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Non-Invasive Monitoring of Hormones in Marine Mammals: Enhancing Steroid Extraction in Beluga (*Delphinapterus leucas*) Saliva

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Societal and governmental concerns about the welfare of animals under human care are on the rise (Tallo-Parra et al. 2023). Animal care facilities have demonstrated a growing commitment to the development of tools and approaches for animal welfare assessments. The paradigm of animal welfare assessment has evolved from a primary focus on appropriate husbandry and environmental provisions to a more integrated approach that includes animal-based indicators, reflecting a growing emphasis on evidence-based assessments (Tallo-Parra et al. 2023). Animal-based welfare indicators are typically categorized in behavioral and physiological measures (Tallo-Parra et al. 2023). Behavioral observations offer a key advantage over physiological measures because of their minimal or non-invasive data collection. Furthermore, the relatively low cost of implementation contributes to their widespread use as indicators for animal welfare assessments (Binding et al. 2020). Nevertheless, continuous behavioral observation is not always possible, especially in free-ranging animals. Alternatively, physiological indicators provide advantages for certain individuals, species, or contexts, particularly when considering data sampling and welfare assessment objectives. Glucocorticoids, or stress hormones, are increasingly employed as physiological indicators of individual and population health or welfare (Ralph and Tilbrook 2016). Cortisol, the main glucocorticoid in marine mammals, is synthesized after the activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to perceived stressors (Atkinson et al. 2015; Ralph and Tilbrook 2016). The resulting secretion of cortisol triggers a progression of behavioral and physiological changes to promote survival. Generally, this response takes from minutes to hours; nevertheless, prolonged or constant activation of this axis can have a negative impact on reproduction and survival (Dickens and Romero 2013). Therefore, the measure of these hormones is particularly valuable for assessing animal welfare across multiple timeframes (Gormally and Romero 2020).

In marine mammals several sample types have proven useful for hormone measurements. Some of these samples can be obtained with minimal disruption to the animal, such as saliva (Monreal-Pawlowsky et al. 2017; Pedernera-Romano et al. 2006), respiratory vapor (Burgess et al. 2018), feces (Hunt et al. 2019), and blubber biopsies (Champagne et al. 2017). Among them, saliva is particularly effective in reflecting free hormone concentrations in blood (Gröschl 2008; Wood 2009). Unlike other sample types, it enables the collection of serial samples over short time intervals (Gormally and Romero 2020). Animals under human care can be trained to open their mouth voluntarily, thus hormone concentrations measured in this matrix should reflect baseline or resting glucocorticoid values (Pedernera-Romano et al. 2006). Previous studies on marine mammals have demonstrated that saliva cortisol levels reflect those in blood (Charlton et al. 2025) and that cortisol in this media fluctuates in accordance with potential stressors (Monreal-Pawlowsky et al. 2017; Pedernera-Romano et al. 2006; Rickert et al. 2022; Ugaz et al. 2013). Nevertheless, the implementation of this technique in marine mammals faces several challenges related to both analytical performance and sample collection. Given the diversity of analytical approaches currently applied to salivary hormones (e.g., ELISA, radioimmunoassay, LC–MS/MS), rigorous analytical validation—including tests of parallelism, accuracy/

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recovery, precision—is essential when adapting assays to new species or matrices (Amaral 2010; López-Arjona et al. 2021). To date, only a limited number of studies in marine mammals have performed analytical validations for salivary steroids, demonstrating acceptable assay performance while highlighting the importance of matrix-specific optimization and validation across different analytical platforms (Charlton et al. 2025; Hogg et al. 2005; Monreal-Pawlowsky et al. 2017; Olsen et al. 2025; Rickert et al. 2022). Equally important is the methodological validation of saliva collection procedures. Although animals can be trained to open their mouth and expel seawater, salivary secretions may still contain variable amounts of seawater, potentially affecting hormone concentrations (Rickert et al. 2022). In addition, in some species, limited sample volume and low sample density can constrain both collection and assay performance. Collection devices may also introduce matrix effects or interfere with steroid measurements (Reckendorf et al. 2021; Thompson et al. 2014). Therefore, these factors must be carefully evaluated and controlled to ensure reliable interpretation of salivary hormone data. Preliminary work has suggested the feasibility of measuring cortisol in beluga whale (*Delphinapterus leucas*) saliva using an enzyme immunoassay (Biancani et al. 2009). Nevertheless, analytical validation of the assay and methodological optimization of sample preparation have not been reported. Accordingly, the aim of this work was to perform assay validation of a commercially available enzyme immunoassay for cortisol quantification in beluga whale saliva, and to optimize the analytical methodology by comparing extraction and non-extraction protocols.

This study was conducted in two female beluga whales trained for voluntary husbandry and sampling procedures under long-term human care. The subjects were two female beluga whales, both 17 and 18 years old at the time of sampling, that moved from a facility in China to a facility in Iceland in the summer of 2019. Two sample-processing methodologies were evaluated to assess the effect of extraction on salivary cortisol measurements: (1) unextracted saliva and (2) methanol-extracted saliva. Animals were sampled from July to December 2023, and from May to July 2024. Saliva samples were collected daily, at consistent time intervals for a period of 5 consecutive days every month. A total of 115 saliva samples were collected (ID 1 = 58; ID 2 = 57). The animals were trained to position themselves vertically against the wall of the pool, head up and mouth open, without any need for restraint, to allow for voluntary saliva sampling. Prior to sample collection, the oral cavity, specifically the tongue, was thoroughly dried using sterile paper towels to eliminate residual water contamination. Animals were not fed during the sampling process to prevent food-borne contamination. A sterile Salivette swab was placed at the base of the tongue for a duration of 10 s. Immediately after, the swab was inserted into a sterile 5 mL syringe, and the plunger was then inserted to manually compress the swab and recover the saliva into a sterile 1.5 mL Eppendorf tube. This procedure was used as centrifugation was not available at the sampling site. After extraction, the Eppendorf tube was labeled with animal identification, date, and time of collection. The Eppendorf tube was subsequently stored at −20°C for long-term preservation. Samples were collected during monthly sampling periods and stored frozen at −20°C until shipment. Samples were then shipped to the laboratory in batches after each sampling period.

Once in the laboratory, saliva samples were left to thaw at room temperature and immediately centrifuged (9.500 × g, 20 min) for supernatant separation. A total amount of 600 μL of the supernatant was transferred to a new microtube and gently mixed with vortex for a few seconds. This volume was then split into two aliquots for the two processing methodologies. For the unextracted saliva protocol, 100 μL were transferred to a new microtube and frozen at −20°C until assay. For the methanol-extracted saliva protocol, the remaining 500 μL were transferred to a new 1.5 mL microtube and a volume of 750 μL of methanol was added to the microtube. The mixture was vigorously vortexed for 15 min, and samples were then centrifuged at 9.500 × g for 10 min. Afterwards, 500 μL of the supernatant were transferred to a new microtube. These microtubes were then placed in an oven at 50°C for 24 h until complete liquid evaporation. Finally, residues were resuspended in 100 μL of enzyme immunoassay (EIA) buffer. Any deviations in volume due to insufficient sample or high viscosity were documented. Samples exhibiting overall high viscosity were noted.

For assay validation, a total of 35 unextracted saliva samples and 35 methanol-extracted samples were pooled separately following the criteria for an immunological validation (Food and Drug Administration 2015; Grotjan and Keel 1996). Precision was calculated with the intra-assay coefficient of variation (CV) given by all duplicated samples measured. Serial dilutions of the pooled samples (1:1, 1:2, 1:4, and 1:8) were used to evaluate assay linearity, which is an indicator of matrix interference and assay specificity. Accuracy was evaluated using the spike-and-recovery test to assess potential matrix effects. Known amounts of cortisol standard solution were added to saliva pool samples at different volumes (25, 75, and 100 μL) and concentrations (0.2, 0.4, and 1.0 ng/mL), and recovery percentages were calculated as the ratio between observed and expected concentrations. Cortisol concentrations from unextracted and methanol-extracted saliva samples, and all the validation tests, were determined by using cortisol EIA detection kits (Neogen Corporation Europe, Ayr, UK).

Data obtained were analyzed using R software (R-project, Version 4.2.2, R Development Core Team, University of Auckland, Auckland, New Zealand) using a p-value < 0.05 as a criterion for significance. Statistical analyses were performed to evaluate assay performance and compare sample-processing methodologies. The assumption of normality was checked using Shapiro–Wilk test. For assay validation, the relationship between expected and obtained values in the dilution test was determined using Pearson's Product correlation test. Accuracy was assessed by calculating the percentage of recovery of known added concentrations (standard + saliva pool). The relationship between expected and observed concentrations was evaluated using Pearson's correlation test. The relationship in cortisol concentrations between methanol-extracted and unextracted saliva samples was evaluated using Spearman correlation test.

This study presents an optimized protocol for extracting steroid hormones from cetacean saliva, demonstrating that incorporating a methanol-extraction step enhances the measurement of steroids in this sample type. We analyzed samples subjected to both processing methods (unextracted and methanol-extracted)

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to obtain comparable data on hormone measurements in two beluga whale individuals.

A total of 115 unextracted and 115 methanol-extracted samples were assayed by EIA. While cortisol could only be detected in 73 unextracted samples, using the methanol-extracted protocol we could detect cortisol in 99 samples. In unextracted samples, 36 were below the detection limit, and 6 samples could not be assayed due to high viscosity, which precluded quantification. Conversely, only 16 methanol-extracted samples were below the detection limit, and density presented no impediment to analysis.

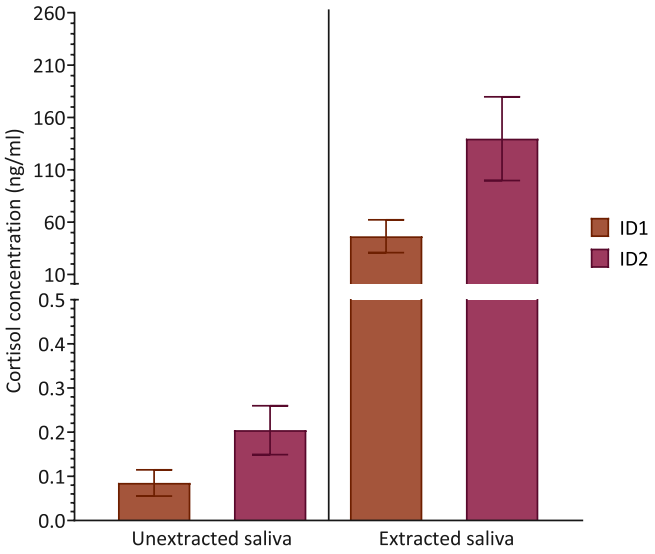


FIGURE 1 | Cortisol concentrations (ng/mL) in unextracted and methanol-extracted saliva samples from two (ID 1 and ID 2) beluga whales. Each bar represents the mean values (±SEM).

Mean hormone concentrations (±SEM) were 0.15 ± 0.03 ng cortisol/ml unextracted saliva and 92.61 ± 21.73 ng cortisol/mL methanol-extracted saliva. Figure 1 shows individual mean cortisol levels (±SEM) in saliva samples processed following the two protocols (unextracted and methanol-extracted).

Performance of the EIA for cortisol analysis in saliva was validated for both protocols, yielding repeatable and reliable results with slight inter-protocol variability. Although the intra-assay CV for the non-extracted samples falls within the range generally considered acceptable during assay development and for measurements in complex biological matrices (Crowther 2001), precision of cortisol measurement displayed improvement when using the methanol-extraction protocol. According to established guidelines for immunoassay performance, CV values below 10% are typically indicative of high analytical precision (Crowther 2001; Grotjan and Keel 1996). For both protocols, serially diluted saliva samples displayed significant correlation coefficients between obtained and expected values ($r = 0.99$, $p < 0.01$; Figure 2 and Table 1), indicating good dilutional linearity and minimal matrix interference across the tested range. Results of the spike-and-recovery test demonstrated acceptable accuracy for cortisol in both protocols (unextracted, $r = 0.93$; methanol-extracted, $r = 0.95$, $p < 0.001$). However, the methanol-extracted samples exhibited higher mean recovery values, closer to 100%, and lower standard deviation compared with the non-extracted protocol, indicating improved analytical accuracy and reduced variability.

These results suggest that the EIA kit used is suitable for the quantification of cortisol in beluga whale saliva processed through both methodologies. However, the enhanced precision and accuracy achieved suggest that methanol-extracted samples may offer improved analytical performance. The observed inter-protocol variability in analytical validation may arise from

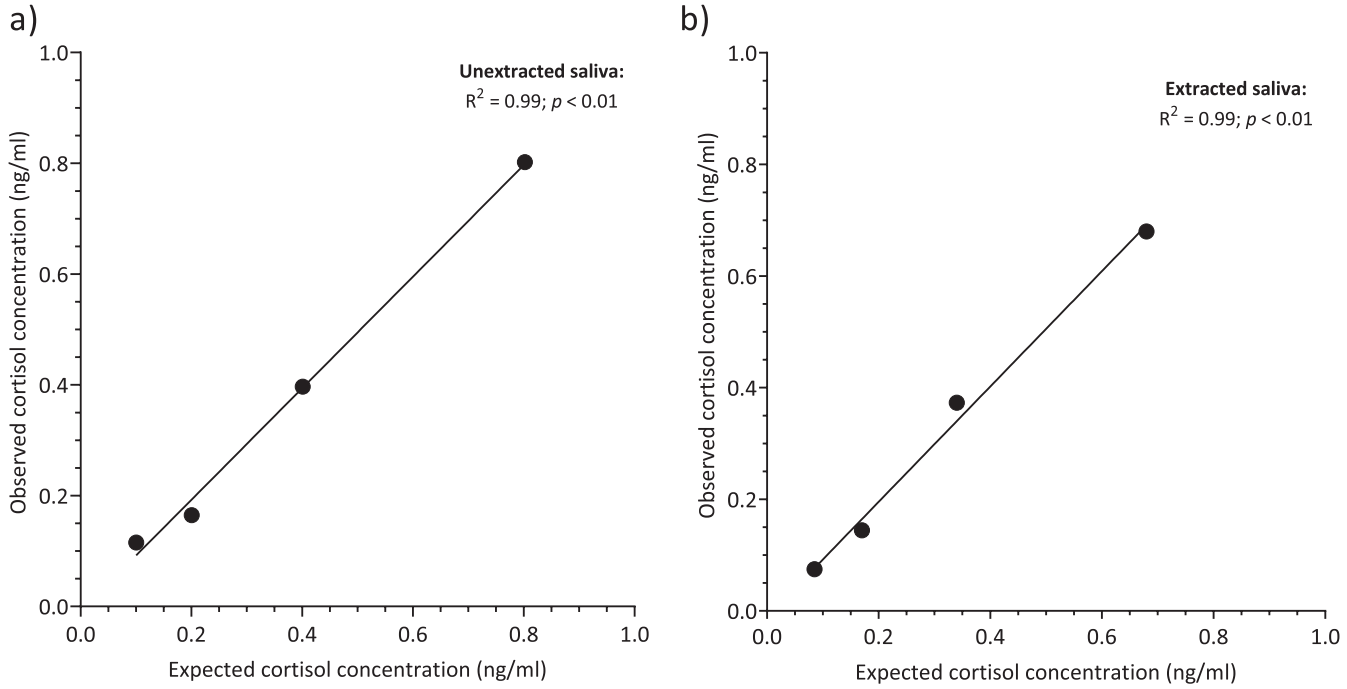


FIGURE 2 | Relationship between observed and expected beluga whale cortisol concentrations (ng/mL) obtained in the dilution test from the (a) unextracted and the (b) methanol-extracted saliva samples.

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TABLE 1 | Analytical validation of the cortisol EIA kit; results show the precision (intra-assay coefficient of variation (CV)), specificity (dilution test), and accuracy (spike-and-recovery test) of the assay.

		Dilution test		Spike-and-recovery test
Protocol type	**Intraassay CV (±SD)**	**R^2**	**Mean error (±SD)**	**Mean recovery (±SD)**
Non-extracted	14.1% (±13.9)	0.99	98.7% (±1.3)	88.1% (±15.5)
Methanol-extracted	6.3% (±8.2)	0.99	94.2% (±5.8)	100.8% (±6.6)

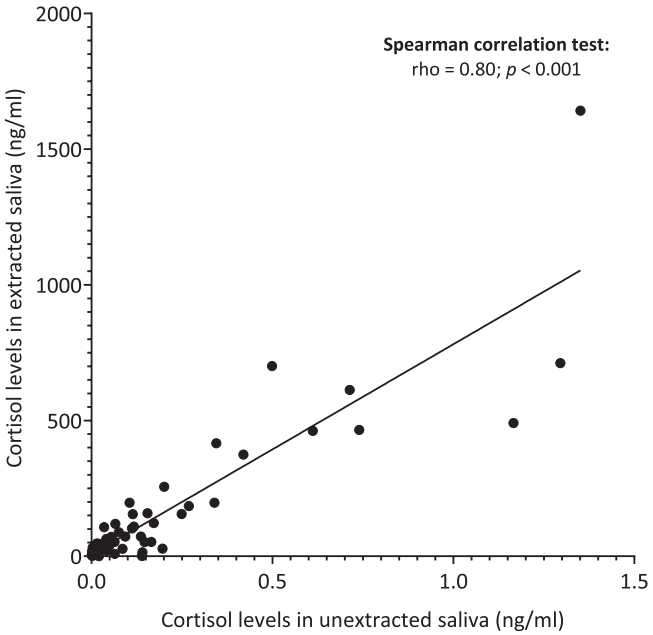


FIGURE 3 | Relationship between beluga whale cortisol concentrations (ng/mL) from unextracted and methanol-extracted saliva samples.

inherent physicochemical characteristics of beluga whale saliva. Specifically, the elevated density could potentially hinder accurate hormone quantification, while low analyte concentrations, possibly due to seawater contamination, may result in levels below the assay's reliable limit of detection affecting the precision of the assay. LC–MS/MS could provide additional confirmation of analyte specificity in complex matrices such as saliva, particularly in marine mammals where potential matrix effects including dilution variability and potential interferents may influence immunoassay-based measurements (Rickert et al. 2022; Wudy et al. 2018).

Finally, we studied the relationship between both methodologies in hormone concentrations. We observed that levels of cortisol in unextracted saliva were positively correlated with those detected in methanol-extracted samples (rho = 0.80, $p < 0.001$; Figure 3).

While saliva represents a promising minimally invasive matrix for monitoring adrenocortical activity in beluga whales, sample collection using swabs showed inherent variability in sample recovery. In some sampling events, limited saliva volume was obtained, likely due to individual variation in salivary secretion and the unavoidable dilution effects associated with the aquatic environment. A recent study on beluga whale blow samples evaluated the use of urea and total protein as potential dilution markers, suggesting that in controlled settings such as aquaria, correction for dilution may be unnecessary due to limited variability in sample water content (Thompson et al. 2026). In saliva, these factors may influence hormone concentrations and should therefore be considered when developing standardized saliva collection protocols for marine mammals. Nevertheless, the results obtained in this study demonstrate that, when combined with an appropriate extraction procedure, saliva can provide a reliable matrix for cortisol assessment in beluga whales.

Overall, the evidence derived from the results obtained in this study strongly supports the methanol-extraction protocol for cortisol quantification in beluga whale saliva. The numerical data, demonstrating a significantly higher rate of cortisol detection in methanol-extracted samples (99 vs. 73), coupled with the reduced number of samples below the detection limit, underscores its enhanced efficacy. Besides, the notably larger standard error of the mean (21.73 ng/mL vs. 0.03 ng/mL) suggests that the methanol extraction protocol revealed a wider distribution of cortisol concentrations among samples. Although the present study was not designed to investigate the biological sources of this variability, the broader range of values may reflect an improved ability to detect differences that could otherwise remain masked. The increased variability in the methanol-extracted samples may provide a broader range of data, which could be valuable in identifying subtle differences in cortisol levels between groups or under different biological conditions. Future studies applying this methodology should evaluate whether the increased variability corresponds to biologically meaningful factors such as individual, temporal, or physiological differences. Moreover, the better precision, accuracy, and sensitivity observed during assay validation, alongside the robust Spearman correlation, further supports the methanol-extraction protocol as the more reliable and effective methodology. Consequently, this protocol is recommended for future studies requiring accurate and precise glucocorticoid measurements in beluga whale saliva as well as that of other marine mammal populations housed under human care.

Author Contributions

Tania Monreal: conceptualization, methodology, project administration, resources, writing – review and editing, supervision. **Annaïs Carbajal:** methodology, data curation, formal analysis, writing – original draft, investigation, software. **Paula Serres-Corral:** methodology, validation, writing – review and editing, software, visualization, investigation. **Enric Horta:** conceptualization, methodology, resources, writing – review and editing, project administration. **Manel López-Bejar:** conceptualization, methodology, supervision, funding acquisition, writing – review and editing, validation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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