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Research Article

Tracking epidermal cortisol and oxytocin in managed killer whales as potential non-invasive physiological welfare indicators

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ABSTRACT

Growing public concern regarding cetacean welfare has emphasized the need for developing robust, science-based tools for welfare assessment applicable across diverse species and settings. Within this context, in a broader effort to advance the validation of novel physiological indicators for monitoring cetacean welfare, this study optimized a non-invasive epidermal sampling method in killer whales (*Orcinus orca*; $N = 5$) and validated AlphaLISA immunoassays for quantifying epidermal cortisol (ECC) and oxytocin (EOC) concentrations. Analysis of body location as a potential confounding factor revealed notable intra-individual variability and lateral asymmetry in hormone concentrations, highlighting the importance of standardized sampling procedures. Significant associations emerged between both hormones and negative welfare indicators, while EOC was also linked to positive behavioral engagement and body weight variation. When considered alongside previous findings, ECC may represent a reliable and feasible biomarker for assessing retrospective, intermediate-term welfare changes in killer whales, particularly when embedded within a broader, multifactorial framework that integrates complementary indicators. By contrast, EOC remains less reliable at this stage, largely due to the limited understanding of the oxytocinergic system and its involvement in both positive and negative affective states. Future research involving larger populations, detailed welfare assessments, and improved understanding of epidermal hormone incorporation dynamics will enhance the practical utility of ECC and EOC as welfare biomarkers in cetaceans.

1. Introduction

Marine ecosystems worldwide are under escalating pressure from anthropogenic activities and climate change, posing critical challenges for the conservation of marine biodiversity (Ramírez et al. 2017). These challenges highlight the urgent need for a robust, scientifically rigorous foundation to understand the impacts of multiple stressors on cetaceans and to assess and improve their welfare. Killer whales (*Orcinus orca*) are particularly susceptible to a range of anthropogenic threats, such as interactions with fisheries (Muto et al., 2017), chemical pollution (Jepson et al., 2016), and noise disturbance (Noren et al., 2009). Moreover, public concern has underscored the ethical dilemmas of housing killer whales under human care, placing the species at the center of animal welfare debates (Bekoff, 2009; Rose and Parsons, 2019). While environmental management, feeding protocols, and veterinary care can enhance certain aspects of cetacean welfare (Almunia and Canchal, 2025; Browning and Veit, 2021), important limitations persist, most notably in the complexity of enclosures, opportunities for

Abbreviations: ACTH, adrenocorticotropic hormone; ECC, Epidermal Cortisol Concentrations; EOC, Epidermal Oxytocin Concentrations; HPA, hypothalamic–pituitary–adrenal axis; KW, Kruskal–Wallis.

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natural foraging, richness of social interactions, and the degree of behavioral choice and control (Brando et al., 2018; Tyack, 2009).

These pressures, together with advances in animal welfare science, have led researchers, institutions, and policymakers to increasingly rely on welfare metrics to guide management decisions and inform policy development (Kagan et al., 2015; Miller et al., 2011). For example, in managed settings, welfare assessment can help define optimal environmental conditions and improve husbandry practices (Clegg et al., 2015; Miller et al., 2021). In the wild, it can support the development of effective conservation strategies and provide tools to evaluate their success (Harvey et al., 2020; Papastavrou et al., 2017). Nevertheless, substantial knowledge gaps persist in defining and assessing killer whale welfare, both under humane care and in the wild (Dierauf and Gaydos, 2018), highlighting the need to develop reliable welfare measures for this species (Avila et al., 2018; Clegg, 2021; Whitham and Wielebnowski, 2013).

Animal welfare science seeks to interpret, in an integrated manner, indicators of biological function, behavior, and environment measured at a given point in time or over a defined period, in order to understand the mental experiences that these indicators are likely to reflect (Beausoleil et al., 2018). Among the diverse approaches to welfare assessment, physiological markers related to health and affective state are often used (Broom and Johnson, 2019; Moberg, 1985). Exposure to stressful stimuli triggers a cascade within the hypothalamic–pituitary–adrenal (HPA) axis: corticotropin-releasing hormone (CRH) prompts the secretion of adrenocorticotropic hormone (ACTH; Chrousos and Kino, 2007), which in turn heightens cortisol synthesis in the adrenal cortex (Serova et al., 2008). Cortisol facilitates adaptive responses by mobilizing metabolic resources in response to acute stress (Sapolsky et al., 2000) while regulating immunity, cardiovascular function, and tissue repair (Ralph and Tilbrook, 2016). However, prolonged or excessive activation, commonly referred to as chronic stress, can disrupt biological functions such as reproduction, immunity, and growth (Atkinson et al., 2015).

Attention is increasingly shifting toward identifying physiological markers that capture positive welfare, such as oxytocin (Rault et al., 2017). This neuropeptide, produced in the hypothalamus and released by the posterior pituitary, is known for its roles in reproduction, lactation, and maternal behavior (Hruby et al., 1990; Lee et al., 2009). Beyond these functions, oxytocin promotes social bonding, reduces aggressive behavior, enhances cognition, and buffers stress in mammals (Burkhart et al., 2022; Insel, 2010; Rault et al., 2017; Ross and Young, 2009). In cetaceans, which form complex social bonds that extend beyond reproduction (Gerber et al., 2022; Seyfarth and Cheney, 2012), measuring peripheral oxytocin may offer insights into their welfare, particularly into aspects related to social and environmental motivation, as suggested in other species (Crockford et al., 2014; Leeds, 2019).

To date, cortisol in killer whales has been measured using matrices such as blood, urine, and feces, each reflecting endocrine activity over different timescales (Steinman et al., 2021; Suzuki et al., 2003; Yehle, 2022; Zaccaroni et al., 2011). In other species, saliva, respiratory droplets, and blubber have also been used (Agustí et al., 2022; Burgess et al., 2018; Champagne et al., 2018; Pedernera-Romano et al., 2006), and recently, keratinous tissues, such as baleen plates, skin or earplugs, have gained attention for capturing hormone levels over extended timeframes (Bechshoft et al., 2015; Hunt et al., 2014; Trumble et al., 2018). Cetacean epidermis, in particular, is characterized by a thick epidermis with a pronounced renewal rate (Harrison and Thurley, 1974), comprising the *stratum basale*, *stratum spinosum*, and *stratum corneum* (Hicks et al., 1985).

In epidermis, cortisol's lipophilic steroid structure may facilitate its diffusion from circulation into cell membranes and its incorporation into the lipid-rich matrix of developing keratinocytes, potentially allowing epidermal cortisol to integrate circulating cortisol signals over time and capture longer-term changes in HPA axis activation (Bechshoft et al., 2020; Henderson, 1993; Wong et al., 2023). Additionally, the epidermis is itself capable of synthesizing and releasing cortisol (Cirillo and Prime, 2011). Together, these properties make epidermal tissue a more accessible, less invasive matrix that may also integrate physiological activity over longer time periods because of its continuous growth.

Oxytocin is a small neuropeptide with limited lipid solubility and a short half-life, making its movement into and persistence within peripheral tissues difficult to interpret. Although passive diffusion across the epidermis is unlikely on the basis of these physicochemical constraints (Quintana et al., 2018), several biologically plausible pathways may account for measurable oxytocin in skin samples. First, circulating oxytocin could reach the epidermis through passive or facilitated transfer from dermal capillaries, particularly if stabilized by carrier proteins or packaged into extracellular vesicles, which can prolong peptide availability (Gröschl, 2008). Second, similar to cortisol, oxytocin may be synthesized locally by epidermal cell types, as keratinocyte-derived oxytocin has been documented in human skin (Deing et al., 2013). Third, oxytocin (either free or bound) has been detected in keratinized matrices such as hair and nails (López-Arjona et al., 2021), and more recently in the epidermis of cetaceans (Agustí et al., 2025a; Agustí et al., 2025b), demonstrating that it can be present in stratified keratinized tissues.

Measuring epidermal cortisol (ECC) and oxytocin (EOC) represents a promising, though still exploratory, approach for evaluating cetacean physiology, welfare and the potential impacts of multiple stressors. While this method is often described as non-invasive or minimally invasive, it still involves direct contact with the animal. However, it is considerably less invasive than standard alternatives such as blood collection or blubber biopsies. In managed settings, where other sample substrates (e.g., blood, urine, saliva, or blubber) are technically accessible, epidermal sampling offers a complementary approach that can be collected repeatedly with minimal disruption to routine management or animal welfare (Agustí et al., 2025b).

As with any emerging matrix, rigorous methodological (i.e., ensuring reliable extraction and assay performance) and physiological (i.e., demonstrating that epidermal concentrations bear a consistent relationship to endocrine activity) validations are essential before these results can be used to support welfare interpretations (Hunt et al., 2013; Koren et al., 2019). Ideally, physiological validation would involve controlled endocrine challenges, such as ACTH stimulation tests, which provide the most robust evidence that circulating glucocorticoid fluctuations are mirrored in epidermal tissue. However, these procedures are invasive, ethically challenging, and generally nor feasible in marine mammals. Consequently, alternative approaches, such as assessing correlations between epidermal hormones and welfare-related conditions (e.g. health or behavioral states) expected to influence welfare in similar ways, offer a practical and ethically acceptable route for preliminary validation (Beaulieu, 2024; Browning, 2023). These welfare-related conditions, which can themselves serve as welfare indicators, can be systematically organized within established evaluative frameworks. The Five Domains Model provides one such structure, categorizing relevant factors across nutritional, environmental, health, and behavioral domains, each contributing to the animal's overall mental state (Mellor et al., 2020; Mellor and Beausoleil, 2015).

In free-ranging killer whales and other cetaceans, meaningful validation of new physiological markers benefits greatly from populations for which detailed demographics, life-history, and health information is available, as these data allow researchers to interpret hormone measurements within an appropriate biological context (Goymann, 2012; Hunt et al., 2013; Touma and Palme, 2005). Several long-term field studies have produced such datasets, enabling robust endocrine validation directly in wild populations (Koopman et al., 1995; Kershaw et al., 2017; Bennett et al., 2024). At the same time, individuals under professional care can contribute complementary advantages, including opportunities for routine health monitoring, controlled sampling conditions, and systematic and reliable documentation of social or environmental changes (Ramirez, 2012; Würsig et al., 2018). Nevertheless, it

is important to recognize that physiological parameters measured in managed individuals may not fully represent those of wild conspecifics. Thus, integrating findings from both managed and free-ranging populations provides the most comprehensive pathway for validating emerging endocrine markers.

While a multi-matrix comparison including blood, urine, or blubber would provide additional validation, our primary objective in this study was to develop and optimize a practical epidermal sampling and analysis method that can be applied repeatedly with minimal disturbance. To this end, and as part of ongoing efforts to validate novel physiological indicators for monitoring cetacean welfare, we aimed to: (i) establish a minimally invasive method for collecting epidermal desquamation samples in managed killer whales; (ii) optimize and validate a protocol for extracting and quantifying cortisol and oxytocin from epidermal samples using an AlphaLISA immunoassay protocol; (iii) examine the effect of sampling body location on ECC and EOC; (iv) assess the influence of welfare-related variables on ECC and EOC, accounting for possible delays between endocrine activity and hormone deposition in the skin, using routine weekly samples collected over an extended period from five individuals, and (v) evaluate the relationship between ECC and EOC, with the overall goal of exploring their potential as retrospective long-term welfare indicators in cetaceans.

2. Materials and methods

2.1. Ethics statement

Skin sampling in this study was performed by specialized trainers authorized by Loro Parque Zoo, as part of the animals' routine training and prophylactic care program. The procedures were integrated into regular husbandry sessions using positive reinforcement training techniques, ensuring that sampling was voluntary, caused no injury, and did not interfere with the killer whales' normal routines. All procedures were carried out in compliance with the requirements established in Articles 3, 4, and 5 of the "Ley 31/2003, de 27 de octubre, de conservación de la fauna silvestre en los parques zoológicos" (BOE-A-2003-19,800), as well as the Council Directive 1999/22/EC of 29 March 1999 on the keeping of wild animals in zoos (EUR-Lex - 31,999 L0022). Likewise, the use of skin samples for this scientific study was expressly authorized by the Director of the Loro Parque zoo.

2.2. Study area and individuals

Five killer whales housed at Loro Parque Zoo in Tenerife, Spain were used in this study. These comprised the entire resident population at the time and consisted of three males aged 27 (M1), 21 (M2), and 11 (M3) years, and two females aged 20 (F1) and an estimated age between 16 and 19 (F2) years. The mean body weights of these individuals were 3729.6 kg (M1), 3000.18 kg (M2), 1780.55 kg (M3), 1937.07 kg (F1), and 2092.99 kg (F2). Four of the individuals were born under human care, while F2 was rescued from the Wadden Sea in 2010, later integrated into the group, and subsequently diagnosed with severe hearing loss (Lucke et al., 2016). In September 2022, during the study period, F1 died due to a heart failure caused by a vascular malformation (Câmara et al., 2024).

The facility consists of an outdoor complex with four interconnected pools ranging from 8 to 12 m in depth. Gates between pools can be variably opened or closed as needed, allowing for flexible management of the animals. The total water volume is approximately 24 million liters. Water temperatures were maintained at around 14 °C throughout the year, ensuring that all individuals remained within their thermal comfort range. Diets were composed of frozen fish, mainly herring (*Cuplea harengus*), sprat (*Sprattus sprattus*) and capelin (*Mallotus villosus*), and individually tailored to meet the nutritional requirements of each animal. Positive reinforcement training served as the primary method for facilitating husbandry, veterinary care, and research procedures.

2.3. Epidermal sampling

Epidermal sampling was conducted using positive reinforcement training, ensuring no injury or disruption to daily routines, locations, or group compositions. Trainers positioned the killer whales with the sampling area exposed above water. A semi-rigid plastic card, previously disinfected with alcohol and air-dried, was used for the epidermal tissue sampling (Fig. 1). Skin was scraped by applying moderate pressure and performing scrapes in multiple directions over healthy areas of approximately 15 cm. The number of scrapes per animal ranged from 3 to 6, depending on the amount of tissue shed, with the goal of filling approximately one-third of a 1.5 mL Eppendorf tube. After collection, samples were immediately stored in a -20 °C freezer located within the facilities adjacent to the pools, minimizing the time between collection and freezing to under 5 min (Fig. 1). The sampling protocol was based on prior experience in a similar study on bottlenose dolphins and belugas (; Agustí et al., 2025b; Agustí et al., 2024) where the number of scrapes required to obtain a sufficient sample varied between individuals.

In a first study examining hormone variation among body regions, the epidermis of each of the five individuals was sampled once, simultaneously across five body regions, considering both body sides (right and left) and pigmentation differences (black vs. grey at the left base of the dorsal fin). This resulted in a total of 11 sampling locations per individual (Fig. 2). The objective was to evaluate the feasibility of the epidermal sampling method, determine the most suitable body region for sampling, and assess whether ECC and EOC varied across body regions, sides, or pigmentation.

Subsequently, in a second study examining hormone variation over time, one sample per individual was collected weekly, every Wednesday, from June 2022 to January 2023, specifically from the base of the left dorsal fin, as this was identified as the optimal sampling location based on the first study, yielding 9, 24, 27, 32 and 32 samples for F1, F2, M1, M2 and M3, respectively (total $N = 124$).

2.4. Sample storage, preparation and hormone extraction

Skin samples previously stored at -20 °C were dried in an oven at 36 °C for 72 h to remove any residual water. To address the previously reported effect of sample mass (Agustí et al., 2024), samples were standardized to 15–20 mg. Samples weighing under 15 mg were excluded from further processing, whereas those exceeding 20 mg were homogenized and subsampled so that 20 mg of tissue entered the extraction procedure. The standardized dried tissue was then ground into a fine powder using a Precellys Evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The powdered epidermis was incubated with 1 mL of methanol for 18 h at room temperature with continuous gentle agitation, ensuring no particles adhered to the tube caps. After incubation, the samples were centrifuged at 16,000*g* for 10 min, and 0.6 mL of the supernatant was carefully pipetted into a new tube. This supernatant was evaporated to dryness over 2 h using an Eppendorf Speed Vac Concentrator 5301 (Hamburg, Germany), and the resulting residue was reconstituted in 100 μL of phosphate-buffered saline (PBS). Reconstituted extracts were then stored at –20 °C until batch analysis to ensure consistency across assay plates.

2.5. Hormone detection and assay validation

Cortisol and oxytocin concentrations were measured using AlphaLISA® immunoassays (PerkinElmer, USA). AlphaLISA was selected over traditional ELISA platforms because its homogeneous, no-wash format minimizes sample loss and matrix-related interference, an important consideration when working with low-volume, keratinized tissues such as epidermis. The oxytocin assay operated in a direct competitive format, while the cortisol assay was conducted in an indirect competitive configuration. Both assays utilize donor and acceptor beads and rely on competition between endogenous and biotinylated hormone for

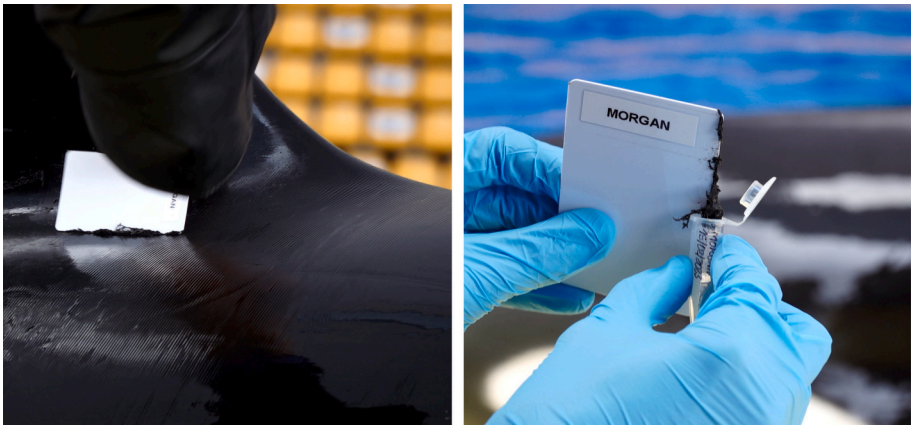

Fig. 1. Collection of epidermis samples of a killer whale (*Orcinus orca*) under human care. The left image illustrates a trainer using a semi-rigid plastic card to scrape the base of the dorsal fin, on the left side of the animal, after the area has been dried. The right image shows the careful placement of the collected epidermis sample into an Eppendorf tube to minimize the risk of contamination.

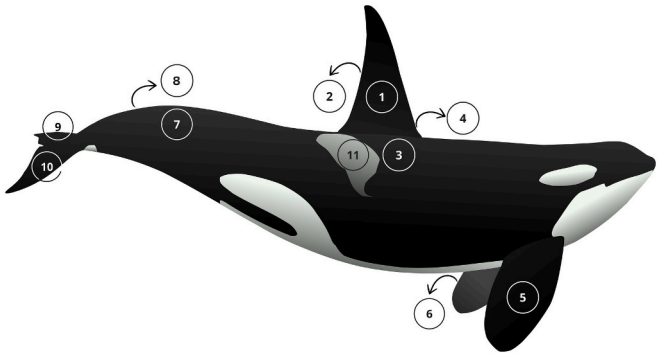

Fig. 2. To study the effect of body location and skin color on epidermal cortisol and oxytocin concentrations, an attempt was made to collect scraped skin samples from eleven body locations in five killer whales (*Orcinus orca*): (1) Right dorsal fin; (2) Left dorsal fin; (3) Right base of the dorsal fin; (4) Left base of the dorsal fin; (5) Right dorsal pectoral fin; (6) Left dorsal pectoral fin; (7) Right dorsal peduncle; (8) Left dorsal peduncle; (9) Dorsal left caudal fin; (10) Dorsal right caudal fin; and (11) Pale grey saddle patch in the right base of the dorsal fin.

binding to a monoclonal antibody specific to each analyte. These antibodies were previously validated for application in keratinized matrices such as sow hair (cortisol: López-Arjona et al., 2020; oxytocin: López-Arjona et al., 2021), where the cortisol antibody displayed 1.03% cross-reactivity with cortisone and the oxytocin antibody showed no measurable cross-reactivity with vasopressin. Following assay validation, all study samples were analyzed in singlet.

Following the validation framework proposed by Reimers and Lamb (1991), assay performance, namely specificity, parallelism, accuracy, precision, and sensitivity was assessed using pooled epidermal extracts from the five individuals (see 2.2. for details on sex and age class). Standard curves were generated by serial dilutions of conjugated oxytocin (Oxytocin–BSA) and cortisol (cortisol-KLH, Cloud-Clone) in AlphaLISA Universal buffer, covering eight concentration levels. Specificity and matrix effects were evaluated through serial dilution of two extracts from 1:2 to 1:128. To verify that sample behavior matched that of the assay standards, we assessed parallelism between serial dilution curves and their respective standard curves by fitting four-parameter log–logistic models to log-transformed concentrations and testing for equality of slopes using an F-test (nonlinear ANCOVA, R package '*drc*'). Accuracy was tested by spiking known quantities of conjugated hormones into extracts with previously quantified levels, and comparing expected versus measured concentrations to calculate recovery percentages. Assay precision was evaluated through assessments of intra- and inter-assay variation using pooled epidermal extracts. For intra-assay precision, five replicates of two pooled extracts (representing high and low hormone concentrations) were measured within a single plate, and coefficients of variation (CVs) were calculated from these replicates. For inter-assay precision, the same two pooled quality-control extracts were included in duplicate on each of the two assay plates used per hormone and were analyzed on different days, from which inter-assay CVs were derived. Sensitivity was defined as the lowest cortisol or oxytocin concentration obtained by serial dilution that could be measured with a coefficient of variation $\leq$20%.

2.6. Characterization of individual welfare state based on zoo reports

In parallel with epidermal sampling, a comprehensive list of potential welfare indicators was developed following the Five Domains Model framework (Mellor et al., 2020), drawing on existing welfare assessment tools for captive bottlenose dolphins (*Tursiops truncatus*), such as C-Well (Clegg et al., 2015) and Dolphin WET (Baumgartner et al., 2024), as well as on welfare evaluation approaches applied in other species (e.g. Boys et al., 2022; Harvey et al., 2023; Table 1).

The indicators were derived from the zoo's established monitoring practices, which are an integral part of the daily care routines for the animals at Loro Parque. As part of these practices, the staff routinely document various aspects of killer whale welfare through reports and observations, focusing primarily on identifying potential health or behavioral concerns. These records encompass activities during training sessions, free periods, and interactions with veterinarians during medical examinations. Daily welfare-related information from trainers' and veterinary records were systematically reviewed, and the data was categorized into indicators scored as either absent (0) or present (1). It is important to note that many welfare indicators remain unvalidated specifically for killer whales due to the limited number of studies conducted on this species. Therefore, validated indicators from other cetacean species, mainly bottlenose dolphins, were used as a reference (Table 1).

Additionally, body weight variation was evaluated as a factor potentially related to epidermal hormonal levels and overall welfare status. Although it was not integrated into the welfare indicator framework, due to the absence of established thresholds for weight change associated with compromised welfare in killer whales, its use as a relative, rather than absolute, measure of body condition follows approaches recommended in recent cetacean's research. Previous studies have shown that temporal variation in body mass provides a more ecologically meaningful indicator of physiological condition than static mass values, which can differ widely across individuals due to age, sex,

Table 1
Killer whale (*Orcinus orca*) potential welfare indicators identified in zoo daily records, organized according to the Five Domains Model (Mellor et al., 2020). Each indicator is assigned to a physical domain (Domains 1–4) and accompanied by a brief description. The final column includes supporting references, mainly based on studies conducted in other species.

Domain	Potential welfare indicator	Description	References
Domain 1: Nutrition	Reduced food intake	Measure of the percentage of fish consumed daily by each animal, defined as days when intake was below 90% of the total food offered (recorded in kg).	Clegg et al. (2019); Johnson et al. (2009); Waples and Gales (2002) (bottlenose dolphins)
Domain 2: Environment	Night social isolation	Records of an individual kept separated from the rest of the group during night, potentially limiting opportunities for affiliative interactions and other social behaviors.	Couquiaud, 2005; Waples and Gales, 2002 (bottlenose dolphins)
	Performance of invasive veterinary procedures	Records of invasive veterinary procedures performed (e.g., ultrasounds, gastric fluid analysis, dental X-rays, gastroscopy, pulmonary ultrasound), either as part of routine monitoring or in response to suspected health issues.	Martelli and Krishnasamy (2023); Morgan and Tromborg (2007) (zoo animals)
Domain 3: Health	Incidence of gastrointestinal diseases/fecal abnormalities	Observations of gastrointestinal dysfunction, including records of abnormal gastric or fecal characteristics, cytological evaluations, microbial cultures, and parasitological examinations. Indicators include pasty or foamy feces, fecal consistency changes, and signs of gastrointestinal discomfort such as abdominal contractions.	St. Leger et al. (2018); Sapolsky (2004) (zoo animals and bottlenose dolphins)
Domain 4: Behavioral Interactions	Motivated and engaged behavior	Observations of an individual displaying a positive attitude and active involvement with its environment. This can include purposeful movements, social interactions, exploration, and prompt reactions to stimuli, along with curiosity, interest in enrichment, and willingness to engage in training or cooperative tasks.	Clegg et al. (2019); Delfour et al. (2021); Huettner et al. (2021); Lauderdale (2017); Shyne (2006) (bottlenose dolphins)
	Agonistic behaviors and social tension	Observations of agonistic interactions, including aggression (e.g., chasing, biting, displacements), social tension (e.g., avoidance, increased vigilance), and dominance-related behaviors.	Clegg et al. (2015); Waples and Gales (2002) (bottlenose dolphins)
	Reduced engagement and activity	Observations of an individual atypically displaying slow movements, reduced responsiveness, or signs of apathy, including decreased engagement in social or environmental interactions, minimal exploratory behavior, or delayed reactions to stimuli.	Baumgartner et al. (2024); Clegg et al. (2019) (bottlenose dolphins)
	Negative behavioral response to social separation	Negative behavioral responses, as interpreted by caretakers, observed during or immediately after a change in social group composition. These may include increased vocalizations, refusal to perform behaviors, or agitation near barriers or gates.	Clegg et al. (2015); Waples and Gales (2002) (bottlenose dolphins)
	Pain-related behavior	Behaviors indicative of pain or physical discomfort, as interpreted by caretakers or veterinarians. These may include lethargy, cautious or stiff swimming, abnormal postures (e.g., arching the body), or avoidance of physical contact in specific areas.	Baumgartner et al. (2024); Clegg et al. (2019) (bottlenose dolphins)
	Presence of low willingness to participate in training (score 1)	Presence of at least one recorded instance of a score of 1 within a training day. Trainers rate each requested behavior throughout the day on a 3-point scale (1 to 3) representing incremental motivation and performance during training sessions.	Clegg et al. (2019); Huettner et al. (2021); Lauderdale (2017); Shyne (2006) (bottlenose dolphins)
	Refusal to perform trained behaviors	Failure or unwillingness to execute a behavior previously trained	Clegg et al. (2019); Delfour et al. (2020); Huettner et al.

(*continued on next page*)

Table 1 (*continued*)

Domain	Potential welfare indicator	Description	References
		and requested by the trainer. This may include disengagement from the session or incomplete responses.	(2021); Lauderdale (2017); (bottlenose dolphins)
	Regurgitation	Observations of individuals ejecting a full fish or partially digested material, often followed by re-swallowing.	Yeater (2005)
	Incidents with the trainers	Records of instances where a whale approaches, interacts, or physically engages with a trainer in an unusual or unexpected manner, without overt aggression.	Clegg et al. (2019); Delfour et al. (2020); Huettner et al. (2021); Lauderdale (2017); (bottlenose dolphins)

or morphology (Karns et al., 2019; Kastelein et al., 2019; Derous et al., 2020). These studies further indicate that monthly changes, including decreases of approximately 5%, may reflect altered energetic status and are therefore more relevant for health assessment than raw mass alone. Body weight was monitored approximately every ten days by the animal care staff using a custom-built stainless-steel platform scale (Emery Winslow Scale, Seymour, CT, USA) installed at the poolside. Killer whales were trained using positive reinforcement to voluntarily beach onto the platform in a controlled position. Daily values were estimated by linear interpolation between consecutive measurements, assuming gradual and continuous changes. Weekly means were then calculated, and the percentage of body weight variation was computed by comparing each week's average with that from four weeks earlier, using the formula: ((week X − week X − 4) / week X − 4) × 100. This approach generated a continuous week-by-week estimate of monthly body weight variation, capturing both increases and decreases relative to individual baseline trajectories. By focusing on relative change, rather than absolute mass, this method provides a biologically interpretable and standardized indicator of variation in body condition, consistent with current recommendations for cetacean health and welfare assessment.

2.7. Data management

Given the large number of variables recorded, it was considered appropriate to simplify the dataset in order to reduce the complexity of the statistical models while retaining those variables most relevant to animal welfare. Many of the welfare-related variables appeared with low frequency (less than 5% of the observed days), potentially introducing noise and limiting the ability to draw meaningful conclusions. To address this, all variables identified as potential negative welfare indicators were grouped into a single cumulative metric named 'Negative welfare indicators' (Table 2). This composite variable, calculated as the sum of 12 individual indicators, ranged from 0 to 12. Daily observations of "Negative welfare indicators" and "Motivated and engaged behavior" were then aggregated by calendar week (Monday to Sunday), with daily values summed to generate weekly totals (Table 2). This temporal aggregation facilitated the interpretation of welfare trends and aligned the behavior data with the corresponding weekly hormone samples.

Because the Negative welfare indicators variable is derived from a summed scale and therefore differs in magnitude from the other predictors, all predictor variables were standardized prior to inclusion in statistical models. Standardization was performed by centering each variable around its mean and scaling by its standard deviation, ensuring comparability across variables and individuals while preserving the relative variation within each indicator.

Table 2
Overview of predictor variables used in statistical models assessing the relationship between epidermal hormone concentrations and welfare-related conditions in killer whales (*Orcinus orca*).

Predictor	Description
Negative welfare indicators (weekly)	Weekly sum of daily scores from the Negative welfare indicators variable, which reflects the cumulative daily presence of behaviors and conditions presumed to negatively affect welfare. This variable includes the following twelve indicators: Reduced food intake, Night social isolation, Performance of invasive veterinary procedures, Incidence of gastrointestinal diseases/ fecal abnormalities, Agonistic behaviors and social tension, Reduced engagement and activity, Negative behavioral response to social separation, Pain-related behavior, Presence of low willingness to participate in training (score 1), Refusal to perform trained behaviors, Regurgitation, and Incidents with the trainers (see Table 1 for details on each indicator)
Motivated and engaged behavior (weekly)	Weekly sum of daily scores of Motivated and engaged behavior (see Table 1 for indicator description)
Body weight variation (% change, 4-week interval)	Weekly percentage change in body weight, calculated by comparing each week's mean weight with that of four weeks earlier (see Section 2.6 for formula and rationale).

To explore the possible delay between activation of the HPA axis or oxytocin system and the appearance of corresponding hormonal signals in the collected epidermis, hormone values were time-shifted backwards by varying durations. This assumed a linear relationship between integrated weekly predictor data and measured hormone levels. Analyses were repeated using multiple lag intervals to capture different potential timelines of hormone incorporation: 20–26, 27–33, 34–40, 41–47, 48–54, 55–61, 62–68, and 69–75 days. These intervals were selected based on epidermal renewal data in bottlenose dolphins (Hicks et al., 1985) and beluga whales (St. Aubin et al. 1990), studies on the delay between serum cortisol peaks and epidermal detection (Bechshoft et al., 2020), and the suggested processes underlying cortisol and oxytocin deposition and production in epidermal tissue (Deing et al., 2013; Henderson, 1993; Quintana et al., 2018; Zmijewski and Slominski 2011).

2.8. Statistical analyses

All analyses were conducted using R (version 4.3.3, R Core Team, 2024), and values are presented as mean ± SD. A significance threshold of p-value <0.05 was applied. Prior to analysis, Shapiro–Wilk tests were performed to assess normality, and several datasets showed deviations from normality. This informed the subsequent use of non-parametric tests for simple comparisons and mixed-effects models (GLMMs and LMMs) with appropriate distributions and random effects, after checking model residuals.

The association between individuals and sample weight categories was assessed using a Chi-square test, implemented via the 'chisq.test' function from the 'stats' package in R. To analyze ECC and EOC across body locations, sides (left vs. right), and coloration (black vs. pale grey), we fitted GLMMs with individual identity included as a random intercept to account for repeated measurements from the same individuals. For each hormone, we specified a single, a priori model including body region, side and coloration as fixed effects; no stepwise model selection was applied, as these predictors were defined a priori based on biological relevance. GLMMs with a gamma distribution were fitted using the

'glmer' function from the 'lme4' package to address the right-skewed distribution of both hormone concentrations. Model fit was evaluated by visual inspection of residuals versus fitted values and Q–Q plots, and by examining conditional and marginal R^2 values. These diagnostics did not indicate strong violations of model assumptions or evidence of overdispersion. When significant differences were detected, post hoc pairwise comparisons were performed using the 'emmeans' package, applying Bonferroni adjustments.

To examine the association between ECC and EOC, we fitted linear mixed-effects models with $\log_{10}$-transformed oxytocin as the response, $\log_{10}$-transformed cortisol as a fixed effect, and individual identity as a random intercept, using the 'lmer' function from the 'lme4' package; model assumptions were checked by visual inspection of residuals and Q–Q plots.

Separate statistical models were used to explore associations between predictors and ECC and EOC, respectively. Before testing associations with welfare-related predictors at different lags, temporal variation in ECC and EOC was examined by fitting LMMs with hormone concentration as the response, 'week' as either a continuous (week index) or categorical fixed effect, and individual identity as a random intercept, using the 'lmer' function from the 'lme4' package. Each hormone was then analyzed independently across several pre-defined time lag intervals: 20–26, 27–33, 34–40, 41–47, 48–54, 55–61, 62–68, and 69–75 days. LMMs were fitted using the lme4 package in R, with individual identity included as a random intercept to capture subject-specific effects. The general model structure was:

$$Yij = \beta 0 + \beta 1X1ij + \cdots + \beta kXkij + uj + \epsilon ij$$

where Yij represents the response variable (ECC or EOC) for observation i of individual *j*, X1ij, …,Xkij are fixed-effect predictors, uj is the random intercept for individual j assumed to be normally distributed with mean zero and variance σu2, and ϵij is the residual error. Coefficients for fixed effects (β) were estimated using restricted maximum likelihood (REML), and predictor significance was evaluated based on standard inferential criteria. Key model assumptions – including the distribution of residuals, homogeneity of variance (homoscedasticity), and absence of multicollinearity among predictors – were assessed. Deviations from normality and variance heterogeneity were minor and are generally accommodated within the LMM framework, which is robust to moderate violations due to the inclusion of random effects and repeated measures structure.

3. Results

3.1. Optimization of the epidermal sampling method

In the first study examining hormone variation among body regions, a total of 55 sampling attempts were conducted across 11 locations per individual in five killer whales, covering five body regions, both body sides (right and left), and pigmentation (black vs. grey at the left base of the dorsal fin; Table 3). Of these, 34 yielded usable samples, while the remaining attempts did not provide sufficient material. The mean ± SD dry weight of the scraped epidermal samples was 20.07 ± 5.44 mg.

Subsequently, in the second study examining hormone variation over time, we attempted to collect one sample per individual each week, every Wednesday, from June 2022 to January 2023, specifically from the base of the left dorsal fin, resulting in the collection of 140 epidermal samples. Most sample collection attempts (79.29% (111/140)) resulted in samples of ≥20 mg of dry epidermis, while 9.29% (13/140) contained between 15 and 20 mg (considered still suitable but close to the limit), and 11.43% (16/140) weighed <15 mg (i.e., unsuccessful sample collections). Individuals participated voluntarily in all sampling attempts and did not show avoidance or discomfort behaviors. Sampling time per individual was around 1 min. Additionally, the Chi-square test revealed a significant association between the individual sampled and the amount of epidermis collected ($\chi^2 = 19.00$, df = 8, $p = 0.0148$).

Table 3
Epidermal sample collection success and dry sample weight across body regions in killer whales (*Orcinus orca*) from the first study (initial body-region sampling). Success in sample collection was determined based on whether the collected sample met the minimum dry weight of 15 mg. Samples below this threshold were considered unsuccessful.

Body Region	Mean dry sample weight ± SD (mg)	% Success in sample collection (n/total)
Base of the dorsal fin	**22.58 ± 5.97**	73.3% (11/15)
Dorsal caudal fin	**20.4 ± 5.6**	30.0% (3/10)
Dorsal fin	**20.4 ± 2.1**	50.0% (5/10)
Dorsal pectoral fin	**19.0 ± 4.3**	70.0% (7/10)
Dorsal peduncle	**16.9 ± 6.5**	70.0% (7/10)

3.2. Assay validation

For ECC, dilution linearity was high (Pearson: r(3) = 0.988, $p = 0.007$). The serial dilutions of pooled skin extract were parallel to the standard curve. A four-parameter log-logistic model with a common slope did not fit significantly worse than a model with separate slopes ($F_{1,6} = 0.17$, $p = 0.69$). Mean intra- and inter-assay coefficients of variation were 9.51% and 7.48%, respectively, confirming good repeatability. Spike-and-recovery tests produced an average recovery of 97.94 ± 11.06%, demonstrating assay accuracy. The assay sensitivity was 25.05 ng cortisol/mL of extract.

For EOC, the dilution linearity test showed a high correlation between expected and measured concentrations (Pearson: r(3) = 0.999, $p < 0.001$). The serial dilutions of pooled skin extract were parallel to the standard curve. A four-parameter log-logistic model with a common slope did not fit significantly worse than a model with separate slopes ($F_{1,6} = 0.04$, $p = 0.85$). Mean intra- and inter-assay CVs were 4.48% and 6.00%, respectively. Spike-and-recovery tests yielded an average recovery of 108.34 ± 15.80%. The assay sensitivity was 32.03 pg oxytocin/mL of extract.

3.3. Effect of body location on epidermal cortisol and oxytocin concentrations

In the first study, the GLMM for ECC (AIC = 171.3; df = 24) did not show significant effects of body location ($p = 0.13$), side ($p = 0.09$), or coloration ($p = 0.22$; Fig. 3; Table S1).

The GLMM for oxytocin concentrations (AIC = 182.9; df = 24) indicated a significant effect of side ($p < 0.001$) but no significant effects of body location ($p = 0.409$) or coloration ($p = 0.323$; Fig. 4; Table S2). Specifically, oxytocin levels were significantly higher on the right side (Estimate = 0.684, $p < 0.001$).

3.4. Descriptive overview, individual variation, and correlation of epidermal cortisol and oxytocin concentrations

In the second study, epidermal cortisol concentrations ranged from 4.1 to 18.7 ng/g across individuals (overall mean ± SD: 9.09 ± 3.30 ng/g; Fig. 5).

Epidermal oxytocin concentrations (EOC) ranged from 2.8 to 21.0 ng/g across individuals (overall mean ± SD: 7.30 ± 4.54 ng/g; Fig. 6).

The relationship between ECC and EOC was examined using a linear mixed-effects model with $\log_{10}$-transformed EOC as the response, $\log_{10}$-transformed ECC as a fixed effect and individual identity as a random intercept. The model indicated a positive association between ECC and EOC (β_logECC = 1.12 ± 0.37 SE, $t = 2.99$, $p = 0.003$; Fig. 7). The marginal R^2 was 0.06, indicating that ECC alone explained a small proportion of the variance in EOC, whereas the conditional R^2 was 0.29, reflecting additional variability attributable to differences among

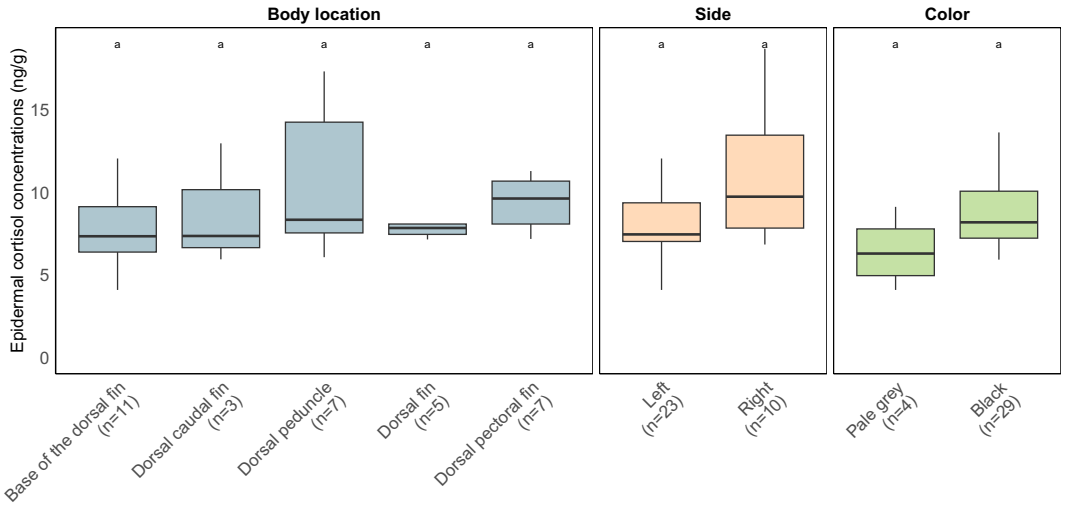

Fig. 3. Epidermal cortisol concentrations across different body locations, sides, and skin color patterns in killer whales (*Orcinus orca*). Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Significance letters ("a") indicate no statistical differences within each comparison group ($p > 0.05$).

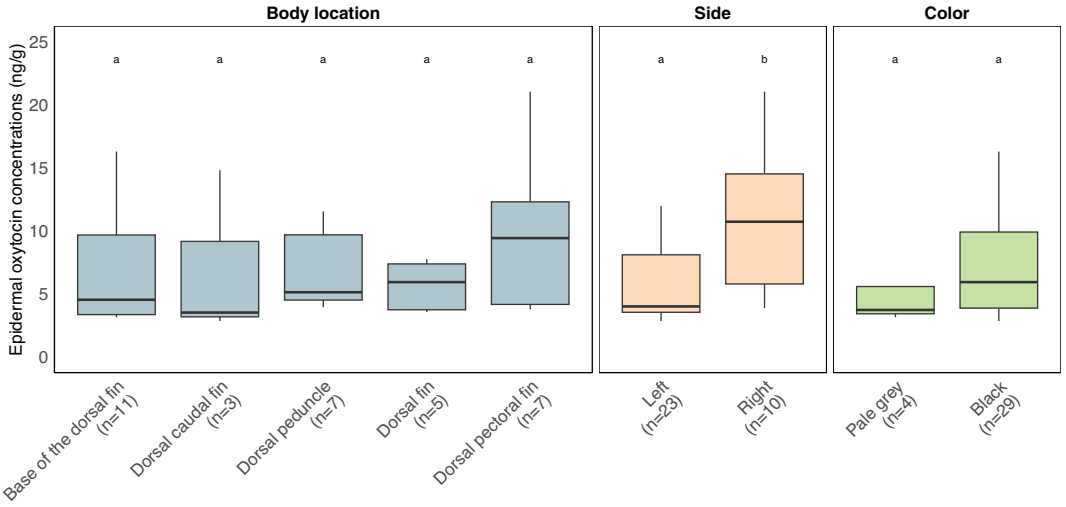

Fig. 4. Epidermal oxytocin concentrations across different body locations, sides, and color patterns of killer whales' (*Orcinus orca*) skin. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate the range of concentrations. Different letters denote statistically significant differences within each comparison group ($p < 0.05$).

individuals. Visual inspection of residuals and Q–Q plots did not reveal major deviations from model assumptions.

3.5. Descriptive analysis of welfare-related predictors

The selected predictors included Negative welfare indicators, Motivated and engaged behavior, and Body weight variation. Negative welfare indicators and Motivated and engaged behavior were assessed on a standardized 0–7 scale and Body weight variation was calculated as the weekly percentage change in body weight over a 4-week interval (see Section 2.6 for details). Table 4 summarizes the descriptive statistics of all predictors considered in the statistical models.

The predictor variables used in the statistical models were only weakly correlated with one another (all Spearman's $\rho < 0.1$), well below commonly accepted thresholds for multicollinearity concerns (e.g., $\rho > 0.6$). Their low intercorrelation and conceptual independence justified retaining all variables in the models.

3.6. Associations of welfare-related predictors on epidermal cortisol concentrations at different time lags

Before examining lagged associations between welfare-related predictors and ECC, temporal patterns in ECC were examined over the study period. In LMMs with week index as a continuous predictor, ECC did not show a significant linear trend over time (βweek $= 0.004$, $p = 0.38$). When "week" was modelled as a categorical factor, only week 38 differed significantly from week 1. These results indicate that ECC showed some temporal variation, but no pervasive systematic pattern across all weeks. Different time lags between predictors and ECC were assessed, covering periods from 20 to 75 days prior to sampling in consecutive 7-day windows. This approach resulted in eight LMMs (Table 5). Among the three predictors assessed, neither Motivated and engaged behavior nor Body weight variation showed statistically significant effects on ECC at any of the examined time lags. Conversely, Negative welfare indicators showed significant positive associations with ECC at multiple time lags prior to sampling (Table 5). Random intercepts for individual identity indicated low-to-moderate between-whale variability in ECC, with intraclass correlation coefficients (ICC)

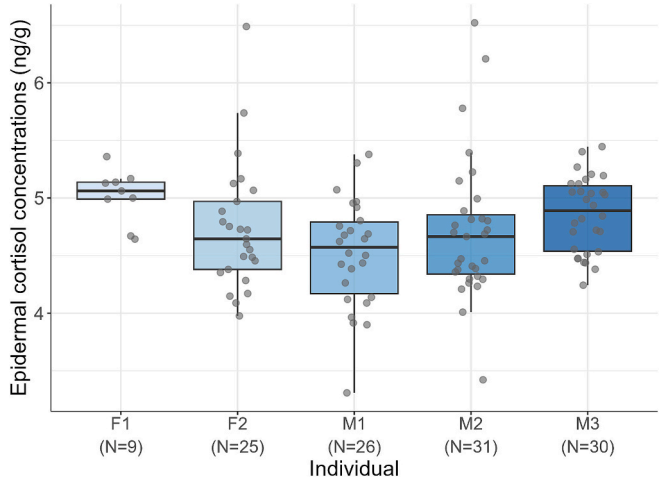

Fig. 5. Epidermal cortisol concentrations across killer whale (*Orcinus orca*) individuals. Boxplots show the distribution of cortisol concentrations for each female (F1, F2) and male (M1, M2, M3) killer whale. Horizontal lines represent the lower quartile, median, and upper quartile values, while whiskers indicate the range.

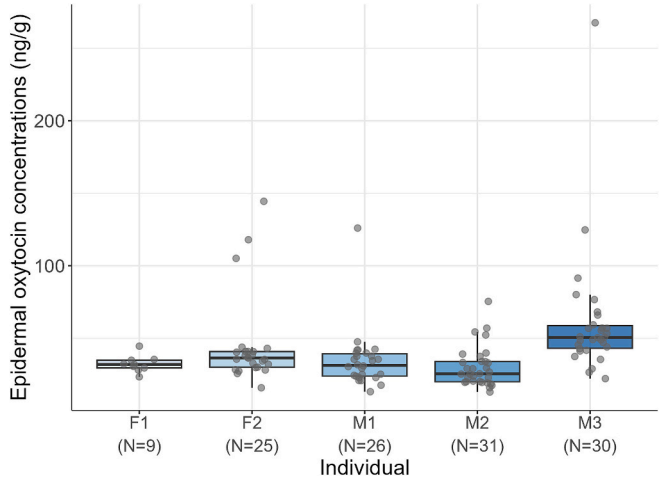

Fig. 6. Epidermal oxytocin concentrations across killer whale (*Orcinus orca*) individuals. Boxplots show the distribution of oxytocin concentrations for each female (F1, F2) and male (M1, M2, M3) killer whale. Horizontal lines represent the lower quartile, median, and upper quartile values, while whiskers indicate the range.

ranging from approximately 4% to 12% across time lags (Supplementary Table S3).

3.7. Effects of welfare-related predictors on epidermal oxytocin concentrations at different time lags

Similarly, before examining lagged associations between welfare-related predictors and EOC, temporal patterns in EOC were examined. In LMMs with week index as a continuous predictor, EOC displayed a modest but significant positive linear trend over time (βweek = 0.63, p = 0.032). When "week" was modelled as a categorical factor, weeks 41 and 48 differed significantly from week 1. Together, these results indicate that EOC exhibited some temporal variation, although not consistently across all weeks. Different time lags between predictors and EOC were assessed, covering periods from 20 to 75 days prior to sampling in consecutive 7-day windows. This approach resulted in eight LMMs (Table 6). Among the three predictors evaluated, Body weight variation

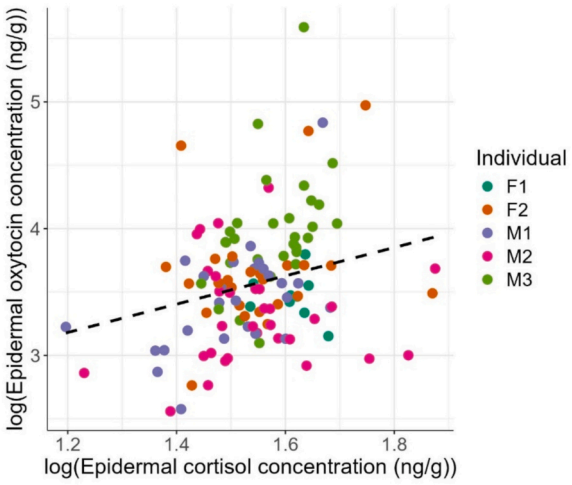

Fig. 7. Relationship between $\log_{10}$-transformed epidermal cortisol (ECC) and oxytocin (EOC) concentrations in killer whales (*Orcinus orca*). Each point represents an individual epidermal sample, and colors indicate different individuals. The marginal R^2 was low ($mR^2 = 0.06$), indicating little association between these hormones.

Table 4
Descriptive statistics of predictor variables used in statistical models relating epidermal hormone concentrations to welfare-related conditions in killer whales (*Orcinus orca*).

Predictor	Mean (scale 0–7)	SD	Median	Min.	Max.
Negative welfare indicators (normalized)*	0.37	0.29	0.33	0	2
Motivated and engaged behavior	0.88	1.47	0	0	6
Body weight variation (%)	-0.25	1.19	-0.16	-6.78	2.01

* Negative welfare indicators were obtained by summing 12 individual measures (possible range: 0–84) and normalized by dividing by 12 to match the 0–7 scale of Motivated and engaged behavior.

was not associated with EOC at any time lag; Motivated and engaged behavior showed positive associations at two time lags; and Negative welfare indicators were positively associated with EOC at one time lag. Random intercepts for individual identity indicated moderate between-whale variability in EOC, with ICC values ranging from approximately 12.5% to 21.0% across time lags (Supplementary Table S4).

4. Discussion

4.1. Optimization of the epidermal sampling method and analytical validation

The proposed method proved feasible for collecting desquamated epidermis from killer whales, as most sampling attempts were successful without causing discomfort to the animals or requiring changes to routine husbandry practices, confirming its minimally-invasive nature and operational feasibility in managed cetaceans. However, sampling success varied by body region. The base of the dorsal fin yielded the most consistent results, likely due to regional differences in epidermal turnover or desquamation. Consequently, the base to the left of the dorsal fin was adopted as the standard sampling site. Weekly collections from this area produced a generally high yield, with only 11% of samples falling below the 15 mg analytical threshold, which were excluded from analysis to avoid potential biases associated with low sample mass, as noted by Agustí et al. (2024).

In free-ranging killer whales, epidermis samples could be obtained

Table 5
Predictor estimates from linear mixed-effects models (LMMs) for epidermal cortisol concentrations in killer whales (*Orcinus orca*) across different time lags. Green cells represent statistically significant ($p < 0.05$) positive relationships while yellow cells indicate trends with p-values between 0.05 and 0.1 ($p > 0.05 < 0.1$).

Predictors	Time lag (estimates)							
	20-26	27-33	34-40	41-47	48-54	55-61	62-68	69-75
(Intercept)	4.74	4.74	4.74	4.74	4.74	4.73	4.73	4.74
Negative welfare indicators	-0.003	**0.11***	0.1.	**0.11***	-0.02	-0.02	**0.11***	0.1.
Motivated and engaged behavior	0.04	0.04	0.02	0.02	0.06	0.02	0.01	-0.02
Body weight variation	-0.03	-0.01	-0.003	0.05	-0.02	-0.06	-0.02	-0.01

***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; .: $0.05 \leq p < 0.1$. Intercepts were significant ($p < 0.001$) in all models but are not marked.

Table 6
Predictor estimates from linear mixed-effects models (LMMs) for epidermal oxytocin concentrations in killer whales (*Orcinus orca*) across different time lags. Green cells represent statistically significant ($p < 0.05$) positive relationships while yellow cells indicate trends with p-values between 0.05 and 0.1 ($p > 0.05 < 0.1$).

Predictors	Time lag (estimates)							
	20-26	27-33	34-40	41-47	48-54	55-61	62-68	69-75
(Intercept)	40.19	41.72	40.52	40.03	39.50	39.31	40.56	41.97
Negative welfare indicators	-0.97	5.95	3.73	-0.75	-3.65	4.95	**15.94*****	6.66.
Motivated and engaged behavior	4.61.	-0.89	3.72	0.57	4.49.	**5.40****	1.5	**5.7****
Body weight variation	4.23	-0.86	-4.91	-6.59.	-6	-4.35	-0.04	6.4.

***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; .: $0.05 \leq p < 0.1$. Intercepts were significant ($p < 0.001$) in all models but are not marked.

using remote biopsy techniques (Barrett-Lennard et al., 1996), a procedure that remains invasive, albeit less so than blubber sampling as it constitutes a more superficial tissue. Samples from stranded individuals or archived in tissue banks also offer valuable opportunities for retrospective analyses (Kellar et al., 2015) although additional studies are needed to evaluate hormone stability in long-term archived material. Importantly, environmental conditions must be carefully considered when planning epidermal sampling in wild killer whales, as cold-water exposure may reduce epidermal turnover and compromise sample yield (Durban and Pitman, 2012; Pitman et al., 2019).

Assay validation suggested that the desquamated epidermis (*stratum corneum*) from killer whales contains quantifiable concentrations of cortisol and oxytocin using our AlphaLISA-based method. These results align well with previous findings highlighting that an assay not explicitly designed for cetacean or epidermal samples can achieve robust linearity, parallelism, recovery, and precision (Agustí et al., 2025a; Agustí et al., 2025b; Robinson et al., 2020). However, measured oxytocin may include immunoreactive degradation products (Szeto et al., 2011), thus, it cannot be excluded that epidermal oxytocin partly reflects oxytocin immunoreactivity rather than intact peptide.

While liquid chromatography-tandem mass spectrometry (LC-MS/MS) typically offers higher analytical sensitivity and specificity (Bechshoft et al., 2015; Bechshoft et al., 2020), immunoassays such as AlphaLISA remain advantageous due to their affordability and lower requirements for specialized equipment and technical skills. These characteristics make immunoassays particularly practical for wildlife and welfare research involving extensive sampling efforts or limited laboratory resources.

A potential limitation of the extraction protocol is that oxytocin is particularly vulnerable to heat degradation, and some loss can occur during evaporation or dry-down steps. While pharmaceutical studies suggest that oxytocin degradation is driven by prolonged (weeks)exposure to elevated temperatures (at >30–50 °C; Nguyen et al., 2019; Kaushal et al., 2012), these data provide only indirect context for endogenous oxytocin in keratinized tissues. Accordingly, before drawing stronger conclusions or further applying this protocol, future studies should prioritize spike–recovery tests to directly assess oxytocin stability across the drying step (i.e. adding a known amount of hormone to skin before drying and extraction, extracting it with the same protocol, and quantifying recovery). Because such tests were not performed in the present study, some degree of oxytocin loss during drying and evaporation cannot be ruled out. In contrast, cortisol is generally reported to be relatively stable in keratinized matrices such as hair or fish scales, with evidence of measurable degradation primarily emerging over much longer timeframes (e.g., multi-year to decade-scale storage) or under more extreme temperature conditions (e.g., Azevedo et al., 2019; O'Toole et al., 2024).

4.2. *Effect of body location on epidermal cortisol and oxytocin concentrations*

The present results indicate that epidermal cortisol and oxytocin concentrations in killer whales do not vary significantly across different body locations or skin coloration patterns. These findings align with previous observations in bottlenose dolphins, where intra-individual variability was noted but no consistent regional distribution emerged (Agustí et al., 2024). Nevertheless, due to our limited sample size and known anatomical variability in cetacean epidermis, such as increased epidermal thickness in ventral regions (Cozzi et al., 2016), variations in dermal papilla height (Jones and Pfeiffer, 1994), and regional differences in melanocyte density (Berta et al., 2015; Perrin, 2009), it remains plausible that cortisol and oxytocin exhibit region-specific variations

across cetacean epidermis. Such structural heterogeneity may introduce biases or inconsistencies in hormone quantification, underscoring the need for standardized sampling locations to ensure the reliability and comparability of results.

Moreover, a lateral asymmetry in EOC was observed, characterized by significantly elevated levels on the right side, accompanied by a similar, though non-significant, trend for cortisol. While rightward behavioral lateralization is well documented in cetaceans (Jaakkola et al., 2021), evidence for physiological lateralization remains limited. Such hormonal asymmetry might reflect regional differences in skin blood flow, localized metabolic activity, or other physiological processes influencing hormone incorporation. It could also potentially result from the order of sample collection between body sides, although sampling was randomized and this factor was not systematically recorded. Further research is necessary to clarify these underlying mechanisms; nonetheless, the present findings underscore the importance of adopting consistent side-specific sampling protocols.

4.3. Descriptive overview and correlation between epidermal cortisol and oxytocin concentrations

ECC measured in killer whales averaged 9.09 ± 3.30 ng/g (range: 4.1–18.7 ng/g). When compared to previously reported values, these concentrations were similar to ranges documented for bottlenose dolphins: 1.58–8.30 ng/g (Agustí et al., 2025b), 0.13–8.09 ng/g (Agustí et al., 2024), and 0.31–16.17 ng/g (Bechshoft et al., 2020); and beluga whales (0.213–8.55 ng/g; Wong et al., 2023). On the other hand, EOC averaged 7.30 ± 4.54 ng/g (range: 2.8–21.0 ng/g). When compared to previously reported values, these concentrations were similar than those documented for the same epidermal layer in striped dolphins (*Stenella coeruleoalba*; 1.09–9.30 ng/g), but lower than concentrations measured in deeper epidermal layers of the same species (1.74–337.33 ng/g; Agustí et al., 2025a). However, both similarities and differences observed across studies and species should be interpreted with caution, as absolute concentrations may be influenced by methodological and analytical variation.

The mixed-effects analysis revealed a positive association between ECC and EOC, although ECC alone explained only a small proportion of the variance in EOC. A similar trend was previously reported by Agustí et al. (2025b) in the stratum corneum of bottlenose dolphins. Although oxytocin is primarily known for its role in promoting social interactions and positive welfare states (Rault et al., 2017), recent research has also emphasized its involvement in stress regulation and adaptive responses. Increases in oxytocin concentrations have been observed in various mammalian species under stress (Chen and Sato, 2016; Sutherland and Tops, 2014), which may contribute to stress buffering by inhibiting activation of HPA axis, thereby facilitating recovery and improving resilience (Chen and Sato, 2016).

4.4. Effects of welfare-related predictors on epidermal cortisol concentrations

There is still limited research on the mechanisms by which glucocorticoids and oxytocin are incorporated into cetacean epidermal tissue, and in other species more broadly, as well as their persistence following deposition. These gaps in knowledge are particularly pronounced in killer whales, for which the epidermal turnover rate remains unknown, complicating accurate temporal interpretation of hormone data. Consequently, we examined a range of biologically plausible intervals (20–75 days) between the activation of the physiological system (HPA or oxytocinergic systems) and hormone detection in the sloughed epidermis. Given these substantial uncertainties, we have maintained a cautious approach when interpreting associations between ECC and welfare-related predictors.

Negative welfare indicators were positively associated with ECC across multiple time lags ranging from 27 to 68 days prior to sampling. Indicators primarily encompassed health impairments, social problems or isolation, performance of invasive veterinary procedures, and aversive human-animal interactions. Health impairments activate the HPA axis to facilitate energy mobilization and modulate immune responses (Silverman et al., 2013), often accompanied by emotional stressors such as pain or social isolation, which can further elevate cortisol levels (Müller and Bossley, 2002). In cetaceans, this relationship is supported by studies reporting increased cortisol concentrations in association with illness or disease across different biological matrices (Agustí et al., 2025a; Biancani et al., 2017; Goldstein et al., 2012; Wong et al., 2023). In addition to physical health problems, social stressors have been consistently shown to activate the HPA axis and elevate basal cortisol concentrations in mammals (De Goeij et al., 1992; Sapolsky, 1983; Tamashiro et al., 2005), a pattern that has also been observed in certain captive cetaceans (Serres et al., 2020). Furthermore, invasive veterinary procedures, while often necessary for animal care, are recognized sources of acute stress across zoo-housed species that can contribute to transient increases in HPA axis activity (Martelli and Krishnasamy, 2023; Morgan and Tromborg, 2007).

The observed association between elevated ECC and Negative Welfare Indicators, encompassing contexts known to activate the HPA axis in mammals and conditions presumed to reflect negative affective states in killer whales, supports the biological validation of ECC as a physiological marker of HPA axis activity and welfare in killer whales. These findings are particularly meaningful and consistent with previous research validating ECC as a relevant physiological welfare indicator in cetaceans (Agustí et al., 2025a, 2025b; Bechshoft et al., 2020; Wong et al., 2023).

In contrast, no relationships were found between ECC and either Motivated and engaged behavior or Body weight variation at any of the examined time lags. Although weight loss has been linked to elevated cortisol levels in several studies on marine mammals (Champagne et al., 2012; Kershaw et al., 2017), the weight fluctuations observed in the present study were markedly more minor, reflecting only slight changes within an otherwise stable body condition.

Overall, limitations arising from the small sample size, the low frequency of certain welfare indicators, and the opportunistic nature of welfare data collection have constrained our ability to detect additional relationships or to disentangle ECC variations in relation to specific welfare indicators. Although caregivers and veterinarians are well positioned to observe and document welfare-related behaviors (Meagher, 2009; Wemelsfelder, 2007; Whitham and Wielebnowski, 2013), the lack of systematic, validated behavioral scoring and the unexamined variability in the biological relevance of different indicators (Sandøe et al., 2019) may have limited the sensitivity and interpretability of the dataset. Future studies aimed at validating ECC as a physiological welfare indicator in killer whales would benefit from larger sample sizes and controlled paradigms designed to assess ECC responses to specific nutritional, health-related, social, or environmental conditions, including responses under more extreme scenarios.

4.5. Effects of welfare-related predictors on epidermal oxytocin concentrations

Unlike cortisol, the interpretation of EOC is still in an early, exploratory stage due to the limited body of prior research. Although oxytocin has been mostly studied in relation to social behaviors (Cavanaugh et al., 2016; Rault et al., 2017), our study was not specifically tailored to monitor the social dynamics that are thought to influence its release. This limitation is compounded by the fact that, in the context of animal welfare research, the oxytocinergic system remains substantially less characterized in mammals than the HPA axis (Rault et al., 2017), and available data in cetaceans are particularly scarce. Methodological uncertainties further complicate interpretation, especially the poorly understood relationship between central and peripheral oxytocin levels (McCullough et al., 2013; Rault, 2016), as well as the

unknown mechanisms by which oxytocin might be transported to or integrated into the skin. Therefore, the associations found between EOC and welfare indicators in this study should be approached with caution.

Negative welfare indicators showed significant positive associations with EOC at a time lag of 62–68 days prior to sampling. Although this may seem counterintuitive given the commonly proposed link between oxytocin and positive welfare states (Chang et al., 2012; Crockford et al., 2014), elevated EOC under presumed negative welfare conditions may reflect oxytocin's role in stress-coping mechanisms (see Section 4.4 for a detailed discussion on the relationship between Negative welfare indicators and stress). This interpretation aligns with oxytocin's documented stress-buffering function (Rault et al., 2017; Tops et al., 2012) and is further supported by the positive correlation observed between EOC and ECC in this study.

Importantly, these findings underscore the potential challenges of using and interpreting EOC as an indicator of positive or negative welfare in killer whales, and probably in cetaceans in general, particularly in the absence of more detailed information on the animals' behavior and welfare. At the same time, the stability and quality of social bonds and affiliative interactions are thought to modulate oxytocin release following stress, thus influencing an individual's capacity to cope with welfare challenges (Rault et al., 2017; Seltzer et al., 2010). Considering this, the findings of the present study open a promising line of research into the role of social support in managing adverse welfare conditions among captive killer whales and other highly social species.

Interestingly, Motivated and engaged behavior showed a positive relationship with EOC at time lags ranging from 55 to 75 days prior to sampling. As previously defined, this indicator captured an individual's positive and active involvement with its environment and willingness to engage in training or cooperative tasks, based on the caregivers' perception. The observed association is thus consistent with evidence linking oxytocin to positive affective states, environmental motivation, and reward-related behaviors (Choleris et al., 2009; Ross and Young, 2009), as well as to positive human–animal interactions (D'Aniello et al., 2022). Nevertheless, given the current absence of direct empirical evidence confirming these relationships either in killer whales or in other cetacean species, this interpretation must be also treated with caution, underscoring the need for further targeted research.

Although oxytocin has been suggested as a potentially valuable physiological indicator of positive welfare, EOC interpretation in cetaceans remains challenging due to methodological uncertainties and the still exploratory stage of epidermal hormone analysis. These difficulties are largely attributable to the interpretative challenges of peripheral oxytocin measurements, which may not reliably reflect central activity (McCullough et al., 2013; Rault, 2016), and to the fact that oxytocin can increase in both positive and negative contexts (Stock and Uvnäs-Moberg 1988) because of its broader role in stress regulation (Buisman-Pijlman et al., 2014). This issue may be particularly pronounced when using integrative matrices such as the epidermis, where the cumulative nature of hormone deposition can obscure distinctions between signals linked to positive affective states and those reflecting stress responses or negative states. To address these challenges, future research should prioritize validating oxytocin responses in blood or saliva within socially relevant contexts, enabling reliable links to short-term behavioral observations or more clearly defined welfare states. In parallel, a better understanding of peripheral oxytocin regulation and the mechanisms driving its incorporation into the skin will be essential to advance the validation of EOC as a potential welfare biomarker in cetaceans.

4.6. Temporal dynamics of cortisol and oxytocin incorporation into the epidermis

To establish the biological validity of ECC and EOC it is also important to determine the time intervals between the activation of the HPA axis or the oxytocin system, respectively, and the subsequent detection of hormonal changes in the collected epidermis. These intervals may differ between cortisol and oxytocin not only because of differences in their molecular properties and mechanisms of incorporation into the skin, but also due to hormone-specific degradation rates in peripheral tissues.Cortisol, a small and highly lipophilic steroid hormone, can diffuse passively through lipid-rich membranes during keratinocyte differentiation, likely facilitating its accumulation in the epidermis (Jeong et al., 2020). In contrast, oxytocin is a larger, hydrophilic peptide hormone that typically requires active transport mechanisms to cross lipid barriers, which may limit its passive deposition in epidermal layers (Gröschl, 2008; Jeong et al., 2020).

In the case of cortisol, its incorporation into cetacean skin is hypothesized to follow a model similar to that proposed for other keratinized tissues in cetaceans, such as whale baleen (Hunt et al. 2016; Noël et al., 2014) and earplugs (Trumble et al., 2018), as well as in the hair of other mammalian species (Russell et al., 2012; Reff et al., 2019). According to this model, hormone concentrations found in deeper epidermal layers reflect more recent physiological activity, whereas concentrations in outer layers, including the stratum corneum, represent earlier physiological states (Bechshoft et al., 2020). This temporal gradient presumably corresponds to epidermal turnover rates, unknown in killer whales but previously estimated at approximately 73 days in bottlenose dolphins (Hicks et al., 1985) and 70-75 days in beluga whales (St. Aubin et al., 1990). Interpreting epidermal hormone dynamics is further complicated by evidence from other mammals suggesting that cortisol and oxytocin are also synthesized locally within the skin, where they play essential roles in skin-specific processes, such as inflammation control, cell proliferation, and local neuroendocrine signaling (Deing et al., 2013; Pondeljak and Lugović-Mihić, 2020; Slominski et al., 2007; Vukelic et al., 2011). Consequently, integumentary hormone synthesis could alter epidermal hormone levels independently of systemic circulation and tissue growth dynamics, potentially affecting the expected temporal patterns. At the same time, hormones produced in the skin may influence central hormonal pathways via feedback mechanisms (Skobowiat and Slominski, 2015), reflecting a complex bidirectional interaction between local and systemic endocrine systems.

In the present study, biologically meaningful relationships between ECC and Negative welfare indicators were identified at time lags between 27 and 68 days prior to sampling. Notable gaps in these associations occurred at intervals of 34-40 and 48-61 days, which is difficult to interpret based on current knowledge. These results partially align with previously reported cortisol detection time lags in the epidermis of beluga whales (68–72 days; Wong et al., 2023) and bottlenose dolphins (40–53 days; Bechshoft et al., 2020; Agustí et al., 2025b), a correspondence that appears plausible considering their phylogenetic relatedness and shared morphological features.

Interestingly, associations between EOC and Negative welfare indicators were detected at a single time-lag window (62–88 days), which overlapped with one of the time lags where significant relationships were also observed for ECC. This convergence supports the potential biological relevance of the findings and reinforces the previously discussed physiological link between oxytocin and cortisol. Additionally, significant associations between EOC and Motivated and engaged behavior were found between 55 and 75 days, again coinciding with time windows in which relationships between ECC, EOC and other welfare indicators had been identified. This temporal alignment across variables, combined with previous findings on ECC time lags (Bechshoft et al., 2020; Wong et al., 2023; Agustí et al., 2025b) and skin turnover rates in other cetacean species (St. Aubin et al., 1990; Hicks et al., 1985), collectively supports the idea that meaningful hormonal responses could be detectable in the epidermis only after longer time lags (approximately $\geq$50 days). Nevertheless, these temporal delays are likely to differ between hormones, because cortisol and oxytocin follow distinct endocrine pathways and may be incorporated into the epidermis at different rates, which in turn may influence when and how strongly they reflect changes in physiological activity.

Another key aspect that requires further investigation is the time

frame over which hormones are incorporated into the epidermis, known as the integration window (Palme, 2019). This likely depends on both the rate of skin renewal and the depth of the tissue sampled. Hormone incorporation may also be influenced by peripheral blood flow and the extent of perfusion into the dermis and epidermis, which can vary with physiological and environmental factors such as stress responses, physical activity, and temperature (Bechshoft et al., 2020; Noren et al., 1999; Champagne et al., 2018). In addition, the ability to detect hormonal changes may vary with the intensity and duration of the stressor that triggered them. Therefore, it is important to assess how sensitive the epidermis is to both adrenocortical and oxytocinergic activity across a range of physiological states varying in intensity and duration, from mild and brief to intense and prolonged (Cook, 2012; Palme, 2019).

While our study does not allow us to confirm this directly, it is generally hypothesized that full-thickness epidermal samples may reflect hormone accumulation over approximately the entire skin turnover cycle, offering a broad overview of physiological status but potentially overlooking short-lived or subtle hormonal fluctuations (Agustí et al., 2025a, 2025b; Bechshoft et al., 2015; Cook, 2012; Touma and Palme, 2005). In contrast, superficial samples restricted to the outer *stratum corneum* may capture a shorter temporal window that, while still being longer than blood, saliva, feces or blubber, makes them potentially more sensitive to more acute and high-magnitude physiological states (Bechshoft et al., 2020).

It should also be taken into account that epidermal turnover rates in cetaceans are not fixed but can vary in response to multiple internal and external factors, hormonal fluctuations and environmental conditions such as temperature shifts (St. Aubin et al., 1990). These factors may influence the timing of hormone incorporation into the epidermis, introducing individual or population-level variability that should be considered when using these indicators to assess welfare. As an extreme example, killer whales inhabiting the cold waters of the Antarctic may temporarily suspend epidermal renewal as a thermoregulatory strategy to reduce heat loss (Durban and Pitman, 2012; Pitman et al., 2019), which could significantly affect the interpretation of ECC and EOC.

Overall, gaining clearer insight into the duration of epidermal renewal and the temporal dynamics of cortisol and oxytocin deposition in the skin remains a key research priority for further validating ECC and EOC. Experiments such as correlations with circulating concentrations, ACTH stimulation tests in animals under human care (Heistermann et al., 2006; Wasser et al., 2000) or studies involving radioactively labeled hormones (Keckeis et al., 2012), are needed to help clarify the temporal dynamics of hormone incorporation into the epidermis. In addition, further observational studies involving a range of affective states, differing in type, duration, and intensity, could help clarify both detection thresholds and the temporal dynamics of hormone deposition, thereby improving the interpretation of epidermal hormone concentrations and better establishing their relevance for welfare assessment.

5. Conclusions

In conclusion, this study optimized a feasible non-invasive method for collecting desquamated epidermis from killer whales under humane care. It also achieved the analytical validation of ECC and EOC quantification using AlphaLISA immunoassays. Although hormone concentrations did not differ significantly across body regions or skin coloration patterns, intra-individual variability and a right-side lateral asymmetry emphasized the need for standardized sampling protocols to avoid confounding factors. The study also provided findings supporting the ongoing biological validation of ECC and EOC, suggesting associations between both hormones and negative welfare indicators, as well as between EOC, positive welfare-related behaviors, and body weight variation. When considered alongside previous findings, our results provide preliminary support for ECC as a promising and feasible candidate biomarker for assessing retrospective, intermediate-term welfare changes in living cetaceans, particularly when embedded within a broader, multifactorial framework that integrates complementary physiological, behavioral, and contextual indicators. By contrast, EOC remains less reliable at this stage, largely due to the limited understanding of the oxytocinergic system and its involvement in both positive and negative affective states, which complicates its interpretation in the context of welfare assessment.

The study also presents several limitations, including small sample size and reliance on welfare data derived from routine monitoring, which have limited the ability to detect associations with specific aspects of welfare. These constraints highlight the need for future research involving larger and more diverse populations, as well as the development of more detailed, standardized tools for welfare assessment. In addition, advancing our understanding of epidermal turnover in this species, along with clarifying how cortisol and oxytocin are incorporated into the epidermis over time, are critical for improving the physiological interpretation and practical application of these biomarkers in cetacean welfare research.

CRediT authorship contribution statement

Clara Agustí: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xavier Manteca:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition. **Javier Almunia:** Writing – review & editing, Resources, Funding acquisition. **Marina López-Arjona:** Writing – review & editing, Methodology, Investigation. **José Joaquín Cerón:** Writing – review & editing, Resources. **Enrique Tejero:** Investigation, Formal analysis. **Nakita Câmara:** Writing – review & editing, Resources. **Laia Guix:** Investigation. **Oriol Talló-Parra:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2026.111993.

Data availability

The data supporting the findings of this study, including the analyses of the effects of body location and welfare-related predictors on epidermal cortisol and oxytocin concentrations, are included in the Supplementary Information files.

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