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Research paper

Optimizing hormone extraction protocols for whale baleen: Tackling questions of solvent:sample ratio and variation

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ABSTRACT

Obtaining endocrine data from alternative sample types such as baleen and other keratinized tissues has proven a valuable tool to investigate reproductive and stress physiology via steroid hormone quantification, and metabolic stress via thyroid hormone quantification in whales and other vertebrates. These alternative sample types provide an integrated measure of plasma levels over the period that the structure was growing, thus capturing months or even years of an individual's endocrine history. Additionally, their robust and stable keratin matrix allows such samples to be stored for years to decades, enabling the analysis and comparison of endocrine patterns from past and modern populations. However, the extraction and analysis of hormones from baleen and other keratinized tissues remains novel and requires both biological and analytical validations to ensure the method fulfills the requirements for its intended use. We utilized baleen recovered at necropsy from southern right whales (*Eubalaena australis*) that died at Península Valdés, Argentina, using a commercially available progesterone enzyme immunoassay (EIA) to address two methodological questions: 1) what is the minimum sample mass required to reliably quantify hormone content of baleen samples analyzed with commercially available EIAs, and 2) what is the optimal ratio of solvent volume to sample mass, i.e., the ratio that yields the maximum amount of hormone with high accuracy and low variability between replicates. We concluded that masses of at least 20 mg should be used whenever possible, and extraction is best performed using an 80:1 ratio of solvent to sample (volume of solvent to sample mass; μl:mg). These results can help researchers to make informed methodological decisions when using a destructive extraction method with rare or unique specimens.

1. Introduction

Whales are exposed to an increasing variety of anthropogenic stressors (e.g., entanglement in fishing gear, vessel strikes, ocean noise, pollution, climate change, etc.), and despite commercial whaling being banned, several whale species and populations are still critically endangered (Thomas et al., 2016). Understanding the effects and identifying the relative importance of multiple anthropogenic and ecological pressures is critical for managing endangered species. In this context, physiological measures can provide valuable tools for informing management and conservation efforts (Madliger et al., 2018).

Conservation physiology is a multidisciplinary field wherein a broad suite of tools and concepts are used to understand how organisms and ecosystems respond to both environmental and anthropogenic change and stressors (Madliger et al., 2020). Monitoring and predicting multiscale responses to change, unraveling disease dynamics, and understanding reproductive biology all help to mitigate human-wildlife conflicts (McCormick and Romero, 2017; Madliger et al., 2020). Conservation endocrinology is a subdiscipline within conservation physiology, which relies on endocrine measurements (hormone

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quantifications) to illuminate the underlying physiological mechanisms by which organisms cope with changing environments, and provide substantial information on the stress response and reproductive status of individual animals. Traditionally, the field of conservation endocrinology has focused on laboratory analyses of plasma samples to assess the physiological status of an organism. However, implementing traditional techniques for monitoring the physiology of mysticetes (baleen whales) is currently not possible because methods for obtaining a blood sample from live large whales with no harm to the animal have not yet been developed or employed (Hunt et al., 2013).

Fortunately, several alternative sample types have proven useful for assessing the endocrine status of large whales which can be used to infer physiological status of individual animals either in real-time or retrospectively. Some of these sample types can be obtained using minimally invasive methods from free-living whales, such as skin and blubber biopsies (Champagne et al., 2017; Galligan et al., 2020; Pallin et al., 2018b, 2018a), respiratory vapor (Browning et al., 2009; Burgess et al., 2018, 2016; Hunt et al., 2014a; Richard et al., 2017), and feces (Ayres et al., 2012; Hunt et al., 2019, 2015; Lemos et al., 2020). Additionally, other samples can be recovered at necropsy, such as earplugs (Crain et al., 2020; Mansouri et al., 2021; Trumble et al., 2018) and baleen (Hunt et al., 2014b, 2017a, 2017b, 2018; Fernández Ajó et al., 2018, 2020; Lysiak et al., 2018). Both earplugs and baleen contain hormones that were deposited into the tissues at various times prior to death, thus providing a record of endocrine status over years or decades preceding the death of the individual. Plasma hormone levels and, it is thought, respiratory vapor, provide an immediate measure of an organism's endocrine status, i.e., at a given day and time, and are essential for the study of peptide and protein hormones. In contrast, some of the alternative sample types can offer a measure of hormone concentrations over a longer timeframe (hours in the case of blubber, days for feces, or months/years for baleen and earplug), providing the advantage of avoiding the influences of capture and handling stress (Cook, 2012; Cyr and Romero, 2008; Hunt et al., 2014b; Karpovich et al., 2020; Kersey and Dehnhard, 2014; Macbeth et al., 2012, 2010; Mingramm et al., 2019; Wasser et al., 2010).

Among these tissues, baleen is particularly useful for retrospective and longitudinal assessments of endocrine status of whales. Baleen is a cornified epithelial structure that functions as a filter-feeding apparatus of the mysticete whales. Like other keratinized epidermal tissues (e.g., feather, fur, claw, hair, and spines), it consists of a growing structure that extends from a well-vascularized zone in the dermis. Steroid and thyroid hormones that are circulating in plasma are deposited in the growing tissue, resulting in a record of the individual's endocrine state at the time the structure was grown. Baleen grows slowly and continuously, and therefore one baleen plate can hold a sequential record of the whale's endocrine history. Baleen thus enables reconstruction of past reproductive cycles and any physiological events that occurred during the period of the baleen growth, which can comprise years (Hunt et al., 2017a, 2018), or even capture the entire lifespan as is the case for young whales (Fernández Ajó et al., 2020, Fernández Ajó et al., 2018). In addition, baleen is routinely recovered at necropsy, and given its strength and durability, hormones are remarkably stable within the keratin matrix and remain detectable in dried samples for decades (Hunt et al., 2017a, 2017b, 2018). Thus, baleen not only captures a multi-year timeframe that has sufficient temporal resolution to determine seasonal endocrine patterns but also, with the use of archival samples, allows for comparisons of present and past populations (e.g., Hunt et al., 2014b, 2018).

However, due to the novelty of the methods for extraction and analysis of hormones from baleen and other keratinized tissue samples, there are multiple technical considerations that require both biological and analytical validations to verify whether hormone profiles recovered from baleen reflect biologically meaningful physiological events (Hunt et al., 2014b; Palme, 2019; Sheriff et al., 2011). Baleen hormone analysis has passed several essential assay validations, including parallelism and accuracy of immunoassays for all steroid and thyroid hormones in all species of whales yet tested (Hunt et al., 2017b). Baleen hormone methods have also passed numerous biological validations, e.g., study of animals with known physiological status (i.e., reproductive status such as a known pregnancy or a known stressful event such as entanglement in fishing gear or the presence of lesions) and assessing the degree to which the endocrine data reflect the physiology of the individual (Fernández Ajó et al., 2020, 2018; Hunt et al., 2018, 2017a; Hunt et al., 2016; Lysiak et al., 2018; Palme, 2019). Several important questions concern the optimization of the laboratory methods, including all steps ranging from tissue collection and storage to hormone extraction and quantification. A question of particular interest for alternative sample types is that of minimum sample mass, as several studies have reported a 'small sample effect' in which samples that fall below a certain threshold of sample mass can produce spuriously inflated hormone data, even though hormone data are routinely corrected for mass of the sample (Millspaugh and Washburn, 2004; Hayward et al., 2010; Ayres et al., 2012; Lemos et al., 2020; Dillon et al, 2021).

Other questions of technical validation include choice and volume of extraction solvent, and the effect of solvent-to-sample ratio (solvent: sample) on extraction yield; that is, the amount of native hormone that can be recovered from these samples with a particular set of extraction parameters. Increasing solvent:sample ratio can often improve extraction efficiency - percentage of native hormone recovered from the sample - particularly if hormone concentration is high enough to saturate the extraction solvent. The ideal solvent:sample ratio for baleen has not yet been determined, with most studies to date using solvent:sample ratios that were originally devised for feces or hair. In this study, we focused on two questions: 1) what is the minimum sample mass of baleen powder required to reliably quantify hormone content of baleen samples analyzed with commercially available EIAs; and 2) what is the optimal ratio of solvent volume to sample mass for steroids extracted from baleen, i.e., the ratio that yields the maximum amount of hormone with high accuracy and low variability between replicates.

We performed technical validations of baleen samples collected from southern right whales (*Eubalaena australis*). We focused on progesterone because of its importance for studies of female reproduction, as well as its role as a precursor in the synthesis of all other steroids (androgens, estrogens, glucocorticoids, mineralocorticoids). We did not address solvent choice, focusing instead on methanol, based on its ubiquity in the literature for use in steroid hormone extractions for almost all keratinized tissues, including baleen (Fernández Ajó et al., 2020; Fernández Ajó et al., 2018, Hunt et al., 2017a; Hunt et al., 2017b, 2014b), as well as its safety profile (i.e., low toxicity and reactivity). We tested extraction yield and precision with a variety of sample masses and tested efficacy of extraction using either constant or variable ratio of solvent volume to sample mass. Ultimately, we provide methodological guidance regarding optimizing sample mass and solvent volume for steroid hormone extraction from powdered baleen.

2. Methods

2.1. Baleen samples

Southern right whale (*Eubalaena australis;* SRW) baleen plates were recovered from necropsied calves at Península Valdés, Argentina by the Southern Right Whale Health Monitoring Program (SRWHMP) team during the calving seasons of 2003 to 2010. We utilized one baleen plate recovered at necropsy that was stored dry at room temperature until prepared for hormone extraction. The plate was cleaned of any remaining soft tissue and freeze dried (lyophilized) in a LabConco Stoppering Tray Dryer lyophilizer (LabConco, Kansas City, MO, USA) until pressure reading of the lyophilizer stabilized (see Fernández Ajó et al., 2018). Following drying, the plate was stored at room temperature in a sealed plastic bag along with a 50 g silica gel desiccant pack (Arbor Assays, Ann Arbor, MI, USA) until pulverization for hormone extraction.

2.2. Pulverization of baleen tissue

At the plate's base (most recent growth), we pulverized a two-by-two cm section with a hand-held electric rotary grinder (Dremel model 395 type 5) fitted with a tungsten ball-tip and flexible extension. Below the plate, abraded baleen powder was collected on a piece of weighing paper, homogenized with a metal stirring rod for 1 min, and weighed with a precision of ± 0.0001 g on a precision scale (Ohaus Explorer Pro EP214C, Pine Brook, NJ, USA) and placed in a 16x100 mm borosilicate glass tube for extraction.

2.3. Hormone extraction

Hormones were extracted from weighed samples with 100% methanol (Methanol HPLC grade, Fisher Chemical™), following previous studies (Hunt et al., 2014b, 2017a, 2017b; Fernández Ajó et al., 2018; 2020). Briefly, absolute methanol was added to the extraction tubes, vortexed for 2 h at room temperature (Large Capacity Mixer, Glas-Col, Terre Haute, IN, USA; speed set on 40) and centrifuged for 1 min at 4025*g*. The resulting supernatant was transferred to a 13 × 100 mm borosilicate glass tube and dried at 45 °C for a minimum of 4 h in a sample evaporator (Speedvac 121P, Waltham, MA) under vacuum. Dried hormone extracts were reconstituted in 0.50 mL assay buffer (X065 buffer; Arbor Assays, Ann Arbor, MI, USA), sonicated for 5 min, vortexed for 5 min, transferred to 1.5 mL vapor proof O-ring-capped cryovials, stored overnight at −80 °C, and decanted to a new cryovial to remove any remaining baleen particulates. This was considered the "1:1″ (full-strength, neat) extract. All extracts were frozen at −80 °C until assay within 2 weeks of extraction.

2.4. Hormone assays and validation

A commercial enzyme immunoassay (EIA; progesterone K025-H1; Arbor Assays, Ann Arbor, MI, USA) was used to quantify immunoreactive progesterone in SRW baleen extracts. As this particular assay has not been used before for this species, a test of parallelism was performed with a pooled SRW baleen extract serially diluted in assay buffer to produce eight dilutions (range 1:1 – 1:128). All dilutions were assayed as unknowns in the progesterone EIA along with serially diluted progesterone hormone standard of known concentration. The linear portions of the two binding curves (i.e., the serially diluted pool vs. known-concentration hormone standards) were compared for equality of slope. Parallelism of these two binding curves within a given assay indicates that the assay antibody binds well to an immunoreactive component in the sample of interest, with very similar affinity as to pure parent hormone; this is considered evidence that the hormone is, in fact, present in the sample (Grotjan and Keel, 1996). An assay accuracy test was conducted to evaluate the "matrix effect," i.e., the potential interference of baleen powder components with the assay. This test was performed by spiking a full standard curve with pooled 1:1 SRW baleen extract and assaying alongside a second standard curve spiked only with assay buffer. The resulting plot of apparent total hormone concentration vs. known standard concentration was assessed for linearity and slope; a slope within the range of 0.7–1.3 (ideal slope = 1.0) indicates the assay correctly discriminates low-dose from high-dose samples without interference from the sample matrix. Following validations, all samples were assayed at 1:1 (i.e., full-strength, neat extract); this dilution was selected for assay because it has the most hormone of any commonly tested dilution, and thus is most likely to saturate the extraction solvent. Assays followed standard QA/QC criteria, including a full standard curve, NSB (non-specific binding), and zero doses ("blank") in every EIA microplate, with the assay of all NSBs, zeros, standards, and unknowns in duplicate. Any sample that exceeded 10% coefficient of variation (CV) between duplicates was re-analyzed. Intra-assay and inter-assay variation for all assays was < 10%. For antibody cross-reactivities, assay sensitivities, and other methodological details, see Hunt et al., 2017a. All assay results are expressed as picograms of immunoreactive hormone per g of baleen powder (pg/g).

2.5. Optimizing assay performance and extraction

2.5.1. Sample mass to solvent volume ratio

Experiment 1 (constant solvent:sample ratio): Pooled and homogenized SRW baleen powder was weighed and aliquoted into 13 × 100 mm borosilicate glass tubes. Five sample masses (75, 50, 25, 10, and 5 mg) were measured and extracted in triplicate. Hormone extractions were performed with a proportional volume of 100% methanol, i.e., 6.0, 4.0, 1.6, 0.8, and 0.4 mL for each respective sample mass. This represents a constant solvent:sample ratio of 80:1 (μl:mg), selected as representing the higher end of the range of solvent:sample ratios reported in the literature for alternative sample types (commonly ranging from 80:1 to 20:1, Rolland et al., 2005; Hunt et al., 2006, 2014b).

Experiment 2 (variable solvent:sample ratio): The same sample masses, in triplicate, were aliquoted as described in Experiment 1, but all hormone extractions were performed using 8 mL of pure methanol (i.e., the maximum volume of solvent that can be vortexed securely in 16 × 100 mm extraction tubes, commonly utilized due to technical aspects of evaporator equipment and throughput) which represents solvent: sample ratios of 107:1, 190:1, 320:1, 800:1, 1600:1 (μl:mg) for each sample mass respectively. For details regarding hormone extraction, See Section 2.3.

2.5.2. Minimum sample mass for hormone detectability / low variability

We evaluated the effect of sample mass on apparent hormone concentration (pg/g) quantified for each sample mass (75, 50, 25, 10, and 5 mg) extracted with both a variable and constant mass:volume ratio. Additionally, we extracted 100 mg of pooled SRW baleen powder using 8 mL of absolute methanol (in triplicate) to evaluate how increasing the sample mass impacts the inter-replicate variability. Insufficient sample powder prevented additional replicates of the 100 mg group, thus precluding inclusion of this sample mass in the full statistical analysis.

2.6. Statistical analysis

2.6.1. Validations

Parallelism results for SRW progesterone were plotted as the percentage of antibody bound vs. log[concentration]. An F test was employed to assess differences between slopes of the resulting binding curve for serially diluted SRW baleen pooled extract and the standard curve. Accuracy results were plotted as apparent total concentration (i.e., standard + SRW baleen pool) vs. known standard concentration and assessed by linear regression, with acceptable accuracy defined as $r^2 \geq 0.99$ and slope within 0.7–1.3 (ideal slope = 1.0). F tests and accuracy tests were performed using two-tailed tests with Prism 7.0c for Macintosh; descriptive statistics (means and coefficients of variation within treatments) were calculated in R (R Core Team, 2018). All differences were considered significant at $p < 0.05$.

2.6.2. Optimizing assay performance and extraction

A two-way ANOVA was performed to evaluate the influence of mass: volume ratio and sample mass on the apparent pg/g of immunoreactive hormone detected in baleen powder extracts. To improve statistical power, a one-way ANOVA, followed by Tukey's post hoc tests, was run to evaluate the effect of mass for the two experiments, i.e., constant and variable solvent:sample ratio. All statistical analyses were performed in R (R Core Team, 2018). All differences were considered significant at $p < 0.05$.

3. Results

3.1. Validations

The progesterone EIA was successfully validated for SRW baleen extracts. Serially diluted samples yielded displacement curves parallel to the respective standard curves, with no significant differences in slope ($F_{(1,6)} = 0.43$; $p = 0.53$) (Fig. 1, panel A). Dilutions 1:1 to 1:16 yielded detectable progesterone in parallelism tests, with higher dilutions falling outside the range of detectability for the test. Accuracy was also acceptable for progesterone assays of SRW baleen extract, as indicated by a linear relationship between observed and expected hormone concentration ($r^2 \geq 0.98$) and a slope within the desired range of 0.7–1.3 (slope = 0.84; Fig. 1, panel B).

3.2. Extraction experiments

The two-way ANOVA showed no effect of solvent:sample ratio on apparent hormone concentration ($p > 0.05$) but the effect of sample mass was highly significant ($p < 0.0001$). The one-way ANOVA indicated a significant effect of the sample mass for both treatments, constant and variable mass:volume ratio. The post hoc Tukey's test identified significantly higher pg/g of apparent hormone in the 5 mg sample mass as compared to all other sample masses in the constant ratio treatment ($p < 0.01$; Fig. 2), and for the variable ratio treatment, significantly higher hormone in both the 5 and 10 mg samples compared to all other sample masses ($p < 0.01$; Fig. 2). Extractions of 100 mg of sample with 8 mL of absolute methanol yielded apparent immunoreactive progesterone concentrations that were similar to those of 20, 50 and 75 mg samples from both treatments. Furthermore, variability between replicates was generally low for 20, 50 and 75 mg samples, but higher for both 5 mg and 10 mg samples (Fig. 2). Data from the 100 mg replicates were slightly less variable than calculated for smaller sample masses (Fig. 2).

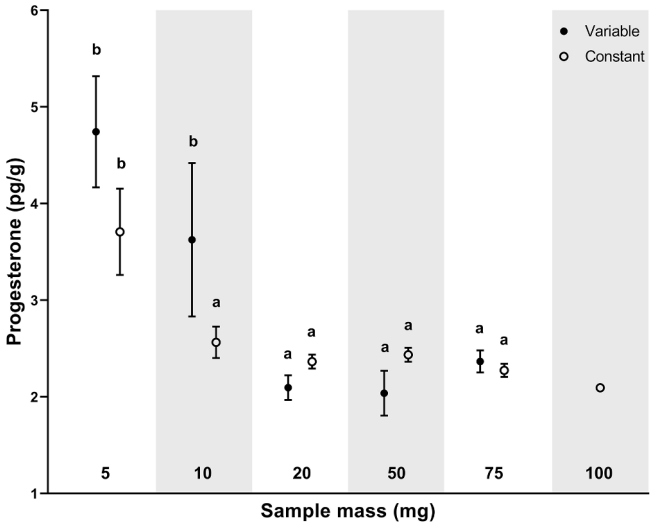

Fig. 2. Extraction experiment results for variable (solid circles) or constant (open circles) solvent:sample ratio (mean progesterone concentration ± SEM). Extractions for 5, 10, 20, 50, and 75 mg of baleen powder were performed in triplicate for each sample mass. Letters indicate significant differences between groups after Tukey's post hoc test. The right-side panel presents a plot for 100 mg of baleen powder extracted with 8 mL of solvent performed in triplicate; error bars are too small to show in the figure.

4. Discussion

We investigated the effect of solvent mass and solvent:sample ratio in performing baleen hormone extractions. We observed that the extractions performed with sample masses ranging from 20 mg to 100 mg yielded repeatable apparent hormone concentrations. However, for the samples below 10 mg, we observed a higher apparent concentration of hormones compared to the rest of the sample masses, even though all hormone concentrations had been corrected for mass of the sample (i.e., data are expressed as pg of hormone per g of baleen powder). This "small sample effect" has been described in the literature for other sample types, including feathers, dermal spines, skin sheds, and feces (Ayres et al., 2012; Berk et al., 2016; Dillon et al., 2021; Millspaugh and Washburn, 2004). The cause of the small sample effect remains unidentified. In experiments with powdered feather, which also reported a small-sample effect, the effect cannot be attributed entirely to extraction efficiency (Berk et al., 2016). It is possible that it represents a systematic error in weighing very small samples. Static effects on dry powdered samples, for example, can substantially influence apparent mass, and it may be that small samples are routinely "under-weighed" (i.e., sample appears to weigh less than it really does). Regardless of the cause of the small-sample effect, our data identify a potential sample mass threshold

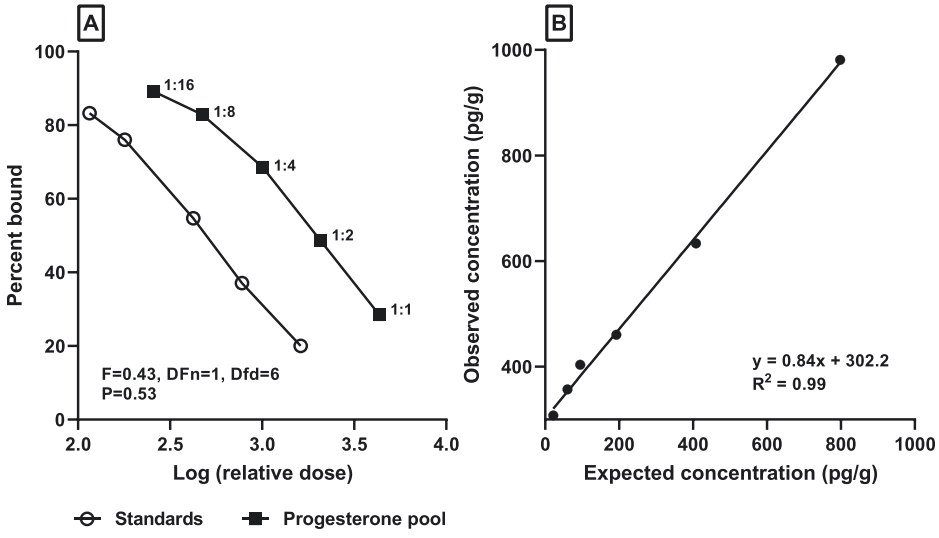

Fig. 1. Parallelism (A) and accuracy (B) for progesterone enzyme immunoassays tested with pooled southern right whale baleen extract. Parallelism results (A) do not include dilutions above 1:16; at these dilutions hormone was not detectable and thus not quantifiable. Statistical results from the F test slope comparison are shown at the bottom of the figure. Accuracy (B) was tested with 1:1 baleen extract; the best-fit regression equation is shown at the bottom of the figure.

at approximately 10 mg, at and below which sample hormone data become both inflated and more variable. Our study did not test any masses between 10 mg and 20 mg; thus, at present we recommend sample masses no lower than 20 mg for baleen hormone extractions.

With regard to the volume of solvent to mass of sample ratio, we found no significant differences in the mean apparent hormone concentration when keeping this ratio constant or variable, i.e., a larger volume of solvent did not improve extraction yield. This indicates that solvent was not becoming saturated with hormone, even at the lowest solvent:sample ratio tested here (80:1). Additionally, when the volume of solvent used was scaled down proportionally to the sample mass, the apparent hormone content showed less inter-sample variation compared with the variable-ratio extractions. This increased variation may be due to the fact that large volumes of solvent, when dried down, can result in variable loss of hormone high on the walls of the dry-down tube. Thus, there may be an optimal volume of solvent above which dry-down artifacts introduce variation to the data.

Deriving endocrine data from baleen (and other keratinized tissues) has proven a valuable tool for conservation physiology studies. These alternative sample types provide an integrated measure of hormone concentrations over the period that the structure was growing and give a timeframe for analysis that can capture months or even years of an individual's hormonal activity. For example, baleen progesterone profiles correspond with reproductive cycles of bowhead whale (*Balaena mysticetus*; Hunt et al., 2014b), and can identify intercalving periods in the North Atlantic right whale (NARW, *Eubalaena glacialis*) (Hunt et al., 2016; Lysiak et al., 2018), and in humpback whales (*Megaptera novaeangliae*) (Lowe *et al.* in review); baleen testosterone patterns correspond with seasonality in reproductive NARW, bowhead, and blue whale (*Balaenoptera musculus*) males (Hunt et al., 2018); and pronounced elevations in baleen glucocorticoids are associated with exposure to chronic stressors (Fernández Ajó et al., 2018, 2020; Lysiak et al., 2018; Gabriele et al., 2021).

While other sample matrices, i.e., earplug, can also provide endocrine data which reflect hormone levels over a much broader time window (i.e., lifespan), usually the information derived is more coarse and lacks the temporal resolution that can be observed in the faster-growing baleen (Hunt et al., 2014b; Trumble et al., 2013). Another advantage of sampling baleen for hormones is that it can be easily collected during necropsy, and it is a durable and stable structure in which, by extension, the endocrine history imbedded in the keratinized matrix is also stable. This allows baleen plates and/or subsampled powder to be stored for years to decades and thus enables analysis and comparison of endocrine patterns from past and modern whale populations (Hunt et al., 2014b). On the other hand, the use of baleen as an alternative sample type for endocrine analysis still remains relatively novel; several technical and biological issues have yet to be addressed (Gormally and Romero, 2020; Palme, 2019; Romero and Wingfield, 2016). This study aimed to address two methodological questions that commonly arise when using a destructive extraction method with rare or unique specimens.

5. Conclusions

Our results suggest that the optimal sample mass for methanol extraction of steroid hormones from baleen samples is 20 mg, and that larger sample masses did not produce either better yield or less variation in the apparent hormone per g of baleen sample. In addition, when the extraction was performed keeping the volume of solvent proportional to the sample mass (namely, a solvent:sample ratio of 80:1), masses as small as 10 mg yielded reliable hormone measurement. Though our study addressed only one hormone in one species, these results suggest that when working with rare, unique, or small samples of baleen, it is possible to obtain reliable quantifications of hormone with less sample than is currently usually employed (75 mg or 100 mg being typical in most studies to date). We emphasize, however, that these results likely depend on precision of the laboratory tools available and the exact methodology employed, which in our laboratory include a digital scale sensitive to 0.0001 g, routine use of an anti-static ionizer next to the scale while weighing all samples, stringent attention to details of pipetting, a vacuum rotary evaporator for dry-down (which tends to confine the dried hormone to the lower part of the tube), and a sonication step during resuspension. In conclusion, we recommend performing extractions with sample masses of at least 20 mg whenever possible and performing the extraction with a volume of solvent proportional to the sample mass. These results indicate how baleen hormone analytic techniques can be more widely employed on small sample masses from rare specimens, such as from natural history museums and stranding archives, thus enabling greater use of this valuable technique to reconstruct the endocrine and physiological history of individual whales over time.

CRediT authorship contribution statement

Alejandro Fernández Ajó: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Kathleen E. Hunt:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Danielle Dillon:** Conceptualization, Methodology, Resources, Writing - review & editing. **Marcela Uhart:** Writing - review & editing, Supervision, Funding acquisition. **Mariano Sironi:** Writing - review & editing, Supervision, Funding acquisition. **Victoria Rowntree:** Writing - review & editing, Supervision, Funding acquisition. **C. Loren Buck:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ayres, K.L., Booth, R.K., Hempelmann, J.A., Koski, K.L., Emmons, C.K., Baird, R.W., Balcomb-Bartok, K., Hanson, M.B., Ford, M.J., Wasser, S.K., 2012. Distinguishing the impacts of inadequate prey and vessel traffic on an endangered killer whale (*Orcinus orca*) population. PLoS One 7. https://doi.org/10.1371/journal.pone.0036842.

Berk, S.A., McGettrick, J.R., Hansen, W.K., Breuner, C.W., 2016. Methodological considerations for measuring glucocorticoid metabolites in feathers. Conserv. Physiol. 4, cow020. https://doi.org/10.1093/conphys/cow020.

Browning, C.L., Rolland, R.M., Kraus, S.D., 2009. Estimated calf and perinatal mortality in western North Atlantic right whales (*Eubalaena glacialis*). Mar. Mammal Sci. 34, 21. https://doi.org/10.1111/j.1748-7692.2009.00361.x.

Burgess, E.A., Hunt, K.E., Kraus, S.D., Rolland, R.M., 2016. Get the most out of blow hormones: validation of sampling materials, field storage and extraction techniques for whale respiratory vapour samples. Conserv. Physiol. 4, cow024. https://doi.org/10.1093/conphys/cow024.

Burgess, E.A., Hunt, K.E., Kraus, S.D., Rolland, R.M., 2018. Quantifying hormones in exhaled breath for physiological assessment of large whales at sea. Sci. Rep. 8, 10031. https://doi.org/10.1038/s41598-018-28200-8.

Champagne, C.D., Kellar, N.M., Crocker, D.E., Wasser, S.K., Booth, R.K., Trego, M.L., Houser, D.S., 2017. Blubber cortisol qualitatively reflects circulating cortisol concentrations in bottlenose dolphins. Mar. Mammal Sci. 33 https://doi.org/10.1111/mms.12352.

Cook, N.J., 2012. Review: minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. Can. J. Anim. Sci. 92, 227–259. https://doi.org/10.4141/CJAS2012-045.

Crain, D.D., Thomas, A., Mansouri, F., Potter, C.W., Usenko, S., Trumble, S.J., 2020. Hormone comparison between right and left baleen whale earplugs. Conserv. Physiol. 8 https://doi.org/10.1093/conphys/coaa055.

Cyr, N.E., Romero, L.M., 2008. Fecal glucocorticoid metabolites of experimentally stressed captive and free-living starlings: implications for conservation research. Gen. Comp. Endocrinol. 158, 20–28. https://doi.org/10.1016/j.ygcen.2008.05.001.

Dillon, D., Fernández Ajó, A., Hunt, K.E., Buck, C.L., 2021. Investigation of keratinase digestion to improve steroid hormone extraction from diverse keratinous tissues. Gen. Comp. Endocrinol. 309, 113795 https://doi.org/10.1016/j.ygcen.2021.113795.

Fernández Ajó, A.A., Hunt, K.E., Uhart, M., Rowntree, V., Sironi, M., Marón, C.F., Di Martino, M., Buck, C.L., 2018. Lifetime glucocorticoid profiles in baleen of right whale calves: potential relationships to chronic stress of repeated wounding by Kelp Gulls. Conserv. Physiol. 6, 1–12. https://doi.org/10.1093/conphys/coy045.

Fernández Ajó, A.A., Hunt, K.E., Giese, A.C., Sironi, M., Uhart, M., Rowntree, V.J., Marón, C.F., Dillon, D., DiMartino, M., Buck, C.L., 2020. Retrospective analysis of the lifetime endocrine response of southern right whale calves to gull wounding and harassment: a baleen hormone approach. Gen. Comp. Endocrinol. 296, 113536 https://doi.org/10.1016/j.ygcen.2020.113536.

Gabriele, C.M., Taylor, L.F., Huntington, K.B., Buck, C.L., Hunt, K.E., Lefebvre, K.A., Lockyer, C., Lowe, C., Moran, J.R., Murphy A Rogers, M.C., Trumble, S.J., Raverty, S., 2021. Humpback whale #441 (Festus): Life, death, necropsy, and pathology, Natural Resource Report NPS/GLBA/NRR-2021/2250. National Park Service, Fort Collins, Colorado.

Galligan, T.M., Boggs, A.S.P., Balmer, B.C., Rowles, T., Smith, C.R., Townsend, F., Wells, R.S., Kellar, N.M., Zolman, E.S., Schwacke, L.H., 2020. Blubber steroid hormone profiles as indicators of physiological state in free-ranging common bottlenose dolphins (*Tursiops truncatus*). Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 239 https://doi.org/10.1016/j.cbpa.2019.110583.

Gormally, B.M.G., Romero, L.M., 2020. What are you actually measuring? A review of techniques that integrate the stress response on distinct time-scales. Funct. Ecol. 1365–2435, 13648. https://doi.org/10.1111/1365-2435.13648.

Grotjan, H.E.; Keel, B.A., 1996. Data Interpretation and Quality Control, in: Immunoassay., Data Interpretation and Quality Control, in: Immunoassay. Elsevier. DOI:10.1111.

Hayward, L.S., Booth, R.K., Wasser, S.K., 2010. Eliminating the artificial effect of sample mass on avian fecal hormone metabolite concentration. Gen. Comp. Endocrinol. 169, 117–122. https://doi.org/10.1016/j.ygcen.2010.08.004.

Hunt, K.E., Rolland, R.M., Kraus, S.D., Wasser, S.K., 2006. Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*). Gen. Comp. Endocrinol. 148, 260–272. https://doi.org/10.1016/j.ygcen.2006.03.012.

Hunt, K.E., Moore, M.J., Rolland, R.M., Kellar, N.M., Hall, A.J., Kershaw, J., Raverty, S. A., Davis, C.E., Yeates, L.C., Fauquier, D.A., Rowles, T.K., Kraus, S.D., 2013. Overcoming the challenges of studying conservation physiology in large whales: a review of available methods. Conserv. Physiol. 1 https://doi.org/10.1093/conphys/cot006.

Hunt, K.E., Rolland, R.M., Kraus, S.D., 2014a. Detection of steroid and thyroid hormones via immunoassay of North Atlantic right whale (*Eubalaena glacialis*) respiratory vapor. Mar. Mammal Sci. 30, 796–809. https://doi.org/10.1111/mms.12073.

Hunt, K.E., Stimmelmayr, R., George, C., Hanns, C., Suydam, R., Brower, H., Rolland, R. M., 2014b. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conserv. Physiol. 2 https://doi.org/10.1093/conphys/cou030.

Hunt, K.E., Rolland, R.M., Kraus, S.D., Prescott, J.H., 2015. Conservation physiology of an uncatchable animal: the north atlantic right whale (*Eubalaena glacialis*). Integr. Comp. Biol. 55, 577–586. https://doi.org/10.1093/icb/icv071.

Hunt, K.E., Lysiak, N.S., Moore, M.J., Rolland, R.M., 2016. Longitudinal progesterone profiles in baleen from female North Atlantic right whales (*Eubalaena glacialis*) match known calving history. Conserv. Physiol. 4, cow014. https://doi.org/10.1093/conphys/cow014.

Hunt, K.E., Lysiak, N.S., Moore, M., Rolland, R.M., 2017a. Multi-year longitudinal profiles of cortisol and corticosterone recovered from baleen of North Atlantic right whales (*Eubalaena glacialis*). Gen. Comp. Endocrinol. 254, 50–59. https://doi.org/10.1016/j.ygcen.2017.09.009.

Hunt, K.E., Lysiak, N.S., Robbins, J., Moore, M.J., Seton, R.E., Torres, L., Loren Buck, C., Buck, C.L., 2017b. Multiple steroid and thyroid hormones detected in baleen from eight whale species. Conserv. Physiol. 5 https://doi.org/10.1093/conphys/cox061.

Hunt, K.E., Lysiak, N.S.J., Matthews, C.J.D., Lowe, C., Fernández Ajó, A., Dillon, D., Willing, C., Heide-Jørgensen, M.P., Ferguson, S.H., Moore, M.J., Buck, C.L., 2018. Multi-year patterns in testosterone, cortisol and corticosterone in baleen from adult males of three whale species. Conserv. Physiol. 6, 1–16. https://doi.org/10.1093/conphys/coy049.

Hunt, K.E., Robbins, J., Buck, C.L., Bérubé, M., Rolland, R.M., 2019. Evaluation of fecal hormones for noninvasive research on reproduction and stress in humpback whales (*Megaptera novaeangliae*). Gen. Comp. Endocrinol. 280, 24–34. https://doi.org/10.1016/j.ygcen.2019.04.004.

Karpovich, S.A., Horstmann, L.A., Polasek, L.K., 2020. Validation of a novel method to create temporal records of hormone concentrations from the claws of ringed and bearded seals. Conserv. Physiol. 8, 1–11. https://doi.org/10.1093/conphys/coaa073.

Kersey, D.C., Dehnhard, M., 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen. Comp. Endocrinol. 203, 296–306. https://doi.org/10.1016/j.ygcen.2014.04.022.

Lemos, L.S., Olsen, A., Smith, A., Chandler, T.E., Larson, S., Hunt, K., Torres, L.G., 2020. Assessment of fecal steroid and thyroid hormone metabolites in eastern North Pacific gray whales. Conserv. Physiol. 8 https://doi.org/10.1093/conphys/coaa110.

Lysiak, N.S.J., Trumble, S.J., Knowlton, A.R., Moore, M.J., 2018. Characterizing the duration and severity of fishing gear entanglement on a North Atlantic right shale (*Eubalaena glacialis*) using stable isotopes, steroid and thyroid hormones in baleen. Front. Mar. Sci. 5, 1–13. https://doi.org/10.3389/fmars.2018.00168.

Macbeth, B.J., Cattet, M.R.L., Stenhouse, G.B., Gibeau, M.L., Janz, D.M., 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): Considerations with implications for other wildlife. Can. J. Zool. 88, 935–949. https://doi.org/10.1139/Z10-057.

Macbeth, B.J., Cattet, M.R.L., Obbard, M.E., Middel, K., Janz, D.M., 2012. Evaluation of hair cortisol concentration as a biomarker of long-term stress in free-ranging polar bears. Wildl. Soc. Bull. 36, 747–758. https://doi.org/10.1002/wsb.219.

Madliger, C. L., Franklin, C. E., Love, O. P., & Cooke, S.J. (Ed.), 2020. Conservation Physiology: Applications for Wildlife Conservation and Management., 1st ed. Oxford University Press. DOI:10.1093/oso/ 9780198836100.001.0001.

Madliger, C.L., Love, O.P., Hultine, K.R., Cooke, S.J., 2018. The conservation physiology toolbox: status and opportunities. Conserv. Physiol. 6, 1–16. https://doi.org/10.1093/conphys/coy029.

Mansouri, F., Winfield, Z.C., Crain, D.D., Morris, B., Charapata, P., Sabin, R., Potter, C. W., Hering, A.S., Fulton, J., Trumble, S.J., Usenko, S., 2021. Evidence of multi-decadal behavior and ecosystem-level changes revealed by reconstructed lifetime stable isotope profiles of baleen whale earplugs. Sci. Total Environ. 757, 143985 https://doi.org/10.1016/j.scitotenv.2020.143985.

McCormick, S.D., Romero, L.M., 2017. Conservation endocrinology. Bioscience 67, 429–442. https://doi.org/10.1093/biosci/bix026.

Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen. Comp. Endocrinol. 138, 189–199. https://doi.org/10.1016/j.ygcen.2004.07.002.

Mingramm, F.M.J., Dunlop, R.A., Blyde, D., Whitworth, D.J., Keeley, T., 2019. Evaluation of respiratory vapour and blubber samples for use in endocrine assessments of bottlenose dolphins (*Tursiops spp.*). Gen. Comp. Endocrinol. 274, 37–49. https://doi.org/10.1016/j.ygcen.2018.12.015.

Pallin, L., Baker, C.S., Steel, D., Kellar, N.M., Robbins, J., Johnston, D.W., Nowacek, D.P., Read, A.J., Friedlaender, A.S., 2018a. High pregnancy rates in humpback whales (*Megaptera novaeangliae*) around the western antarctic peninsula, evidence of a rapidly growing population. R. Soc. Open Sci. 5, 180017 https://doi.org/10.1098/rsos.180017.

Pallin, L., Robbins, J., Kellar, N., Bérubé, M., Friedlaender, A., 2018b. Validation of a blubber-based endocrine pregnancy test for humpback whales. Conserv. Physiol. 6 https://doi.org/10.1093/conphys/coy031.

Palme, R., 2019. Non-invasive measurement of glucocorticoids: advances and problems. Physiol. Behav. 199, 229–243. https://doi.org/10.1016/j.physbeh.2018.11.021.

Richard, J.T., Robeck, T.R., Osborn, S.D., Naples, L., McDermott, A., LaForge, R., Romano, T.A., Sartini, B.L., 2017. Testosterone and progesterone concentrations in blow samples are biologically relevant in belugas (*Delphinapterus leucas*). Gen. Comp. Endocrinol. 246, 183–193. https://doi.org/10.1016/j.ygcen.2016.12.006.

Rolland, R.M., Hunt, K.E., Kraus, S.D., Wasser, S.K., 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. Gen. Comp. Endocrinol. 142, 308–317. https://doi.org/10.1016/j.ygcen.2005.02.002.

Romero, M.L., Wingfield, J.C., 2016. Oxford series in behavioral neuroendocrinology. Tempests, poxes, predators, and people: stress in wild animals and how they cope 1–2.

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–887. https://doi.org/10.1007/s00442-011-1943-y.

Thomas, P.O., Reeves, R.R., Brownell, R.L., 2016. Status of the world's baleen whales. Mar. Mammal Sci. 32, 682–734. https://doi.org/10.1111/mms.12281.

Trumble, S.J., Robinson, E.M., Berman-Kowalewski, M., Potter, C.W., Usenko, S., 2013. Blue whale earplug reveals lifetime contaminant exposure and hormone profiles.

Proc. Natl. Acad. Sci. U. S. A. 110, 16922–16926. https://doi.org/10.1073/pnas.1311418110.

Trumble, S.J., Norman, S.A., Crain, D.D., Mansouri, F., Winfield, Z.C., Sabin, R., Potter, C.W., Gabriele, C.M., Usenko, S., 2018. Baleen whale cortisol levels reveal a physiological response to 20th century whaling. Nat. Commun. 9, 4587. https://doi.org/10.1038/s41467-018-07044-w.

Wasser, S.K., Azkarate, J.C., Booth, R.K., Hayward, L., Hunt, K., Ayres, K., Vynne, C., Gobush, K., Canales-Espinosa, D., Rodríguez-Luna, E., 2010. Non-invasive measurement of thyroid hormone in feces of a diverse array of avian and mammalian species. Gen. Comp. Endocrinol. 168, 1–7. https://doi.org/10.1016/j.ygcen.2010.04.004.