Genetics and preliminary hormone analyses in Western Gray whale biopsy samples collected off Sakhalin Island in 2011

[1]JOHN W. BICKHAM, [2]JACQUELINE DOYLE, [2]J ANDREW DEWOODY, [3]REBECCA PEZDEK, [3]KIM WOOTEN, [4]NICHOLAS KELLAR, [4]MARISA TREGO, [5]JENNIFER DUPONT, [6]TODD O'HARA, AND [3]CÉLINE A.J. GODARD-CODDING

[1]*Batelle Memorial Institute, Houston, TX, USA*
[2]*Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN, USA*
[3]*The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX, USA*
[4]*Southwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, La Jolla, CA*
[5]*ExxonMobil Upstream Research Company, Houston, TX, USA*
[6]*University of Alaska Fairbanks, Fairbanks, AK, USA.*
Contact email: bickhamj@battelle.org

ABSTRACT:

The western gray whale subpopulation is listed as critically endangered, as designated by the IUCN. The IWC Scientific Committee has stressed the value of reproductive status and genetic analyses for this subpopulation. Here, we report on 1) the molecular sexing of six individuals biopsied off Sakhalin Island in 2011, 2) mtDNA control region (CR) and cytochrome b (cyt b) haplotype sequences, and 3) the preliminary optimization of hormone analyses in gray whale. Total genomic DNA was extracted from each biopsy and molecular sexing was determined by amplification of three primer sets via multiplex PCR. We used both Zfy/x and SRY protein encoding genes according to previously described methods for gray whales. Data for the two mtDNA genes were compared to previously described haplotypes using NCBI BLAST. The above methods identified a single male and five females with 4 CR haplotypes and 3 cyt b haplotypes. The 4 CR haplotypes (A, AI, B, E) have all been previously reported in western gray whales, and they are also known in eastern gray whales. Pregnancy detection via blubber progesterone analysis has been previously validated in several cetacean species but not yet in gray whales. Blubber samples from five eastern gray whales were obtained from one euthanized female calf and four stranded animals (one female calf, one male juvenile and two female juveniles). Two 150 mg sections of blubber were analyzed for each animal. One section was used as a positive control and spiked with 300 ng/g of progesterone. This progesterone level is within the range reported in pregnant cetaceans. Samples were homogenized using a FastPrep® 24, extracted for steroids, and assayed for progesterone via an ELISA kit. Further optimization is currently underway and includes 1) increasing extraction efficiency, 2) measuring progesterone in adult female of known reproductive status, and 3) reducing minimum sample size required for assay.

Introduction:

Two North Pacific populations of gray whales (*Eschrichtius robustus*) are currently recognized; the large eastern gray whale population (ca. 19,000, Laake et al., 2009) and the small critically endangered western gray whale population (ca 155; IUCN, 2012). Western gray whales inhabit summer feeding grounds in the Sea of Okhotsk, off the northeast coast of Sakhalin Island and the southeastern coast of the Kamchatka Peninsula. Their wintering grounds are poorly known but include the South China Sea (Weller and Brownell, 2012). Recent photographic and genetic matches along with satellite tagging reports have also indicated the presence of 23 western gray whales in North America (Lang et al., 2011; Weller et al., 2011; Urban et al., 2012). Due to its current IUCN status, the IWC Scientific Committee has stressed the value of reproductive status and genetic analyses for the western gray whale population. Six western Gray whale biopsy samples (WGW 011, 019, 119, 129, 139, 141) collected off Sakhalin Island, Russia, in the fall of 2011were analyzed for genetics including molecular sexing and mitochondrial DNA analysis. Pregnancy detection via blubber progesterone analysis has been previously validated in several cetacean species (Mansour et al., 2002, Kellar et al. 2006; Perez et al. 2011) but not yet in gray whales. Optimization of pregnancy testing in this species, using a progesterone enzyme immunoassay, is underway using eastern gray whale samples. Biopsy samples collected from western gray whales will be analyzed upon completion of assay optimization and validation.

Methods:

Genetic analyses

DNA was extracted from each tissue sample using standard potassium acetate extraction (Sambrook and Russell, 2001). Primers for the gray whale control region amplification were 5’-TACCAAATGTATGAAACCTCAG-3’ and 5’- CCTCCCTAAGACTCAAGGAAG-3’ (Alter and Palumbi 2009). Amplification conditions were as follows: denaturation for 2.5 minutes at 90°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 1 minute and a 1.5 minute extension at 72C. Primers for gray whale cytochrome b were 5’-CCTCATGATGAAACTTCGGTTCCC-3’ and 5’- AAGAGGAAGTAGAGGATGGATGCG-3’ (Alter and Palumbi 2009). Amplification conditions were as follows: denaturation for 2 minutes at 94°C, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds and a 1.5 minute extension at 72°C. Low-sodium clean-up was conducted on all PCR products. Sequencing reactions were performed with the primers given above. Additionally, two internal primers were used in cytochrome b sequencing reactions: 5’-ATATCATTCTGAGGCGCAACCGTCA-3’ and 5’-CCCAGATTCATTCGACTAGGGTAG-3’. Amplified products were cleaned using Sephedex and sequenced in both directions on an automated sequencer. Sequence data were edited and aligned in Sequencher 5.0. NCBI BLAST was used to align sequences to previously described haplotypes from Alter and Palumbi (2009).

Several primer sets were used to molecularly sex the whales. The following primers from Fain and LeMay (1995) were used: 5’- ATAATCACATGGAGAGCCACAAGCT-3’ and 5’-

GCACTTCTTTGGTATCTGAGAAAGT-3' (Zfy/x gene) and 5'-CCCATGAACGCATTCATTGTGTGG-3' and 5'- ATTTTAGCCTTCCGACGAGGTCGATA-3' (SRY gene). Two products (bands) are amplified in males and one product (band) is amplified in females. Methods used previously for sexing gray whales were generously provided by Dr. Aimee Lang (NOAA). Amplification conditions were as follows: denaturation for 3 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and a 1 minute extension at 72°C. The primers 5'-TTCAGCTTGCWGCTAGRTTCCTCT-3' and 5'- AWGRTGAAGRACAAGGCCCATCT-3' were developed by Dr. John Patton (Purdue University) for use in bowhead whales. Two products (bands) are amplified in males, and one product (band) is amplified in females. The primers 5'- GCATTGTGCATTCTACTCCGTCAC-3' and 5'-MACTTCCCTTCTSAGGAGATTTARYACTG-3' were also developed by Dr. John Patton for use in bowhead whales. A single product is amplified only in males. Amplification conditions were as follows: denaturation for 2 minutes at 94°C, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 57°C for 30 seconds and a 2 minute extension at 72°C.

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). Epidermis and blubber samples were obtained from Dr. Frances Gulland (The Marine Mammal Center) for five eastern gray whales including two female juveniles, two female calves, and one juvenile male. All samples were obtained from stranded animals except for one female calf, which was euthanized. Two 150 mg sections of blubber were analyzed for each animal. One section was 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). Both 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 (MP Biomedicals, Solon,OH). The supernatant was pipetted off 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 vortexed, 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 plates were read on a Synergy 4 plate reader (BioTek, Winooski, VT) with an absorbance of 405 nm with a correction of 570 nm.

Results:

Genetic analyses

The above methods identified a single male (WGW139) and five females with 4 CR haplotypes and 3 cyt b haplotypes. The 4 CR haplotypes (A, AI, B, E) have all been previously reported in western gray whales, and they are also known in eastern gray whales (Lang et al., 2011).

Biochemistry optimization

Female juvenile (n=2) P4 levels averaged 0.515 ng/g, male juvenile (n=1) P4 level was 0.615 ng/g, and female calf (n=2) P4 levels averaged 0.961 ng/g. These P4 values detected in immature eastern gray whales are within the low end of P4 concentrations seen in immature animals of other marine mammal species, including the short-beaked common dolphin *D. delphis* (0.92-48.2 ng/g) and the Pacific white-sided dolphin *L. obliquidens* (0.11-34.4 ng/g), and just below the northern right-whale dolphin *L. borealis* (0.98-33.1 ng/g) (Kellar et al., 2006). The P4 level detected in the male eastern gray whale was slightly below the range reported in male minke whales *B. acutorostrata* (0.81-3.33 ng/g) (Mansour et al., 2002). Further optimization is currently underway and includes 1) increasing extraction efficiency, 2) measuring progesterone in adult female of known reproductive status, and 3) reducing minimum sample size required for assay. Biopsy samples collected from western gray whales will be analyzed upon completion of assay optimization and validation in eastern gray whale samples.

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