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INTERNATIONAL WHALING COMMISSION

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ABSTRACT

The western gray whale (*Eschrichtius robustus*) subpopulation is currently listed as critically endangered, as designated by the IUCN. The IWC Scientific Committee previously stressed the value of studies on reproductive status and feeding ecology for this subpopulation. Here, we report analyses conducted on thirty-five individuals biopsied off Sakhalin Island in 2011(6 whales), 2012 (20 whales) and 2013 (9 whales), including the optimization of progesterone assays in that species for the purpose of determining reproductive fitness and pregnancy and the analysis of C and N stable isotopes to assess chemical feeding ecology.

Pregnancy detection via blubber progesterone analysis has been previously validated in several cetacean species but not in gray whales. Using stranded eastern gray whale samples: 1) the extraction of steroid hormones and analysis of progesterone has been successfully validated, and 2) the amount of blubber needed for the progesterone extraction and pregnancy determination has been successfully reduced and optimized from 150 mg to 50 mg (wet weight). Western gray whale blubber samples were obtained from three female whales of unknown life-stage in 2011 and from three adult male and two adult female whales in 2013. Only small epidermis samples stored in ethanol were obtained in 2012, they did not prove suitable for progesterone or stable isotope analyses, as expected from the literature. Sections of blubber (no epidermis) weighing 50 mg were analyzed for all 2011 and 2013 animals. Female progesterone levels (n=5) ranged from levels below the detection limit of the ELISA kit (0.01ng/g) up to 3.02 ng/g, and male progesterone levels (n=3) ranged from 0.02-0.39 ng/g. Progesterone levels in pregnant gray whales are currently unknown but the female western gray whale progesterone values detected were below those reported in non-pregnant mature cetacean species, including the short-beaked common dolphin, *D. delphis,* (6.75-33.3 ng/g) and the Pacific white-sided dolphin, *L. obliquidens,* (3.75-20.5 ng/g), and at the low end of the range reported in non-pregnant northern right-whale dolphins, *L. borealis,* (2.11-34.7 ng/g) (Kellar *et al*. 2006). Extraction efficiency was within the range previously reported for the steroid hormone extraction used (Kellar *et al*. 2006, 2009), which allows distinction to be made between pregnant and non-pregnant female cetaceans. Thus, analyses indicated the female gray whales were likely not pregnant when biopsied. Further optimization is currently underway and includes 1) measuring progesterone in adult female gray whales of known reproductive status, and 2) further reducing minimum sample size required for assay from 50 mg to 30 mg for the determination of pregnancy.

Isotopic ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were determined in 10 western gray whale epidermis biopsies (3 from 2011, 7 from 2013) (>10mg) with underlying tissues removed. $\delta^{13}C$ ranged from -17.66 to -14.97, while $\delta^{15}N$ ranged from 11.21 to 14.26, indicating a relatively broad range of values that may reflect variation in feeding ecology (e.g., feeding areas, trophic levels). Validation studies using stranded eastern gray whales indicated that stable isotope ratios in gray whale epidermis is largely independent of epidermal layer (depth) despite differences in function and morphology.

Introduction

The western gray whale (*Eschrichtius robustus*) subpopulation is currently listed as critically endangered, as designated by the IUCN. The IWC Scientific Committee previously stressed the value of studies on reproductive status and feeding ecology for this subpopulation. Here, we report analyses conducted on thirty-five individuals biopsied off Sakhalin Island in 2011(6 whales), 2012 (20 whales) and 2013 (9 whales), including the optimization of progesterone assays in that species for the purpose of determining reproductive fitness and pregnancy and the analysis of C and N stable isotopes to assess chemical feeding ecology.

Western gray whale biopsy samples were obtained from The International Whaling Commission (IWC, 2011 samples), the Institute of Ecology and Evolution of the Russian Academy of Sciences (IPEE RAS), and the A.V. Zhirmunsky Institute of Marine Biology Far Eastern Branch (IBM FEB RAS) for three females of unknown life-stage and one male of unknown life-stage from 2011, eleven adult females, five adult males, one juvenile female and three juvenile males from 2012, and three adult females, four adult males, one juvenile female and one juvenile male from 2013.

Methods

Collection and Storage: Western Gray Whale Biopsies

Biopsy samples were obtained through collaborations with the IWC, IPEE RAS, and IBM FEB RAS. Frozen samples were shipped on ice packs and samples stored in ethanol were shipped at room temperature to The Institute of Environmental and Human Health, Lubbock, TX. Frozen samples were subsequently shipped on dry ice to the Wildlife Toxicology Laboratory, Fairbanks, AK for stable isotope analyses. Frozen samples were stored in -80^{o}C freezer until analyses. A detailed inventory of samples is presented below in Table 1.

Collection and Storage: Eastern Gray Whale Samples

Frozen biopsy samples and tissues from stranded animals were obtained through a collaboration with The Universidad Autónoma de Baja California Sur (UABC) and The Marine Mammal Center (TMMC), respectively. All samples were shipped on dry ice and stored in -80^{o}C freezer until processing and analyses. Inventory and hormone analyses were performed at The Institute of Environmental and Human Health, Lubbock, TX and stable isotope analyses were conducted at The Wildlife Toxicology Laboratory, Fairbanks. Due to the nature of the sample collections, subsamples with ample amounts (mass) of dermis and hypodermis were received from stranded animals. Thus, sample mass is not reported in the detailed inventory of eastern gray whale samples presented below in Table 2.

Table 1: Western gray whale inventory

SOURCE ID	TTU ID	SPECIES	YEAR	LOCALITY	TISSUE	STORAGE	MASS (mg)
2011							
R042	ER-12-925	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	80.00
R044	ER-12-926	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin epidermis	ETOH	117.00
R045	ER-12-927	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin epidermis	ETOH	96.00
R048	ER-12-928	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin epidermis	ETOH	104.00
R049	ER-12-929	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin epidermis	ETOH	43.00
M401	ER-12-930	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin epidermis	ETOH	86.00
b1-2	ER-12-931	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	45.00
b2-2	ER-12-932	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	115.00
b3-2	ER-12-933	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	185.00
b4-2	ER-13-934	*Eschrichtius robustus*	2011	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	179.00
2012							
1	ER-14-0147	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	36.60
2	ER-14-0148	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	21.45
3	ER-14-0149	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	13.02
4	ER-14-0150	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	41.22
5	ER-14-0151	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	44.31
6	ER-14-0152	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	28.00
7	ER-14-0153	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	5.90
8	ER-14-0154	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	18.47
9	ER-14-0155	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	12.92

10	ER-14-0156	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	19.98
11	ER-14-0157	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	27.19
12	ER-14-0158	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	25.25
13	ER-14-0159	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	38.57
14	ER-14-0160	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	24.04
15	ER-14-0161	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	8.47
16	ER-14-0162	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	25.15
17	ER-14-0163	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	27.27
18	ER-14-0164	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	13.19
19	ER-14-0165	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	44.50
20	ER-14-0166	*Eschrichtius robustus*	2012	Piltun, Sakhalin Island, Russia	Skin epidermis	ETOH	14.20
2013							
1	ER-14-0167	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	4.58
2	ER-14-0168	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	582.81 (290.72 skin)
3	ER-14-0169	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	569.08
4	ER-14-0170	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	410.42 (288.05 skin)
5	ER-14-0171	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	406.70 (252.90 skin)
6	ER-14-0172	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	132.20 (69.90 skin)
7	ER-14-0173	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	264.43 (129.5 skin)
8	ER-14-0174	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	80.81
9	ER-14-0175	*Eschrichtius robustus*	2013	Piltun, Sakhalin Island, Russia	skin/blubber biopsy	FROZEN	193.56 (98.65 skin)

Table 2: Eastern gray whale tissue inventory, data provided courtesy of The Marine Mammal Center and The Universidad Autónoma de Baja California Sur

Whale ID	TTU ID	Sex	Age	Tissue Freshness	Species	Examination Date	COD	Collection Location
C-117	ER-13-088	M	Adult	Moderate	Gray Whale	5/5/2000	Unknown	Sausalito, CA
C-126	ER-13-093	M	Juvenile	Fresh	Gray Whale	5/31/2000	Unknown	Richmond, CA
C-139	ER-13-092	M	Calf	Moderate	Gray Whale	2/25/2001	Maternal Separation	Morro Bay, CA
C-341	ER-13-090	M	Adult	Fresh	Gray Whale	4/19/2011	Boat strike	San Francisco, CA
C-317	ER-13-029	M	Juvenile	Moderate	Gray Whale	4/21/2010	Unknown	Richmond, CA
LOL-18Mar14-110-ER	ER-14-278	F	Adult	N/A (biopsy)	Gray Whale	3/18/2014	N/A (biopsy)	Laguna Ojo de Liebre, Mexico
LOL-21Mar14-139-ER	ER-14-295	F	Adult	N/A (biopsy)	Gray Whale	3/21/2014	N/A (biopsy)	Laguna Ojo de Liebre, Mexico

Biochemistry Analyses

Pregnancy testing in gray whale was optimized following methodologies established in other cetacean species (Mansour *et al*. 2002, Kellar *et al*. 2006; Perez *et al*. 2011). Eastern gray whale blubber samples obtained from stranded animals were received from The Marine Mammal Center (F. Gulland and others) and were used for the validation and optimization of this methodology in the gray whale species. Due to the small size of biopsy samples, reduction of tissue amount for pregnancy determination and biochemistry analyses from 150 mg to 50 mg wet weight (ww) was necessary. Four blubber sections (no epidermis) were analyzed from each eastern gray whale sample: two 150 mg sections and two 50 mg sections. One 150 mg and one 50 mg sections were used as a positive control and spiked with 300 ng/g of progesterone (P4). This progesterone level is within the range reported in pregnant cetaceans (Kellar *et al*. 2006). Detailed sample processing procedures are described below. After calculating amounts of progesterone following the instructions provided within the ELISA kit (Enzo Life Sciences, Farmingdale, NY), extraction efficiencies were calculated by subtracting the amount of progesterone in a sample from the amount of progesterone in the same-weight spiked sample and dividing by spike amount (300 ng/g).

Progesterone analysis was conducted on all western gray blubber samples with sufficient frozen material (50 mg ww) for assay requirements: i.e. samples from three female whales of unknown life-stage in 2011 and from three adult male and two adult female whales in 2013. Samples excluded from the analyses included one sample from 2011 (male) due to inadequate amounts of blubber for sample processing and pregnancy determination (<<50 mg ww), any of the samples from 2012 because of ethanol storage, and four samples from 2013 (one adult female, one adult male, one juvenile female, and one juvenile male) due to inadequate amounts of blubber for sample processing and pregnancy determination (<<50 mg ww). Two adult female eastern gray whale samples collected in 2014 were also analyzed because they had recently given birth and elevated levels of progesterone was suspected. Due to their small size, neither the western gray whale samples nor the two female eastern gray whale samples contained enough material to analyze a spiked control for the purpose of determining extraction efficiency. To address this issue, two 50 mg ww blubber sections (one from each of two eastern adult male gray whale samples collected in 2013) were analyzed alongside the western gray whale samples. One section was used as a positive control and spiked with 300 ng/g of progesterone and extraction efficiency was calculated as mentioned above. A blank and a blank spiked at 300 ng/g were also run as quality control samples and for the purpose of determining extraction efficiency. 50 mg sections were placed into separate homogenization tubes and homogenized for eight 45 s periods at a speed of 6.5 m/s on a FastPrep® 24 benchtop homogenizer (MP Biomedicals, Solon,OH). The homogenate was washed using ethanol and run through a series of steroid extractions using ethanol, acetone, diethyl ether, acetonitrile, and hexane. After the addition of each chemical(s), samples were mixed by vortex, centrifuged, and evaporated with nitrogen gas using a Biotage TurboVap® LV (Biotage, Charlotte, NC). After the final extraction P4 levels were quantified

via an ELISA assay kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. Samples and standards (100µl) were placed into 96 well plates treated with goat anti-mouse IgG. Progesterone alkaline phosphatase conjugate and progesterone monoclonal antibody were added to all wells, excluding blanks and specified control wells. Plates were incubated at room temperature on a plate shaker for 2 hours then washed in triplicate with tris buffered saline. P-nitrophenyl phosphate in buffer (200 µl) was added to each well and incubated for 45 min at room temperature without shaking. A final stop solution of 50µl of trisodium phosphate in water was added to each well and the plate was read on a Synergy 4 plate reader (BioTek, Winooski, VT) with an absorbance of 405 nm with a correction of 570 nm.

Stable Isotope Analyses

Due to the small nature of biopsy samples, full thickness epidermis samples from eastern gray whales were used to evaluate potential variability of stable isotope ratios based on sampling depth in gray whale species. Gray whale epidermis has three distinct morphological and functional layers: the protective outer layer, stratum corneum; the middle layer, stratum spinosum; and the regenerative layer, stratum basale (Reeb *et al.* 2007). Distinct sampling layers for stable isotope analysis included C (stratum corneum), D1 (only stratum spinosum), D2 (primarily stratum spinosum) and D3 (primarily stratum basale). 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 blubber from D3.

Biopsies from epidermis of western gray whales (frozen, not stored in ethanol) were very small (4-40mg). Most samples had a small amount of blubber 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 stable isotopes. Epidermal samples from both eastern gray whales (subsampled by layer) and western gray whales (biopsies) were freeze dried for 48 hours and homogenized using a tissue mill or Wig-L-Bug™ in the Wildlife Toxicology Laboratory at the University of Alaska Fairbanks (UAF). Isotopic ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) 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 (δ) notation relative to an international standard (Vienna PeeDee Belemnite for $\delta^{13}C$ and atmospheric air for $\delta^{15}N$) in parts per thousand (‰).

Isotopic ratios of carbon and nitrogen of different epidermal layers in eastern gray whales were compared using repeated measures ANOVA; in the case of $\delta^{13}C$ using a Friedman repeated measures ANOVA by rank, followed by a Tukey test to isolate groups that were statistically different ($p < 0.05$); in the case of $\delta^{15}N$ using a one-way repeated measures ANOVA as assumptions of normality and equal variance were met.

Results and Discussion

Biochemistry Analyses

A summary of the biochemical analyses for the eastern gray whale sample size optimization is presented below in Table 3:

Table 3: Eastern gray whale analyses for sample size optimization

Sample	Sex	Life-Stage	Progesterone (ng/g ww)	Sample Weight	Extraction Efficiency
ER-13-093	M	Juvenile	0.27	150 mg	71.87%
ER-13-093	M	Juvenile	0.23	50 mg	42.48%
ER-13-092	M	Calf	0.34	150 mg	54.38%
ER-13-092	M	Calf	0.34	50 mg	40.76%
ER-13-029	M	Juvenile	3.19	150 mg	33.42%
ER-13-029	M	Juvenile	1.27	50 mg	90.63%
ER-13-088	M	Adult	0.39	150 mg	36.78%
ER-13-088	M	Adult	0.13	50 mg	95.32%
ER-13-090	M	Adult	0.21	150 mg	48.36%
ER-13-090	M	Adult	0.09	50 mg	90.30%

Progesterone was detectable and quantifiable in the 50 mg ww blubber samples analyzed. It was noted that the 50 mg ww blubber samples homogenized much better in the lysing matrix, and the 150 mg ww blubber samples often did not homogenize completely and tended to overwhelm the lysing matrix. As the technician became more skilled at the protocol, extraction efficiencies using 50 mg ww blubber samples reached or exceeded 90%. Progesterone levels detected in the 50 mg ww blubber samples were similar or close to half those detected in the corresponding 150 mg ww blubber samples. In all cases, the progesterone levels were very low and far below levels known to indicate pregnancy in other cetacean species (Kellar *et al.* 2006, Mansour *et al.* 2002). High variation in replicate samples associated with the ELISA methodology has been reported before but has been shown to be inconsequential in pregnancy determination due to the large gap existing between progesterone levels in pregnant and non-pregnant animals (Kellar *et al*. 2006). Thus a 50 mg ww blubber sample size was selected for all subsequent analyses.

A summary of the biochemical analyses conducted on the western gray whale samples (50 mg) is presented in Table 4 below. Progesterone levels detected ranged from below the limit of detection (LOD, 0.01ng/g ww) to 3.02 ng/g ww in females and from 0.02 to 0.39 ng/g ww in males with a mean of 0.22 ng/g ww. The two adult male eastern gray whale samples analyzed as controls had a mean progesterone level of 0.30 ng/g ww. The two female eastern gray whale biopsy samples had progesterone levels of 0.07 and 0.39 ng/g ww. The extraction efficiency for these analyses ranged from 52.8% to 72.5%, and the mean extraction efficiency of 64.1% was

used to determine level of P4 for all samples. These extraction efficiencies are slightly lower but within the range of extraction efficiencies from Kellar *et al.* 2006 (63.3%-95.9%; mean: 71.1%) and Kellar *et al.* 2009 (60.0%-98.9%; mean: 72.1%) which both utilize the same steroid hormone extraction protocol.

Table 4: Western gray whale analyses with associated Eastern gray whale controls

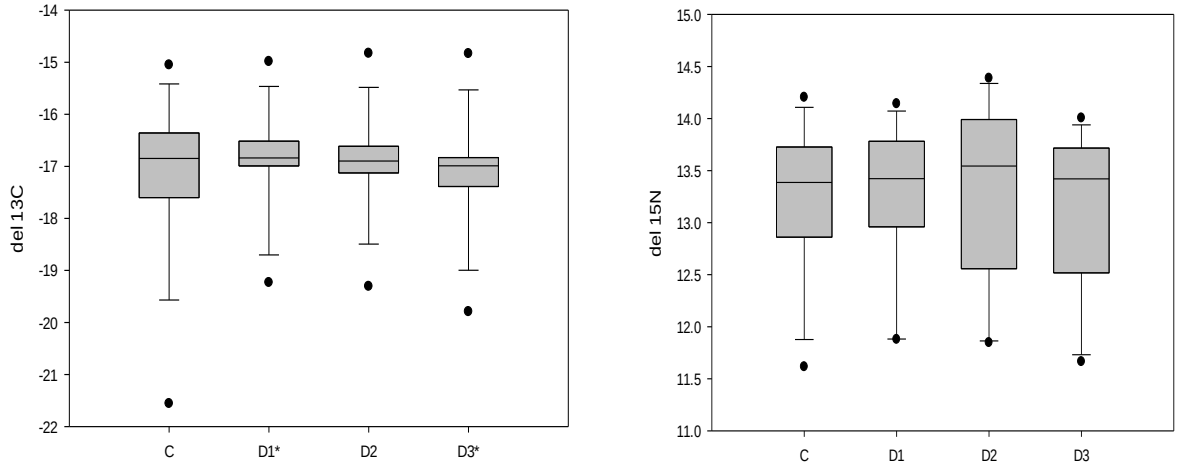
Sample	Sex	Life-Stage	Progesterone (ng/g ww)	Collection Location
ER-12-932	F	Unknown	3.02	Piltun, Sakhalin Island, Russia
ER-12-933	F	Unknown	0.46	Piltun, Sakhalin Island, Russia
ER-12-934	F	Unknown	<LOD	Piltun, Sakhalin Island, Russia
ER-14-0168	M	Adult	0.24	Piltun, Sakhalin Island, Russia
ER-14-0170	M	Adult	0.39	Piltun, Sakhalin Island, Russia
ER-14-0171	F	Adult	<LOD	Piltun, Sakhalin Island, Russia
ER-14-0173	F	Adult	<LOD	Piltun, Sakhalin Island, Russia
ER-14-0175	M	Adult	0.02	Piltun, Sakhalin Island, Russia
ER-13-088	M	Adult	0.26	Sausalito, CA
ER-13-090	M	Adult	0.33	San Francisco, CA
ER-14-0278	F	Adult	0.39	Laguna Ojo de Liebre, Mexico
ER-14-0295	F	Adult	0.07	Laguna Ojo de Liebre, Mexico

Progesterone levels in pregnant gray whales are currently unknown but the female western (and eastern) gray whale progesterone values detected were below those reported in non-pregnant mature cetacean species, including the short-beaked common dolphin, *D. delphis*, (6.75-33.3 ng/g) and the Pacific white-sided dolphin, *L. obliquidens*, (3.75-20.5 ng/g), and at the low end of the range reported in non-pregnant northern right-whale dolphins, *L. borealis*, (2.11-34.7 ng/g) (Kellar *et al*. 2006). Extraction efficiency was within the range previously reported for the steroid hormone extraction used (Kellar *et al*. 2006, 2009), which allows distinction to be made between pregnant and non-pregnant female cetaceans. Reported progesterone levels in confirmed pregnant female cetaceans (*D. delphis* (132-415 ng/g), *L. borealis* (196-402 ng/g), *L. obliquidens* (161 ng/g), and *B. acutorostrata* (22.8-454 ng/g)) are significantly different and show no overlap when compared to non-pregnant female cetaceans of the same species (*D. delphis* (6.75-48.2 ng/g), *L. borealis* (0.98-34.7 ng/g), *L. obliquidens* (0.11-34.4 ng/g), and *B. acutorostrata* (1.36-3.43 ng/g) (Kellar *et al.* 2006, Mansour *et al.* 2002). Thus, analyses indicated the female western gray whales were likely not pregnant when biopsied. Further optimization is currently underway and includes 1) measuring progesterone in adult female eastern gray whales of known reproductive status, and 2) further reducing minimum sample size required for assay from 50 mg to 30 mg for the determination of pregnancy.

Stable Isotopes

Isotopic ratios of $\delta^{13}C$ and $\delta^{15}N$ in eastern gray whale epidermis at different depths are presented in Figure 1 below. The only statistically significant difference in isotopic ratios based on epidermal depth in eastern gray whales was between D1 and D3 for $\delta^{13}C$ (Chi-square = 12.6000, 3DF, P = 0.006). Overall, the D1 layer was slightly more enriched for ^{13}C, but the difference was small and likely not biologically significant (mean difference between paired $\delta^{13}C$ for D1 and D2 for was 0.22 ± 0.21). Most of the biopsies from the western gray whales had small amounts of blubber attached, so they were compared to values from eastern gray whales collected from the layer adjacent to the blubber (D3). However, given the lack of statistically or biologically significant differences in stable isotopes in different epidermal layers, it seems reasonable to compare biopsies even if they have been collected from different epidermal layers.

Figure 1: Isotopic ratios of carbon (del 13C; $\delta^{13}C$) and nitrogen (del 15N: $\delta^{15}N$) in ‰ at different depths in epidermis of eastern gray whales - C (stratum corneum), D1 (only stratum spinosum),



D2 (primarily stratum spinosum) and D3 (primarily stratum basale). Statistically significant differences between layers ($p < 0.05$) are indicated by an asterisk.

Median $\delta^{13}C$ and $\delta^{15}N$ for western and eastern gray whale epidermis are presented in Table 5. While there are similarities between the two groups, the relationships between $\delta^{13}C$ and $\delta^{15}N$ were different in each group (Fig. 2).

Table 5: Isotopic ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in western and eastern gray whale epidermis. D3 represents the layer of epidermis adjacent to the blubber.

	$\delta^{13}C$		$\delta^{15}N$	
	Mean (± S.D)	Median	Range	Mean (± S.D)
Western gray whale biopsy (n = 10)	-16.52 (±0.82)	-16.64	-17.66 – -14.97	12.20 (±0.85)
Eastern gray whale D3(n = 15)	-17.10 (±1.07)	-16.99	-19.79 – -14.85	13.15 (±0.76)

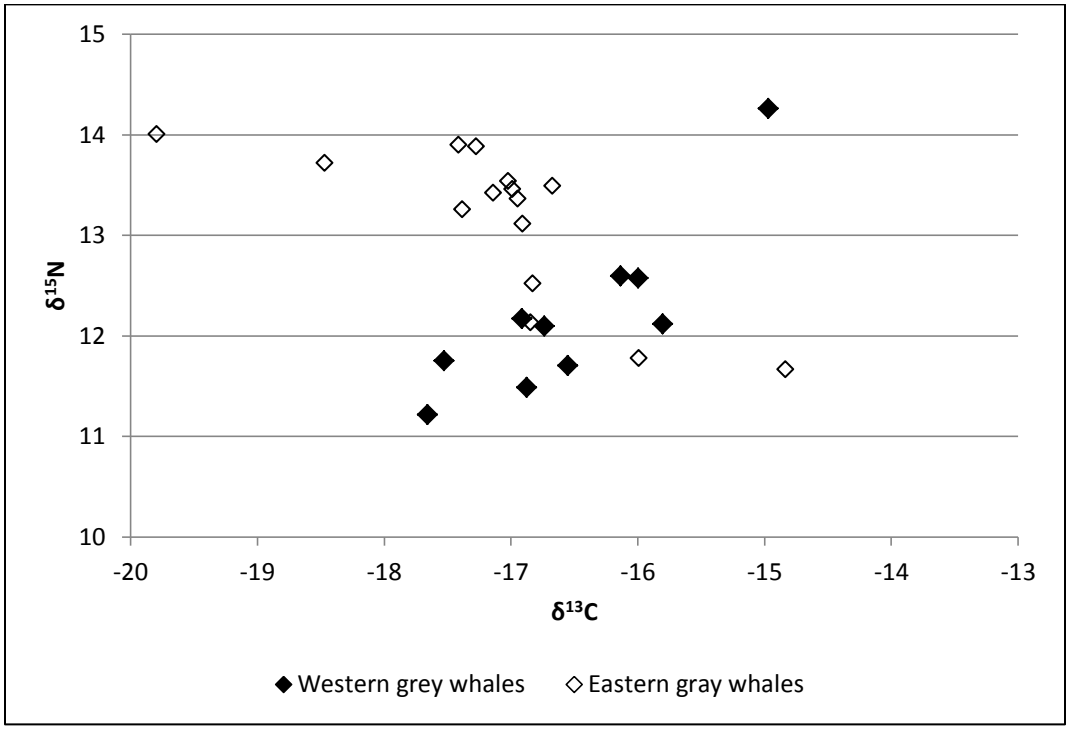


Figure 2: Relationships between $\delta^{13}C$ and $\delta^{15}N$ values in epidermis from western and eastern gray whales.

For the eastern gray whales higher $\delta^{15}N$ were associated with moderate to low $\delta^{13}C$ values, while in the western gray whales the opposite occurred. In general, western gray whales had relatively low $\delta^{15}N$ compared to the eastern population, with all but one value below 12.6, likely indicating lower trophic level feeding. For one individual (ER-12-933, female) $\delta^{15}N$ was comparable to the higher values in the eastern population. The wide range of $\delta^{13}C$ (2.5 – 5 units) indicates potentially broad foraging areas for both groups. It is important to note that eastern gray whale samples came from carcasses of stranded individuals, while samples from western gray whales were biopsies collected from free ranging animals with varying locations of sampling. Thus, comparisons are somewhat limited.

Conclusions

Biochemistry Analyses

Female P4 levels (n=5) ranged from levels below the detection limit of the ELISA kit (0.01ng/g) up to 3.02 ng/g, and male P4 levels (n=3) ranged from 0.02-0.39 ng/g. From these data, it was determined that the female whales analyzed for progesterone were not pregnant. This conclusion was met by comparing the gray whale progesterone levels to other confirmed pregnant female cetaceans such as the short-beaked common dolphin, *D. delphis*, (132-415 ng/g), the northern right-whale dolphin, *L. borealis*, (196-402 ng/g), the Pacific white-sided dolphin, *L. obliquidens*, (161 ng/g), and the minke whale, *B. acutorostrata*, (22.8-454 ng/g) (Kellar *et al.* 2006, Mansour *et al*. 2002). The P4 values detected in the female western gray whales are just below the P4 concentrations seen in other non-pregnant mature marine mammal species, including the short-beaked common dolphin, *D. delphis*, (6.75-33.3 ng/g) and the Pacific white-sided dolphin, *L. obliquidens*, (3.75-20.5 ng/g), and within the range detected in northern right-whale dolphin, *L. borealis*, (2.11-34.7 ng/g) (Kellar *et al*. 2006). Our detected P4 levels for males were within the range reported for minke whales, *B. acutorostrata*, (0.81-3.33 ng/g) (Mansour *et al.* 2002).

Further optimization is currently underway and includes 1) measuring progesterone in adult gray whale females of known reproductive status 2) further reducing minimum sample size required for assay. Once progesterone levels in pregnant or recently pregnant females are measured, we will investigate the suitability of further reducing the amount of blubber needed for pregnancy determination from 50 mg to 30 mg ww. We hypothesize that this smaller amount of material may be suitable for pregnancy determination but not absolute progesterone quantitation due to inherent limitations associated with ELISA methodology. We are currently investigating a Liquid Chromatography/Mass Spectrometry methodology that may be suitable to both pregnancy determination and absolute progesterone quantitation using small blubber samples.

Stable isotopes

Stable isotopes of C and N values were determined in epidermis biopsies that were >10mg, where it was possible to remove blubber and maintain a sufficient mass of sample. In smaller samples, it was very difficult to fully remove blubber, which would confound stable isotope analyses. Validation studies from the larger eastern gray whale samples indicate that C and N isotope ratios in epidermis are largely independent of the layer from which the sample is taken, in spite of morphological and functional differences. Thus, it seems reasonable to compare biospies even if they have been collected from different epidermal layers. However, we cannot comment on different biopsy locations. There was a relatively wide range of both $\delta^{13}C$ and $\delta^{15}N$ values in western gray whale epidermis that may reflect variation in feeding ecology (e.g.

feeding areas, trophic levels). The patterns of $\delta^{13}C$ and $\delta^{15}N$ in the epidermis of western gray whales was substantially different from those of eastern gray whales, though one must consider that the samples from the western gray whales came from free ranging animals, while those from the eastern population came from carcasses of stranded animals.

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Literature Cited

IUCN. 2012. Report of the western gray whale advisory panel at its eleventh meeting, February 2012, Geneva, Switzerland. [Available from https://cmsdata.iucn.org/downloads/wgwap_11_report_eng.pdf]

Kellar, N.M., M.L. Trego, C.I. Marks, A.E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22(1): 1-16.

Kellar, N.M., M.L. Trego, C.I. Marks, S.J. Chivers, K. Danil, and F.I. Archer. 2009. Blubber testosterone: A potential marker of male reproductive status in short-beaked common dolphins. Marine Mammal Science, 25:1-16.

Laake, J., A. Punt, R. Hobbs, M. Ferguson, D. Rugh, and J. Breiwick. 2009. Re-analysis of gray whale southbound migration surveys 1967-2006. NOAA Technical Memorandum. NMFS-AFSC-203. 55pp.

Lang, A. R., D.W. Weller, R. LeDuc, A. M. Burdin, V.L. Pease, D. Litovka, V. Burkanov, and R.L. Brownell, Jr. 2011. Genetic analysis of stock structure and movements of gray whales in the eastern and western North Pacific. Paper SC/63/BRG10 presented to the International Whaling Commission Scientific Committee (Unpublished). 20 pp. [Available at http;//www.iwcoffice.org]

Mansour, A., D. McKay, J. Lien, J. Orr, J. Banoub, N. Oien, and G. Stenson. 2002. Determination of pregnancy status from blubber samples in minke whales (Balaenoptera acutorostrata). Marine Mammal Science 18(1):112-120.

Perez, S., A. Garcia-Lopez, R. De Stephanis, J. Gimenez, S. Garcia-Tiscar, P. Verborgh, et al. 2011. Use of blubber levels of progesterone to determine pregnancy in free-ranging live cetaceans. Marine Biology 158(7): 1677-1680.

Rea, L.D., J.M. Castellini, L. Correa, B.S. Fadely, and T.M. O'Hara. 2013. Maternal Steller sea lion diets elevate fetal mercury concentrations in an area of population decline. Science of the Total Environment 454-455: 277-282.

Reeb, D., P.B. Best, and S.H. Kidson. 2007. Structure of the integument of southern right whales (Eubalaena australis). The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology. 956-613.

Urbán R.J., D. Weller, O. Tyurneva, S. Swartz, A. Bradford, Y. Yakovlev, O. Sychenko, H. Rosales N., S. Martínez A., A. Burdin and A. Gómez-Gallardo U. 2012. Report on the photographic comparison of the western and Mexican gray whale catalogues. SC/64/BRG13

Weller, D. W. and R. L. Brownell, Jr. 2012. A re-evaluation of gray whale records in the western North Pacific. Paper SC/64/BRG10 presented to the International Whaling Commission Scientific Committee (Unpublished). 4 pp. [Available at http;//www.iwcoffice.org]

Weller et al. 2011. Movements of western gray whales from the Okhotsk Sea to the eastern North Pacific. Paper SC/63/BRG6 presented to the International Whaling Commission Scientific Committee (Unpublished). 5 pp. [Available at http;//www.iwcoffice.org]