



# Assessing $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$ and Total Mercury Measures in Epidermal Biopsies From Gray Whales

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The gray whale (*Eschrichtius robustus*) is recognized as two geographically isolated and somewhat genetically distinct populations, with recent evidence showing exchange that questions the degree of isolation. The western gray whale subpopulation is currently listed as critically endangered, as designated by the IUCN, whereas the eastern North Pacific population is listed by the IUCN as “Least Concern.” The International Whaling Commission has stressed the value of studies on feeding ecology and contaminants for the western subpopulation. Our objective was to test for differences among epidermal tissue strata as part of an optimization effort to best use and interpret data from small biopsy samples. Fresh to moderately decomposed full thickness samples of epidermis and dermis were collected from stranded eastern gray whales along the California coast, United States (N: 14), and biopsy samples (N: 10) were collected from free-ranging western gray whales. We determined  $^{13}\text{C}$  and  $^{15}\text{N}$  stable isotope ratios ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ); and total Hg concentrations ([THg]) from histologically different strata of epidermal tissue samples from the eastern gray whale population. The information from these assessments (stranded whale samples) were directly applied for determining  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  from biopsies collected remotely from free-ranging western gray whales, as well as evaluating [THg]. We found a significant difference in [THg] values between the different strata. This has implications for analyzing Hg, and possibly other elements, from an epidermal biopsy from a free-ranging gray whale, and we recommend identifying which stratum/strata the biopsy represents. There were no significant differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values between the different strata, making epidermal biopsies from free-ranging gray whales an ideal sampling method to investigate  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios regardless of strata representation.

**Keywords:** biopsy, stranded marine mammal, contaminant, epidermis, gray whale, mercury, stable isotopes

## INTRODUCTION

Gray whales (*Eschrichtius robustus*) inhabit shallow coastal waters in the eastern and western coastlines of the North Pacific and are recognized as two geographically isolated and somewhat genetically distinct populations (Bradford et al., 2008; Bickham et al., 2013; Brüniche-Olsen et al., 2018a), with recent evidence showing some exchange (Weller et al., 2012; Lang et al., 2014; Mate et al., 2015; Brüniche-Olsen et al., 2018a). The western gray whale subpopulation is currently listed as critically endangered, as designated by the IUCN, whereas the eastern North Pacific population is listed by the IUCN as “Least Concern” (IUCN, 2012). The International Whaling Commission (IWC) and the IUCN have stressed the value of studies on factors that could impact recovery of the western subpopulation (Bickham et al., 2013). A research program to evaluate the western population’s biology was initiated in 1997 as a collaboration between Russian and United States scientists. The study is focused around the primary western gray whale feeding ground, which is located off the northeastern coast of Sakhalin Island, Russia, in the Okhotsk Sea, and is considered to provide access to most of the western gray whale population (Bradford et al., 2008; Bickham et al., 2013; Brüniche-Olsen et al., 2018b).

Some contaminants are known to bioaccumulate and biomagnify in marine mammals in the North Pacific and Arctic (Dietz et al., 2013). Hg concentrations have increased in Arctic environments during the past 150 years, leading to more than 92% of Hg body burden in higher trophic level species possibly originating from anthropogenic activities (Dietz et al., 2009). The relative longevity of gray whales sets the stage for bioaccumulation, but gray whales primarily feed on lower trophic levels of benthic invertebrates (Dehn et al., 2006a,b; Horstmann-Dehn et al., 2012) as compared to piscivorous cetaceans well known to have much higher Hg tissue concentrations. It is challenging to measure adverse effects from Hg in free-ranging species, but reports from pinnipeds (Van-Hoomissen et al., 2015; Kennedy et al., 2019) suggests an increase in abnormal behavior and a decrease in haptoglobin levels, associated with increased Hg concentrations. Previous reports have documented Hg concentrations in subsistence hunted gray whales (Tilbury et al., 2002; Dehn et al., 2006a), and from stranded gray whales (Varanasi et al., 1994). In contrast, there is no documentation of Hg concentrations sampled from living free-ranging gray whales, possibly biasing the reported Hg concentrations for this species. Biopsies collected remotely by boat from live animals will typically provide only a small sample of skin (epidermis and dermis). Thus, it is important to know difference in Hg concentration for the different epidermal layers as the proportion of strata may vary biologically based on sample collection dynamics (e.g., full thickness epidermis or not, angle of biopsy through the epidermis). The structure of cetacean epidermis is different from other mammals. The epidermis consists of three strata, in contrast to five strata in most terrestrial mammals. Strata granulosum and lucidum which are found in most mammals are absent in cetaceans (Sokolov, 1962; Reeb et al., 2007). Evidence shows that epidermal conditions alter Hg deposition in bottlenose dolphin (*Tursiops truncatus*), with

a positive relationship between Hg concentrations and age, and a negative relationship with height of stratum spinosum (Miller et al., 2011). Hg analysis in different epidermal strata was previously done in beluga (*Delphinapterus leucas*) and narwhal (*Monodon monoceros*) (Wagemann and Kozłowska, 2005), but to our knowledge it has not been previously analyzed in gray whales.

Assessments of feeding ecology are also important in long-lived cetaceans. Ratios of stable isotopes of carbon-13 and nitrogen-15 have been used extensively to trace pathways of organic matter and complexity of trophic levels (Rounick and Winterbourn, 1986; Caraveo-Patino and Soto, 2005; Dehn et al., 2006b; Horstmann-Dehn et al., 2012) as well as movement (migration) between regions with varying isotopic signatures (Hoekstra et al., 2002). The eastern gray whale population migrates from their summer feeding grounds in Alaska to their winter grounds in Baja California Sur, Mexico. The western gray whale population has its summer feeding grounds in the Okhotsk Sea, and migrates south along the eastern coast of northern Asia to their winter breeding grounds thought to be along the coast of southern China (Bradford et al., 2008; Weller et al., 2012; Brüniche-Olsen et al., 2018b). Gray whales are benthic feeders filtering sediment for their primary prey of ampeliscid amphipods (*Ampelisca macrocephala*) (Caraveo-Patino and Soto, 2005; Dehn et al., 2006a; Caraveo-Patino et al., 2007). It had been generally accepted that gray whales only feed during summer, but  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values have shown large variability in stable isotope composition during the winter months (Caraveo-Patino et al., 2007) indicating feeding behavior may be more complicated and worthy of temporal and spatial assessment for both eastern and western gray whales.

The critically endangered western gray whale population is dangerous to approach and difficult to study in the remote marine environment of the subarctic and Arctic. Biological studies of this species rarely provide robust sample materials unless a stranding occurs near an established network of responders (e.g., central California) or from well-coordinated sampling of subsistence harvested whales (Russia). Biopsy samples collected by remote sampling from boats often result in small samples of epidermal tissue with varying amounts of underlying tissue (“blubber”). Gray whale epidermis samples were morphologically and functionally divided into three distinct layers for this study: stratum corneum, stratum spinosum, and stratum basale. The epidermis and dermis interdigitate for structural support resulting in mixing of the stratum spinosum and stratum basale (Reeb et al., 2007).

In this study, we determined  $^{13}\text{C}$  and  $^{15}\text{N}$  stable isotope ratio values ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) and total Hg concentrations ([THg]) from histologically different strata of epidermal tissue samples from the eastern gray whale population where sample mass was generous. Our aim was to assess if obtaining a full depth proportionally representative biopsy sample is critical in preventing biases for Hg, and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measurements that may be introduced from a partial depth epidermis sample. In addition, these represent background data for this region and time of year. Previous studies on gray whales found that lipid extractions changes  $\delta^{13}\text{C}$  ratios in the epidermis (Horstmann-Dehn et al., 2012), and we conducted our stable isotope analysis both before

and after lipid extraction for the eastern gray whale samples. The information gained from assessing full depth biopsies from stranded gray whales, is directly applied when we report  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  from biopsies collected remotely from free-ranging western gray whales of expected extremely limited epidermal mass.

## MATERIALS AND METHODS

### Collection and Storage: Eastern Gray Whale Samples

From 2000–2011 fresh to moderately decomposed (code 2–3, Raverty et al., 2018) full thickness samples of epidermis and dermis were collected from stranded eastern gray whales along the California coast, United States ( $N = 15$ ). The animals ranged from calves to adults, with 9 males, 5 females and one unknown sex. The samples were obtained through a collaboration with The Marine Mammal Center (TMMC, Sausalito, CA, United States; MMPA permit No. 932-1905-00/MA-009526 to Frances Gulland). Samples were stored at  $-80^\circ\text{C}$  prior to and after shipping and shipped on dry ice. Total mercury concentration ([THg]) and carbon (C) and nitrogen (N) stable isotope analyses were conducted at the University of Alaska Fairbanks (UAF), Fairbanks, Alaska (United States). Histologic examination was conducted at the University of Tennessee (UT). Samples and specifically targeted subsamples (e.g., individual layers) provided ample amounts (mass) with full thickness epidermis, dermis and hypodermis abundantly available from these stranded animals relative to biopsies. Thus, this investigation allowed for sharing among investigators, validation (repeated analyses), and archiving that are not possible with valuable and hard to obtain smaller biopsy samples (described next). Sample collection site on whales could not be standardized because of variation in access based on position of the stranded whales.

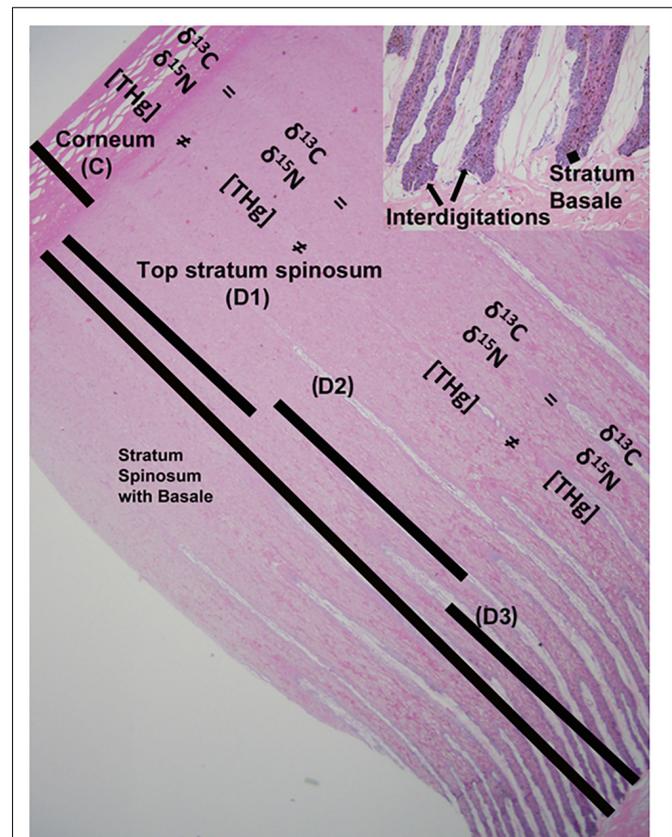
### Collection and Storage: Western Gray Whale Biopsies (Russia)

Biopsy samples ( $N = 10$ ) were collected from free-ranging western gray whales off the northeast coast of Sakhalin Island, Russia. Briefly, biopsies were obtained using a 150 lb. draw weight compound crossbow (Barnett RC-150) with 40 mm by 7 mm internal diameter tips arrows (Brüniche-Olsen et al., 2018b). Samples collected in 2011 were obtained through a collaboration with the IWC, *Russian Academy of Sciences Severtsov Institute of Ecology and Evolution* (IPEE RAS), and National Scientific Center of Marine Biology of the Far East Branch of the Russian Academy of Science (NNTsMB DVO RAN). The 2011 samples were imported frozen from Russia to the NOAA Southwest Fisheries Science Center, La Jolla, CA by the IWC and then to Texas Tech University under CITES permit 11US774223/9. Samples collected in 2013 were obtained through a collaboration with the Western Gray Whale Monitoring Program that Exxon Neftegas Limited and Sakhalin Energy Investment Company have conducted since 2002. These samples were imported frozen from Russia to Texas Tech University under CITES permit

13US082589/9. All frozen subsamples were subsequently shipped from Texas Tech University on dry ice to UAF for analyses. Frozen samples were stored at  $-80^\circ\text{C}$  until analysis. Research was completed under NMFS permits No. 932-1905-00/MA-009526 and No. 932-1905-01/MA-009526-01.

### Histology

Previously frozen tissues from the eastern population (stranded animals) were fixed in 10% formalin, routinely processed, sectioned at approximately  $5\ \mu\text{m}$ , placed on glass slides, stained with Hematoxylin and Eosin (H&E), and viewed using light microscopy. Photomicrographs of the sections were taken using an Olympus dp71 camera (Olympus America, Inc., 3500 Corporate Parkway, Center Valley, PA, United States), layers were identified as stratum corneum, stratum spinosum, and stratum basale, with significant interdigitation between stratum spinosum and stratum basale near the base of the epidermis (**Figure 1**).



**FIGURE 1** | Gray whale epidermis is morphologically and functionally divided into three distinct layers for this study: stratum corneum (C), stratum spinosum, and stratum basale. Depth 1 (D1) includes only stratum spinosum and is equal to the top layer of the spinosum. Depth 2 (D2) is the distal portion of the epidermal-dermal interdigitation area and is composed of primarily stratum spinosum, with little stratum basale. Depth 3 (D3) is the proximal portion of the epidermal-dermal interdigitation area and is composed of primarily stratum basale, with little stratum spinosum.

## Chemical Analyses; Mercury

Total mercury (THg) analysis was only conducted for the eastern gray whales due to limitation in biopsy sample size from the western gray whales. Determination of THg concentration ([THg]) was performed at the Wildlife Toxicology Laboratory (WTL) at UAF. Gray whale epidermis has three distinct morphological and functional layers for our purposes: the protective outer layer, stratum corneum (C); the middle layer, stratum spinosum; and the regenerative layer, stratum basale (Reeb et al., 2007). Based on structural analysis (histologic), samples of epidermis from 15 stranded whales were sectioned to provide the corneum and 3 equal depths for the rest of the epidermis – C (stratum corneum), D1 (only stratum spinosum), D2 (primarily stratum spinosum) and D3 (primarily stratum basale, **Figure 1**). The stratum corneum was easily identified visually and was removed with a clean scalpel. The remaining epidermis was then divided into three equal layers (D1–D3), removing all traces of underlying hypodermis from D3.

Subsamples from each depth were freeze-dried for 48 h and water content was calculated by subtraction of dry mass from wet mass. Dried samples were ground to a fine, homogenous powder with a cryomill. The [THg] was measured in triplicate using a direct mercury analyzer (DMA80; Milestone Inc., 25 Controls Drive, Shelton, CT, United States) according to EPA method 7473 (Castellini et al., 2012; Rea et al., 2013). Data for [THg] are reported on both a wet weight (ww) and dry weight (dw) basis. Quality control included measurement of blanks, liquid standards (3 ng THg at 0.100 µg/g) and certified reference materials IAEA 086 Hair (0.573 µg/g) and DORM 3 (0.355 µg/g) with each analytical run. Percent recoveries (mean ± SD) for liquid standards, IAEA 086, and DORM 3 were 96.7 ± 2.7, 102.4 ± 3.5 and 114.5 ± 2.6, respectively. Detection limit for [THg]<sub>dw</sub> was 0.008 µg/g for a 30 mg dry sample (0.25 ng THg). Three individuals had [THg] below the detection limit and have not been included in comparisons of epidermal layers. Thus, [THg] data represents 12 of 15 whales.

## Chemical Analyses; C and N Stable Isotope Ratios

Isotopic ratios of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) were analyzed at the Alaska Stable Isotope Facility at UAF by combustion using a continuous flow isotope ratio mass spectrometer according to methods described in Rea et al. (2013). Isotopic analyses are expressed in delta ( $\delta$ ) notation relative to an international standard (Vienna PeeDee Belemnite for  $\delta^{13}\text{C}$  and atmospheric air for  $\delta^{15}\text{N}$ ) in parts per thousand (‰, Bond and Hobson, 2012). The mean analytical precision was determined using working laboratory standards (Sigma Chemical Co., Highland, IL, United States; mean  $\delta^{13}\text{C}$  = -15.82 ‰ and mean  $\delta^{15}\text{N}$  = 7.02 ‰) across separate sample runs and instrument precision was 0.1 ‰ for  $\delta^{13}\text{C}$  and 0.2 ‰ for  $\delta^{15}\text{N}$ . Enrichment of the heavy isotope of a particular element was reported using the following notation and equation:

$$\delta R \text{ ‰} = \left( \left( \frac{R \text{ sample}}{R \text{ standard}} \right) - 1 \right) \times 1000$$

Where the differential notation ( $\delta R$ ) represents the relative difference between isotopic ratios of the sample and standard gases (i.e.,  $^{13}\text{C}/^{12}\text{C}$ ,  $^{15}\text{N}/^{14}\text{N}$ ).

Due to the limited amount of tissue available from biopsy samples (western gray whales), full thickness epidermis samples from stranded eastern gray whales were used to evaluate potential variability of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values based on epidermal depth in gray whale populations. Distinct sampling layers for C and N stable isotope analysis were the same as those used for THg analysis described above.

Biopsies from epidermis of western gray whales (frozen, not stored in ethanol) were very small (4–40 mg). Most samples had a small amount of hypodermis attached which was removed with a clean scalpel, being careful to preserve as much epidermis as possible. Enough tissue was recovered from 10 individuals (3 from 2011, 7 from 2013) to measure C and N stable isotopes from the D3 layer (based on presence of hypodermis). Epidermal samples from both eastern gray whales (subsamped by layer) and western gray whales (biopsies) were freeze-dried for 48 h and homogenized using a tissue mill or Wig-L-Bug™ (depending on sample size) in the WTL. Stable isotope ratios of C and N were measured before and after lipid extractions for eastern gray whales as sample mass was abundant. For western gray whales, tissue samples were too small and stable isotope analysis could only be done once without lipid extractions. Lipid extractions followed the method described in Cyr et al. (2019), where 1.0–2.0 g of freeze-dried sample muscle was rinsed three times in a mixture of 2:1 chloroform: methanol for 15 min, air dried overnight, then freeze-dried for 24 h.

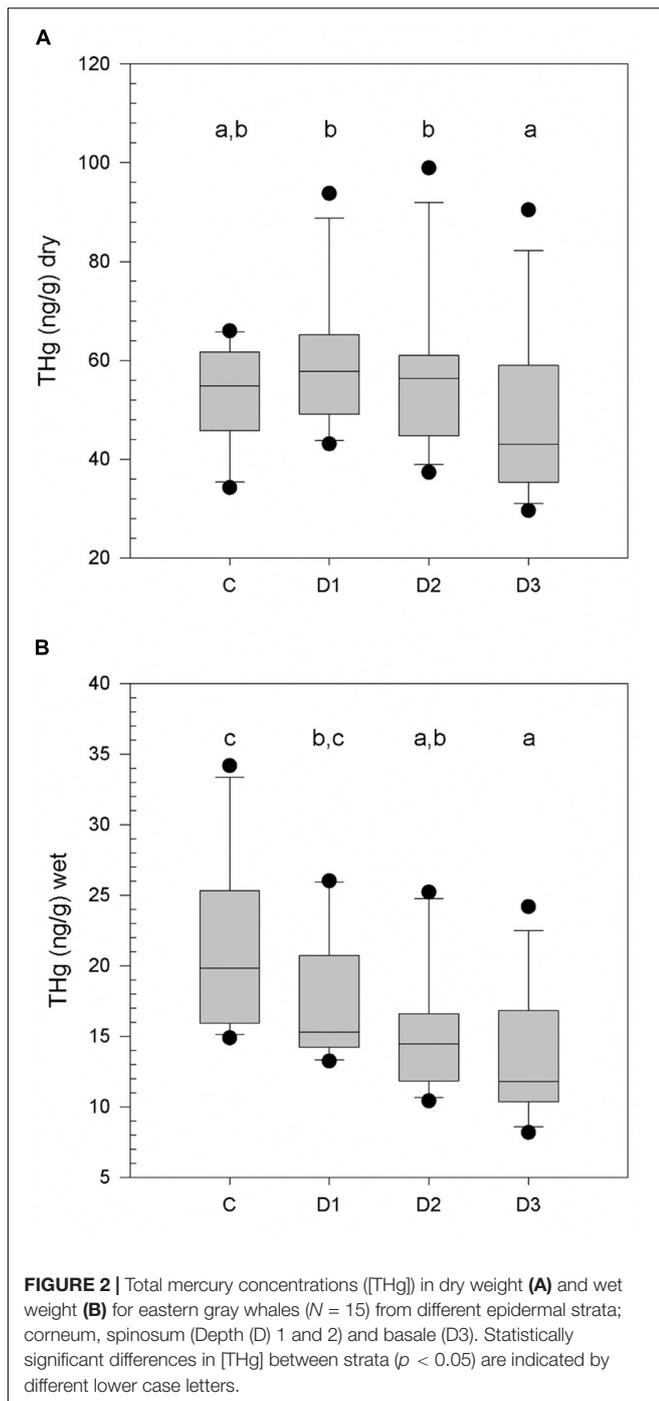
## Statistical Analysis

All statistical analyses were performed as linear models in the statistical software R, version 3.1.1 (R Development Core Team, 2014) and SigmaPlot 11.0 (Systat Software, Inc.). Tissue variables analyzed included [THg]<sub>dw</sub>, [THg]<sub>ww</sub>,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  from the three different epidermal strata, sectioned in four different layers (C, D1, D2, and D3), both before and after lipid extraction. We tested for normality with histogram, fitted values and theoretical quantiles (Normal Q-Q plot). Water content, [THg]<sub>dw</sub> and [THg]<sub>ww</sub> from different epidermal layers were analyzed using Friedman repeated measures ANOVA on ranks followed by *post hoc* Tukey multiple pairwise comparison tests. Comparisons of C and N stable isotope ratios between the two populations were made with ANOVA. Comparison of  $^{13}\text{C}$  and  $^{15}\text{N}$  stable isotopes before and after lipid extractions were made using repeated measures ANOVA followed by *post hoc* Holm-Sidak multiple pairwise comparison tests. Summary statistics for all variables were calculated with Microsoft Excel®. For all statistical analysis, we considered *p* values less than 0.05 significant.

## RESULTS

### Total Mercury

Total mercury concentration was measured in four different epidermal depths for the eastern gray whale samples (**Figure 2**).



The mean and standard deviation (SD) of [THg] measured in wet weight and dry weight, water content and lipid content, from four different layers (for the eastern population) are reported in **Table 1**. There was a significant difference in water content between sectioned epidermal layers ( $\bar{O}^2 = 25.10$ , 3DF,  $p < 0.001$ ), with corneum having significantly lower water content than D2 or D3 ( $p < 0.05$ ). There was a significant difference in [THg] represented as both dry weight (dw) and wet weight (ww) concentrations from the different epidermal layers ( $X^2 = 25.2$ ,

3DF,  $p < 0.001$  and  $X^2 = 19.6$ , 3DF,  $p < 0.001$ , respectively). The pattern of significant differences ( $p < 0.05$ ) between layers was different for [THg]<sub>dw</sub> compared to [THg]<sub>ww</sub>, as illustrated in **Figures 2A,B**, respectively.

The greatest individual [THg]<sub>dw</sub> measured was 98.92 ng/g (0.098  $\mu\text{g/g}$ ) in D2. There were three calves in the data set, and all had [THg]<sub>dw</sub>  $< 8.0$  ng/g. In contrast, the lowest value measured in an adult was 29.58 ng/g measured in D3.

## Stable Isotopes (Carbon-13 and Nitrogen-15; $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ )

For the eastern population the mean and SD of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measured from four different layers are reported in **Table 1**. The chemical analyses for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in eastern gray whales were conducted with and without lipid extraction. For samples that were not lipid extracted there was no significant difference in  $\delta^{15}\text{N}$  ( $F_{3,42} = 1.84$ ,  $p = 0.15$ ) or  $\delta^{13}\text{C}$  ( $F_{3,42} = 1.77$ ,  $p = 0.17$ ) values measured from the different epidermal layers for the eastern gray whale samples. These findings were consistent after lipid extraction with no significant difference in  $\delta^{15}\text{N}$  values ( $F_{3,41} = 0.652$ ,  $p = 0.586$ ) or  $\delta^{13}\text{C}$  values ( $F_{3,41} = 1.838$ ,  $p = 0.155$ ) measured from the different epidermal layers. As expected, there was a significant association between both  $\delta^{15}\text{N}$  (slope = 1.017,  $R^2 = 0.93$ ,  $p < 0.01$ ) and  $\delta^{13}\text{C}$  (slope = 1.22,  $R^2 = 0.86$ ,  $p < 0.01$ ) before and after lipid extraction (**Figure 3**) and both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were significantly enriched in the heavy isotope in lipid extracted versus non-lipid extracted samples in all epidermal layers ( $p < 0.001$  for all pairwise comparisons). The absolute difference between lipid and non-lipid extracted samples was relatively small for  $\delta^{15}\text{N}$  ( $0.34 \pm 0.20$  ‰, maximum = 1.08 ‰), while effect of lipid extraction on  $\delta^{13}\text{C}$  was more variable ( $0.44 \pm 0.58$  ‰, maximum = 3.16 ‰).

Due to very small tissue volume the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for the western gray whale population were only measured in the D3 as non-lipid extracted samples (**Table 1**). We found no differences in  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  values as measured in D3 ( $F_{1,8} = 1.24$ ,  $p = 0.29$  and  $F_{1,8} = 0.86$ ,  $p = 0.39$ , respectively) when comparing eastern and western gray whale populations (non-lipid extracted samples).

## DISCUSSION

We report [THg],  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values from stranded animals from the eastern gray whale population; and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values from the biopsy sampled western population. The analyses were performed on the histologically distinct epidermal strata to investigate differences between epidermal layers in [THg] and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  for the eastern population. This evaluation was directly applied to assist with analyzing and reporting  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values from biopsies of living free-ranging gray whales from the western population as we considered use of the small but valuable biopsy material.

Mercury levels in arctic marine habitats have increased 92% in the last 150 years, and it continues to be important to conduct biomonitoring for Hg in long-lived marine mammals

**TABLE 1** | Water and lipid content and values of carbon and nitrogen stable isotopes ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) from different epidermal strata; corneum, spinosum (Depth (D) 1 and 2) and basale (D3)<sup>1</sup> of eastern and western gray whales.

Variable	Top layer spinosum			
	Corneum	D1	D2	D3
<b>Eastern gray whales</b>				
Water content	60.0 ± 0.07% <sup>a</sup>	70.8 ± 2.9% <sup>a,b</sup>	73.0 ± 2.4% <sup>b</sup>	72.0 ± 2.9% <sup>b</sup>
Lipid content	12.06 ± 6.77%	9.96 ± 1.93%	10.71 ± 2.05%	13.93 ± 2.64%
$\delta^{15}\text{N}$ (non-lipid extracted)	13.22 ± 0.74	13.27 ± 0.71	13.30 ± 0.81	13.15 ± 0.74
$\delta^{15}\text{N}$ (lipid extracted)	13.54 ± 0.73 <sup>‡</sup>	13.60 ± 0.63 <sup>‡</sup>	13.61 ± 0.73 <sup>‡</sup>	13.52 ± 0.78 <sup>‡</sup>
$\delta^{13}\text{C}$ (non-lipid extracted)	-17.09 ± 1.42	-16.88 ± 0.95	-16.91 ± 0.93	-17.10 ± 1.04
$\delta^{13}\text{C}$ (lipid extracted)	-16.20 ± 0.87 <sup>‡</sup>	-16.22 ± 0.77 <sup>‡</sup>	-16.09 ± 0.85 <sup>‡</sup>	-16.09 ± 0.88 <sup>‡</sup>
THg dry weight (ppb)	43.34 ± 21.1 <sup>†</sup>	48.53 ± 25.64 <sup>†</sup>	46.79 ± 25.88 <sup>†</sup>	38.79 ± 23.19 <sup>†</sup>
THg wet weight (ppb)	17.00 ± 9.29 <sup>†</sup>	14.20 ± 7.67 <sup>†</sup>	12.62 ± 7.05 <sup>†</sup>	10.86 ± 6.36 <sup>†</sup>
<b>Western gray whales</b>				
$\delta^{15}\text{N}$ (non-lipid extracted)				12.20 ± 0.77
$\delta^{13}\text{C}$ (non-lipid extracted)				-16.52 ± 0.74

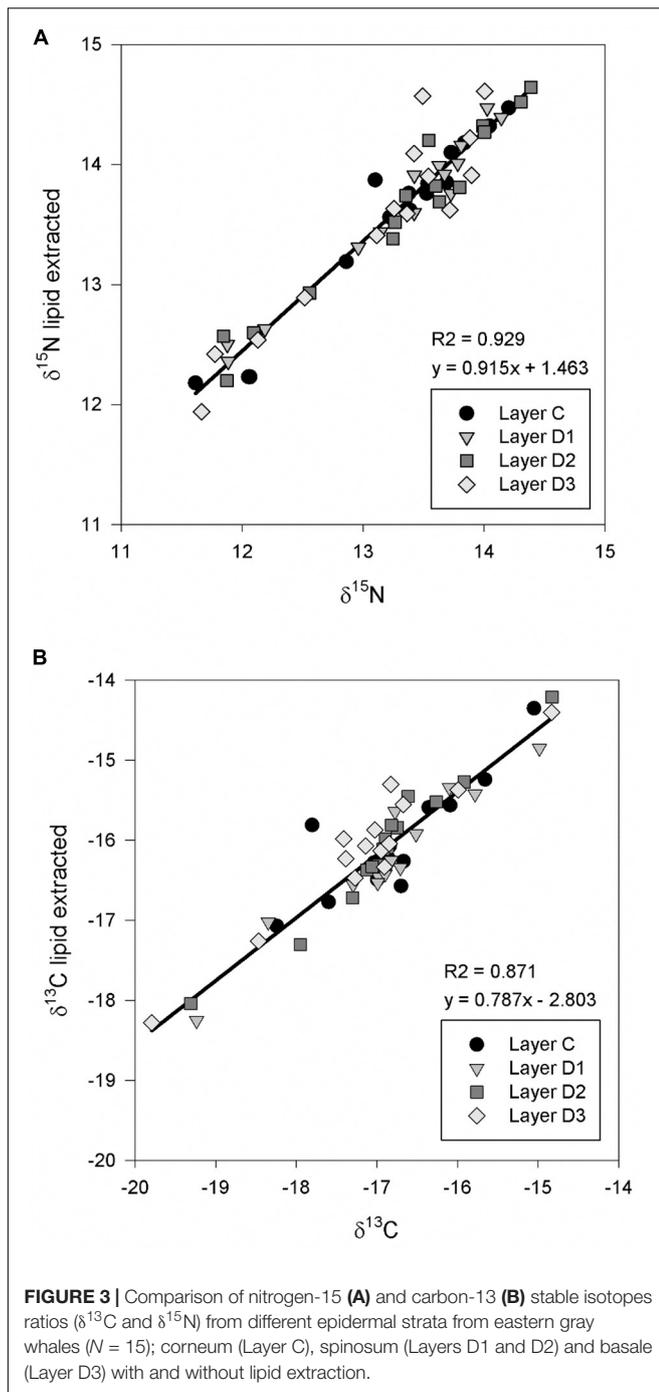
Measurements reported as mean ± standard deviation (SD). Statistically significant differences ( $p < 0.05$ ) in water content between different strata are indicated by lower case letters. Statistically significant differences ( $p < 0.001$ ) between lipid and non-lipid extracted samples for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  are noted with ‡. †Statistical differences ( $p < 0.05$ ) in THg between epidermal strata are noted with †. Additionally,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for D3 from the western gray whale population ( $N = 10$ ) are listed. Due to the very small biopsy size, only stable isotope ratios for non-lipid extracted samples were measured. <sup>1</sup>Corneum is the protective outer layer. Spinosum includes the entire layer from the corneum to the proximal basale and for mercury and stable isotope analysis spinosum was divided into 3 equal depths (D1, D2, D3). D1 includes only stratum spinosum. D2 is composed of primarily stratum spinosum, with little stratum basale. D3 is the proximal portion of the epidermal-dermal interdigitation area and is composed of primarily stratum basale.

(Dietz et al., 2013; Peterson et al., 2016). The [THg] found in epidermal tissues in this study are higher than reported by Dehn et al. (2006a), but lower than reported by Varanasi et al. (1994) and Tilbury et al. (2002). As expected, calves had the lowest values. Using a small epidermal biopsy collected from a free-ranging animal for Hg biomonitoring in cetaceans is a valuable approach. This is analogous to use of hair and fur in pinnipeds to monitor Hg exposure (Rea et al., 2013; Van-Hoomissen et al., 2015; Peterson et al., 2016). However, as we have shown here, there are significant differences in [THg] between the different epidermal strata, with greatest [THg]<sub>dw</sub> occurring in D1 and D2. The relatively high [THg]<sub>wet</sub> in the corneum reflects lower water content in that layer (60.0 ± 0.07% water compared to 70.8 ± 2.9, 73.0 ± 2.4, and 72.0 ± 2.9% water in layers D1–D3 of the spinosum, respectively) and demonstrates the utility of comparing [THg] on both a wet and dry mass basis. Thus, determining which strata and representation of the concentration (wet or dry weight) best correlates with other tissues would be a logical next step. A previous study found that epidermal levels of Hg were weakly correlated with blubber, kidney, muscle, and liver concentrations in bowhead whales (*Balaena mysticetus*) (O'Hara et al., 2008). In bottlenose dolphins, epidermal Hg was closely correlated with whole blood making epidermal samples valuable for biomonitoring in this species (Woshner et al., 2008), and epidermal condition drives [THg] deposition in this tissue (Miller et al., 2011). For gray whales, further sampling should be conducted to establish correlations between epidermis and other tissues and among populations and cohorts (calves, subadults, adults) where access to stranded and subsistence harvested (Russia) whales allows a multiple tissue study design to improve interpretation of epidermal biopsies. These proposed studies could include other elements of

interest such as zinc, cadmium, selenium, and lead when tissue sample weight allows.

When the distinct epidermal strata were compared in samples collected from stranded whales, we found no difference in  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  ratios. We do not expect the stable isotope ratios for carbon-13 and nitrogen-15 to change between fresh to moderately decomposed samples and living animals (Payo-Payo et al., 2013). This indicates biopsy sampling, allowing only for marginal tissue size, from free-ranging gray whales to study these isotopes does not require consideration of the anatomic structure of epidermis for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . We also found similar significant associations between lipid extracted and non-lipid extracted samples, although the lipid extracted samples were significantly enriched in both the <sup>15</sup>Nitrogen and <sup>13</sup>Carbon across strata compared to non-lipid extracted samples.

Gray whales have higher  $\delta^{13}\text{C}$  values compared to other baleen whales, likely due to their feeding habit of filtering out invertebrates from sediment in the benthic ecosystems (Dehn et al., 2006b; Horstmann-Dehn et al., 2012). The  $\delta^{15}\text{N}$  values were similar to values reported previously (Dehn et al., 2006a; Caraveo-Patino et al., 2007; Horstmann-Dehn et al., 2012), demonstrating a low trophic level and prey based on benthic gammaridean amphipods (Dehn et al., 2006a). Hoekstra et al. (2002), Gendron et al. (2015) demonstrated how tissue measures of  $\delta^{13}\text{C}$  were seasonally variable related to the migration of bowhead whales, and studies of  $\delta^{13}\text{C}$  in eastern gray whales have shown that this population likely feed during their time at the winter breeding grounds (Caraveo-Patino and Soto, 2005; Caraveo-Patino et al., 2007). Future studies might elucidate the western population of gray whales' geographical winter range and possible temporal and spatial changes with extended monitoring and research using C and N stable isotope ratios. Our finding that



$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios do not vary in the different epidermal strata, makes a small biopsy from the endangered free-ranging western gray whales suitable for such analyses.

To maximize information obtained from each small biopsy sample from the free-ranging gray whales, histologic analyses were performed on samples collected from stranded eastern gray whale epidermis. We found that the protective corneum layer was about one tenth of the spinosum layer. In the D3 layer rete pegs interdigitate with papillae from the dermis for support, much

like other cetacean epidermal tissues (Reeb et al., 2007; Miller et al., 2011). Structural studies of cetacean epidermal tissues are important for studies of free-ranging whale biopsies to assess such things as contaminants, skin lesions/disease, body condition, etc (Wagemann and Kozłowska, 2005; Woshner et al., 2008; Miller et al., 2011). Epidermal biopsies are difficult to obtain and very valuable, and the information gained helps determine whether biopsy testing may target specific strata to minimize waste of tissue and possibly enhance comparisons among whales (reduced variability in values that may be driven by strata type).

## CONCLUSION

Epidermal tissues collected from stranded eastern gray whales were used to evaluate [THg] measures, and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios between the corneum, stratum spinosum and stratum basale. We found a significant difference in [THg] between the different strata, represented as both ww and dw. This has implications for analyzing Hg from an epidermal biopsy from a free-ranging gray whale, and we recommend identifying which stratum the biopsy represents. For these comparisons of Hg concentrations between strata it is important the comparison is done on a wet mass basis as % water is one of the main factors driving the [THg] differences between the epidermal layers. There were, however, no significant difference in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values between the different strata, making epidermal biopsies from free-ranging whales an ideal sampling method to investigate  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  relationships that can be conducted independent of strata identification.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

This animal study was reviewed and approved by the International Whaling Commission and then to Texas Tech University under CITES permit 11US774223/9. NMFS permits Nos. 932-1905-00/MA-009526 and 932-1905-01/MA-009526-01.

## AUTHOR CONTRIBUTIONS

All authors contributed to manuscript review and preparation. Additionally, TO'H, CG-C, DM, JD, and KB designed the study. TO'H and DM supervised experiments. ML conducted stable isotope analyses, statistical analyses and prepared the first draft of the manuscript. JC performed the Hg analyses. DM and BG prepared and analyzed the histological analyses. VV conducted fieldwork and sample collection. CG-C and VV supervised sample processing and distribution of samples to collaborators and secured all permits. All authors contributed to the writing, critically revised the manuscript, approved the final version and agreed to be held accountable for the content herein.

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## REFERENCES

- Bickham, J. W., Doyle, J., DeWoody, A., Pezdek, R., Wooten, K., Kellar, N., et al. (2013). Genetics and preliminary hormone analyses in Western Gray whale biopsy samples collected off Sakhalin Island in 2011. *Paper SC/65a/BRG23 presented to the IWC Scientific Committee*, (Cambridge, MA: International Whaling Commission).
- Bond, A. L., and Hobson, K. A. (2012). Reporting stable-isotope ratios in ecology: recommended terminology, guidelines and best practices. *Waterbirds* 35, 324–331. doi: 10.1675/063.035.0213
- Bradford, A., Weller, D., Wade, P., Burdin, A., and Brownell, R. Jr. (2008). Population abundance; and growth rate of western gray whales *Eschrichtius robustus*. *Endang Species Res* 6, 1–14. doi: 10.3354/esr00106
- Brüniche-Olsen, A., Urban, R. J., Vertyankin, V. V., Godard-Codding, C. A. J., Bickham, J. W., and DeWoody, J. A. (2018b). Genetic data reveal mixed stock aggregations of gray whales in the North Pacific Ocean. *Biol. Lett.* 14:20180399. doi: 10.1098/rsbl.2018.0399
- Brüniche-Olsen, A., Westerman, R., Kazmierczyk, Z., Vertyankin, V. V., Godard-Codding, C., Bickham, J. W., et al. (2018a). The inference of gray whale (*Eschrichtius robustus*) historical population attributes from whole-genome sequences. *BMC Evol. Biol.* 18:87. doi: 10.1186/s12862-018-1204-3
- Caraveo-Patino, J., Hobson, K., and Soto, L. (2007). Feeding ecology of gray whales inferred from stable-carbon and nitrogen isotopic analysis of baleen plates. *Hydrobiologia* 586, 17–25. doi: 10.1007/s10750-006-0477-5
- Caraveo-Patino, J., and Soto, L. (2005). Stable carbon isotope ratios for the gray whale (*Eschrichtius robustus*) in the breeding grounds of Baja California Sur, Mexico. *Hydrobiologia* 539, 99–107.
- Castellini, J. M., Rea, L. D., Lieske, C. L., Beckmen, K. B., Fadely, B. S., Maniscalco, J. M., et al. (2012). Mercury concentrations in hair from neonatal and juvenile Stellar sea lions (*Eumetopias jubatus*): implications based on age and region in this Northern Pacific marine sentinel piscivore. *Ecohealth* 9, 267–277. doi: 10.1007/s10393-012-0784-4
- Cyr, A., López, J. A., Rea, L., Wooler, M. J., Loomis, T., McDermott, S., et al. (2019). Mercury concentrations in marine species from the Aleutian Islands: spatial and biological determinants. *Sci. Total Environ.* 664, 761–770. doi: 10.1016/j.scitotenv.2019.01.387
- Dehn, L., Follmann, E., Rosa, C., Duffy, L., Thomas, D., Bratton, G., et al. (2006a). Stable isotope and trace element status of subsistence-hunted bowhead and beluga whales in Alaska and gray whales in Chukotka. *Mar. Pollut. Bull.* 52, 301–319. doi: 10.1016/j.marpollbul.2005.09.001
- Dehn, L., Follmann, E., Thomas, D., Sheffield, G., Rosa, C., Duffy, L., et al. (2006b). Trophic relationships in an Arctic food web and implications for trace metal transfer. *Sci. Total Environ.* 362, 103–123. doi: 10.1016/j.scitotenv.2005.11.012
- Dietz, R., Outridge, P., and Hobson, K. (2009). Anthropogenic contributions to mercury levels in present-day Arctic animals—A review. *Sci. Total Environ.* 407, 6120–6131. doi: 10.1016/j.scitotenv.2009.08.036
- Dietz, R., Sonne, C., Basu, N., Braune, B., O'Hara, T. M., Letcher, R., et al. (2013). What are the toxicological effects of mercury in Arctic biota? *Sci. Total Environ.* 443, 775–790. doi: 10.1016/j.scitotenv.2012.11.046
- Gendron, M., Castellini, J. M., O'Hara, T., Klein, D., Wooten, K., Pezdek, R. J., et al. (2015). Hormone and stable isotope of C and N analyses in Western Gray Whale (*Eschrichtius robustus*) biopsy samples collected off Sakhalin Island in 2011, 2012 and 2013. *Paper SC/66a/BRG/10 (Rev1) presented to the International Whaling Commission, Scientific Committee*, San Diego, CA.
- Hoekstra, P. F., Dehn, L. A., George, J. C., Solomon, K. R., Muir, D. C. G., and O'Hara, T. M. (2002). Trophic ecology of bowhead whales (*Balaena mysticetus*) compared with that of other arctic marine biota as interpreted from carbon-, nitrogen-, and sulfur-isotope signatures. *Can. J. Zool.* 80, 223–231. doi: 10.1139/Z01-229
- Horstmann-Dehn, L., Follmann, E. H., Rosa, C., Zelensky, G., and George, C. (2012). Stable carbon and nitrogen isotope ratios in muscle and epidermis of arctic whales. *Mar. Mam. Sci.* 28, E173–E190.
- IUCN (2012). *Report of the Western Gray Whale Advisory Panel at Its Eleventh Meeting*. Geneva: IUCN.
- Kennedy, S. N., Castellini, J. M., Hayden, A. B., Fadely, B. S., Burkanov, V. N., Dajles, A., et al. (2019). Regional and age-related variations in haptoglobin concentrations in Steller sea lions (*Eumetopias jubatus*) from Alaska, USA. *J. Wildl. Dis.* 55, 91–104. doi: 10.7589/2017-10-257
- Lang, A., Calambokidis, J., Scordino, J., Pease, V., Klimek, A., Burkanov, V., et al. (2014). Assessment of genetic structure among eastern North Pacific gray whales on their feeding grounds. *Mar. Mam. Sci.* 30, 1473–1493. doi: 10.1111/mms.12129
- Mate, B. R., Ilyashenko, V. Y., Bradford, A. L., Vertyankin, V. V., Tsidulko, G. A., Rozhnov, V. V., et al. (2015). Critically endangered western gray whales migrate to the eastern North Pacific. *Biol. Lett.* 11:20150071. doi: 10.1098/rsbl.2015.0071
- Miller, D. L., Woshner, V., Styer, E. L., Ferguson, S., Knott, K. K., Gray, M. J., et al. (2011). Histologic findings in free-ranging Sarasota Bay bottlenose dolphin (*Tursiops truncatus*) skin: mercury, selenium, and seasonal factors. *J. Wildl. Dis.* 47, 1012–1018. doi: 10.7589/0090-3558-47.4.1012
- O'Hara, T. M., Hanns, C., Woshner, V., Zeh, J., Bratton, G., and Taylor, R. (2008). Essential and non-essential elements in the bowhead whale: epidermis-based predictions of blubber, kidney, liver and muscle tissue concentrations. *J. Cetacean Res. Manag.* 10, 107–117.
- Payo-Payo, A., Ruiz, B., Cardona, L., and Borrell, A. (2013). Effect of tissue decomposition on stable isotope signatures of striped dolphins *Stenella*

- coeruleoalba* and loggerhead sea turtles *Caretta caretta*. *Aquat. Biol.* 18, 141–147. doi: 10.3354/ab00497
- Peterson, S. H., McHuron, E. A., Kennedy, S. N., Ackerman, J. T., Rea, L. D., Castellini, J. M., et al. (2016). Evaluating hair as a predictor of blood mercury: the influence of ontogenetic phase and life history in pinnipeds. *Arch. Environ. Contam. Toxicol.* 70, 28–45. doi: 10.1007/s00244-015-0174-3
- Raverty, S., Duignan, P. J., Jepson, P. D., and Morell, M. (2018). “Marine Mammal Gross Necropsy,” in *CRC Handbook of Marine Mammal Medicine*, eds F. M. D. Gulland, L. A. Dierauf, and K. L. Whitman (Boca Raton, FL: CRC Press), 249–265.
- R Development Core Team (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: <http://www.R-project.org/> (accessed 2016).
- Rea, L. D., Castellini, J. M., Correa, L., Fadely, B. S., and O’Hara, T. M. (2013). Maternal Stellar sea lion diets elevate fetal mercury concentrations in an area of population decline. *Sci. Total Environ.* 454–455, 277–282. doi: 10.1016/j.scitotenv.2013.02.095
- Reeb, D., Best, P., and Kidson, S. (2007). Structure of the integument of southern right whales, *Eubalaena australis*. *Anat. Rec.* 290, 596–613. doi: 10.1002/ar.20535
- Rounick, J., and Winterbourn, M. (1986). Stable carbon isotopes and carbon flow in ecosystems. *Bioscience* 36, 171–177. doi: 10.2307/1310304
- Sokolov, V. (1962). Adaptations of the mammalian skin to the aquatic mode of life. *Nature* 195, 464–466. doi: 10.1038/195464a0
- Tilbury, K., Stein, J., Krone, C., Brownell, R. Jr., Blokhin, S., Bolton, J., et al. (2002). Chemical contaminants in juvenile gray whales (*Eschrichtius robustus*) from a subsistence harvest in Arctic feeding grounds. *Chemosphere* 47, 555–564. doi: 10.1016/s0045-6535(02)00061-9
- Van-Hoomissen, S., Gulland, F. M. D., Greig, D. J., Castellini, J. M., and O’Hara, T. M. (2015). Blood and hair mercury concentrations in the Pacific Harbor Seal (*Phoca vitulina richardii*) Pup: associations with neurodevelopmental outcomes. *Ecohealth* 12, 490–500. doi: 10.1007/s10393-015-1021-8
- Varanasi, U., Stein, J., Tilbury, K., Meador, J., Sloan, C., Clark, R., et al. (1994). Chemical contaminants in gray whales (*Eschrichtius robustus*) stranded along the west coast of North America. *Sci. Total Environ.* 145, 29–53. doi: 10.1016/0048-9697(94)90296-8
- Wagemann, R., and Kozłowska, H. (2005). Mercury distribution in the skin of beluga (*Delphinapterus leucas*) and narwhal (*Monodon monoceros*) from the Canadian Arctic and mercury burdens and excretion by moulting. *Sci. Total Environ.* 351–352, 333–343. doi: 10.1016/j.scitotenv.2004.06.028
- Weller, D., Klimek, A., Bradford, A., Calambokidis, J., Lang, A., Gisborne, B., et al. (2012). Movements of gray whales between the western and eastern North Pacific. *Endang Species Res.* 18, 193–199. doi: 10.3354/esr00447
- Woshner, V., Knott, K. K., Wells, R., Willer, C., Swor, R., and O’Hara, T. M. (2008). Mercury and selenium in blood and epidermis of bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, FL: interaction and relevance to life history and hematologic parameters. *Ecohealth* 5, 360–370. doi: 10.1007/s10393-008-0164-2

**Conflict of Interest:** JD was employed by the company ExxonMobil Upstream Research Company. KB was employed by the company Shell Global Solutions.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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