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Humpback Whale (*Megaptera novaeangliae*) Blubber Steroid Hormone Concentration to Evaluate Chronic Stress Response from Whale-Watching Vessels

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Abstract

A booming whale-watching industry in Juneau, Alaska, is raising concerns over potential impacts to humpback whales (*Megaptera novaeangliae*) and the sustainability of this growing industry. In this study, we investigated the physiological response of these whales to chronic vessel disturbance by measuring hormone concentrations (cortisol, progesterone, testosterone, and estradiol) that have been sequestered in blubber throughout the whale-watching season. We focused our analysis on cortisol, a steroid hormone associated with stress response, and hypothesized that cortisol in biopsy samples would be positively correlated with the amount of vessel traffic in the 3 to 4 months prior to sampling. Humpback whales in the Juneau area were compared with whales from control areas with far less vessel traffic in both Southeast Alaska and the western Gulf of Alaska using biopsies collected late in the tour season. We did not find elevated cortisol in whales sampled in the Juneau area relative to the Southeast Alaska control area ($p = 0.14$) or sites in the western Gulf of Alaska, which had higher cortisol levels ($p < 0.001$). The cause of the regional cortisol differences is not known but could be representative of regional differences in baseline hormone concentrations or be linked to predator or nutritional stressors. The lack of elevated cortisol in Juneau-area whales suggests high vessel traffic is not resulting in chronic cortisol sequestration in whales and may be indicative of whales near Juneau being habituated to vessel traffic.

Key Words: cortisol, blubber, humpback whale, *Megaptera novaeangliae*, whale watching, stress response, ecotourism

Introduction

Vessel disturbance, both from the physical presence of boats and the associated vessel noise, has at least short-term behavioral and physiological impacts on marine mammals (Bejder & Samuels, 2003; New et al., 2015). Many studies have documented behavioral changes as a result of vessel disturbance, including reduced foraging and resting, increased respiration and travel, reduced vocalizations, and vessel evasion (Bejder & Samuels, 2003; Quakenbush et al., 2010; Campana et al., 2015; Meissner et al., 2015; Senigaglia et al., 2015; Blair et al., 2016; Cosentino, 2016; Culloch et al., 2016; Dunlop, 2016; Pérez-Jorge et al., 2016). Moreover, elevated underwater noise (such as noise from excessive vessel traffic) can result in increased cortisol levels in fishes and marine mammals (Spreng, 2000; Wright et al., 2007; Rolland et al., 2012; Nichols et al., 2015).

Whale-watching tourism is a growing industry worldwide, with increased vessel disturbance potentially negatively impacting whales (Bejder & Samuels, 2003). Several studies have highlighted short-term behavioral responses specific to vessel disturbance from whale-watching tourism. Examples of short-term behavioral responses to vessel disturbance include increased respiration, movement (vessel evasion), and surface activity; reduced resting and foraging; and so on. These types of responses have been documented in humpback whales (*Megaptera novaeangliae*; Corkeron, 1995; Stamation et al., 2010; Avila et al., 2015), killer whales (*Orcinus orca*; Trites & Bain, 2000; Jelinski, et al., 2002), minke whales (*Balaenoptera acutorostrata*; Christiansen et al., 2013), sperm whales (*Physeter macrocephalus*; Cosentino, 2016), and North Atlantic right whales

(*Eubalaena glacialis*; Argüelles et al., 2016). Yet, how these short-term behavioral responses by cetaceans translate into long-term impacts remains poorly understood. However, it is important to consider long-term impacts to better understand if these disturbances are persisting and potentially threatening the survival and/or fitness of affected individuals through repeated exposure (Bejder & Samuels, 2003; Wright et al., 2009; Hunt & Moore, 2013; Scarpaci & Parsons, 2014; Atkinson et al., 2015; King et al., 2015; New et al., 2015; Senigaglia et al., 2015). For the purposes of this study, we consider long-term impacts to be those that persist beyond acute encounters and define *long-term* as 3 or more months.

Long-term stress response is correlated with physiologic markers such as the concentration of cortisol in certain tissues. Cortisol is a glucocorticoid steroid hormone that is produced when the hypothalamic-pituitary-adrenal axis is activated by stimuli that are perceived to be urgent or threatening (Sapolsky et al., 2000; Wingfield & Romero, 2011). Like all steroid hormones, cortisol is lipophilic and sequesters in the lipid-rich blubber of cetaceans (Deslypere et al., 1985; Hunt et al., 2013). Blubber, once thought to be only a reservoir for storing energy, is now believed to be a complex and metabolically dynamic tissue, which is responsible, in part, for regulating production of hormones and glucose (Kershaw & Flier, 2004). For example, relative blubber cortisol concentrations in beluga whales (*Delphinapterus leucas*) were measured in groups entrapped in ice flows vs non-entrapped individuals harvested for subsistence use. Blubber cortisol concentrations for entrapped whales were approximately seven times higher than in non-entrapped whales (Trana et al., 2016). Kellar et al. (2015) investigated short-beaked common dolphins (*Delphinus delphis*) incidentally killed as bycatch in a gillnet fishery (presumably a relatively quick death) and compared them with stranded animals that have a greater likelihood of prolonged stress prior to their death. These authors found that stranded animals had mean blubber cortisol concentrations that were over six times higher than animals killed as bycatch (Kellar et al., 2015). Both studies support the notion that cortisol in blubber is a useful measure of relative stress response in cetaceans.

The process of extracting and measuring steroid hormones in tissues and excretions of free-ranging cetaceans is useful in assessing long-term hormone levels and has been validated in many other studies. Examples include the use of blubber (e.g., Mansour et al., 2002; Kellar et al., 2006, 2013, 2015; Pérez et al., 2011; Noren & Mocklin, 2012; Trego et al., 2013; Trana et al., 2015, 2016; Vu et al., 2015), lung mucus from blow samples (Hogg et al., 2009; Dunstan et al., 2012; Hunt et al., 2013), and feces (Wasser et al., 2000; Rolland et al., 2005; Hunt et al., 2006; Burgess et al., 2013). Kellar et al. (2013) evaluated progesterone concentrations (also a steroid hormone) in urine, serum, and blubber of bowhead whales (*Balaena mysticetes*) and provided evidence that steroid hormone levels are mirrored among these media. Further, these authors noted that urine and serum steroid hormone concentrations fluctuate on hourly to daily scales, while blubber steroid hormone concentrations reflect fluctuations occurring on the scale of weeks to months. In another study, cortisol concentrations in harbor seal (*Phoca vitulina*) blubber and serum were compared, and similar conclusions were made on the longer (multi-month) retention of cortisol in blubber (Kershaw & Hall, 2016).

Foraging humpback whales near Juneau, Alaska, are the focus of a thriving seasonal tourism industry that operates from May through September. Approximately one-quarter of Juneau's summer visitors, over 250,000 travelers, purchase trips on whale-watching excursions (Alaska Department of Commerce, Community, and Economic Development [ADCCED], 2012). Ticket sales alone from Juneau whale-watching tours generate more than 30 million U.S. dollars of annual revenue (based on a conservative estimate of $120 average ticket price). Because this ecotourism industry focuses on humpback whales and is the largest (ADCCED, 2012) and most lucrative whale-watching industry in the State of Alaska, Juneau-area humpback whales are among Alaska's most economically important marine wildlife species.

Humpback whales were once considered to be on the brink of extinction from commercial exploitation (Johnson & Wolman, 1984); however, they have been recovering throughout much of their range (Calambokidis et al., 2008). Humpback whales are protected under the Marine Mammal Protection Act, as are all marine mammals in the U.S., and the Endangered Species Act (ESA). Recently (September 2016), the U.S. National Marine Fisheries Service designated humpback whales into 14 distinct population segments (DPSs) for management purposes. While all humpback whales were previously considered endangered (globally) under the ESA, after defining the finer DPS management units, only five of the 14 DPSs remain listed under the ESA as endangered or threatened (National Oceanic and Atmospheric Administration [NOAA], 2016). The remaining DPSs are no longer listed under the ESA (NOAA, 2016), including the Hawaii DPS, which makes up approximately 94% of Southeast Alaska's summer population (Wade et al., 2016). The ESA explicitly states the need for post-delisting monitoring of factors that could threaten the continued recovery of listed or recently delisted ESA species. One such potential threat to recovering humpback whale populations is vessel disturbance (Bejder & Samuels, 2003).

Whale-watching pressure in the Juneau area has been steadily increasing over the last two decades as the whale-watching industry has grown to include a high number of whale-watching vessels (A. Jensen, Alaska Regional Office, NMFS, pers. comm., 18 February 2016). There are now growing concerns for the sustainability of the whale-watching industry near Juneau because of the increase in disturbance to whales in the area. The Juneau tour area is relatively small, roughly 30 × 15 km and part of an archipelago system made up of narrow passageways between islands. During the summer season, there are between two to 30 whales foraging in the tour area, typically clustered in hot spots where prey is presumed to be abundant. In 2016, there were 60 tour boats operating out of Juneau's main port, Auke Bay, that participated in whale watching (both whale-watching-specific and charter-fishing boats; Teerlink, unpub. data, 2016). At times, when there are many whales dispersed throughout the area, the whale-watching effort can be distributed among whales. However, when whale abundance is low or highly aggregated, it is common for up to 30 whale-watching, charter, and recreational craft to follow a single group of whales. This is especially true for groups of whales engaged in coordinated bubble-net feeding activity. These large aggregations make for particularly exciting whale watching, and tour and recreational boats rarely pass up the opportunity to stop and watch, even if it means sharing the space with several other boats. Consequently, bubble-net feeding groups are often surrounded by dozens of vessels and associated vessel noise throughout the day for the entirety of the tour season (Teerlink, unpub. data, 2010-2017).

The main objective of this study was to determine if there is evidence of a long-term physiological stress response in humpback whales to the high vessel densities found in the Juneau area during the summer tour season. For the purposes of this study, we consider *long-term* to be effects lasting beyond acute encounters and define it as a 3 to 5 mo window. We hypothesized that cortisol in blubber is positively correlated with the amount of vessel traffic in the 3 to 5 mo leading up to sampling and, therefore, would be significantly higher in Juneau-area whales in late summer than in whales from other areas at the same time of year. For this study, we sampled humpback whales in the Juneau tour area and compared their cortisol concentrations to whales in Stephens Passage in Southeast Alaska and in Kodiak Island and Shumagin Islands in the western Gulf of Alaska, all areas with far less vessel traffic.

Because blubber reflects steroid hormone levels sequestered over longer temporal scales (Kellar et al., 2013; Kershaw & Hall, 2016), we believe that measuring blubber cortisol concentrations is a unique way to evaluate chronic stress response from persistent vessel presence relative to more "natural" stressors experienced by all whales. We expect that sex and life history status can affect cortisol levels as is seen in other studies (e.g., Steinman et al., 2015) but do not have data on sex, maturity, and reproductive status of whales sampled for this study. In lieu of these data, we evaluated sex steroid hormone concentrations, specifically testosterone, progesterone, and estradiol, as proxies for sex, maturity, and reproductive status and used them to ensure that there were no underlying trends in life history status biasing our samples.

Methods

Photo Identification

Humpback whales in this study were tracked using photo identification. Humpback whales are individually identifiable by the unique combination of shape, pigmentation, and scarring on their ventral fluke surface that is visible when a whale descends on a sounding dive (Katona & Whitehead, 1981). The process of photographing these markings is a trusted and cost-effective method for obtaining sighting histories to track individual humpback whales (Katona & Whitehead, 1981; Friday & Smith, 2000; Calambokidis et al., 2008; Straley et al., 2008; Teerlink et al., 2015).

Photo-identification data were collected on regular surveys of the tour area from May through September during 2014, and by a subset of whale-watching industry participants who collected fluke photographs on their tours and submitted them to us as part of a citizen science program (Teerlink, 2017). The combined pool of photo-identification data provides sighting history information that was used to (1) identify individual humpback whales being sampled; (2) identify whales that use the Juneau area and, therefore, are exposed to high densities of whale-watching vessel traffic; (3) provide relevant information on life history; and (4) help to avoid inadvertently double sampling the same individual whale.

Field Methods and Study Design

Biopsy samples were collected from whales in the Juneau tour area (N = 17) and control areas with far less vessel traffic: Stephens Passage in Southeast Alaska (N = 12) and the western Gulf of Alaska (N = 20; Table 1; Figure 1). Samples from the Juneau area were collected from within the established Juneau tour area, the predominant whale-watching waters used by ~60 whale-watching vessels

Table 1. Summary of humpback whale (*Megaptera novaeangliae*) biopsy samples analyzed for steroid hormones, including the area and years in which they were collected

Area	# whales sampled	Collection years
"Experimental" area – Juneau	17	2014
Control 1 – Stephens Passage	12	2014
Control 2a – Kodiak Island	12	2010-2014
Control 2b – Shumagin Islands	8	2007-2012

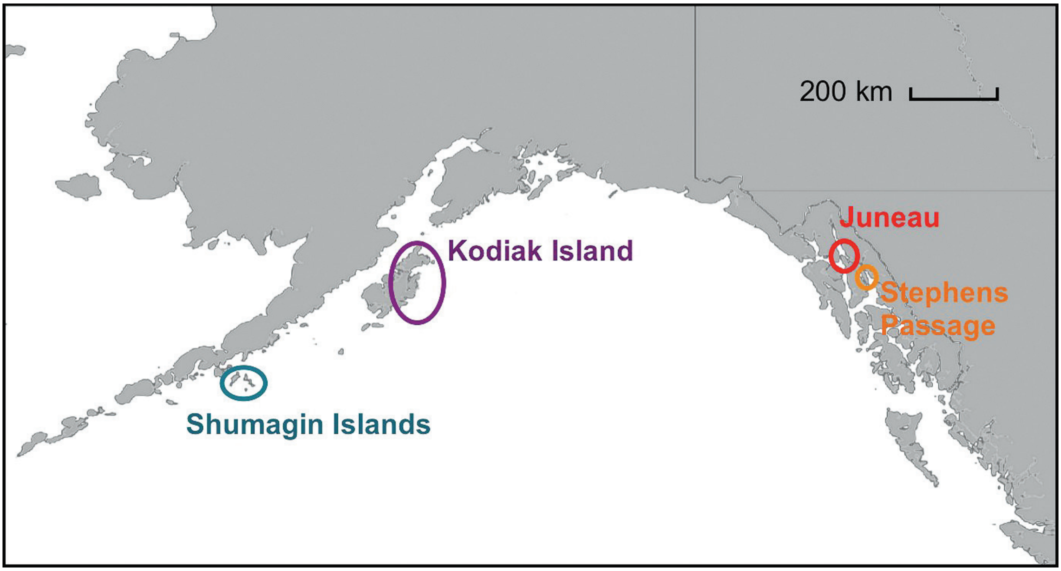

Figure 1. Locations of humpback whale (*Megaptera novaeangliae*) biopsy sampling. Biopsies were used to measure cortisol concentrations in whales found in multiple areas in the Gulf of Alaska with differing vessel disturbance. Juneau, with high vessel exposure, was compared to control areas with far less vessel traffic: Stephens Passage in Southeast Alaska, and Kodiak Island and Shumagin Islands in the western Gulf of Alaska.

operating out of Auke Bay. In Stephens Passage, samples were collected from whales in the southern extent in Seymour Canal and Gambier Bay. This area is geographically close to Juneau (~115 km south) but has far less vessel traffic. Beyond the absence of a whale-watching industry, Seymour Canal and Gambier Bay are secluded from through-traffic and shipping and transportation vessels, and are limited to occasional recreational boaters. In the western Gulf of Alaska, samples were taken near Kodiak Island (N = 12) and Shumagin Islands (N = 8), which are geographically farther from Juneau (~1,200 km) but found at a similar latitude to the Southeast Alaska sites (Figure 1). The vessel traffic near Kodiak Island and Shumagin Islands is much lower than in the Juneau area and is generally limited to fishing, shipping, and recreational vessels (little or no whale-watching tourism). Samples from Southeast Alaska (Juneau and Stephens Passage) were collected specifically for this study (authorized by the University of Alaska Institutional Animal Care and Use Committee #474034-1 and #642456-2), and samples from the western Gulf of Alaska (Kodiak Island and Shumagin Islands) were taken from tissue archives (Witteveen et al., 2015).

Remote biopsy sampling is a commonly used field method in marine mammal research and has been practiced for over 30 years to collect tissue samples (e.g., Aguilar & Nadal, 1984; Witteveen et al., 2011). Studies measuring cetacean responses to biopsy sampling indicate that any adverse effects are minimal (Noren & Mocklin, 2012). Biopsies were collected via a modified 0.22 rifle (PneauDart) that shoots an untethered dart with a biopsy-coring tip and collects a sample approximately 0.5 g and about 15 mm deep and 5 mm in diameter. Darts bounce off the animal (with the skin and blubber core sample intact) and float until they can be retrieved. There is some expected lipid loss as a result of the biopsy process (sampling effect; Ryan et al., 2013), but we assumed that this was relatively consistent among biopsy-collected samples, regardless of sampling location. The amount of time a biopsy is in water can also impact the amount of lipid retained in a sample (Ryan et al., 2013; C. Allen, Southwest Fisheries Science Center, NMFS, pers. comm., 13 December 2015). All biopsy samples used in this study, regardless of area or year, were collected in the same way with a relatively consistent retrieval time for all samples (1 to 2 min), and we believe any sampling effect or lipid loss from retrieval time to therefore have a minimal effect in our study. Biopsy samples were only taken from animals that had been previously photo identified so that samples could be linked to individual sighting histories. Samples were stored within the biopsy dart tip in plastic bags on ice until return to the lab where the sample could be removed from the dart, packaged in glass vials, and frozen at -80°C for later processing. Field and storage methods were the same for all samples in this study, including the archived samples. The only known difference between archived and recent samples is the amount of time they were stored. Some of the western Gulf of Alaska samples were collected earlier than the Southeast Alaska samples (outlined in Table 1). While duration of frozen storage varied, this factor had no impact on cortisol concentration in beluga whale blubber (Trana et al., 2015). Therefore, we believe that storage duration is not a major factor affecting steroid hormone concentrations.

Steroid hormone concentrations in blubber may vary based on tissue depth. In cross-section analyses, blubber tissue closer to the muscle has higher cortisol concentrations than blubber located closer to the skin (Trana et al., 2015). This is consistent with known stratification and differences in metabolic turnover of lipids (Koopman et al., 1996; Bagge et al., 2012), where the inner layer is highly metabolically active and the outer layer contains lipid classes with insulative properties with slower turnover. Because all samples in this study were from biopsies where the sample was from a comparable blubber depth (the outer layer of blubber closest to the skin that is less dynamic), we did not consider variability with blubber depth as a factor.

We limited all biopsy sampling, including control areas, to late in the tourism season (August to early October) because this would theoretically reflect the whale-watching "treatment" exposure of the prior weeks and months (Kellar et al., 2013; Kershaw & Hall, 2016). A summary of sighting histories of individual whales sampled in the Juneau area is given in Table 2. From photo-identification data collected over several years in Juneau, Kodiak Island, and Shumagin Islands, and the 2014 identifications from Stephens Passage, none of the whales sampled in the control areas were seen in the Juneau area (or vice versa), indicating that movement of individual whales among the experimental area and control areas was unlikely. We expect that any stress response from our research vessel approach and/or biopsy collection was not reflected in blubber samples because blubber steroid hormone levels are not thought to reflect real-time circulating blood/serum levels but, rather, longer-term cumulative steroid hormone levels (Kellar et al., 2013; Kershaw & Hall, 2016).

Laboratory Methods – Steroid Extraction and Analysis

While the focus of this study was the assessment of cortisol concentrations in humpback whale biopsy samples, we also measured the concentrations of three sex steroid hormones (testosterone, progesterone, and estradiol) in each sample to better understand steroid hormone compositions in the different study regions. Blubber biopsies were subsampled to 0.2 g (±0.025 g) from biopsy cores, and lipids were extracted from the subsample using a method modified from Hunt et al. (2006, 2014). The sample was added to 2.8 mL ceramic bead homogenizer cryovials, and 10 µL of deuterated hormone was added as internal standard for each of the four hormones evaluated: d_4-cortisol, d_9-progesterone, d_5-estrodiol, and $^{13}C_3$-testosterone. Then, 1,460 µL of 100% MeOH (methanol) was added to bring the solution to 2 mL. Vials were vortexed for 8 min and then rocked for 24 h at room temperature. Homogenized samples were centrifuged for 20 min at 10,000 RPM before the supernatant was transferred to 2 mL glass vials, and the methanol was evaporated under nitrogen gas. The resulting lipid extract was sealed, frozen, and shipped in liquid nitrogen dry shippers to the Metabolite Profiling Facility at Purdue University in West Lafayette, Indiana. There, each sample was reconstituted with 200 µL of methanol, then split into two equal aliquots and dried again using an Eppendorf-Vacufuge rotary evaporating device.

The first aliquot of each extract was derivatized with dansyl chloride (dansyl Cl) according to Zhang et al. (2009) to assess estradiol. To each sample, 20 µL of 10 mM Na_2CO_3 and 50 µL of freshly prepared dansyl Cl solution (3 mg/mL acetone) was added. The samples were heated at 60°C for 10 min. Samples were transferred to autosampler vials and immediately analyzed. An Agilent 1200 Rapid Resolution Liquid Chromatography (LC) system coupled to an Agilent 6460 series QQQ Mass Spectrometer (MS) was used to analyze all samples *post* derivatization. For the dansyl Cl derivatives, the following conditions were used with a Waters

Table 2. Summary of sighting history for humpback whales sampled in the Juneau tour area. The total number of sightings in the 2014 season from surveys and data collected by whale-watching boats is given to demonstrate a proxy for exposure to whale-watching pressure. The "Min. residency days" indicates an additional proxy measure for residency in the tour area. This measure is the number of days lapsed between the first and last sighting of an individual and assumes that the whale did not leave between sightings. Sightings are also broken down by month to show how sightings were distributed throughout the summer. "Date biopsied" is provided to show the relationship between timing of sightings to sampling. There were no sightings of the control area (Stephens Passage, Kodiak Island, and Shumagin Islands) whales in the Juneau tour area during this study or vice versa.

Whale ID	Total # sightings	Min. residency days	May	June	July	Aug	Sept	Oct	Date biopsied (Year: 2014) (d/mo)
1879_calf_2011	11*	48	0	0	0	3	7	1	10 Sept
UAF-20140910-365	1	1	0	0	0	0	1	0	10 Sept
2348	6*	68	0	0	1	2	3	0	10 Sept
1434	21*	75	0	0	4	11	6	0	10 Sept
UAF-20140910-468	4	54	0	0	0	1	3	0	10 Sept
1443	13*	98	0	2	5	5	1	0	10 Sept
2006	18*	124	3	0	0	7	8	0	10 Sept
2171	21*	113	0	2	0	9	9	1	12 Sept
1538	51*	141	3	12	2	21	13	0	12 Sept
1820	16*	66	0	0	6	4	6	0	12 Sept
1447	37*	122	3	3	5	15	11	0	12 Sept
1879	23*	137	5	5	5	5	3	0	12 Sept
UAF-20140913-136	1	1	0	0	0	0	1	0	13 Sept
276	1	1	0	0	0	0	1	0	29 Sept
2258	7*	39	0	0	0	2	5	0	29 Sept
1429	1	1	0	0	0	0	0	1	7 Oct
1612	1	1	0	0	0	0	0	1	7 Oct

*Indicates that this individual also has sightings in the tour area in other years

Xbridge C18 2.1 × 100 mm, 3 µm column for LC separation: Buffers were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was as follows: time 1 min = 90% A and 10% B, time 5 min = 0% A and 100% B, time 15 min = 0% A and 100% B, time 15.5 min = 90% A and 10% B, and time 18 min = 90% A and 10% B. The flow rate of buffers through the High Performance Liquid Chromatography (HPLC) column was 0.3 mL/min. Multiple Reaction Monitoring (MRM) was used to target the specific steroid hormones of interest. Data were acquired in positive Electrospray Ionization (ESI) mode by monitoring the following transitions in atomic mass: Estradiol (dansyl Cl) 506.1→171 (30V), 155.8 (40V); d_5-Estradiol (dansyl Cl) 511.1→171 (30V), 155.8 (40V); and Estriol (dansyl Cl) 522→171 (30V), 155.8 (40V). The ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/min, nebulizer pressure of 45 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/min, capillary voltage of 3,500 V, and nozzle voltage of 1,500 V.

The second sample aliquot was derivatized with the AB Sciex Keto derivatization kit (AB Sciex, Framingham, MA, USA) to assess testosterone, cortisol, and progesterone. To each sample, 50 µL of reagent was added. The reaction time was 60 min at room temperature. The samples were transferred to autosampler vials and immediately analyzed. An Agilent Zorbax 80Å Extend-C18 4.6 × 150 mm, 5 µm column was used with the buffers (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was the same as for the first aliquot. MRM was used to target the specific steroid hormones of interest. Data were acquired in positive ESI mode by monitoring the following transitions in atomic mass: Testosterone 403.1→344.1 (20V), 164 (40V); $^{13}C_3$-Testosterone 406.1→347.1 (20V), 167 (40V); Cortisol 477.1→418.3 (15V), 388.2 (35V); d_4-Cortisol 481.1→422.3 (15V), 392.3 (35V); Progesterone 429.1→370.0 (20V), 126 (30V); and d_9-Progesterone 438.1→379.0

(20V), 132 (30V). The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/min, nebulizer pressure of 45 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/min, capillary voltage of 4,000 V, and nozzle voltage of 1,000 V.

Extraction efficiency was evaluated by comparing known volumes of deuterated internal standards (and without added sample) that had been through the extraction process ($N = 8$, "Blank – Extracted") with the same volume of deuterated internal standard that had not been through the extraction process ($N = 5$, "Blank – Not Extracted"). The percent of deuterated internal standard recovery was the ratio of average hormone concentration in the "Blank – Not Extracted" samples and average hormone concentration in the "Blank – Extracted" samples. The extraction efficiencies for each hormone were as follows: cortisol = 71.5%, testosterone = 107.4%, progesterone = 51.1%, and estradiol = 79.4%. Extraction efficiencies are provided for reference; results were not altered to correct for inefficiencies in extraction. Steroid hormone measurements are reported as concentrations, ng/g (divided by the wet adipose mass of the extracted sample).

Evaluation of Chronic Stress Response from Vessel Disturbance

Cortisol concentrations were compared pairwise between collection areas using a two sample *t* test at a significance level of $\alpha = 0.05$ to evaluate potential chronic stress response from vessel disturbance in Juneau whales compared with whales from the control areas. An *ad hoc* power analysis was conducted in *R*, Version 3.2.5 (R Core Team, 2016) to assess the statistical power given to sample sizes. Effect size was estimated using Rosnow & Rosenthal (1996).

Juneau-area trends in cortisol concentration were further investigated by correlating the cortisol concentration for each individual whale with the total number of sightings of that animal prior to sampling (a proxy for relative exposure to high vessel "treatment") with a Pearson's linear correlation at a significance level of $\alpha = 0.05$.

Evaluation of Sex Steroid Hormones

We analyzed additional steroid hormones to assess potential site-specific differences in demographics of sampled individuals. Data on sex, maturity, and pregnancy status of individual animals sampled were not available; however, these factors could influence cortisol concentrations and confound our results. Testosterone, progesterone, and estradiol are sex hormones that vary with life history status and were used as *ad hoc* measures to determine if our samples had an equal representation of life history status among regions.

Hormone concentrations from all samples were tested for correlations in a pairwise analysis. Both Pearson correlation coefficients and Spearman rank correlation coefficients were generated for each pairwise comparison. The ranked analysis was included to eliminate the bias that outliers may have on coefficients. Estradiol was removed from all analyses as more than half of the sample concentrations were below the detection threshold (approximately 75 ng/g). Spatial patterns in testosterone and progesterone concentrations were then evaluated to reveal potential bias in life history status of sampled whales. These comparisons were done using a one-way ANOVA at a significance level of $\alpha = 0.05$, same as for analysis of cortisol concentrations. We would expect that progesterone would be substantially elevated in pregnant whales (e.g., Kellar et al., 2013), which may complicate any correlations. To eliminate the bias that these "outliers" would have in evaluating regional differences in progesterone, we also ran a one-way ANOVA using ranked data.

Results

Evaluation of Chronic Stress Response from Vessel Disturbance

Cortisol concentrations from samples varied among regions ($F = 9.56$, $df = 3$, $p < 0.001$). However, cortisol concentrations of samples from the Juneau area were not higher than those from control areas. In particular, there was no significant difference (*t* test: $t = 1.6$, $p = 0.13$) in mean cortisol concentration between the treatment area, Juneau, and the nearby Southeast Alaska control area, Stephens Passage (observed mean difference = 7.4 ± 9.8 ng/g [95% CI]; Table 3). The associated statistical power was 0.27, given an estimated effect size of 0.5 ($\alpha = 0.05$). Humpback whales in the western Gulf of Alaska, however, had significantly higher blubber cortisol concentrations than whales in Southeast Alaska (*t* test: $t = -4.5$, $p < 0.001$; observed mean difference = 37.2 ± 17.1 ng/g [95% CI]; Figure 2). The associated statistical power was 1.00, given an estimated effect size of 2.8. Cortisol concentration was not significantly correlated with the frequency of Juneau area sightings (Pearson's linear correlation: $R = 0.35$, $p = 0.17$).

Evaluation of Sex Steroid Hormones

Significant positive correlations were found between each pair of steroid hormones analyzed using both the Pearson correlation coefficients and the Spearman rank correlation coefficients

(Table 4). There were no regional differences in testosterone concentration (ANOVA: F = 0.875, *df* = 3, *p* = 0.46). Progesterone concentration, however, did vary by region, seen both with actual concentration values (ANOVA: F = 3.76, *df* = 3, *p* = 0.017) and with ranked data (ANOVA: F = 8.82, *df* = 3, *p* = 0.001).

Table 3. Steroid hormone concentrations (ng/g) in humpback whale blubber samples summarized by area. Values are means (with 1 SD given in parentheses) and medians (less susceptible to potential outliers than are means).

		Cortisol (ng/g)	Testosterone (ng/g)	Progesterone (ng/g)	Estradiol (ng/g)*
Juneau	*Mean (1 SD)*	22.3 (14.0)	4.7 (1.9)	85.3 (77.6)	178.9 (29.0)
	Median	18.6	3.9	50.5	188.9
Stephens Passage	*Mean (1 SD)*	14.9 (11.5)	5.2 (3.5)	43.2 (35.7)	295.5 (182.2)
	Median	13.0	4.8	27.4	179.8
Kodiak Island	*Mean (1 SD)*	53.5 (39.1)	6.3 (3.5)	89.3 (22.6)	202.8 (85.4)
	Median	52.5	5.0	81.4	236.2
Shumagin Islands	*Mean (1 SD)*	61.5 (25.6)	6.2 (3.3)	128.3 (52.6)	121.5 (NA)
	Median	51.3	6.1	118.6	121.5

*Estradiol values are reported here but were excluded from analysis because most (~60%) of the samples had concentrations below the detection threshold (~75 ng/g).

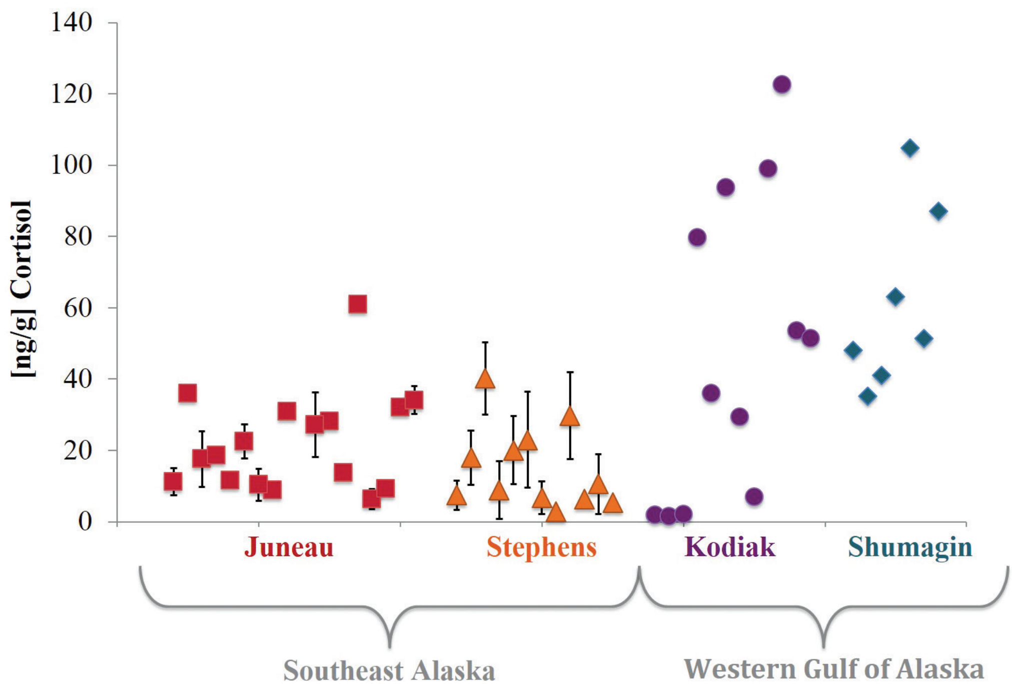

Figure 2. Cortisol concentration (ng/g) in blubber biopsies in humpback whales by region. Symbols mark the mean value, and error bars represent two standard deviations and are present only for samples with enough excess tissue to analyze in duplicate. There was no significant difference in Juneau and Stephens Passage samples (*t* test: $t = 1.6, p = 0.13$), but there was a significant difference between concentrations in samples collected from Southeast Alaska and the western Gulf of Alaska (*t* test: $t = -4.5, p < 0.001$).

Table 4. Correlations between steroid hormone concentrations in humpback whale blubber samples. Correlation values shown to the left (gray background) are Pearson correlation coefficients, and those to the right (white background) of the diagonal are Spearman rank correlation coefficients. Each coefficient is followed by a *p* value, shown in parentheses. All correlations shown are significant ($\alpha < 0.05$).

	Cortisol	Testosterone	Progesterone
Cortisol	--	0.29 (0.04)	0.39 (0.01)
Testosterone	0.43 (0.0025)	--	0.36 (0.005)
Progesterone	0.21 (0.16)	0.24 (0.10)	--

Discussion

The main purpose of this study was to determine if humpback whales that were subjected to high densities of whale-watching vessel traffic expressed physiological signs of increased stress response compared with whales in more remote regions. We hypothesized that humpback whales that are exposed to high whale-watching pressure would have significantly higher cortisol concentrations in their blubber toward the end of the tour season than whales in areas with low vessel traffic. We compared samples from humpback whales during August, September, and late October near Juneau (high whale-watching) with control areas (low-to-no whale-watching activity): Stephens Passage in Southeast Alaska, and Kodiak Island and Shumagin Islands in the western Gulf of Alaska. We found no evidence of whales moving between the study areas during the course of the study (from reviewing photo-identification data collected previous and concurrent to sampling). We found no difference in tissue cortisol concentrations between samples collected in Juneau and Stephens Passage but did find significantly higher levels in samples from the western Gulf of Alaska (Kodiak Island and Shumagin Islands), indicating that there is no evidence for cumulative elevated cortisol levels in whales sampled from areas with high levels of whale-watching. However, regional differences (i.e., higher cortisol concentrations in the western Gulf of Alaska) are considered in the discussion below.

Evaluation of Chronic Stress Response from Vessel Disturbance

We found no evidence to support our original hypothesis that humpback whales in the Juneau area have higher cortisol concentrations relative to the other areas sampled due to a stress response from chronic vessel disturbance. However, it is important to note that our sample sizes are small, and the statistical power to detect differences is low (0.27). Still, the 95% CI included zero, thus we cannot rule out the possibility that there is no difference between groups. Further, results from other studies suggest that biologically meaningful differences may be larger and possible to detect from small sample sizes. For example, Trana et al. (2016) documented cortisol differences between populations and report a large difference in means (~7×) and a high percentage of non-overlap between groups, which is indicative of high effect sizes (~1.7; Cohen, 1988). If effect sizes are indeed high for identifying biologically significant differences in mean cortisol concentrations, lower sample sizes may be acceptable. We saw small differences in means (7.4 ng/g) and a low percentage of non-overlap (23%) in Juneau compared with Stephens Passage samples. However, when all Southeast Alaska samples were pooled and compared with the western Gulf of Alaska, we saw larger differences in the means (37.2 ng/g) and a higher percentage of non-overlap (42%) in Southeast Alaska compared with the western Gulf of Alaska. Thus, while we have low statistical power between Southeast Alaska sites, there are no obvious (large) differences in blubber cortisol concentration means between Juneau and Stephens Passage, and the estimated effect size (0.5) may not be biologically significant. When we looked specifically at samples from Juneau-area whales, we saw no indication that frequency of sightings was correlated with cortisol concentration ($p = 0.17$).

Given our findings, we conclude that Juneau-area humpback whales are likely habituated to vessel presence. We define habituation as in Cyr & Romero (2009): ". . . with repetition the animal learns to perceive that *stimulus* as *innocuous*, and thus reduces the intensity of their stress response to that particular stimulus" (p. 297). Anecdotally, humpback whales in the Juneau area are less skittish of boats compared with whales in other areas. Indeed, whales appear to be quite comfortable moving and feeding among boats in this area, and it is not uncommon to have whales surface within a few feet of vessels and continue feeding even as more and more boats move into an area (J. Moran, Alaska Fisheries Science Center, NMFS, pers. comm., 7 May 2015). In a study of whale reactions to vessel disturbance, Watkins (1986) noted that humpback whales near Cape Cod became habituated to tour boat activity. These authors reported that whales' reactions to boats changed from "negative," where whales abruptly changed behavior and evaded close interaction with boats, to "positive," where whales would permit close approaches and even appear to be curious of boats while continuing to vocalize.

These behavior changes, or habituation to vessel disturbance, occurred quickly: "Sometimes only a few encounters were needed to transform a whale's wariness to apparent unconcern" (Watkins, 1986, pp. 253-254). Given the regional differences in cortisol concentration found in our study, we believe that it is unlikely that adrenal exhaustion, where adrenals become so overstimulated that they no longer produce the cortisol (Cadegiani & Kater, 2016), is a factor. Instead, we believe it is more likely that whales in this area are simply not perceiving vessel disturbance as a threat and, thus, are not eliciting a stress response (habituation). Therefore, we believe that a lack of an elevated cortisol signal in our samples indicates that whales feeding in the Juneau area do not exhibit a chronic physiological response to vessel disturbance due to habituation.

Habituation in Juneau-area humpback whales does not necessarily mean that whale-watching practices are benign. First, habituation can be problematic for wild animals as it tends to make them less cautious of humans and vessels and could lead to a higher susceptibility to collisions and propeller strikes (Watkins, 1986; Bejder & Samuels, 2003; Cyr & Romero, 2009; Harris et al., 2012). Second, while we did not find evidence of a chronic stress response in whales in the Juneau area, we suspect that not all whales are habituated to high boat densities. We hypothesize that tolerance to vessel disturbance is variable by individual and that whales likely retreat to outlying areas with less boat traffic when their individual tolerances for boat traffic are exceeded. Bottlenose dolphins (*Tursiops truncatus*), for example, evade tour boats when vessel densities exceed thresholds (Pérez-Jorge et al., 2016). In this scenario, whales would not be accumulating cortisol in their tissues as a result of high vessel disturbance because they simply leave the area (and, therefore, the stimulus).

In our study area, this is supported by anecdotal observations during times of low whale abundance, where the humpback whales present appear to be limited to the ones most commonly seen in the tour area. In total, there are between 70 to 85 individual humpback whales seen near Juneau each year. Of these, half are transient, moving into and out of the area within a few days. However, the other half exhibits varying degrees of site fidelity, including approximately 15 whales that are seen regularly (10 or more sightings per season) and have high inter-annual site fidelity, reliably returning to the Juneau area each summer after their tropical migration (Teerlink, 2017).

Intuitively, humpback whales with the highest site fidelity should have the most experience feeding among high vessel densities and are more likely to be habituated. In the absence of knowledge on tolerance of individual whales to vessel disturbance, we advise a precautionary approach to tourism and boat traffic increase. We also recommend studies that focus on characterizing soundscapes to better understand the ways in which sound from vessels can impact humpback whales. Lastly, we recommend continued studies to monitor whales under the existing whale-watching levels to ensure sustainability in industry practices, particularly at times when whale abundance is low or whales aggregate, which then can cause increased vessel crowding. In particular, we recommend individual (confirmed by photo identification) behavioral analyses in future studies to allow for additional parameters (e.g., breathing intervals, dive times, resting, travel, etc.) to be evaluated in the presence and absence of whale-watching vessels.

Our results indicate differences in cortisol concentration between Southeast Alaska and western Gulf of Alaska samples that may reveal regional differences in steroid hormone levels. Humpback whales sampled in the western Gulf of Alaska had significantly higher levels of cortisol than did their Southeast Alaska counterparts. Samples from the western Gulf of Alaska were collected by different researchers, but the methods and equipment used were the same. The only other differences in the samples is in the years that they were collected and the storage duration. Samples from the Gulf of Alaska were collected earlier (2007-2014) than the Southeast Alaska samples (2014 only). However, we do not see a temporal trend in the western Gulf of Alaska data and interpret this to mean that the higher tissue cortisol concentrations in western Gulf of Alaska samples were not a result of the collection method or temporal differences in sample collection.

The cause of higher cortisol concentrations in humpback whales in the western Gulf of Alaska (Kodiak and Shumagin Islands) is unknown. It could be due to differences in prey resources, less favorable environmental conditions, increased predation threat, or some other unknown factor(s). However, data on prey availability and humpback whale prey preferences were collected in these areas during the years when biopsy samples were taken, and there is no evidence to suggest limited prey quantity or quality (Witteveen et al., 2015; Wright et al., 2015, 2016). Further, transient killer whales (the only predator of humpback whales in Alaskan waters) are common in both Southeast Alaska (Dahlheim & White, 2010) and the western Gulf of Alaska (Zerbini et al., 2007). That said, humpback whales are not considered to be regular killer whale prey in Southeast Alaska (Dahlheim & White, 2010); whereas in the western Gulf of Alaska, gray whale (*Eschrichtius robustus*) calves (similarly sized to humpback whale calves) are a regular target prey for killer whales (Matkin et al., 2007). It is possible that killer whales could be more of a threat, and potentially a chronic stressor, to humpback whales in the western Gulf of Alaska than in Southeast Alaska but that there are fewer observers in the western Gulf of Alaska to document attacks (Neilson et al., 2012). Both Southeast Alaska and the western Gulf of Alaska are predominantly comprised of Hawaii DPS individuals (94 and 89%, respectively; Wade et al., 2016), a management unit that is considered healthy (not listed as threatened or endangered under the ESA). Therefore, we believe that it is unlikely that the regional differences in cortisol concentration are indicative of underlying differences in DPS status.

Despite the statistical difference in cortisol concentrations detected between regions, this difference may not be biologically significant. Other studies of marine mammal blubber cortisol concentrations show much wider ranges—for example, Trana et al. (2016) documented a seven-fold increase in cortisol concentration in ice-entrapped beluga whales. Harbor seal blubber cortisol concentration increases by two orders of magnitude when they molt (Kershaw & Hall, 2016); whereas, the average range documented in humpback whale blubber in this study was only two-fold between regions. Therefore, it is important that future studies continue to investigate humpback whale blubber cortisol concentrations to further our knowledge of baselines and regional variability to understand what cortisol concentration differentials are biologically meaningful.

Evaluation of Sex Steroid Hormones

Testosterone and progesterone concentrations were analyzed as a proxy for sex, maturity, and reproductive status of sampled humpback whales. Concentrations of testosterone, progesterone, and cortisol were significantly positively correlated between one another in pairwise analyses. Because testosterone is higher in males than females (Kellar et al., 2009; Vu et al., 2015), but did not vary among regions, we interpret this finding to indicate that there was no sampling bias toward males among the regions. Progesterone, however, did vary by region. Even when progesterone concentration data were ranked to eliminate the "outlier" effect of pregnant individuals (discussed below), cortisol and progesterone were correlated, and regional differences in progesterone concentration were apparent. This difference was primarily driven by higher progesterone concentrations in Shumagin Islands vs Stephens Passage (Table 3). These differences could indicate that pregnancy and/or female maturity were not equally represented in the samples collected among regions. Progesterone is higher in mature females than in immature females or males (Kellar et al., 2013). Because we did not see testosterone differences among regions but did see differences in progesterone between the two areas, we believe that this likely indicates that Shumagin Island samples over-represent mature and (potentially) pregnant females. As cortisol covaries with progesterone (Steinman et al., 2015), this may be a confounding factor in our analysis. However, while this could be a factor driving elevated cortisol in Shumagin Islands (where progesterone concentration was highest), it cannot explain elevated cortisol concentrations in the Kodiak region (where progesterone concentrations are not elevated relative to the other areas in this study). Therefore, we believe that the regional patterns in cortisol concentration discovered in this study are actually present and not confounding factors caused by differences in life history status among sampling areas.

Conclusion

To our knowledge, this is the first study to measure cortisol concentrations as a way of evaluating chronic impacts from whale-watching activities on humpback whales. We did not find evidence to support our hypothesis that there would be a correlation between cortisol concentration and vessel traffic; however, our sample sizes and statistical power were low. This finding may be indicative of habituation to vessel traffic in this area, which may be an important factor at play in the dynamics between this booming tourism industry and the humpback whales that summer near Juneau, Alaska.

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