Multi-year longitudinal profiles of cortisol and corticosterone recovered from baleen of North Atlantic right whales (*Eubalaena glacialis*)

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ABSTRACT

Research into stress physiology of mysticete whales has been hampered by difficulty in obtaining repeated physiological samples from individuals over time. We investigated whether multi-year longitudinal records of glucocorticoids can be reconstructed from serial sampling along full-length baleen plates (representing ~10 years of baleen growth), using baleen recovered from two female North Atlantic right whales (*Eubalaena glacialis*) of known reproductive history. Cortisol and corticosterone were quantified with immunoassay of subsamples taken every 4 cm (representing ~60 d time intervals) along a full-length baleen plate from each female. In both whales, corticosterone was significantly elevated during known pregnancies (inferred from calf sightings and necropsy data) as compared to intercalving intervals; cortisol was significantly elevated during pregnancies in one female but not the other. Within intercalving intervals, corticosterone was significantly elevated during the first year (lactation year) and/or the second year (post-lactation year) as compared to later years of the intercalving interval, while cortisol showed more variable patterns. Cortisol occasionally showed brief high elevations ("spikes") not paralleled by corticosterone, suggesting that the two glucocorticoids might be differentially responsive to certain stressors. Generally, immunoreactive corticosterone was present in higher concentration in baleen than immunoreactive cortisol; corticosterone:cortisol ratio was usually >4 and was highly variable in both individuals. Further investigation of baleen cortisol and corticosterone profiles could prove fruitful for elucidating long-term, multi-year patterns in stress physiology of large whales, determined retrospectively from stranded or archived specimens.

HIGHLIGHTS

- Immunoreactive cortisol and corticosterone are detectable in whale baleen
- Single baleen plates contain multi-year longitudinal time series of glucocorticoids
- Glucocorticoids tend to be elevated in baleen growth coincident with pregnancy
- Corticosterone was present in baleen at much greater concentration than cortisol

KEYWORDS: marine mammals, *Cetacea*, baleen hormones, cortisol, corticosterone, stress

1. Introduction

Many populations of large whales are experiencing increased exposure to a variety of stressors, such as global climate change, vessel strikes, increases in ocean noise (e.g., vessel noise, military sonar, seismic exploration), entanglement in fishing gear, toxins, and food limitation (reviewed in Thomas and Reeves, 2015). Differentiating the potential physiological impacts of these various stressors has proven difficult, partly due to a relative dearth of techniques for assessing adrenal activity and stress physiology in large cetaceans (Atkinson et al., 2015; Hunt et al., 2013). In terrestrial wildlife, physiological effects of natural and anthropogenic stressors are often monitored via assay of glucocorticoids (GCs) — cortisol, corticosterone and related hormones. Generally, GC secretion from the vertebrate adrenal gland is stimulated by the hypothalamo-pituitary-adrenal axis in response to a wide variety of stressors, including natural states of heightened energetic needs (e.g., pregnancy, lactation, migration) as well as unpredictable stressors (e.g., predator presence, injury, human disturbance, noise exposure, etc.; Romero and Wingfield, 2015). Together the GCs coordinate an adaptive stress response that includes heightened energy availability (via gluconeogenesis, lipolysis, etc.), inhibition of nonessential systems (reproduction, growth, immune response), and alteration of behavior toward "escape" or "emergency" behaviors (reviewed in Romero and Wingfield, 2015).

Most vertebrates secrete both cortisol and corticosterone from the adrenal gland; the two hormones are similar in structure and can be interconverted with a single enzymatic step.

Abbreviations:
B = corticosterone; BGR = baleen growth rate; EIA = enzyme immunoassay; F = cortisol; GC = glucocorticoid; HPLC = high performance liquid chromatography; NARW = North Atlantic Right Whale; RIA = radioimmunoassay; SI = stable isotopes

Different taxa are traditionally classed as either "cortisol-dominant" (most mammals) or "corticosterone-dominant" (birds, reptiles, a few mammalian taxa such as rodents, etc.) based on which hormone is usually more abundant in plasma (Hancock, 2010). The two hormones are often assumed to parallel one another; however, some data indicate that cortisol and corticosterone may respond differently to acute vs. chronic stressors (Koren et al., 2012).

Serial plasma samples collected over time from the same individuals (i.e., longitudinal sampling) have been particularly informative for clarifying relationships of GCs with a multitude of potentially important natural and anthropogenic stressors. Longitudinal sampling enables detailed study of cycles in GC secretion, relationships of GCs to long-lasting reproductive states such as pregnancy or lactation, and can help discriminate cases of chronic vs. acute stress events (e.g., Bettendorf et al., 1998; Grant et al., 2016; Koester et al., 2015; Reeder et al., 2004). Wildlife GC research increasingly employs alternative (non-plasma) sample types such as feces and hair for such studies (e.g., Lafferty et al., 2015; Putman et al., 2015; Terwissen et al., 2014). Not only do these alternative sample types eliminate influences of capture stress, but they also integrate hormone secretion over longer time periods (hours to weeks) and may be preferable for studies of chronic and anthropogenic stress (Dantzer et al., 2014; Dickens and Romero, 2013). Continuously-growing tissue types such as claw, nail, and hair may also have the unique advantage of capturing a longitudinal time series of GC secretion that spans the period of tissue growth, which may be months to years depending on species- and tissue-specific growth rate (D'Anna-Hernandez et al., 2011; Mack and Fokidis, 2017; Malcolm et al., 2013; Matas et al., 2016; Meyer and Novak, 2012; Izawa et al., 2015; Yang et al.,1998).

It is presently not possible to collect plasma samples from free-living large whales — in fact, no method exists for live capture of large whales — and thus basic information on their

adrenal activity is extremely limited. However, some alternative sample types can be collected from whales, including feces, blubber biopsy dart samples, and respiratory vapor (e.g., Hogg et al., 2005, 2009; Hunt et al., 2006, 2013, 2014a; Kellar et al., 2006; Rolland et al., 2005; Vu et al., 2015). For example, fecal GCs of North Atlantic right whales (*Eubalaena glacialis*, NARW) show significant increases corresponding with energetically-demanding states such as pregnancy and lactation, as well as with anthropogenic stressors of chronic ocean noise and entanglement (Corkeron *et al.*, 2017; Hunt et al., 2006; Rolland et al. 2005, 2012, *in revision*). Unfortunately, longitudinal glucocorticoid profiles from individual whales have been extremely difficult to obtain, the best exemplar being one study of GC content of annual cerumen (earwax) layers from a single blue whale (Trumble et al., 2013). Whale earwax plugs, however, are relatively rarely collected, decompose rapidly in stranded specimens, and their temporal resolution appears limited to 6-month periods.

Baleen, which is routinely collected at necropsy, may enable such longitudinal analyses of GC patterns over multiple years in individual whales. Baleen is the filter-feeding apparatus of the mysticete whales, and consists of flexible, long, thin strips ("plates") suspended in parallel from the upper palate, each plate growing continuously from the upper end (base) and wearing away at the distal tip. Baleen is composed of long filaments of α-keratin (suspected to be homologous to hair of other mammals) embedded in a hard, but flexible, matrix of calcium salts (St. Aubin et al. 1984; Young et al., 2015). In most species a baleen plate represents several years of continuous growth, and in the *Balaenidae* (bowhead, *Balaena mysticetus*, and right whales, *Eubalaena spp.*) a single plate can represent more than a decade of the whale's life (Best et al., 1996; Lee et al., 2005; Lubetkin et al., 2008; Lysiak, 2009; Matthews and Ferguson, 2015). In many migratory species, the season and year of growth of each point on a baleen plate can be

estimated via analysis of stable-isotope (SI) ratios, which often vary seasonally due to shifts in prey base as whales migrate between summer and winter feeding grounds (Best and Schell, 1996; Lee et al., 2005; Matthews and Ferguson, 2015). Baleen of bowhead whales (*Balaena mysticetus*) has recently been found to contain progesterone and cortisol, with concentrations of both hormones at the base of the plate (most recently grown baleen) corresponding to sex and reproductive condition of the whales at the time of death (Hunt et al., 2014b). In a recent study of two adult female North Atlantic right whales (NARW, *Eubalaena glacialis*) that had been killed by vessel strike, baleen progesterone was found to be dramatically elevated in regions of baleen grown during known pregnancies (Hunt et al., 2016). These results suggest that baleen plates might also contain longitudinal, multi-year, time series of other steroid hormones. Information on glucocorticoids is particularly desirable given management needs for methods of assessing effects of natural and anthropogenic stressors in large whales (Atkinson et al., 2015; Hunt et al., 2013). The National Academies of Sciences has recently recommended further research on baleen hormones for assessment of stress in cetaceans (National Academies of Sciences, Engineering, and Medicine, 2016).

In order to assess baleen GC profiles in individual whales, we used the same two NARW plates analyzed previously for progesterone (Hunt et al., 2016), i.e., from two adult females of known calving history, to reconstruct decade-long cortisol and corticosterone profiles. Mysticete whales are assumed to be cortisol-dominant; we studied both cortisol and corticosterone based on relative lack of information on circulating glucocorticoids in NARW (though see Rolland et al., *in revision*), and also due to the possibility that the two glucocorticoids may offer differing and perhaps complementary information on acute vs. chronic stress (Koren et al., 2012). Methodological testing was also necessary to address issues such as assay performance and

potential hormone degradation over the 9-10 year time period of baleen growth. Specific research questions were: (1) Are cortisol and corticosterone detectable and quantifiable in NARW baleen with commercial immunoassays, with good assay performance (parallelism and accuracy validations)? (2) Does either hormone show any indication of degradation in older baleen at the tip of the plate (i.e., poorer assay parallelism)? (3) Does either glucocorticoid show elevations that coincide with known energetically-expensive states, such as pregnancy and lactation (Fortune et al., 2013, van der Hoop et al., 2017)? (4) What are the relative concentrations of cortisol and corticosterone in baleen?

2. METHODS

2.1 Study animals

We studied baleen plates recovered from necropsy of two NARW females: Female 1 (Eg #1004, "Stumpy") and Female 2 (Eg #1014, "Staccato"). NARW are a critically endangered species (total population size ca. 500 individuals; Pettis and Hamilton, 2016) that have been under intensive long-term study for more than three decades. Baleen plates from females of known reproductive history (i.e., confirmed via calf sightings) are quite rare; only two such NARW plates were available for this study. Sightings data for both females are from the North Atlantic Right Whale Identification and Sightings Catalog (www.rwcatalog.neaq.org), which uses photo-identification of individual whales. Photo-identification is performed by an external team following clearly established and well tested photo-identification protocols; see Hamilton et al. (2007) and Frasier et al. (2009) for details. The resulting sightings records for Female 1 and Female 2 are summarized in Hunt et al. (2016). Briefly, Female 1 was first sighted in 1975 and was found dead on February 7, 2004 with injuries consistent with vessel strike. In the ten years

prior to death (i.e., the interval captured by her baleen plate) she had two known pregnancies, resulting in a calf born in December 1996 and a full-term fetus discovered at necropsy in February 2004 (North Atlantic Right Whale Consortium, 2015). Female 2 was first sighted in 1974 and was found dead on April 20, 1999, also with injuries consistent with vessel strike, though in her case there were also indications of possible previous injury or disease prior to the vessel strike; the necropsy found traumatic injury sustained on the right side fracturing the mandible, lingual ulcerations, epidermal papillomatosis and ulceration, hemorrhagic colitis, and suspected septicemia precipitated by trauma and complicated by chronic illness (Moore et al., 2005). In the decade prior to death, Female 2 calved twice, in December 1990 and December 1996 (Moore et al., 2005; North Atlantic Right Whale Consortium, 2015). She also acquired several entanglement scars during the last four years of her life, thought to represent at least two separate incidents of entanglement in fishing gear (P. Hamilton, pers. comm.). Fishing gear entanglements are a common source of morbidity and some mortality in adult NARW, and entail substantial energetic burden and physiological stress (Moore, 2014; Moore and van der Hoop, 2012; Rolland et al., *in revision*; van der Hoop et al., 2017). Female 1 did not acquire any known entanglement scars during the time period represented by her baleen plate. Female 1's plate was from the left side of the mouth and Female 2's from the right side. Both plates were stored at room temperature for multiple years between necropsy (in 2004 for Female 1 and 1999 for Female 2) and hormone extraction (in 2015).

Previously published baleen progesterone profiles for both females showed good correspondence between dates of calf sightings (or, in Female 1's case, size of fetus at necropsy) and locations of extremely high progesterone content in baleen grown the year prior, based on an estimated gestation length of 12-13 mo (Best, 1994) and an estimated baleen growth rate (BGR)

of 1 cm per 15 days (derived from stable-isotope data and sightings records; Hunt et al., 2016; Lysiak, 2009). Baleen progesterone remained very low during the intercalving intervals of seven years for Female 1 and six years for Female 2, likely indicating that there were not any undetected pregnancies (Hunt et al., 2016). NARW intercalving intervals averaged 3.7 years in the 1980s, but lengthened (for unknown reasons) to 5.8 years in the 1990s (Kraus et al., 2007), the time period captured by both baleen plates in this study. The first year of an intercalving interval represents lactation, which lasts 12-13 mo in NARW (Hamilton et al., 1995; Kraus et al., 2007).

2.2. Measurement of baleen plates and estimating date of growth

Baleen plates were measured with a tape measure permanently attached to the posterior face of each plate on the labial edge, following the natural curve of the plate (see Hunt et al., 2016). The proximal end of the base of the plate (newest baleen) was designated as the "zero cm" point and was assigned an estimated growth date of the day before the whale was found dead. Other points along the baleen plate were then assigned an estimated date-of-growth based on the distance from the zero-cm point and the estimated BGR (1 cm / 15 d).

2.3. Pulverization of baleen and extraction of hormones

This study used archived baleen extracts stored from the prior study on progesterone (see Hunt et al., 2016). In brief, the extracts were prepared using a hand-held electric rotary grinder (Dremel Model 395 Type 5) to drill a short (4 cm) transverse groove across the posterior face of the plate, starting at the labial edge, with baleen powder collected on a piece of weigh paper. Successive samples were drilled every 4 cm along the complete length of the plates, with each 4 cm span

representing ca. 60 days of baleen growth. Total number of baleen samples was $n = 55$ for Female 1 and $n = 58$ for Female 2. Hormones were extracted from samples with 4.00 mL 100% methanol added to 100.0 mg of well-mixed baleen powder, followed by 20 h vortexing, centrifugation, two rinses (each with 1.0 mL methanol) and dry-down of the combined supernatant under air blow; for details see Hunt et al. (2016). Extracts were reconstituted in 0.50 mL assay buffer ("progesterone assay buffer" #X065, Arbor Assays, Ann Arbor, MI, USA), vortexed 1 min, sonicated 1 min, transferred to cryovials, spun in a minifuge for 10 s and decanted to a new cryovial to remove any remaining particulates. All extracts were stored at -80°C and assayed for glucocorticoids within one year.

2.4. Hormone assays

Immunoreactive cortisol was quantified with a commercial enzyme immunoassay (EIA) kit, selected based on previous successful use with bowhead whale baleen (catalog #K003-H1, Arbor Assays, Ann Arbor, MI; Hunt et al., 2014b). The cortisol EIA has six standards spanning 100-3200 pg/mL. The manufacturer's reported sensitivity limit is 17.3 pg/mL, average intra-assay precision is 8.8%, average inter-assay precision is 8.1%, and cross-reactivity to corticosterone is 1.2%; for other cross-reactivities and further details see Hunt et al. (2014b). 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.

Immunoreactive corticosterone was quantified using a double-antibody ^{125}I radioimmunoassay (RIA) kit selected based on previous successful use in NARW fecal extracts as well as feces of other mammals (MP Biomedicals [previously ICN Biomedicals] catalog #07-

120102, Costa Mesa, CA, USA; Hunt et al., 2006; Wasser et al., 2000). The manufacturer provides six standards spanning 125-5000 pg/ml; an additional low-dose standard of 62.5 pg/ml was added to the standard curve, created by mixing equal volumes of the lowest standard and the manufacturer's assay buffer ("steroid diluent", MP Biomedicals, Costa Mesa, CA, USA). Assay sensitivity is reported by the manufacturer to be 200 pg/mL; however, addition of the extra standard lowers the sensitivity to an estimated 17 pg/mL, calculated in-house from the 95% confidence interval of twenty-four blanks run in six separate assays. Intra-assay variation is 7.2%, inter-assay variation is 6.9%, and cross-reactivity to cortisol is <1.0%; for other assay details including other cross-reactivities, see Wasser et al. (2000). The manufacturer's protocol was followed except that all reagents, standards and samples were pipetted at half-volume. Prior to assay, samples were diluted four-fold in the same buffer used to prepare the assay standards ("steroid diluent," MP Biomedicals, Costa Mesa, CA, USA).

Assay validations for both hormones employed standard parallelism and accuracy tests (Grotjan et al., 1996). In order to test the possibility that hormones might degrade in older baleen, parallelism was tested for both assays with two pools, one made from "new" baleen at the base of the plates (0-20 cm region, consisting of unerupted baleen within the gum tissue, i.e., never directly exposed to seawater), and the other from older baleen at the tip of the plates in the >180 cm region (i.e., baleen that had been directly exposed to seawater for ~9-10 years). Potential degradation of hormone in older baleen could theoretically result in poorer parallelism of the old-baleen pool as compared to the new-baleen pool, assuming the degraded hormone does still bind partially to the antibody (see Discussion). Both pools were created by combining equal volumes of ten samples, five from each female. The pools were serially diluted eight times

in appropriate assay buffer, after which all dilutions were assayed for both hormones, with the slope of percent-bound vs. log[relative dose] compared to the standard curves.

Accuracy (aka "matrix effect"; Grotjan et al., 1996) was tested to assess whether the assays can distinguish low from high doses with acceptable mathematical accuracy despite the presence of unusual sample matrix, i.e., baleen extract. This test used a sample pool produced by combining equal volumes of samples from intercalving intervals (defined from sightings records, i.e., multiple years when no calf was sighted with the female), again using five samples from each female (n = 10 total). Intercalving intervals were selected due to previous findings that glucocorticoids may be elevated during pregnancy (Rolland et al., 2005), since accuracy tests require a pool with relatively low concentration of the hormone of interest. Based on parallelism results, this pool was assayed at 1:1 (full strength) for the cortisol assay but was diluted to 1:4 for the corticosterone assay, so as to fall close to 50% bound on the standard curves (i.e. area of greatest assay precision). In each assay, a set of hormone standards was spiked with an equal volume of pool and assayed as unknowns, with concentration calculated in reference to a second standard curve spiked only with an equal volume of assay buffer. Observed dose of the spiked standards was then compared to known standard dose.

After validations were complete, all baleen samples were assayed individually at 1:1 for cortisol, and at 1:4 for corticosterone. Each whale's samples were grouped in a single assay for each hormone. All samples, standards, and low and high controls were assayed in duplicate, and non-specific binding samples and blank samples (i.e. zero dose) in quadruplicate. Any samples with >10% coefficient of variation between duplicates, or with anomalous results compared to adjacent samples, were re-assayed to confirm results. Final results were converted to ng of immunoreactive hormone per g of baleen powder.

2.5. Statistical analyses

Assay parallelism results from the new-baleen and old-baleen pools were analyzed with F-tests comparing slopes of the linear portions of all three curves (new-baleen, old-baleen, and standards). Accuracy results were graphed as observed vs. known standard dose and assessed against the following criteria: slope of the best-fit regression line within 0.7–1.3, coefficient of determination close to 1.0, nonsignificant deviation from linearity (via runs test) and y-intercept of the regression line within 50% of the apparent concentration of the pool when assayed alone. For glucocorticoid profiles across time within an individual whale, concentrations of corticosterone and cortisol during pregnancies vs. intercalving intervals were compared using unpaired *t*-tests for each whale and each hormone. To assess potential patterns within intercalving intervals, different calendar years (year 1 = lactation year, year 2 = post-lactation or "resting" year, followed by years 3-6) were compared to each other with ANOVA, followed by post-hoc comparisons using Tukey's multiple comparisons tests. Within each individual, cortisol-corticosterone correlations were assessed in three separate analyses: pregnancies only, intercalving intervals only, and pregnancies + intercalving intervals combined (i.e. all samples from that individual). Corticosterone:cortisol ratio (B/F ratio) was calculated for all samples in both whales, and coefficient of variation was determined for each whale's set of B/F ratios. Pregnancies and intercalving intervals were defined from sightings records and necropsy data (see Hunt et al., 2016). Statistical tests were performed with Prism 6 for Mac OSX. All tests were two-tailed with alpha set at 0.05.

3. RESULTS

3.1. Assay Validations

3.1.1. Parallelism. There was good parallelism for cortisol and corticosterone in both newer (base) and older (tip) baleen, with no significant difference in slope among the linear portions of the three curves (new baleen, old baleen, and standards; cortisol, $F_{2,9} = 0.2215$, $P = 0.8055$; corticosterone, $F_{2,9} = 0.7752$, $P = 0.4890$; Fig. 1, left panels).

3.1.2. Accuracy (matrix effect). Accuracy slopes (Fig. 1, right panels) of observed vs. expected dose were good for both assays, with slopes very near the ideal of 1.0 (cortisol slope = 1.060, corticosterone slope = 1.036) and coefficients of determination > 0.90. There was no significant deviation from linearity in either assay (cortisol, $P = 0.5000$; corticosterone, $P = 0.9714$), though the corticosterone accuracy curve was slightly more linear (compare straightness of lines in Fig. 1, right panels). In both assays, the y-intercept of the regression equation was acceptably close to the concentration of the pool when measured alone (cortisol, y-intercept = 0.190 ng/ml, pool alone = 0.136 ng/ml; corticosterone, y-intercept = 0.198 ng/ml, pool alone = 0.145 ng/ml).

3.2. Glucocorticoid patterns during known pregnancies

In Female 1, both glucocorticoids were significantly higher during pregnancies than during intercalving intervals (cortisol, $P < 0.0001$, $t_{53} = 4.374$; corticosterone, $P = 0.0002$, $t_{53} = 4.073$; Fig. 2). In Female 2, however, cortisol was not significantly different during pregnancies as compared to intercalving intervals, but corticosterone was significantly elevated during pregnancies (cortisol, $P = 0.2895$, $t_{56} = 1.069$; corticosterone, $P < 0.0001$, $t_{56} = 4.680$; Fig. 2). The lack of correlation of cortisol with pregnancy in Female 2 may be related to a series of high "cortisol spikes" that dominant the later half of her baleen plate and that often occurred during

intercalving intervals (Fig. 2, "spikes" [prominent brief elevations that are not associated with late pregnancy] are marked with arrows; see section 3.3, next).

3.3. Glucocorticoid patterns during intercalving intervals

During intercalving intervals, baleen corticosterone declined significantly in both females (Fig. 2; Female 1, $P = 0.0057$; Female 2, $P = 0.0029$), while baleen cortisol declined significantly in Female 1 but showed no significant change in Female 2 (Female 1, $P < 0.0001$; Female 2, $P = 0.0822$). Post-hoc pairwise comparisons of calendar years revealed that in Female 1, the second year of the intercalving interval (post-lactation year) had significantly higher baleen corticosterone than all other years, while the first and second year both had significantly higher baleen cortisol than all other years ($P<0.05$). In Female 2, the first and second year both had significantly elevated corticosterone compared to all other years ($P < 0.05$) while cortisol showed no difference.

Baleen plates from both females also had “spikes” — sporadic, very brief, elevations of either or both glucocorticoids that were not obviously connected to known reproductive events. Most of these occurred during intercalving intervals (Fig. 2, arrows). The second (more recent) half of Female 2's cortisol profile was dominated by a series of at least seven cortisol spikes (excluding the expected elevation in late pregnancy), most of which were not associated with any concurrent corticosterone spike (Fig. 2, lower panel, blue arrows). However, the last and most prominent cortisol spike coincided with a prominent corticosterone spike, several months before death. Finally, Female 1 had a prominent corticosterone spike in the very oldest section of the plate; cortisol was mildly elevated in the same sample (Fig. 2, upper panel, black arrow).

3.4. Corticosterone compared to cortisol

3.4.1. Relative abundance and B/F ratio. Cortisol and corticosterone were detectable in all samples from both whales, but the corticosterone assay consistently detected much greater concentrations of immunoreactive hormone. B/F ratio was > 1.0 (i.e., more corticosterone detected than cortisol) in all 55 samples from Female 1 and 56 of 58 samples from Female 2. In Female 1, mean B/F ratio (± SEM) was 4.83 ± 0.27 (median = 4.2, range 1.8–9.4); in Female 2, mean B/F ratio was 4.35 ± 0.43 (median = 3.35, range 0.40–15.6). Additionally, B/F ratio was highly variable, even between adjacent samples (Fig. 3); overall, the coefficient of variation for B/F ratio was 41% for Female 1 and 75% for Female 2.

3.4.2. Correlations of corticosterone with cortisol. Across the entire baleen plate, corticosterone and cortisol were significantly and positively correlated in Female 1 ($P < 0.0001$, $r^2 = 0.5683$) but not in Female 2 ($P = 0.3038$, $r^2 = 0.0192$). Relationships between the two hormones were most consistent during pregnancies. During Female 1's pregnancies (all pregnancy samples combined), corticosterone and cortisol were significantly and strongly correlated with each other ($P < 0.0001$, $r^2 = 0.6834$, $n = 18$), likely reflecting the fact that the two glucocorticoids had roughly similar temporal profiles during a pregnancy, i.e., elevating in the second half of the pregnancy and dropping just prior to birth. However, in Female 2's pregnancy samples, the two glucocorticoids were not significantly correlated ($P = 0.4532$, $r^2 = 0.0477$, $n = 14$). The lack of correlation in Female 2 appears primarily due to the second pregnancy, which had two cortisol peaks rather than one. If this pregnancy is excluded and the analysis is limited to Female 2's first pregnancy, the corticosterone-cortisol correlation becomes significant and strong ($P = 0.0099$, $r^2 = 0.7661$, $n = 7$).

During intercalving intervals of both females, the two glucocorticoids remained significantly correlated but the coefficient of determination (r^2) was weaker than during pregnancies. In both females, during intercalving intervals only 15% of variation in one glucocorticoid during intercalving intervals was explainable by the other glucocorticoid (Female 1, $P = 0.0204$, $r^2 = 0.1482$, $n = 36$; Female 2, $P = 0.0114$, $r^2 = 0.1462$, $n = 43$).

4. DISCUSSION

4.1. Detectability and assay validations

Immunoreactive cortisol and corticosterone were both readily detectable along the full length of NARW baleen plates, an encouraging result given that the plates were grown over a ~9-10 yr time period, were continuously exposed to seawater for most of that time, and were subsequently stored at room temperature for over a decade post-necropsy.

For both hormones, the similar parallelism seen in the tip (older baleen) as compared to the base (newer baleen) indicates that hormone degradation may not be a significant effect in older baleen. We emphasize, however, that the parallelism test presented here in old vs. new baleen, which we believe to be a novel approach to assessing degradation, does not conclusively prove that no degradation has occurred. For example, if some portion of immunoreactive hormones degrade so much that the resulting degraded products do not bind to the assay antibody at all, parallelism in older baleen might still appear good. However, theoretically this should also result in an average decrease in apparent concentration of hormone, from base (newest baleen) to tip (oldest baleen); no such decline in hormone content is apparent in Female 1, the individual that did not suffer entanglements (Female 2's profile cannot be assessed in this manner due to the fact

that she was known to have suffered entanglements in the second half of her baleen plate). Alternatively, degradation might result in a metabolite(s) that has increased affinity to the antibody; this should result in an increase in apparent hormone concentration with age, but again, no such trend is apparent in Female 1. Overall, the limited evidence available suggests degradation is not a serious concern, especially in that it does not obscure the biological patterns of interest (e.g., adrenal activity of pregnancies). Nonetheless, we recommend further studies on the possibility of hormone degradation in baleen. Such studies could include studies of fresh baleen that are sampled as soon as possible after death and re-sampled for multiple years afterward; comparisons of average hormone content in plates stored for different periods of time and different storage conditions; and additional comparisons of base vs. tip baleen hormone concentrations within individual whales. Further, the chemical identity of immunoreactive baleen steroids has not been definitively established. High performance liquid chromatography (HPLC), mass spectrometry or similar approaches will be necessary to conclusively determine the identity of the immunoreactive compounds detected by the assays.

4.2. Baleen GCs are elevated during pregnancies

Corticosterone was elevated during pregnancies in both females, and cortisol was significantly elevated during pregnancies in Female 1 (though not in Female 2, where patterns in cortisol may have been obscured by cortisol spikes). This result is in agreement with previous findings that fecal GCs are routinely elevated in pregnant NARW (Rolland et al., 2007). Another adrenal hormone, aldosterone, has also recently been reported to be elevated in feces from pregnant female NARW (Burgess et al., *in revision*). In bowhead whales, single samples of fresh baleen from four pregnant females had higher mean immunoreactive cortisol compared to six

nonpregnant females; this difference was not statistically significant, but sample size was very low in that pilot study (Hunt et al, 2014b). Elevated GC concentrations in pregnancy have also been reported across a wide variety of other mammals, with varied sample types including plasma, saliva, feces and hair (Bentley, 1998; Carnegie et al., 2011; D'Anna-Hernandez et al., 2011; Dettmer et al., 2015; Fanson et al., 2012; Grant et al., 2014; Meyer and Novak, 2012; Pukazhenthi and Wildt, 2004; Scarlata et al., 2012; Van Meter et al., 2009). Given that the patterns reported here for baleen GCs parallel findings from whale fecal samples as well as from many other taxa, and especially given the excellent temporal alignment of elevations in baleen GCs with known pregnancies, it appears likely that baleen glucocorticoid profiles may indeed represent a multi-year time series of adrenal activity in large whales.

4.3. Intercalving intervals

In both whales, corticosterone was significantly elevated during the first year (lactation year) and/or second year (post-lactation or "resting" year; see Rolland et al., 2005). Elevated baleen corticosterone concentration could be related to the energetic burden of lactation or of recovery during the following year; lactation is known to be an energetically expensive state for large whales (Fortune et al., 2013; van der Hoop et al., 2017). Cortisol showed a similar pattern in Female 1 but not in Female 2, suggesting some independence of the two hormones (see section 4.4, next).

4.4. Corticosterone compared to cortisol

4.4.1. Relative abundance. Immunoreactive corticosterone was present in far higher concentrations than immunoreactive cortisol, frequently four-fold higher or more. This pattern

was unexpected given that cetaceans are thought to be cortisol-dominant. Hormone ratios in baleen might not be reliable indicators of ratios in plasma, i.e., corticosterone may be preferentially deposited in baleen. It is also possible that NARW, and possibly other cetaceans, may not consistently be cortisol-dominant. Relatively little direct data exist on circulating glucocorticoid ratios in most cetaceans; some odontocete (toothed whales) species do have greater plasma cortisol than corticosterone (e.g., bottlenose dolphin, *Tursiops truncatus*, Ortiz and Worthy, 2000; killer whale, *Orca orcinus*, O'Brien et al., 2017), but adrenal glands recovered from stranded pygmy sperm whale (*Kogia breviceps*) and Gervais' beaked whale (*Mesoplodon europaeus*) were found *in vitro* to produce three times more corticosterone than cortisol (Carballeira et al., 1987). Mysticete plasma samples have been obtained only very rarely, and typically only cortisol has been measured (e.g., fin whale, *Balaenoptera physalus*, Kjeld, 2001). A single plasma sample from a live-stranded NARW did have more cortisol than corticosterone (Rolland et al., *in revision*). However, all plasma data reported from mysticetes to date represent situations of either stranding or hunting, both of which might be expected to cause elevations in glucocorticoids. It may be that ratios of the two hormones shift during stressful events, particularly acute stress, since cortisol reportedly elevates more rapidly during acute stress than does corticosterone (Koren et al., 2012).

4.4.2. Corticosterone/Cortisol Ratio. An additional surprise was the relative independence of corticosterone from cortisol, as indicated by high variability in B/F ratio. In cortisol-dominant species, it has been assumed that corticosterone functions only as an intermediate in the biosynthetic pathway to cortisol, correlates very tightly with cortisol, and does not provide any additional information (reviewed in Hancock, 2010; Koren et al., 2012). In this study, however,

B/F ratio varied dramatically from sample to sample, so much so that the two hormones were often not significantly correlated or only very weakly correlated. Lack of correlation of corticosterone and cortisol, both within and across individuals, has recently been documented in multiple other mammalian species traditionally classed as cortisol-dominant, calling into question both the concept of cortisol-dominance generally and also that of the less abundant hormone "paralleling" the more abundant one (Hancock, 2010; Koren et al., 2012). Koren et al. (2012) found that in four of 13 “cortisol-dominant” mammalian species tested, including three of eight artiodactyls (relatives of cetaceans), there was not any consistent relationship of cortisol to corticosterone. Even in the species that did have a significant relationship between the two hormones, there was only weak repeatability of B/F ratio within individuals, with coefficient of variation of the B/F ratio commonly as high as 50%. Koren et al. (2012) suggest that "dual glucocorticoid signaling” may be a possibility, i.e. cortisol and corticosterone may respond differentially to different types of stressors and might even trigger different downstream responses in target tissues. The two hormones have differing affinities for corticosteroid binding globulin, transporters and receptors (Funder, 2013; MacKenzie, 2015; Nixon et al., 2016; Sacta et al., 2016). In some species, cortisol is a better index of acute stress than corticosterone, while corticosterone can sometimes show stronger relationships to chronic stress (Gong et al., 2015, mouse, *Mus musculus*; Hancock, 2010, human, *Homo sapiens,* and guinea pig, *Cavia porcellus*; MacKenzie, 2015, human; McCorkell et al., 2013, bison, *Bison bison*; Vera et al., 2011, tuco-tuco, *Ctenomys talarum*). The NARW profiles presented here suggest that the dual signaling model could be considered for the large whales as well. This theoretical framework is only speculative at present, particularly given the extremely low sample size of individuals, but can be

tested in future studies employing more baleen plates from many more individual whales, ideally of known history and known cause of death.

4.5. Conclusions

The data presented here suggest that baleen glucocorticoid profiles could be used to retrospectively assess stress physiology of large whales. A drawback of this tissue type is that baleen cannot be recovered from live individuals, but a large archive of stored samples exists in stranding-response networks, historical archives of baleen in museums, and archeological sites. Results of this study and prior studies (Hunt et al., 2014b, 2016) suggest that steroids remain detectable in baleen for at least two decades, in agreement with findings that steroid hormones show good longevity in mammalian hair (thought to be homologous to baleen filaments) for decades or even centuries (Bechshøft et al., 2012, 2013; Webb et al., 2014). Thus, archived baleen samples may contain historic information on stress physiology that has heretofore been extremely difficult to obtain for large whales. It must be emphasized that baleen hormone analysis is still in its infancy. The chemical identity of baleen steroids must be determined, the ideal extraction method assessed, and there are important unresolved questions regarding variability in baleen growth rate, temporal resolution of each sampling point on the baleen, and variation within and across sampling points. Once these methodological issues are addressed, analyses of longitudinal profiles of glucocorticoids in baleen may prove useful for disentangling the complex effects of natural and anthropogenic stressors on physiology of individual large whales.

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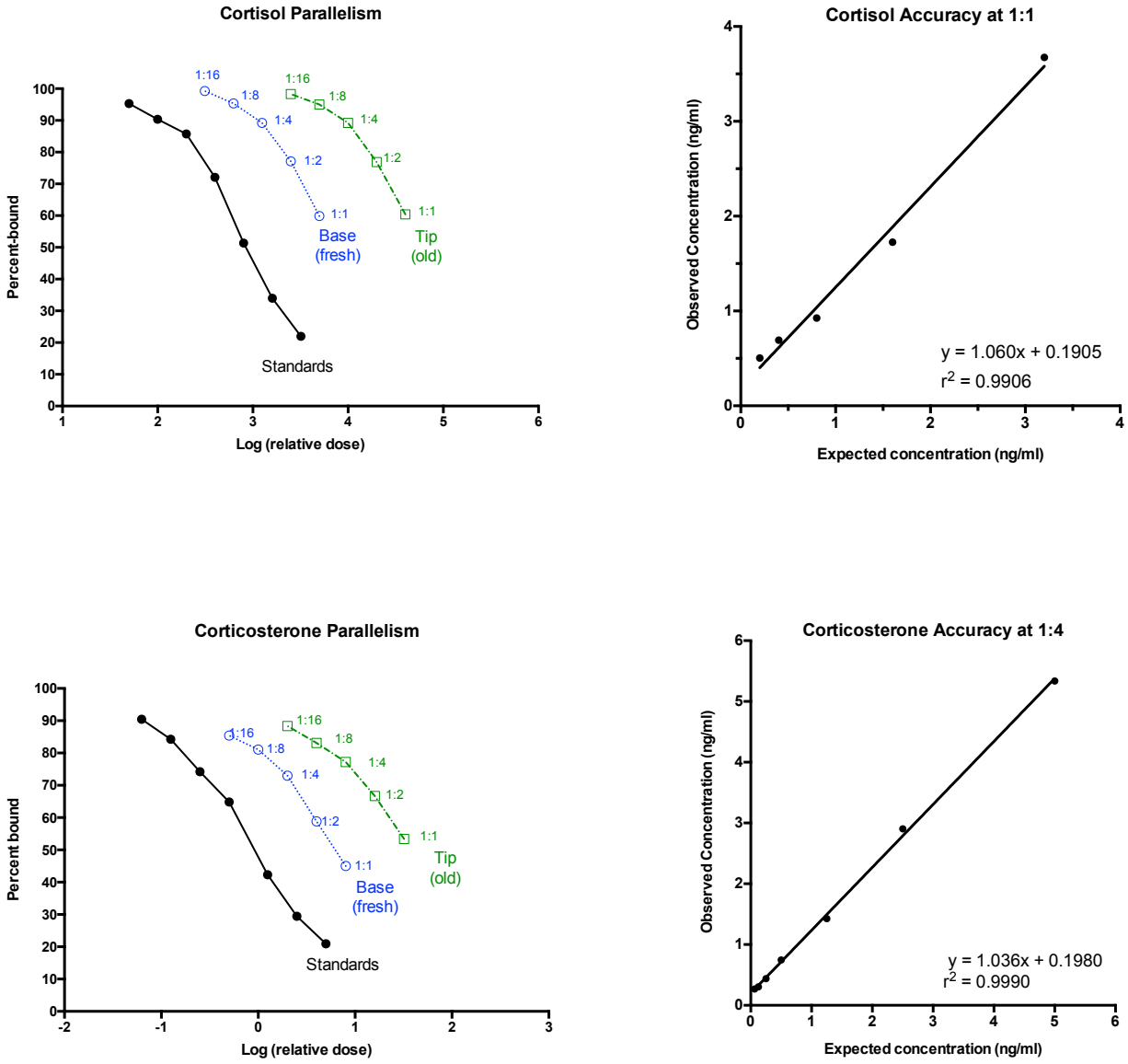

Figure 1. Parallelism and accuracy results for a cortisol enzyme immunoassay (top) and a corticosterone radioimmunoassay (bottom) tested with pooled extract from North Atlantic right whale baleen. Parallelism (left) was tested both for older baleen at the tip of the baleen plate (green lines), and recently grown baleen embedded in gum tissue at the base of the plate (blue lines). Accuracy graphs (right) show best-fit linear regression equation and the associated coefficient of determination (r^2).

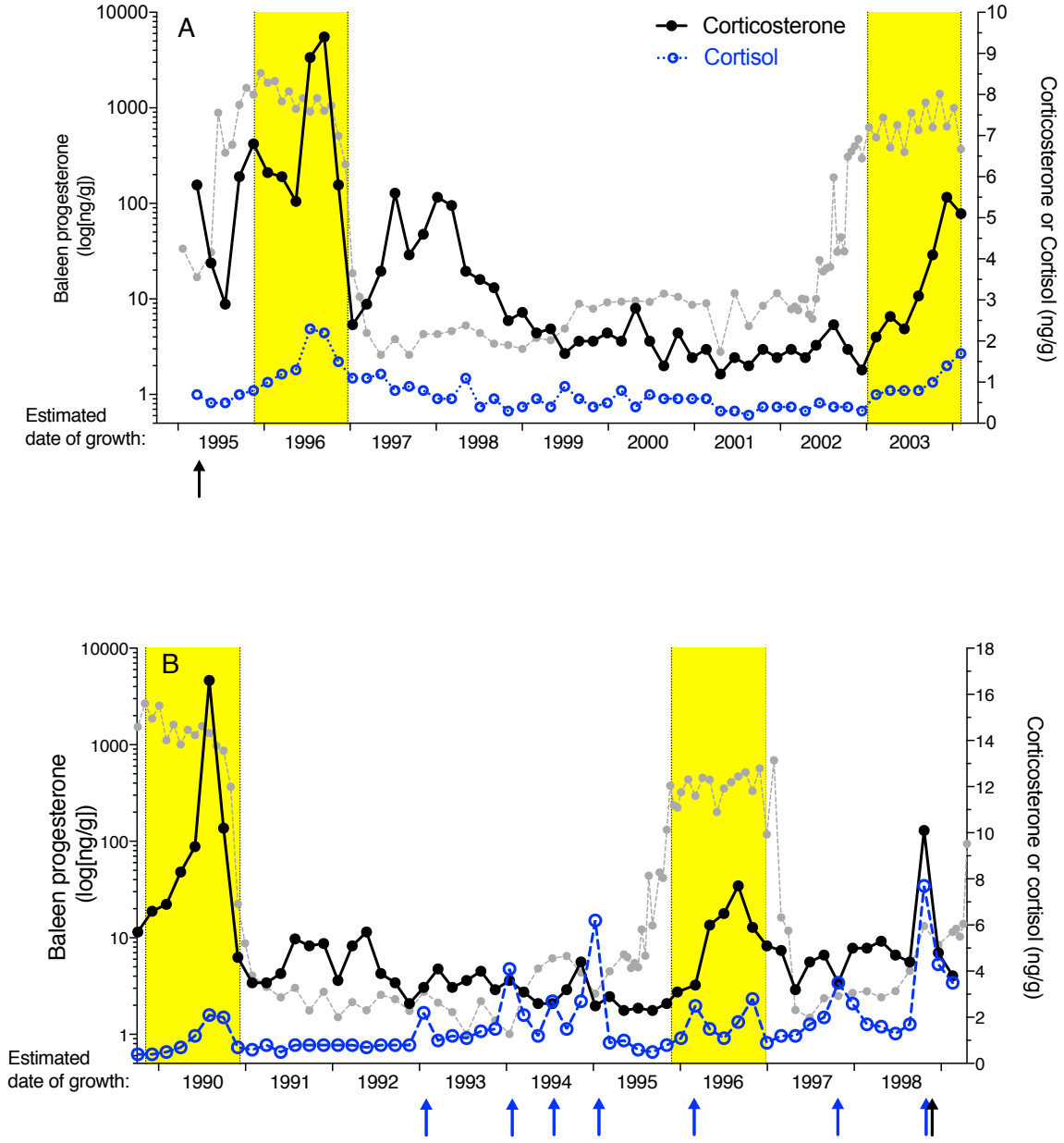

Figure 2. Immunoreactive corticosterone (black dots and solid lines) and immunoreactive cortisol (blue circles and dashed lines) across the full length of baleen plates from two adult female North Atlantic right whales, Female 1 (panel A, top) and Female 2 (panel B, bottom). Yellow bars mark known pregnancies, inferred from sightings of neonate calves and/or size of fetus found at necropsy (see Hunt et al. 2016 for sightings records) combined with an estimated gestation duration of 13 mo. Previously published progesterone data (used with permission from Hunt et al., 2016) is shown in gray for reference; note that progesterone is displayed on a logarithmic scale. Arrows indicate "spikes", brief elevations in glucocorticoids that are not associated with known reproductive events; blue arrows indicate prominent cortisol spikes and black arrows corticosterone spikes.

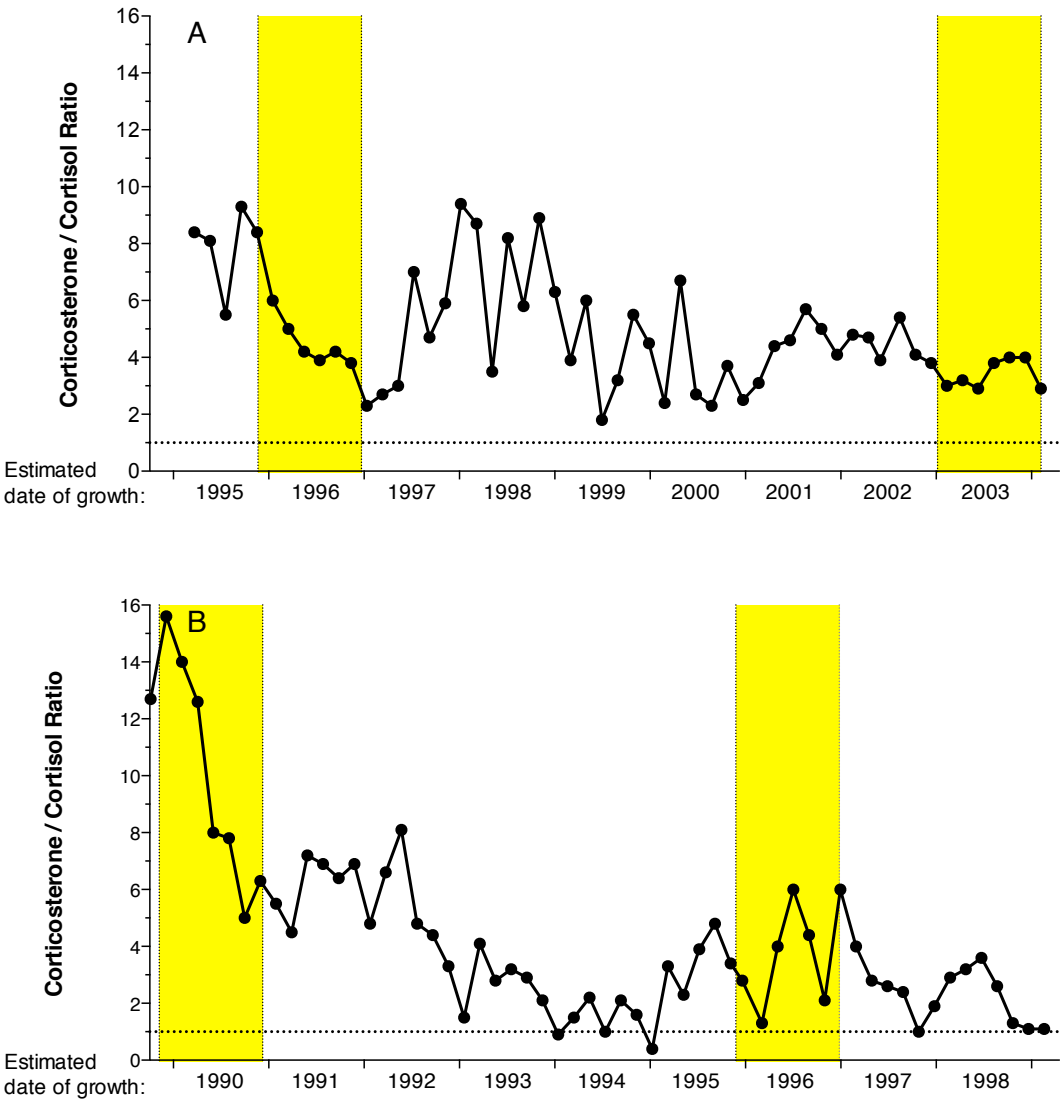

Figure 3. Ratio of immunoreactive corticosterone to immunoreactive cortisol across the full length of baleen plates from two adult female North Atlantic right whales, Female 1 (panel A, top) and Female 2 (panel B, bottom). Yellow bars mark known pregnancies, inferred from sightings of neonate calves and/or size of fetus found at necropsy (see Hunt et al. 2016 for sightings records) combined with an estimated gestation duration of 13 mo. Dashed line indicates parity, e.g. a 1:1 ratio of the two hormones.