 HB, PerkinElmer, Hopkinton, MA USA) were coated with 100 µL per well of 1 µg/mL of Protein A (Pierce, Thermo Scientific, Ward Hill, MA, USA) in a 0.5M carbonate buffer (Alfa Aesar, Tewksbury, MA, USA) and incubated for 2 h at 37 °C, and then washed 3 times using 300 µL per well of wash buffer (0.1M PBS and 0.05% Tween-20). Plates were blocked with 300 µL per well of blocking buffer containing 1% Bovine Serum Albumin (BSA, Lot#1357C442, VWR, Amnesco Life Sciences, Radnor, PA, USA), 5% NaCl (Lot#2127CC500, VWR, BDH Chemicals, Radnor, PA) 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) in 0.01M PBS and incubated at 37 °C for 1 h. During incubations, serum samples were diluted in 1mL of ELISA buffer (1% BSA, 0.1% Tween-20, 0.1% Triton, 5% NaCl in 1× PBS) and serially diluted 3 to 1 in Eppendorf tubes. An 11-point standard curve (ranging from 1.8 µg/mL to 32 µg/mL) and an internal control of known concentration (9 µg/mL) were prepared from purified unlabeled canine IgG (#0129-01 Southern Biotech, Birmingham, AL, USA) in ELISA buffer. Spike and percent recoveries were determined from adding an additional 100 µL of purified canine IgG (3 µg/100 µL) standard into the internal standard IgG control (3 µg/mL) and the SSL serum matrix control. Percent recoveries of 80 to 120 percent and relative standard deviation among sample replicates of less than 15 were deemed acceptable. Inter-plate and intra-plate variability was calculated using measurements of the internal standard control, and the SSL serum matrix control.

4.3.3 Lymphocyte counts

Differential lymphocyte counts were quantified for young free-ranging SSL pups using the Abaxis Vet Scan Autoanalyzer (Union City, CA USA) in the field (Ulak Island and Agattu Island) using methods from Lander et al (2013), or submitted to the Alaska Sea Life Center for quantification via their ProCyte Dx Hematology Analyzer (Westbrook, ME, USA) (Ugamak Island, Chiswell Island, and Graves Rock).

4.3.4 Whole blood total mercury

Whole blood total mercury concentrations [THg] (reported as μg/g, wet weight) were previously reported for free-ranging animals (Kennedy et al., 2019). Briefly, samples were analyzed at the Wildlife Toxicology Laboratory at the University of Alaska Fairbanks, Fairbanks, Alaska, USA using a Milestone DMA-80 direct mercury analyzer (Milestone, Monroe, Connecticut, USA) and methodology that was previously described (Castellini et al., 2012; McHuron et al., 2014; Peterson et al., 2016; Rea et al., 2013). Certified reference materials, calibration verifications, and method blanks were included in each run for quality control and assurance.

4.3.5 Statistics

The distribution of immunoglobulin concentrations was tested for normality using a Shapiro-Wilk test, however, they did not meet the assumptions of parametric statistical analysis. Immunoglobulin concentrations were transformed (1/X) to meet the assumptions of normality for parametric testing. No statistical differences were detected among sample years ($P>0.05$), therefore subsequent analysis was performed with combined data. For free-ranging SSL pups, one-way ANOVA and a multiple comparison Tukey honest significance difference test was used for identifying differences in the variation of immunoglobulin concentrations among rookeries and year (if more than two sampling years), whereas t-tests were used to assess differences between sexes, and year for rookeries with two sampling years. Linear regression was used to determine if immunoglobulin concentrations varied with body condition (as

calculated in Kennedy et al. 2019), age (days), lymphocyte counts, and whole blood [THg]. The cut-off for grouping the data into high [THg] and low [THg] was 0.11 μg/g and was based on the statistical findings of Kennedy et al. (2019). Outliers were defined as greater than two standard deviations above or below the mean, and were removed if they were determined to influence the results of statistical analysis. Mean immunoglobulin concentrations were also compared between the captive pups for the pre-weaning and then post-weaning periods, and then mean immunoglobulin concentrations during pre-weaning were compared with the post-weaning period. All statistical analyses and graphical representations of the data were performed using R computing (R Core Team 2014), and differences were considered significant at an alpha level of less than 0.05. Arithmetic mean (± Standard Deviation) are reported for captive and free-ranging SSL adult females and young pups.

4.4 Results

4.4.1 Immunoglobulin assay performance

Percent recoveries for the SSL serum matrix spike, the canine standard IgG spike, and the internal standard control were 105. 37%, 91.79%, and 93.50% respectively. The average variability among triplicates was 7.28%. The inter-plate and intra-plate variability of the enhanced protein A ELISA was 8.43% and 7.28% respectively.

4.4.2 Mercury quality assurance and quality control

Briefly, recoveries for measuring [THg] in blood samples were 95.66 ± 0.03% for liquid standard calibration verifications (1ppm HgCl2), and 90.44 ± 0.11% and 96.77 ± 0.04% for certified reference materials (Seronorm and DORM-3 respectively).

4.4.3 Maternal antibody investment by year, region, sex, body condition, and age

Mean immunoglobulin concentrations in young free-ranging pups varied regionally, and the greatest concentrations were observed in young pups sampled from Chiswell Island ($\bar{X}$ = 2447.65 ± 869 μg/ml) followed by Graves Rock ($\bar{X}$= 2200.58±893 μg/ml), and Ulak Island ($\bar{X}$ = 2082.09 ± 1680 μg/ml)

whereas the lowest mean IgG concentrations were observed in Agattu Island ($\bar{X}$ = 1648.40 ± 695 μg/ml) and Ugamak Island ($\bar{X}$ = 1589.15 ± 552 μg/ml). Following the removal of influential outliers, significant differences in the variability in immunoglobulin concentrations were detected (ANOVA, F4, 77 = 4.081, P = 0.005), and the differences were driven by young pups sampled in Agattu Island and Ugamak Island that were observed to have the lowest mean immunoglobulin concentrations, especially compared with Chiswell Island (Figure 4.1). Mean immunoglobulin concentrations differed among male and female pups at Agattu Island, with no sex differences detected in other rookeries. No relationship was found between immunoglobulin concentrations and body condition within rookeries (P > 0.05), or age in days for young free-ranging Chiswell Island pups (Figure 4.2 A; P > 0.05).

4.4.4 Lymphocyte counts, whole blood total mercury and immunoglobulin

To account for endogenous immunoglobulins produced by the pup, the relationship between lymphocyte counts and immunoglobulin concentration was assessed, however, no significant relationship was found (Figure 4.2 B; P > 0.05). Whole blood [THg] were log-transformed to meet assumptions of normality. Immunoglobulin concentrations were not related to THg in free-ranging pups or adult females (Figure 4.3; P > 0.05). Additionally, no statistical differences were detected when comparing mean immunoglobulin concentrations among sampling years, or between high and low mercury groups (P > 0.05) for Agattu Island and Ulak Island. The [THg] measured in young free-ranging pups from other islands were within the low mercury group (THg < 0.11 μg/g) therefore, comparisons between [THg] groups could not be made.

4.4.5 Variation in dam and pup immunoglobulin concentrations

Maximum immunoglobulin concentrations for the captive dam were observed in the month of August for both sampling years (2013 & 2014), and the dates coincided with early post parturition for the first captive pup and late stages of pregnancy for the second captive pup. Immunoglobulin concentrations of the captive dam were greater than her two captive pups during early months of the pre-weaning period

while the pups were still nursing (Figure 4.4, Table 4.1). Thereafter, the dam's immunoglobulin concentrations decreased with time during the late pre-weaning period until the pups were weaned. After the first captive pup (P1) was weaned, immunoglobulin concentrations increased in the captive dam to peak concentration at a similar time point that immunoglobulin concentrations increased in her P1. Once weaned, the average immunoglobulin concentrations of P1 increase up to concentrations similar to the average immunoglobulin concentrations of the dam ($\bar{x}$ = 3,498 μg/ml), and the dam immunoglobulin concentrations remain at a maximum until the second pup is born. Patterns of maternal antibody investment are similar among the two pups, and follow similar pre- and post-weaning temporal trends. However, following the birth of the second captive pup (P2), immunoglobulin concentrations of the dam did not return to peak concentrations. The mean immunoglobulin concentrations of the captive dam for the month of October (n = 2) for the two sampling years (2013 & 2014, $\bar{X}$ = 3704.00 ± 933 μg/ml) were comparable to mean concentrations of the five free-ranging adult females with dependent pups sampled from the Aleutian Islands in the same month and years ($\bar{X}$ = 4394.79 ± 570 μg/ml). Given the limited number of samples for the month of October for the captive dam, statistical comparisons could not be made. There were no statistical differences found, however, when comparing the mean pre-weaning (P1, $\bar{X}$ = 2302.3 ± 201 μg/ml and P2, $\bar{X}$ = 2410 ± 146 μg/ml; t = -0.86, P = 0.43) or post-weaning (P1, $\bar{X}$ = 3341.33 ± 112 μg/ml and P2, $\bar{X}$ = 3373 ± 827 μg/ml; t = -0.06, P = 0.95) immunoglobulin concentrations among the two captive pups (Table 4.1). When pooling pre- and post-weaning immunoglobulin measurements for both pups, the mean immunoglobulin concentration during the pre-weaning period ($\bar{X}$=2356.01 ± 172 μg/ml) was significantly lower than the mean immunoglobulin concentrations post-weaning ($\bar{X}$ = 3357.33 μg/ml; P=0.002).

4.5 Discussion

The contribution of maternal humoral immunity for passive protection of neonates is essential for fighting off antigenic challenges during the first stages of life until the immune system of the newborn becomes sufficiently competent (Prabhudas et al., 2015; Ross et al., 1994). Although mechanisms of

maternal shaping of the offspring's immunity remain to be discovered in marine mammals, the role of linking ecology with transgenerational contributions to immunity is critical to understand immune status in neonates (Roth et al., 2018), especially for wildlife (Hasselquist and Nilsson, 2009) like the SSL. Passive humoral immunity in neonatal pinnipeds can vary during different stages of lactation (Frouin et al., 2013; Ross et al., 1994) and with changing environmental conditions, as observed for California sea lions (Banuet-Martínez et al., 2017) and Galapagos sea lions (Brock et al., 2013a, 2013b). Significant findings from this study include regional differences observed in passive maternal humoral immunity in free-ranging young SSL pups, and that maternal antibody investment in captive SSLs varied with the timing of pregnancy and weaning. Additionally, we consider how region and mercury influence the variation in IgG in SSLs. Regional differences were observed in free-ranging young SSL pups and these findings corroborate with regional trends observed for other immune biomarker measurements from young SSL pups, as was noted for haptoglobins (Kennedy et al., 2019). However, we did not observe a relationship of passive immunity (as measured by IgG) with mercury concentrations in young free-ranging SSL pups. These collective findings support that there may be regional differences in the immune status of free-ranging young SSLs. We are unable to determine if this translates into a significant functional difference by region. In general, this work contributes to understanding maternal humoral immunity in developing SSL pups, and highlights the potential for the dam to incur energetic costs associated with providing maternal investment of humoral immunity during consecutive births.

There are many challenges to investigating the immune status of wildlife species (Boughton et al., 2011; Garnier et al., 2017), yet some of these challenges can be overcome by including captive animals and using taxonomically appropriate methodology for measuring immunoglobulins (Frouin et al., 2013; Kennedy et al., 2018; Ross et al., 1994). Assessing maternal antibody investment in free-ranging dam and pup pairs for the frequency and duration of repeated measurements as the captive SSLs in this study, in most instances, is logistically and ethically challenging. However, the IgG measured in the captive SSL dam and her two pups from samples collected periodically over the duration of multiple years provided

preliminary information regarding temporal variation in maternal investment of humoral immunity for SSLs. Although this dataset is limited to a single dam and her two pups, it is possible that dam's investment of passive humoral immunity was consistent for her two pups. As the pups suckle, their IgG increase slightly, and then decrease just before weaning, and this phenomenon has been reported in other marine mammals. Immunoglobulin levels in the pups were significantly higher post weaning, and this is to be expected as the pup gains endogenous immunity, and becomes immunocompetent. Immuno-competence was not addressed in this study, however, we can speculate the ontogeny of immunity in the post weaning period as seen in other pinnipeds (Ferreira et al., 2005; Keogh et al., 2010; Ross et al., 1994). The greatest IgG in the captive dam were from samples collected in August through October in 2013 and again in 2014 before the second pup was born. These findings support that there is a seasonal component to the dam's humoral immunity, and may relate to other physiological changes during early pregnancy as seen in other pinnipeds (Ferreira et al., 2005; Kovacs and Lavigne, 1992). Following the birth and nursing of the second pup (especially after the pup was weaned), the IgG of the dam declined, and did not return to expected peak concentrations. This could indicate the potential for costs and trade-offs associated with consecutive pupping that may negatively affect the dam's own humoral immunity (as measured by IgG), yet this did not affect the maternal investment of passive immunity in the second born pup.

Rearing male offspring has been shown to be more energetically costly compared with females in grey seals and elephant seals (Anderson and Fedak, 1987; Le Boeuf et al., 1989; Ono and Boness, 1996), and considering the link between metabolism and immunity (Demas et al., 2013; French et al., 2011), it is possible that there is a difference in the cost of maternal antibody investment between rearing a female pup versus a male pup. This notion may help explain the decline in immunoglobulin concentrations observed in the captive dam after nursing the second male pup. However, additional captive dam and pup pairs would need to be sampled to adequately address this relationship. Free-ranging adult female SSLs were also sampled in this study, yet, the nature of the energetics involved with maternal antibody

investment, or the potential sex bias in rearing offspring relating to the variation in maternal antibody investment cannot be determined from our data. Regardless, immunoglobulin concentrations from the five free-ranging adult females with dependent pups from the Aleutian Islands sampled were comparable to measurements from the healthy captive dam during similar months. However, we are careful to draw inference from our comparisons of IgG between captive and free-ranging adult females, especially considering free-ranging bottled nosed dolphins (*Tursiops truncatus*) were observed to have significantly greater immunoglobulin levels compared with different populations of captive dolphins (Ruiz et al. 2009). Additional sampling of adult females that would include additional rookeries is warranted.

In other species of marine mammals, environmental conditions (Vera-Massieu et al., 2015), adequate nutrition and foraging opportunities for the dam (Pitcher et al., 1998; Sigler et al., 2009), parasite burden (Acevedo-Whitehouse et al., 2009; Bustnes and Galaktionov, 2004), anthropogenic inputs (Atkinson et al., 2008; Brock et al., 2013a; Muller et al., 2013; Vera-Massieu et al., 2015), and contaminants, such as mercury (Desforges et al., 2016; Dórea, 2015), can impact changes to immunity. In humans, pregnant mothers consuming contaminated fish from regions impaired by gold mining activity had high whole blood [THg] that were significantly associated with IgG (Nyland et al., 2011). Methylmercury bio-accumulates in marine food webs and biomagnifies in top trophic level predators, like SSLs, at concentrations that are greater than most mammals (AMAP, 2018; Castellini et al., 2012; Rea et al., 2013). Despite the potential for adverse effects of mercury on immune status and response of mammals (Das et al., 2008; Desforges et al., 2013; Dupont et al., 2016; Scheuhammer et al., 2014), and associations of mercury with the acute phase response protein, haptoglobin, in SSLs (Kennedy et al. 2019), it does not appear that mercury was associated with maternal antibody investment as measured by immunoglobulin concentrations in young free-ranging SSL pups in this study. The effects of [THg] may have been masked by small sample sizes, considering the individual with the greatest [THg] had the lowest IgG. Regardless of mercury, free-ranging SSL pups sampled from Agattu Island and Ugamak Island had significantly lesser concentrations of IgG compared with Chiswell Island and Graves Rock

pups, and these differences in maternal antibody investment in free-ranging pups may be indicative of changes in the immune status in young pups (or dams) among these rookeries.

An important dietary factor not measured herein that may relate to regional variations in mercury and immunoglobulins, is selenium. Selenium is a dietary derived essential element that varies with concentrations of mercury in pinnipeds (Frouin et al., 2012; Hosnedlova et al., 2017; Lailson-Brito et al., 2012; McHuron et al., 2014; Woshner et al., 2001), including SSLs (Correa et al., 2014). Mucosal immunity is an important component of the development of passive immunity in the colostrum and transport of immunoglobulins to the lumen of the milk ducts (Hasselquist and Nilsson, 2009; Hurley and Theil, 2011; Koch et al., 2016), and dietary selenium enhances the absorption of circulating concentrations of maternally derived IgG in the intestine of nursing neonates of terrestrial species (Burk et al., 2013; Erdoğan et al., 2017). Selenium to mercury molar ratios might be indicative of selenium deficiency in specific tissues in SSLs (Correa et al., 2014) and this is an important nutritional factor to consider for neonatal immunity as it relates to mercury. Inadequate selenium may have negative impacts on the physiological aspects of maternal immunoglobulin absorption in pups, and the metabolic activity and mucosal immunity of pregnant or lactating dams. Given mercury binds irreversibly with selenium, greater concentrations of mercury in pups might mean there is less selenium available for immunoglobulin absorption in pups, although this has never been empirically tested. Although no relationship was found between mercury concentrations and IgG in young free-ranging pups, we cannot rule out the interactive effects of selenium and mercury on maternal antibody investment in young free-ranging pups. To fully understand the propensity for adverse effects of mercury on maternal antibody investment selenium status must be taken into consideration; especially in regions where mercury concentrations in SSL forage fish are, in general, significantly greater.

4.6 Conclusions

We measured IgG in captive and free-ranging SSL dams and pups as a proxy of maternal antibody investment in SSLs. In free-ranging pups, regional differences in maternal antibodies were

observed, however, the reason for the difference remains unclear. We found no association between whole blood [THg] and maternal IgG. Many factors that were not measured here may account for the regional differences observed. Subsequent studies should aim to identify mechanisms involved with variation in maternal antibody investment. If logistically feasible, future studies should investigate the contribution of other nutrients (selenium), hormones, and metabolic activity to variations in humoral immunity of dams and pups. These might be important factors to consider given the observation that immunoglobulin concentrations varied temporally during periods of lactation, weaning, and pregnancy in the captive SSLs in this study. Temporal variation in IgG was observed in the captive SSLs, and corresponded to life history events such as lactation and/or early gestation of the dam, or weaning of the pups. Although the IgG were consistent between captive pups, the captive dam's IgG did not return to levels expected after birthing and nursing the second male pup. This observation may be preliminary evidence to indicate an energetic trade-off, or cost of birthing a second pup, especially if male pups are more costly to rear, although a sex bias for maternal investment in SSLs has not been investigated. Additional factors that may contribute to varying IgG, especially in free-ranging SSLs, are disease burden, nutritional status, and success (or failure) of passive maternal immunoglobulin transfer. Lastly, a reference range of concentrations of maternally derived immunoglobulins that provide protection against antigenic challenge and positive outcomes for survival during phases of early life in SSL pups also need to be defined. Until then, the current study provides preliminary evidence for temporal and regional variation in maternal antibody investment in adult females and young SSL pups, and serves to contribute to our nascent understanding of the immune status of young free-ranging SSL pups born on geographically distinct rookeries.

4.7 Acknowledgements

We would like to thank Renae Sattler and the staff and volunteers at the Alaska Sea Life Center (ASLC) for paired samples collected from captive animal under National Marine Fisheries Service permit #18534 and IACUC (R12-03-02). We also thank the field crews of ASLC, the Alaska Department of Fish

and Game (ADF&G), and the Marine Mammal Laboratory for support to collect of samples from Steller sea lions pups from Agattu Island, Ulak Island, Ugamak Island, and Chiswell Island (ADF&G Permit # 18537, ASLC Permit #18438-00, IACUC #2015-38B, and UAF IACUC #883669-5) and Graves Rock (ADF&G Permit# 18537, National Parks Permit #GLBA-2016-SCI-0011, and IACUC 2015-38B), and adult females from Kiska Island, Kiska Island, Adak Island, Kanaga Island, and Ulak Island (ADF&G Permit 18438-03 and UAF IACUC #883669-5).

4.8 Figures

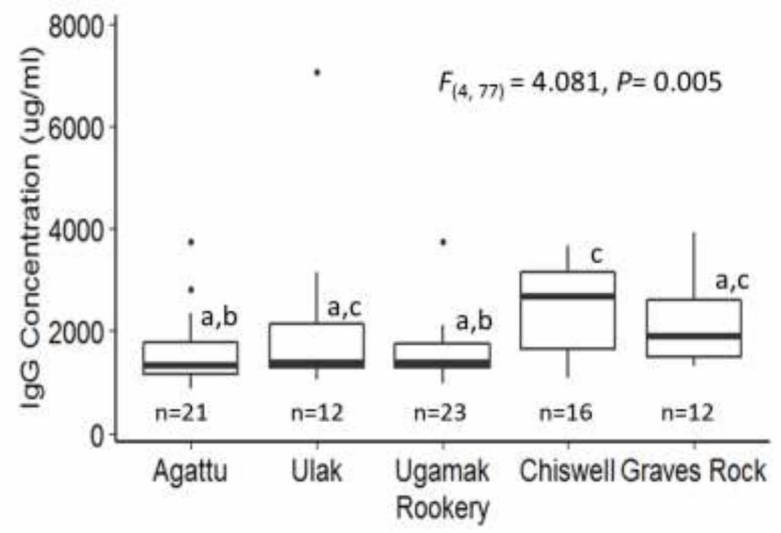
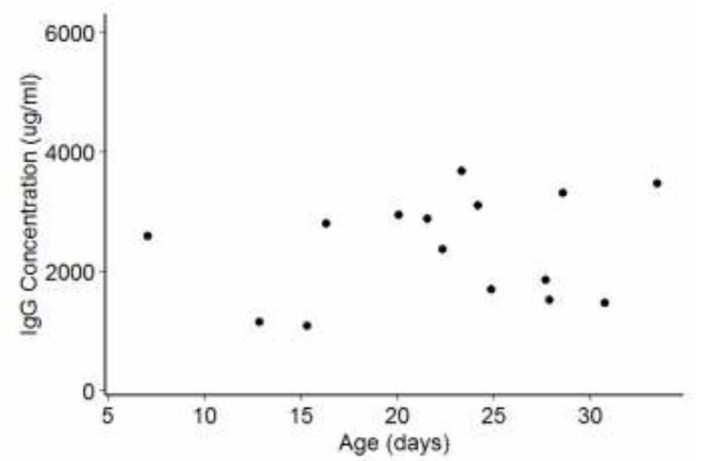


Figure 4.1 - Median, interquartile range, and sample size of total immunoglobulin G (IgG) concentrations measured in young free-ranging pups sampled from various rookeries in Alaska. Immunoglobulin concentrations varied significantly among regions, and multiple comparison group assignments are noted a-c.

A.



B.

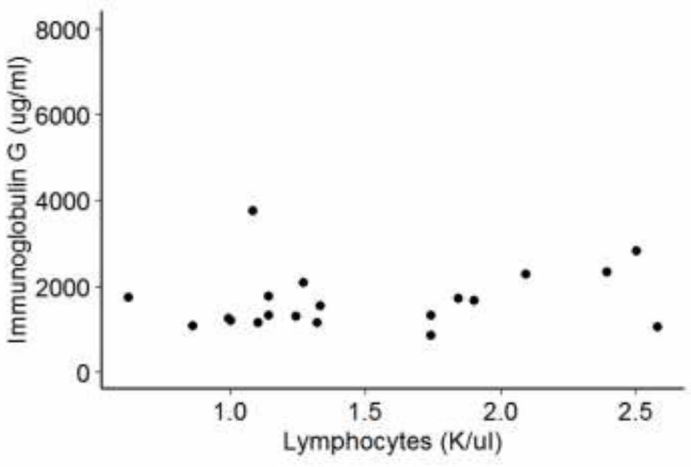


Figure 4.2 - Immunoglobulin concentrations did not vary with known age (days) in young free-ranging pups sampled from Chiswell Island (A), or with lymphocyte counts in free-ranging pups sampled from any of the rookeries (B). Note: the graph in (B) is from data collected from Agattu Island pups as an example.

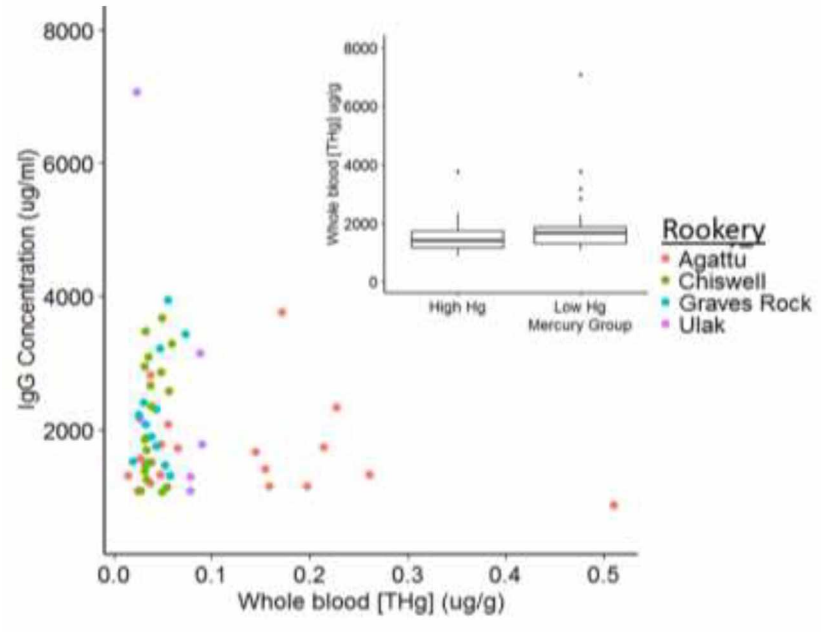


Figure 4.3 - Immunoglobulin concentrations and whole blood total mercury concentrations did not relate to one another, nor was there a difference in the variation of immunoglobulin concentrations between high (≥ 0.11 µg/g) and low (< 0.11 µg/g) level whole blood total mercury ([THg]) groups in young free-ranging pups from Agattu Island (inset graph), $P>0.05$.

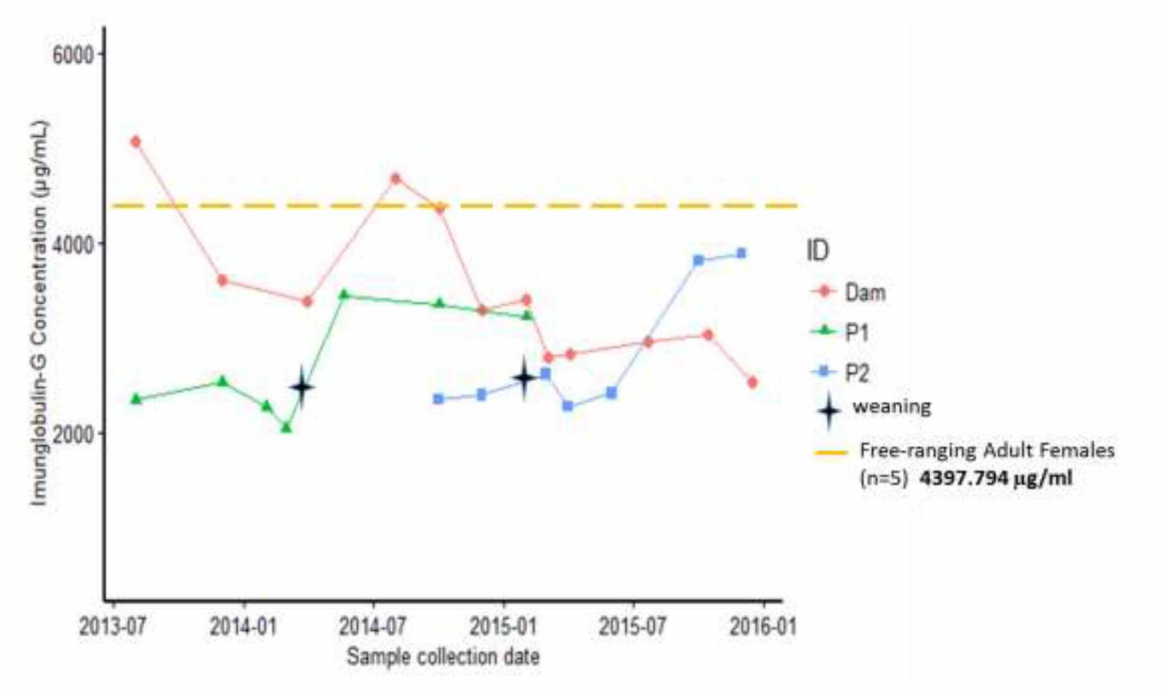


Figure 4.4 - Temporal variation in immunoglobulin concentrations measured in a captive dam and her two neonatal pups after birth through weaning. The first pup (P1) was born on June 20, 2013, whereas the second pup (P2) was born on July 20, 2014. The stars indicate the time point of weaning for each pup (switched to a fished based diet). The average concentration of five free-ranging adult females (observed with dependent pups) sampled within the Aleutian Islands in October of 2014 and 2015 is also shown (dotted line).

4.9 Tables

Table 4.1 - Range, sample size (n), mean, and standard deviation (±SD) are presented for total immunoglobulin G concentrations ([IgG]), automated lymphocyte counts (LYM), and whole blood total mercury concentrations ([THg]) measured in free-ranging SSL adult females and young pups, and in a captive Steller sea lion (SSL) dam and her two pups.

	[IgG] Range (n)	[IgG] Mean (±SD)	LYM Range (n)	LYM Mean (±SD)	[THg] Range (n)	[THg] Mean (±SD)
Captive SSLs[a]						
Adult Female	866.80-3760.00 (12)	3498.00 (±802)	NA	NA	NA	NA
Pre-weaning[b]	2050.00-3890.00 (14)	2356.01 (±173)	NA	NA	NA	NA
Post-weaning	2420.00-3890.00 (6)	3357.33 (±528)	NA	NA	NA	NA
Free-ranging SSLs						
Adult Females[c]	3752.80-4932.00 (5)	4397.79 (±570)	NA	NA	0.12-0.24 (5)	0.18 (±0.05)
Pre-weaning pups						
Agattu Island	866.8-3760.0 (21)	1648.40 (±695)	0.62-2.58 (20)	1.49 (±0.57)	0.02-0.51 (20)	0.12 (±0.12)
Ulak Island	1057.80-7064.96 (12)	2082.09 (±1680)	0.64-2.56 (7)	1.71 (±0.78)	0.02-0.13 (8)	0.06 (±0.04)
Ugamak Island	979.00-3755.92 (23)	1589.15 (±552)	0.58-3.09 (20)	1.63 (±0.65)	0.01-0.04 (15)	0.03 (±0.01)
Chiswell Island	1081.60-3676.12 (16)	2447.65 (±869)	1.93-4.28 (13)	3.18 (±0.68)	0.03-0.06 (14)	0.04 (±0.01)
Graves Rock	1316.80-3946.0 (12)	2200.58 (±893)	1.49-6.61 (11)	3.51 (±1.46)	0.02-0.07 (11)	0.04 (±0.02)

[a]Sample size (n) represents repeated sample measurements from a captive dam and her two pups housed at the ASLC

[b]Mean pre-weaning immunoglobulin concentrations for both captive pups were significantly lower than the mean immunoglobulin concentrations post-weaning

[c]Free-ranging adult females were sampled from rookeries in the Aleutian Islands

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Chapter 5 – **Regional variations in cytokine profiles and *in utero* mercury exposure in Steller sea lions (*Eumetopias jubatus*)**[5]

[5]Kennedy, S.N., Keogh, M., Levin, M., Maniscalco, J., Castellini, J.M., Lian, M., Fadely, B., Ferrante, A., Rea, L.D., O'Hara, T.M. (*In Prep*). Regional variations in cytokine profiles and *in utero* mercury exposure in Steller sea lions (*Eumetopias jubatus*). Conservation Physiology.

5.1 Abstract

Steller sea lions (SSL) in the western distinct population segment experienced a significant population decline followed by stable or increasing population trends in rookeries in the Gulf of Alaska (GOA) while rookeries within the Aleutian Islands (AI) have failed to recover. Rookeries in Southeast Alaska (SEA) have shown a steady increase over time. Regional differences were found in whole blood mercury concentrations ([THg]) with greater than 20% of young pups in certain rookeries in the AI having [THg] above critical adverse effects thresholds established for piscivorous mammals whereas rookeries in the eastern GOA and SEA have lower levels of [THg]. Some pups in the AI are exposed to mercury concentrations *in utero* above critical thresholds leaving them at risk of adverse immunological outcomes. Given the evolutionarily conserved aspects of mammalian immunity, immune cell-signaling proteins can be used to evaluate the immune status of marine mammals. We compared cytokine and chemokine concentrations in pups among regions (AI, eastern GOA, SEA), and associations among cytokines, chemokines, white blood cell counts (WBC), and [THg]. The liver is an important target organ for immune system development and mercury accumulation therefore liver enzymes, aspartate and alanine aminotransferase, were also investigated with [THg]. Regional differences in cytokines and chemokines, and immune protein associations were observed. Total WBC and [THg] were positively associated in AI pups whereas lymphocytes and [THg] in SEA pups were negatively associated. No associations between mercury and cytokines, chemokines, or AST and ALT were found. We provide evidence for regional differences in immune status, and report concentration ranges of a suite of cytokine and chemokines which may prove to be a new tool for assessing the immune system in pinnipeds. Changes to the cytokine and chemokine milieu during early development in SSL pups could lead to an imbalance in downstream hematopoietic progenitor cell differentiation that could impact immunological resiliency at the population level.

5.2 Introduction

Immunomodulation by means of *in utero* toxicant exposure could render developing young at risk of impaired immunity and subsequent population-level effects in species that are vulnerable to population decline (e.g., endangered, lack resilience). Mercury is a persistent environmental toxicant that bioaccumulates in upper trophic organisms and can induce adverse physiological and immunological consequences when concentrations exceed critical thresholds for some species (Basu and Head, 2010; Dietz *et al.*, 2013). Mercury concentrations have been on the rise in circumpolar north (Dietz *et al.*, 2013), and Steller sea lions (SSLs) are an emerging sentinel for the environmental health of the North Pacific (Phillips *et al.*, 2011; Castellini *et al.*, 2012). Given their trophic feeding ecology and life history, SSLs can be exposed to concerning levels of mercury (Basu *et al.*, 2007; Castellini *et al.*, 2012; Peterson *et al.*, 2016). In particular, some young SSL pups are exposed to concentrations of mercury *in utero* that are above critical adverse effects thresholds defined for piscivorous mammals (Castellini *et al.*, 2012; Rea *et al.*, 2013b); therefore, they may be at risk of adverse neurocognitive and immunological outcomes (Basu *et al.*, 2007; Kennedy *et al.*, 2019). Twenty to thirty-five percent of SSL pups sampled from various rookeries within regions of decline in the Aleutian Islands had total mercury concentrations that were above critical adverse effects thresholds (Basu *et al.*, 2007; Kennedy *et al.*, 2019) leading to the hypothesis that mercury may be a contributing factor for the lack of population recovery in those regions (Castellini *et al.*, 2012; Rea *et al.*, 2013b). Further, mercury accumulation in target organs like the liver may have the greatest impact on developing young considering the fetal liver is the origin of nascent hematopoietic stem cells that subsequently develop into their respective precursor innate immune cell lineages during gestation (Levy, 2007; Correa *et al.*, 2014; Dórea, 2015). This notion warrants our investigation to understand the potential physiological and immunological repercussions of elevated mercury concentrations in young SSL pups from a regional perspective.

Clinical adverse effects thresholds of mercury have yet to be defined for SSLs, although many studies report on the effects of mercury on immune response in other species. For instance, mercury is

linked with inflammation and autoimmunity in other mammals (Somers *et al.* 2015; Crowe *et al.* 2017), and immunosuppressive effects were reported for marine mammals (Das *et al.* 2008; Desforges *et al.* 2016). *In vitro* exposure studies that exposed marine mammal lymphocytes to various concentrations of monomethylmercury demonstrated that changes to immune cell function and response may occur at concentrations ranging from 0.2 to 0.5 ppm (Das *et al.* 2008; Kakuschke *et al.* 2009; Desforges *et al.* 2016; O'Hara and Hart 2018). Adverse immunological changes resulting from mercury exposure may compound the risk of disease susceptibility, particularly in developing young with naïve and nascent immune defenses (Neale *et al.*, 2005; Eagles-Smith *et al.*, 2018). A recent study by Kennedy *et al.* (2019) reported that young SSL pups from rookeries within the western AI had mercury concentrations that were associated with changes in the acute phase immune response protein, haptoglobin, supporting a plausible link between mercury and the innate immune system in developing SSLs. It remains to be determined whether transplacental mercury exposure adversely affects immune system development and response in young SSLs during critical immunological time points of gestation, or if variations in mercury concentrations (or a combination of both) are associated with significant changes in immunity after birth. Regardless of the cause of immunomodulation, insufficient or dysfunctional immune response when subjected to pathogens may have detrimental population-level effects in threatened and endangered species such as the SSL. Therefore, comparing the immune status of young SSL pups from rookeries within regions of decline with those from rookeries with stable population growth might offer insight to understanding immunological contributions to resiliency among regions.

Assessing the immune status and response of wildlife populations is challenging with limited species-specific methodology, especially for marine mammals that spend the majority of their lifetime in remote areas at sea. Dynamics of marine diseases are also governed by factors that differ from terrestrial systems adding to the difficulty in identifying threats to immune homeostasis and predicting immune status in marine mammals (Lafferty 2017). Given the caveats and paucity of available tools for immunological screening in wildlife (Boughton *et al.* 2011; Garnier *et al.* 2017), including marine

mammals, it is necessary to establish a suite of measurable prospective biomarkers to evaluate the immune status of marine mammal populations with respect to immune system development, and pathogen and/or toxicant exposure (Gulland and Hall 2007). Many components of immune cell signaling pathways are evolutionarily conserved (Liongue *et al.*, 2016) and this allows for the application of immune protein biomarkers for assessing the immune status of various wildlife species, including pinnipeds (Levin *et al.*, 2014). The mammalian immune system is a dynamic, complex network thereby requiring an intricate communication system among cells and tissues for eliciting a concerted immune response and maintaining homeostasis to preserve overall health. Cytokines and chemokines function as the primary communication messengers among cells of the immune system to direct immune development. Specifically, cytokines and chemokines work in concert to elicit and facilitate immune system development, and they control cell-mediated and humoral immune responses. Some cytokines and chemokines are essential for signaling cell growth and differentiation (i.e. IL2, IL7, IL15, IL18, and GM CSF), and acute phase protein production (IL6). Others moderate the differentiation of specific lymphocytes into their respective lineages, like the T-cell subtypes Th1 (IFN-γ, IL-2, TNF-α) or Th2 (IL-10), that serve to provide a specific immune response that is tailored to the type of antigen/pathogen present. The advancement of multiplex molecular methodology has provided simultaneous detection and quantification of multiple cytokines and chemokines allowing for a broader understanding of innate and adaptive immune responses in mammalian wildlife, including pinnipeds (Levin *et al.*, 2014). Quantifying cytokines and chemokines can provide important information regarding changes to immune cell lineages during development and subsequent proliferation, differentiation, and directionality of immune response. When taken into consideration with clinical health indices (i.e. hematology and serum chemistry), and potential environmental stressors, like mercury, cytokine and chemokine profiles can shed light on identifying changes to specific ontogenic immune pathways that may not be detected solely from traditional complete blood cell counts in a non-model and endangered species, such as the SSL.

From an environmental and conservation perspective, monitoring the immune status of SSLs from regions that have experienced significant population decline and lack of recovery (i.e. the AI of the western distinct population segment) in the context of neonatal mercury exposure can identify changes to immune system that may be significant to individuals, certain cohorts, and/or populations. Cytokines and chemokines are useful biomarkers of immune response and their use might circumvent some of the current difficulties of monitoring immune status in marine mammals. In this study, we quantitatively assessed immune status in SSL pups using the novel biomarkers cytokines and chemokines, and traditional hematology in pups from regions lacking population recovery in the western (Agattu Island) and central AI (Ulak Island) and compared to pups from reference rookeries with stable or increasing pup production in the eastern Gulf of Alaska (Chiswell Island) and the Southeast Alaska (Graves Rock). Specifically, we report concentrations of cytokines and chemokines in young SSL pups and made regional comparisons of variations and accounted for the sample year, and sex of the individuals. Considering leukocyte counts vary during postnatal development (Keogh *et al.*, 2010), we investigated postnatal changes of the immune response during the first weeks of life by assessing the relationship of cytokines, chemokines, total and differential white blood cells with age for young pups with known birth dates (± 4 h) sampled from Chiswell Island. Cytokines often regulate cellular response in a paracrine or autocrine fashion, therefore, we also investigated associations among the various cytokines, chemokines and hematology measurements to understand how these immune proteins may work in concert during postnatal development in SSLs from different regions. Given the concerning levels of mercury in SSL pups (Castellini *et al.*, 2012; Rea *et al.*, 2013b) and the association of *in utero* exposure to mercury (>0.11 ppm) with lower liver-derived innate immune protein haptoglobin in the young SSL pups from Agattu Island (Kennedy *et al.*, 2019), we also investigated associations of mercury with cytokines, chemokines, total white blood cell counts (WBC), and measurements of two liver enzymes, aspartate (AST) and alanine aminotransferase (ALT), to elucidate potential regional differences in development of immune pathways and immune status with respect to *in utero* [THg].

5.3 Methods

5.3.1 Sample collection

We sampled free-ranging, young SSL pups (n=59, <1.5 mo.) at natal rookeries from Agattu Island (n=24) in 2013 (n=6) and 2015 (n=12), Ulak Island in 2013 (n=6), and Chiswell Island (n=15) and Graves Rock (n=20) in 2016 (Figure 5.1). Ages were estimated based on date of capture and size. Exact age (days) was determined for most of the Chiswell Island animals born on the rookery from dams that were previously branded or identifiable with natural markings based on video recordings of births from the Alaska Sea Life Center monitoring program (Maniscalco *et al.*, 2010). Routine capture, restraint, and sampling methodology was used to collect whole blood as previously described (Raum-Suryan *et al.*, 2004; Castellini *et al.*, 2012; Lander *et al.*, 2013). Demographic data such as age class, sex, and rookery were recorded for each individual.

5.3.2 Hematology and serum aminotransferases

Total and differential white blood cell counts were quantified using the Abaxis Vet Scan Autoanalyzer (Union City, CA USA) in the field (Ulak Island and Agattu Island) using methods from Lander et al (2013), or submitted to the Alaska SeaLife Center for quantification via their ProCyte Dx Hematology Analyzer (Westbrook, ME, USA) (Chiswell Island and Graves Rock). AST and ALT were quantified (U/L) from frozen serum by Phoenix Central Laboratories (Mukilteo, WA, USA) using previously reported methods (Lander *et al.*, 2013) in a subset of young pups (n=13; Agattu Island).

5.3.3 Quantification of serum cytokines

Serum samples were stored at -80°C until analysis (< 3 years) and prior to notable protein degradation is known to occur (de Jager *et al.*, 2009). We quantified (pg/ml) serum cytokines (GM-CSF, IL-6, IL-7, IL-8, IL-15, IL-18), including those representing Th1 (IFN-γ, IL-2, TNF-α) and Th2 (IL-10) response, and chemokines (MCP1 and IP10) according to the manufacturers' instruction using the Millipore Canine Cytokine/Chemokine Magnetic Bead Panel and the Bio-Plex® 100/200™ System at the

University of Connecticut. All samples were analyzed on a single Millipore plate to reduce experimental error for comparing the protein measurements. The assay quantification protocol followed validated methodology reported for other pinniped species (Levin *et al.*, 2014). All quality control values were within the manufacturer's specified concentration ranges for each run.

5.3.4 Whole blood total mercury concentrations [THg]

A Milestone DMA-80 direct mercury analyzer (Milestone, Monroe, Connecticut, USA) at the Wildlife Toxicology Laboratory at the University of Alaska Fairbanks, Fairbanks, Alaska, USA was used to measure whole blood [THg] (reported as ppm, wet weight) using methods previously reported (Castellini *et al.*, 2012; McHuron *et al.*, 2014; Peterson *et al.*, 2016). Calibration verifications, certified reference materials, and system and method blanks were included in each run for quality control and assurance. Recoveries for measuring [THg] in blood samples were 95.66 ± 0.03% for liquid standard calibration verifications (1ppm HgCl2), and 90.44 ± 0.11% and 96.77 ± 0.04% for certified reference materials (Seronorm and DORM-3 respectively).

5.3.5 Statistical Analyses

Mean, median, and standard deviation are reported for each hematology and immune protein measurement for young pups. Cytokine and chemokine data did not meet normality assumptions using a Shapiro-Wilk test, and normality assumptions were not met following log transformation. Given the distribution of the data, parametric comparisons could not be established, and non-parametric statistics were employed (Whitcomb and Schisterman, 2008; Zhang *et al.*, 2009; LaFleur *et al.*, 2011). For each cytokine and chemokine measured, if at least three or more individuals from each rookery had measurements greater than the minimum detection concentration limit (mdl) that immune protein was included for statistical analysis. Of those included in statistical analysis, if the cytokines or chemokines were below the minimum detection concentration limit, half of the mdl value was used (Appendix A; (Cohen and Ryan, 1989)) Non-parametric Wilcoxon–Mann–Whitney tests were then used to analyze

differences in cytokine concentrations between two factors (sex and year), while Kruskal-Wallis tests were used to identify differences in concentrations among rookeries. When significant differences were detected among more than two factors, a Dunn's Test of multiple comparisons was performed to identify which of the factors were driving the observed differences. For the subset of pups from Chiswell Island with known ages, associations of cytokines/chemokines and differential white blood counts with age were examined using Pearson's correlation. Associations of cytokines and chemokines, and white blood cell counts with cytokines/chemokines were also assessed using Pearson's correlations.

We tested regional differences in [THg] using a Kruskal-Wallis and Dunn's multiple comparisons tests. To test for differences in cytokine and chemokine concentrations with [THg], young SSL pups from Agattu Island were binned into high (>0.11 ppm) or low (≤0.11 ppm) mercury group based on a statistically derived mercury concentration associated with changes to the innate immune protein, haptoglobin, in SSLs (Kennedy *et al.*, 2018). Given the distribution of the data, parametric relationships between cytokines and chemokines with mercury could not be established, and non-parametric statistics were employed. Statistical comparisons of median protein concentrations between high and low [THg] groups were made using data from Agattu Island pups given they were the only rookery with pups exceeding the ≥0.11 ppm cut-off threshold. Wilcoxon–Mann–Whitney tests were performed to assess if median cytokine/chemokine concentration differed among the assigned high and low mercury groups. Associations of white blood cell counts and serum aminotransferases with [THg] were assessed using Pearson's correlations. Differences were considered significant at an alpha value greater than 0.05.

5.4 Results

5.4.1 Regional differences in immune proteins and their associations

No differences were detected for the distribution of cytokine and chemokine concentrations between sampling years (2013 and 2015) for young pups from Agattu Island ($p > 0.05$), therefore further analysis was performed on the combined data. Variation in cytokine and chemokine concentrations in young pups was independent of sex ($p > 0.05$), therefore, males and females were pooled for Agattu Island, Chiswell Island, and Graves Rock. Given the limited sample size of young pups from Ulak Island, testing differences between sexes was not possible so data were also pooled. The age of Chiswell Island pups with known birth dates ranged from 6.2 to 33.4 days, and age was not significantly correlated with any of the cytokines or chemokines, total WBC counts, or differential WBC counts. All of the cytokines and chemokines measurable using the canine kit were detected in at least one or more SSL serum samples (Table 5.1). The protein that had concentrations greater than the minimum detection concentration in every serum sample tested from pups from all locations was KC-like protein. At least 1-2 individuals from Chiswell Island and Graves Rock had measurable concentrations of IL2, MCP1 and TNFα. However, these three proteins were not detected in any samples collected from young pups from Agattu or Ulak Islands. Lastly, IFNγ was detected in Chiswell Island and Agattu Island, but not Ulak Island or Graves Rock (Table 5.1). Of the proteins measured, IL6, IL7, IL8, IL10, IL15, IL18, GM-CSF, and KC-like proteins were used for statistical analysis to investigate variability in protein concentrations among rookeries (Table 5.1). For young SSL pups, significant differences in protein concentrations among rookeries were observed for IL6 (chi-squared = 11.07, df = 3, $p = 0.01$) , IL7 (chi-squared = 23.50, df = 3, $p < 0.001$), IL15 (chi-squared = 10.67, df = 3, $p = 0.01$), IL18 (chi-squared = 20.4582, df = 3, $p < 0.001$), GM-CSF (chi-squared = 16.02, df = 3, $p = 0.001$) and KC-like (chi-squared = 14.1558, df = 3, $p = 0.003$) proteins. The Dunn's multiple comparison's tests revealed that young pups from Graves Rock had significantly greater concentrations of IL6, IL7, IL15, IL18, and GM-CSF than any other rookery (Figure 5.2). Agattu Island, Ulak Island and Chiswell Island had median concentrations of GM-CSF, IL15, and

IL6 that were not significantly different, and all were significantly lower than the median concentrations observed in pups from Graves Rock. Young pups from Agattu Island had the lowest concentrations of IL7 and IL18 and mean and median concentrations of these two proteins were greater the further east the young pups were sampled (Figure 5.1, Figure 5.2). Even though the immunosuppressive cytokine IL10 was greater on average for Chiswell Island pups compared with other rookeries, the median concentrations of IL10 (and IL8) did not vary significantly (p>0.05) among rookeries (Figure 5.2). Of the possible associations among cytokines and chemokines, young pups from Graves Rock had positively correlated concentrations of IL6, IL7, IL8, IL15, IL18, and GM-CSF and these associations (n=10) were significant (Table 5.3). Young pups from Chiswell Island also had positive correlations (n=8) for the same proteins with the exception that no correlation was found between IL7 and IL8 (Table 5.3). In comparison, only one significant correlation was found between IL6 and IL15 in pups from Agattu Island. The sample size of Ulak Island pups was insufficient for assessing protein associations.

5.4.2 Regional associations of white blood cell counts with immune proteins

Total WBCs in Graves Rock pups had a significant, and positive correlation with concentrations of IL6 (r = 0.77, p = 0.001), IL7 (r = 0.55, p = 0.04), IL15 (r =0.61, p = 0.02), IL18 (r = 0.59, p = 0.03) and GM-CSF (r = 0.59, p = 0.03) (Figure 5.3). Neutrophil counts were the only differential WBC correlated with cytokines in Graves Rock pups. Aside from IL7, neutrophils were significantly and positively correlated with IL6 (r = 0.71, p < 0.01), IL15 (r = 0.65, p = 0.01), IL18 (r = 0.57, p = 0.03), and GM-CSF (r = 0.66, p = 0.01) in Graves Rock pups. In comparison, the anti-inflammatory IL10 cytokine was significantly correlated with total WBC (r = 0.59, p = 0.02) in Chiswell Island pups, but no other significant correlations were found for differential counts with proteins for this rookery. For Agattu Island pups, neither total WBCs nor differential counts were significantly correlated (p > 0.05) with cytokine or chemokine concentrations.

5.4.3 Whole blood total mercury [THg] and regional associations with immune measures

Whole blood [THg] in young pups differed significantly among rookeries. Agattu Island pups had significantly greater [THg] concentrations (median = 0.10 ppm, range = 0.01- 0.51 ppm; chi-squared = 12.39, df = 3, $p = 0.01$) than other rookeries followed by Ulak Island (median = 0.06 ppm, range = 0.02 - 0.09 ppm), Chiswell Island (median = 0.04 ppm, range = 0.03 - 0.06 ppm) and Graves Rock (median = 0.04 ppm, range = 0.02 - 0.07 ppm) shared similarly lower [THg]. Only Agattu Island had pups that were above the 0.11 ppm cut-off for comparing the variation of immune proteins between high and low [THg] mercury groups. However, no significant differences were detected for median cytokine and chemokine concentrations between the high and low [THg] groups of Agattu Island pups. Of the tests performed to assess correlations of whole blood [THg] with total and differential WBC counts in young pups among rookeries, two significant correlations were observed. Total WBC counts were positively correlated (t = 0.85, df = 16, r = 0.41, $p = 0.05$,) with whole blood [THg] in Agattu Island pups (Figure 5.4A), likely being driven by neutrophil counts (t = 2.03, df = 16, $p = 0.06$). While lymphocyte counts were negatively correlated (t = -3.32, df = 12, $p = 0.01$, r = -0.69) with whole blood [THg] in Graves Rock pups (Figure 5.4B). All other comparisons made between blood cells counts and [THg] were not significant. Mean ALT and AST values in pups from Agattu Island were 37.15 ± 17.43 U/L and 22.85 ± 9.37 U/L, respectively, and values for Ulak Island pups were 29.2 ± 9.97 U/L and 23.7 ± 7.55 U/L, respectively. However, neither serum ALT or AST measurements had a significant relationship with [THg] for Agattu Island pups.

5.5 Discussion

We compared cytokine and chemokine measurements and identified associations among immune measures across geographically distinct groups of young SSL pups. The use of cytokines and chemokines in conjunction with immunological and physiological parameters proved to be useful to describe immune profiles, infer general immune status, and to determine if a link could be made between immune status and *in utero* exposure of mercury. A key finding was that cytokine and chemokine concentrations varied

among young SSL pups from different rookeries likely indicating regional differences in immune status. We also identified regional differences in the associations among cytokines, chemokines, and hematological measurements. Cytokines and chemokines often work in concert to regulate immune response, therefore, the lack of associations between cytokines, chemokines, and cell counts in Agattu Island pups compared with Chiswell Island and Graves Rock further supports the regional differences in immune status during early stages of development. Regional variation in immune proteins in young SSL pups is a significant finding and may be indicative of differences in hematopoietic progenitor cell-signaling pathways and/or immune response to antigenic challenges during early stages of immune system growth and development. The associations found between hematological measures and [THg] in SSL pups provides some support for the possibility that transplacental mercury exposure may influence immune cells that are responsible for the production of cytokines and chemokines. However, this evidence does not substantiate the hypothesis of adverse effects of mercury on immune status in SSL pups, especially considering no associations were found between [THg] and cytokines/chemokines or liver enzymes.

The concentrations of important pro-inflammatory and hematopoietic cell stimulating cytokines (GM_CSF, IL7, IL15, IL18, and IL6) were significantly lower in young pups from Agattu and Ulak Islands compared with Chiswell Island and Graves Rock. This indicates regional differences in immune response and status and gives rise to the concern that some cytokine pathways (hematopoiesis, lymphocyte differentiation, and acute phase response) may be disrupted in Agattu and Ulak Islands compared with the reference group, Graves Rock, with stable or increasing population growth. Varying concentrations of cytokines involved in immune system growth and development is indicative of differences in hematopoietic progenitor cell-signaling pathways among SSL rookery pups. Cytokines and chemokines are categorized based on their functionality, and they elicit and facilitate an appropriate immune response to stimuli and are important for directing the development of the immune system in young. Cytokines influence the growth and development of blood cells, coordinate cell differentiation,

and mediate cell-to-cell interactions and proliferation during immune response whereas chemokines are responsible for chemotaxis of cells during inflammation and angiogenesis. The cytokine protein concentrations in young SSL pups are likely distinct from the dam, given there is evidence that fetal and maternal myeloid bone marrow cells and CD8 T cells have a differential phenotype and gene expression (Krow-Lucal *et al.*, 2014; Wang *et al.*, 2016). At the beginning of hematopoietic development, the cytokine GM_CSF plays a significant role in stimulating the production of hematopoietic progenitor cells and enhances their mobilization from the bone marrow into circulation. The cytokine IL7 enhances the development of precursor B cells in the bone marrow and T cells in the thymus and is produced by stromal cells, hepatocytes, and neurons. The cytokine IL7 is also a major co-factor for the rearrangement of genes encoding the T-cell receptors involved in cell mediated immunity, plays a role in angiogenesis, and regulates the differentiation of cytotoxic cell lineages that are involved in pathogen clearance. Similarly, IL15 influences hematopoietic division of T cells by skewing the maturation process to favor CD8 T-cells that are vital for protection against intracellular pathogens. IL15 can also influence the development of natural killer cells. The pro-inflammatory cytokine IL6 can induce B-cells to differentiate into plasma cells (after B-cell activation) and can upregulate the production of protective acute phase response proteins (i.e. C-reactive protein, and haptoglobin). Further, IL6 is an important regulator of immune activation in fetal and newborn immunity (Krow-Lucal *et al.* 2014) and was significantly lower in Agattu Island, Ulak Island, and Chiswell Island pups when compared to Graves Rock. Most of these important cytokines were found to be significantly lower in rookeries that experienced significant population decline farther west (Agattu Island, Ulak Island, and in some cases, Chiswell Island) compared with the reference group (Graves Rock). Considering the role of IL6 in the haptoglobin pathway (Alayash, 2011), our finding of lesser IL6 concentrations in pups from Agattu and Ulak Islands (Aleutian Islands) compared to Graves Rock within Southeast Alaska is in agreement with the trend observed for regional differences in haptoglobin concentrations (Kennedy *et al.* 2019).

Positive associations among redundant or pleiotropic cytokines, chemokines, and leukocyte counts are expected, however, the number of associations observed for Agattu Island pups were vastly different when compared to Chiswell Island and Graves Rock. More positive associations were found in Graves Rock and Chiswell Island pups when compared to Agattu Island pups. Many of the cytokines that were measured in SSLs instruct developing immune cells in tissue specific microenvironments to divide and differentiate (e.g., IL6, IL7, IL15, IL18), and the positive associations of cytokines and leukocyte counts observed in Graves Rock indicates proliferation and differentiation of hematopoietic cells in those pups. Although many of these cytokines were positively associated with one another and with leukocytes in Graves Rock pups, only one association was seen (WBCs and IL10) for Chiswell Island pups, and this association was not observed in Graves Rock pups. Parasites and viral burden can influence heightened IL10 expression (Duignan *et al.*, 2014; Redpath *et al.*, 2014), and parasites have been documented in densely populated SSL rookeries (Hughes *et al.* 2004). Although direct comparisons of mean hematology values could not be compared due to a difference in methodology, it should be noted that the eosinophil counts in Chiswell Island pups were the greatest on average compared to the other sites. Regional differences in parasite or viral burden could explain greater eosinophil counts, greater concentrations of IL10, and the positive association of IL10 with WBCs in Chiswell Island pups.

Mercury concentrations did not correlate with circulating concentrations of cytokines in young SSL pups. In SSL pups from Agattu Island (location with greatest concentrations of [THg]) no significant differences in cytokine concentrations were found between the high and low [THg] groups (based on the statistical threshold of 0.11 ppm) (Kennedy *et al.*, 2019). This is in contrast to the differential cytokine patterns seen in children exposed to mercury above EPA thresholds (29 nmol/L or 5.83 ppm), namely a negative association with IL6 (the primary cytokine signal for acute phase protein production, including haptoglobin), and a positive association with an immunosuppressive cytokine, IL10 (Hui *et al.*, 2016). Fetal blood concentrations of [THg] can differ slightly from post birth [THg] in children that may give rise to a differences in correlative associations with immune protein measures (Hui *et al.*, 2016). For

young SSL pups, this is unlikely the case considering circulating whole blood [THg] in young pups compared with [THg] from their natal coat that was developed *in utero* are tightly correlated, therefore, whole blood [THg] is representative of *in utero* exposure concentrations of mercury in dependent pups (Rea *et al.*, 2013a; Peterson *et al.*, 2016). Additional studies report associations of mercury and other cytokines (Gardner *et al.*, 2009; Nyland *et al.*, 2011; Motts *et al.*, 2014), however, the direction of association (positive or negative) between mercury and some of the cytokines measured are not in agreement among the studies. The lack of association between [THg] and cytokines/chemokines could be due to the disproportionate number of individuals in the high [THg] group, that the cut-off of [THg] selected may not be relevant for changes to cytokine concentrations, or, that mercury does not influence cytokine/chemokine production in SSLs. Proposed ranges of [THg] that relate to adverse effects in immune measure in pinnipeds fall between 0.2 and 0.5 ppm (O'Hara and Hart, 2018). Individuals from in the high [THg] group were above the lower limit (0.10 ppm) benchmark for adverse effects in humans (National Research Council 2006) and above the level of mercury (>0.11 ppm) associated with lower haptoglobin protein in young SSL pups from Agattu Island (Kennedy *et al.*, 2019). Two out of the six Ulak Island pups had [THg] close to the high [THg] group cut-off (0.11 ppm; Kennedy *et al.*, 2019), but all fell within the low mercury group category whereas Chiswell Island and Graves Rock did not have any pups that fell into the high [THg] group. The overall mean [THg] for Agattu pups was also slightly above this benchmark (0.12 ppm) and they also had significantly lesser mean/median concentrations of most cytokines and chemokines compared to the other regions. Interestingly, one young pup from Agattu Island with the greatest concentration of [THg] (0.51 ppm) did not have measurable amounts of cytokines and chemokines, aside from the neutrophil attractant, KC-like protein (and one of the greatest WBC). The [THg] in that pup exceeded (5X) the maximum adverse effects concentration established for piscivorous mammals (Basu *et al.*, 2007) and the statistical threshold relating to decreased innate immune protein, haptoglobin (Kennedy *et al.*, 2019). Therefore, the pup with 0.51 ppm of [THg] may have experienced immunomodulatory effects as demonstrated by the absence of detectable levels of cytokines and chemokines and the greatest WBCs compared to other individuals. This individual may have had a

current infection yet was unable to elicit the appropriate response to the infection due to immunomodulation effects of mercury on cytokine production, and possibly, progenitor cell development and cell-mediated cytotoxicity. Mercury may indirectly suppress or interrupt the cell-signaling pathways necessary for normal growth and development of the neonatal immune system in SSL pups by a different mechanism than what is represented by the biomarkers measured herein. However, a specific threshold for mercury inducing changes to cytokine production for young SSLs *in vivo* is unknown therefore additional sampling and in vitro assessments should be made.

Mercury relates to specific populations of cells in a concentration dependent manner (Das *et al.*, 2008; Desforges *et al.*, 2016; Dupont *et al.*, 2016; Oulhote *et al.*, 2017), and the potential for a biphasic response may explain the contrasting relationships of [THg] and immune cell counts for rookery pups subjected to regionally distinct concentrations of mercury *in utero*. For example, in mice fed a dose of 50μM versus 100 μM of mercury chloride ($HgCl_2$), IFN and the activity of hematopoietic stem cells was activated in the lower dose, yet repressed in the higher dose and the effect of the lower dose was absent in a different mouse strain; this indicates that inorganic mercury can influence immune response and their respective functions in a concentration and strain dependent manner (Li *et al.*, 2018). Mercury can elicit a biphasic response in other cell signaling pathways (Unoki *et al.*, 2016), and the discrepancies in the directionality of [THg] associations with different immune cells (positive vs. negative) seen in SSL pups might be explained by this phenomenon. Graves Rock pups had the lowest [THg] on average, and a negative association of mercury with lymphocyte counts. This is consistent with an *in vitro* pinniped study conducted with harbor seals (Das *et al.*, 2008). Agattu Island pups had an average THg above a certain threshold and showed an increase in total WBCs (the majority were neutrophils) with [THg], and the same pup with the greatest [THg] (0.51ppm) with nearly all cytokines and chemokines non-detect also had one of the greatest total white blood cell counts (21.34 K/ul, nearing the upper reference threshold) and eosinophils (0.19 K/ul) while lymphocyte counts were nearing the lower threshold and well below average (Lander *et al.*, 2013). Similarly in other mammals, bats with greater [THg] had more neutrophils

and fewer monocytes and reduced bacteria killing ability, and demonstrated lower innate functions (Becker *et al.*, 2017). Mercury is one possible environmental pressure (among other contaminants like PCBs) (Neale *et al.*, 2005), and the disparate associations of [THg] and blood cell counts could reflect an *in utero* mercury related imbalance of cell populations during development. Although, immunological effects thresholds of [THg] need to be defined for cellular activity and response in SSL with cytokine/chemokine measurements as an endpoint during early stages of development.

Although [THg] in SSLs likely effects populations of immune cells (and indirectly, the production of immune cell signaling proteins), it does not appear that the majority of concentrations observed lead to changes in liver enzymes. Mercury effects on liver function in SSLs are unknown, but given the ALT and AST were within statistically defined reference thresholds of Lander *et al.* (2013), it is assumed no hepatic damage occurred. In mice, mercury was shown to have negative effects on the production of IL6 in mouse liver without causing hepatotoxicity (Kim and Sharma, 2005), and this might be the case for SSL pups regarding the haptoglobin pathway in SSL pups (Kennedy *et al.*, 2019). Measurements of AST and ALT in Agattu and Ulak Island pups did not relate to [THg] concentrations. Further, liver enzymes for the pup with the greatest whole blood THg (0.51 ppm) from Agattu Island this individual also remained within the reported reference range (Lander *et al.*, 2013). However, the mean ALT and AST values in pups from Agattu Island (37.15 units and 22.85, respectively) and Ulak Island (29.2 U/L and 23.7 U/L, respectively) were below the mean reference values previously reported (43.5 U/L and 37.3, respectively) (Lander *et al.*, 2013). It is notable that many of the AST measurements in individual Agattu Island pups were below the lower reference threshold. One out of 6 pups from Ulak Island, and 5 out of 13 pups from Agattu Island were below the lower reference threshold for AST (Lander *et al.*, 2013). The physiological implications of this finding are not likely to be directly related with immune status, but rather it could relate to a nutritional deficit. For example, infants that exhibit consistently low values of AST (or ALT) can have an underlying vitamin B6 deficiency (Ono *et al.*, 1995; D'Agata and Balistreri, 1999). More relevant to SSL pups might be a deficiency in an essential element

that relates to Vitamin B6, inflammation, and immunity, like selenium (Se) (Ralston *et al.*, 2008; Huang *et al.*, 2012; McHuron *et al.*, 2014; Hosnedlova *et al.*, 2017). Selenium is an important component of protective antioxidant enzymes (selenoproteins) that is transferred via the placenta (Burk *et al.*, 2013). An additional essential nutrient, Vitamin B6, is associated with selenium retention in the heart, liver, and kidneys (Jin and Yin, 2005). If the pups with below threshold AST measurements have a deficiency in B6, then selenium may not be retained in the appropriate tissues during development. Selenium binds with mercury typically in a 1:1 molar ratio and differential binding and molar ratios can occur in different tissue compartments (Koeman *et al.*, 1975; Correa *et al.*, 2014). Therefore, deviations from a 1:1 ratio associated with greater relative concentrations of mercury and inadequate Se could indirectly impact immune related processes involving selenoproteins (Brummer, 2012; Huang *et al.*, 2012; Narayan *et al.*, 2015; Das *et al.*, 2016).

There are many factors other than mercury that could explain the regional differences in immune measurements and associations observed. Significant changes in concentrations of specific groups of cytokines is often the result of developmental changes, however, a targeted response to a specific type of antigenic stimulus, differences in gene expression, or, a diseased state that leads to an aberrant immune response (Zimmerman *et al.*, 2014) could lead to differences in immune response at the population level. Agattu and Ulak Island pups sampled in this study had lesser cytokine and chemokine concentrations compared to Chiswell Island and Graves Rock pups. The magnitude of the observed differences might be the result of location specific pressures on immune status including density dependent diseases (i.e. *Uncinaria* sp.) in rookeries where pup production has been increasing. Increased parasite or viral burden in rookeries with greater densities (increased pup production) could also explain the notably greater counts of eosinophils in Chiswell Island and Graves Rock rookeries compared with Agattu and Ulak Island rookeries. Genetic variation and differences among haplotypes of SSLs from different regions (Baker *et al.*, 2005; Phillips *et al.*, 2011), and differential expression of immune genes (Bowen *et al.*, 2006) could also explain the differences in cytokine and chemokine concentrations that were observed

among the rookeries. Other considerations include dietary preferences of the dam (Doll *et al.*, 2018) during gestation that could lead to the accumulation of other immunomodulating contaminants (or antigens) from forage fish during pregnancy.

5.6 Conclusion

The immune status of young SSL pups varied regionally, and pups from Agattu and Ulak Islands in the western AI may have altered immune cell-signaling response compared with the Chiswell Island and Graves Rock pups. Although the cause of the differences observed remain to be determined, our findings support the use of cytokines as a new tool for studying the immune status in wild populations of SSLs. The difference in cell-signaling protein concentrations (cytokines and chemokines) among rookery pups is likely not impacted directly from *in utero* exposure to mercury, although there is some evidence that mercury could lead to changes among the source cells that manufacture immune proteins. The relationship of *in utero* mercury exposure with immune cells of SSL pups during early development is likely concentration dependent where greater [THg] concentrations positively associate with total leukocytes (as seen in Agattu Island) and at lesser [THg] concentrations (as seen in Graves Rock pups) are negatively associated with lymphocytes, but this may not influence cell-signaling protein production. Therefore, mercury may interact with hematopoietic stem cell activity in a concentration dependent manor (Li *et al.*, 2018). However, it remains to be determined how mercury influences immune status in young SSL pups. In this study, we provide evidence for regional differences in cytokine and chemokine profiles and associations of immune cells with mercury in young SSL rookery pups. Therefore, individual pups born in regions where the dams' dietary intake of mercury is much greater (i.e., western AI, (Rea *et al.*, 2013a)) may be at greater risk for altered immune response when compared to reference rookeries (Chiswell Island and Graves Rock). Changes to cytokine and chemokine milieus during early development likely gives rise to an imbalance in downstream hematopoietic progenitor cell differentiation that could impact cell- signaling pathways and immunological resiliency in young pups. The mechanism by which mercury (and other environmental pressures) can impact immune pathways that are responsible

for the differentiation of hematopoietic and somatic cells (i.e. stromal cells, epithelia, hepatocytes) that produce regulatory cytokines during early stages of immune system development requires further investigation.

5.7 Acknowledgements

We would like to thank the staff and volunteers at the Alaska Sea Life Center (ASLC) for paired samples collected from captive animal under National Marine Fisheries Service permit #18534 and IACUC (R12-03-02). We also thank the field crews of ASLC, the Alaska Department of Fish and Game (ADF&G), and the Marine Mammal Laboratory for support to collect of samples from Steller sea lion pups from Agattu Island, Ulak Island, Ugamak Island, and Chiswell Island (ADF&G Permit # 18537, and ASLC Permit #18438-00, and IACUC #2015-38B) and for Graves Rock (ADF&G Permit# 18537, National Parks Permit #GLBA-2016-SCI-0011, and IACUC 2015-38B).

5.8 Figures

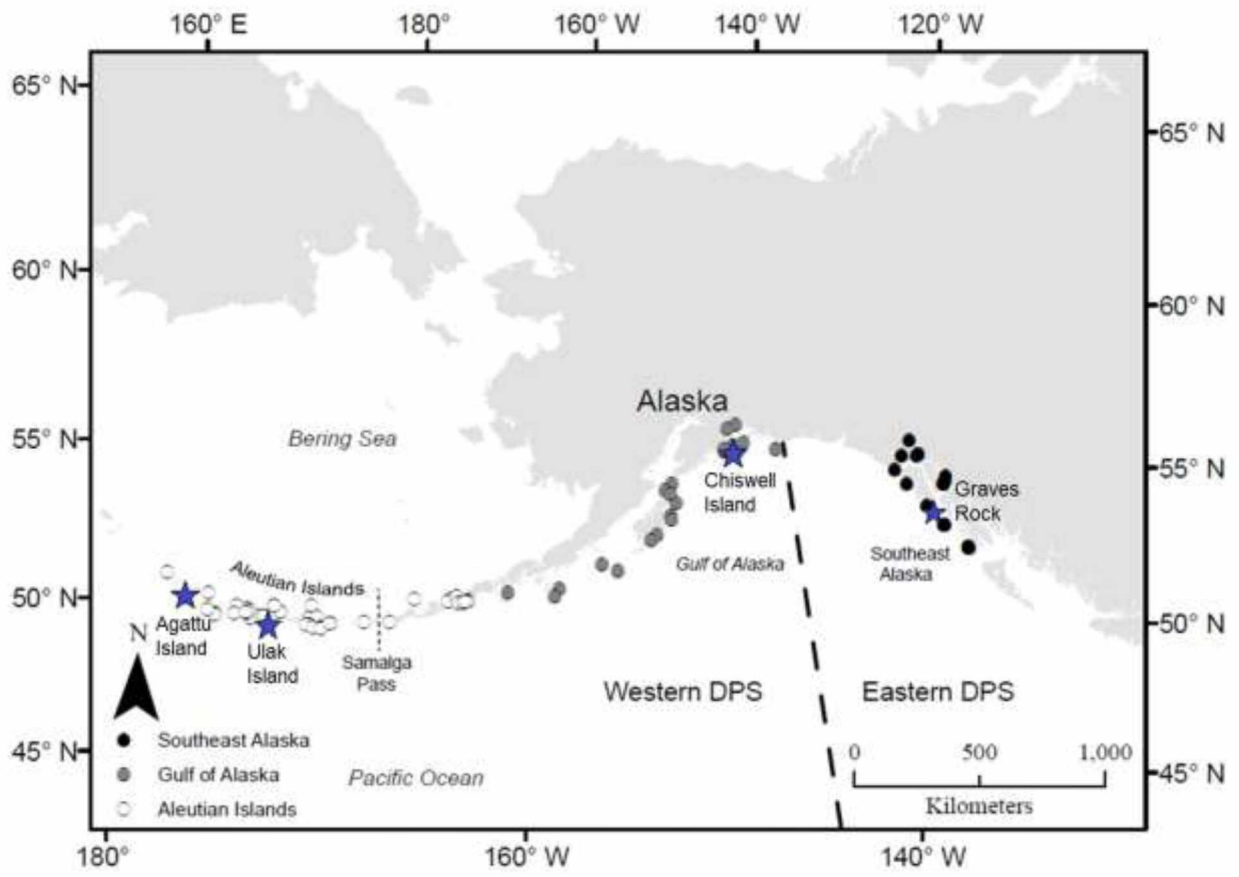


Figure 5.1 - Map of the distribution of Steller sea lion (SSL) rookeries along the coast of Alaska showing the longitude delineation (144ºW) for the western and eastern distinct population segments (DPS) and the regional classifications for Aleutian Islands (AI), Gulf of Alaska (GOA), and Southeast Alaska (SEA). Samples were collected from young SSL pups from rookeries (indicated by stars) on Agattu Island and Ulak Island (western AI), Chiswell Island (eastern GOA), and Graves Rock (SEA).

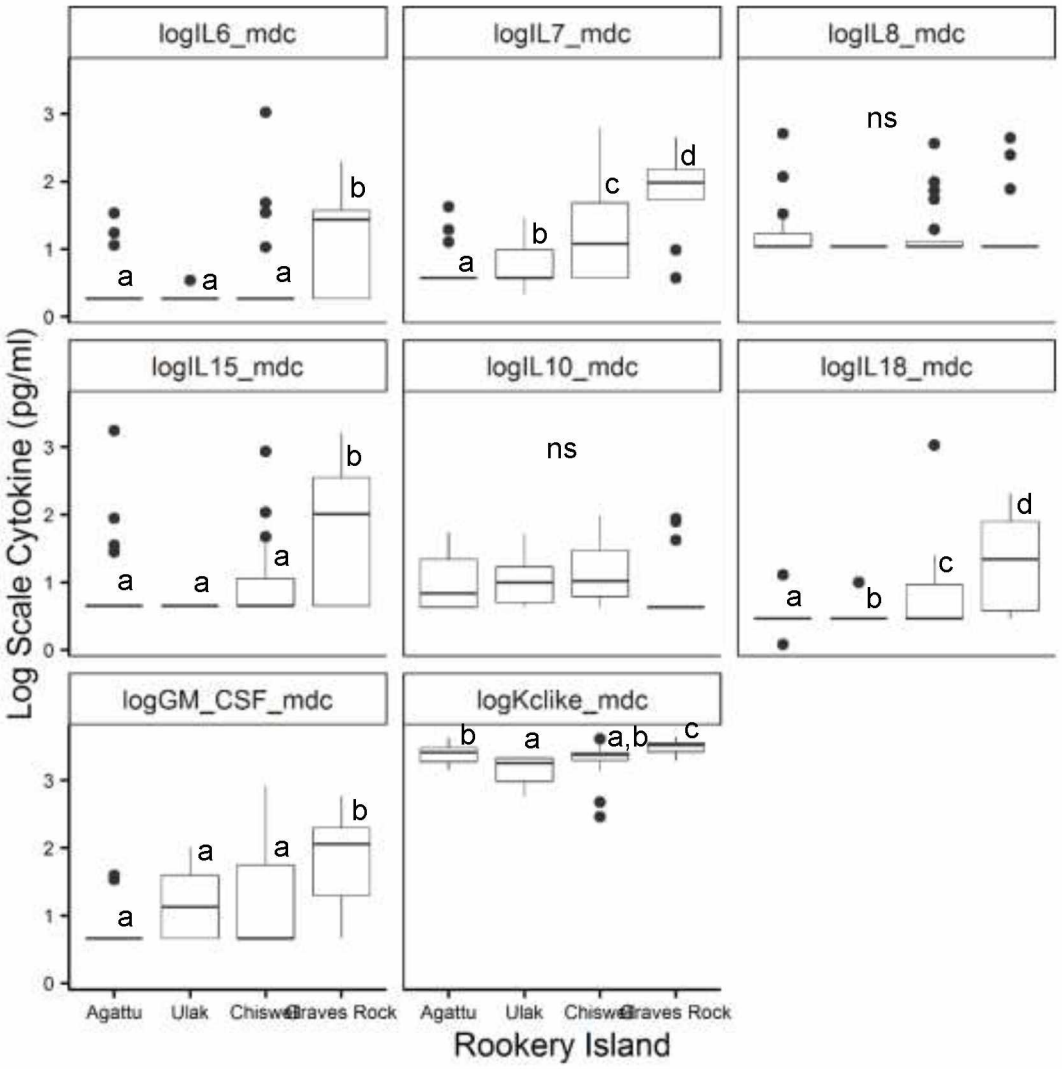
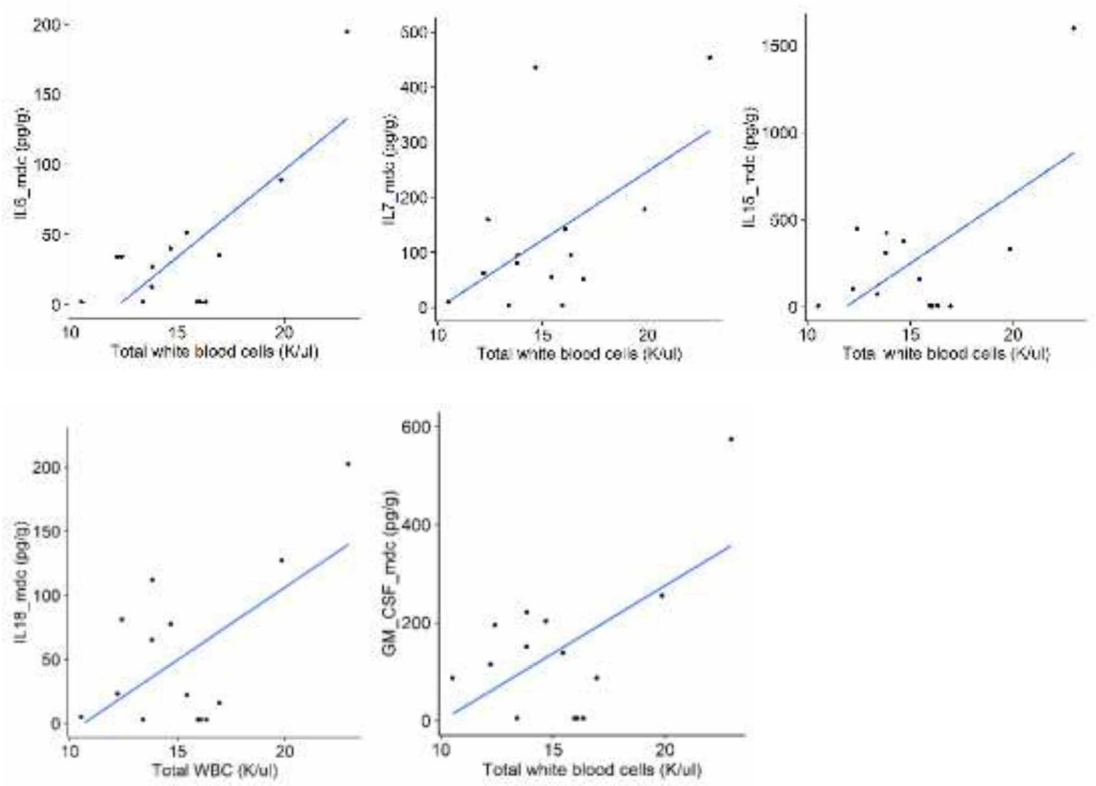


Figure 5.2 - Regional comparisons of median cytokine and chemokine concentrations (pg/ml) in young SSL pups from Agattu Island, Ulak Island, Chiswell Island, and Graves Rock. Data are presented in log scale for comparison.

A). Graves Rock (WBC & cytokines)



B) Graves Rock (Neutrophils & cytokines)

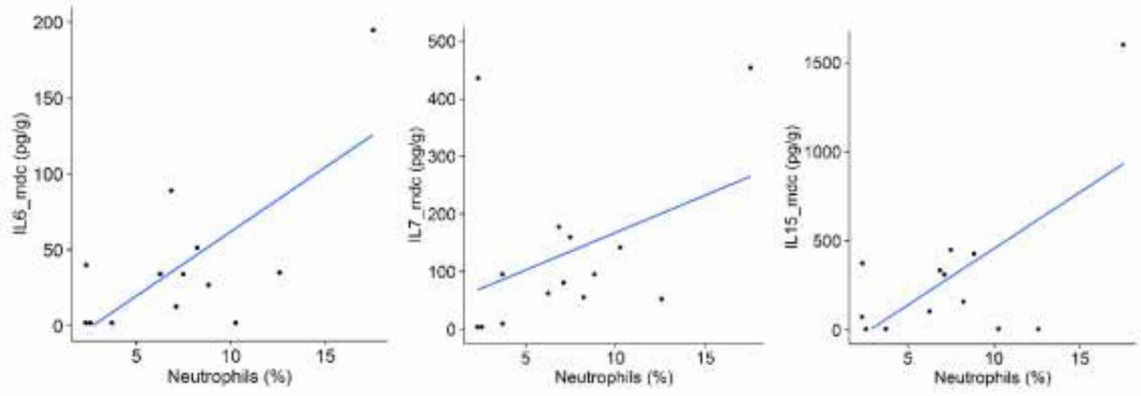
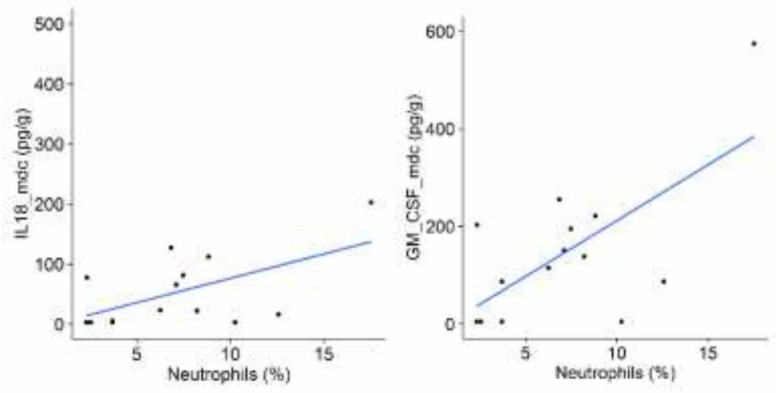


Figure 5.3 - Significant correlations between serum cytokines and chemokines and A) total white blood cell counts (WBC) and B) neutrophils for pups sampled from Graves Rock, and C) between total white blood cell counts and IL10 for pups sampled from Chiswell Island.



C. Chiswell Island (WBC & IL10)

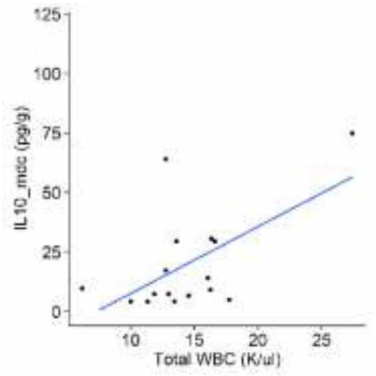


Figure 5.3 (Continued)

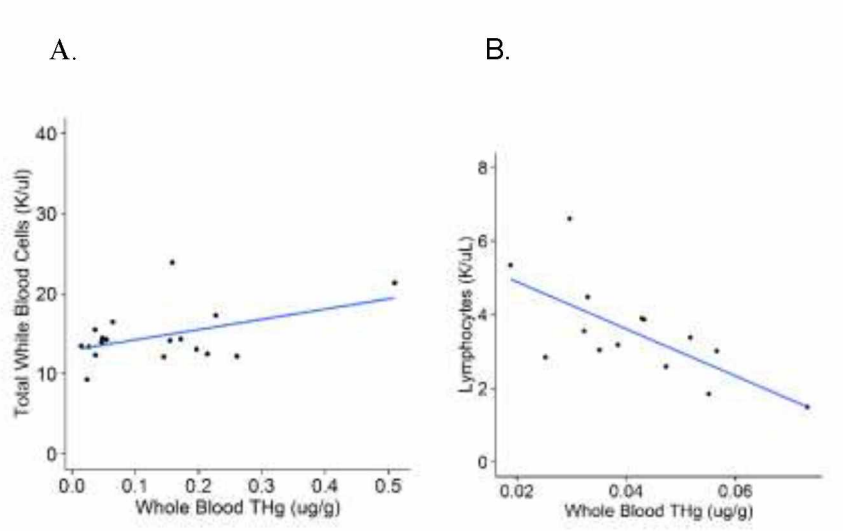


Figure 5.4 - Significant correlations between total white blood cell counts (WBC) and whole blood total mercury (THg) in young pups from Agattu Island (A), and lymphocyte counts and whole blood total mercury (THg) in young pups from Graves Rock pups (B).

5.9 Tables

Table 5.1 - Sample size, mean, median, standard deviation (SD), and range for immune cell signaling proteins, cytokines and chemokines, for young SSL pups sampled from rookeries on Agattu Island, Ulak Island, Chiswell Island, and Graves Rock, Alaska.

Location	Agattu Island, Western Aleutian Islands			Ulak Island, Central Aleutian Islands			Chiswell Island, Eastern Gulf of Alaska			Graves Rock, Northern Southeast Alaska		
Sample size			*n* = 18			*n* = 6			*n* = 15			*n* = 15
Conc. (pg/ml)	n[b]	mean (SD)	median (range)	n[b]	mean (SD)	median (range)	n[b]	mean (SD)	median (range)	n[b]	mean (SD)	median (range)
GM-CSF	3	9.81 (12.05)	4.6 (4.60-39.62)	3	32.62 (38.36)	4.6 (4.60-102.66)	8	79.60 (186.69)	4.6 (4.60-824.09)	11	142.89 (145.60)	114.38 (4.60-574.27)
IFNg	1	12.91 (16.60)	9 (9.00-79.44)	0	9.00[a]	9.00[a]	1	11.46 (9.45)	9 (9.00-51.04)	1	9.00[a]	9.00[a]
IL2	0	1.75[a]	1.75[a]	0	1.75[a]	1.75[a]	2	66.17 (287.10)	1.75 (1.75-1285.94)	4	25.10 (75.31)	1.75 (1.75-291.42)
IL6	3	5.03 (8.35)	1.85 (1.85-34.05)	1	2.11 (0.65)	1.85 (1.85-3.43)	3	58.69 (233.90)	1.85 (1.85-1051.04)	11	35.26 (50.63)	26.91 (1.85-194.70)
IL7	3	4.1 (9.58)	3.75 (3.75-41.95)	2	9.39 (10.65)	3.75 (2.13-29.46)	11	56.47 (137.50)	11.9 (3.75-623.15)	13	130.13 (138.54)	95.18 (3.75-453.48)
IL8	5	47.46 (118.48)	10.85 (10.85-511.24)	0	10.85[a]	10.85[a]	4	38.61 (80.07)	10.85 (10.85-362.42)	5	59.38 (121.32)	10.85 (10.85-436.68)
IL10	12	15.79 (16.21)	7.61 (4.25-54.80)	4	16.60 (18.15)	10.04 (4.25-51.88)	14	22.94 (26.19)	10.345 (4.25-96.12)	5	17.34 (28.62)	4.25 (4.25-87.47)
IL15	4	109.5 (411.81)	4.5 (4.50-1757.50)	0	4.50[a]	4.50[a]	7	57.34 (190.63)	4.5 (4.50-860.23)	11	257.08 (408.79)	103.24 (4.50-1599.00)
IL18	3	3.36 (2.40)	2.9 (1.20-12.82)	2	4.08 (2.89)	2.9 (2.90-9.99)	8	59.38 (236.39)	2.9 (2.90-1063.37)	12	50.79 (59.55)	22.12 (2.90-202.59)
IP10	1	2.59 (4.20)	1.6 (1.60-19.41)	0	1.60[a]	1.60[a]	1	3.26 (7.42)	1.6 (1.6-34.8)	2	1.60[a]	1.60[a]
KC-like	18	2624.00 (837.44)	2596 (1409-4246)	6	1563.33 (715.60)	1819.515 (564.43-2201.78)	15	2286.67 (895.17)	2390.17 (288.20-4101.52)	13	3170.79 (764.77)	3297.82 (1925.66-4364.30)
MCP1	0	10.5[a]	10.5[a]	1	10.50[a]	10.50[a]	1	32.83 (99.86)	10.5 (10.50-457.0)	3	23.12 (48.90)	10.5 (10.50-199.87)
TNFa	0	3.05[a]	3.05[a]	1	3.05[a]	3.05[a]	1	15.35 (55.02)	3.05 (3.05-249.10)	4	8.41 (20.78)	3.05 (3.05-83.52)

a= all samples were below the minimum detection concentration
b=the number of individuals above the minimum detection concentration

bold=met criteria for statisticial analysis

Table 5.2 - Correlations between cytokines and chemokines in serum collected from young SSL pups from Agattu Island, Chiswell Island, and Graves Rock (note: Ulak Island did not have a sufficient number of samples with measurements above the minimum detection limit for analysis).

Location	*t*	df	*p*-value	Pearson's Correlation Coefficient
Agattu Island, Western Aleutian Islands				
IL6 & IL15	7.03	16	<0.001	0.87
Chiswell Island, Eastern Gulf of Alaska				
IL7 & IL6	18.84	18	<0.001	0.98
IL7 & GM_CSF	10.31	18	<0.001	0.93
IL6 & IL18	85.42	18	<0.001	0.99
IL6 & IL15	32.07	18	<0.001	0.99
IL6 & GM_CSF	12.07	18	<0.001	0.94
IL15 & IL18	39.41	18	<0.001	0.99
IL15 & GM_CSF	11.84	18	<0.001	0.94
IL18 & GM_CSF	11.81	18	<0.001	0.94
Graves Rock, Northern Southeast				
IL7 & IL18	3.87	13	0.002	0.73
IL7 & IL6	3.58	13	0.003	0.71
IL7 & GM_CSF	4.16	13	0.001	0.76
IL6 & IL18	5.99	13	<0.001	0.86
IL6 & IL15	7.77	13	<0.001	0.91
IL6 & GM_CSF	9.14	13	<0.001	0.93
IL15 & IL7	4.18	13	0.001	0.76
IL15 & IL18	7.98	13	0.001	0.91
IL15 & GM_CSF	11.16	13	<0.001	0.95
IL18 & GM_CSF	11.21	13	<0.001	0.95

Table 5.3 - Sample size, mean, median, standard deviation (SD) are given for total and complete white blood cell counts (WBCs), serum aminotransferase concentrations, and whole blood total mercury concentrations for young pups sampled at rookeries on Agattu Island, Ulak Island, Chiswell Island, and Graves Rock.

	Vetscan					Vetscan					Procyte					Procyte				
	Western Aleutian Islands, Agattu Island					Central Aleutian Islands, Ulak Island					Eastern Gulf of Alaska, Chiswell Island					Northern Southeast, Graves Rock				
	n = 18					*n* = 6					*n* = 20					*n* = 15				
	n	Mean	Median	SD	Range	*n*	Mean	Median	SD	Range	*n*	Mean	Median	SD	Range	n	Mean	Median	SD	Range
Lymphocytes (K/ul)	18	1.44	1.33	0.52	0.62-2.50	5	1.26	1.28	0.61	0.64-2.23	16	3.31	3.27	0.69	1.93-4.44	13	3.51	3.28	1.33	1.49-6.61
Neutrophils (K/ul)	18	12.6	11.93	3.16	8.00-22.00	5	10.7	11.15	1.93	8.59-13.03	16	5.34	4.75	3	1.69-9.84	13	7.13	6.98	4.32	2.32-17.53
Monocytes (K/ul)	18	0.59	0.58	0.18	0.35-0.97	5	0.41	0.39	0.11	0.30-0.58	16	1.37	1.28	0.46	0.76-2.49	13	1.64	1.49	0.79	0.80-4.04
Basophils (K/ul)	18	0.01	0	0.02	0.00-0.07	5	0	0	0	0	16	0.02	0.01	0.03	0.00-0.11	13	0.07	0.03	0.11	0.00-0.39
Eosinophils (K/ul)	18	0.02	0.01	0.04	0.00-0.19	5	0	0	0	0	16	4.35	4.52	4.4	0.04-14.43	13	2.97	0.80	3.58	0.02-9.10
Total WBC (K/ul)	18	14.65	14.05	3.42	9.28-23.88	5	12.4	12.74	1.8	9.84-14.24	16	14.38	13.52	4.52	6.21-27.42	13	15.32	15.07	3.20	10.53-22.95
ALT (U/L)	13	37.15	33	17.43	22-89	6	29.2	29	9.97	13-41	0	NA	NA		NA	0	NA	NA		NA
AST (U/L)	13	22.85	19	9.37	15-46	6	23.7	24	7.55	10--32	0	NA	NA		NA	0	NA	NA		NA
THg (ppm)	18	0.12	0.1	0.11	0.01- 0.51	6	0.06	0.05	0.03	0.02-0.09	18	0.04	0.04	0.01	0.03-0.06	15	0.04	0.04	0.01	0.02-0.07

5.10 Appendix

Appendix Table 5.1 - The minimum concentration detected using the Millpore canine multiplex kit, and the value of the half the minimum detection concentration assigned for SSL serum samples that were below the minimum detection limit.

Cytokine	Minimum Detection Limit pg/ml	Half mdc
GM_CSF	9.2	4.6
IFNg	18	9
IL2	3.5	1.75
IL6	3.7	1.85
IL7	7.5	3.75
IL8	21.7	10.85
IL15	9	4.5
IP10	3.2	1.6
KClike	5.3	2.65
IL10	8.5	4.25
IL18	5.8	2.9
MCP1	21	10.5
TNFa	6.1	3.05

Source from Millipore Canine Kit Insert

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Chapter 6 - **Conclusions** [6]

[6]Stephanie Kennedy

6.1 Overview

Although the functional capabilities of the proteins measured in this dissertation (haptoglobin, cytokines, and immunoglobulins) have not been addressed for Steller sea lions (SSLs), a general understanding of the changes in immune status can be inferred from this work. It is apparent from the findings of this dissertation that the immune status (as assessed by the immune protein networks and pathways investigated herein) of young developing SSL pups differs regionally. The reasons for the apparent statistically significant regional differences in these immune parameters in young developing SSL pups are explored. *In utero* exposure to mercury may influence innate, acute phase response pathways (as seen in Chapter Two), but the mechanism of the association between haptoglobin and mercury in young pups requires further investigation on a cellular, molecular, and thermodynamic basis. This notion is highlighted by similar associations of contaminants and haptglobin biomarkers found in a captive feeding study with sled dogs (Sonne et al., 2016). Additionally, maternal antibody investment (Chapters Three and Four) is an important component of neonatal immune protection while cytokines (Chapter Five) dictate the development and function of immune cells to mediate the immune response in developing young. Relatively lower concentrations of these important immune proteins observed in pups from regions lacking population recovery might indicate that those individuals are immunologically challenged. One could argue, however, that antigenic challenge is greater among denser rookeries with stably increasing pup production in the eastern regions, although disease screening efforts do not support a clear distinction for regional differences in disease burden in SSLs (Atkinson et al., 2008; Burek et al., 2005a, 2005b). Protective immunoglobulin concentration ranges have yet to be defined for SSLs, and the function of the cytokine network has yet to be described. However, it is likely that insufficient concentrations of maternal immunity and cell-signaling proteins might lead to reduced resiliency to disease (Hall et al., 2009, 2002). Long term assessment of survival rates for the animals included in this study is underway, yet it will take many years to compile sufficient data for analysis. It is my hope that the immune data parameters measured in these chapters will be overlaid with survival estimates. Additionally, archived samples could be analyzed for a

continuation of conservation efforts to understand changes to immune status that relate to regional and temporal changes in population dynamics.

6.2 Future directions

The intricacies of the immune system and the ontogeny of immune function in SSLs awaits to be discovered. Further, causative agents attributing to the differences in immune proteins measured were not identified in this dissertation. In order for causative agents to be identified, advanced eco-immunological toolkits must be developed with systematic monitoring programs in place for a more in depth investigation of other physiological and environmental factors that likely influence immunity in SSLs. Most of all, it will take ingenious creativity to circumvent the current pitfalls of studying the immune system of marine mammals. This is especially the case when attempting to understand changes to immunity with the pervasive cumulative effects of changing environmental conditions, physiological status, disease, and the multiplicity of contaminant cocktails working in concert to ultimately shape the current dynamics of SSL ecology. Differences in animal handling, sample collection, and laboratory methodology should also be taken into consideration; standard operating protocols should be developed and validated for a species-independent approach to comparative studies that include sympatric species when applicable. Future work should focus on developing methods to identify antigenic challenges in SSL dams and pups, and to investigate the maternal fetal interface in more detail. The most prominent advances to the fields of marine mammal eco-immunology and toxicology (that would significantly enhance conservation efforts for SSLs) awaits the discovery of specific mechanisms behind the apparent associations between immune status and toxicants like mercury in developing young. The biological meaning behind the differences in the immune status of young SSL pups that I observed in my dissertation work rests on this notion.

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