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STUDENT AWARDEE PAPER

Reproductive Steroid Hormone Patterns in Baleen of Two Pregnant Humpback Whales (*Megaptera novaeangliae*)

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Synopsis Understanding reproductive physiology in mysticetes has been slowed by the lack of repeated samples from individuals. Analysis of humpback whale baleen enables retrospective hormone analysis within individuals dating back 3–5 years before death. Using this method, we investigated differences in four steroid hormones involved in reproduction and mating during confirmed pregnant and non-pregnant periods in two female humpback whales (*Megaptera novaeangliae*) with known reproductive histories based on sightings and necropsy data. Cortisol, corticosterone, testosterone, and estradiol concentrations were determined via enzyme immunoassay using subsamples of each baleen plate at 2 cm intervals. There were no significant differences in cortisol or corticosterone during pregnancy when compared to non-pregnancy (inter-calving interval), but there were significant differences between the two whales in average glucocorticoid concentrations, with the younger whale showing higher values overall. For testosterone, levels for the younger female peaked at parturition in one pregnancy, but also had spikes during non-pregnancy. The older female had three large spikes in testosterone, one of which was associated with parturition. Estradiol had large fluctuations in both whales but had generally lower concentrations during non-pregnancy than during pregnancy. There were peaks in estradiol before each pregnancy, possibly coinciding with ovulation, and peaks coinciding with the month of parturition. Both estradiol and testosterone could be useful for determining ovulation or impending birth. Using baleen to investigate retrospective steroid hormone profiles can be used for elucidating long-term patterns of physiological change during gestation.

Lay summary Case studies of two pregnant humpback whales whose hormones were analyzed in baleen may illuminate when humpback whales ovulate, gestate, and give birth. These physiological metrics could assist in accurate population growth assessments and conservation of the species. This study shows that baleen hormone analysis can be a useful tool for understanding whale reproductive physiology.

Introduction

Understanding pregnancy physiology is critical for the recovery of endangered and threatened whale species whose populations were decimated during commercial whaling and are currently under threat from anthropogenic impacts (e.g., noise, entanglement, ship strikes; Thomas et al. 2016). However, conservation of many vulnerable large whale populations has been hindered by lack of data regarding basic reproductive patterns. Determining physiological changes during reproduction in mysticetes is important for understanding causes of decline and projections of population growth (Lanyon et al. 2019). However, it is difficult to sample individuals over time because whales live underwater and are often only in one location for a short period; additionally, no method exists for live capture or blood sampling of large whales, challenging efforts to collect repeated samples for endocrine analysis.

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Issues with repeated sample collection have spurred the development of methods for analysis of novel (non-plasma) tissues, providing a way to assess physiological profiles in these cryptic species (Hunt et al. 2013). Previous studies analyzed hormone concentrations in novel sample types such as respiratory vapor (Hunt et al. 2013; Hunt et al. 2014a; Hunt et al. 2016b), feces (Rolland et al. 2005; Hunt et al. 2006; Rolland et al. 2017; Hunt et al. 2019), blubber (Pallin et al. 2018; Mingramm et al. 2020), baleen (Hunt et al. 2016a; Hunt et al. 2017a; Hunt et al. 2017b; Hunt et al. 2018; Fernández Ajó et al. 2020; Lowe et al. 2021b), and cerumen (ear plugs) (Trumble et al. 2018; Crain et al. 2020). Baleen, in particular, offers potential for retrospective assessment of endocrine patterns over multiple years, while still providing sufficient temporal resolution to illuminate seasonal patterns within a year (Hunt et al., *in press)*. Baleen consists of a series of overlapping vertical keratinous plates that hang from both sides of the upper jaw of a mysticete whale, used for filter-feeding (Hunter 1788; Tullberg 1883; Fudge et al. 2009). Each baleen piece ("plate") grows continuously and slowly, incorporating hormones from a highly vascularized root region as it grows, and thus a single plate of baleen can yield longitudinal data spanning years. All circulating steroid and thyroid hormones appear to be deposited in growing baleen. Available data indicate that baleen endocrine data likely have weekly to monthly temporal resolution, depending on species-specific baleen length and growth rate as well the sampling interval (number of cm between sampling points on the plate), thus making it an ideal tissue type for examination of long-term physiological trends (Best and Schell 1996, Hunt et al. 2013; Hunt et al. 2016a; Hunt et al. 2016b; Werth et al. 2020). Annual cycles in stable isotopes can help determine baleen growth rate and therefore the estimated date of growth of each point on the plate, since the stable isotopes change seasonally as the whales migrate and change prey types or begin fasting (Hobson and Schell 1998, Hobson et al. 2004; Lubetkin et al. 2008). Unfortunately, baleen cannot be collected from living whales and thus samples can only be obtained opportunistically from necropsies, that is, specific individuals cannot be targeted for sample collection. Baleen samples from individual whales of known history are therefore rare. In these rare cases when baleen is available from a whale with an associated sighting history, it offers the unique benefit of detailed longitudinal information on an individual that can be matched to known life history events (e.g., independently confirmed pregnancy or lactation). Such individual case studies can help to provide physiological validations for the baleen-hormone analytic method (i.e., do patterns in hormone across a baleen plate truly reflect individual endocrine history) as well as casting light on the physiology of life history stages that otherwise are exceedingly difficult to study. These multi-year analyses of individual hormone data can be an important step to determine how the reproductive physiology of large whales changes during pregnancy.

The adrenal steroid hormones known as glucocorticoids (GCs; cortisol and corticosterone) are of particular interest for studies of physiological stress in wildlife, as both GCs tend to increase during response to stress. Identification and interpretation of unusual elevations of GCs, however, relies on the ability to discriminate normal elevations of GCs that occur predictably during certain various life history stages (e.g., pregnancy) with stressors that occur due to unpredictable anthropogenic impacts (e.g., entanglement). As many mammals exhibit increases in circulating GCs during normal pregnancies, information on the GC profile across both pregnancy and non-pregnancy would therefore be valuable for interpreting the causes and consequences of non-pregnancy-related increases in GCs. Increases in maternal circulation of GCs during pregnancy are thought to be related to the increased energetic demands of pregnancy, fetal development, and parturition (Dupouy et al. 1975; Liggins 1994). Single-point fecal sampling suggests that GCs elevate, at least at some point during gestation, in several mysticetes including bowhead whales (Hunt et al. 2014b), North Atlantic right whales (NARW, Hunt et al. 2006), humpback whales (Hunt et al. 2019), and blue whales (Valenzuela-Molina et al. 2018). Individual profiles of baleen GC profiles across full pregnancies have been published for only two individual NARWs (Hunt et al. 2017a) and showed an increase in corticosterone in both whales during pregnancy but an increase in cortisol during pregnancy in only one whale. The two GCs that have been assessed, cortisol and corticosterone, possibly respond differently to various types of stressors (Koren et al. 2012; Hunt et al. 2017a), therefore, analyzing both hormones is valuable to determine how these two GCs vary in baleen whales over multiple years. Assessing GCs during pregnancy may enable greater understanding of the stresses and physiological changes that pregnancy entails, as well as improved discrimination of stress responses to anthropogenic sources (Baker et al. 2013; Palme 2019). By understanding baseline GC patterns that occur during pregnancy, it could be possible to determine if anthropogenic impacts are increasing GCs during pregnancy or if GCs from stress obscure pregnancy signals.

Another type of steroid hormones, androgens, are often considered male hormones but are important in female physiology. In female mammals, androgens are secreted by the ovaries and adrenal glands, although generally in lower concentrations than in males (Squires

2010). One of these androgens, testosterone, is responsible for primary sexual development in males but studies have shown that it has behavioral and physiological roles in females as well (reviewed in Staub and De Beer 1997). Fecal and respiratory testosterone in North Atlantic right whales was significantly higher in pregnant females than in non-pregnant females (Corkeron et al. 2017; Burgess et al. 2018) and 36% of humpback whale females approaching the breeding grounds had high levels of two androgens, androstenedione and testosterone, suggesting that these hormones could be a sign of impending birth (Dalle Luche et al. 2020). Indeed, there are reports of female mammals having high testosterone levels during pregnancy (e.g., cows, *Bos taurus* (Mostl et al. 1981), humans (Bammann et al. 1980), and marmosets, *Callithrix jacchus* (Chambers and Hearn 1979)) but the androgen patterns that occur in whales over the duration of a pregnancy are currently unknown. Using baleen to assess patterns in testosterone could help determine whether androgen assessment of pregnant females might confirm pregnancy or imminent birth, but as yet no studies have assessed androgens across the duration of a pregnancy in any species of whale.

The third major category of reproductive steroids, the estrogens, includes several related hormones (17b-estradiol, estrone, estriol, etc.) involved in sexual development, follicular development, and ovulation in female mammals (Levasseur 1984). 17b-estradiol (“estradiol” hereafter) peaks at ovulation, and, additionally, it has been shown to peak shortly before birth in some mammals [e.g., buffalo, *Bubalus bubalis* (Arora and Pandey 1982); marmosets, *Callithrix jacchus* (Chambers and Hearn 1979)], but it is unknown if baleen whales also exhibit this pattern. Fecal estradiol is higher in pregnant female NARW compared to all other reproductive classes, and is also elevated in lactating females compared to non-lactating, non-pregnant females (Rolland et al. 2005); an elevation in estradiol was also found during pregnancy in NARW baleen of an entangled female (Lysiak et al. 2018). Thus, better information on patterns in estradiol across pregnancy and lactation could inform efforts to discriminate the reproductive status of females via endocrine profile.

In prior case studies of two female humpback whales of known history, we demonstrated that baleen contains patterns of elevated progesterone corresponding to periods when the whales were known to be pregnant, suggesting that baleen endocrine profiles do accurately reflect individual endocrine history in this species (Lowe et al. 2021b). To this prior dataset on progesterone, we here add longitudinal data from both whales on four steroid hormones also important to reproduction: cortisol, corticosterone, estradiol, and testosterone. To our knowledge, these are the first longitudinal data presented for female humpback whales for any of these four hormones, and the first to cover known pregnancies. By using baleen, it was possible to quantify these hormones before, during, and after documented pregnancies. Specifically, we asked whether GCs, estradiol, or testosterone increases in humpback whales before, during or after gestation, and if any of the four hormones could be useful for the determination of pregnancy or reproductive state. Examining these four hormones in tandem allows for a better understanding of the physiology of humpback whales during reproduction.

Materials and methods

The Arctos museum database, National Oceanographic and Atmospheric Administration stranding databases and other scientific entities, totaling 197 strandings and museum collections, were queried for baleen of humpback whales with known histories. Two North Pacific baleen specimens from Glacier Bay National Park were located that met the following criteria: available for semi-destructive sampling, known to be from females of adult body length, and with associated individual sightings records that included documented pregnancies in the 3 years prior to death (the likely period of growth of the baleen). Baleen was recovered during necropsy by members of the US Marine Mammal Standing Network.

Study animals

Both whales are from the North Pacific population and were found dead in Southeast Alaska (SEAK) and are referred to by their SEAK catalog numbers: SEAK 68 and SEAK 1473.

SEAK 68 was first reported in the Alaska humpback whale fluke catalog in 1975 when she was spotted twice in Glacier Bay, Alaska during the summer feeding season (Gabriele et al. 2010). She was subsequently spotted in Alaska from 1979 until 2000 and was also spotted in Hawaii in 1981 and 1986. According to sighting history, she was seen with a calf five times from 1982 to 2000. She was found dead in 2001 after being killed from a cruise ship strike. She was ∼44.5 years old based on earplug analysis, and was pregnant at the time of death (fetus 39.2 cm, estimated to be four months old based on size, i.e., one-third through the presumed ∼12 months gestation length of humpbacks) (Gabriele et al. 2010).

SEAK 1473 was first spotted in 1997 in Glacier Bay, Alaska, and was only ever sighted in this location, where she was seen every year from 1997 until 2008. She was found dead near Glacier Bay in 2010 and was pregnant at the time of death (fetus 168 cm). SEAK 1473 was only seen once with a calf, in 2007, and was described as "small" from 1998 to 2000 (Gabriele et al. 2017). She strongly resembled a calf that was documented in Glacier Bay in 1996 but it was not possible to confirm this match, or her exact age, and therefore her age was estimated to be ∼13 years old. Due to advanced decomposition, her cause of death is unknown. Although SEAK 1473 was found dead in May 2010, she was suspected to have died in fall 2009 based on fetus size. The age of fetus was estimated to be eight months old based on size, that is, two-thirds through the presumed ∼12 months gestation length of humpbacks (Berta et al. 2016).

Measurement of baleen plates

Baleen was cleaned of any remaining gum tissue and then allowed to dry. Baleen plates were measured with a tape measure permanently attached to the posterior face of each plate approximately 2 cm from the labial edge (see Hunt et al. 2016a). The proximal end of the base of the plate (newest baleen) was designated as the "zero cm" point.

Estimating date of growth using stable isotope analysis

Stable isotope (SI) analysis was previously performed on baleen of both females to estimate date of growth of each point on the baleen plate. For complete methodology see Lowe et al. (2021a). Briefly, 1.0 ± 0.2 mg of baleen powder from each sampling location was weighed directly into tin capsules and then analyzed for relative abundance of stable isotopes of carbon and nitrogen [expressed as δ^{13}C and δ^{15}N, respectively, and are given as per mil values (‰)]. using a Thermo FlashSmart elemental analyzer in line with a ThermoFinnigan DeltaPlus XP continuous-flow isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Analysis of hormonal periodicity focused on nitrogen isotope ratios due to the fact that nitrogen isotopes have more pronounced and predictable annual variation in mysticete whales than do other elements (Best and Schell 1996; Lysiak 2008; Busquets-Vass et al. 2017; Lysiak et al. 2018). The growth rate of the baleen plate was determined via counting between the peaks of the isotope nitrogen-15 versus air (δ15N). Estimated date of growth of each point on the plate was then calculated based on peaks and troughs of annual SI cycles, as in Lowe et al. (2021b).

Pulverization of baleen and extraction of hormones

Extracts were prepared using a hand-held electric rotary grinder (Dremel Model 395 Type 5) to drill a short (1.5 cm) transverse groove across the posterior face of the plate, starting at the "zero point" (root of plate; most recently grown baleen), with baleen powder collected on a piece of weigh paper. Successive samples were drilled every 2 cm along the complete length of the plates, a sampling interval that has been previously shown to delineate seasonal patterns in baleen whales (Best and Schell 1996; Hunt et al. 2018), with each 2 cm increment estimated to represent a time interval of roughly 30–45 days in adults (Lowe et al. 2021a; Lowe et al. 2021b). SEAK 1473 had limited baleen available at some sampling sites due to use of this plate in prior assay validations (Lowe et al. 2021b), and thus cortisol was assayed every odd centimeter while corticosterone was analyzed every even centimeter. In total, the number of samples for SEAK 1473 were corticosterone ($n = 44$), cortisol samples ($n = 31$), 17β-estradiol ($n = 32$), and testosterone ($n = 12$). The number of SEAK 68 samples were: corticosterone ($n = 24$), cortisol ($n = 24$), 17β-estradiol ($n = 22$), and testosterone ($n = 20$). The differences in number of samplings for each plate and hormone were due to extensive sampling that prevented enough powder from being analyzed at each location.

Hormones were extracted with a modification of methods of Hunt et al. (2016b), that is, 4.00 mL 100% methanol added to 75.0 mg of well-mixed baleen powder, followed by 2 h vortexing, centrifugation, extraction of 3.00 mL of supernatant, dry down overnight at 45°C in a rotary evaporator. Dried extracts were reconstituted in 0.50 mL assay buffer (buffer "X065" Arbor Assays, Ann Arbor, MI, USA), sonicated 5 min, shaken for 5 min, transferred to cryovials, cooled, and then decanted to new cryovials to remove any remaining particulates. All extracts were stored at −80°C and assayed for GCs within 1 year.

Hormone assays

Cortisol, corticosterone, testosterone, and 17β-estradiol were quantified using enzyme immunoassays (EIA) previously validated for baleen (Hunt et al. 2017b; Hunt et al. 2018). All samples were run at 1:4 dilution, a dilution that kept samples close to the middle of the binding curve (area of greatest precision). Samples over 95% bound on initial assay ($n = 13$) were re-assayed at 1:2 dilution, and if still over 95% bound, they were re-assayed at 1:1 (full-strength extract, $n = 3$). Any samples under 5% binding were re-assayed at a higher dilution, generally 1:10 ($n = 6$). Any samples with coefficients of variation between duplicates >10% were rerun for quality assurance ($n = 27$).

Commercially available kits were selected based on previous successful use on mysticete baleen (Hunt et al. 2014b; Hunt et al. 2016a; Hunt et al. 2016b; Hunt et al. 2017a; Hunt et al. 2017b; Fernández Ajó et al. 2018; Hunt et al. 2018) and previously validations described in Hunt et al. (2017b) that used sets of standards spiked with pooled 1:1 baleen extract run alongside a set of standards spiked only with buffer. The cortisol assay (#K003-H1, Arbor Assays, Ann Arbor, MI, USA) has six standards, from 100 to 3200 pg/mL. An additional low standard was created by mixing equal parts buffer with the 100 mL standard to create a 50 pg/mL standard. The cortisol EIA has a reported sensitivity limit of 45.4 pg/mL with a cross reactivity to corticosterone of 1.2%. The average inter-assay precision is 8.1% and the average intra-assay precision is 8.8%. The manufacturer's protocol was followed except that the cortisol standards were brought up in the same buffer previously used to prepare the extracts (buffer #X065, Arbor Assays, Ann Arbor, MI, USA), based on technical advice from the assay manufacturer. The corticosterone EIA (#K014-H1, Arbor Assays, Ann Arbor, MI, USA) has six standards spanning 78.125 to 10,000 pg/mL according to the manufacturer's protocol; an additional low standard was added by diluting the lowest standard by 1:2 to capture low values that were seen in pilot studies (Hunt et al. 2017a; Hunt et al. 2018). The sensitivity limit for pooled baleen corticosterone in baleen whales (see Hunt et al. 2017b) is 16.9 pg/mL, average intra-assay precision is 5.2% and average inter-assay precision is 7.9%. The estradiol EIA (#K030-H5, Arbor Assays, Ann Arbor, MI, USA) has five standards ranging from 39 to 10,000 pg/mL and a reported sensitivity limit of 26.5 pg/mL with a cross reactivity to estrone of 0.73%. The average inter-assay precision is 5.1% and the average intra-assay precision is 8.4%. The testosterone (#K032-H1, Arbor Assays, Ann Arbor, MI, USA) EIA has seven standards ranging from 41 to 10,000 pg/mL and a reported sensitivity limit of 30.6 pg/mL with a cross reactivity to dihydrotestosterone of 56.8%. The average inter-assay precision is 10.9% and the average intra-assay precision is 9.3%.

Statistical analysis

To investigate how pregnancy status correlates with baleen hormone levels, both between the two whales and within the baleen growth period of each whale, we used a mixed linear effects model (LMM) (nlme package, Bates et al. 2015). The model was set using total hormone concentration (for each of the four hormones) as dependent variables and pregnancy status as an explanatory variable (categorical with two levels: pregnant or non-pregnant). We included the plate centimeter (cm) and individual (ID: SEAK 68 or SEAK 1473) as random factors. The model was constructed as follows:

$$\mathrm{Hormoneconcentration} \sim \mathrm{Pregnancystatus} +,$$

$$\mathrm{random} = \mathrm{list}\,(\sim 1\,|\mathrm{whaleID}, \sim 1|\,\mathrm{cm})\,.$$

To obtain the significance of the explanatory variable (pregnancy status) fixed effect, we used the "summary(model)" function in R, and consider the factor as significant when *t*-values higher than 2 and smaller than −2.0 and the *P*-value <0.05 (Luke 2017) using R version 4.0.2 (R Core Team 2013) Correlations were run between each hormone as well as progesterone, which was analyzed during a previous study (Lowe et al. 2021b) using the "corrplot" package (R Core Team 2020) (see Supplementary Figs. S1 and S2).

Results

Cortisol and corticosterone

Both females had GC "spikes," brief elevations lasting two or fewer sampling points, in one or both GCs at various points throughout the baleen growth period (Figs. 1 and 2); these spikes occurred during both pregnant and non-pregnant periods. SEAK 68 showed two large spikes in cortisol occurring approximately eight months apart along during a non-pregnant period in the older baleen growth with many smaller spikes throughout the baleen growth period (Fig. 1). Besides these spikes, cortisol and corticosterone were often at similar levels (cortisol $\bar{X} = 1.84 \pm 0.60$, corticosterone $\bar{X} = 1.78 \pm 0.36$, mean ± standard deviation; Fig. 3, Supplementary Table S2). SEAK 1473 had much more variation in both hormones; her peak cortisol level was more than three times the highest level seen in SEAK 68 (cortisol $\bar{X} = 4.36 \pm 3.35$, corticosterone $\bar{X} = 4.69 \pm 1.45$). This large cortisol spike occurred during a non-pregnant period. SEAK 1473's cortisol levels ranged from 0.03 to 13.39 ng/g while SEAK 68's cortisol levels ranged from 0.80 to 3.62 ng/g; SEAK 1473's corticosterone levels ranged from 2.26 to 9.38 ng/g while SEAK 68's cortisol levels ranged from 1.12 to 2.44 ng/g (Supplementary Table S2).

Testosterone

Testosterone showed high variability in both females during pregnant and non-pregnant periods (Figs. 1 and 3). For the older female (Fig. 1), values ranged from 2.24 to 9.06 ng/g ($\bar{X} = 5.66 \pm 1.70$) while the younger female (Fig. 2) had a larger range, from 5.35 to 22.45 ng/g ($\bar{X} = 13.40 \pm 5.66$). Testosterone spiked at parturition in one pregnancy (21.93 ng/g) in the younger female (Fig. 2) but also spiked during a non-pregnant period at roughly the same level (22.45 ng/g). The older female had three

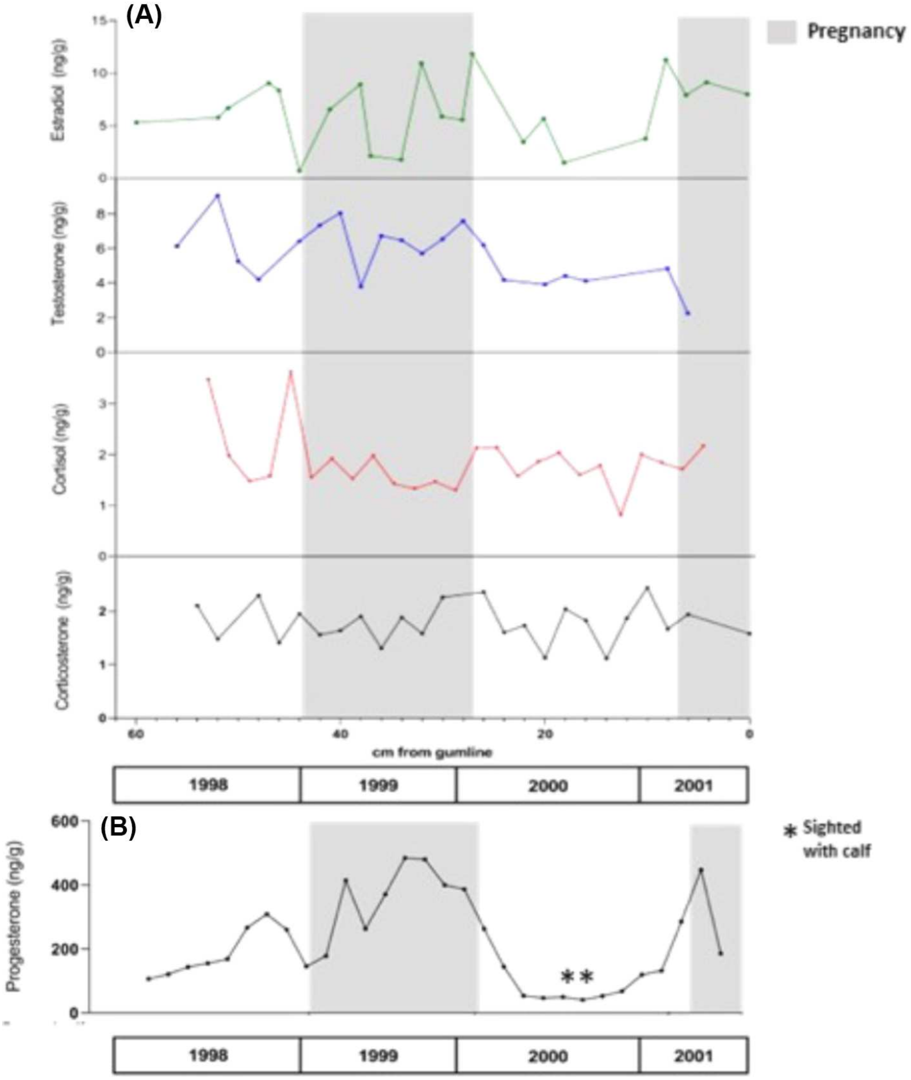

Fig. 1 Humpback whale (*Megaptera novaeangliae*) SEAK 68 steroid hormone levels over the baleen growth period, including corticosterone, cortisol, testosterone, and estradiol (**Panel A**) and comparison with progesterone (**Panel B**, adapted from Lowe et al. 2021b). SEAK 68 was estimated to be 44.5 years old and died from a ship strike. Y-axis is in nanograms of hormone per gram of baleen powder. Gray-shaded areas are inferred pregnancies based on sighting history and fetus at necropsy. Fetus length was 39.2 cm at time of maternal death. Dates were extrapolated using stable isotopes and are an estimate only.

large spikes in testosterone but none of them were associated with parturition (Fig. 1).

Estradiol

Estradiol concentrations were also highly variable across the baleen plates of the two whales, but unlike testosterone, the older female had higher values than the younger female (Figs. 1 and 2). The older female had estradiol values ranging from 0.71 to 11.84 ng/g ($\bar{X} = 6.37 \pm 3.23$, Fig. 2, Supplementary Table S2), while the younger female's estradiol concentrations ranged from 0.91 to 6.87 ng/g ($\bar{X} = 3.32 \pm 1.39$, Fig. 1, Supplementary Table S2). Estradiol was generally lower during the intercalving intervals than during pregnancy ($P = 0.12$) and there were peaks in estradiol before each pregnancy.

Hormone patterns during pregnancy versus non-pregnancy

Neither whale differed in concentration cortisol, corticosterone, estradiol, or testosterone between reproductive states ($P > 0.1$ for all comparisons).

Correlations between hormones

SEAK 1473, the 13 years old, had a very strong negative relationship between estradiol and progesterone (−0.9, Supplementary Fig. S1). Most of the other relationships were weak (<0.4). The two strongest relationships after

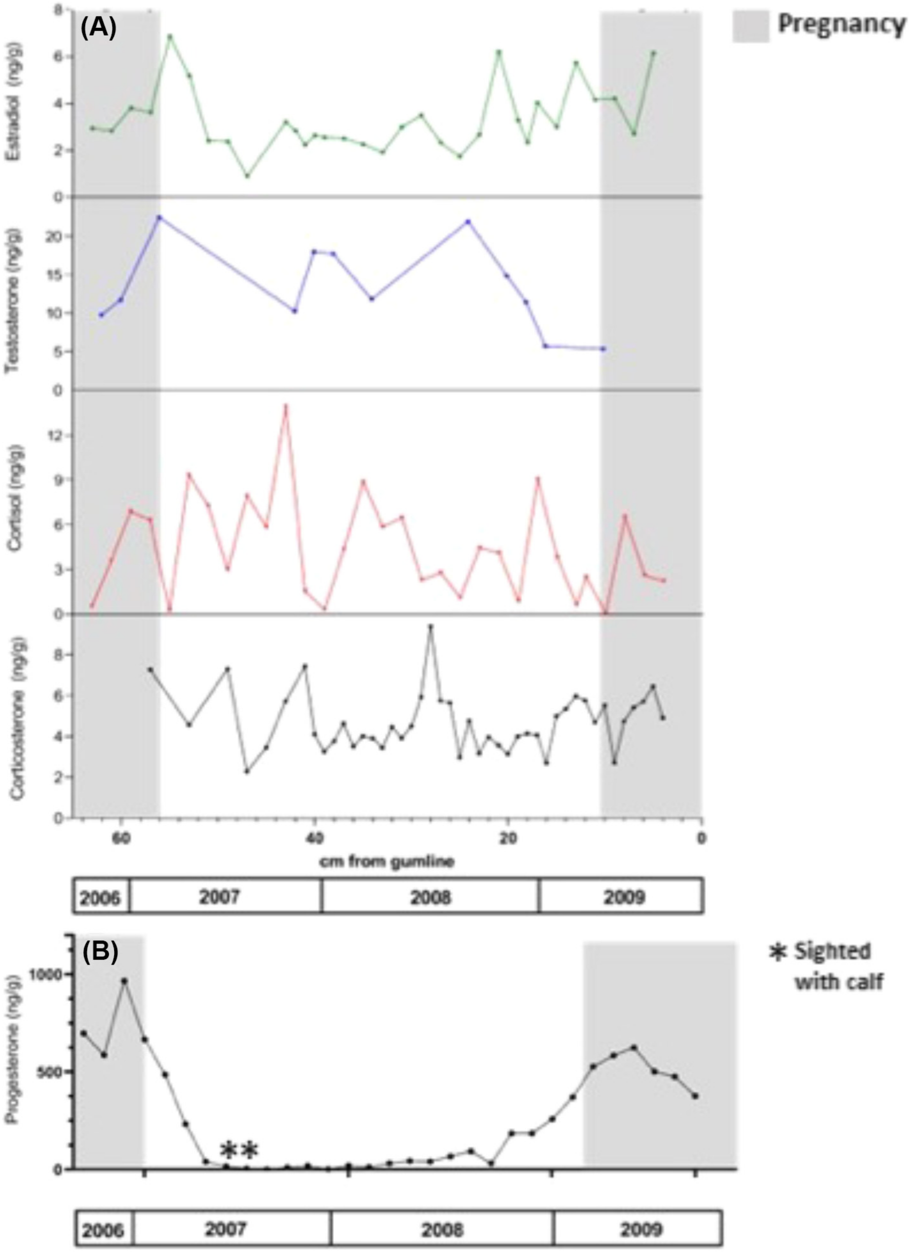

Fig. 2 Humpback whale (*Megaptera novaeangliae*) SEAK 1473 steroid hormone levels over the baleen growth period, including corticosterone, cortisol, testosterone, and estradiol (**Panel A**) and comparison with progesterone (**Panel B**, adapted from Lowe et al. 2021b). SEAK 1473 was estimated to be ∼13 years old and died from unknown causes. Y-axis is in nanograms of hormone per gram of baleen powder. Gray-shaded areas are inferred pregnancies based on sighting history and fetus at necropsy. Fetus length was 168 cm at time of maternal death. Dates were extrapolated using stable isotopes and are an estimate only.

estrogen–progesterone were testosterone–progesterone (−0.37) and cortisol–progesterone (−0.32). The correlation between cortisol and corticosterone was −0.17.

SEAK 68, the 44.5 years old, did not show a strong negative relationship between estradiol and progesterone like the other whale; rather, it was slightly positive (0.29, Supplementary Fig. S2). The strongest correlations between any of the hormones were between corticosterone–estradiol (−0.38) and testosterone–progesterone (0.32). The relationship between cortisol and corticosterone could not be assessed through correlation due to a lack of sufficient data from the same sampling points due to previous analysis.

Discussion

All four hormones were readily detectible along the baleen plate, showing that longitudinal studies on stress

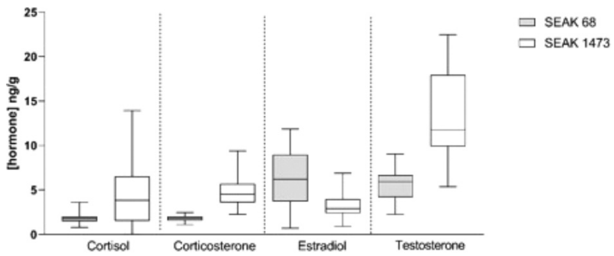

Fig. 3 Steroid hormone concentrations in nanograms of hormone per gram of baleen powder for two pregnant humpback whales whale (*Megaptera novaeangliae*). Gray-shaded boxes represent SEAK 68 (44.5 years old) and white boxes represent SEAK 1473 (~13 years old).

and reproductive hormones during pregnancy using baleen are possible. Overall, the younger whale often showed higher concentrations of hormones than the older whale (~30 years older). GCs did not show clear patterns associated with pregnancy and might therefore be better indicators of other types of stress (e.g., nutritional or anthropogenic stressors) in this species. Both whales had spikes of estradiol and testosterone associated with pregnancy but also had peaks that were not coincident with gestation.

Baleen GCs did not increase during pregnancy

Corticosterone and cortisol were assessed along the baleen plate of two whales before, during, and after known pregnancies. Neither hormone was significantly higher during pregnancy for either whale, which contrasts with previous research showing elevated GCs during pregnancy in other baleen whales (Rolland et al. 2007; Hunt et al. 2014b; Hunt et al. 2017a; Hunt et al. 2019). It is possible that the different GC profiles of pregnancy might be obscured in these two individuals by cortisol and corticosterone spikes due to unpredictable stressful events. That is, if multiple stressful events (e.g., entanglement, increased noise, etc.) occurred during the non-pregnancy period, any increase in GCs due to pregnancy would be difficult to discern. Increased GCs during pregnancy were also possibly not observed because of the low sample size of two individuals; baleen collection from whales with known histories is extremely rare, thus sample sizes for these types of studies are unavoidably low, and the two individuals studied here might have had anomalous patterns of GCs.

Another possible explanation for the lack of increase in GCs during pregnancy seen here is that humpback whales might not exhibit elevations in GCs during pregnancy, possibly in any sample type (e.g., plasma) or specifically in baleen. GCs increase in late pregnancy in most mammalian taxa (Edwards and Boonstra 2018) but this pattern is not seen in all terrestrial artiodactyls, which are whales' closest relatives, and it may be that there is variation among the cetaceans in the degree to which GCs change during pregnancy. In humpback whale feces, however, GC metabolites were higher in pregnant females than non-pregnant females and males (Hunt et al. 2019), suggesting that different tissue types can yield different GC patterns during gestation. Further analysis using multiple sample types from the same whale (e.g., feces and baleen both collected at necropsy) could help clarify how these hormones vary between different parts of the body.

In other mysticetes, GCs elevations during pregnancy have been documented in North Atlantic right whale (*Eubalaena glacialis*; baleen, feces; Hunt et al. 2017a), bowhead whale (*Balaena mysticetus*; baleen; Hunt et al. 2014b), and blue whale (*Balaeoptera musculus*; feces; Valenzuela-Molina et al. 2018). In the newest grown baleen of two NARW (*Eubalaena glacialis*), corticosterone was significantly elevated during pregnancies but cortisol was only significantly elevated during pregnancies in one female (Hunt et al. 2017a), demonstrating the possibility of a pregnancy response in one GC only. In bowhead whale (*Balaena mysticetus*) baleen, pregnant females had higher cortisol than non-pregnant females and males (Hunt et al. 2014b). It is possible that the exceedingly long baleen of NARW and bowhead whales allows for a longer and clearer assessment of GCs during pregnancy, as those baleen plates might contain more pregnancies for analysis since NARW baleen contains upwards of 10–20 years of growth versus 3–5 years of growth for humpbacks (Hunt et al. 2016a; Hunt et al. 2017a; Lysiak et al. 2018). In this study, the correlation between GCs and pregnancy were not significant, but this pattern might change as more baleen plates are analyzed from a range of differently aged females. Finally, GCs elevations in pregnancy have also been documented in some odontocetes, for example, captive killer whales (Steinman et al. 2016; Robeck et al. 2017). Thus, the general cetacean pattern appears to be for GCs to elevate during pregnancy, but humpback data demonstrate that this pattern does not always occur in all individuals or all sample types.

Corticosterone compared to cortisol

Most studies of cetacean physiology have only measured cortisol, based on an assumption that cortisol is the main GC and that corticosterone, if detectable, would merely parallel cortisol and might be relatively uninformative. However, newer data indicate that some cetacean sample types have higher corticosterone than cortisol (e.g., Hunt et al., 2017b) and that the two GCs might have quite different patterns (Fernández Ajó *et al.* 2018; Hunt et al. 2018; Fernández Ajó *et al.* 2020; Lowe et al. 2021a). This study also found that cortisol and corticosterone did not strongly correlate. When examining GCs across the entire plate length, corticosterone and cortisol showed various points of dominance (greater concentration) along the baleen, highlighting the need for more research to determine if humpback whales are cortisol or corticosterone dominant, or whether, in fact, the concept of GC "dominance" (one GC always being at greater concentration than the other) is relevant for cetaceans. It is possible that relative concentrations of the two GCs in baleen may not be directly comparable to plasma concentrations; for example, corticosterone could be preferentially deposited in baleen (Hunt et al. 2017a). Additionally, some tissues are steroidogenic (including some epithelial tissues, such as, potentially, the root of a growing baleen plate—thought to be a homolog of epithelial hair follicles of other mammals). Previous mammalian studies testing the relationship between these two hormones have questioned both the concept of dominance (i.e., one GC consistently being more abundant than the other GC) as well as the concept of parallelism between the "dominant" and "subordinate" hormones, that is, the concept that the less abundant GC correlates so consistently with the more abundant GC that assay of the less abundant GC would provide no additional information (Hancock 2010; Koren et al. 2012). Hunt et al. (2017a) found that the two GCs had markedly different patterns in baleen of two female NARW, with the two hormones sometimes elevating in parallel (e.g., late pregnancy) but at other times providing quite different information (corticosterone showing broad, prolonged elevations during lactation; cortisol showing brief "spikes" corresponding to periods of entanglement). Validation tests of GC assays in baleen of eight different whale species revealed that most, but not all, mysticetes had more corticosterone than cortisol in baleen powder (Hunt et al. 2017b). Dalle Luche et al. (2019) found that cortisol in humpback whales is higher in blubber than other GCs (five females, five males) but also had the largest range; other studies with humpback whales have concentrated on only one hormone so comparison cannot be done (Kershaw et al. 2013; Teerlink et al. 2018; Mingramm et al. 2019; Cates et al. 2020; Mingramm et al. 2020). Koren et al. (2012) suggested that some mammals may have "dual glucocorticoid signaling," wherein each hormone reacts to different types of stressors and causes different downstream effects, and, ultimately, provides different information to wildlife researchers. For example, in captive bottlenose dolphins, cortisol was mainly affected by season (highest in fall) while corticosterone was only affected by reproductive state, with significant increases observed in pregnant females as compared to non-pregnant females (Robeck et al. 2017). Koren et al. (2012) additionally found that in three of eight artiodactyls (relatives of cetaceans) studied, there was not any consistent relationship of cortisol to corticosterone. In this study, each GC exhibited a unique profile across time—that is, the two GCs were not in parallel for either whale. Thus, the humpback whale profiles presented here suggest that cortisol dominance in baleen cannot be assumed for large whales. We suggest that when feasible, both hormones should be analyzed.

Testosterone

Testosterone showed peaks near areas that coincided with birth in both whales, although the younger female's values were almost twice those of the older female. Previous research has shown that testosterone might be an indicator of impending birth in humpback whales migrating to the breeding grounds (Dalle Luche et al. 2020), and our research shows similar patterns. Previous studies using fecal samples found higher androgens in pregnant female NARW when compared to all other reproductive classes (Rolland et al. 2005; Corkeron et al. 2017). However, that study primarily used fecal samples collected in August and September, thought to represent mid-gestation for pregnant females; it is not possible to assess testosterone via fecal samples in NARW females shortly prior to birth because female NARW fast (and do not produce fecal samples) during the calving season. We also note that in case when testosterone elevates concurrently with the estrogens (as seen here in late gestation), it cannot be assumed that the testosterone is playing a direct physiological role, since testosterone is a precursor to the estrogens and thus could be expected to elevate in plasma whenever the estrogens elevate. However, it is now generally believed that androgens do play a direct physiological role in most female mammals, that is, binding to androgen receptors and exerting androgen-specific effects (Hammes and Levin 2019). Generally, we encourage researchers to consider routine assay of testosterone and other androgens in females, rather than restricting testosterone assays to just males.

Estradiol

Estradiol concentrations varied during the various reproductive phases in both whales, although there were generally lower values during non-pregnancy periods. There were large differences between the two individuals in the range of estradiol values and peaks were observed prior to pregnancy and could be an indicator of ovulation. Estradiol could also be important in determining impending birth, along with testosterone, as both hormones peak during the period that we expected the whales to give birth. However, there were also other peaks that occurred that were not associated with birth. With a sample size of only two we cannot yet conclude that estradiol and testosterone generally peak at the end of gestation in all humpback whales; follow-up studies with more individuals are clearly needed. Because of this individual variation, changes in baseline in multiple hormones (i.e., estradiol and testosterone) could be a better indicator of reproductive class change rather than a threshold, which has been proven successful for other hormones, such as progesterone (Pallin et al. 2018).

Conclusion

Our study shows that steroid hormone analysis along the baleen growth period can be used to determine physiological patterns during pregnant and non-pregnant periods in humpback whales but, due to the limited sample size, are not clear enough on their own to determine pregnancy status. Further exploration may find more consistent patterns with pregnancy periods that could prove useful to determining gestational state without the need for additional analyses (e.g., progesterone or sighting history). When assessing GCs for assessing causes and consequences of anthropogenic stress in baleen whales, it is important to know if internal factors, such as pregnancy, are increasing GC concentrations. Results of this study and prior studies show that GCs in baleen whales are highly variable among individuals (Rolland et al. 2007; Hunt et al. 2017a). Testosterone and estradiol showed large variations along the plate but had peaks that were associated with birth, showing promise for these two hormones to be evaluated for their role in late pregnancy and parturition. Future studies should examine all available sample types (baleen, blubber, feces, and respiratory vapor) in females with known history to determine if and how these various steroid hormones change in various tissues throughout pregnancy states.

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Conflict of interest statement

The authors declare no conflicts of interest.

Data availability statement

Data will be uploaded to an online open database and also available upon request.

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