Marine Mammal Science

MARINE MAMMAL SCIENCE, 00(00): 1–21 (Month 2018)

DOI: 10.1111/mms.12546

Examination of relationships between stable isotopes and cortisol concentrations along the length of phocid whiskers

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Abstract

Alaskan seals are found in remote and sometimes inaccessible locations, making it difficult to collect time-series information. This study explores a novel method to examine temporal changes in diet and physiological status of ringed (*Pusa hispida*), spotted (*Phoca largha*), and harbor (*Phoca vitulina*) seals using cortisol concentrations and $\delta^{15}N$ and $\delta^{13}C$ stable isotopes (SIs) measured in serial sections of whiskers. As whiskers grow, whisker tissue is deposited sequentially making these measurements temporally aligned. Whisker cortisol presented in a distinct pattern with elevated concentrations at the root section followed by a curvilinear decline moving toward the tip of most whiskers. Comparing SIs at the root to the rest of the whiskers, $\delta^{13}C$ values were slightly lower in ringed and harbor seal whiskers and $\delta^{15}N$ values were slightly higher in harbor seal whiskers. The data were modeled controlling for the observed trends in cortisol concentrations and further associations between cortisol concentrations and SIs were detected in spotted and harbor seal whiskers. Additional research examining the source and stability of whisker cortisol is warranted. However, the methods presented here demonstrate that whiskers could prove valuable to gather long-term and naturally aligned dietary and physiological information.

Key words: whisker, vibrissa, cortisol, stable isotope, $\delta^{13}C$, $\delta^{15}N$, *Pusa hispida*, *Phoca largha*, *Phoca vitulina*.

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Alaskan pinnipeds are experiencing changes in climate and sea ice extent, and these changes are predicted to intensify over time (*e.g.*, Douglas 2010). Unfortunately, the current status of ringed and spotted seal populations are poorly understood due to the difficulty associated with obtaining accurate counts of seals dispersed across the vast and sometimes inaccessible sea ice. Each pinniped species will be uniquely affected by the ongoing and future changes to their habitats resulting from differences in prey and habitat requirements, therefore it is essential to develop methods to assess changes in diet and physiological status in difficult to study species.

As marine environments are altered, prey species will likely be affected leading to changes in phocid diets. Direct methods of diet investigations *via* stomach and scat content include biases such as underrepresentation of partially consumed or soft-bodied prey (Harvey and Antonelis 1994, Arim and Naya 2003). More recently indirect methods, such as stable isotope (SI) analyses, have been used to assess diet in many species (Hobson 1999, Dehn *et al.* 2007, Herreman *et al.* 2009). Variations in isotope ratios of N and C can provide information on trophic level and foraging area. Within a food web, the ratios of naturally occurring stable nitrogen isotopes (expressed as $\delta^{15}N$) are predictably enriched in a stepwise fashion with trophic level (Newsome *et al.* 2010). Stable carbon isotopes (expressed as $\delta^{13}C$) are enriched to a lesser extent by trophic level; instead the largest differences are associated with habitat use or foraging locations as carbon is more depleted in offshore pelagic prey than nearshore benthic prey (*e.g.*, Newsome *et al.* 2010). SI values in most animal tissues represent an average of diet consumed over a tissue-specific period, *e.g.*, days to months due to different rates of tissue regeneration or turnover (Tieszen *et al.* 1983, Hobson *et al.* 1996). Therefore, methods commonly used to describe diets provide information over distinct periods, and repeated sampling is required to characterize temporal changes in diet. In remote areas or during winters in Alaska, repeated sampling of pinnipeds is time consuming, expensive, and at times impossible. Therefore, whiskers that contain metabolically inert information deposited during the previous ≥1 yr for phocids and several years for otariids (Hall-Aspland *et al.* 2005, Stricker *et al.* 2015, Beltran *et al.* 2016, Lübcker *et al.* 2016, McHuron *et al.* 2016) are becoming an increasingly popular tool to create dietary reconstructions (Hobson *et al.* 1996, Newland *et al.* 2011, de la Vega *et al.* 2016). These studies emphasize the utility of whiskers for tracking diet, yet to date there are no methods that pair physiological parameters with the stable isotope information gained from whiskers.

The current and predicted changes in habitat and prey will have negative, positive, or neutral effects on seal species, depending on their life history and ecology; therefore it is essential to develop tools to assess physiological responses to changing conditions, particularly diet. Cortisol, the primary glucocorticoid in pinnipeds (DeRoos and Bern 1961), is released when animals perceive a stressor leading to a suite of behavioral and physiological changes (*e.g.*, Sapolsky 1990, Wingfield 2003) including mobilizing fatty acids and increasing gluconeogenesis (Gil *et al.* 1985, Atkinson *et al.* 2015). Cortisol also plays a significant role during phocid fasting, lactation (Engelhard *et al.* 2002, Ortiz *et al.*

2003), and molt (Riviere *et al.* 1977, Ashwell-Erickson *et al.* 1986, Kershaw and Hall 2016). Further, cortisol is associated with energy balance (Strack *et al.* 1995), and changes in body condition or diet have been associated with circulating stress-related hormones in birds (Kitaysky *et al.* 1999, Cockrem *et al.* 2006), terrestrial mammals (Barboza *et al.* 2004, George *et al.* 2014), and seals (Bartsh *et al.* 1992). Therefore, elevated cortisol concentrations can indicate energetic demand or deviation from physiological homeostasis (Bonier *et al.* 2009). Consequently, a method that tracks cortisol concentrations in association with SI values will be beneficial for understanding how animals are responding to a changing environment.

Steroid hormones, including cortisol, accumulate in keratinized mammalian tissues such as hair (Macbeth *et al.* 2010, 2012; Meise *et al.* 2016) and baleen (Hunt *et al.* 2014), suggesting that it may be possible to use these keratinized tissues to evaluate stress during the period the keratin was deposited. For example, hair cortisol concentrations were elevated following administration of adrenocorticotropic hormone (ACTH) in lynx (*Lynx canadensis*) (Terwissen *et al.* 2013), and in response to chronic stress in rhesus monkeys (*Macaca mulatta*) (Davenport *et al.* 2008) and domestic dogs (*Canis lupus familiaris*) (Siniscalchi *et al.* 2013). Hair cortisol concentrations were also used to assess environmental stressors and contaminant levels in polar bears (*Ursus maritimus*) (Bechshøft *et al.* 2012*a*, 2012*b*, 2013, 2015) and nutritional stress in brown bears (*Ursus arctos*) (Bryan *et al.* 2013). These findings signify that cortisol, measured in hair, can be used as an index of circulating concentrations, even though the mechanisms of cortisol incorporation into keratin are not understood.

An advantage of examining cortisol concentrations in metabolically inert tissues, such as hair or whiskers, is the ability to avoid the acutely elevated serum cortisol concentrations associated with capture or harvest that can obscure baseline circulating levels (Harcourt *et al.* 2010, Champagne *et al.* 2012, Keogh *et al.* 2013). However, in phocids, hair growth occurs over about 2.5 mo (Ashwell-Erickson *et al.* 1986), which is a relatively short period compared to whiskers that may continue to grow for a year or more (Zhao and Schell 2004, Beltran *et al.* 2015, Lübcker *et al.* 2016, McHuron *et al.* 2016). Due to the similarity between whiskers and hair and the utility of other keratin tissues, such as baleen, to measure steroid hormones (Hunt *et al.* 2014); it is likely that cortisol is incorporated into whiskers during growth, and analyses of sections along the length of the whisker will allow researchers to recreate a timetable of changes in cortisol concentrations over time.

This study measured cortisol concentrations and stable carbon and nitrogen values in serially sampled sections of whiskers from three species of phocids that inhabit Alaskan waters: ringed seals (*Pusa hispida*), spotted seals (*Phoca largha*), and harbor seals (*Phoca vitulina*). The objectives of this study were: (1) determine whether measurable levels of cortisol were present in phocid whiskers, (2) compare whisker cortisol concentrations among and within three phocid species, (3) determine whether cortisol concentrations varied along the length of whiskers, and (4) characterize associations between $\delta^{13}C$ and $\delta^{15}N$ values and cortisol

concentrations from serial sections of whiskers. It is not understood by which process cortisol is incorporated into keratin, a nonlipid matrix. As a result, the relationships shown in this study are largely a result of exploratory analyses and we do not endeavor to explain the factors driving differences in cortisol concentrations or stable isotope values measured among or within phocid whiskers. Instead, results are presented to show that this novel method, using serial sections of whiskers, has the potential to describe concurrent changes in diet (SIs) and physiological homeostasis (cortisol) over time.

Methods

Whisker Collection and Handling

During 2009 and 2010 the longest whiskers were collected from adult and subadult (age estimate > 3 yr) ringed (n = 20,), spotted (n = 20), and harbor seals (n = 28) (Table 1). The ringed and spotted seal whiskers were obtained from animals harvested for subsistence purposes. The spotted seal whiskers were collected at Shishmaref in the Chukchi Sea, the ringed seal whiskers were collected at Shishmaref, Diomede in the Bering Strait, and Gambell and Hooper Bay in the Bering Sea. Whole cheek pads were collected and stored at −20ºC for ∼2–3 yr until whiskers were extracted and stored in paper envelopes at room temperature for <1 yr prior to analysis. Harbor seals were live-captured in Tracy and Endicott Arms, Southeast Alaska, sedated (Diazepam 0.25 mg/kg), and manually restrained. Whiskers were extracted with pliers so that the root portion remained intact and stored in whirl-paks or paper envelopes at room temperature for 2–4 yr until analyzed.

Whisker Preparation and Sectioning

Whiskers were decontaminated using methods similar to those used for hair preparation in Macbeth *et al.* (2010). Some whiskers had small amounts of follicle tissue that remained adhered to the proximal (root) end, which was removed using fine forceps under a dissecting microscope. Whole whiskers were washed in 100% methanol then sonicated in deionized water for 30 min and dried in an oven at 60ºC for ≥ 12 h. Whiskers were then serially sectioned (Fig. S1) and individual sections were analyzed separately for concentrations of cortisol or SI values (Table 1). To track locations along the whisker (*Lw*), zero was assigned to the root end and each 1 mm increment along the whisker shaft was assigned a sequential number. For SI analysis, 8–17 sections (1–4 mm) were excised intermittently along the whisker shaft. The spacing between SI sections was shorter at the root than at the tip since whisker growth slows over time and a longer period is represented per unit of length at the root for phocids (Zhao and Schell 2004, Beltran *et al.* 2015, Lübcker *et al.* 2016, McHuron *et al.* 2016). Generally, four sections were removed from the first 11 mm at 0–1 mm, 4–5 mm, 7–8 mm, and 10–11 mm. Distal to 11 mm, 1–4 mm sections were removed at about every 10 mm until the tip was reached (Fig. S1). Depending on the

Table 1. Sample sizes, mean ($\overline{x}$), SD, and ranges of whisker lengths, collection day, cortisol concentrations, and $\delta^{15}N$ and $\delta^{13}C$ values from whisker sections for each species.

	Ringed	Spotted	Harbor
Whiskers			
sample size (whole whiskers)	20	20	28
whisker length $\overline{x}$ ± SD (mm)	95 ± 12	108 ± 18	109 ± 13
whisker length range (mm)	69–124	60–130	72–130
collection day of year $\overline{x}$ ± SD	30 Nov ± 49 d	8 Oct ± 7 d	15 May ± 26 d
collection day of year range	3 Oct–27 Apr	2 Oct–25 Oct	29 Apr–14 Jul
collection years	2009–2010	2009–2010	2009–2010
Cortisol			
combined sections (lost *via* error)	1 (7)	0 (0)	32 (5)
sections analyzed	67	112	115
sections below detectable limit	5	4	18
concentration $\overline{x}$ ± SD (pg/mg)	6.0 ± 5.8	3.1 ± 2.0	2.5 ± 1.9
concentration range (pg/mg)	0.6–28.4	0.3–10.9	0.3–12.6
Stable isotopes			
sections analyzed	245	257	445
$\delta^{15}N$ $\overline{x}$ ± SD (‰)	17.2 ± 1.3	17.3 ± 1.0	15.6 ± 0.7
$\delta^{15}N$ range (‰)	13.6–20.8	13.7–19.3	13.7–17.8
$\delta^{13}C$ $\overline{x}$ ± SD (‰)	−17.6 ± 1.3	−16.6 ± 0.7	−14.4 ± 0.5
$\delta^{13}C$ range (‰)	−21.3–−15.2	−20.8–−14.9	−17.0–−12.9

degree of narrowing at the whisker tip, distal SI section lengths were usually increased to 2–4 mm. After sections for SI analysis were removed, the remaining pieces were combined to form 3–7 larger sections. Samples that were analyzed for cortisol concentrations, from single or combined sections, were ≥15 mm and ranged from 0.7 and 15.5 mg (Fig. S1).

Cortisol Extraction

Whisker sections (n = 294, Table 1) designated for cortisol analysis were homogenized to a fine powder using a mixer mill (Retsch model

MM301, Retsch Inc., Newtown, PA) or a Wig-L-Bug Almalgamator (Crescent Dental MFG Co.). Cortisol was extracted by adding 0.5 mL of HPLC-grade methanol to each powdered sample (0.7–15.5 mg) and placing samples on a slow rotator for 24 h at room temperature. Samples were then centrifuged for 15 min at 2,150 g at 20ºC and the supernatant was collected. To ensure all extracted cortisol was recovered, the powdered samples were rinsed twice more with 0.5 mL methanol, vortexed for 1 min, centrifuged, and the supernatant from each methanol rinse was combined in the same tube and dried under a gentle stream of nitrogen gas at 38ºC. The extract was reconstituted with 0.2 mL phosphate buffer for 24 h at 4ºC. Sample cortisol concentrations were analyzed in triplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Oxford EA-65 Cortisol EIA kit, Oxford Biomedical, Lansing, MI). To ensure sufficient sample mass for cortisol extraction two (n = 29) or three (n = 4) adjacent sections were combined prior to analysis, 12 samples produced no results due to lab error, and 27 were below the detection limit (BDL) of the assay (Table 1). If the result was BDL, 50% of the detectable limit was used to estimate cortisol concentrations for that section (Cohen and Ryan 1989).

Performance characteristics of the ELISA were determined using five harbor seal whiskers. Intraassay and interassay percent coefficients of variation (% CV) were 7.36% (n = 5) and 9.41% (n = 10), respectively. Parallelism between serially diluted harbor seal whisker extracts and the ELISA kit standard curve was observed (r^2 = 0.998, $P < 0.001$). Cortisol extraction efficiency was 99.8% ± 4.8% (± SEM, n = 3). Optimal sample mass was determined to be 5 mg, approximately 15 mm of whisker length, but some distal sections were longer to accommodate for the narrowing of the whisker toward the tip and some samples had lower masses from material loss when transferring between containers.

Stable Isotopes

Whisker sections (n = 947, Table 1) designated for SI analysis were prepared using modified methods from Nash *et al.* (2009). Each section was placed individually into a vial, sonicated in a 2:1 chloroform and methanol solution for 30 min, dried at 60ºC for 30 min, sonicated in deionized water for 30 min, dried again, weighed, and sealed into a tin capsule for SI analysis. Stable carbon and nitrogen isotope analyses of whisker sections were conducted at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks using an elemental analyzer (Costech Analytical Technologies, Inc., ESC 4010) interfaced to an isotope ratio mass spectrometer (ThermoFisher, DeltaPlusXP). Typical precision for sample analysis was SD ≤ 0.05‰ for $\delta^{13}C$ values and SD ≤ 0.07‰ for $\delta^{15}N$ values. Results are presented in the conventional delta (δ) notation as parts per thousand (‰) deviation from the international standards VPDB (carbon) and N_{AIR} (nitrogen), using the equations $\delta^{15}N = [(\text{sample } {}^{15}N/{}^{14}N)/(\text{standard } {}^{15}N/{}^{14}N) - 1] \times 1{,}000$ or $\delta^{13}C = [(\text{sample } {}^{13}C/{}^{12}C)/(\text{standard } {}^{13}C/{}^{12}C) - 1] \times 1{,}000$.

Table 2. Generalized additive mixed models (GAMs) describing differences in cortisol concentrations, $\delta^{13}C$, or $\delta^{15}N$ along the length of whiskers (*Lw*) and between sexes (*Sx*). Data for each species were modeled separately and predictor variables were considered statically significant. Adjusted proportions of variance explained (r^2_{adj}), model log-likelihoods [log($\mathscr{L}$)], second order Akaike information criterion (AICc), delta AIC (Δ_{ic}), and model weights (w_i) are shown. See Tables S1–S3 for a list of the top five models.

Measure	Seal sp.	Model terms	r^2_{adj}	log($\mathscr{L}$)	AICc	Δ_{ic}	w_i
Cortisol	Ringed	*Lw*	0.46	−69.4	163.7	0.00	0.55
Cortisol	Spotted	*Lw*	0.72	−27.1	103.9	0.00	0.55
Cortisol	Harbor	*Sx* + *Lw* + *Lw* × *Sx*	0.59	−63.3	193.3	0.00	0.93
$\delta^{13}C$	Ringed	*Lw*	0.53	−303.5	657.0	0.00	0.48
$\delta^{13}C$	Spotted	null	0.15	−252.4	539.1	1.41	0.19
$\delta^{13}C$	Harbor	*Lw*	0.45	−168.7	407.5	0.00	0.61
$\delta^{15}N$	Ringed	null	0.29	−357.0	755.0	1.68	0.16
$\delta^{15}N$	Spotted	null	0.39	−283.8	611.3	0.00	0.41
$\delta^{15}N$	Harbor	*Sx* + *Lw*	0.49	−249.9	573.9	0.00	0.57

Factors influencing whisker cortisol and SIs—To estimate trends in whisker cortisol, $\delta^{13}C$, and $\delta^{15}N$ values (response variables) with respect to seal sex (*Sx*) and the location along the whiskers where sample sections were obtained (*Lw*), generalized additive mixed models (GAMs) were used. Models were run independently for each species. For each response variable, a global model was constructed that included sex as a factor variable, location along the whisker as a penalized cubic regression spline (CRS) smoother term, and a sex and location along the whisker (*Lw* × *Sx*) interaction term as a CRS smoother term. Smoother terms were limited to five degrees of freedom ($k = 6$). Statistically significant predictors were chosen using AIC model selection (Burnham and Anderson 2002) (Table 2, see Appendix S1 for all model results).

Associations between cortisol and SIs—SI sections were both smaller than and unevenly distributed within cortisol sections (Fig. S1). As a result, the timescale represented by SIs was mismatched with respect to that of cortisol concentrations. To address this, trends in $\delta^{13}C$ and $\delta^{15}N$ values were modeled with respect to location along the whisker of each whisker using least-squares second-degree local polynomial regressions (Cleveland and Devlin 1988). Optimal fit was achieved by adjusting the degree of smoothing (*i.e.*, "span" parameter, 0.33 for all splines) to ensure suitable visual fit to SIs. To allow a comparison between cortisol concentrations and SIs on the same time scale, estimates for $\delta^{13}C$ and $\delta^{15}N$ values, temporally aligned with cortisol concentrations, were calculated as the mean of 100 evenly spaced predicted values along the polynomial trend curves within lengths of whisker representing each section that was homogenized and analyzed for cortisol concentrations.

After the trends in cortisol and $\delta^{13}C$ and $\delta^{15}N$ values were described, we then used GAMs to estimate the strength of associations between cortisol and averaged $\delta^{13}C$ and $\delta^{15}N$ values while controlling trends in

cortisol concentrations associated with sex and location along the whisker (described above in *Factors influencing whisker cortisol and SIs)*. A global model was constructed with cortisol as the response variable, and sex, location along the whisker, $\delta^{13}C$, and $\delta^{15}N$ values included as predictor variables both as main effects and in two-way interactions. Sex was included as a factor variable while all other effects were included as smoother terms (CRS, $k = 6$).

All GAM analyses were conducted using R software version 3.3.1 (R Core Team 2015) and model fitting was performed using R package "gamm4" (Wood and Scheipl 2016). All GAMs used an identity link function, assumed a Gaussian (*i.e.*, normal) distribution for residuals, and were fit by maximum likelihood. Models included a whisker-specific random intercept and *Lw* slope term to account for sample correlations. Model residuals were visually inspected to detect nonlinear trends and ensure that they were normally distributed against all predictors. During preliminary inspections, it was found that cortisol concentrations (response variable) needed to be transformed using a natural logarithm to meet modeling assumptions. For more detailed modeling information, see Appendix S1.

Results

Whisker Cortisol Concentrations

Cortisol concentrations were highest near the root and declined in subsequent sections moving toward the tip (Fig. 1A, 2B, 3A, and 3B). However, all three species had some notable exceptions to the observed patterns. For example, whiskers from five ringed (Fig. 1A), two spotted (Fig. 1B), and two harbor seals (Fig. 2B) had the highest cortisol concentrations in sections distal to the root, and two of these whiskers had the highest concentrations in the distal half of the whiskers (Fig. 1B, 2B). The highest cortisol concentrations and the largest degree of variation were found in ringed seal whiskers (Table 1). Average cortisol concentrations in spotted and harbor seal whisker sections were lower than ringed seal whiskers by 2.9 pg/mg and 3.1 pg/mg, respectively (Table 1).

Whisker SIs

There was a high degree of inter- and intrawhisker variability in whisker SI values. Harbor seal whisker $\delta^{15}N$ values were lower than ringed and spotted seal whiskers by 1.7‰ and 1.6‰, respectively (Table 1). Harbor seal whiskers had the highest average $\delta^{13}C$ value, spotted and ringed seal whiskers averages were 2.2‰ and 3.2‰ lower, respectively (Table 1).

Trends in Whisker Cortisol Concentrations

Location along the whisker was a significant predictor of cortisol concentrations in the whiskers of both ringed ($F = 44.6$, $P < 0.001$; estimated degree of freedom, edf = 1.0) and spotted seals ($F = 23.9$, $P < 0.001$, edf = 2.9), however, sex was not (Table 2). In harbor seal whiskers, sex ($\beta_{\text{males}} = -0.04$, SE = 0.15), location along the whisker

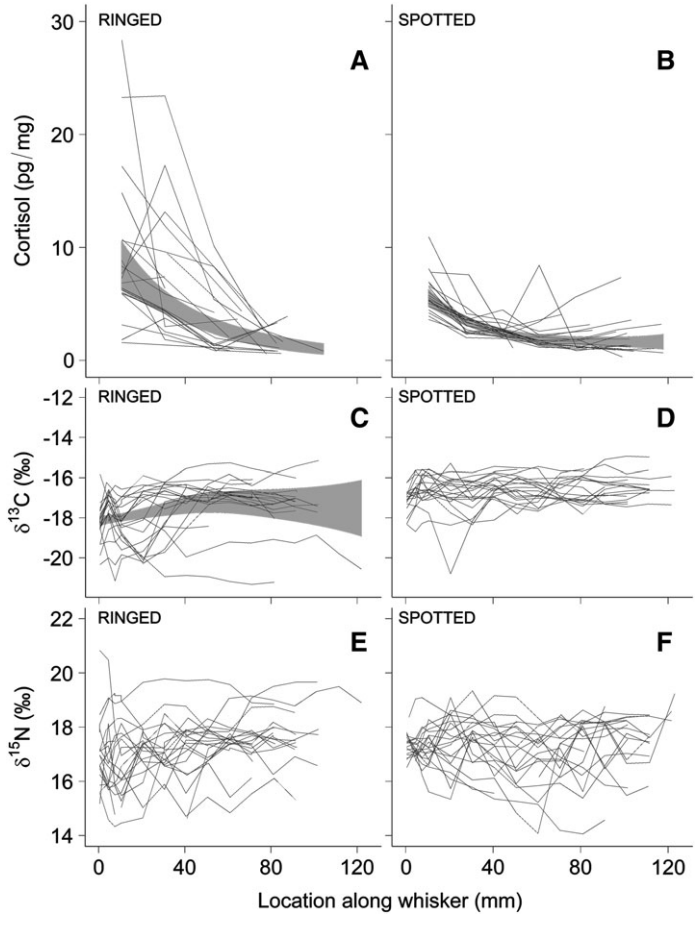

Figure 1. Measured values of cortisol concentrations, δ^{13}C, and δ^{15}N along the length of whiskers of ringed and spotted seals (black lines) and the 95% confidence intervals for model predicted responses (gray regions). Predicted response trends were based on best fit generalized additive mixed models. Cortisol concentrations declined curvilinearly across the length of whiskers of both ringed (A) and spotted (B) seals while δ^{13}C values exhibited a slight increase from root to about 50 mm (C). There was no significant trend in δ^{13}C along whiskers of spotted seals (D). Additionally, no significant trends were found for δ^{15}N along whiskers of ringed (E) or spotted seals (F).

($F = 20.8$, $P < 0.001$, edf = 2.1), and an interaction between the two (*Lw* × *Sx)* ($F = 4.94$, $P = 0.029$, edf = 1.0), were statistically significant predictor variables for cortisol concentrations (Table 2). The shape and amplitude of the trends in cortisol concentrations along the length of the whiskers differed markedly between species. Cortisol concentrations declined sharply from root to tip in ringed seal whiskers (Fig. 1A), from root to about 80 mm in spotted seal whiskers (Fig. 1B), and gradually from root to tip in harbor seal whiskers (Fig. 2A, B). Compared to

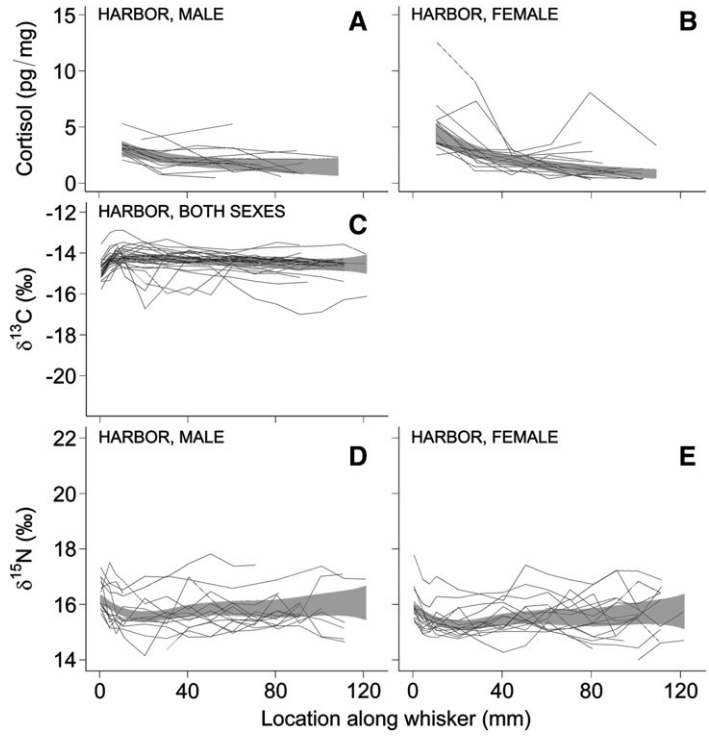

Figure 2. Measured values of cortisol concentrations, $\delta^{13}C$, and $\delta^{15}N$ along the length of whiskers of male and female harbor seals (black lines) and 95% confidence intervals for the predicted response (gray region). Predicted response trends were based on the best fit generalized additive mixed models. Along the length of harbor seal whiskers, cortisol concentrations declined in both sexes but the degree of decline was less steep in males (A) compared to females (B). Values of $\delta^{13}C$ exhibited a short and steep rise followed by a long gradual decline over the length of whiskers. Overall whisker $\delta^{15}N$ values were higher for males (D) than for females (E) but both sexes exhibited a similar trend of a short decline and then gradual rise along the lengths of whiskers.

female harbor seals, whisker cortisol was slightly lower near the root for males (Fig. 2A, B). GAM trends suggested that cortisol concentrations in harbor seal whiskers were lower near the root and declined less steeply over whisker length compared to ringed and spotted seal whiskers.

Trends in Whisker $\delta^{13}C$ Values

Location along the whisker was a significant predictor of the $\delta^{13}C$ value trends in the whiskers of both ringed ($F = 4.23$, $P = 0.005$, edf = 2.7) and harbor seals ($F = 5.84$, $P < 0.001$, edf = 5.07) but not spotted seals (Table 2). Additionally, $\delta^{13}C$ values did not vary by sex in any species (Table 2). In ringed seal whiskers, $\delta^{13}C$ values increased gradually from the root to about 50 mm but then exhibited no significant trend toward the tip (Fig. 1C). In harbor seal whiskers, $\delta^{13}C$ values increased

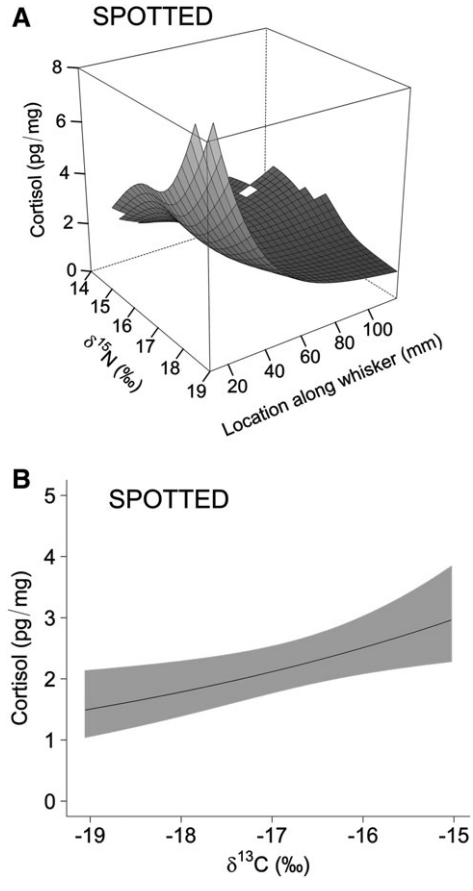

Figure 3. Response plots showing changes in spotted seal whisker cortisol concentrations in association with SI values. Trends in cortisol over the length of spotted seal whiskers varied with values of $\delta^{15}N$ (A) while cortisol was positively associated with $\delta^{13}C$ values independent of seal sex or location along the whiskers (B) with 95% confidence intervals for the predicted response (gray region).

sharply from the root to about 10 mm and then declined gradually toward the tip (Fig. 2C). No significant trends were found for $\delta^{13}C$ values in spotted seal whiskers because values, with some exceptions, appeared to remain relatively stable within and across whiskers (Fig. 1D).

Trends in Whisker $\delta^{15}N$ Values

Neither location along the whisker nor sex adequately explained variation in spotted or ringed seal whisker $\delta^{15}N$ values (Fig. 1E, F). In harbor seal whiskers, both location along the whisker ($F = 5.83$, $P < 0.001$, edf = 5.1) and sex ($\beta_{males} = 0.31$, SE = 0.08) were significant predictors of $\delta^{15}N$ values (Table 2). Male harbor seal whiskers had slightly higher

Table 3. Generalized additive mixed models (GAMs) describing associations between cortisol concentrations and interpolated $\delta^{13}C$ and $\delta^{15}N$ values after controlling for variation along the length of whiskers (*Lw*) and between sexes (*Sx*). Results are presented separately for each species. The model with only *Lw* was the best fit for ringed seal whisker data, which indicated no significant association between whisker cortisol concentrations and SI values. See Table 2 for complete description and definitions for statistics abbreviated below and Table S4 for a list of the top five models.

Seal sp.	Model terms	r^2_{adj}	log()	AICc	Δ_{ic}	w_i
Ringed	*Lw*	0.62	−53.5	149.9	0	0.32
Spotted	$Lw + \delta^{13}C + \delta^{15}N + \delta^{15}N \times Lw$	0.68	−32.5	126.0	0	0.36
Harbor	$Sx + Lw + Lw \times Sx + \delta^{15}N + \delta^{15}N \times Lw$	0.58	−62.7	197.0	0	0.62

$\delta^{15}N$ values overall, and for both sexes, $\delta^{15}N$ values declined from the root to 20 mm and then gradually increased toward the tip (Fig. 2D, E).

Associations Between Cortisol and SIs

After controlling for the previously found significant trend between whisker cortisol concentrations and location along the whisker, no additional associations were found between cortisol concentrations and SIs in ringed seal whiskers (Table 3).

For spotted seal whiskers, additional variation in cortisol concentrations was explained by $\delta^{13}C$ values ($F = 6.93$, $P = 0.01$, edf = 1.0), $\delta^{15}N$ values ($F = 1.87$, $P = 0.19$, edf = 3.0), and an interaction between $\delta^{15}N$ values and location along the whisker ($\delta^{15}NxLw$) ($F = 9.94$, $P = 0.002$, edf = 1.0) (Tables 3, S4). This indicates that once the trend in cortisol concentrations associated with location along the whisker (Fig. 1B) was accounted for, cortisol concentrations were highest in sections that also had higher $\delta^{15}N$ values near the whisker root, and cortisol concentrations near the root diminished in magnitude with respect to middle $\delta^{15}N$ values and disappear at the lowest $\delta^{15}N$ values (Fig. 3A). About 5 pg/mg of variation in cortisol concentrations could be attributed to $\delta^{15}N$ values near spotted seal whisker roots. At the tips of spotted seal whiskers, the association between cortisol concentrations and $\delta^{15}N$ values reverses and cortisol concentrations were lowest, instead of highest, in sections that had higher $\delta^{15}N$ values, accounting for a difference of ~1 pg/mg of variation in cortisol concentrations. Cortisol concentrations in spotted seal whiskers were positively associated with $\delta^{13}C$ values. This indicates that after the trend in cortisol associated with location along the whisker (Fig. 1B) was accounted for, about 1 pg/mg of increase in cortisol was associated with a 4‰ increase in $\delta^{13}C$ values (Fig. 3B).

Harbor seal whisker cortisol concentrations were associated with $\delta^{15}N$ values ($F = 2.43$, $P = 0.06$, edf = 2.41) and an interaction between $\delta^{15}N$ values and location along the whisker ($\delta^{15}N \times Lw$) ($F = 4.80$, $P = 0.01$, edf = 2.0). This means that after the trends in cortisol concentrations

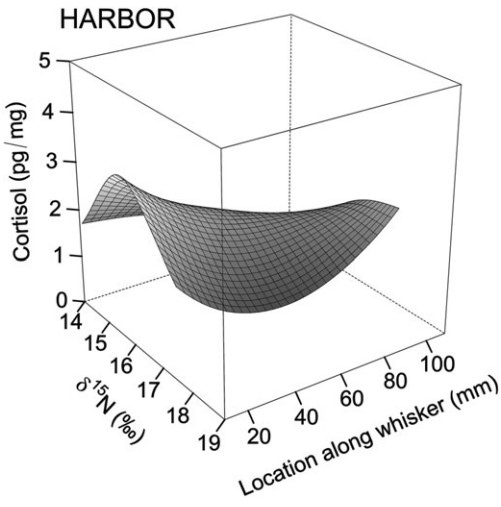

Figure 4. Three-dimensional predicted response of harbor seal whisker cortisol concentration trends in association with $\delta^{15}N$ values along the length of the whiskers.

associated with location along the whisker and sex (Fig. 2A, B) were accounted for, cortisol concentrations in harbor seal whisker root sections were highest in the mid-range of $\delta^{15}N$ values, and at the tip, cortisol concentrations were highest at the highest $\delta^{15}N$ values (Fig. 4).

Discussion

To our knowledge, this is the first study to show that steroid hormones can be measured in mammalian whiskers. Furthermore, we showed that all three phocid species had a persistent pattern of elevated cortisol near the whisker roots; and after controlling for this trend in cortisol concentrations across the whisker lengths and by sex, associations exist between cortisol concentrations and $\delta^{15}N$ and $\delta^{13}C$ values. These findings demonstrate that whiskers could provide important information about associations between diet and physiological status over time because SI values and cortisol concentrations are deposited sequentially with whisker growth and are therefore temporally aligned.

Cortisol

The general premise is that as hair or whiskers grow, cortisol is deposited into the keratin, concentrations measured in keratinous tissues are an index of circulating concentrations, and cortisol remains biochemically unchanged once deposited (Kirschbaum *et al.* 2009). We found that for all three species, the majority of cortisol concentrations measured in serial sections of whiskers exhibited a general pattern with the highest concentrations near the root followed by a curvilinear decrease moving towards the tip. The reason for this observed pattern in cortisol

concentrations is unknown, and possible causes include: (1) temporal changes in circulating cortisol concentrations, (2) differing rates of cortisol inclusion associated with nonlinear whisker growth rates (*i.e.*, concentration or dilution), (3) water immersion causing leaching of cortisol out of the whisker tissue, or (4) inclusion of nonkeratin tissues near the root. Therefore, future research should focus on the mechanisms of cortisol deposition and retention in growing whiskers.

To interpret cortisol concentrations in whiskers, it is necessary to understand when the whisker material included in each section was deposited. Phocid whiskers grow in an asymptotic pattern with very rapid growth that slows to little or no growth within a few months (Beltran *et al.* 2015, Lübcker *et al.* 2016, McHuron *et al.* 2016). Spotted seal whiskers in the McHuron *et al.* (2016) study initiated growth in April and reached asymptotic length within 4 mo. Assuming the whiskers in this study grew and molted in a similar fashion, only a small proportion of the proximal section would be added during the period after asymptotic length was acquired which could be up to 8 mo depending on collection date. It is possible that the elevated cortisol concentrations near the root of ringed, spotted, and harbor seal whiskers reflects a period of high cortisol concentrations in circulation that occurred during the time that the section was deposited. However, the timing associated with the elevated cortisol is difficult to estimate due to the slow growth rate at the whisker root section, the wide range of whisker collection dates, and the fact that the proximal section represents an average of cortisol over 20 mm of whisker material.

It is also possible that cortisol is incorporated into the whisker material at different rates. During the initial rapid whisker growth, cortisol concentrations may be diluted by the large amount of material being deposited, and then more concentrated when whisker growth slows near the root. The whiskers in this study were collected at or near asymptotic lengths, and thus all of the root sections represent the slowest portion of the growth curves; consequently we could not determine if cortisol is deposited differentially during different whisker growth rates.

Another possibility is that persistent immersion in sea water could cause the cortisol to leach out of the whisker material over time. In serial sections of hair from monkeys and humans, cortisol concentrations decreased significantly with repeated exposure to soap and water (Hamel *et al.* 2011, Li *et al.* 2012). This would suggest that phocid whiskers could lose cortisol with repeated exposure to sea water. However, the permeability of the cuticle of hair or whiskers may be substantially different when comparing hair or whiskers handled in the lab to those on live animals. For example, archived harbor seal whiskers, when dyed with commercially available hair dye, readily absorbed the dye and turned a dark black color that was maintained during several months of exposure to moving seawater; yet when the same dye was applied to the whiskers of live seals, the whiskers turned slightly gray and no apparent coloration could be detected after 24 h (Alaska Department of Fish and Game, unpublished data). It seems that the dye readily permeated the cuticle of archived whiskers but could not penetrate the

whisker cuticle on live animals. This suggests that permeability of the whisker cuticle may have changed after the whisker was removed from the animal. Therefore, leaching may occur more readily in a laboratory setting when the hair or whisker cuticle may be different, perhaps more desiccated, than when on a live animal.

Lastly, blood or skin was manually removed from the exterior of whiskers in this study to avoid affecting the results. However, nonkeratin material may be included in the whisker medulla because reddish spots have been observed in the interior of some phocid whiskers root sections (Alaska Department of Fish and Game, unpublished data). This tissue would not be removed by cleaning the outer surface prior to analyses, and the inclusion of blood-related material would cause cortisol concentrations to be elevated due to the stress-related release of cortisol into circulation during capture or harvest (*e.g.*, Sapolsky 1990, Wingfield 2003). Further, inclusion of blood or plasma in the whisker root would result in lower $\delta^{13}C$ but unchanged $\delta^{15}N$ compared to the rest of the whisker (Beltran *et al.* 2016). Lower $\delta^{13}C$ and higher $\delta^{15}N$ at the root of southern elephant seal (*Mirounga leonina*) whiskers was attributed to the inclusion of molecules other than keratin at the whisker root (Lübcker et al. 2016). In this study, spotted seal whiskers had no SI trends; ringed seal whiskers had lower $\delta^{13}C$ at the root compared to the rest of the whisker length and no trend in $\delta^{15}N$; and harbor seal whiskers had lower $\delta^{13}C$ but higher $\delta^{15}N$ at the root compared to the rest of the whisker. Thus, it is unclear from the results of this study if the elevated cortisol concentrations and the SI values at the root of the whiskers sampled could reflect the inclusion of blood-related or other compounds at the root.

Associations Between Cortisol and SIs

The approach used in this study revealed associations between whisker cortisol concentrations and $\delta^{13}C$ and $\delta^{15}N$ values after controlling for variation in cortisol across sexes and along the length of whiskers, but, we do not endeavor to explain these trends. However, associations between these measurements could provide evidence about how life history and behavior can influence individual physiology, and thus success and survival. For example, higher $\delta^{13}C$ is associated with consumption of nearshore/benthic prey and lower $\delta^{13}C$ with offshore/pelagic prey (*e.g.*, Hobson and Welch 1992). Extending that understanding, one might hypothesize that changes in foraging locations, prey availability, or both (as described by changes in $\delta^{13}C$) contributed to different levels of physiological stress for seals (as indicated by changes in cortisol concentrations).

The associations between cortisol concentrations and $\delta^{15}N$ values in spotted and harbor seal whiskers include an interaction with location along the whisker indicating that the association changes throughout the period of whisker growth. For spotted seals, during the period that the most proximal whisker section was deposited, cortisol concentrations were much higher (4 pg/mg) at 19‰ compared to 14‰ $\delta^{15}N$; suggesting that foraging at higher trophic levels contributed to

physiological changes resembling stress (*i.e.*, higher cortisol concentrations). Alternately, the elevated $\delta^{15}N$ values and cortisol concentrations could represent a period of fasting or nutritional stress when protein stores were being mobilized. While seals initially utilize blubber stores to meet energetic needs, there is a point that they switch to protein mobilization to maintain a layer of blubber for insulation (Rosen and Renouf 1997, Mellish *et al.* 2007), and use of body protein stores would increase $\delta^{15}N$ values (*e.g.*, Hobson *et al.* 1993). In spotted seal whisker sections distal to the root, the association between cortisol concentration and $\delta^{15}N$ values flattens, and near the tip the association reverses and higher cortisol is associated with feeding at a lower tropic level. If the lower tropic level prey is of lower quality or more difficult to access, this would agree with previous studies on mammals and birds showing stress-related hormones are inversely correlated food availability (Kitaysky *et al.* 1999) and body mass and condition (Bartsh *et al.* 1992, Barboza *et al.* 2004, Cockrem *et al.* 2006, George *et al.* 2014).

For harbor seals, during the period that the most proximal whisker section was deposited, cortisol concentrations were slightly higher (~1 pg/mg) when $\delta^{15}N$ was 15‰ and 16‰ compared to both higher and lower $\delta^{15}N$ values. This association is lower in magnitude than the spotted seal whiskers. Furthermore, moving toward the tip of harbor seal whiskers, higher cortisol concentrations were associated with higher $\delta^{15}N$ values; this could mean that during the period that the whisker tip was grown, higher stress was associated with feeding on higher trophic level prey or foraging in a manner or area that allows consumption of higher trophic level prey.

The associations described here demonstrate the potential utility of extracting naturally aligned dietary and physiological information from whiskers. The methods presented in this study could facilitate future research tracking how marine or terrestrial mammals are responding to habitat alterations associated with changes in climate or anthropogenic influences.

Conclusions and Recommendations

To understand how cortisol and SIs in whiskers are related, controlled studies should be conducted. Skin or blood-related tissues in whiskers could contribute to elevated cortisol concentrations at the proximal sections, nonlinear whisker growth could cause concentration or dilution of cortisol, and leaching could cause depressed cortisol concentrations in distal sections. It is, therefore, important to determine whether cortisol concentrations relate to seal physiology in a similar way throughout the whisker and, if not, what factors affect the relationship between the concentration of cortisol at the time of deposition and the measurements of cortisol and SIs obtained from whiskers in the laboratory.

For future studies, we recommend several approaches for studying trends in whisker cortisol. First validations of whisker cortisol should be conducted using captive seals *via* exposure to different stress scenarios or ACTH injections, paired with marking of the whiskers to track whisker growth. Whisker collections should be conducted across all months,

and paired measurements of cortisol concentrations in whisker roots and circulation should be conducted. Additionally, to determine if whisker growth rate affects cortisol deposition, cortisol concentrations in phocid whiskers collected prior to being fully grown (less than at asymptotic lengths) and whiskers from other species with constant growth rates should be examined. Studies should be conducted to assess if skin or blood-related tissues are incorporated at the root of whiskers and if cortisol is lost through leaching. Finally, in this study we assumed the laboratory validation of harbor seal whiskers was sufficient for ringed and spotted seal whiskers. However, prior to using whisker cortisol concentrations, laboratory validations should be conducted on whiskers from each species.

The cortisol concentrations presented here for phocid whiskers raise many questions. However, this study has determined that cortisol is present in measurable concentrations in phocid whiskers, varies along the length of the whiskers, and can be measured with commercially available ELISAs. Furthermore, after the unexplained curve of cortisol concentrations was controlled for, we found associations between $\delta^{13}C$ and $\delta^{15}N$ values and cortisol concentrations in spotted and harbor seal whiskers. Analysis of whisker cortisol concentrations could be a powerful tool to gather long-term physiological information from marine and terrestrial animals, but further research is needed to gain a better understanding of the source and stability of the cortisol in whiskers.

Acknowledgments

This work was supported by the National Fish and Wildlife Federation (grant number 28217), the National Marine Fisheries Service (NMFS) (grant number NA11NMF4390200), the North Pacific Research Board (grant number 1528), and the US Fish and Wildlife Service (grant number AK-22). The samples included in this study were collected under NMFS permits issued to ADF&G (permit #s 1000, 358-1585, and 358-1787). Harbor seals were live captured and handled under ADF&G IACUC approvals 07-16 and 09-08. The authors would like to thank all of the participants from the harbor seal capture trips for collecting harbor seal whiskers and the ADF&G Arctic Marine Mammal Program for providing ringed and spotted seal whiskers. We acknowledge Mandy Keogh, Lori Quakenbush, Patrick Charapata, Mark Nelson, and three anonymous reviewers for providing manuscript edits. Although the research described has been funded in part by the National Oceanic and Atmospheric Association (NOAA), it has not been subjected to NOAA review and therefore does not necessarily reflect the views of NOAA, and no official endorsement should be inferred. Reference to trade names does not imply endorsement by NMFS, NOAA, or ADF&G.

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Received: 14 February 2017
Accepted: 27 June 2018

Supporting Information

The following supporting information is available for this article online at http://onlinelibrary.wiley.com/doi/10.1111/mms.12546/suppinfo.

Appendix S1. This appendix contains additional details about the model selection process, tables showing the top five candidate models for each comparison, and a map of the sections removed from each whisker for analyses.