Title: **Regional variations and relationships among cytokine profiles, white blood cell counts, and blood mercury concentrations in Steller sea lion (*Eumetopias jubatus*) pups**

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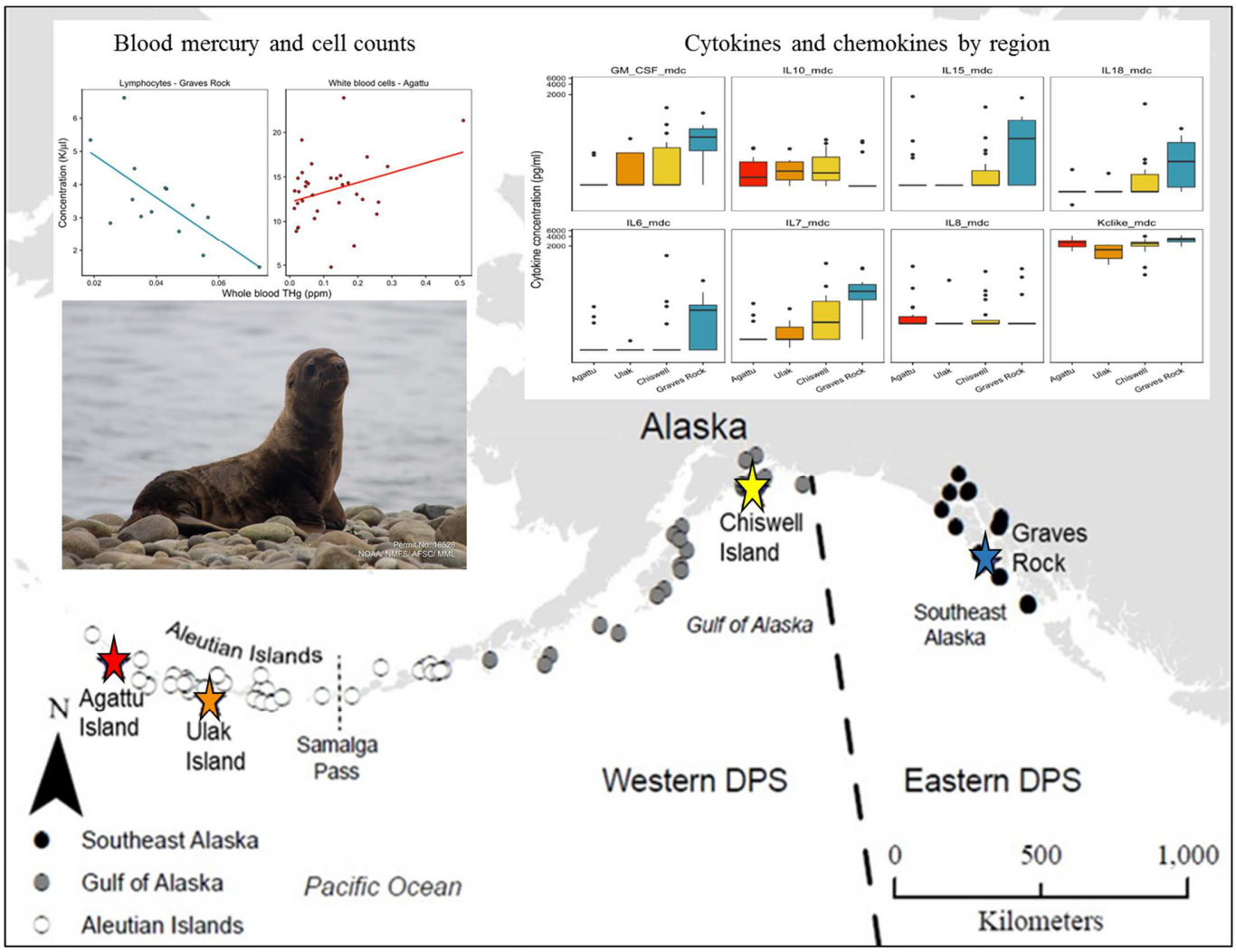
Highlights (85 characters or less):

- Immune measures were compared with blood mercury in Alaskan Steller sea lion pups.
- Serum cytokine and chemokine concentrations differed regionally.
- Cytokines and chemokines were associated with select blood cell counts.
- Blood mercury was associated with blood cell counts, not cytokines or chemokines.
- Cytokine and chemokine profiles may indicate pup immune status differs by region.

Graphical Abstract



Abstract (300/300 word limit)

The Steller sea lion (SSL) population west of 144˚W longitude experienced a significant population decline. While there appears to be a stable or increasing population trend in rookeries in the Gulf of Alaska (GOA) and Southeast Alaska (SEA), some rookeries within the Aleutian Islands (AI) have failed to recover. Previous studies found regional differences in whole blood total mercury concentrations ([THg]) showing more than 20% of AI pups had [THg] above critical thresholds for increased risk of immunological effects and other adverse outcomes. Measurements of immune cell-signaling proteins can be used to evaluate the immune status of marine mammals in relation to [THg]. We compared serum cytokine and chemokine concentrations in pups among regions (AI, eastern GOA, SEA), and examined associations among cytokines, chemokines, white blood cell (WBC) counts, and [THg]. Considering liver is an important target organ for mercury and immune protein synthesis we additionally examined the relationship of [THg] with liver-related enzymes serum aspartate (AST) and alanine aminotransferase (ALT). We observed regional differences in cytokine and chemokine measurements and immune protein associations. There was a positive association between total WBC counts and [THg] in AI pups, whereas a negative association between lymphocytes and [THg] in SEA pups. These findings may indicate regional variation in proliferation and differentiation of hematopoietic cells, differences in immune system development, and/or a difference in antigenic stimuli. No associations between [THg] and cytokines, chemokines, AST or ALT were found. Observed regional differences in cytokine and chemokine milieu during gestational and early development in SSL pups could lead to an imbalance in cell differentiation that could impact immunological resiliency in juvenile and adult life stages. We report concentration ranges of a suite of cytokines and chemokines which may prove to be a useful metric for ecotoxicology and risk assessment studies in SSLs and other wildlife.

1. Introduction

In utero toxicant exposure could render developing young at increased risk for impaired immunity with subsequent population-level effects in vulnerable species. Mercury (Hg) is a persistent environmental toxicant that bioaccumulates and biomagnifies in upper trophic level organisms and can cause adverse physiological and immunological consequences for some species, especially fish-eating mammals (Basu and Head, 2010; Dietz *et al.*, 2013). Physiological effects often involve the accumulation of Hg in target organs such as the brain and liver, and these effects are particularly concerning for developing young. In particular, fetal and neonatal hepatic exposure to Hg may impact the immune system of developing young considering the hepatic origin of nascent hematopoietic stem cells that subsequently develop into their respective precursor innate immune cell lineages during gestation (Levy, 2007; Dórea, 2015; Zhang *et al.*, 2017).

Since the industrial age, Hg concentrations have been on the rise in the circumpolar north (Dietz *et al.*, 2009, 2013), and Steller sea lions (SSLs) are an emerging sentinel for the environmental health in the North Pacific (Phillips *et al.*, 2011; Castellini *et al.*, 2012; Kennedy *et al.*, 2019). The western stock population of SSLs declined by about 80% between the late 1970s and 2000 (Loughlin and York, 2000; Burkanov and Loughlin, 2005). Although the population has increased overall since 2003, regional variation in population trends limits recovery (Fritz *et al.*, 2016) with sustained negative trends west of Samalga Pass in the western and central Aleutian Islands (AI) (Sweeney *et al.*, 2018). Twenty percent of SSL pups sampled from rookeries within regions of population decline in the AI had total mercury concentrations ([THg]) above adverse effects benchmarks (0.1-0.5ppm) (O'Hara and Hart, 2018), which may contribute to the lack of population recovery in those regions (Rea *et al.*, 2013, 2020). Adult females absorb monomethyl mercury ($MeHg^+$) from their diet during pregnancy and can expose pups *in utero* to $MeHg^+$ above critical adverse effects threshold concentrations defined for other piscivorous mammals; therefore, SSL pups may be at risk for adverse neurocognitive and immunological outcomes (Castellini *et al.*, 2012; Rea *et al.*, 2013, 2020; O'Hara and Hart, 2018; Kennedy *et al.*, 2019; Levin *et al.*, 2020).

The mammalian immune system is a dynamic, complex network requiring an intricate communication system among cells and tissues for eliciting a concerted immune response and maintaining homeostasis to preserve overall health. Many components of immune cell signaling pathways, including cytokines and chemokines, are evolutionarily conserved (Liongue *et al.*, 2016) and can be used to help assess the immune status of various wildlife species, including pinnipeds (Levin *et al.*, 2014), as well as assess the potential immunotoxic effects of environmental stressors such as Hg (Levin *et al.*, 2020). Cytokines and chemokines (Table S1) elicit and facilitate immune system development, control cell-mediated and humoral immune responses, and are essential for signaling cell growth and differentiation (i.e. IL-2, IL-7, IL-15, IL-18, and GM-CSF), and acute phase protein production (IL-6). The balance of T-cell helper (Th) subtypes Th1 (IFN-γ, IL-2, TNF-α) and Th2 (IL-10) cytokines help direct lymphocytes into their respective cellular effector functions, serving to provide a specific immune response tailored to the type of antigen/pathogen present. The advancement of multiplex molecular methodology has made simultaneous detection and quantification of cytokines and chemokines possible in a suite, or panel using a commercialized kit (Levin *et al.*, 2014). Cytokine and chemokine measurements may enhance interpretation of immunological effects of environmental stressors, including Hg, when considered with traditional clinical health indices (*i.e.*, hematology and serum chemistry) in a non-model and endangered species, such as the SSL.

Adverse immunological effects thresholds for Hg have yet to be defined for SSL pups, although many studies report on the effects of Hg on immune response in other species. For instance, Hg has profound effects on the immune system in humans (Somers *et al.*, 2015; Crowe *et al.*, 2017), and immunosuppressive effects were reported for marine mammals (*e.g.*, harbor seals, *Phoca vitulina*) (Das *et al.*, 2008; Desforges *et al.*, 2016). *In vitro* studies that exposed marine mammal lymphocytes to various concentrations of Hg demonstrated changes to immune cell function, with responses that occurred at concentrations ranging from 0.1 to 0.5 ppm (Das *et al.*, 2008; Kakuschke *et al.*, 2009; Desforges *et al.*, 2016; O'Hara and Hart, 2018; Levin *et al.*, 2020). Adverse immunological changes resulting from Hg

106 exposure may increase the risk of disease susceptibility, particularly in developing young with naïve and
107 nascent immune defenses (Neale *et al.*, 2005; Eagles-Smith *et al.*, 2018). A recent study by Kennedy *et*
108 *al*. (2019) reported SSL pups from rookeries within the western AI had whole blood [THg] associated
109 with changes in concentrations of the acute phase immune response protein, haptoglobin, supporting a
110 plausible link between Hg and the innate immune system in developing SSLs. It remains unknown
111 whether transplacental $MeHg^{+}$ exposure adversely affects immune system development and response in
112 SSLs pups during critical immunological time points of gestation, or if variations in [THg] (or a
113 combination of both) are associated with significant changes in immunity after birth. Regardless of the
114 cause of immunomodulation, insufficient or dysfunctional immune response when subjected to pathogens
115 may have detrimental population-level effects in endangered species (Grogan *et al.*, 2018), including the
116 SSL.

117 The aims of this study were 1) to investigate the association of whole blood [THg] (acquired
118 during gestation) and critical immunological and physiological parameters of SSL pups, and 2) evaluate
119 the regional differences in immune status in SSL pups. Immunological measures (e.g. cytokines and white
120 blood cell (WBC) counts) and [THg] in blood were examined and compared in SSL pups from the
121 western AI (Agattu Island) and central AI (Ulak Island), the eastern Gulf of Alaska (GOA; Chiswell
122 Island) and Southeast Alaska (SEA; Graves Rock). Considering the liver is involved with cytokine-
123 mediated acute phase response and detoxification (Cray, 2012), we also examined relationships between
124 liver enzymes, aspartate (AST) and alanine aminotransferase (ALT), and whole blood [THg]. We also
125 investigated if other physiological parameters (age and/or sex) would relate to postnatal changes of the
126 immune response during the first weeks of life (Maniscalco *et al.*, 2008; Keogh *et al.*, 2010). We
127 hypothesized that the immune status of SSL pups would differ regionally, and that varying [THg] is likely
128 associated with differences in immune status.

129

2. Methods

2. 1 Sample collection

Free-ranging SSL pups (n = 59, <1.5 mo.) were sampled at four natal rookeries; Agattu Island 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 1). Exact age (± 4 h) was determined for the Chiswell Island animals born by dams previously branded or identifiable with natural markings based on video recordings of births from the Alaska Sea Life Center monitoring program (Maniscalco *et al.*, 2008, 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 sex and rookery were recorded for each individual.

2.2 Hematology and serum aminotransferases

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

2.3 Quantification of serum cytokines

Serum samples were stored at -80°C until analysis (< 3 years) and analyzed prior to the timeframe when 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 (MCP-1 and IP-10), 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 (Table S1). The assay quantification

protocol followed validated methodology reported for other pinniped species (Levin *et al.*, 2014). All cytokines with quality control values that were within the manufacturer's specified concentration ranges for each run were included for statistical analysis.

2.4 Whole blood total Hg concentrations ([THg])

Whole blood [THg] (reported as ppm, wet weight) were measured using a Milestone DMA-80 direct Hg analyzer (Milestone, Monroe, Connecticut, USA) in the Wildlife Toxicology Laboratory at the University of Alaska Fairbanks, Fairbanks, Alaska, USA, 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 $HgCl_2$), and 90.44 ± 0.11% and 96.77 ± 0.04% for certified reference materials (Seronorm and DORM-3 respectively).

2.5 Statistical Analyses

Statistical analyses were computed using the statistical program R version 3.1.2 (R Development Core Team, 2014). Mean, median, and standard deviation are reported for each hematology and immune protein measurement. 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, non-parametric statistics were employed. Within each rookery, if at least 3 individuals or more had concentrations greater than the minimum detection concentration limit (MDL; Table S1) for each cytokine and chemokine measured, those data were included for statistical analysis. Of those immune proteins included in statistical analysis, if the cytokines or chemokines were below the minimum detection concentration limit, half of the MDL value was used (Table S1) (Cohen and Ryan, 1989; Whitcomb and Schisterman, 2008; Zhang *et al.*, 2009).

177 Non-parametric Wilcoxon–Mann–Whitney tests were used to analyze differences in cytokine and
178 chemokine concentrations between factors with two levels (sex, year, and high *vs*. low [THg]), while
179 Kruskal-Wallis tests were used to identify differences in cytokine, chemokine, and [THg] among multiple
180 rookeries. To test for differences in cytokine and chemokine concentrations with high *vs*. low [THg], data
181 were binned into high (> 0.11 ppm) or low (≤ 0.11 ppm) Hg group based on a statistically derived Hg
182 concentration associated with changes in the innate immune protein, haptoglobin concentration, in SSLs
183 (Kennedy *et al.*, 2019). Statistical comparisons of median protein concentrations between high and low
184 [THg] groups were made using data from Agattu Island pups given they were the only rookery with pups
185 exceeding the ≥ 0.11 ppm threshold. When significant differences were detected among more than two
186 factors, a Dunn's Test of multiple comparisons was performed to identify which factors were associated
187 with the observed differences (adjusted *p* values using the Holm method are reported).

188 Associations of cytokines and chemokines, WBC counts and [THg], and serum aminotransferases
189 with [THg] were assessed using Pearson's correlations. For the subset of pups from Chiswell Island with
190 known ages (n = 19), associations of cytokines/chemokines and differential WBC counts with age (days)
191 were also examined using Pearson's correlation. Suspect outliers were statistically identified using the
192 "Horn" method (referenceIntervals R package) and removed from analysis if they were deemed
193 influential (Horn *et al.*, 2001). Differences were considered significant at an alpha value less than 0.05.

194

195 **3. Results**

196 *3.1 Regional differences in immune proteins*

197 All measurable cytokines and chemokines were detected in at least one or more SSL serum
198 samples. Quality control values were acceptable for all cytokines and chemokines except for IFN_{Υ} (Table
199 1), therefore, data for that measure were excluded from the dataset. No differences between sampling
200 years (2013 and 2015) for pups from Agattu Island ($p > 0.05$) or between sex ($p > 0.05$) for Agattu Island,

Chiswell Island, and Graves Rock were detected, therefore further analysis was performed on pooled data. Given the limited sample size of pups from Ulak Island, testing differences between sexes was not possible and data were pooled. Chiswell Island pups ranged in age from 6.20 to 33.40 days, and age was not significantly correlated with any of the cytokine or chemokine concentrations, total WBC counts, or differential WBC counts ($p > 0.05$).

Of the proteins measured, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, GM-CSF, and KC-like proteins were used for statistical analysis to investigate differences in protein concentrations among rookeries (Table 1). Unlike other proteins, KC-like protein had concentrations greater than MDL in all serum samples. Only 1-2 individuals from Chiswell Island and Graves Rock (yet no individuals from Agattu or Ulak Islands) had measurable concentrations of IL-2 (range = 1.75 – 1,285.94 pg/ml), MCP-1 (range = 10.50 – 457.00 pg/ml) and TNF-α (range = 3.05 – 249.00 pg/ml) therefore no statistical comparisons were made for these measures (Table 1). For SSL pups, significant differences in protein concentrations among rookeries were observed for IL-6 (range = 1.85 – 1,051.04 pg/ml, chi-squared = 9.72, df = 2, $p = 0.008$) , IL-7 (range = 3.75 – 623.15 pg/ml, chi-squared = 21.34, df = 2, $p < 0.001$), IL-15 (range = 4.50 – 1,757.54 pg/ml, chi-squared = 6.95, df = 2, $p = 0.031$), IL-18 (range = 2.90 – 1,063.37 pg/ml, chi-squared = 18.42, df = 2, $p < 0.001$), GM-CSF (range = 4.60 – 824.09 pg/ml, chi-squared = 16.02, df = 3, $p = 0.001$) and KC-like (range = 288.20 – 4,364.30 pg/ml, chi-squared = 14.16, df = 3, $p = 0.003$) proteins. The Dunn's multiple comparison's tests revealed that pups from Graves Rock had significantly greater concentrations of IL-6, IL-7, IL-15, IL-18, and GM-CSF than other rookeries (Figure 2). Median concentrations of IL-6 (and GM-CSF) were not significantly different between pups sampled on Agattu Island and Chiswell Island while also being significantly lower than the median concentrations observed in pups from Graves Rock ($p < 0.020$ and $p < 0.010$). Pups from Agattu Island had the lowest concentrations of IL-7 ($p < 0.001$), IL-15 ($p = 0.03$), and IL-18 ($p < 0.001$) and mean and median concentrations of these two proteins were greater the further east the pups were sampled (Figure 1, Figure 2). Even though the immunosuppressive cytokine IL-10 (range = 4.25 – 96.12 pg/ml) was greater on

226 average for Chiswell Island pups compared with other rookeries, the median concentrations of IL-10 (and
227 IL-8, range = 10.85 - 511.24 pg/ml) did not vary significantly ($p > 0.05$) among rookeries (Figure 2).

228 Of the possible associations among cytokines and chemokines, pups from Graves Rock had
229 positively correlated concentrations of IL-6, IL-7, IL-8, IL-15, IL-18, and GM-CSF and these associations
230 (n = 10) were significant (Table 2). Pups from Chiswell Island also had the same positively correlated (n
231 = 9) concentrations between proteins except IL-7 and IL-8 (Table 2). In comparison, only one significant
232 correlation was found between IL-6 and IL-15 in pups from Agattu Island. The sample size of Ulak Island
233 pups was insufficient for assessing protein associations.

234 *3.2 Regional associations of white blood cell counts with immune proteins*

235 The WBC counts for all individuals fell within reported reference ranges (90% confidence
236 interval) for SSL pups from other studies (Lander *et al.*, 2013). However, three individuals with the
237 greatest WBC counts (Agattu Island = 23.88 K/µl, Chiswell Island= 27.42 K/µl, and Graves Rock = 22.95
238 K/µl) were suspect outliers within our data set using a conservative statistical approach for outlier
239 detection (Horn *et al.*, 2001). Total WBC counts in Graves Rock pups had a significant positive
240 correlation with concentrations of IL-6 ($r = 0.77$, $p = 0.001$), IL-7 ($r = 0.55$, $p = 0.039$), IL-15 ($r = 0.61$, p
241 $= 0.020$), IL-18 ($r = 0.59$, $p = 0.025$) and GM-CSF ($r = 0.59$, $p = 0.027$) (Figure 3). Neutrophil counts
242 were the only differential WBC correlated with cytokines in Graves Rock pups. Neutrophils had
243 significant correlations with IL-6 ($r = 0.71$, $p = 0.004$), IL-15 ($r = 0.65$, $p = 0.011$), IL-18 ($r = 0.57$, $p =$
244 0.032), and GM-CSF ($r = 0.66$, $p = 0.011$) in Graves Rock pups. In comparison, the anti-inflammatory IL-
245 10 cytokine was significantly correlated with total WBC counts ($r = 0.59$, $p = 0.016$) in Chiswell Island
246 pups, but no other significant correlations were found for differential counts with proteins for this
247 rookery. Following the removal of suspect outliers, all significant associations between WBC counts and
248 differential cell counts with cytokines or chemokines for Graves Rock and Chiswell became insignificant.
249 For Agattu Island pups, neither total WBC counts nor differential counts were significantly correlated

with cytokine or chemokine concentrations ($p > 0.05$), and the removal of the outlier did not affect these results.

3.3 Whole blood total mercury concentration ([THg]) and regional associations with immune measures

Whole blood [THg] in pups differed significantly among rookeries (Table 3). Agattu Island pups had significantly greater [THg] (median = 0.10 ppm, range = 0.01- 0.51 ppm; chi-squared = 9.99, df = 3, $p = 0.019$) than other rookeries, followed by Ulak Island (median = 0.07 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) pups shared similar [THg] that were less than concentrations observed for Agattu Island and Ulak Island.

Of the tests performed to assess correlations of whole blood [THg] with total and differential WBC counts in pups within the different rookeries, two significant correlations were observed. To assess influence of data points from individuals with the greatest WBC counts on observed associations, analyses were performed with and without those individuals. Total WBC counts were positively correlated (df = 16, r = 0.46, $p = 0.053$; Figure 4A) with whole blood [THg] in Agattu Island pups and this association is likely driven by neutrophil counts (df = 16, r = 0.45, $p = 0.059$). The associations remained following the removal of one Agattu Island pup with the greatest WBC count (df = 15, r = 0.58 $p = 0.014$, and df = 15, r = 0.59, $p = 0.013$ respectively). Lymphocyte counts were negatively correlated (df = 12, r = -0.69, $p = 0.006$; Figure 4B) with whole blood [THg] in Graves Rock pups, and this association remained following the removal of the pup with the greatest WBC counts (df = 11, r = - 0.70, $p = 0.008$). All other comparisons made between blood cells counts and [THg] were not statistically significant (Table 3).

Only Agattu Island had pups above the 0.11 ppm (n = 9) cut-off for comparing variation of immune proteins between high and low [THg] groups relative to this threshold. No significant associations were found between cytokines or chemokines and [THg], and no significant differences were

detected for median cytokine and chemokine concentrations between the high and low [THg] groups of Agattu Island pups.

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 29.20 ± 9.97 U/L and 23.70 ± 7.55 U/L, respectively (Table 3). However, neither serum ALT nor AST measurements had a significant relationship with [THg] for Agattu Island pups.

4. Discussion

Blood cytokine and chemokine concentrations, measured in conjunction with traditional immunological (e.g. WBC counts) and physiological (*e.g.*, serum liver enzymes) biomarkers, are increasingly useful to describe immune profiles, infer general health status, and to determine if links between immune status and environmental toxicants exist (Nyland *et al.*, 2011; Buchmann, 2014; Zimmerman *et al.*, 2014; Hui *et al.*, 2016; Becker *et al.*, 2017; Ahn *et al.*, 2018). In this study, we identified associations among cytokine and chemokine concentrations across geographically distinct groups of SSL pups. A key finding was that the concentrations and associations of cytokines and chemokines varied among SSL pups from different rookeries, thereby indicating differences in their immune status. Cytokines and chemokines help drive a proper immune response to pathogens, or other insults, but may be impacted by environmental stressors, such as toxicants, including Hg. The associations found between [THg] and WBC counts provide some support for the possibility that transplacental Hg exposure may influence immune cells responsible for the production of and response to cytokines and chemokines. However, additional evidence for *in vivo* effects of Hg on cellular immune response is needed to support the hypothesis of direct adverse effects of Hg on immune status in SSL

296 pups considering no associations were found between circulating [THg] and cytokines/chemokines (or
297 serum liver enzymes).

298 Significantly lower concentrations of pro-inflammatory and hematopoietic cell stimulating
299 cytokines (*e.g.*, IL-6, GM-CSF) observed in SSL pups from regions of decline, Agattu and Ulak Islands,
300 raises new inquiries about the regulation of their cytokine and chemokine protein expression pathways
301 and potential effects on immune system development. Cytokines and chemokines are categorized based
302 on their functionality and are important for the development of the immune system for a context specific
303 immune response following antigenic stimuli. In addition, cytokines influence the growth and
304 development of blood cells, coordinate cell differentiation, and mediate cell-to-cell interactions and
305 proliferation during immune response whereas chemokines are responsible for chemotaxis of cells during
306 inflammation and angiogenesis. Specific cytokines involved with hematopoiesis, lymphocyte
307 differentiation and development, and acute phase response (GM-CSF, IL-6, IL-7, IL-15, Table S1) were
308 found to be significantly lower in rookeries that experienced significant population decline farther west
309 (Agattu Island, Ulak Island, and in some cases, Chiswell Island) compared with Graves Rock. Further, IL-
310 6 is involved with immune activation in neonatal immunity (Krow-Lucal *et al.*, 2014) and stimulates the
311 haptoglobin pathway (Alayash, 2011) and both functional networks may be impaired in pups from Agattu
312 and Ulak Islands (AI) given their lower IL-6 concentrations compared to Graves Rock pups within SEA.
313 The observation of lower IL-6 concentrations in western AI is also in agreement with the trend previously
314 observed for regional differences in haptoglobin concentrations (Kennedy *et al.*, 2019).

315 Associations among redundant or pleiotropic cytokines, chemokines, and leukocyte counts can
316 occur during mammalian development (Sarandakou *et al.*, 1998; Levy, 2007; Sood *et al.*, 2012; Zhang *et*
317 *al.*, 2017), however, fewer significant correlations between immune proteins were observed in Agattu
318 Island than Chiswell Island and Graves Rock. Many of the cytokines that were measured in SSL pups
319 instruct developing immune cells in tissue specific microenvironments to divide and differentiate (e.g.,
320 IL-6, IL-7, IL-15, IL-18). The significant associations between cytokines (and associations of cytokines

and chemokines with leukocyte counts) we observed could represent proliferation and differentiation of hematopoietic cells in Graves Rock and Chiswell Island pups, and the lack of positive associations in pups from Agattu Island may indicate differences in immune system development, or a difference in antigenic stimuli. It is possible that antigenic challenges that result in high WBC counts may alter cytokine concentrations in SSL pups considering positive associations of cytokine and leukocyte counts for Chiswell and Graves Rock pups were influenced by individuals with high WBC counts (and neutrophils). This notion is further supported by the finding that when individuals with the greatest WBC counts were removed, the relationships became insignificant.

Location-specific factors could explain the regional differences in the variation in cytokine and chemokine concentrations and the nature of the associations that were observed. A tolerogenic immune status is necessary for commensal microbiota establishment during early immune system development (Zhang *et al.*, 2017). Therefore, the variation in some cytokines and chemokines may indicate geographic differences in the timing of immune system development in SSL pups. More likely, the differences we observed may correspond to a targeted response to a location specific antigenic stimulus (Monastero and Pentyala, 2017). For example, location-specific pressures that alter immune status may include density dependent diseases like parasites (*i.e.*, *Uncinaria* sp.) and viruses (*i.e.*, Phocine distemper virus) in rookeries like Chiswell Island. Parasites, viruses, fungi, and bacterial burden can influence IL-10 expression and leucocyte counts (Sood *et al.*, 2012; Duignan *et al.*, 2014; Redpath *et al.*, 2014; Peñaloza *et al.*, 2016) and greater IL-10 also downregulates the Th1 response that is necessary to combat intracellular pathogens (Couper *et al.*, 2008). Parasites have been documented in densely populated SSL rookeries (Hughes *et al.*, 2004), and increased pathogen burden in Chiswell Island rookeries with increased pup production may explain the heightened IL-10 concentrations and notably greater eosinophil counts in pups from that location. This notion highlights the possibility for using cytokine and chemokine measurements to identify general trends of antigenic challenge by marine-derived pathogens that may not

345 be captured by traditional screening methodologies. However, the application of cytokine and chemokine
346 measurements for this purpose would need clinical validation.

347 Factors other than those described above may influence the regional variation in cytokines and
348 chemokines, leucocytes, and [THg] in SSL pups such as differences in population genetics (Hoffman *et*
349 *al.*, 2009; Jemison *et al.*, 2013; O'Corry-Crowe *et al.*, 2014) and/or immune gene expression (Bowen *et*
350 *al.*, 2006), congenital diseases that lead to an aberrant immune response (Zimmerman *et al.*, 2014), or
351 dietary preferences of the dam (Scherer *et al.*, 2015; Doll *et al.*, 2018). Regional differences in dietary
352 preferences of the dam could lead to the accumulation of other contaminants like organochlorines
353 (Beckmen *et al.*, 2016) and other trace elements (arsenic, aluminum, cadmium, and lead) (Holmes *et al.*,
354 2008), biotoxins, antigens, and/or antioxidants and immuno-protective nutrients (*e.g.*, Se) from forage
355 fish that may have interactive effects with Hg and immune system development during gestation (Neale *et*
356 *al.*, 2005; Correa *et al.*, 2014; Hosnedlova *et al.*, 2017; Lian *et al.*, 2020; Rea *et al.*, 2020). Further, fetal
357 blood [THg] can differ slightly from post birth [THg] in children and that may give rise to differences in
358 correlative associations with immune protein measures (Hui *et al.*, 2016). This may be the case for older
359 SSL pups, however, this phenomenon is unlikely for newborn pups considering circulating whole blood
360 [THg] and [THg] from their natal coat that was developed *in utero* are tightly correlated (Rea *et al.*,
361 2013). Therefore, whole blood [THg] is representative of *in utero* exposure concentrations of Hg in
362 dependent pups (Rea *et al.*, 2013; Peterson *et al.*, 2016).

363 Cytokine expression is sensitive to the immunotoxic effects of *in vitro* Hg exposure of SSL pup
364 lymphocytes (Levin *et al.*, 2020), but a clear relationship between [THg] and cytokine concentrations *in*
365 *vivo* (following natural, transplacental exposure) was not found in this study. Some of the pups in the
366 present study had [THg] greater than concentrations associated with immunotoxic effects *in vitro* (>0.1
367 ppm) (Levin *et al.*, 2020), decreased haptoglobin protein (>0.11 ppm) (Kennedy *et al.*, 2019), and the
368 lower limit (0.10 ppm) benchmark for increased risk of adverse effects in pinnipeds (O'Hara and Hart,
369 2018). Previous studies report associations of Hg and cytokine concentrations in humans (Gardner *et al.*,

2009; Nyland *et al.*, 2011; Motts *et al.*, 2014). In children, [THg] above EPA thresholds (29 nmol/L or 5.83 ppm) corresponded to differential cytokine patterns such as a negative association with IL-6 (the primary cytokine signal for acute phase protein production, including haptoglobin), and a positive association with an immunosuppressive cytokine, IL-10 (Hui *et al.*, 2016). It is possible effects of [THg] on cytokine production in SSL pups *in vivo* occur at concentrations greater than benchmarks (0.1 and 0.5 ppm) previously suggested for SSL pups (O'Hara and Hart, 2018; Levin *et al.*, 2020). Although Agattu Island pups had significantly lower mean/median concentrations of most cytokines and chemokines compared to the other regions, the overall mean [THg] (0.12 ppm) for Agattu pups was also near previously reported benchmarks. It is plausible that the lack of significant associations between cytokines, chemokines, and [THg] may be coincidentally due to the limitations of conducting a natural exposure study where the SSL pups with greater [THg] might be too few to statistically capture the true effects of greater [THg] with cytokines *in vivo*. Alternatively, Hg may indirectly suppress or interrupt the cell-signaling pathways necessary for normal growth and development of the neonatal immune system by a different mechanism than what is represented by the biomarkers measured herein. Although direct relationships between [THg] and cytokines measures were not observed, the consequences of *in utero* exposure to greater concentrations of Hg in SSL pups should not be dismissed, especially in pups from Agattu Island where *in utero* [THg] were greatest (Rea *et al.*, 2020).

The discrepancies in significant associations (positive vs. negative) of [THg] with different types of immune cells (lymphocyte *vs*. WBC counts) for Graves Rock and Agattu Island might be explained by the phenomenon that Hg can elicit a dose-dependent, biphasic cellular response (Unoki *et al.*, 2016). Graves Rock pups had the lowest [THg] on average, and a negative association of Hg with lymphocyte counts. This is further supported by an *in vitro* assessment of monomethylmercury effects on SSL pup lymphocytes that demonstrated decreased lymphocyte proliferation, and that these effects may be more pronounced at greater Hg concentrations (Levin *et al.*, 2020). In contrast with our findings from Graves Rock, Agattu Island pups in the current study had an average [THg] above the highest concentration for

immunotoxic effects *in vitro* (>0.1ppm) and showed an increase in total WBC counts (the majority were neutrophils) with [THg]. Similarly in other marine mammals, greater Hg was associated with an increase in neutrophils (Schaefer *et al.*, 2011). Bats with greater [THg] also had more neutrophils and fewer monocytes and reduced bacteria killing ability, and demonstrated lower innate functions (Becker *et al.*, 2017). Considering Hg can attenuate the inflammatory behavior of cells (Ahn *et al.*, 2018), it is intriguing to reason that *in utero* Hg exposure has immunotoxic effects on cellular activity of innate immune cell networks including cytokine production in SSL pups *in vivo* during early development. Effects of greater [THg] on cytokine and chemokine production in SSL pups cannot be ruled out since there is some evidence that Hg could lead to changes among populations of the source cells that manufacture immune proteins. This notion should be explored further. Regardless of the mechanism behind the associations of [THg] and immune cells, these findings highlight the concern for an imbalance of innate immune networks that may lead to impaired pathogen detection and clearance, and increased vulnerability of SSL pups in the western AI to diseases (Penta *et al.*, 2014).

Although [THg] in SSLs likely effects populations of immune cells (and indirectly, the production of immune cell signaling proteins), it did not appear that observed concentrations lead to detectable changes in serum liver enzymes. The effects of Hg on liver function in SSLs are unknown but given the ALT and AST of pups with the greatest concentrations of [THg] were within statistically defined reference thresholds of Lander *et al.* (2013), it is assumed no detectable hepatic damage occurred. In mice, Hg was shown to have negative effects on the production of IL-6 in mouse liver without causing hepatotoxicosis (Kim and Sharma, 2005), and this might be the case for SSL pups regarding the haptoglobin pathway in SSL pups (Kennedy *et al.*, 2019).

5. Conclusion

Immune status of SSL pups varied among rookeries, and pups from Agattu and Ulak Islands in the western AI had different immune cell-signaling protein concentrations compared with Chiswell Island

and Graves Rock pups. Although the cause(s) of the differences observed remain to be determined, our findings support the use of cytokines as an additional metric for studying the immune status in wild populations of SSLs. The difference in cell-signaling protein (cytokines and chemokines) concentrations among rookery pups is likely not impacted directly from *in utero* exposure to Hg concentrations for previously proposed benchmark concentrations. In this study, we provide evidence for regional differences in 1) cytokine and chemokine profiles (and their associations) and 2) the nature of the associations of immune cells (hematology) with [THg] in SSL rookery pups. Changes to cytokine and chemokine milieus during early development may give rise to an imbalance in downstream hematopoietic progenitor and somatic cell (i.e. stromal cells, epithelia, hepatocytes) differentiation that could impact cell-signaling pathways and immunological resiliency in pups. The mechanisms leading to the observed regional differences in the immune status of SSLs pups should be further investigated.

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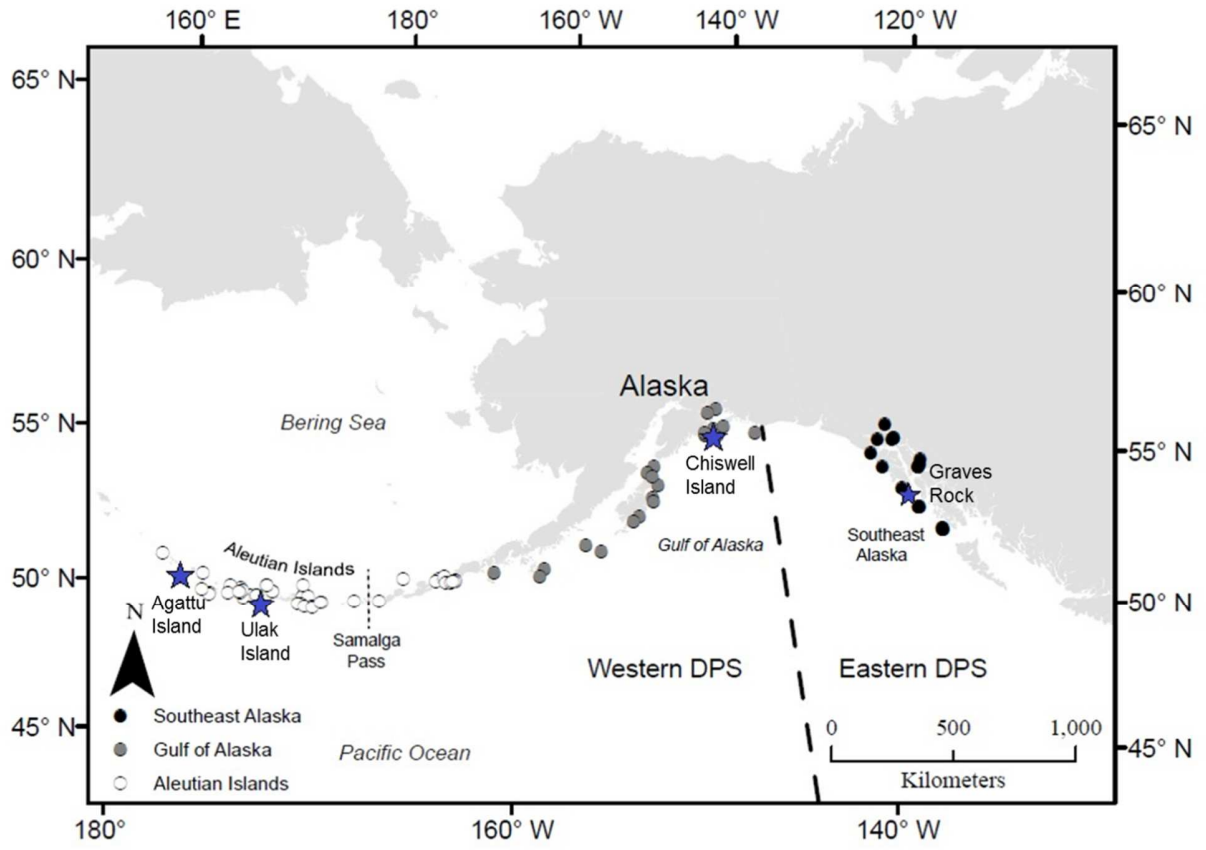
458 **Figures**



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460 **Figure 1** - Distribution of Steller sea lion (SSL) rookeries and haulouts (indicated by dots) along the coast
461 of Alaska showing the longitude delineation (144ºW) for the western and eastern distinct population
462 segments (DPS) and the regional classifications for Aleutian Islands (AI), Gulf of Alaska (GOA), and
463 Southeast Alaska (SEA). Samples were collected from SSL pups from rookeries (indicated by stars) on
464 Agattu Island and Ulak Island (western AI), Chiswell Island (eastern GOA), and Graves Rock (SEA).

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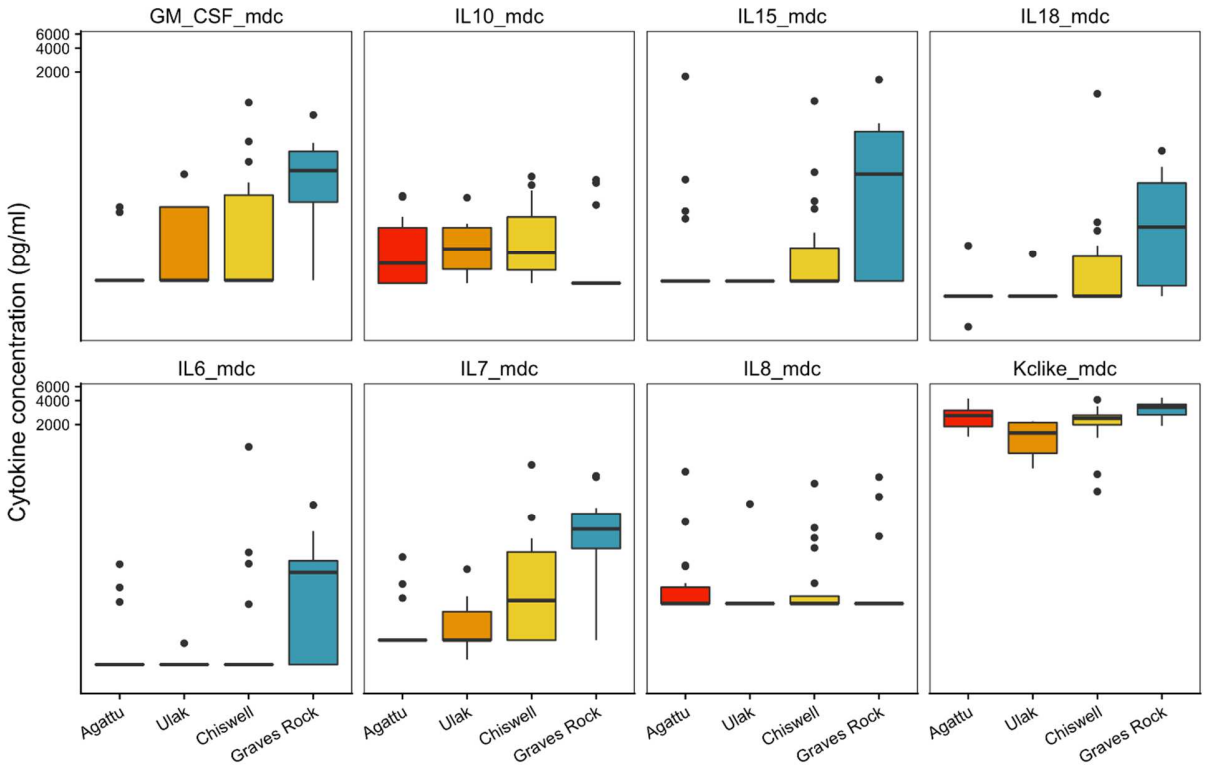
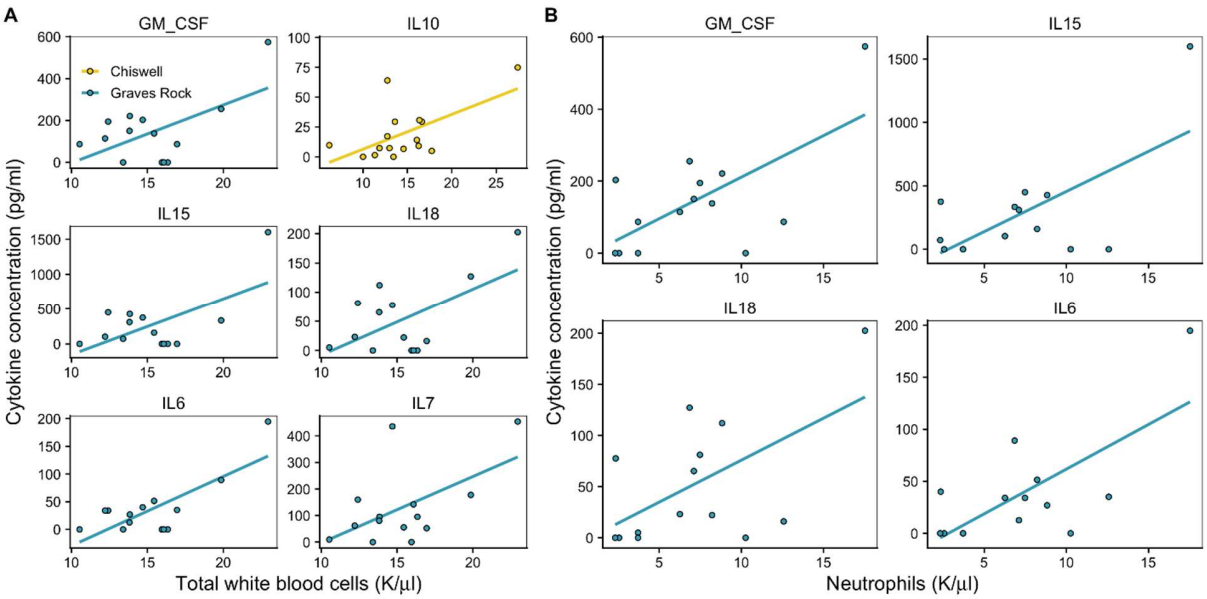


Figure 2 – Rookery comparisons of median cytokine and chemokine concentrations (pg/ml) in Steller sea lion pups from Agattu Island, Ulak Island, Chiswell Island, and Graves Rock. Median concentrations are indicated by the lines within the quartile boxes.



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484 **Figure 3** - Significant correlations between serum cytokines and chemokines (pg/ml) and A) total white
485 blood cell counts (K/µl) and B) neutrophil counts (K/µl) for pups sampled from Graves Rock (blue)
486 Chiswell Island (yellow) with outliers included.

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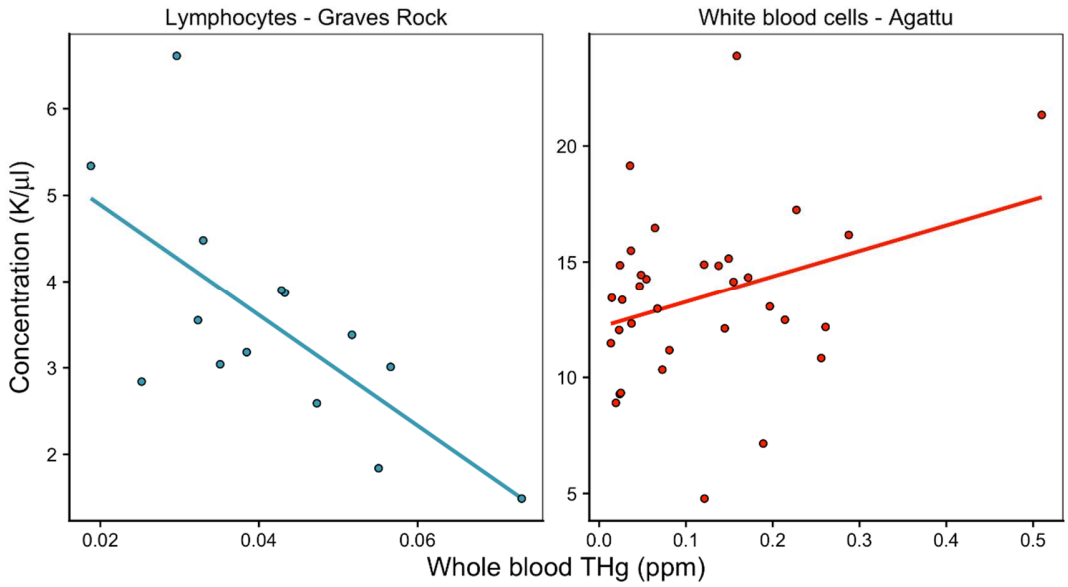

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502 **Figure 4** - Significant correlations between lymphocyte counts and whole blood total mercury (THg)
503 (ppm) in Steller sea lion pups from Graves Rock (blue) and total white blood cell counts (K/µl) and THg
504 in pups from Agattu Island (red).

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Table 1 - Sample size, mean, median, standard deviation (SD), and range for immune cell signaling proteins, cytokines and chemokines, for Steller sea lion pups sampled from rookeries on Agattu Island, Ulak Island, Chiswell Island, and Graves Rock, Alaska.

Location (Sample size)	Agattu Island, Western Aleutian Islands (n = 18)			Ulak Island, Central Aleutian Islands (n = 6)			Chiswell Island, Eastern Gulf of Alaska (n = 15)			Graves Rock, Southeast Alaska (n = 15)		
Conc. (pg/ml)	*a*	mean (SD)	median (range)	*a*	mean (SD)	median (range)	*a*	mean (SD)	median (range)	*a*	mean (SD)	median (range)
GM-CSF	3	9.81 (12.05)	4.60 (4.60-39.62)	3	32.62 (38.36)	22.11 (4.60-102.66)	8	79.60 (186.69)	4.60 (4.60-824.09)	11	142.89 (145.60)	114.38 (4.60-574.27)
IL-2	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)
IL-6	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)
IL-7	3	7.23 (9.58)	3.75 (3.75-41.95)	2	9.39 (10.65)	3.75 (2.13-29.46)	11	56.47 (137.50)	11.90 (3.75-623.15)	13	130.13 (138.54)	95.18 (3.75-453.48)
IL-8	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)
IL-10	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)	10.35 (4.25-96.12)	5	17.34 (28.62)	4.25 (4.25-87.47)
IL-15	4	109.50 (411.81)	4.50 (4.50-1757.54)	0	4.50[a]	4.50[a]	7	57.34 (190.63)	4.50 (4.50-860.23)	11	257.08 (408.79)	103.24 (4.50-1599.00)
IL-18	3	3.36 (2.40)	2.90 (2.90-12.82)	2	4.08 (2.89)	2.90 (2.90-9.99)	8	59.38 (236.40)	2.90 (2.90-1063.37)	12	50.79 (59.55)	22.12 (2.90-202.59)
IP-10	1	2.59 (4.20)	1.60 (1.60-19.41)	0	1.60[a]	1.60[a]	1	3.26 (7.42)	1.60 (1.60-34.80)	0	1.60[a]	1.60[a]
KC-like	18	2623.90 (837.44)	2596.47 (1409.00-4246.11)	6	1563.33 (715.60)	1819.52 (564.43-2201.78)	15	2286.67 (895.17)	2390.18 (288.20-4101.52)	13	3170.79 (764.77)	3297.82 (1925.66-4364.30)
MCP-1	0	10.50[a]	10.50[a]	1	10.50[a]	10.50[a]	1	32.83 (99.86)	10.50 (10.50-457.0)	3	23.12 (48.90)	10.50 (10.50-199.87)
TNF-α	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=the number of individuals above the minimum detection concentration

bold=met criteria for statistical analysis

509 **Table 2** - Statistically significant correlations between cytokine and chemokine concentrations in serum
510 collected from Steller sea lion pups from Agattu Island, Chiswell Island, and Graves Rock (note: Ulak
511 Island did not have a sufficient number of samples with measurements above the minimum detection limit
512 for analysis).

Location	n	*p*-value	Pearson's Correlation Coefficient
Agattu Island, Western Aleutian Islands			
IL-6 & IL-15	16	<0.001	0.87
Chiswell Island, Eastern Gulf of Alaska			
IL-6 & IL-7	18	<0.001	0.98
IL-6 & IL-15	18	<0.001	0.99
IL-6 & IL-18	18	<0.001	0.99
IL-6 & GM-CSF	18	<0.001	0.94
IL-7 & IL-18	18	<0.001	0.97
IL-7 & GM-CSF	18	<0.001	0.93
IL-15 & IL-18	18	<0.001	0.99
IL-15 & GM-CSF	18	<0.001	0.94
IL-18 & GM-CSF	18	<0.001	0.94
Graves Rock, Southeast Alaska			
IL-6 & IL-7	13	0.003	0.71
IL-6 & IL-15	13	<0.001	0.91
IL-6 & IL-18	13	<0.001	0.86
IL-6 & GM-CSF	13	<0.001	0.93
IL-7 & IL-15	13	0.001	0.76
IL-7 & IL-18	13	0.002	0.73
IL-7 & GM-CSF	13	0.001	0.76
IL-15 & IL-18	13	0.001	0.91
IL-15 & GM-CSF	13	<0.001	0.95
IL-18 & GM-CSF	13	<0.001	0.95

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516 **Table 3 -** Sample size, mean, median, standard deviation (SD) are given for total and complete white blood cell counts (WBCs), serum aspartate
517 (AST) and alanine aminotransferase (ALT) concentrations, and whole blood total mercury concentrations ([THg]) for Steller sea lion pups
518 sampled at rookeries on Agattu Island, Ulak Island, Chiswell Island, and Graves Rock.

	Vetscan					Vetscan					Procyte					Procyte				
	Agattu Island, Western Aleutian Islands					Ulak Island, Central Aleutian Islands					Chiswell Island, Eastern Gulf of Alaska					Graves Rock, Southeast Alaska				
	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	12.60	11.93	3.16	7.79-21.54	5	10.70	11.15	1.93	8.59-13.03	16	5.34	4.75	3.00	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	0.00	0.02	0.00-0.07	5	0.00	0.00	0.00	0.00	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	0.00	0.00	0.00	0.00	16	4.35	4.52	4.40	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	12.36	12.74	1.80	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	33.00	17.43	22.00-89.00	6	29.17	29.00	9.97	13.00-41.00	0	NA	NA		NA	0	NA	NA		NA
AST (U/L)	13	22.85	19.00	9.37	15.00-46.00	6	23.70	24.00	7.55	10.00-32.00	0	NA	NA		NA	0	NA	NA		NA
[THg] (ppm)	18	0.13	0.10	0.12	0.01- 0.51	6	0.06	0.08	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

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