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Physiological welfare indicators in wild cetaceans: Epidermal cortisol and oxytocin concentrations in stranded striped dolphins

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ABSTRACT

Anthropogenic pressures and climate change present growing challenges for cetaceans, as the combined effects of multiple stressors can jeopardize their welfare and survival. In this context, validating reliable individual welfare indicators is crucial for quantifying these impacts. This study aimed to validate a method for measuring cortisol and oxytocin from the epidermis of stranded striped dolphins (*Stenella caeruleoalba*) using enzyme immunoassays, while accounting for confounding factors such as epidermal layer and body location. The effects of different causes of death—'Peracute Underwater Entrapment' and 'Distress Associated'— along with biological factors, were examined in relation to epidermal hormone levels. Furthermore, the relationship between these hormone levels and markers suggesting an impaired welfare, was explored. Validation tests indicated that the method was effective in quantifying both epidermal cortisol and oxytocin concentrations. Specifically, epidermal cortisol levels showed strong correlations with both serum and blubber levels and were 6 times higher in emaciated individuals and 14 times higher in those with distress-associated deaths, supporting its use in assessing hypothalamic-pituitary-adrenal activity. Interestingly, results supported the validity of epidermal cortisol levels as markers of impaired welfare in dolphins, as they consistently increased across conditions assumed to negatively affect welfare but varying in terms of severity and duration. In contrast, epidermal oxytocin levels could not be validated as an indicator of the general oxytocin system nor as an indicator of welfare in this species. In conclusion, this study successfully validated epidermal cortisol as a reliable physiological indicator of welfare in striped dolphins, providing a promising tool for assessing individual and population-level welfare impacts. However, further research is needed to fully explore the potential role of oxytocin as a welfare biomarker in cetaceans.

1. Introduction

Anthropogenic pressures and climate change pose significant challenges to marine biodiversity conservation (Ramírez et al., 2017). Cetaceans face nowadays a growing array of stressors, such as ship strikes, entanglement in fishing gears, pollution, noise, and the broader impacts of climate change (de Vere et al., 2018; Kebke et al., 2022). These cumulative stressors can compromise cetacean welfare and survival, which could lead to decreased fitness — characterized by reduced reproductive output and increased mortality (Broom and Johnson, 2019). Consequently, assessing individual welfare is critical to understanding and mitigating the broader impact of anthropogenic disturbances and climate change on animal populations (Pirotta et al., 2022).

However, practical challenges, such as tracking and repeatedly sampling free-ranging cetaceans, pose significant barriers to effective welfare assessment (Hunt et al., 2013). Despite these difficulties, the use of biological tissues from both free-ranging and stranded cetaceans individuals has provided valuable insights (Clegg and Delfour, 2018; Derous et al., 2020; Hunt et al., 2013; Kellar et al., 2015).

Cetacean skin, in particular, has emerged as a potential key tissue for

Abbreviations: HPA, hypothalamic–pituitary–adrenal axis; EIA, enzyme immunoassay; CeMV, cetacean morbillivirus; PUE, peracute underwater entrapment; SC, stratum corneum; SSB, stratum spinosum and basale.

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studying health and physiology. Cetacean skin is structured into three distinct layers: the epidermal, dermal, and hypodermal layers. Notably, it features a marked thick epidermis, a trait that enhances resistance and supports homeostasis (Eckhart et al., 2019; Harrison and Thurley, 1974). The epidermis itself contains three distinct layers: stratum basal, stratum intermedium, and stratum corneum; and exhibits constant mitotic activity and sloughing, showing a very high cellular turnover rate (Geraci et al., 1986; Hicks, 1985). In bottlenose dolphins, the epidermal renewal period is reported to span approximately 73 days (Hicks, 1985).

The epidermis has recently been identified as a viable matrix for measuring glucocorticoid hormone levels in cetaceans (Bechshoft et al., 2020; Bechshoft et al., 2015). Typically, exposure to stressful stimuli leads to a heightened secretion of glucocorticoid hormones subsequent to the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Mostl and Palme, 2002). Cortisol, the primary glucocorticoid in mammals, has thus been employed as a stress biomarker across various matrices for numerous cetacean species (Atkinson et al., 2015; Kellar et al., 2015; Kershaw et al., 2017; Trana et al., 2016) but how it relates to the welfare of vertebrates remains controversial (Ralph and Tilbrook, 2016).

Cortisol in cetaceans has been studied using diverse samples such as blood, saliva, respiratory vapor, urine, feces, blubber, epidermis, and baleen (Bechshoft et al., 2015; Champagne et al., 2018; Champagne et al., 2017; Fair et al., 2014; Hart et al., 2015; Hunt et al., 2014; Mingramm et al., 2019; Pedernera-Romano et al., 2006; Suzuki et al., 1998). Alternatives to the use of blood offer the possibility of using less invasive approaches and allow the consideration of different temporal windows. For instance, while cortisol levels in blubber and feces have been suggested to reflect mid-term activation of the HPA axis (i.e. occurring within hours; Champagne et al., 2018), cortisol levels in keratinous materials seem to be indicative of longer-term activation (Bechshoft et al., 2015; Trumble et al., 2018).

On the other hand, the assessment of pleasure and stress tolerance is becoming increasingly important in animal welfare science. In this context, a promising biomarker which is attracting a lot of interest is oxytocin (Rault et al., 2017). Oxytocin is a neuropeptide known for its role in reproduction, lactation, and maternal behavior (Lee et al., 2009), which also plays an important role in social bonding and cognition (Ross and Young, 2009). Moreover, it has important stress-buffering effects (Rault et al., 2017). In cetaceans, social bonds beyond mating and maternity offer fitness benefits through enhanced cooperation and resource access (Gerber et al., 2022; Seyfarth and Cheney, 2012). Measuring oxytocin in cetaceans could thus gauge social and environmental motivation, akin to findings in primate studies (Crockford et al., 2014). In contrast to cortisol concentrations, oxytocin concentrations have only been measured in the blood of free-ranging dolphins (Robinson et al., 2020). Yet, recent findings advocate for the feasibility of assessing oxytocin within keratinous matrices such as mammalian hair (López-Arjona et al., 2021).

Cortisol, produced by the adrenal gland, likely diffuses into skin cells due to its lipophilic nature (Chrousos, 1995). Nevertheless, the epidermis itself can synthesize and release cortisol, suggesting that skin acts similarly to the HPA axis (Cirillo and Prime, 2011). In contrast, oxytocin, synthesized in the hypothalamus and released into the bloodstream, is unlikely to diffuse in the skin due to its peptide structure (Quintana et al., 2018). However, similar to cortisol, oxytocin is produced in epidermal cells (Deing et al., 2013), suggesting it may also be measurable in the skin.

Overall, epidermal cortisol and oxytocin levels could serve as biomarkers of the HPA axis and the oxytocin system activity, respectively, in living cetaceans. This method could thus become a useful tool, in combination with other methods, to understand the cumulative impacts of multiple stressors on the welfare of cetaceans. However, methodological (i.e. whether the full protocol can distinguish low from high hormone concentrations in the matrix) and physiological (i.e. whether the measured hormone concentrations truly reflect the general physiological state of the animal) validations need to be performed for every species (Hunt et al., 2013).

Moreover, if we aim to infer into the welfare of individual animals, validation is also required to ensure indicators are measuring the intended target states (i.e., their affective states). Establishing a valid indicator requires a demonstrated relationship with the affective state of animals, which can manifest either as the cause or effect (Beaulieu, 2024). To confirm this relationship, validation involves initially formulating assumptions about the causal connections (e.g., specific conditions impair the welfare of animals), which are then scrutinized through multiple independent lines of evidence (Browning, 2023). To establish a structured approach to welfare evaluation, frameworks like the Five Domains Model are commonly applied. This model includes four interacting physical/functional domains—nutrition, environment, health, and behavior—and a fifth domain focused on mental state (Mellor et al., 2020; Mellor and Beausoleil, 2015).

In free-ranging cetaceans, the validation of markers related to the HPA axis, as well as welfare indicators, requires studying populations with well-documented physiological and welfare states. Once validated successfully, the technique can then be applied more broadly to other populations where validated indicators can be measured (e.g. Trana et al., 2016; followed by Kucheravy et al., 2022). Stranded cetaceans, subjected to necropsies and detailed anatomopathological studies, therefore provide precious resources for validating physiological welfare indicators (e.g. Agusti et al., 2022; Lowe et al., 2021; Rolland et al., 2017).

On the Catalonian coast (western Mediterranean Sea), the primary causes of mortality in striped dolphins (*Stenella coeruleoalba*), the most abundant cetacean in the Mediterranean, include peracute underwater entrapment (PUE), maternal separation, cetacean morbillivirus (CeMV), and brucellosis (Cuvertoret-Sanz et al., 2020). While diseases, injuries, maternal separation, and emaciation can be related to prolonged activation of the HPA axis and diminished quality of life (i.e. welfare during a period of more than a few days (Broom, 2007, Broom, 2006; Broom and Johnson, 2019), most dolphins experiencing PUE are typically in better welfare, suffering from acute hypoxia and subsequent asphyxiation within minutes. These animals are generally in good body condition as they die suddenly, contrasting with other animals experiencing health deterioration due to disease or starvation (Cuvertoret-Sanz et al., 2020; Moore et al., 2013; De Quirós et al., 2018). This sudden event may result in acute activation of the HPA axis, indicating poor welfare during a very brief period until death (Agusti et al., 2022; Kershaw et al., 2017).

In a general effort to validate cetacean welfare physiological markers, the specific objectives of this study were the following ones: (i) to validate a protocol for extracting cortisol and oxytocin from the epidermis of striped dolphins and for analyzing the concentrations of these hormones using enzyme immunoassays (EIA); (ii) to identify potential confounding factors affecting hormone concentrations, including epidermal layer and body location; (iii) to evaluate the association between cortisol levels in the epidermis, blubber, and serum; (iv) to evaluate the relationship between epidermal cortisol and oxytocin concentrations; (v) to examine the effects of different causes of death and biological features on epidermal cortisol and oxytocin levels; and (vi) to explore the relationship between the duration and severity of welfare compromise with epidermal cortisol and oxytocin levels.

2. Methods

2.1. Ethics statement

For this study, all samples were obtained post-mortem, following the recovery of carcasses by the Marine Fauna Stranding Network, with the support of the Catalan Government. No animals were specifically targeted for this research or any related studies. The handling and analysis of carcasses were conducted in accordance with official governmental

permits, including AG-2015-474, PTOP-2016-663, and PTOP-2021-14.

2.2. Study area, individuals and causes of death

Epidermis samples were collected from 49 stranded striped dolphins along the northwestern Mediterranean Sea over the period from June 2012 to February 2024. Necropsies were conducted on all recovered carcasses at the Veterinary School of Universitat Autònoma de Barcelona by pathologists certified by the European College of Veterinary Pathologists, adhering to protocols established by Kuiken and Baker (1991). The causes of death were determined through necropsy and histopathology, and ancillary laboratory techniques.

The sample comprised females and males, categorized into three life history stages in accordance with the criteria established by previous research (Calzada et al., 1997; Gómez-Campos et al., 2011; Table 1): (1) calves (including neonates and individuals up to one year of age); (2) juveniles (defined as females <187 cm in length and males <190 cm); and (3) adults. Body condition was classified as either 'good condition' or 'emaciated' (Table 1) based on anatomical observations such as visibility of certain bones, the dorso-axial muscle mass, and the presence or scarcity of fat reserves (Joblon et al., 2014). Finally, the state of carcass preservation was evaluated using a scale from 1 (very fresh) to 5 (very autolytic), as outlined by Jauniaux et al. (2005), with the distribution of preservation states 1 through 4 being 8.16 %, 79.6 %, 8.16 %, and 4.08 %, respectively.

The primary presumed cause of death identified was 'Peracute underwater entrapment' (PUE; $N = 13$ (27 %)). This condition was characterized by individuals exhibiting no signs of disease and maintaining a healthy body condition. These individuals had full forestomachs and presented with indicators associated with fisheries interaction, such as evidence of net entanglement or decompression-associated gas bubble formation (Cuvertoret-Sanz et al., 2020; Moore et al., 2013; De Quirós et al., 2018). Other presumed causes of death included the separation of calves from their mothers ($N = 7$ (14 %)), infection with CeMV (N = 7 (14 %)), infection with *Brucella ceti* ($N = 5$ (10 %)), septicemia ($N = 4$ (8 %)), severe parasitism coupled with emaciation ($N = 1$ (2 %)), pulmonary angiomatosis (N = 1 (2 %)), tension pneumothorax (N = 1 (2 %)), and cases of diseased o injured animals where the cause of death remained undetermined ($N = 10$ (20 %)). Except for PUE, all other causes of death were collectively classified as 'Distress associated' ($N = 36$), including those individuals experiencing an aversive state caused by an inability to adapt to stressors, due to various factors including disease, injury, and/or social stress (Moberg and Mench, 2000).

2.3. Characterization of individual welfare state based on necropsy measurements

A comprehensive list of markers measured in necropsy reports, following the Five Domains Model framework, was compiled (Table 2). This list includes markers potentially reflecting a deterioration of at least one of the domains underlying the affective states of animals, drawing on the works by Boys et al. (2022), Harvey et al. (2023), and Mellor et al. (2020). Each indicator was scored as 0 (absent) or 1 (present) for each individual dolphin.

Table 1
Distribution of individuals across life history stage, sex and body condition.

Sex	Body condition	Life history stage			Total
		Calves	Juveniles	Adults	
Females	Good condition	1	3	9	13
	Emaciated	4	3	2	9
	Total	5	6	11	22
Males	Good condition	4	8	9	21
	Emaciated	0	3	3	6
	Total	4	11	12	27

Due to the nature of the data and the focus on specific markers observed in necropsy reports, a balanced set of measures encompassing all welfare domains (e.g., environmental domain) was not available. Therefore, the goal of the intra-individual aggregation process presented here was not to derive an overall welfare score for individual dolphins but to compare the severity of welfare compromise between individuals and relate it to skin hormonal levels. Moreover, as noted by Sandøe et al. (2019), the aggregation of welfare indicators should be approached cautiously, recognizing significant differences in the validity and importance of various indicators.

To assign a severity score to the welfare compromise of each individual, all markers suggesting an impaired welfare (binary value 0 or 1 based on absence or presence, respectively) were summed to provide a severity score ranging from 0 to 4. Markers appearing in multiple domains (e.g. 'Emaciated body condition') were counted only once, and 'Peracute underwater entrapment' was excluded due to its short duration, which is unlikely to correlate with epidermal cortisol levels (Agusti et al., 2022). Finally, each individual was categorized as having short-term, medium-term, or long-term welfare compromise based on the indicator observed with the longest duration. For instance, if an individual exhibited one long-term indicator and one medium-term indicator, the individual was classified as experiencing long-term welfare compromise.

2.4. Sample collection and preparation

Full depth integument samples (2 × 2 cm; i.e. epidermis ($N = 49$) and blubber ($N = 41$)) were collected from the left lateral flank and stored at −80C°. Cardiac blood samples ($N = 28$) were collected from some individuals via puncture at the midpoint of a coronal line from the sternum to the axillar fold, using a 20 mL syringe with a 16 G, 1.7 × 133 mm catheter needle (AngiocathTM). Samples were transferred to sterile tubes, centrifuged at 1372*g* for 6 min, and the serum was aliquoted into 1 mL cryotubes, then stored at −80 °C for analysis.

To study the correlation between epidermal cortisol concentrations and those in serum and blubber, epidermal samples were obtained concurrently with a related study focusing on cortisol levels in blubber and serum (Agusti et al., 2022). Results from the cortisol analyses in blubber and serum were used in this study. Cortisol quantification was performed using the same EIA technique than in this study, as described in section 2.6. and detailed in Agusti et al. (2022) and Carbajal et al. (2022).

To examine the relationship between hormone concentrations across different epidermal layers, each skin sample was divided into two subsamples: stratum corneum (SC) and stratum spinosum and basale (SSB). For each sample, SC was separated from adjacent epidermal layers employing fine-pointed tweezers for manual separation. This approach hinged on a generalized SC's capacity for straightforward detachment under controlled force, allowing for its efficient isolation with low tissue damage (Fig. 1).

To study the effect of sample body location in SC and SSB cortisol and oxytocin concentrations, epidermal samples from 10 of the individuals were collected from five distinct anatomical regions: anterior and posterior relative to the dorsal fin, as well as dorsal, medial, and ventral positions adjacent to the pectoral fin (Fig. 2). These samples were surgically excised before necropsy using a scalpel and were further subdivided into SC and SSB as detailed before.

2.5. Epidermal samples washing and hormones extraction

For both cortisol and oxytocin extraction, we adapted and validated a protocol drawing upon methodology previously outlined by Agustí et al. (2024). These methods, originally developed for cortisol extraction from the scraped epidermis of bottlenose dolphins and beluga whales (*Delphinapterus leucas*), began with washing each SC and SSB sample using 1.5 mL (for SC) or 2 mL (for SSB) of isopropanol (99.5 % purity,

Table 2
Markers suggesting impaired welfare, potential physical/functional impairments (domains one to four of the Five Domains Model), and their associated inferred negative affects (domain five of the Five Domains Model) identified in necropsy reports of stranded striped dolphins.

Domain	Necropsy markers	Potential physical/functional impacts (Domains 1–4)	Potential negative affects inferred (Domain 5)	Duration of the welfare compromise[1]
Domain 1: Nutrition	Emaciated body condition	Energy deficit	Hunger, weakness of starvation	Long-term
	Mother separation	Energy deficit	Hunger, weakness of starvation	Medium-term
Domain 3: Health	Acute trauma or injury[2]	Disruption of integument, muscle, joints or bones, impaired musculoskeletal activity	Pain, malaise, dizziness, exhaustion, and/or breathlessness	Medium-term
	Chronic trauma or injury[3]	Disruption of integument, muscle, joints or bones, impaired musculoskeletal activity		Long-term
	Acute functional or health impairment[4]	Systemic or localized pathology or disfunction, metabolic disturbances		Medium-term
	Chronic functional or health impairment[5]	Systemic or localized pathology or disfunction, metabolic disturbances		Long-term
	Peracute underwater entrapment	Struggling, asphyxiation, disruption of integument, muscle, joints or bones	Breathlessness, anxiety, malaise, physical exhaustion	Short-term
Domain 4: Behavioral Interactions	Emaciated body condition	Muscular weakness, metabolic disturbances	Malaise, exhaustion	Long-term
	Mother separation	Mother-calf relationship and affiliative interactions impeded, social isolation	Loneliness, fear, insecurity	Medium-term

[1] Short-term: welfare compromise occurring within minutes; Medium-term: welfare compromise occurring within hours to weeks; Long-term: welfare compromise occurring over months to years.
[2] Acute trauma or injury: observations such as fresh lacerations, recent fractures, acute blunt force trauma, and other signs of severe physical injuries occurring within hours to weeks.
[3] Chronic trauma or injury: observations such as old, healed fractures, long-standing entanglement scars, chronic wounds, indicating long-term or recurring physical injuries.
[4] Acute functional or health impairment: observations such as acute infections, sudden metabolic disturbances, or rapidly developing disease conditions, significantly impairing health and functionality within hours to weeks.
[5] Chronic functional or health impairment: observations such as chronic infections, long-term parasitic infestations, or degenerative diseases, indicating long-standing health issues leading to persistent dysfunction and metabolic disturbances.

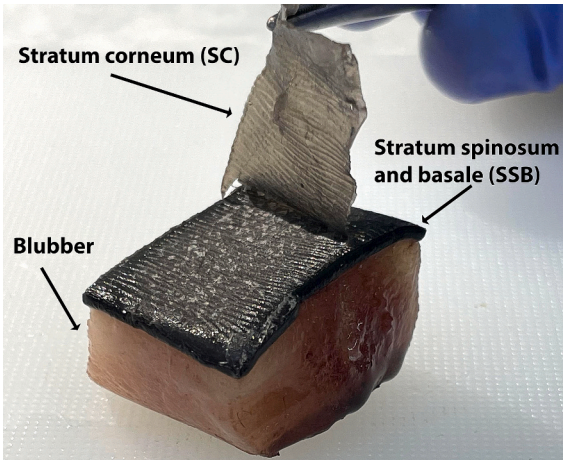

Fig. 1. Manual dissection of the stratum corneum. The image displays the mechanical separation of the stratum corneum layer using fine-pointed tweezers. The figure illustrates the application of controlled force to detach the stratum corneum from the underlying epithelial layers (black tissue) and blubber (white-pink tissue), showcasing the ease of layer isolation without compromising tissue integrity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

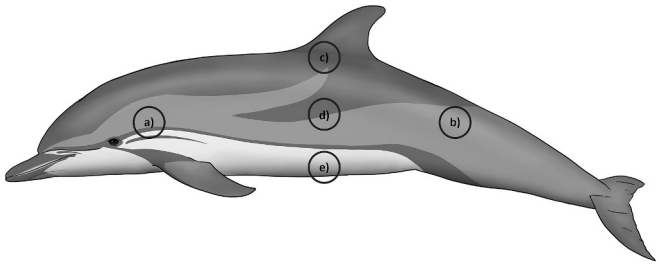

Fig. 2. Epidermal sampling sites collected on striped dolphins: cranial (a) and caudal (b) relative to the dorsal fin, alongside dorsal (c), medial (d), and ventral (e) positions near the pectoral fin. Photo credit: Emma Abad García, 2020.

Sharlab). Samples were vortexed at 9500 ×g for 30 s to remove exogenous steroid contaminants while preserving endogenous steroids, as recommended by Davenport (2006). This washing was repeated three times for thorough cleansing.

Samples were then transferred to Petri dishes and subjected to a drying phase. This was accomplished by placing the samples in an oven (Heraeus model T6; Kendro® Laboratory Products, Langenselbold, Germany) and maintaining a temperature of 40 °C for a period of 48 h. Upon completion of the drying phase, the samples were prepared for homogenization by being sectioned into smaller fragments. This preparatory step was followed by a grinding process using a ball mill (Model MM200, Retsch, Haan, Germany), ensuring the uniform consistency of the sample material. In order to minimize sample loss during this procedure, SC samples were placed into 2 mL Eppendorf tubes, each containing five 0.7 mm stainless-steel balls, and secured within 10 mL stainless-steel grinding jars. Conversely, SSB samples were processed directly within 10 mL stainless-steel grinding jars, utilizing two 12 mm stainless-steel grinding balls. The grinding operation was uniformly conducted for all samples for a duration of 5 min at a frequency of 25 Hz. Samples were then normalized to specific masses to account for a previously observed effect of sample mass (Agustí et al., 2024). Specifically, SC samples were adjusted to a mass of 15 to 20 ± 0.5 mg, while SSB samples were standardized to 50 ± 0.5 mg. With the methodology described in section 2.4., we collected enough SC sample mass (≥ 15 mg of dry SC) in 69.39 % of sampling attempts.

For each sample, 1.5 mL of pure methanol was added, followed by vortex mixing. The samples were then incubated with gentle agitation for 18 h at 30 °C using a G24 Environmental Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, NJ, USA) to facilitate steroid extraction. Post-extraction, the samples underwent centrifugation at 10,000 rpm for 10 min at room temperature to separate the supernatant from the pellet. A volume of 1.2 mL of the supernatant was carefully transferred to a 2-mL Eppendorf tube and subsequently dried in a Heraeus model T6 oven (Kendro Laboratory Products, Langenselbold,

Germany) at 38 °C. After approximately 48 h, once the methanol had fully evaporated, the dry extracts were re-suspended in EIA buffer (1 M phosphate buffered saline) and vortexed for 30 s.

2.6. Hormones detection and assays validation

Cortisol concentrations from SC and SSB samples were determined by using competitive EIA kits (Neogen® Corporation Europe, Ayr, UK) and following manufacturer's instructions. According to the manufacturer, cross-reactivity of the ELISA cortisol antibody with other steroids is as follows: prednisolone 47.4 %, cortisone 15.7 %, 11-deoxycortisol 15.0 %, prednisone 7.83 %, corticosterone 4.81 %, 6-hydroxycortisol 1.37 %, 17- hydroxyprogesterone 1.36 %, and deoxycorticosterone 0.94 %. Oxytocin concentrations from SC and SSB samples were determined by using competitive EIA kits (Arbor Assays®, Ann Arbor, USA). The cross-reactivity reported by the manufacturer is as follows: Oxytocin 100 %, isotocin 94.3 %, mesotocin 88.4 %, Lys8-Vasopressin 0.14 %, Arg8-Vasotocin 0.13 % and Arg8-Vasopressin 0.12 %.

In line with Reimers and Lamb's (1991) EIA validation criteria, our assays' accuracy, precision, specificity, and sensitivity were rigorously evaluated using 80 pooled SSB samples. Specificity was assessed through dilution tests (1:1, 1:2, 1:5, 1:10 with EIA buffer), accuracy via spike-and-recovery tests, precision by calculating intra-assay and inter-assay coefficients of variation (CV), and sensitivity by determining the lowest detectable hormone concentration.

2.7. Statistical analysis

All analyses were done using R (version 4.3.3, R Core Team, 2024) used All the values were presented as mean ± SD. A p-value <0.05 was considered for significance. Prior to analysis, a Shapiro-Wilk test was performed to check for normality of all data, and Levene's test was used to assess the homogeneity of variances. Data that did not follow a normal distribution were transformed using square root and logarithmic (log 10) transformations, and normality was re-evaluated. If normality was not improved, non-parametric tests were applied for the analysis.

2.7.1. Assays validation

Correlations of the dilution tests (expected values and obtained values) were determined using Pearson's correlation tests, performed with the 'cor.test' function from the 'stats' package.

2.7.2. Methodological aspects

Spearman's rank correlation coefficients, performed with the 'cor.test' function from the 'stats' package, were used to evaluate the relationships between SC and SSB cortisol concentrations, as well as between SC and SSB oxytocin concentrations. Considering the high correlation found between SC and SSB cortisol concentrations and the incomplete availability of SC samples for all individuals, subsequent analyses of cortisol were conducted using only SSB results.

Generalized Linear Mixed Effect Models (GLMMs) were used to examine SSB cortisol and both SC and SSB oxytocin concentrations across body locations, considering the repeated measurements from the same individuals, which were treated as random effects (Bolker et al., 2009). A log link function was applied to transform the response variables, allowing them to better meet the requirements of the model. The 'glmer' function from the 'lme4' package was used to fit GLMMs with a gamma distribution, addressing the right skew in the concentration data for both hormones. When significant differences were detected, post hoc pairwise comparisons were conducted using the 'contrast' function from the 'emmeans' package, applying Bonferroni adjustments.

Finally, Spearman's rank correlation coefficients, performed with the 'cor.test' function from the 'stats' package, were used to assess the relationships between SSB and blubber cortisol concentrations, SSB and serum cortisol concentrations, as well as the correlations between SC cortisol and oxytocin concentrations, and SSB cortisol and oxytocin concentrations.

2.7.3. Epidermal cortisol and oxytocin concentrations as a function of cause of death and biological features

Linear regression models were used to examine the effects of various predictors on SSB cortisol and SC and SSB oxytocin in the studied individuals. The predictors included emaciation, presumed cause of death ('PUE' or 'Distress associated'), sex, age category, and season. Both SSB cortisol and oxytocin concentrations values were log-transformed to meet the assumptions of normality and homoscedasticity. The linear regression model was fitted using the 'lm' function from the 'stats' package, with stepwise backward elimination performed using the 'stepAIC' function from the 'MASS' package to identify the most explanatory variables for the final model. Model fit was assessed using Akaike's Information Criterion (AIC), with the model having the lowest AIC value selected for further interpretation.

Model assumptions were checked using diagnostic plots, and the normality of residuals was confirmed by the Shapiro-Wilk test using the 'shapiro.test' function from the 'stats' package. Multicollinearity was assessed using the variance inflation factor (VIF), calculated with the 'vif' function from the 'car' package, and was found to be negligible. The 95 % confidence intervals for the final model parameters were calculated with the 'confint' function from the 'stats' package, and likelihood-ratio tests (LRT) were performed with the 'lrtest' function from the 'lmtest' package. Additionally, Mann-Whitney U tests, performed with the 'wilcox.test' function from the 'stats' package, were conducted to compare the SSB cortisol concentrations between emaciated individuals and those in good condition, as well as between individuals categorized under 'PUE' and those with 'Distress associated' causes of death, as specified before.

2.7.4. Epidermal cortisol and oxytocin concentrations in relation to the duration and severity of welfare compromise

Mann-Whitney U tests were employed to examine differences in SSB cortisol, and SC and SSB oxytocin levels, between individuals with and without specific markers suggesting impaired welfare, due to the non-normal distribution of the cortisol concentration data. To explore the correlations between welfare compromise severity scores and SSB cortisol, and SC and SSB oxytocin levels, Spearman correlation analysis were conducted. Further, the effect of welfare compromise severity on SSB cortisol, and SC and SSB oxytocin levels, was evaluated using Kruskal-Wallis tests using the 'kruskal.test' function from the 'stats' package. The differences in on SSB cortisol, and SC and SSB oxytocin levels, among short-term, medium-term, and long-term welfare compromise durations were also analyzed using the Kruskal-Wallis test.

3. Results

3.1. Assay validation

For SSB cortisol, the mean intra- and inter-assay coefficients of variation determined were 6.22 % and 5.24 %, respectively, indicating high repeatability of the test. In the linearity of the dilution, the obtained cortisol concentrations were strongly correlated with the expected cortisol values (Pearson: $r(3) = 0.99$, $p = 0.007$; Fig. S1). The average recovery percentage from the spike recovery test was 108.12 ± 8.12 %. The assay sensitivity was 0.02 ng of cortisol / mL of extract.

For SSB oxytocin, the mean intra- and inter-assay coefficients of variation were 7.59 % and 10.16 %, respectively, indicating good repeatability of the test. In the linearity of the dilution, the obtained cortisol concentrations were strongly correlated with the expected cortisol values (Pearson: $r(3) \geq 0.999$, $p < 0.001$; Fig. S1). The average recovery percentage from the spike recovery test was 107.66 ± 6.9 %. The assay sensitivity was 13.04 ng of oxytocin / mL of extract.

3.2. Methodological aspects

A significant positive correlation was identified between the cortisol concentrations in the SC and the SSB (Spearman: r(32) = 0.939, p < 0.001; Fig. 3). Considering this correlation and the incomplete availability of SC samples for all individuals, subsequent analyses of cortisol were conducted using only SSB results. Notably, excluding the two outliers (Fig. 3) from the analysis did not substantially impact the correlation (Spearman: r(30) = 0.92, p < 0.001). Contrarily, no significant correlation was identified between the oxytocin concentrations in the SC and the SSB (Spearman: r(32) = 0.036, $p = 0.835$).

Further statistical analysis indicated a marginally significant difference in absolute cortisol levels between SC and SSB (Wilcoxon signed-rank test: V = 196, $p = 0.051$; Fig. 4). Moreover, a significant difference was observed in oxytocin levels between SC and SSB (Wilcoxon signed-rank test: V = 21, p < 0.001; Fig. 4).

The model for SSB cortisol concentrations (AIC = 180.3; df = 44) indicated significant differences based on body location. Specifically, the ventral body location showed significantly lower cortisol concentrations compared to the other locations (Estimate = −0.201, $p = 0.013$; Fig. 5; Table S2). Post hoc pairwise comparisons showed significantly lower cortisol concentrations in ventral samples compared to dorsal samples ($p = 0.032$; Table S5). Additionally, the model revealed considerable variance among individuals in SSB cortisol concentrations (Variance = 0.626, SD = 0.791).

The model for SC oxytocin concentrations (AIC = 183.4; df = 34) did not indicate significant differences based on body location (Table S3). Furthermore, the model revealed some variance among individuals in SC oxytocin concentrations, although the individual random effect had a relatively low variance (Variance = 0.004, SD = 0.064). Contrary to SC oxytocin, the model for SBB oxytocin concentrations (AIC = 610.0; df = 43) identified significant differences in one of the analyzed locations (Table S4). Specifically, only the cranial body location showed lower cortisol concentrations (Estimate = −1.104; $p = 0.010$; Fig. 6). However, post hoc pairwise comparisons did not reveal any significant differences (Table S5). In addition, the SBB model demonstrated considerable variance among individuals, showing a higher variance compared to the SC model (Variance = 0.05; SD = 0.223).

Results from the correlation of epidermal cortisol concentrations with those in other tissues revealed a significant positive correlation between SSB cortisol concentrations (Fig. 4) and blubber cortisol concentrations (26.26 ± 23.55 ng/g; Spearman: r(40) = 0.871, $p < 0.001$; Fig. 7). Furthermore, a significant positive correlation was observed between SSB cortisol concentrations (Fig. 4) and serum cortisol concentrations (52.63 ± 37.42 ng/g; Spearman: r(26) = 0.813, p < 0.001; Fig. 7).

Finally, the Spearman correlation analysis revealed different relationships between cortisol and oxytocin across the two distinct layers. For the SC, although result suggested a tendency for cortisol and oxytocin to increase together, the correlation was not statistically significant (rho(32) = 0.253, $p = 0.142$). For the SSB, the Spearman correlation coefficient was much lower (rho(47) = 0.011, $p = 0.939$), indicating no relationship between SSB cortisol and oxytocin levels.

3.3. Epidermal cortisol and oxytocin concentrations as a function of cause of death and biological features

For SSB cortisol, the final reduced linear regression model was significant (p < 0.001), with an adjusted R^2 of 0.667 (Table S6). The model retained emaciation and presumed cause of death as significant predictors. The Shapiro-Wilk test confirmed the normality of the residuals (W = 0.966, $p = 0.185$). Sex, age category, and season therefore did not contribute to the explanation of the variability in the SSB cortisol concentrations. All predictors had VIF values less than 2, indicating no significant multicollinearity. The likelihood-ratio test indicated that the final model significantly improved the fit to the data (Chisq = 54.853, $p < 0.001$).

Specifically, emaciated individuals had SSB cortisol values almost six times as high as those measured in individuals in good condition (Mann-Whitney U test: W(46) = 104, p < 0.001; Fig. 8). Individuals categorized with 'Distress associated' causes of death had SSB cortisol values 14 times as high as those measured in individuals categorized under 'PUE' (Mann-Whitney U test: W(46) = 6, $p < 0.001$; Fig. 8).

For SC oxytocin, the analysis showed that none of the predictors significantly explained the variability in log-transformed SC oxytocin concentrations (Table S7). The adjusted R^2 of the model was negative, suggesting that the model did not explain any variability in the data. The Shapiro-Wilk test confirmed the normality of the residuals in the final model (W = 0.992, $p = 0.995$). The likelihood-ratio test indicated that the final model did not significantly improve the fit to the data (Chisq =

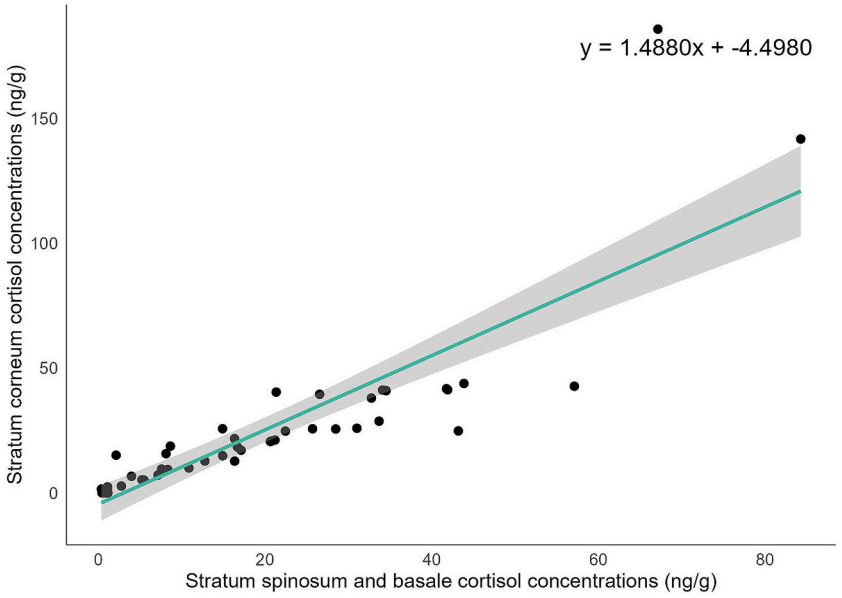

Fig. 3. Correlation between stratum spinosum and basale and stratum corneum cortisol concentrations (Spearman: r(32) = 0.939, p < 0.001). The potential asymptotic trend at higher concentrations warrants further investigation. Shaded areas indicate the 95 % confidence intervals.

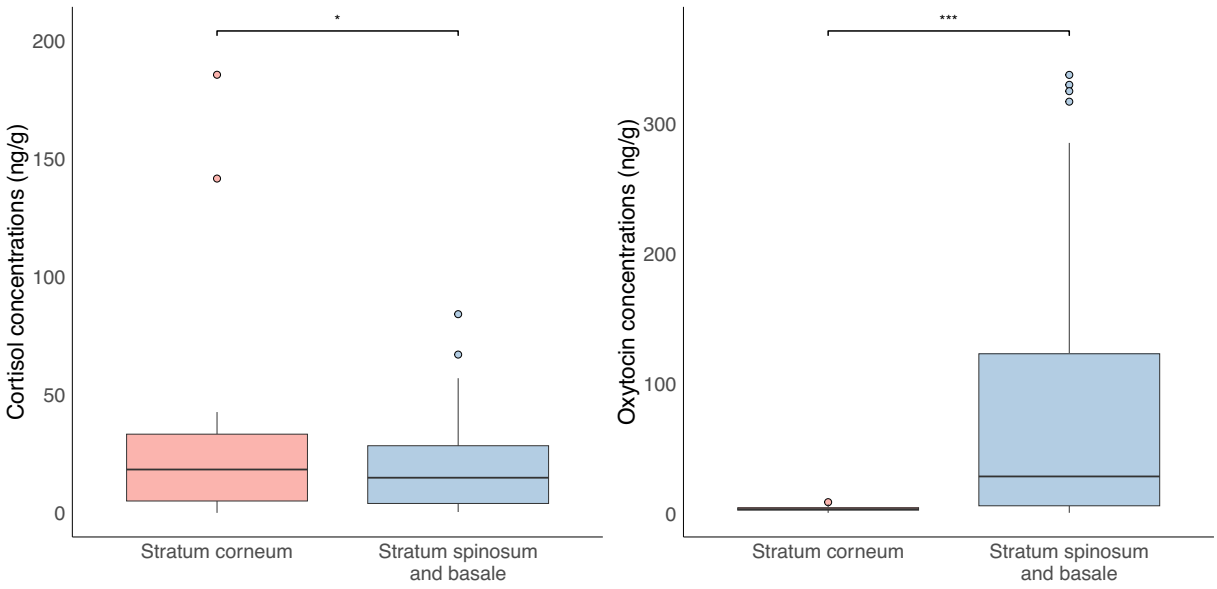

Fig. 4. Cortisol and oxytocin concentrations in stratum corneum ($N = 34$) and stratum spinosum and basale (SN = 49) layers. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Asterisks indicate statistically significant differences between the layers based on the Wilcoxon signed-rank test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Note: The difference in sample sizes is due to stratum corneum sampling yielding sufficient mass (≥15 mg) in 69.39 % of attempts.

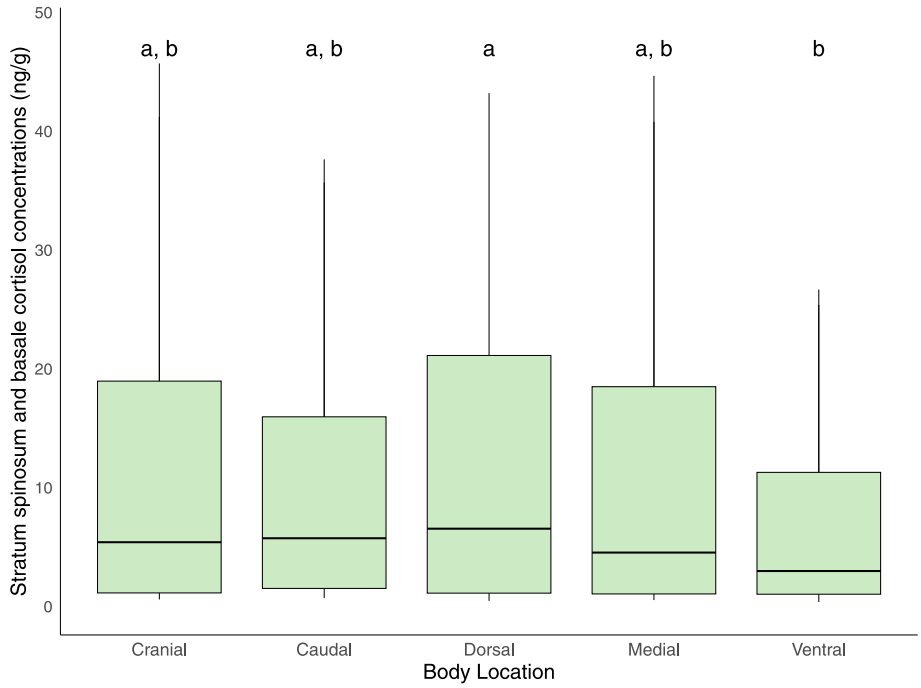

Fig. 5. Stratum spinosum and basale (SSB) cortisol concentrations across different body locations. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Different letters indicate significant differences between body locations based on pairwise comparisons ($p < 0.05$).

0, $p = 1$). Similarly, no predictors significantly explained the variability in log-transformed SSB oxytocin concentrations (Table S8). The model had a negative adjusted R^2, indicating no explanatory power. Residuals deviated slightly from normality (Shapiro-Wilk: $W = 0.935$, $p = 0.009$). Emaciation showed a trend toward significance ($p = 0.073$) but was not statistically meaningful.

3.4. Epidermal cortisol and oxytocin concentrations in relation to the duration and severity of welfare compromise

The Mann-Whitney *U* tests revealed several significant relationships between the presence of markers suggesting impaired welfare and SSB cortisol levels. Specifically, presence of 'Chronic trauma or injury', 'Chronic functional or health impairment', 'Emaciated body condition', and 'Mother separation', and absence of 'Peracute underwater entrapment' showed significant or marginally significant ('Mother separation')

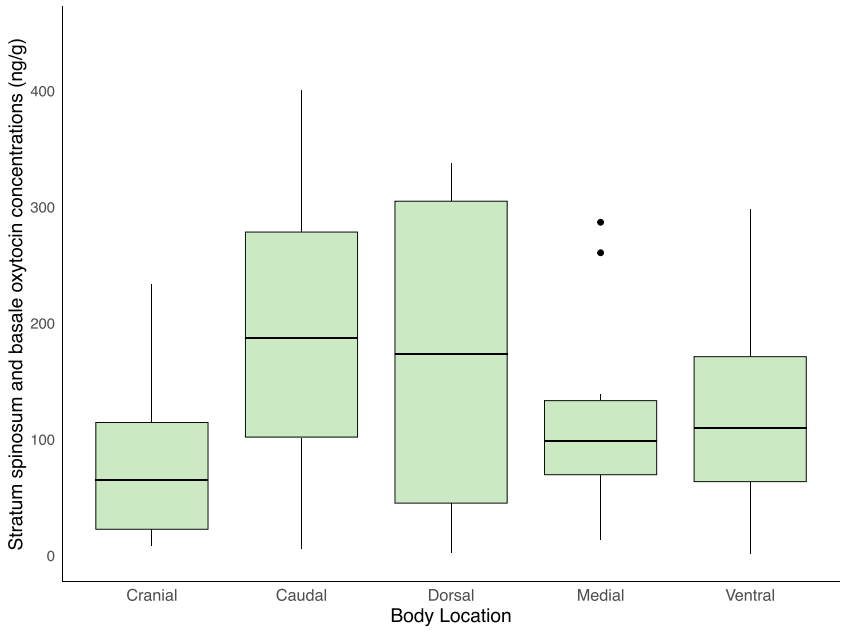

Fig. 6. Stratum spinosum and basale (SSB) oxytocin concentrations across different body locations. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Post hoc pairwise comparisons did not reveal significant differences between body locations.

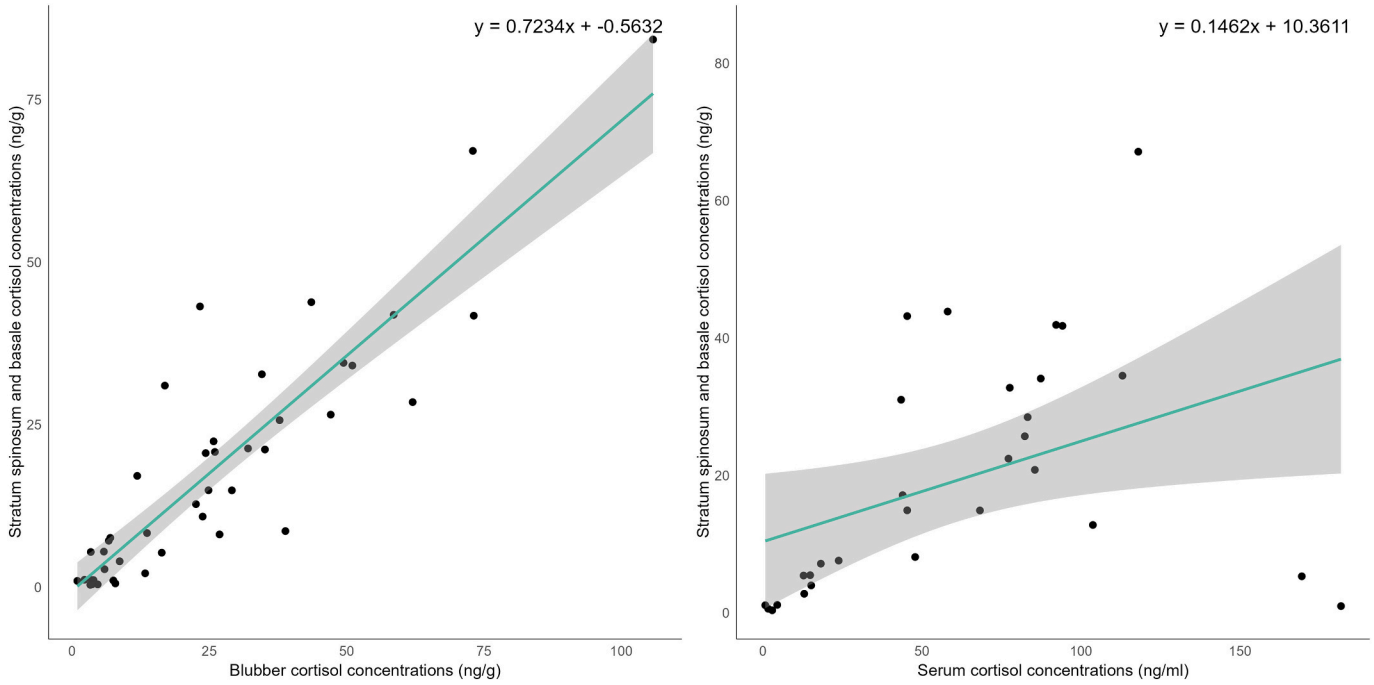

Fig. 7. Correlation between stratum spinosum and basale (SSB) cortisol concentrations and blubber and serum cortisol concentrations. Left: SSB cortisol concentrations (18.56 ± 18.94 ng/g) and blubber cortisol concentrations (26.26 ± 23.55 ng/g; Spearman: $r(40) = 0.871$, $p < 0.001$). Right: SSB cortisol concentrations (27.84 ± 18.94 ng/g) and serum cortisol concentrations (52.63 ± 37.42 ng/g; Spearman: $r(26) = 0.813$, $p < 0.001$).

associations with increased cortisol levels. In contrast, the markers 'Acute trauma or injury' and 'Acute or subacute functional or health impairment' did not show a significant relationship with SSB cortisol concentrations (Table S9).

The Spearman correlation analysis indicated a significant positive correlation between the welfare compromise severity score and SSB cortisol levels (rho = 0.7, $p < 0.001$). The Kruskal-Wallis test further confirmed the effect of the welfare compromise severity score on skin cortisol levels ($\chi^2(3) = 25.184$, $p < 0.001$), demonstrating a significant difference in cortisol concentrations across different levels of welfare compromise severity (Fig. 9).

Finally, the Kruskal-Wallis test for differences in SSB skin cortisol levels among short-term, medium-term, and long-term welfare compromise (duration of the welfare compromise) revealed significant differences ($\chi^2(2) = 2$, $p < 0.001$), with the highest levels observed in the long-term category (Fig. 10).

For SC oxytocin, the Mann-Whitney U tests showed that individuals presenting 'Acute trauma or injury' had significantly higher oxytocin

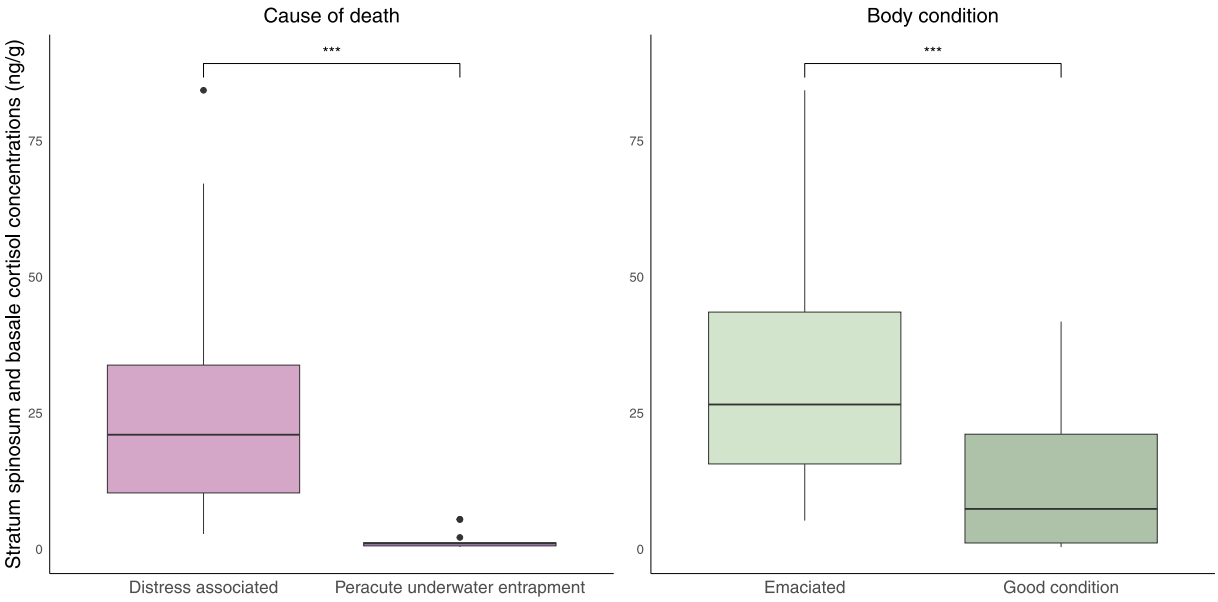

Fig. 8. Stratum spinosum and basale cortisol concentrations in emaciated individuals and those in good condition (left), and in individuals categorized by different causes of death (right). Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Asterisks indicate statistically significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

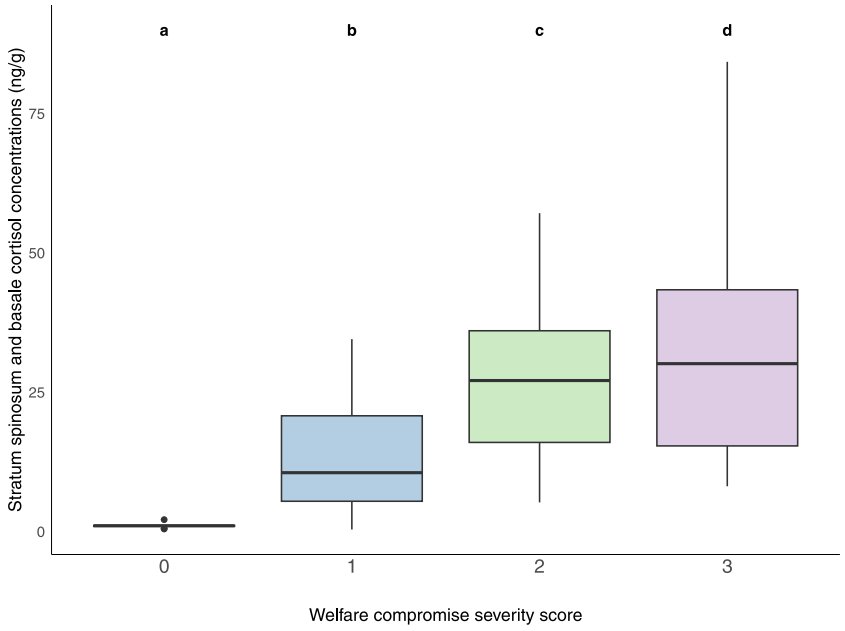

Fig. 9. Stratum spinosum and basale cortisol concentrations across different levels of welfare compromise severity score. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Different letters indicate statistically significant differences between severity scores.

levels (5.25 ± 1.70 ng/g) compared to those not presenting it (3.91 ± 1.77 ng/g, $p = 0.047$; Table S10). For SSB oxytocin, individuals presenting 'Chronic trauma or injury' had significantly lower levels (11.1 ± 12.31 ng/g) compared to those not presenting it (91 ± 104.01 ng/g, $p = 0.05$; Table S11). Additionally, individuals presenting 'Mother separation' had marginally higher SSB oxytocin levels (150.51 ± 110.53 ng/g) compared to those not presenting it (6.22 ± 2.40 ng/g, $p = 0.055$; Table S11).

The spearman correlation analysis for oxytocin levels indicated no significant correlation between the welfare compromise severity score, either for SC oxytocin (rho = 0.117, $p = 0.501$) or for SSB oxytocin (rho = 0.072, $p = 0.621$) levels. Similarly, the Kruskal-Wallis tests for both SC oxytocin ($\chi^2(2) = 1.118$, $p = 0.572$) and SSB oxytocin ($\chi^2(2) = 0.618$, $p = 0.734$) did not reveal significant differences in hormone concentrations across different welfare compromise severity scores. Finally, the Kruskal-Wallis tests for both SC oxytocin ($\chi^2(2) = 2.450$, $p = 0.294$) and SSB oxytocin ($\chi^2(2) = 1.145$, $p = 0.564$) did not reveal significant differences in hormone concentrations across different durations of welfare compromise.

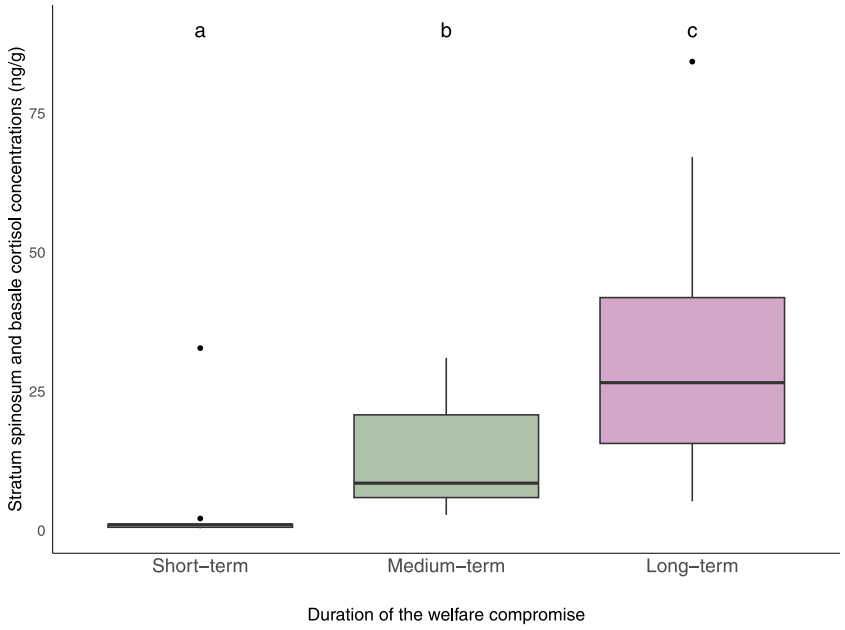

Fig. 10. Stratum spinosum and basale cortisol concentrations across different durations of welfare compromise. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Different letters indicate statistically significant differences between durations.

4. Discussion

4.1. Assay validation

The validation procedure demonstrated that the epidermis of striped dolphins contains detectable concentrations of cortisol and oxytocin, measurable with the methodology employed here, i.e. an EIA kit not specifically intended for epidermal or cetacean samples.

For SSB cortisol, intra-assay CVs confirmed good repeatability within assays, and the inter-assay CVs indicated consistent repeatability across different assays. Spike-and-recovery tests confirmed the successful recovery of various cortisol concentrations, suggesting minimal interference from other sample components. Additionally, serial dilutions of pooled extracts exhibited dose-dependent binding to assay antibodies, parallel to the assay's standard curves, indicating that other substances within the epidermal matrix did not disrupt cortisol-antibody binding. Similarly, SSB oxytocin validation also displayed good repeatability with low intra- and inter-assay CVs. The spike-and-recovery test also demonstrated the successful recovery of a wide range of oxytocin concentrations, and serial dilutions of pooled extracts exhibited dose-dependent binding to the oxytocin assay's antibodies, paralleling the standard curve. The range of cortisol concentrations observed in healthy striped dolphins' epidermal SC (Fig. 8) aligns closely with previously reported values by Bechshoft et al. (2020) in bottlenose dolphins (0.31 to 16.17 ng cortisol/g of dry SC), suggesting that methodological modifications had low impact on the quantification of the hormone (Agustí et al., 2024).

Nonetheless, the potential for cross-reactivity with uncharacterized cortisol and oxytocin metabolites, or conjugated molecules, which are not specified by the respective manufacturers cannot be entirely excluded (cortisol: Schultheiss et al., 2018; oxytocin: López-Arjona et al., 2021). High-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), as detailed by Bechshoft et al. (2020, 2015), would provide a more accurate and sensitive approach than EIA (Schultheiss et al., 2018). However, due to its cost-effectiveness and lower expertise requirements, EIA remains the method of choice in many research contexts, especially in wildlife studies.

4.2. Methodological aspects

Numerous factors influencing blood hormone levels are likely to be reflected in the concentrations of those hormones found in the epidermis. These factors include individual differences, seasonal variations, sex, age, and life-history stages, among others. Additionally, specific factors related to skin samples, such as the layer of the skin and the location on the body where the sample was collected, should be considered to ensure the results are meaningful and biologically relevant.

A significant and strong positive correlation was identified between cortisol concentrations in the SC and SSB, suggesting that cortisol levels in these two distinct epidermal layers could reflect the same information. Although an asymptotic trend was observed at higher concentrations, driven by two markedly high cortisol values, this did not compromise the overall correlation, which remained strong even after excluding these outliers. Future studies with larger sample sizes are essential to better understand this phenomenon. Moreover, a marginally significant difference in absolute cortisol levels between SC and SSB was found. These variations could potentially be attributed to the distinct physical or biochemical characteristics of each layer, as well as the fact that they may represent different physiological time windows.

Epidermal growth begins in the basal layer, where matrix cells proliferate to renew tissue (Rommel and Lowenstine, 2001). Cells then migrate through the stratum spinosum to the SC before being shed as sloughed epidermis (Geraci et al., 1986), with skin renewal rates estimated at approximately 73 days in bottlenose dolphins (Hicks, 1985). Cortisol is synthesized in the adrenal gland and released into the bloodstream (Chrousos, 1995), and is likely incorporated into skin cells through passive diffusion. Keratinous tissues may thus trap hormones, preserving a record of physiological changes, as shown in studies on whale baleen and earplugs (Hunt et al., 2016; Noël et al., 2014; Trumble et al., 2018). This concept supports the hypothesis that full-depth epidermis reflect cortisol levels accumulated over the epidermal growth period, while stratum basale sections indicate more recent cortisol levels, and the SC reflects older physiological states (Bechshoft et al., 2020). Findings in bottlenose dolphins have shown that cortisol peaks in blood are reflected in the SC with a 45–60 days delay (Bechshoft et al., 2020; Kellar et al., 2018), potentially supporting the

'accumulation' hypothesis and explaining the differences in absolute cortisol levels observed between SSB and SC samples.

If cortisol accumulates in cells during growth, resulting in a time-stratified matrix, cortisol levels in the SC and SSB would not necessarily be correlated. Therefore, the high correlation observed in this study could be attributed to relatively stable physiological states in most individuals during the skin's growth period. However, unlike baleen or earplugs, the epidermis is a metabolically active tissue, which demands caution when interpreting measured hormone concentrations. For example, some studies have questioned the validity of hair cortisol as a long-term marker (Kalliokoski et al., 2019), as it can spike due to acute stress (Cattet et al., 2017; Cattet et al., 2014) and may shift along the hair shaft, converting to other metabolites (Kapoor et al., 2018). Similarly, there is no solid evidence that cortisol remains locked in growing skin (Agustí et al., 2024), supporting an alternative explanation for the high correlation found in this study between SC and SSB cortisol concentrations. Additionally, the epidermis can produce cortisol by expressing elements of the HPA axis (Cirillo and Prime, 2011; Slominski et al., 2007; Zmijewski and Slominski, 2011), so we should consider that our findings likely encompass both adrenal activity and local skin production sources of cortisol.

Nevertheless, our findings suggest both SC and SSB layers are suitable for analyzing cortisol levels in cetacean epidermal samples. Epidermal samples can be collected from stranded animals, tissue banks (Bechshoft et al., 2015), biopsied individuals (Noren and Mocklin, 2011), and during tagging, health assessments, and disentanglement efforts. Additionally, SC samples can be collected from epidermis naturally sloughed off at the sea surface (Engelhaupt, 2004), or from animal's bow riding boats (Harlin et al., 1999). Importantly, ensuring sufficient SC sample mass is crucial, as smaller samples (<15 mg) can increase variability and reduce data reliability (Agustí et al., 2024); however, obtaining adequate sample sizes can sometimes be challenging. Overall, although SC shows promising potential for use, further research is needed to better understand the kinetics of cortisol incorporation and removal in the skin, influencing factors, and the time window represented by SC and SSB.

In contrast to cortisol, no significant correlation was found between SC and SSB oxytocin concentrations. Moreover, oxytocin levels were substantially lower in the SC than in the SSB. These findings suggest that oxytocin distribution may exhibit greater variability, potentially influenced by distinct functional roles of oxytocin and rates of turnover within specific epidermal layers, thereby challenging the validity of using SC as a representative measure of overall epidermal oxytocin levels in live animals.

The effect of body location on hormone concentrations was also examined. For SSB cortisol concentrations significant differences were found, with lower cortisol concentrations observed at the ventral body location compared to dorsal samples. Individual variation also contributed considerably to the observed differences. While SC oxytocin showed no variation across locations, the model for SSB oxytocin indicated lower values at the cranial location, though pairwise comparisons did not confirm these differences. In both cases, minimal individual variation was observed. In the same individuals, blubber cortisol concentrations also differed between body regions (Carbajal et al., 2022), as described previously in other species (Kellar, 2013). In contrast to our findings, Carbajal et al. (2022) found that the dorsal area of the blubber in striped dolphins had higher cortisol levels than the caudal, cranial, and medial regions, while the ventral area had higher cortisol concentrations than the medial region. In bottlenose dolphins, SC cortisol concentrations did not vary significantly across body locations but showed high variability between regions, (Agustí et al., 2024).

Although not finding specific trends as a function of body locations across studies, results suggest that cortisol is not uniformly distributed across dolphin epidermis. The ventral part of cetacean epidermis is typically thicker (Cozzi et al., 2016), and there are regional differences in dermal papilla height (Jones and Pfeiffer, 1994) and melanocyte concentration (Berta et al., 2015; Perrin, 2009). These variations may explain regional differences in cortisol distribution, potentially leading to erroneous results or comparisons between studies. Thus, we recommend sampling the same specific body location within a study.

Neither sex, age category, nor season predicted the concentrations of cortisol and oxytocin in the epidermis, aligning with findings of other studies in dolphins (Agustí et al., 2024; Kellar et al., 2015; St. Aubin et al., 1996). Contrary to our results, some studies have identified sex-related variations (Houser et al., 2021a, 2021b; Mingramm et al., 2019) as well as seasonal fluctuations (Funasaka et al., 2011; Houser et al., 2021a, 2021b) in dolphin's circulating cortisol concentrations. Finally, age-related differences in circulating cortisol concentrations have been noted in dolphins (Houser et al., 2021a, 2021b). These discrepancies between studies may be explained by species-specific differences in hormone regulation, life history traits, or environmental factors such as behavior and food availability. Therefore, further research should continue controlling variables like sex, reproductive state, and all possible relevant factors for accurate hormone assessments.

Finally, although valuable insights into marine mammal physiology have been derived from studies measuring hormones in stranded individuals, the effects of stranding and decomposition on tissue hormone levels are not well understood, potentially leading to inaccurate ecological interpretations (Mello et al., 2017; Trana et al., 2015). Therefore, we suggest that hormone analysis from well-preserved skin of stranded animals is feasible, but further research is needed to assess the effects of post-mortem changes across different preservation states.

4.3. *Validity of epidermal cortisol concentrations as a measure of the HPA activity*

Before being applied as a measure of the HPA activity, it is crucial to validate that cortisol values obtained provide relevant physiological information (Behringer and Deschner, 2017). Validation can be achieved by demonstrating a good correlation between circulating cortisol levels and their concentrations in the corresponding matrix (Touma and Palme, 2005). However, the diurnal rhythms and episodic fluctuations in circulating cortisol levels, coupled with the time delay in cortisol incorporation into the skin, make it challenging to establish a meaningful correlation. Despite this, we still found that epidermal cortisol concentrations were highly correlated with both serum and blubber cortisol concentrations. While there are no previous studies correlating these matrices, significant correlations between other keratinized matrices (e.g. hair) and circulating cortisol concentrations have been documented (Koren et al., 2019; Koren et al., 2006; Tallo-Parra et al., 2017).

As discussed in section 4.2, cortisol likely diffuses from circulation and accumulates into epidermis cells during its growth, potentially resulting in a time-stratified matrix (Agustí et al., 2024; Bechshoft et al., 2020). Consequently, cortisol levels in blood and epidermis may not be always directly correlated. In fact, cortisol peaks in blood and blubber have been detected in the SC with a delay of 45–60 days (Bechshoft et al., 2020). Therefore, the strong correlation of cortisol concentrations observed between blood, blubber and epidermis could be attributed to relatively stable blood cortisol levels over the skin's growth period (Goymann, 2005). These results were unexpected in the case of individuals that died due to PUE, as we could expect an acute increase in circulating cortisol levels due to entrapment that should not be reflected in the skin. However, hypoxia and asphyxiation occur in dolphins within minutes of capture in bottom-set nets (Ijsseldijk et al., 2021; Soulsbury et al., 2008), leaving limited time for cortisol levels to rise significantly during the acute stress response (Agusti et al., 2022), which may explain the low cortisol levels observed.

Physiological validation can also be achieved by comparing cortisol levels in individuals exposed to stressors known to elevate glucocorticoids before and after the event, or against a control group under

baseline conditions (Behringer and Deschner, 2017; Hunt et al., 2013; Touma and Palme, 2005). Accordingly, in our study, 'Distress associated' deaths and emaciation were compared to 'PUE' and higher SSB cortisol values compared to those in good condition. In pinnipeds, extended periods of food restriction have been associated with elevated cortisol concentrations in the bloodstream (Champagne et al., 2012; Guinet et al., 2004). Moreover, low body condition has been related to increased blubber cortisol concentrations in stranded dolphins and porpoises (Agusti et al., 2022; Kershaw et al., 2017). During periods of reduced food intake or fasting, cortisol stimulates lipolysis and enhances gluconeogenesis to maintain energy homeostasis (Bergendahl et al., 1996; Exton et al., 1972), likely resulting in elevated cortisol levels in the skin of striped dolphins in poor body condition.

'Distress-associated' causes of death were linked to higher SSB cortisol levels, whereas individuals classified as 'PUE' exhibited lower SSB cortisol levels. Individuals classified as 'PUE' were thought to suffer an acute stress response before death, but an increase in blood cortisol levels due to entrapment would not be expected to be reflected in the epidermis (Agusti et al., 2022). These individuals showed no signs of disease and maintained a good body condition (Cuvertoret-Sanz et al., 2020; Moore et al., 2013; De Quirós et al., 2018), which would be typically associated with lower cortisol concentrations compared to those with 'Distress-associated' causes of death. 'Distress-associated' deaths involved individuals experiencing an aversive state, including disease, injury, and/or social stress (Moberg and Mench, 2000).

The emergence of different diseases and injuries is typically associated with an enhanced activity of the HPA axis (e.g. Stubsjøen et al., 2015; Corradini et al., 2013; Burnett et al., 2015). During illness, elevated levels of circulating cortisol are crucial for mobilizing stored energy resources to support the immune response, especially under conditions of energetic stress (Silverman et al., 2013). At the same time, glucocorticoids' immunosuppressive properties regulate immune cells and mediators that could become harmful if unregulated (Keller et al., 1991; McEwen et al., 1997). Moreover, in the long term, this suppression can render individuals more susceptible to disease or exacerbate pre-existing distress conditions (Leonard and Miller, 1995; Levine, 1993). Elevated cortisol levels during illness may also be influenced by affective factors such as pain or isolation (Müller and Bossley, 2002). Moreover, young separated from their mothers typically display stress-related physiological responses (e.g. Coe et al., 1978; Pérez-Torres et al., 2016).

Overall, our findings suggest that epidermal cortisol concentrations can reflect differences in circulating cortisol levels in cetaceans and may therefore serve as a valuable sample type for comparative studies. However, to achieve thorough physiological validation, further research is necessary to bolster the evidence and increase sample sizes. Further studies could use adrenocorticotropic hormone (ACTH) challenge to stimulate cortisol release and integration into the matrix matrix (e.g. Heistermann et al., 2006; Wasser et al., 2000), ideally in captive animals, that would also help to determine the lag between endocrine stimulation and hormone appearance in the skin.

4.4. Validity of epidermal cortisol concentrations as a physiological welfare indicator

To validate epidermal cortisol as a physiological welfare indicator, we analyzed the relationship between SSB cortisol concentrations and a range of necropsy markers presumed to reflect negative welfare states. Results showed that SSB cortisol concentrations consistently increased in animals presenting markers suggesting long-term impaired welfare but not in those presenting markers suggesting short-term or medium-term impaired welfare. Furthermore, when categorizing the duration of welfare compromise and analyzing its relationship to SSB cortisol concentrations, the highest levels were observed in individuals experiencing longer term welfare compromise, while the lowest levels were observed in those with short-term welfare compromise. As discussed in section 4.2, this distinction may be attributed to the peripheral nature of the skin matrix, with longer-term welfare compromise potentially leading to greater cortisol accumulation. In contrast, the more recent a welfare compromise is, the less likely it would be detectable using this physiological indicator.

Moreover, results demonstrated a positive relationship between the apparent severity of welfare compromise and epidermal cortisol levels, with individuals apparently experiencing more severe welfare compromise exhibiting higher SSB cortisol concentrations. This suggests that SSB cortisol may serve as a sensitive biomarker, reflecting various gradations in the severity of welfare compromise. Similarly, in dairy cows, hair cortisol was negatively correlated with a welfare estimation score based on nutrition, housing conditions, health status, and behavior (Eerdenburg et al., 2021). In pigs, post-mortem plasma lactate and creatine kinase activity positively correlated with welfare indices based on behavioral, clinical, and physiological measures (Brandt et al., 2015). And in horses, a significant negative relationship was found between neutrophil:lymphocyte (N:L) ratio and individual welfare score, assessed by a protocol that included health and behavioral parameters (Popescu and Diugan, 2017). However, a significant limitation of our study is that, due to its necropsy-based nature, only available markers from stranded dolphins were used, limiting the assessment to specific welfare aspects and domains. Furthermore, aggregating welfare indicators entails challenges, as combining measures can reduce accuracy due to varying validity and relevance (Sandøe et al., 2019).

Overall, building on the recommendations of Browning (2023) and Beaulieu (2024) for validating welfare indicators, our results suggest that elevated SSB cortisol concentrations may be a reliable indicator of negative welfare states in striped dolphins, within a specific time window ranging from hours to months. Firstly, a range of conditions observed in striped dolphins, mainly related to chronic health or nutritional problems, were assumed to be associated with negative affective states and were therefore identified as markers of impaired welfare. Secondly, the presence of necropsy markers suggesting long-term impaired welfare was linked to higher SSB cortisol values. Finally, the rise in SSB cortisol levels appeared consistent across various markers suggesting long-term impaired welfare, with higher concentrations observed as both the apparent severity and duration of the welfare compromise increased.

However, it is important to note that while some conditions may have elevated epidermal cortisol by influencing affective states, others likely increased cortisol by impacting different physiological processes— such as immune function or the metabolic response to starvation (Beaulieu, 2024). This complexity arises from the intricate interplay between affective states and peripheral physiological markers, which is far more complex than the ideal of a direct, causal link, due to constant interactions between various physiological functions (Armstrong and Boonekamp, 2023; Costantini, 2022; Heidinger et al., 2022). Therefore, to further investigate the validity of SSB cortisol concentrations as a welfare indicator, it would be worthwhile conducting additional studies on healthy individuals exposed to welfare-impairing conditions that have less effects on homeostasis (e.g., boredom, frustration, isolation).

4.5. Validity of epidermal oxytocin concentrations as a measure of the oxytocin system and as a physiological welfare indicator

The design of this study did not allow for a proper physiological validation of epidermal oxytocin concentrations as a measure of the oxytocin system in striped dolphins. First, circulating oxytocin levels were not measured, which limits direct comparisons between blood and skin concentrations. Moreover, the mechanisms by which oxytocin transfer from blood into the skin remain unclear. Unlike lipophilic steroid hormones, oxytocin's peptide structure, being larger and lipid-insoluble, likely limits its diffusion and deposition in the skin (Quintana et al., 2018). This may explain why studies in other species report weak correlations between oxytocin concentrations in plasma and those in saliva or urine (Feldman et al., 2011; Horvat-Gordon et al.,

2005; Martins et al., 2020). Furthermore, oxytocin and its receptors are also produced in epidermal keratinocytes and dermal fibroblasts, and the oxytocin system plays a role in modulating the HPA axis (Deing et al., 2013). Thus, measuring oxytocin in non-plasma matrices may not provide a reliable reflection of current circulating oxytocin levels, but could still reflect certain aspects of the oxytocin system.

Neither emaciation nor presumed cause of death predicted SC oxytocin levels. In contrast, emaciated individuals had higher SSB oxytocin concentrations compared to those in good condition, though this difference was not statistically significant. The relationship between oxytocin, health and markers of energy homeostasis is not well understood. However, our findings differ from other studies, which have shown higher plasma oxytocin levels correlate with better body condition scores (Schorr et al., 2017; Skinner et al., 2019; Szulc et al., 2016), likely due to oxytocin decreasing as a physiological adaptation to stimulate caloric intake and conserve energy (Lawson, 2024). Additionally, SSB oxytocin was significantly lower in individuals with chronic trauma or injury, and higher in calves presumably separated from their mothers, approaching statistical significance. However, overall results do not support the validity of skin oxytocin concentrations as a reliable indicator of welfare.

Although oxytocin is often signaled as an indicator of positive welfare, its relationship with positive affective states remains unclear. Oxytocin is also mobilized in response to non-social activities like exercise and eating (Mitsui et al., 2011), as well as non-social stressors (Nishioka et al., 1998; Olff et al., 2013). Indeed, during a homeostatic challenge, the release of glucocorticoids is accompanied by the production of oxytocin, which plays a key role in reducing anxiety and promoting emotional regulation (Beaulieu, 2024). However, in our study, no significant relationship was observed between oxytocin and cortisol concentrations in either skin layers. Generally, oxytocin is believed to have evolved as part of social coping mechanisms (Buisman-Pijlman et al., 2014; Cavanaugh et al., 2016), and rather than indicating strictly positive or negative states, oxytocin's role appears to be adaptive. Its effects are thought to be primarily influenced by environmental and social conditions (Tops et al., 2014), which, in this study, were not known.

Finally, we cannot rule out the possibility that oxytocin measurements may be artefactual, given the challenges and discrepancies often reported in studies, particularly related to sample extraction methods (Bienboire-Frosini et al., 2024). For instance, certain extraction protocols may generate oxytocin fragments, which can reduce recovery rates and lead to underestimation of oxytocin concentrations. Furthermore, these fragments may interfere with immunoassays, potentially resulting in inaccurate measurements (Bienboire-Frosini et al., 2024). To overcome these issues, future studies should address these methodological challenges and aim toward defining the kinetics of oxytocin incorporation, production, and clearance in both plasma and skin. Additionally, variability in oxytocin secretion should be explored in animals in different contexts and welfare conditions, with a focus on aspects like social bonding, environmental motivation, or stress relief.

5. Conclusion

In conclusion, this study validated the measurement of cortisol and oxytocin in the epidermis of striped dolphins using enzyme immunoassays, with cortisol proving to be a reliable biomarker of the HPA axis and welfare in these animals. The findings demonstrated good assay repeatability, with cortisol levels in both the SC and SSB layers correlating strongly with serum and blubber concentrations. Furthermore, cortisol levels in the epidermis were linked to markers of long-term impaired welfare, reflecting both the severity and duration of negative welfare states. However, the study also revealed that oxytocin measurements in the epidermis were less reliable, likely due to methodological limitations. The observed oxytocin concentrations in epidermis did not consistently correlate with markers of impaired welfare, nor were they associated with cortisol levels. These findings highlight the need for improved extraction techniques and a deeper understanding of oxytocin kinetics in cetacean skin. Advancing methodological and physiological validations will be crucial for enhancing our understanding of cetacean welfare in the face of growing anthropogenic and environmental challenges.

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CRediT authorship contribution statement

Clara Agustí: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laia Guix:** Writing – original draft, Methodology, Investigation, Formal analysis. **Annaïs Carbajal:** Writing – review & editing, Resources, Methodology, Investigation. **Mariano Domingo:** Writing – review & editing, Resources. **Manel López-Béjar:** Writing – review & editing, Resources. **Xavier Manteca:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition. **Oriol Talló-Parra:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111793.

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