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Assessing oxidative stress in Steller sea lions (*Eumetopias jubatus*): Associations with mercury and selenium concentrations

Marianne Lian[a,b,*], J. Margaret Castellini[a], Thomas Kuhn[b], Lorrie Rea[c], Louise Bishop[d], Mandy Keogh[e], Stephanie N. Kennedy[b,f], Brian Fadely[g], Edwin van Wijngaarden[h], John M. Maniscalco[i], Todd O'Hara[a,j]

[a] Department of Veterinary Medicine, University of Alaska Fairbanks, 2141 Koyokuk Dr, Fairbanks, AK 99775-7750, USA
[b] Department of Chemistry and Biochemistry, University of Alaska Fairbanks, 900 Yukon Dr Rm. 194, Fairbanks, AK 99775-6160, USA
[c] Water and Environmental Research Center, Institute of Northern Engineering, University of Alaska Fairbanks, 1764 Tanana Loop, Fairbanks, AK 99775, USA
[d] Department of Biology and Wildlife, University of Alaska Fairbanks, 2090 Koyokuk Dr, Fairbanks, AK 99775, USA
[e] Division of Wildlife Conservation, Alaska Department of Fish and Game, P.O. Box 110024, Douglas, AK 99811-0024, USA
[f] Harvard Medical School and Division of Gastroenterology, Boston Children's Hospital, 300 Longwood Ave. Enders 6th Floor, RM 650, Boston, MA 02115, USA
[g] Marine Mammal Laboratory, Alaska Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 7600 Sand Point Way NE, Seattle, WA 98115, USA
[h] University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, USA
[i] Alaska SeaLife Center, 301 Railway Avenue, P.O. Box 1329, Seward, AK 99664, USA
[j] Veterinary Integrative Biosciences, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA

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ABSTRACT

Selenium (Se) bioavailability is required for synthesis and function of essential Se-dependent antioxidants, including the enzyme glutathione peroxidase (GPx). Strong interactions between monomethyl mercury and Se impair the critical antioxidant role of Se. Approximately 20% of Steller sea lion (*Eumetopias jubatus*, SSL) pups sampled in the western Aleutian Islands, Alaska, had total Hg concentrations ([THg]) measured in hair and whole blood above thresholds of concern for adverse physiologic effects in pinnipeds. Importantly, low molar ratios of TSe:THg, in some cases < 1 in several tissues (hair, liver, pelt, muscle, spleen, intestine, heart, lungs, brain) were documented for one SSL pup with [THg] above threshold of concern, which may lead to antioxidant deficiency. Our aim with this study was to evaluate the relationship between circulating [THg], [$MeHg^+$], [TSe] and TSe:THg molar ratio status relative to oxidative stress and antioxidants measured during general anesthesia in free-ranging SSL. We captured, anesthetized and sampled newborn SSL pups at rookeries located in the Aleutian Islands or Gulf of Alaska. Biomarkers analyzed for oxidative stress included 4-hydroxynenonal and thiobarbituric acid reactive substances (4-HNE and TBARS, respectively, lipid peroxidation), protein carbonyl content (PCC, protein oxidation), and GPx activity as a key indicator for Se-dependent antioxidant defense levels. We found a negative association between TBARS and [TSe], and SSL with low [TSe] had higher concentrations of 4-HNE than those with intermediate [TSe]. These results suggest that SSL with lower [TSe] experience increased lipid peroxidation potentially associated with [THg] status.

1. Introduction

The Steller sea lion (*Eumetopias jubatus*, SSL) is a marine apex predator recognized as an important sentinel species for One Health in the North Pacific (Castellini et al., 2012). The One Health concept focuses on the intricate linkages between human, animal and environmental health. SSLs share coastal ecosystems with humans, and consume fish and seafood species commonly harvested for subsistence and commercial use (Cyr et al., 2019; Fritz et al., 2019). For SSL, two distinct population segments (DPS) are recognized within the US, divided into a

* Corresponding author at: Department of Veterinary Medicine, University of Alaska Fairbanks, 2141 Koyokuk Dr, Fairbanks, AK 99775-7750, USA.
E-mail addresses: mlian@alaska.edu (M. Lian), jcastellini@alaska.edu (J.M. Castellini), tbkuhn@alaska.edu (T. Kuhn), ldrea@alaska.edu (L. Rea), lrbishop@alaska.edu (L. Bishop), mandy.keogh@alaska.gov (M. Keogh), Stephanie.Kennedy@childrens.harvard.edu (S.N. Kennedy), brian.fadely@noaa.gov (B. Fadely), Edwin_van_Wijngaarden@urmc.rochester.edu (E. van Wijngaarden), johnm@alaskasealife.org (J.M. Maniscalco), tmohara@tamu.edu (T. O'Hara).

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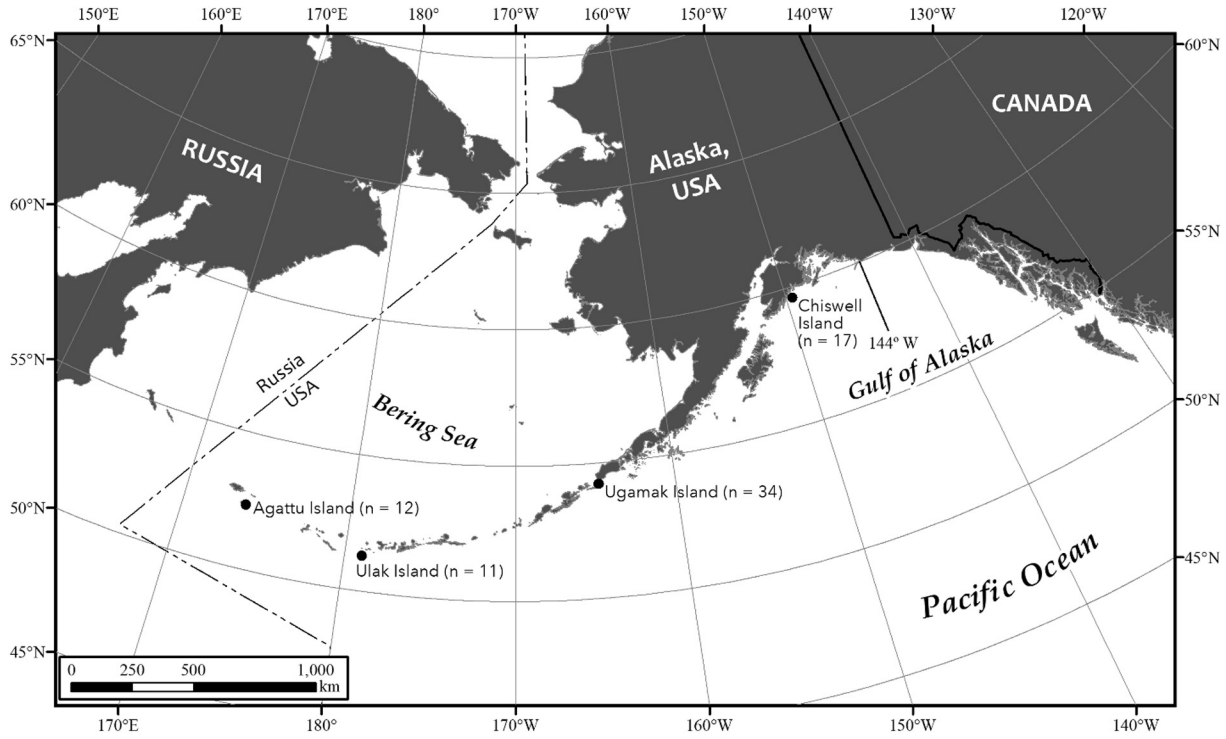

Fig. 1. Steller sea lion pups sampled on rookeries from four different regions; Western Aleutian Islands (Agattu Island), Central Aleutian Islands (Ulak Island), Eastern Aleutian Islands (Ugamak Island) and Gulf of Alaska (Chiswell Island), along coastal Alaska, USA.

western DPS and eastern DPS at 144°W latitude (Fig. 1). The National Marine Fisheries Service (NMFS) has listed the western DPS endangered under the US Endangered Species Act (ESA), whereas the eastern DPS was delisted in 2013 following population recovery (NMFS, 1997). The reasons for the population decline, and subsequent failure of some regions in the western DPS to recover are still uncertain (Atkinson et al., 2008; Altukhov et al., 2015; Fritz et al., 2019).

High total mercury concentrations ([THg]) found in animals in the western and central Aleutian Islands (Rea et al., 2013) have led to concern over potential health and reproduction impacts to these metapopulations. Approximately 20% of sampled pups in these regions (Rea et al., 2013) had [THg] above suggested lower toxicologic threshold (> 0.2 μg/g for whole blood and 20 μg/g for hair) of concern for adverse effects in pinnipeds (O'Hara and Hart, 2018; McHuron et al., 2019). Few studies have investigated if relatively high [THg] have adverse effects on SSLs, but Kennedy et al. (2019) found lower haptoglobin levels in SSLs with [THg] above thresholds of concerns. Levin et al. (2020) using SSL samples and in vitro approaches indicated potential adverse effects of $MeHg^+$ on the immune system. Another possible adverse effect from relatively high [THg] are relatively low molar ratios of total selenium (TSe) and THg (TSe:THg) in some SSL pups (Correa et al., 2014), potentially making them vulnerable to Hg toxicicosis and/or Se deficiency. Indeed, Correa et al. (2014) documented one SSL pup (with [THg] measured in hair to 59.17 μg/g) with TSe:THg molar ratios < 1.0 in several organs (liver, brain, heart, spleen and lungs). Se modulates Hg toxicity, and animals with low molar ratios may be functionally Se deficient (O'Hara and Hart, 2018).

Se is an essential element and a component of several critical biomolecules that function as antioxidants and immunomodulators including the antioxidant family of glutathione peroxidases (GPx) which comprises four distinct mammalian selenoproteins (Brigelius-Flohe, 1999; Farina et al., 2011). The interaction between Se and Hg partly involves strong affinity for binding with selenol (—SeHg) groups of endogenous molecules. In the case of selenol-containing proteins and enzymes, this formation of the Se—Hg bond can impair protein function (Farina et al., 2011). Monomethylmercury ($MeHg^+$)–selenocysteine interaction may impair Se bioavailability affecting synthesis and function of the Se-dependent antioxidant GPx (Ralston and Raymond, 2018). Due to strong binding affinity, Se is an important biochemical target for $MeHg^+$ toxicity. When Se is in relatively high excess of $MeHg^+$, $MeHg^+$ is biologically inactive (Berry and Ralston, 2008; Ralston and Raymond, 2018).

Pinnipeds are adapted to cope with oxidative stress, mainly due to the piscivorous diet containing high amounts of Se and other antioxidants (Zenteno-Savin et al., 2002). Breath holding during dives leads to hypercapnia and hypoxia in peripheral tissues and vital organs. The reperfusion of hypoxic tissues and organs with oxygenated blood post-dive leads to formation of reactive oxygen species, free radical formation and produces oxidative stress in several organ systems. The antioxidant effect of Se and selenol-containing proteins minimizes reperfusion damage (Vázquez-Medina et al., 2007) as part of a complicated antioxidant system. If antioxidant capacities are inadequate, the diving pinniped is vulnerable to oxidative stress derived from numerous interacting sources (Vázquez-Medina et al., 2012). The high [THg] in free-ranging SSLs in the Aleutian Islands are concerning in this context. While some adverse effects from Hg may be counteracted by Se, the relatively low molar ratios of TSe:THg found in some SSL pups with relatively high [THg] suggests these animals are more vulnerable to Hg toxicicosis and oxidative damage. A significant association between Hg and oxidative stress has been established in free-ranging blue shark (*Prionace glauca*) (Barrera-García et al., 2012) and in laboratory studies (Su et al., 2007; Farina et al., 2011). In this study, we investigate associations between biomarkers of oxidative and antioxidant status, and whole blood [THg] and [TSe] in free ranging SSL pups in areas of population decline and recovery.

Capture and anesthesia of SSL are necessary tools for conservation, research, and management of this species (Lian et al., 2017). Pinnipeds may experience similar physiological responses during capture, restraint, and anesthesia as occur during diving and other activities, including breath holding, bradycardia, shunting of blood from peripheral tissues and elevated CO_2 levels (Haulena, 2014; Lian et al., 2017). Specifically, this study evaluated the relationship between circulating

[THg], [$MeHg^+$], [TSe] and TSe:THg molar ratio status relative to oxidative stress and antioxidants measured during general anesthesia in free-ranging SSL. With a mutual link between oxidative stress and chronic inflammatory stress (Angelini et al., 2017), it is imperative to also assess inflammatory stress biomarkers such as haptoglobin (Kennedy et al., 2019), tumor necrosis factor-alpha (TNF-α, Sharick et al., 2015), and interleukin-6 (IL-6). Oxidative damage can be associated with a number of biomarkers already validated for pinnipeds, including protein carbonyl content (PCC, protein oxidation), 4-hydroxynonenal (4-HNE, lipid oxidation) and, thiobarbituric acid reactive substances (TBARS, lipid oxidation, Vázquez-Medina et al., 2006; Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011). The negative effects of oxidative stress may be prevented by GPx activity which is a Se-dependent antioxidant, also validated for pinnipeds (Vázquez-Medina et al., 2007).

2. Methods

2.1. Restraint and sampling

All SSL pups included in this study were anesthetized for general management and research needs, and blood samples were collected opportunistically for this study (IACUC 883669-1, ADF&G Permit # 18537-01, and ASLC Permit #18438-00). Young SSL pups were sampled at Chiswell Island in June 2016 (N = 17); and at Agattu Island (N = 12), Ulak Island (N = 11) and Ugamak Island (N = 34), during the last week of June/first week of July 2017 (Fig. 1).

Pups were physically restrained, weighed and anesthetized with isoflurane gas via mask induction for branding (Lian et al., 2017). The anesthesia lasted ≤10 min for each animal. Due to logistical constraints, the pups at Chiswell Island were sampled while anesthetized, whereas the pups in Aleutian Islands were sampled immediately following anesthesia, while the pup was recovering, still in a sedate state. Blood samples were collected from the caudal gluteal plexus using Vacutainer® assemblage with 20 gauge × 1½″ needle (Becton–Dickinson, Franklin Lakes, NJ, USA) and collected into three different evacuated blood collection tubes containing either sodium heparin (trace element free tubes), ethylenediaminetetraacetic (EDTA) anticoagulants or into serum tubes with no additive. Within 20 min of sampling, EDTA tubes were centrifuged (battery operated clinical centrifuge) to separate plasma from the packed cells. EDTA plasma samples were immediately aliquoted into 0.5 mL cryovials and frozen on dry ice on the rookery and subsequently stored in nitrogen gas. Remaining packed cells in EDTA tubes, whole blood samples in sodium heparin tubes, and serum separator tubes (SST) were all stored chilled in a cooler for 3–10 h until processing.

2.2. Sample processing

Within 12 h of sampling at the rookery, the chilled packed cells were washed 3× with cold phosphate buffered saline (PBS) (1:2, vol:vol) by centrifugation (10,000 ×*g*, 10 min) and supernatants (PBS with remnant white blood cells) discarded. Erythrocytes (1 mL) were transferred to 1 mL cold distilled water (10 mL glass tube), resulting cell lysates centrifuged (10,000 ×*g*, 10 min), and supernatants (intracellular content) aliquoted into cryovials (0.5 mL). Whole blood samples collected in trace element free tubes were aliquoted into 2 mL cryovials, whereas SSTs were centrifuged, and serum samples pipetted into 2 mL cryovials. All aliquots were stored initially in nitrogen gas (−78.5 °C) and transferred to a − 80 °C freezer until analysis.

2.3. Chemical and biomarker analyses

We analyzed oxidative stress biomarkers from EDTA plasma (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011) at the Wildlife Toxicology Laboratory (WTL), University of Alaska Fairbanks (UAF), using commercially available assay kits. These included OxiSelect™ HNE Adduct Competitive ELISA kit for 4-hydroxynonenal (4-HNE, Cell Biolabs, Inc., Vázquez-Medina et al., 2011), thiobarbituric acid reactive substances (TBARS) measured with Cayman Chemicals' TBARS (TCA Method) Assay Kit (Vázquez-Medina et al., 2010), and OxiSelect™ protein carbonyl content ELISA kit (PCC, Cell Biolabs, Inc., Vázquez-Medina et al., 2011) using spectrophotometry (Spectramax, 340PC, Molecular Devices, San Jose, California, USA). Total protein was determined in EDTA plasma samples analyzed for PCC (Quick Start™ Bradford Protein Assay, Bio Rad) to normalize PCC values per mg total protein (Vázquez-Medina et al., 2007). Erythrocyte lysates were analyzed for GPx activity using a commercially available colorimetric assay (GPx, Analytical Sciences Laboratory University of Idaho). Briefly, the catalysis of peroxide to water by GPx in the presence by glutathione (GSH) was quantified at 340 nm via coupled NADPH oxidation by the enzyme glutathione reductase (GSH-RED). The Biomate™ 3 spectrophotometer detected the absorbance change per 15 s and the average absorbance change per minute was calculated. Catalytic activity of GPx was normalized per gram of hemoglobin. Hemoglobin (Hb) was determined by recording the absorbance at 540 nm as follows:

$$Hb = \frac{\text{Abs450} \times \text{MWHb} \times \text{DF}}{\in \times \text{l} \times \text{conversion factor}}$$

$$Y\ mU\ GPX = 273.3 \times \frac{\Delta Sample\ abs}{min} - \frac{\Delta Blank\ abs}{min}$$

$$\text{Specific activity} = Y\ mU\ GSH - \frac{Y\ mU\ GPX}{mg\ [Hb]}$$

Whole blood [THg] was measured in duplicate using a Direct Mercury Analyzer (DMA80; Milestone Inc., 25 Controls Drive, Shelton, CT 06484, USA) following Kennedy et al. (2019), including quality control (QC) standards and certified reference materials in each run. Percent recoveries for quality control samples were 94.1 ± 0.1% for liquid standard (0.1 μg/g $HgCl_2$), 101.2 ± 0.5% for seronorm L-3 (Accurate Chemical and Scientific Corp.) and 103.1 ± 0.5% for DORM4 (National Research Council, Canada). For [$MeHg^+$] analysis whole blood aliquots (200 μl) were digested in 10 mL 25% KOH in methanol for 24–36 h, followed by addition of 20 mL methanol and analysis by cold vapor atomic fluorescence spectroscopy (CVAFS) using a Brooks Rand MERX-M automated system, according to modified EPA method 1630 (Woshner et al., 2008). Percent recoveries for QC samples were 97.7 ± 8.9% for blank spike (1 μg/g MeHgOH), 108.1 ± 7.0 for Toxic Elements in Caprine Blood L-3 (NIST 955c), 94.7 ± 9.8% for DORM4, 91.1 ± 16.2% for sample spikes. Relative standard deviation (%RSD) for duplicate digests was 5.8 ± 2.7%.

For [TSe] analysis whole blood aliquots (400–500 μl) were digested in 8 mL nitric acid and 2 mL of 30% hydrogen peroxide using a Milestone ETHOS UP high-performance microwave system at WTL (UAF). Samples were digested at 170 °C for 15 min and diluted with ultrapure water to a final volume of 25 mL. For samples from 2016 (Chiswell) aliquots of the first digest were reduced (Se^{6+} to Se^{4+}) in the presence of excess HCl at 95 °C for 60 min and analyzed using mercury/hydride flow injection atomic spectroscopy (MHS-FIAS, Perkin Elmer Analyst 800, Knott et al., 2011, Correa et al., 2013). For samples from 2017 (Aleutian Islands) aliquots (5 mL) of the first digest were shipped with ice packs to the Washington Animal Disease Diagnostic Laboratory (WADDL) and analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Quality control included analysis of standard reference material seronorm L-3, DORM4, blank spikes (1 μg/g), sample spikes, and duplicate digests for each digestion batch.

Percent recovery of the sample spike and certified reference materials (CRM) were high for samples run by ICP-MS relative to those run by MHS-FIAS, particularly for Seronorm L-3, which is the matrix-matched CRM (Table 1). For each digestion batch we calculated a correction factor for ICP-MS samples based on the average % recovery for the blank spike, Seronorm L-3 and DORM4 where Correction

Table 1
Percent recovery of quality control samples using mercury/hydride flow injection atomic spectroscopy (MHS-FIAS), inductively couple plasma mass spectrometry (ICP-MS) and ICP-MS with a correction factor applied (ICP-MS_{corr}) for each digestion batch. Correction factor = 100 / Average % Recovery for 3 quality control sample types (blank spike, seronorm, DORM4) within each digestion batch. Values represent the mean ± SD for all digestion batches. Combined recovery represents the mean ± SD % recovery for the combined control sample types. Statistically significant differences between analytical methods were determined by one-way analysis of variance followed by Holm-Sidak pairwise comparison ($p < 0.05$) and are indicated by different letters (A, B) for each QC sample type.

% recovery Mean ± SD	MHS-FIAS	ICP-MS	ICP-MS_{corr}
Blank spike (1 μg/g)	107.0 ± 9.6 B	112.7 ± 3.3 B	92.2 ± 3.3 A
Seronorm L-3	94.4 ± 2.7 A	128.9 ± 4.5 C	105.1 ± 2.9 B
DORM4	108.7 ± 7.9 AB	116.4 ± 6.2 B	94.9 ± 2.9 A
Sample spike	90.7 ± 11.0 A	118.5 ± 2.3 B	97.2 ± 5.1 A
Combined recovery Blank spike, seronorm, DORM4	**103.4 ± 6.7 A**	**122.7 ± 4.5 B**	**97.4 ± 6.8 A**

Factor = 100/Average %Recovery for the 3 QC samples. The correction factor (0.796–0.881) was applied to each sample within that digestion batch. Percent recoveries for FIAS, ICP-MS and ICP-MS_{corr} are reported in Table 1.

Immune biomarkers analyzed included EDTA plasma samples for haptoglobin (University of Miami Miller School of Medicine, Department of Pathology) and serum for interleukin-6 and tumor necrosis factor-a (IL-6 and TNFα) were measured using the commercially available Millipore Canine Cytokine/Chemokine Magnetic Bead Panel and the Bio-Plex® 100/200™ System (University of Connecticut) previously validated to measure cytokines in several pinniped species (Levin et al., 2014).

2.4. Statistical analyses

All statistical analyses were computed with the statistical program R version 3.5.2 (R Development Core Team, 2014). A p value < 0.05 was considered significant for all analyses. To test the hypothesis that [$MeHg^+$], [THg], [TSe] or TSe:THg molar ratio are associated with markers of oxidative stress under simulated oxidative stress conditions of anesthesia, we used multiple linear regression with a normal (Gaussian) linear model. The response variables tested (4-HNE, TBARS, PCC and GPx) and the predictor variables ([$MeHg^+$], [THg], [TSe] and TSe:THg molar ratio) were analyzed for normality with histogram and theoretical quantiles (Normal Q-Q plot). Assumptions for normality were fulfilled with well-fitted and normally distributed residuals for the model with homoscedasticity and linearity. A correlation matrix showed an expected correlation between [THg] and [$MeHg^+$] ($r^2 = 1.0$), indicating collinearity, and [THg] was removed from the model. In addition, the response variables of interest (4-HNE, TBARS, PCC and GPx) were tested with all variables measured. To test these covariates we used backwards selection, ultimately providing a final model of significant variables. The variables tested included: sex, rookery, [THg], [$MeHg^+$], [TSe], TSe:THg molar ratio, IL-6, TNFα, GPx, and haptoglobin. A correlation matrix showed that in addition to [THg] and [$MeHg^+$] being correlated, IL-6 and TNFα were correlated ($r^2 = 0.83$). TNFα was only measurable in 6/74 animals, all which were also elevated in IL-6, explaining this correlation. Hence, [THg] and TNFα were removed from the full model.

The oxidative stress variables were additionally compared between different concentration categories of [$MeHg^+$], [TSe] and TSe:THg molar ratio. Whole blood [$MeHg^+$] was defined as low (< 0.055 μg/g), intermediate (IM, 0.055–0.099 μg/g) and high (> 0.099 μg/g), and these assessments were based on thresholds of concern for whole blood [THg] (AMAP, 2018; O'Hara and Hart, 2018; McHuron et al., 2019). Similar information is not available for [TSe], and the categories were based on a box plot and quartiles of whole blood [TSe] for all animals in this study, where high concentrations were the top 25%, intermediate concentrations were the middle 50%, and low concentrations were the bottom 25% of the animals. Accordingly, [TSe] was defined as low (< 0.30 μg/g), intermediate (0.30–0.41 μg/g), and high (> 0.41 μg/g). The categories for TSe:THg molar ratios were defined in a similar manner to [TSe] from a box plot of TSe:THg for all animals in the study and TSe:THg molar ratio was defined as low (< 18), intermediate (18–41) and high (> 41).

Differences in oxidative stress and antioxidants, [THg], [$MeHg^+$], [TSe] and TSe:THg molar ratio among the different rookeries were assessed. These comparisons, in addition to the different categories of [$MeHg^+$], [TSe] and TSe:THg molar ratio, were conducted with an ANOVA or a Kruskal Wallis test. For these analyses, the dataset was tested for normality with a Shapiro Wilks test. If a variable did not meet the requirements for normality, it was log-transformed, and the test for normality repeated. Variables that did not comply with normal distribution were analyzed using non-parametric tests. A post hoc test was conducted on all significant tests with either a Tukey Honest Significant Difference test or a Kruskal Multiple Comparison test. Statistically significant differences between analytical methods were determined by one-way analysis of variance followed by Holm-Sidak pairwise comparison (Table 1).

3. Results

Our study included 74 samples from 35 male and 39 female SSL pups. Of these, all were analyzed for oxidative stress biomarkers, 61 SSL pups were analyzed for [THg] and [$MeHg^+$], and 53 for [TSe]. Of the 61 pups analyzed for [THg], six categorized as having high (> 0.099) [THg] and [$MeHg^+$], and out of the 53 pups analyzed for [TSe], 11 categorized as having low [TSe]. None of the pups studied were categorized as both high [THg] and low [TSe]. We noted significant differences in [THg] (chi-squared = 17.171, df = 3, $p < 0.001$), among rookeries, with higher [THg] in Agattu, Ulak, and Chiswell, than Ugamak. A similar observation was made for [$MeHg^+$] (chi-squared = 21.88, df = 3, $p < 0.001$), where Agattu and Chiswell had higher concentrations than Ugamak (Table 2). Rea et al. (2013) also showed the highest [THg] at Agattu in the western Aleutian Islands. There was no significant difference (chi-squared = 4.0758, df = 3, $p = 0.25$) in [TSe] among rookeries, but there was a significant difference ($F_{3,49} = 6.4$, $p < 0.001$) in TSe:THg molar ratio. The lowest TSe:THg molar ratios were at Agattu, with higher molar ratios at Ugamak and Chiswell. All biomarkers summarized in Table 2.

3.1. Correlates of oxidative stress and glutathione peroxidase

TBARS was significantly negatively correlated with [TSe] (slope = −11.59, $R^2 = 0.15$, $F_{1,50} = 6.09$, $p = 0.017$, plotted with quantile regression in Fig. 2), TSe:THg molar ratio (slope = 0.08, $R^2 = 0.15$, $F_{1,50} = 5.51$, $p = 0.023$), and GPx (slope = 0.17, $R^2 = 0.11$, $F_{1,66} = 7.76$, $p = 0.007$). No significant model for 4-HNE or PCC was identified while testing predictor variables related to the hypothesis. The model including 4-HNE and all predictor variables showed a significant association between 4-HNE and rookery ($R^2 = 0.25$, $F_{3,68} = 7.65$, $p < 0.001$), and between PCC and rookery ($R^2 = 0.34$, $F_{3,69} = 12.08$, $p < 0.001$). The full suite of statistical results for hypothesis testing is summarized in Table 3.

When all predictor variables were included in the model, there was a significant association between GPx, rookery ($R^2 = 0.34$, $F_{3,66} = 9.32$, $p < 0.001$), and sex, with GPx activity higher in females

Table 2
Biomarkers for lipid peroxidation (4-hydroxynonenal (4-HNE), and thiobarbituric acid reactive substances (TBARS)), protein oxidation (protein carbonyl content (PCC)), antioxidant (glutathione peroxidase (GPx)), elements ([THg], [MeHg+], [TSe] and TSe:THg molar ratio), and immune system (interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), and haptoglobin), measured in Steller sea lion pups sampled in Aleutian Islands at Agattu, Ulak and Ugamak, and in the Gulf of Alaska at the Chiswell Islands.

Biomarker	Unit	Rookery							
		N	Agattu	N	Ulak	N	Ugamak	N	Chiswell
Oxidative stress									
4-HNE	μg/mL	12	8.9 ± 3.3 (3.1–14.6)	11	8.7 ± 4.1 (4.0–17.3)	34	21.0 ± 9.2 (3.1–32.5)[¤]	17	3.9 ± 33.4 (0.9–134.1)[¤]
TBARS	μM/mL	12	16.9 ± 1.5 (14.8–19.5)	11	16.1 ± 3.6 (12.1–23.4)	33	19.5 ± 5.4 (9.85–32.5)[¤]	16	14.2 ± 3.2 (8.9–20.9)[¤]
PCC	nmol/mg	12	1.6 ± 0.7 (0.6–2.8)*	10	2.3 ± 1.3 (0.7–4.5)[#]	34	1.4 ± 1.1 (0.5–5.3)[¤]	17	0.3 ± 0.2 (0–0.8)*,#,¤
Antioxidant									
GPx	mUnits/mg Hgb	10	70.9 ± 7.7 (56–82)*	10	63.4 ± 3.2 (58–67)	33	68 ± 7.7 (49–85)[¤]	17	59 ± 6.7 (51–76)*,¤
Metals and selenium									
[THg]	μg/g	12	0.12 ± 0.09 (0.014–0.28)*	11	0.04 ± 0.02 (0.01–0.07)[#]	23	0.02 ± 0.01 (0.01–0.06)*,#,¤	17	0.04 ± 0.01 (0.03–0.06)[¤]
[MeHg+]	μg/g	12	0.11 ± 0.08 (0.014–0.26)*	11	0.04 ± 0.01 (0.02–0.06)	21	0.03 ± 0.01 (0.01–0.04)*,¤	17	0.04 ± 0.01 (0.03–0.07)[¤]
[TSe]	μg/g	12	0.40 ± 0.12 (0.30–0.67)	10	0.38 ± 0.07 (0.28–0.58)	18	0.30 ± 0.15 (0.24–0.89)	15	0.36 ± 0.22 (0.22–1.07)
TSe:THg molar ratio		12	20.3 ± 18.9 (3.9–60.4)*,¤	10	26.9 ± 13.6 (12.7–65.0)	18	36.1 ± 19.5 (18.9–90.9)*	15	25.7 ± 22.6 (14.6–99.5)[¤]
Inflammation									
IL-6	pg/mL	12	19.5 ± 20.6 (0–68.6)	11	55.1 ± 110.5 (0–401.6)[#]	34	9.5 ± 50.0 (0–256.9)[¤]	17	0 ± 246.5 (0–1051.0)#,¤
TNF-α	pg/mL	12	3.7 ± 12.3 (0–44.4)	11	21.7 ± 48.6 (0–155.5)	34	0 ± 18.9 (0–112.0)	17	0 ± 58.6 (0–249.1)
Haptoglobin	mg/mL	12	2.7 ± 1.2 (1.3–4.8)	11	2.7 ± 1.0 (1.7–4.3)	33	1.9 ± 1.0 (0.9–4.4)	17	2.4 ± 2.2 (0.9–9.1)

*,#,¤Significant ($p < 0.05$) difference between rookeries.

than males. ($R^2 = 0.34$, $F_{1,69} = 6.71$, $p = 0.012$).

3.2. Categories of [MeHg$^+$], [TSe] and TSe:THg molar ratio

There was a significant difference in 4-HNE between the [TSe] categories (chi-squared = 6.75, df = 2, $p = 0.034$, Fig. 3). Animals with low [TSe] had higher 4-HNE concentrations than pups with intermediate [TSe]. The categories of [MeHg$^+$] and TSe:THg molar ratio had no significant differences in oxidative stress biomarkers.

3.3. Differences among rookeries

All biomarkers of oxidative stress differed among rookeries. Ugamak had higher lipid oxidation biomarkers than Chiswell; both 4-HNE (chi-squared = 18.39, df = 3, $p < 0.001$), and TBARS ($F_{3,67} = 3.74$, $p = 0.01$). All rookeries in the Aleutian Islands also had higher PCC than found at Chiswell Island (chi-squared = 38.34, df = 3, $p < 0.001$). GPx varied significantly ($F_{3,66} = 8.09$, $p < 0.001$) among rookeries, with Agattu and Ugamak being higher than Chiswell. The immune biomarker IL-6 was also higher on Ulak and Ugamak Islands than on Chiswell Island (chi-squared = 13.89, df = 3, $p = 0.003$).

4. Discussion

Our study revealed that animals with relatively low [TSe] exhibited increased concentrations of lipid peroxidation biomarkers (4-HNE and TBARS), possibly due to decreased protection against oxidative stress. The importance of Se-metabolism is evident through the functions of selenoproteins, including GPx enzymes and thioredoxin reductases (Lu and Holmgren, 2014; Bjørklund et al., 2017). For example: GPx intercept and detoxify hydroxyl radicals, while thioredoxins restore vital cellular redox molecules that have become oxidized back into their

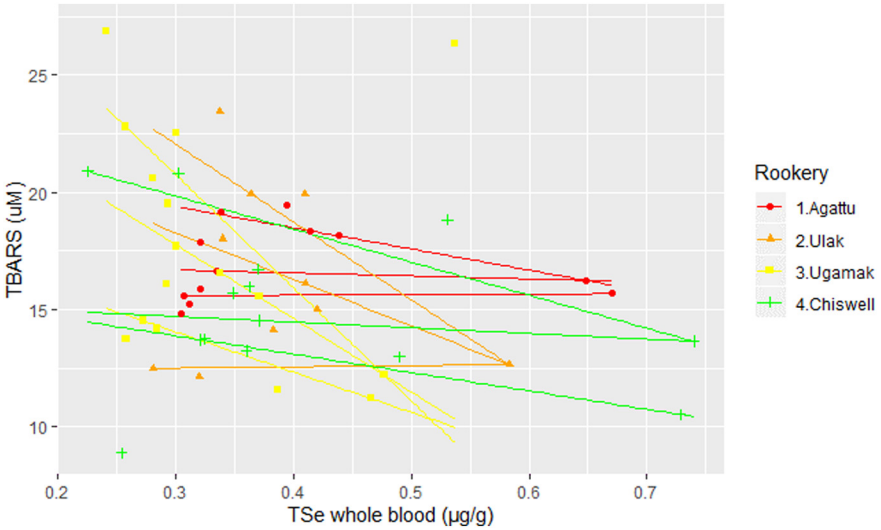

Fig. 2. A significant negative association (slope = −11.59, $R^2 = 0.15$, $F_{1,50} = 6.09$, $p = 0.017$) between [TSe] in whole blood and TBARS μM measured in plasma collected from Steller sea lion pups at three different rookeries in the Aleutian Islands (Agattu, Ulak and Ugamak Islands) and in the Gulf of Alaska (Chiswell Island).

Table 3
For 4-hydroxynonenal (4-HNE), thiobarbituric acid reactive substances (TBARS), protein carbonyl content (PCC), and glutathione peroxidase (GPx), hypotheses testing was done with multiple linear regression, where each of these variables were tested with the predictor variables monomethyl mercury ($MeHg^+$), total selenium (TSe) and the molar ratio between TSe and total Hg (TSe:THg molar ratio). For each model slope, R^2, F, degrees of freedom and *p* is reported.

Statistical variables	Slope	R^2	F	Degrees of freedom	*p*
4-HNE					
MeHg	−23.08	0.038	0.48	1, 49	0.491
TSe	0.13	0.038	< 0.01	1, 49	0.991
TSe:THg molar ratio	0.04	0.038	0.17	1, 49	0.68
TBARS					
MeHg	23.50	0.149	3.24	1, 50	0.078
TSe*	−11.59	0.149	6.09	1, 50	**0.017**
TSe:THg molar ratio*	0.08	0.149	5.51	1, 50	**0.023**
PCC					
MeHg	0.76	0.033	0.02	1, 50	0.864
TSe	−1.41	0.033	0.77	1, 50	0.382
TSe:THg molar ratio	0.01	0.033	0.78	1, 50	0.379
GPx					
MeHg	47.08	0.053	2.42	1, 48	0.126
TSe	−9.23	0.053	0.72	1, 48	0.399
TSe:THg molar ratio	0.11	0.053	1.75	1, 48	0.192

* Significant ($p < 0.05$) predictor variable in bold font.

functional forms for the prevention of oxidative damage in the cell (Branco et al., 2012; Branco et al., 2014). The Hg-dependent sequestration of Se, and irreversible inhibition of selenoenzymes required to prevent and reverse oxidative damage, seems to be primarily responsible for the characteristic effects of Hg toxicity (Ralston and Raymond, 2018). This may describe the observed association with increased lipid peroxidation in animals with lower [TSe] concentrations in this study.

Pinnipeds may have a higher tolerance for $MeHg^+$ exposure than non-piscivorous terrestrial mammals. This is possibly linked to evolution of biochemical mechanisms involving Se and other antioxidant systems needed to handle everyday oxidative stress (e.g. diving, fasting) (O'Hara and Hart, 2018). Se sequesters $MeHg^+$ making it biologically inactive, and thereby protect against Hg toxicity in pinnipeds (Ralston and Raymond, 2018). The high capacity for antioxidant defenses in pinnipeds (Vázquez-Medina et al., 2012), along with the protective role of Se (Berry and Ralston, 2008), probably ameliorated some of the oxidative stress expected in animals with low TSe:THg molar ratios. This is supported by the significant association between GPx and TBARS found in this study, suggesting increased GPx activity with increased lipid peroxidation. Our study did not find a direct relationship between [THg] concentration and oxidative stress. While 6 of 74 animals had high [THg] (> 0.099 μg/g), none of these animals had low [TSe] (< 0.30 μg/g), supporting the supposition that [TSe] or [TSe]:[THg] are key to oxidative stress responses.

Lipid peroxidation results from many external and internal stressors (Halliwell and Gutteridge, 2015). The susceptibility of polyunsaturated fatty acids (PUFA) to peroxidation corresponds to the number of double bounds in the lipid molecules. Some of the primary substrates for peroxidation in the lipid membrane are also mainly responsible for maintenance of physiologically crucial properties including permeability and fluidity (Zoidis et al., 2018). The newborn pups are suckling lipid-rich milk, interrupted with bouts of fasting while the dam is foraging (Pitcher et al., 2001), and the lipid peroxidation detected in these pups may be related to fasting status (Sharick et al., 2015) and/or plasma lipid content (Zoidis et al., 2018). The levels of lipid peroxidation measured in this study by 4-HNE analysis are comparable with levels found in breeding northern elephant seals (*Mirounga angustirostris*, NES, Sharick et al., 2015), but higher than found in resting and voluntarily submerged NES (Vázquez-Medina et al., 2011). The TBARS levels found in this study are comparable with levels found in other otariids (Righetti et al., 2014). Pinnipeds are repeatedly exposed to apnea-induced protein and lipid peroxidation, but are evolutionarily adapted to avoid oxidative damage via several antioxidant mechanisms (Zenteno-Savin et al., 2002; Vázquez-Medina et al., 2011). The protein oxidation measured in this study averaged between 1.39 and 2.25 nmol/mg among pups at the rookeries in the Aleutians Islands, all significantly higher than pups at Chiswell Island. Other studies have found protein oxidation in resting and voluntarily submerged NES (Vázquez-Medina et al., 2011) and ringed seal (*Pusa hispida*) (Vázquez-Medina et al., 2007) tissues to be comparable with the animals at Chiswell Island, and ten times lower than the values presented from the Aleutians Islands SSL pups in this study. Although our study noted relatively high levels of lipid and protein oxidation, a possibly apnea-induced link between oxidative stress associated with capture and anesthesia physiologic (and oxidative) stress, was not evident.

There was a significant difference in all oxidative stress biomarkers among the different rookeries with the highest lipid peroxidation at Ugamak (Eastern Aleutian Islands), and higher protein peroxidation at all rookeries in the Aleutian Islands, compared with Chiswell Island in the Gulf of Alaska. A similar difference was noted for GPx, with higher

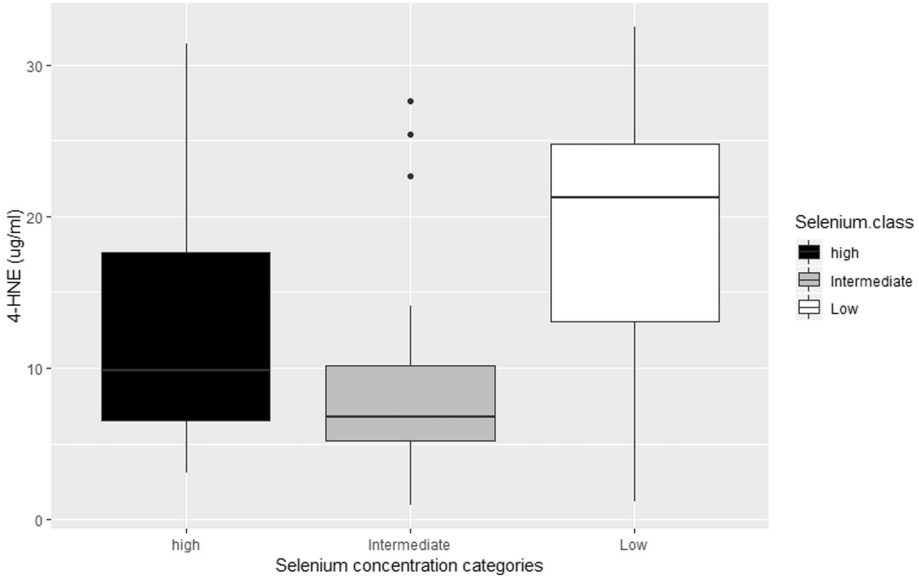

Fig. 3. Significantly higher concentrations of 4-hydroxynenonal (4-HNE) in animals with low [TSe] compared animals with intermediate [TSe] (chi-squared = 6.75, df = 2, $p = 0.034$). Categories based on distribution of whole blood [TSe] for all animals in the study, where high concentrations were the top 25%, intermediate concentrations were the middle 50%, and low concentrations were the bottom 25% of the animals. Accordingly, [TSe] was defined as low (< 0.30 μg/g), intermediate (IM, 0.30–0.41 μg/g), and high (> 0.41 μg/g). All samples collected from Steller sea lion pups at three different rookeries in the Aleutian Islands (Agattu, Ulak and Ugamak Islands) and in the Gulf of Alaska (Chiswell Island).

activities at Agattu and Ugamak Islands, compared to Chiswell Island. For immune biomarkers, there was a difference among rookeries with significantly increased concentrations of IL-6 at Ulak and Ugamak, compared with pups on Chiswell Island. Noting this repeated geographic difference in several biomarkers emphasizes the multiple drivers for the differences among rookeries, including possible differences in external and internal stressors (Kennedy et al., 2019), but also in diet (Scherer et al., 2015; Fritz et al., 2019), and possibly other environmental and ecological factors. Which specific factors contribute to higher levels of lipid and protein oxidation in the Aleutian Islands compared with the Gulf of Alaska is not clear.

In summary, we found a significant relationship between declining [TSe] and increased lipid peroxidation. There was a negative association between TBARS and [TSe], and animals with low [TSe] had higher concentrations of 4-HNE than animals with intermediate [TSe]. The significant association between GPx and TBARS found in this study suggest increased GPx activity with increased lipid peroxidation, possibly protecting pups with relatively high [THg] and low TSe:THg molar ratios from oxidative stress.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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