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INTERNATIONAL
WHALING COMMISSION

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[1]MARY HAYDEN, [2]RUCHIKA BHAWAL, [2]JOHN ESCOBEDO, [2]CLINTON HARMON, [1]DAVID KLEIN, [2]SUSAN SAN-FRANCISCO, [2]MASOUD ZABET-MOGHADDAM, [3]TODD O'HARA, [4]JOHN BICKHAM, AND [1]CÉLINE A.J. GODARD-CODDING

[1]*The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX, USA*

[2]*Center for Biotechnology and Genomics, Texas Tech University, Lubbock, TX, USA*

[3]*Department of Veterinary Medicine, University of Alaska Fairbanks, Fairbanks, AK, USA.*

[4]*Batelle Memorial Institute, Houston, TX, USA and Texas A&M University, College Station, TX USA*

Contact email: celine.godard@ttu.edu

ABSTRACT

The value of studies regarding the reproductive and stress status of the IUCN critically endangered western gray whale (*Eschrichtius robustus*) subpopulation has been repeatedly stressed by the IWC Scientific Committee. Biopsy samples from western gray whales off Sakhalin Island have been collected in collaboration with Russian scientists since 2011. These biopsy samples have continually provided invaluable yet limited material to assess hormone concentrations, genetics and stable isotopes of C and N, as previously reported by our team (Hayden *et al.* 2016, Gendron *et al.* 2015, Bickham *et al.* 2015, Bickham *et al.* 2013). Here we report on the progesterone and testosterone analysis of 14 western gray whale biopsies collected in 2014 (2 immature males, 1 adult male, 2 males of unknown life-stage, 2 immature females, 1 adult male, and 6 females of unknown life-stage) using ELISA methodologies. Based on progesterone levels reported in other cetacean species, the females analyzed here are likely not pregnant. Concentrations of testosterone in gray whale blubber biopsies have never been assessed. The values reported here are in the lower end of values reported in pubertal and immature males for the short-beaked common dolphin (Kellar *et al.* 2009).

We previously validated reproductive hormone profiling in gray whales using ELISA (Gendron *et al.* 2015) and, more recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Hayden *et al.* 2016). Here we also report on the further optimization and validation of using LC-MS/MS with nano electrospray ionization for hormone analysis in blubber samples. A nanoLC/MS/MS method was validated for the analysis of three steroid hormones, without derivatization, extracted from 50 mg blubber samples. Data was acquired with an LTQ XL ion trap mass spectrometer in positive ion mode, using single reaction monitoring. All three steroids were analyzed in a single run with cholic acid used as a surrogate internal standard for quantitation due to its steroidal structure and lack of measurable endogenous levels in blubber. The lowest limits of quantitation for progesterone, testosterone, and hydrocortisone were significantly improved compared to previous studies using conventional LC-MS/MS. The lowest limit of detection was 7 fg/µL using a 1 µL injection volume. Calibration curves for steroid quantification showed good linearity (r^2>0.99) between 14 - 3,620 fg/µL. After validation, the method was successfully applied to quantification of steroids in eastern gray whale blubber samples. Using our nanoLC-MS/MS method, three steroid hormones were successfully quantitated in three eastern gray whale 50 mg blubber samples. Progesterone was detected in two samples, and the concentration quantitated in the female blubber sample was higher than the progesterone concentration of the male blubber sample. Testosterone was detected in two male blubber samples with the adult male having a higher concentration than the juvenile. Hydrocortisone was detected in two of the three blubber samples.

Future studies include 1) further validating the nanoLC-MS/MS methodology by comparing it to a paired ELISA analysis, 2) analyzing other cetacean blubber samples to validate a cetacean-wide protocol, and 3) incorporating additional steroid hormones to the current panel to produce a more complete reproductive and fitness/stress assessment. Also, the extracts from the 14 2014 western gray whale samples will be analyzed using the nanoLC-MS/MS methodology for the method comparison study.

INTRODUCTION

Information on stress, reproductive fitness and overall health, while difficult to obtain, is critical for the conservation and management of wild marine mammal populations. There is extensive literature reporting on the relevance and significance of hormone analyses in providing fundamental information on health and fitness status in wildlife (Keay *et al.* 2006, Soldin and Soldin 2009). However this area of research is quite recent in the marine mammal field. Analyses of steroid hormones from marine mammals has been conducted in a variety of matrices including serum (Kellar *et al.* 2013, Kellar *et al.* 2015, Champagne *et al.* 2017), muscle (Yoshioka *et al*. 1994), urine (Kellar *et al.* 2013), feces (Rolland *et al*. 2005, Hunt *et al*. 2006, Biancani *et al*. 2009, Burgess *et al*. 2012, Champagne *et al.* 2017), earwax (Trumble *et al*. 2013), baleen (Hunt *et al*. 2014), blow (Hogg *et al.* 2009, Hunt *et al.* 2014, Thompson *et al.* 2014), skin including blubber (Mansour *et al*. 2002, Kellar *et al*. 2006, Kellar *et al*. 2009, Perez *et al.* 2011, Kellar *et al.* 2013, Trego *et al.* 2013, Bechshoft *et al.* 2015, Kellar *et al*. 2015, Trana *et al.* 2015, Clark *et al*. 2016, Champagne *et al*. 2017). Reproductive status, more specifically pregnancy status, has been successfully evaluated using progesterone analyses in several species including the minke whale, *Balaenoptera acutorostrata*, the short-beaked common dolphin, *Delphinus delphis*, the northern right-whale dolphin, *Lissodelphis borealis*, the Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, the common bottlenose dolphin, *Tursiops truncatus*, the dugong, *Dugong dugon*, the bowhead whale, *Balaena mystiatus*, the long-beaked common dolphin, *Delphinus capensis*, the pantropical spotted dolphin, *Stenella attenuata*, the eastern spinner dolphin, *Stenella longirostris*, the Dall's porpoise, *Phocoenoides dalli*, and the humpback whale, *Megaptera novaeangliae* (Mansour *et al*. 2002, Kellar *et al*. 2006, Burgess *et al.* 2012, Perez *et al.* 2011, Kellar *et al.* 2013, Trego *et al.* 2013, Clark *et al.* 2016). One study in the short-beaked common dolphin, *D. delphis*, investigated the use of testosterone analysis as a potential marker of male reproductive status (Kellar *et al.* 2009). Stress and fitness status has been assessed in the harbor porpoise, *Phocoena phocoena*, the short-beaked common dolphin, *D. delphis*, the common bottlenose dolphin, *T. truncates*, and the beluga whale, *Delphinapterus leucas*, via glucocorticosteroid analyses (Fair *et al.* 2014, Thompson *et al.* 2014, Bechshoft *et al.* 2015, Kellar *et al.* 2015, Champagne *et al.* 2017).

Skin biopsy collection provides the most reliable access to samples from live wild cetacean populations due to the sampling logistics associated with collecting serum, muscle, urine, feces, earwax, baleen, and blow. Steroid hormone analyses in the blubber matrix have primarily been conducted in animals that were stranded, by-caught, or killed in ship strike or native hunt (Mansour *et al*. 2002, Kellar *et al*. 2006, Kellar *et al*. 2009, Kellar *et al.* 2013, Trego *et al.* 2013, Kellar *et al*. 2015, Trana *et al.* 2015) with only three studies conducted in skin biopsies (Perez *et al.* 2011, Clark *et al.* 2016, Champagne *et al.* 2017). Hormone information from stranded, by-caught, or incidentally killed individuals is valuable but not necessarily reflective of population reproductive rates, fitness, or stress. Similarly, the stress experienced by an individual prior to stranding, capture, or death would likely influence glucocorticosteroids levels in the skin, thus making extrapolation to population problematic.

Current steroid hormone quantitation techniques in the blubber matrix include various immunoassays such as enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA). These assays are accompanied with multiple limitations including the requirement of a large blubber

sample (75-150 mg w/w) for each hormone and the variability of data generated from these assays due to the cross-reactivity of kit antibodies with steroid hormones of similar structure (Boots *et al.* 1998, Whitehead *et al.* 2013). The sample mass requirement makes these analyses less compatible with skin biopsy samples than with the large samples that can be obtained from necropsied individuals. Furthermore, skin biopsies are often divided for multiple additional studies such as genetic, stable isotope, fatty acid, biomarker, and contaminant analyses (Godard *et al.* 2004, Trujillo *et al.* 2004, Herman *et al.* 2005, Dehn *et al.* 2006, Fair *et al.* 2010, Godard-Codding *et al.* 2011, Balmer *et al.* 2015). We recently developed a liquid chromatography-tandem mass spectrometry with nano-electrospray ionization (nanoLC-MS/MS) method where multiple hormones can be analyzed from a single 50 mg w/w blubber sample (Hayden *et al.* 2017). With this method, questions regarding pregnancy rates, male reproductive rates, stress levels, and baseline hormone data can be answered simultaneously. NanoLC-MS/MS also allows for additional analyses as adequate blubber sample remains. Additionally, the use of analytical techniques such as nanoLC-MS/MS may help standardize the analysis of steroid hormones in marine mammals by generating accurate and precise quantitation data that is comparable between laboratories and species.

We report here on 1) ELISA blubber hormone analyses for both progesterone and testosterone in 14 western gray whales biopsied off Sakhalin Island in 2014 and 2) the validation of a nanoLC-MS/MS method for simultaneous blubber hormone analysis of progesterone, testosterone and hydrocortisone in samples obtained from eastern gray whales. Additionally, method comparison between ELISA and nanoLC-MS/MS blubber hormone analysis are underway in the 2014 western gray whale samples.

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 to The Institute of Environmental and Human Health, Lubbock, TX. Frozen samples were stored in -80^{o}C freezer until analyses. A detailed inventory of samples is presented below in Table 1.

Table 1: Western Gray Whale Sample Inventory

Whale ID	TTU ID	Sex	Age	Species	Collection Date	Collection Location
2	ER-16-0055	F	Unknown	Gray Whale	8/4/2014	Piltun, Sakhalin Island, Russia
10	ER-16-0046	M	Immature	Gray Whale	8/9/2014	Piltun, Sakhalin Island, Russia
13	ER-16-0045	F	Immature	Gray Whale	8/10/2014	Piltun, Sakhalin Island, Russia
14	ER-16-0054	F	Unknown	Gray Whale	8/16/2014	Piltun, Sakhalin Island, Russia
15	ER-16-0069	F	Unknown	Gray Whale	8/16/2014	Piltun, Sakhalin Island, Russia
19	ER-16-0070	M	Adult	Gray Whale	8/22/2014	Piltun, Sakhalin Island, Russia
22	ER-16-0049	M	Immature	Gray Whale	8/27/2014	Piltun, Sakhalin Island, Russia
23	ER-16-0059	F	Unknown	Gray Whale	8/28/2014	Piltun, Sakhalin Island, Russia
25	ER-16-0065	F	Adult	Gray Whale	8/28/2014	Piltun, Sakhalin Island, Russia
28	ER-16-0058	F	Immature	Gray Whale	9/3/2014	Piltun, Sakhalin Island, Russia
31	ER-16-0060	M	Unknown	Gray Whale	8/28/2014	Piltun, Sakhalin Island, Russia
32	ER-16-0063	F	Unknown	Gray Whale	8/28/2014	Piltun, Sakhalin Island, Russia
33	ER-16-0064	F	Unknown	Gray Whale	9/3/2014	Piltun, Sakhalin Island, Russia
34	ER-16-0051	M	Unknown	Gray Whale	9/3/2014	Piltun, Sakhalin Island, Russia

Collection and Storage: Eastern Gray Whale Samples

Tissues from stranded animals were obtained through collaboration with The Marine Mammal Center (TMMC). 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. 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 as they were relatively robust.

Table 2: Eastern Gray Whale Sample Inventory

Whale ID	TTU ID	Sex	Age	Tissue Freshness	Species	Collection Date	COD*	Collection Location
C-68	ER-13-031	F	Calf	Fresh	Gray Whale	10/10/1997	Maternal Separation	Point Arena, 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

*COD = cause of death

Steroid Hormone Extraction

Steroid hormone extraction was performed as described previously in other cetacean blubber samples (Mansour *et al*. 2002, Kellar *et al*. 2006; Perez *et al*. 2011). 50 mg samples from the eastern gray whales were analyzed via nanoLC-MS/MS. 75 mg samples from the western gray whales were analyzed via progesterone and testosterone ELISAs. For steroid extraction, either 50 or 75 mg sections were placed into separate homogenization tubes and homogenized 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 solvent, samples were mixed by vortex, centrifuged, and evaporated with dry nitrogen gas using a Biotage TurboVap® LV (Biotage, Charlotte, NC).

Samples for nanoLC-MS/MS analysis were filtered by syringe and cellulose filter, evaporated, centrifuged for 15 minutes at 3750 g, and dissolved in 50 µL of a 60:40 solution of LC-MS grade water:acetonitrile with 0.1% formic acid. Samples were stored in the -80^{o}C freezer until nanoLC-MS/MS steroid hormone analysis. Samples were spiked with a surrogate internal standard of 5 nM cholic acid for the purposes of quantitation. Samples were sonicated for 5 min and vortexed until dissolved to form a clear solution. Prior to injection, samples were diluted 10-fold with the same 5 nM cholic acid solution and 1 µL of diluted sample was injected for nanoLC-MS/MS analysis.

ELISA Steroid Hormone Analysis

The same sample extract was used for both progesterone and testosterone quantiation via ELISA. Samples were reconstituted in 500 µL of 1X Phosphate buffered saline: 1% bovine serum albumin. Progesterone levels were quantified via an ELISA assay kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. After calculating amounts of progesterone following the instructions provided with the ELISA kit, extraction efficiencies were calculated by subtracting the amount of progesterone in a blank sample from the amount of progesterone in the spiked blank sample and dividing by spike amount (300 ng/g). Progesterone concentrations were corrected according to the corresponding extraction efficiency.

Testosterone levels were quantified via an ELISA assay kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's protocol. Concentrations of testosterone were calculated following the instructions provided with the ELISA kit. The same extraction efficiency determined using the blank and spiked blank samples spiked with progesterone was used to correct the testosterone concentrations.

nanoLC-MS/MS Steroid Hormone Analysis

A Dionex Ultimate 3000 nano LC system (autosampler, degasser, two binary pumps, and an Acclaim Pepmap RSLC analytical column (75 µm x 15 µm, nanoviper, C18, 2µm, 100 A) from Thermo Scientific) was employed in the separation of progesterone, testosterone, hydrocortisone and cholic acid. MS detection was performed in the positive ionization mode on a Thermo LTQ XL linear ion trap mass spectrometer equipped with nano ESI spray. Selected reaction monitoring (SRM) method was used for analysis of steroids, and the steroids eluted at different retention times based on hydrophobicity: progesterone (23.24 min), testosterone (18.94 min), hydrocortisone (14.95 min), cholic acid (18.40 min). The Collision Induced Dissociation (CID) experiments were performed with ultrapure helium gas as a collision gas. The optimized conditions for the SRM were as follows: ion spray voltage, 1.90 kV and source temperature 220^{o}C. The precursor to product ion transitions used for the SRM transitions are summarized in Table 3.

Table 3: SRM transitions selected for three steroids and cholic acid as a surrogate internal standard. Positive ion mode was used for the analysis.

Steroid	Molecular Weight (Da)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CID (%)	Source Fragmentation Energy (V)
Progesterone	314.47	315.21	297.11	35	0
Testosterone	288.42	289.20	271.20	35	35
Hydrocortisone	362.46	363.21	327.10	35	35
Cholic Acid	408.51	373.00	355.00	35	25

Calibration curves were generated from six concentrations of analyte (with constant concentration of surrogate internal standard). The curves for progesterone, testosterone and hydrocortisone quantitation were generated with the following concentrations: 0.05 nM (16 fg/µL), 0.1 nM (31 fg/µL), 0.5 nM (157 fg/µL), 1 nM (314 fg/µL), 5 nM (1.572 pg/µL), and 10 nM (3.14 pg/µL). Cholic acid concentration was held constant at 5 nM, and concentrations were reported as peak area ratios. Surrogate internal standards for quantitation can be very useful (e.g. Kunze, *et.al* 2015), and cholic acid was selected as it is similar in structure to steroid hormones and lack of measurable accumulation in the blubber samples tested.

RESULTS

Quantification of steroids in 2014 western gray whale blubber samples using ELISA

Progesterone concentrations range from below the limit of detection (8.57 pg/mL) to 0.21 ng/g. Progesterone levels in pregnant gray whales have not yet been determined, but the female western gray whale progesterone values detected were below those reported in non-pregnant mature cetacean species, including the humpback whale, *Megaptera novaeangliae* (0.13-0.32 ng/g), 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 the northern right-whale dolphins, *L. borealis*, (2.11-34.7 ng/g) (Clark *et al.* 2016, Kellar *et al*. 2006). Pregnant female cetacean progesterone levels (*M. novaeangliae* (46.05-286.53 ng/g), *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 higher and show no overlap when compared to non-pregnant female cetaceans of the same species (Clark *et al.* 2016, Kellar et al. 2006, Mansour et al. 2002). Therefore, it is likely the female western gray whales shown here were not pregnant at the time of sample collection. Progesterone detected in male western gray whales is in the range reported in male humpback whales, *M. novaeangliae* (0.05-0.46 ng/g) (Clark *et al.* 2016).

Testosterone concentrations range from below the limit of detection (5.67 pg/mL) to 1.36 ng/g. Testosterone analysis in cetaceans has been utilized in the short-beaked common dolphin to determine male reproductive status in mature males (1.8-83.0 ng/g), pubertal males (1.2-6.7 ng/g), and immature males (0.6-5.6 ng/g) (Kellar *et al.* 2009). Concentrations of testosterone in gray whale blubber biopsies have never been assessed. The values reported here are in the lower end of values reported in pubertal and immature males for the short-beaked common dolphin.

Table 4: Progesterone and Testosterone concentrations measured in western gray whale blubber samples (LOD = Limit of Detection)

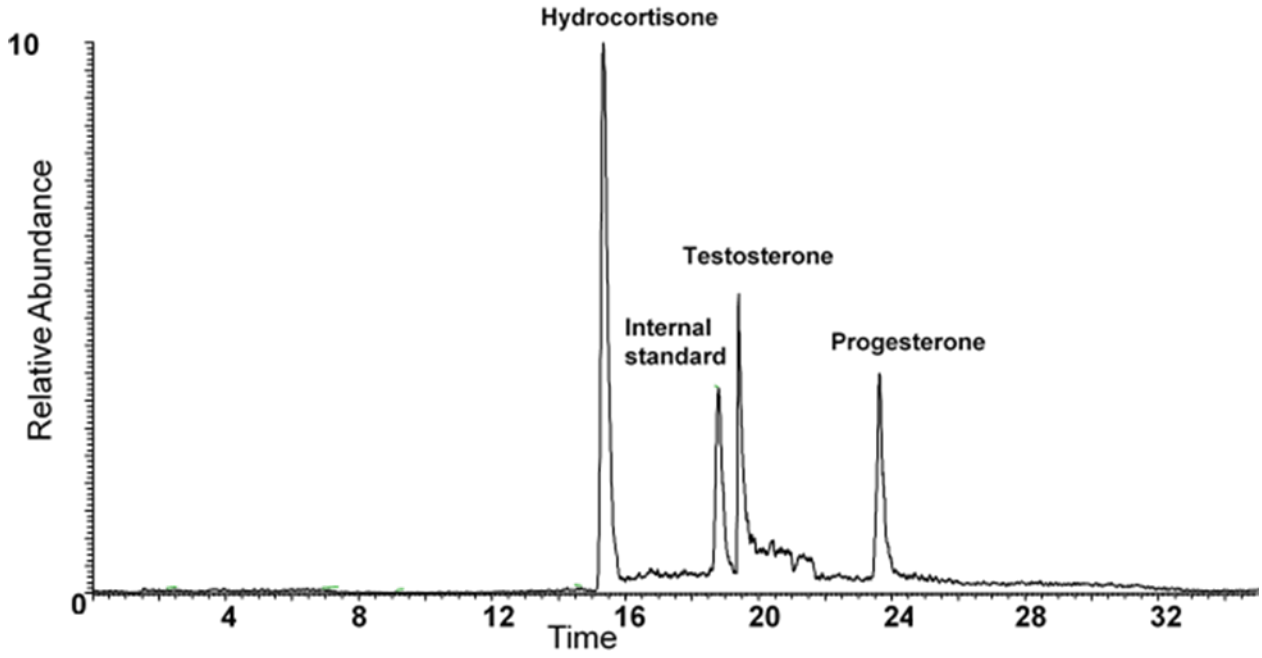
Sample ID	Sex	Age	Pregnancy Status	Sample Weight (g)	Progesterone ng/g	Testosterone ng/g
ER-16-0051 (34)	M	Unknown	N/A	0.075	0.07	1.36
ER-16-0045 (13)	F	Immature	N/A	0.075	<LOD	0.11
ER-16-0055 (2)	F	Unknown	N/A	0.075	<LOD	0.08
ER-16-0058 (28)	F	Immature	N/A	0.075	<LOD	<LOD
ER-16-0046 (10)	M	Immature	N/A	0.075	0.21	0.21
ER-16-0060 (31)	M	Unknown	N/A	0.075	<LOD	0.15
ER-16-0063 (32)	F	Unknown	N/A	0.075	<LOD	0.03
ER-16-0065 (25)	F	Adult	N/A	0.075	<LOD	<LOD
ER-16-0059 (23)	F	Unknown	N/A	0.075	<LOD	0.15
ER-16-0070 (19)	M	Adult	N/A	0.075	<LOD	0.20
ER-16-0069 (15)	F	Unknown	N/A	0.075	<LOD	0.05
ER-16-0049 (22)	M	Immature	N/A	0.075	0.04	0.09
ER-16-0064 (33)	F	Unknown	N/A	0.075	0.13	0.19
ER-16-0054 (14)	F	Unknown	N/A	0.075	<LOD	0.09

Qualitative analysis of steroids by nanoLC/MS/MS

This study reports the application of nanoLC-MS/MS to determine progesterone, testosterone, hydrocortisone, and cholic acid (as a surrogate internal standard) in gray whale blubber. An ion chromatogram of the four analytes of interest separated using this methodology is below (Figure 1).

The Limit of Detection (LOD) with this method was 7.8 fg/µL for progesterone, 7.2 fg/µL for testosterone and 9 fg/µL for hydrocortisone. This LOD is an improvement compared to that reported for the analysis of urinary steroids using conventional liquid chromatography where the LOD was 50 fg/µL (Allende *et al.* 2014). Using this methodology, no sensitivity or method limit of detection is lost when compared to ELISA methodology for progesterone (LOD = 8.57 fg/µL) and testosterone (LOD = 5.87 fg/µL), but this method shows great method limit of detection when compared to hydrocortisone ELISA methods (LOD = 56.72 fg/µL).

Figure 1. Total Ion Chromatogram of four analytes separated using the nanoLC-MS/MS method.



Quantitation of steroids using nanoLC-MS/MS

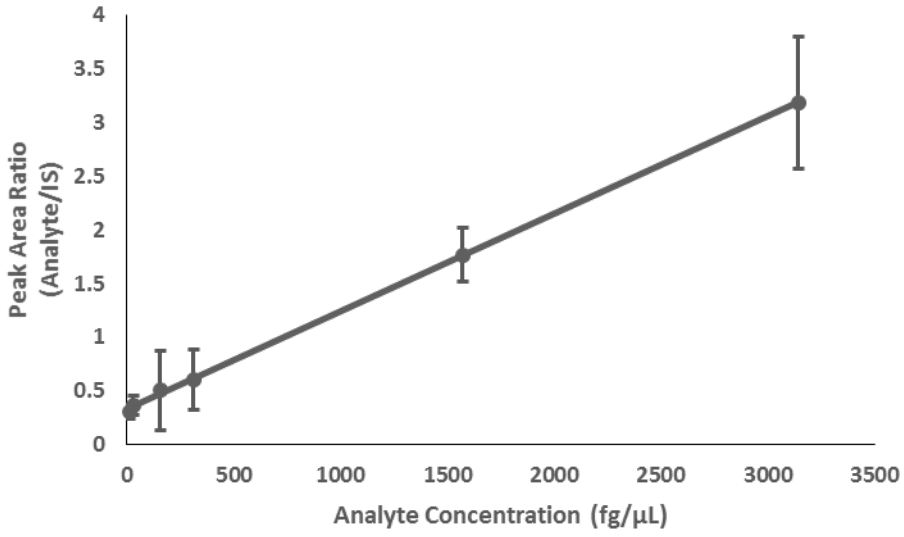
A good correlation was observed between the concentration of analyte used in the experiment and the ratio of peak area of analyte/IS expressed by r^2 of the calibration curves (Figure 2, Table 4). All three steroid calibration curves exhibited linearity over the wide range of 0.05 - 10 nM (Table 2). The Lower Limit of Quantification (LLOQ) was determined using the lowest analyte concentration giving a minimum signal-to-noise ratio (S/N) > 10. Determination of the S/N is performed by comparing measured signals from a sample with a known low

concentration of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. Based on that, the LLOQ of each steroid was calculated and found to be 16 fg/µL for progesterone, 14 fg/µL for testosterone and 18 fg/µL for hydrocortisone.

Table 4. Data of progesterone, testosterone, and hydrocortisone calibration curves

	Progesterone	**Testosterone**	**Hydrocortisone**
Molar range	0.05 -10 nM (16 -3,140 fg/µL)	0.05 - 10 nM (14 -2,890 fg/µL)	0.05 - 10 nM (18 -3,620 fg/µL)
r^2	0.992	0.991	0.997
Slope	0.0009	0.001	0.0007
LLOQ (fg/µL)	16	14	18

Figure 2. Calibration curve for progesterone (16 -3,140 fg/µL). Concentration points represent the average of three replicate injections of 1 µL.



Effect of matrix on recovery using nanoLC-MS/MS

The effect of the blubber matrix on recovery and quantitation of steroids was assessed as a part of validating the nanoLC-MS/MS methodology. Sample extracts from an adult male blubber sample with no detectable endogenous progesterone were diluted 10 times with equal amounts of surrogate IS and various amounts of progesterone ranging from 31.45 to 3145 fg/µL. After nanoLC-MS/MS analysis, progesterone concentrations were calculated and recovery of progesterone was assessed (Table 5). Recoveries ranged from 88-118% and demonstrate acceptable recovery with method precision of less than 20% from actual concentration. This is comparable to data previously reported (Zhang *et al.* 2011, Kunze *et al.* 2015).

Due to sample mass limitations of blubber with no detectable testosterone and/or hydrocortisone, matrix effects studies for those two steroid hormones were not able to be conducted. Currently, an appropriate blubber analog matrix is not available to conduct these types of studies, and we recommend they be conducted opportunistically when suitable and adequate blubber samples are obtained.

Table 5. Recovery of progesterone measured in five gray whale blubber extracts spiked with known amounts of progesterone

Spiked Progesterone samples	**Spiked concentration fg/µL**	**Net mean value fg/µL**	**Mean recovery %**
1	31.45	37.1	118 ± 10.6
2	157.23	163.5	104 ± 9.3
3	314.47	306.6	97.5 ± 7.9
4	1572.00	1530.8	97.4 ± 6.7
5	3145.00	2786.2	88.6 ± 16.7

Quantification of steroids in eastern gray whale blubber samples using nanoLC-MS/MS

The nanoLC-MS/MS method was used to quantitate the three steroid hormones (progesterone, testosterone, and hydrocortisone) in 50 mg eastern gray whale samples. Calibration curves were used to quantify the steroids in the sample using the SRM method. The IS cholic acid did not interfere with this detection and provided improved quantitation of steroid hormones from blubber matrix.

Progesterone concentrations for the eastern gray whale blubber samples were detected in two of the three samples and ranged from 1.5-52.0 ng/g. Concentrations were higher in the adult female than in the adult male. Testosterone concentrations were detected in both male blubber samples ranging from 3.1-20.5 ng/g. The adult male had a higher testosterone concentration than the juvenile male. Hydrocortisone was detected in two of the three samples ranging from 3.8-3.9 ng/g.

Table 5. Concentration of three steroid hormones detected in 50 mg gray whale blubber samples using nanoLC-MS/MS (ND = non-detectable)

Gray whale blubber sample	**Sex**	**Hydrocortisone ng/g**	**Testosterone ng/g**	**Progesterone ng/g**
ER-13-092 (C-139)	Adult male	3.8	20.5	1.5
ER-13-031 (C-68)	Adult female	ND	ND	52.0
ER-13-093 (C-126)	Juvenile male	3.9	3.1	ND

CONCLUSIONS

14 western gray whale samples collected off Sakhalin Island in 2014 were analyzed for progesterone and testosterone via ELISA. Based on progesterone levels reported in other cetacean species, the females analyzed here are likely not pregnant. Obtaining a blubber sample from a confirmed pregnant female gray whale would provide a definitive pregnancy diagnostic for this species. Concentrations of testosterone in gray whale blubber biopsies have never been assessed. The values reported here are in the lower end of values reported in pubertal and immature males for the short-beaked common dolphin (Kellar *et al.* 2009). Samples analyzed for testosterone from gray whales of known sexual maturity would aid in interpretation of data.

Using our nanoLC-MS/MS method, three steroid hormones were successfully quantitated in three eastern gray whale 50 mg blubber samples. Progesterone was detected in two samples, and the concentration quantitated in the female blubber sample was higher than the progesterone concentration of the male blubber sample. Testosterone was detected in two male blubber samples with the adult male having a higher concentration than the juvenile. Hydrocortisone was detected in two of the three blubber samples.

Our nanoLC-MS/MS method for quantitation of steroids delivered significant improvement of LLOQ in comparison to previous studies where conventional LC-MS/MS was used, regardless of species. Keski-Rakhonen *et al.* 2011 reported an LLOQ of 100 fg/µL for numerous steroids including progesterone using an injection volume of 40 µL for each sample from human serum. The injection volume reported for this method is 1 µL per sample. These results indicate that the nanoLC-MS/MS method reported here is more than a 200-fold improvement with regards to LLOQ compared to that reported using conventional LC-MS/MS. Kunze *et al*. 2015 indicate an even higher LLOQ for testosterone (4400 fg/µL) and progesterone (2500 fg/µL). Zhang *et al.* 2011 report comparable LLOQ for testosterone (10 fg/µL) in human cell line samples but employ the use of steroid derivatization. Chemical derivatization is time consuming and adds another step to the analysis which could be a source of additional variation. The direct nanoLC-MS/MS method reported here is much easier for analyses of complex biological samples without the use of chemical derivatization.

Other advantages of this method are high specificity and the small sample requirement (50 mg) with an injection volume of 1 µL. It provides significant improvement in the field of marine mammal research, where steroid hormone analysis is currently performed using an ELISA which requires larger sample mass, is less specific due to cross-reactivity, and generates data only on a single hormone. The nanoLC-MS/MS analysis is ideal for the small sample masses associated with blubber biopsy collection in free-swimming marine mammals as it only requires 50 mg and provides a more complete health assessment with multiple hormone analysis. With the efficient use of the tissue, this hormone analysis method allows for additional analyses to be conducted in biopsy samples that may have been previously precluded due to the limited blubber mass of biopsy samples. This methodology will prove especially valuable for endangered and protected species for which only small samples are available and can be applied to both liquid and solid matrices. This will benefit the reproductive fitness and stress assessments of the IUCN-listed critically endangered Western gray whale population as well as other marine mammal populations. Future directions of this research will also involve the inclusion of additional biologically relevant steroid hormones in the analyses, such as estradiol and other glucocorticosteroids.

Future studies for this project also include the nanoLC-MS/MS analysis of the 14 western gray whale extracts analyzed for progesterone and testosterone using ELISA for a methodology comparison. Another goal of this study is to analyze steroid hormones in confirmed pregnant female gray whales.

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