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Blubber under stress: *ex vivo* cortisol exposure induces an anti-inflammatory state in precision-cut adipose tissue slices from humpback whales

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Cetaceans are exposed to multiple anthropogenic stressors that may disrupt hormonal balance, notably through elevated release of circulating stress hormones, such as catecholamines and glucocorticoids. While stress response mechanisms are vital in the short term, prolonged stress can have serious health implications including impaired immunity. Yet, cause-effect relationships and mechanistic understanding about the impacts of elevated stress hormones on large cetacean health remain poorly understood due to the ethical and logistical challenges of studying free-ranging whales. To address this knowledge gap, the present study established an *ex vivo* precision-cut adipose tissue slice (PCATS) model from blubber biopsies collected from 13 humpback whales (*Megaptera novaeangliae*) in Norway. Metabolic viability of PCATS was assessed and confirmed by measuring ΔO_2 saturation in the culture medium, as a proxy for mitochondrial respiration. PCATS of humpback whales were further used to investigate the effects of stress hormone exposure on the relative mRNA levels of a set of 5 stress and immunoregulatory genes: heat-shock protein 70 (*HSP70*), toll-like receptor 4 (*TLR4*), peroxisome proliferator activated receptor gamma (*PPARG*), interleukin 10 (*IL10*) and tumor necrosis factor alpha (*TNF*). PCATS were incubated for 48 hours with 400 nM cortisol introduced every 12 hours to mimic a chronic stress response alone and combined with 10 µM epinephrine in the final 12 hours to simulate an acute stress response. Relative gene expression assessed through RT-qPCR, revealed that both cortisol alone and combined with epinephrine significantly upregulated *PPARG* and downregulated *TNF* and *TLR4* expression, suggesting that cortisol induces an anti-inflammatory state. The present findings call for in-depth transcriptomic analyses to identify which biological pathways may be compromised by stress hormones in cetaceans. Our study also opens new research avenues for using PCATS as an *ex vivo* model to investigate the effects of multiple stressors on free-living cetaceans.

KEYWORDS
cetacean, epinephrine, gene expression, immunotoxicity, Norway, oxygen consumption, stress hormones, viability

1 Introduction

Cetaceans play key roles as marine ecosystem engineers, promoting carbon sequestration, shaping trophic dynamics and facilitating nutrient transport for primary production (Kiszka et al., 2022; Roman et al., 2014). They are also sentinel species of ocean and human health (Bossart, 2011). Despite these essential functions, cetaceans are increasingly exposed to a suite of anthropogenic stressors including chemical pollution, resource depletion, noise, pathogens, and accidental catches and fishing gear interactions (Avila et al., 2018). These threats have the potential to increase the concentrations of stress hormones, such as glucocorticoids (Hing et al., 2016; Agustí et al., 2025). For instance, positive correlations have been observed between ship traffic and blubber cortisol concentrations in humpback whale (*Megaptera novaeangliae*) (Pallin et al., 2022), as well as fecal cortisol metabolites in North Atlantic right whales (*Eubalaena glacialis*) (Rolland et al., 2012).

The physiological stress response driven by hormones is an essential function to respond and adapt to environmental stressors. The mammalian acute stress response is characterized by a rapid and short-lived stimulation of the adrenal medulla by the sympathetic nervous system, that releases catecholamines (primarily epinephrine and norepinephrine) (Romero, 2004). In parallel, the hypothalamus-pituitary-adrenal (HPA) axis, cause the secretion of glucocorticoids (e. g., cortisol), peaking around 30 min post stimuli, to mediate the longer-term stress response. Cortisol binds to the glucocorticoid or mineralocorticoid receptors and regulates the expression of genes involved in key metabolic functions such as immunity, inflammation and energy homeostasis (Gerber et al., 2021; Timmermans et al., 2019). In captive beluga whales (*Delphinapterus leucas*), transportation and out-of-water examinations were associated with increased circulating cortisol concentrations and a concurrent downregulation of immune-related gene expression (Unal and Romano, 2021). Similarly, in captive bottlenose dolphins (*Tursiops truncatus*), exposure to high-level impulsive noise triggered stress responses, evidenced by elevated cortisol levels and increased transcription of an anti-inflammatory cytokine, despite the absence of behavioral changes (Yang et al., 2021).

While acute stress responses are transient and typically adaptive, enabling rapid coping with environmental challenges, persistent or unpredictable stressors that prevent recovery to baseline can lead to chronic stress (Romero and Butler, 2007; Sapolsky et al., 2000). A prolonged activation of the HPA axis can disturb hormonal regulation, impair energy balance, suppress immune function, and ultimately compromise individual reproduction and survival (Romero, 2004). For example, studies on wild bottlenose dolphins revealed that impaired stress hormone production through a reduction of adrenal cortices caused by the Deepwater Horizon oil spill, combined with thermal stress and heightened vulnerability to pathogens, likely contributed to high mortality within affected populations (Smith et al., 2017; Venn-Watson et al., 2015).

Blubber, the subcutaneous adipose tissue layer in cetaceans, is a multifunctional organ composed of adipocytes, preadipocytes, endothelial and immune cells. It plays key endocrine functions associated with energy balance, reproduction, immunity and inflammation, through the secretion of signaling molecules such as cytokines and adipokines (Coelho et al., 2013; Kershaw and Flier, 2004). In cetaceans, cortisol concentrations in blubber reflect circulating levels, suggesting that this tissue can serve as a reliable matrix for assessing systemic stress responses (Agusti et al., 2022; Champagne et al., 2017). Recent proteomics investigations on blubber biopsies from harbor porpoises (*Phocoena phocoena*) and minke whales (*Balaenoptera acutorostrata*) revealed that proteins associated with immunity and inflammation represented one of the largest functional groups in the tissue (Kershaw et al., 2024, 2018).

Inflammation is an essential defense mechanism of the immune system and is characterized by the recruitment of immune cells to the site of infection, as well as the activation of the adaptive immune system in lymphoid tissues (Abbas et al., 2016). Induction of inflammation is caused by pathogen- or damage-associated molecular patterns through pattern recognition receptors, such as toll-like receptor 4 (TLR4). Activation of these receptors induces the activation of a pro-inflammatory cascade, leading to the release of signaling molecules, including pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF). In adipose tissue of studied model species (i.e., humans and rodents), stress hormones act as immunosuppressants by inhibiting the expression of pro-inflammatory cytokines, and by activating anti-inflammatory polarization of adipocytes and macrophages. This process occurs through the upregulation of anti-inflammatory nuclear receptors such as peroxisome proliferator activated receptor gamma (PPARG), and increased secretion of anti-inflammatory cytokines, such as interleukin 10 (IL10) (Mogensen, 2009). The impacts of stress hormones have been extensively studied in humans and rodents and now, glucocorticoids are commonly prescribed for their immunosuppressive properties (Vandewalle et al., 2018). However, for marine mammals, there are significant data gaps regarding key immune endpoints, such as immunoregulatory genes and mechanisms of specific immune alterations (Desforges et al., 2016). Marine mammals are increasingly threatened by infectious diseases such as morbilliviruses and herpesviruses (Di Guardo et al., 2019; Costa et al., 2025), in addition to detrimental anthropogenic activities (e.g. accidental collisions with vessels and acoustic disturbances) which are known to induce a stress response in exposed populations (Koch et al., 2018). In wildlife, immunosuppression can further increase susceptibility to infectious diseases, impair wound healing (Wright et al., 2011; Atkinson et al., 2015) and may only become apparent once a population faces immune challenges (e.g. infectious diseases and injuries) potentially worsening the severity of an outbreak (Bossart and Duignan, 2019; Brousseau et al., 2012).

Correlative field studies have long been used to explore associations between stressor exposure and health in marine mammals (Buckman et al., 2011; Jewson, 1992; Linsky et al., 2024; Müller et al., 2013). However, these studies face inherent limitations, including the inability to establish causality, confounding factors, lack of control and logistical challenges. To overcome these limitations, *in vitro* and *ex vivo* approaches have emerged as promising alternatives (Vazquez et al., 2024). Blubber

biopsies, which are minimally invasive (Hunt et al., 2013) have enabled the development of precision-cut adipose tissue slices (PCATS), recently applied successfully on northern elephant seal (*Mirounga angustirostris*) blubber biopsies (Debier et al., 2020). This innovative approach, which prior to the current study, had not yet been tested or applied to cetaceans, represents a significant advancement in elucidating the mechanisms underlying chronic stress in large whales.

The present study aimed to establish an *ex vivo* model of precision-cut adipose tissue slices (PCATS) for cetaceans, using freshly collected blubber biopsies from free-living humpback whales. Traditional and established methods for assessing cell viability are often ineffective for thicker tissue cultures, disturbed by the slicing process (e.g., release of lactate dehydrogenase) or require the destruction of the tissue itself (e.g., the ATP assay). Thereby, the metabolic viability of the PCATS was assessed by measuring the oxygen consumption of the blubber tissue over time (ΔO_2 saturation) in the culture medium as a proxy for mitochondrial respiration. The PCATS model was then used to test the response to stress hormone exposure on the expression of 5 genes, commonly used as biomarkers of health and stress in cetaceans: heat shock protein 70 (*HSP70*), toll-like receptor 4 (*TLR4*), peroxisome proliferator activated receptor gamma (*PPARG*), interleukin 10 (*IL10*) and tumor necrosis factor alpha (*TNF*) (Hofstetter et al., 2020; Linsky et al., 2024; Müller et al., 2013; Unal et al., 2018). We anticipated that exposure to stress hormones would shift the tissue towards an anti-inflammatory balance, through an upregulation of *PPARG* and *IL10*, and a downregulation of *TNF* and *TLR4*. In addition, we expected the stress hormones would further upregulate *HSP70* expression as part of a cellular adaptive mechanism to mitigate proteotoxic stress.

2 Materials and methods

2.1 Biopsy sampling and PCATS preparation

A total of 13 free-ranging adult humpback whales were sampled in northern Norway (Kvænangen) in November 2024 (Supplementary Table 1, Supplementary Materials). The outer blubber biopsies were collected and rinsed in 1X phosphate-buffered saline (PanReac AppliChem, Darmstadt, Germany) prewarmed to 25 °C and placed into pre-oxygenated prewarmed sampling medium according to Debier et al. (2020). The biopsy samples were transported to the laboratory and processed in sterile conditions under a biological safety cabinet (ESCO® Lifesciences Group, Changi North, Singapore), within 24 to 48 hours after collection. Biopsies were first cut longitudinally to fit in the groove of the slicing device originally designed for slicing rat spinal cord (Mouse and Rat Spinal Cord Matrices, Ted Pella Inc., Redding, CA, United States), as described by Debier et al. (2020). Then, 1 mm-thick PCATS were prepared using razor blades (detailed in Supplementary Materials). Mean weight of one PCATS was 6.45 ± 1.97 mg (n=312 PCATS from 13 individuals).

2.2 Metabolic viability assay through oxygen consumption measurement

The metabolic viability of humpback whale PCATS (N = 6 individuals) was assessed by measuring ΔO_2 saturation in the culture media as a proxy for mitochondrial respiration using a respiratory microplate system (LoligoSystem, Viborg, Denmark). The ΔO_2 saturation was compared between freshly collected (fresh PCATS) and non-viable PCATS (negative control), and saturation for both treatments was also measured across three biopsy segments (innermost, middle, and outermost) to examine potential variations along the biopsy core. The negative control consisted of PCATS exposed to 3 cycles of flash freezing in liquid nitrogen for 10 min followed by thawing in a warm water bath (ED-13, Julabo GmbH, Seelbach, Germany) at 37 °C for 15 min. The assay was conducted in triplicate for each treatment and biopsy depth, for each individual. Two PCATS were transferred into each 500 µL wells in the closed system respirometry microplate with pre-warmed fresh culture media (37 °C) supplemented with 0.6% HEPES (Life Technologies) to enhance the buffering capacity of the culture medium. The microplates were sealed according to manufactures protocol and then placed into the incubator (Stuart Orbital Incubator 51500; Cole-Parmer, Staffordshire, UK) at 37 °C. Oxygen saturation in the culture medium was measured every 30 seconds for 20 hours of incubation. Values of culture medium alone were subtracted from the result to obtain ΔO_2 saturation (%).

2.3 Exposure to stress hormones

To mimic a chronic stress response, PCATS were incubated for 48 hours under repeated cortisol exposure, either alone or in combination with a short-term exposure to epinephrine to simulate an acute stress response. Due to a limited amount of subcutaneous adipose tissue available per individual, no additional treatment involving a short-term exposure to epinephrine alone could be tested. Exposure durations were selected to ensure PCATS metabolic viability throughout the entire culture and represent relatively short-term stress responses, as opposed to chronic effects typically associated with long-term exposure beyond tissue viability (Romero, 2004). The concentration of 400 nM of cortisol was selected based on cortisol levels measured in blubber of bycaught or stranded cetaceans and captive dolphins (Birukawa et al., 2005; Champagne et al., 2017; Guo et al., 2021; Mingramm et al., 2020). The concentration of 10 µM epinephrine was selected based on humpback whale PCATS response to lipolytic stimuli (Lambilotte et al., unpublished data).

Following preparation of PCATS, four PCATS from each whale sampled for the exposure study (N = 7 individuals) were placed into each culture well containing 1 mL of culture medium, following the protocol outlined by Debier et al. (2020). A minimum of 18 wells were prepared, ensuring that each condition (control, cortisol, and cortisol + epinephrine) included at least 6 wells. These wells contained PCATS collected along the biopsy depth from the innermost to the outermost.

The plates were placed on an orbital shaker (IKA KS 130 B S1, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 75 rpm in an incubator (MCO-18AIC, SANYO Electric Co., Ltd., Osaka, Japan)

at 37 °C with 5% CO_2. After 30 min of incubation, the culture medium was collected to remove cellular debris from slicing and renewed according to treatments: i) 400 nM cortisol applied every 12 hour, for 48 hours, ii) 400 nM cortisol applied every 12 hour for 48 hours, supplemented with 10 µM epinephrine for the last 12 hour of culture and iii) a solvent (vehicle) control without any hormone. Hydrocortisone (98%; Thermo Scientific, Waltham, MA, USA) was dissolved in ethanol (96%; VWR) and diluted in water, resulting in a final ethanol concentration of 0.002% in the culture medium. The equivalent concentration of ethanol was used in the control condition as a solvent (vehicle) control. Epinephrine Bitartrate (>98%; Thermo Scientific) was dissolved in water. After the experiment, PCATS were rinsed twice in RNase free 1X phosphate-buffered saline (Thermo Scientific Chemicals), snap frozen and stored at -80 °C.

2.4 mRNA expression analysis

2.4.1 RNA extraction

PCATS were pooled to reach ~ 65 mg, in duplicates per treatment. The pooled tissues was placed in 450 µL of QIAzol Lysis Reagent (QIAGEN GmbH, Hilden, Germany) with 2% Triton x-100 (SigmaAldrich, St. Louis, MO, USA), with a steel bead (4.5 mm, 0.35 g; BLASTER®, ActionSportGames A/S, Skanderborg, Denmark), and homogenized at 30 Hz in a Qiagen tissue lyzer II (QIAGEN GmbH) for 4 min. An additional 450 µL of QIAzol with 2% Triton x-100 were added and samples were incubated at room temperature for 30 min, according to recommendations from Linsky et al. (2022). The RNA was isolated with 200 µL of chloroform-isoamyl alcohol (24:1, Sigma-Aldrich), centrifuged at 4 °C in an Eppendorf 5418 R centrifuge (Eppendorf, Hamburg, Germany) for 15 min at 14,000 rpm. The duplicate isolates were pooled and cleaned according to manufacturer's protocol, using the Qiagen lipid mini kit (RNeasy Lipid Tissue Mini Kit, QIAGEN) with on-column DNase treatment (RNase-free DNase Set, QIAGEN). The RNA was eluted in 50 µL of nuclease-free water (QIAGEN) and purified with 1X volume of purification beads (NebNext purification beads, New England Biolabs, Ipswich, MA, USA). The final volume of 20 µL RNA held an average yield of 62.01 ± 35.57 ng/µL, with the purity of 2.00 ± 0.08 (260/280) and 1.69 ± 0.23 (260/230), measured with spectrophotometer (NanoDrop, Thermo Scientific). The RNA integrity number (RIN) was measured according to manufacturer kit (Bioanalyzer, Agilent 2100), with average RIN of 8.00 ± 0.82 (Supplementary Table S3, Supplementary Materials). RNA was reverse transcribed into complementary DNA (cDNA) according to manufacturer's protocol (High-capacity cDNA reverse Transcription Kit, applied biosystems).

2.4.2 RT-qPCR

Tyrosine 3-monooxygenase (*YWHAZ*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were selected as housekeeping genes based on previous application in skin biopsies from striped dolphin (Spinsanti et al., 2006). The target genes, *HSP70*, *TLR4*, *PPARG*, *IL10* and *TNF*, were selected based on three criteria: i) existing literature on gene expression in marine mammal blubber (Buckman et al., 2011; Kashiwabara et al., 2023; Linsky et al., 2024), ii) their use as biomarker of stress and immunoregulatory responses in correlative studies on cetaceans (Buckman et al., 2011; Hofstetter et al., 2020; Linsky et al., 2024; Müller et al., 2013; Unal et al., 2018), and iii) their mechanistic connection in terrestrial model species (da Cruz Nascimento et al., 2022; Daynes and Jones, 2002; Du et al., 2012). Primers were designed using the National Center for Biotechnology Information (NCBI) from the genome of blue whale (*Balaenoptera musculus*) as the genome of humpback whale was not available. The alignment of the blue whale primers to the humpback whale genome (megNov1 genomic assembly (GCA_004329385.1) and ASM4183430v1 (GCA_041834305.1)) was verified with Basic Local Alignment Search Tool (Altschul et al., 1990). Primers were supplied by Merck (Life Science, Darmstadt, Germany) (Supplementary Table S4, Supplementary Materials). The RT-qPCR was run in a QuantStudioTM 6 Pro (Applied Biosystems) with FAST SYBR Green detection Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol (Supplementary Figure S2: Melt curves, Supplementary Materials). The fold change was calculated according to the Pfaffl method (Pfaffl, 2001), between treatments and control, and the relative gene expression (RGE) was normalized to the gene expression in the control and set around 1 for interpretability.

2.5 Statistical analyses

All statistical analyses were conducted on R (version 4.3.0). To investigate ΔO_2 saturation in culture medium, a linear mixed-effects model (lme4 (Bates et al., 2015)) was applied with log transformed ΔO_2 saturation as response variable, and the interaction term of time and living status (fresh PCATS or negative control), biopsy depth (inner, middle, outer) as predictors and ID as a random effect. The significance threshold was set as $p < 0.05$ (lmeTest) (Kuznetsova et al., 2017)). The values were transformed back from log1p for better interpretability, and the time was converted to hourly intervals instead of 30-second intervals. The effect of treatments on gene expressions in PCATS of humpback whales was investigated with the same model using log-transformed relative gene expression as response variable, treatment (control, cortisol, cortisol with epinephrine) as predictor, and sample ID as a random effect. Due to challenges to meet the model assumptions for *TNF*, the Wilcoxon non-parametric test was also applied as a cross-validation by using the "wilcox.test" function in R, run twice (i.e., parameterized with paired samples = "True" and "False"). The effect on each gene was tested separately. Diagnostic plots (i.e., residuals vs fitted, scale-location, QQ-plot) were investigated to assess whether the data met model assumptions. The final values of relative gene expression are presented as mean ± standard deviation along with a 95% CI [lower; upper].

3 Results

3.1 Metabolic viability through oxygen saturation measurements

The ΔO_2 saturation in culture medium holding the fresh PCATS, decreased significantly over the 20 hours (Table 1 and Figure 1, $p <$

0.001). While the ΔO_2 saturation of the negative control remained within the value of culture medium itself. The depth of the biopsy significantly affected the ΔO_2 saturation in the fresh PCATS, with the greatest decrease observed in PCATS derived from the outermost biopsy section, followed by the middle section, and the smallest decrease occurring in the innermost section (Table 1, $p < 0.001$; Supplementary Figure 1, Supplementary Materials). Inter-individual variance in oxygen saturation measurement was low (Supplementary Table S5, Supplementary Materials).

3.2 mRNA transcript levels in stress hormone-exposed PCATS

The expression of key genes encoding biomarkers of health and stress in cetaceans was measured (Figure 2). *PPARG* was significantly upregulated in PCATS following exposure to cortisol by 1.69-fold change ($p < 0.01$) as well as cortisol with epinephrine by 1.35-fold change ($p = 0.034$) compared to control. Conversely, *TLR4* was significantly downregulated in PCATS following exposure to cortisol, by 0.65-fold change ($p < 0.01$) and cortisol with epinephrine by 0.70-fold change ($p = 0.038$) compared to control. Correspondingly, *TNF* was also downregulated by 0.48-fold change ($p = 0.014$) by cortisol, and by 0.51-fold change ($p = 0.017$) by cortisol with epinephrine. The exposure to stress hormones did not significantly impact the expression of *HSP70* and *IL10* ($p > 0.36$). Finally, there were no significant differences between mRNA expressions of the selected genes between PCATS exposure to cortisol compared to cortisol with epinephrine ($p > 0.28$). As represented in Figure 2, a high variance in the expression of target genes was noted between individuals (Supplementary Table S5, Supplementary Materials).

4 Discussion

4.1 Metabolic viability of PCATS

This study is the first to establish an *ex vivo* precision-cut adipose tissue slice (PCATS) model from cetacean blubber biopsies. The saturation values show that PCATS from humpback whales consume oxygen and thus suggest they can remain viable for at least 20 hours after slicing, and 48 hours after being collected in the field. This constitutes important knowledge, as immediate access to laboratory facilities is often unavailable following biopsy sampling of cetaceans, requiring a delay in processing. In contrast, tissue slices in other species (e.g., northern elephant seal or humans) are typically prepared immediately upon collection, using a similar cutting method to the present work (Debier et al., 2020; Schopow et al., 2020). A similar approach using different instruments has been applied previously to track temporal oxygen consumption and confirm viability of blubber explants in grey seals incubated over 24 hours (using Fibox 4 trace oxygen meter and oxygen sensitive optodes PreSens Precision Sensing; Robinson et al., 2021), as well as in human subcutaneous adipose tissue explants incubated for 1.5 to 2.5 hours (using Oroboros Instruments; Kraunsøe et al., 2010). The approach applied in the current study is promising because, unlike more established viability methods on cells, the measurement is not overshadowed by the destruction of peripheral cells during PCATS preparation, as seen with the lactate dehydrogenase assay, nor does it require the destruction of the PCATS itself, as with the ATP assay. This allows the tissue to be preserved and utilized further. Consequently, oxygen saturation measurements emerge as a relevant method for assessing metabolic viability in PCATS. The closed system respirometry microplate used in the present study (Microplate system (core), Loligo® Systems) is an easy to use, readily transportable, high-throughput instrument originally designed for measuring individual continuous respiration rates in tiny aquatic and terrestrial organisms (Daase et al., 2018; Renner et al., 2022).

The ΔO_2 saturation showed high intra-individual variations, which may be caused by differences in metabolic activity along the blubber core rather than differences in metabolic viability (Supplementary Figure 1, Supplementary Materials). In grey seal blubber explants, the innermost part of the full-depth biopsy had higher consumption than the outermost (Robinson et al., 2021). In contrast, the present study found the opposite trend with higher consumption in PCATS derived from the outer part of biopsies. The

TABLE 1 Summary of the linear mixed-effects model with the response variable log1p (ΔO_2 saturation per 30 seconds) with the interaction between time (hour) and status (fresh PCATS vs. negative control) as predictors, along with depth of biopsy (inner, middle or outer).

	Log1p (ΔO_2 per 30 seconds)					ΔO_2 per hour	
Predictor	Estimate	SE	Degrees of freedom	t-value	p-value	Estimate	SE
Intercept	-1.177	0.167	5.04	-7.03	< 0.01	0.069	0.18
$\Delta O_{2\%}$ over time	-0.0008	< 0.0001	85129	-107.74	< 0.001	-0.096	<0.001
Negative control intercept	0.82	0.01	85129	57.17	< 0.001	1.28	0.02
Time:Negative control	0.0009	< 0.0001	85129	80.89	< 0.001	0.10	< 0.01
Depth middle:inner	0.07	< 0.01	85129	7.69	< 0.001	0.07	< 0.01
Depth middle:outer	-0.30	< 0.01	85129	-34.20	< 0.001	-0.26	< 0.01
Depth inner:outer	-0.37	< 0.01	85129	-41.89	< 0.001	-0.31	< 0.01

Humpback whale ID was included as random effect. The ΔO_2 per hour correspond to the estimates and standard error (SE) of time scaled to every hour and transformed back from log1p for interpretability. The ΔO_2 saturation decreased over the 20 hours with an estimated slope of -0.096 per hour ($p < 0.001$). The negative control remained close to 0, the value of culture medium itself. The depth of the biopsy also influenced the ΔO_2 saturation in the fresh PCATS, with ΔO_2 saturation decreasing at a higher rate for PCATS from the outermost biopsy section, followed by the middle and innermost respectively ($p < 0.001$).

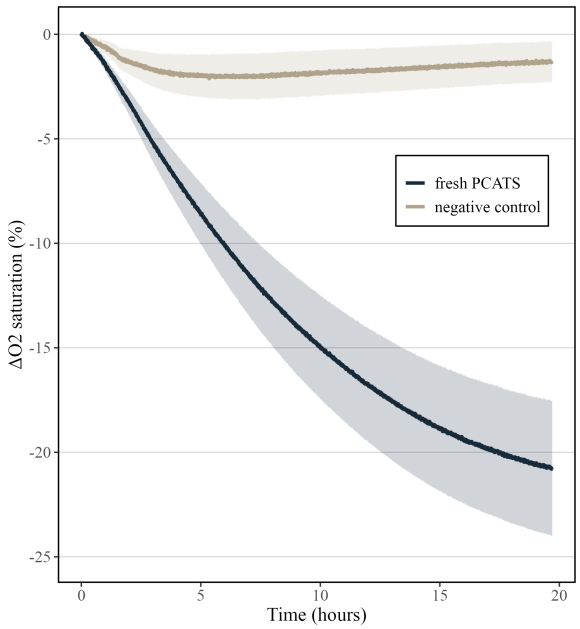

FIGURE 1
The ΔO_2 saturation (%) in culture medium containing fresh and negative control precision-cut adipose tissue slices (PCATS) of humpback whales (N = 6 individuals) incubated at 37 °C and measured every 30 seconds for 20 hours. Shaded area represents the standard error. The PCATS were made from blubber from all sections of ~6 cm long biopsies.

innermost part of marine mammal blubber is known to have higher vascularization than the middle and outermost sections (Khudyakov et al., 2022; McClelland et al., 2012; Robinson et al., 2021). Importantly, the biopsies obtained from humpback whales in the present study measured approximately ~ 6 cm in length (including the skin part). However, considering that the average blubber depth of humpback whales prior to migration has been reported to be 12.1 ± 1.0 cm (Waugh et al., 2014), the biopsies collected in the present study were only including middle and outer layers. Also, compared to biopsies collected directly with control from the animals on land (e.g. seals), cetacean biopsies are remotely collected from a distance with an air gun, which can cause mechanical deformation to the tissue, potentially compromising the integrity of the innermost parts of biopsies.

Robinson et al. (2021) also observed higher oxygen consumption rates in explants from seals in feeding state compared to those in fasting state, emphasizing the need to account for feeding state into the model. The humpback whales sampled for the current study are long-range migratory whales using the Norwegian Sea as a migration corridor. They travel between their summer feeding area characterized by high primary productivity mainly located in the northern Barents Sea east of the Svalbard archipelago (Leonard and Øien, 2019) and their breeding ground located in warm tropical waters of the West Indies or Cape Verde with low food availability (Kettemer et al., 2022). Humpback whales gather during the wintertime in northern Norway to feed on the abundant schooling Norwegian spring-spawning (NSS) herring (*Clupea harengus*). Some individuals might have been feeding intensively for weeks while other might have just arrived from their migration departure. This could potentially contribute to variations of ΔO_2 saturation between individuals. The application of respirometry to confirm metabolic viability of PCATS from humpback whales represents a steppingstone. We encourage future studies to refine the current protocol by increasing the amount of PCATS placed in each culture well to narrow confidence intervals and better distinguish potential differences between treatments, as well as extending the duration of the assay (at least up to 48 hours). Finally, we encourage the application of additional assays to complement the present viability assessment of PCATS (e.g. ATP and MTT assays) (Van de Bovenkamp et al., 2006), and to demonstrate its functionality in several cetacean species. Finally, cellular damage and apoptosis measured through lactate dehydrogenase assay (Debier et al., 2020) and immunofluorescence histology (Schopow et al., 2020) are relevant to complement the present respirometry assay applied on PCATS.

4.2 Stress hormone exposure

PPARG, which encodes a master regulator transcription factor involved in anti-inflammatory activation and lipid metabolism, was upregulated by cortisol alone and in combination with epinephrine in humpback whale PCATS. Available literature on the regulation of *PPARG* in cetaceans mainly refers to toxicologic impacts of environmental contaminants on its expression (Lühmann et al., 2020; Sun et al., 2023). On the other hand, data on the effects of stress hormones on *PPARG* expression in marine mammals remain limited. An *in vivo* study conducted on northern elephant seal showed that *PPARG* was upregulated in blubber two hours after adrenocorticotropic hormone (ACTH) injection, which triggered a rise in circulating cortisol levels (Khudyakov et al., 2017). These results are consistent with the present findings. However, in PCATS of northern elephant seal exposed to 2 µM cortisol every 12th hour for 48 hours, either alone or in combination with 100 nM of epinephrine for the last 12 hours, transcriptomic analysis revealed that cortisol alone had no effect on the expression of *PPARG* or its upstream regulators (Kashiwabara et al., 2023). Interestingly, in PCATS exposed to 100 nM epinephrine alone, there was an upregulation of the PPARG co-activator *PPARGC1A* and a downregulation of the PPARG corepressor *ASXL1*. When cortisol and epinephrine were combined, expression of the PPARG co-activator was also upregulated in PCATS of northern elephant seal (Kashiwabara et al., 2023). In the present work, the single effect of epinephrine could not be tested on humpback whales PCATS due to limitations in the amount of tissue available. The present findings align with studies conducted on humans and rodents, showing an upregulation of *PPARG* in cortisol- and dexamethasone-exposed adipocytes (Lahnalampi et al., 2010). Beyond its role in anti-inflammatory processes, *PPARG* is a key regulator of energy homeostasis, especially fat differentiation and maturation of pre-adipocytes. The upregulation of *PPARG* by cortisol suggests enhanced preadipocyte maturation as well as increased lipolytic activity in differentiated adipocytes, as observed in terrestrial models (i.e., humans and rodents) (Bolsoni-Lopes and Alonso-Vale, 2015; Lefterova et al., 2014; Tontonoz and Spiegelman, 2008). Follow-up studies are needed to simultaneously measure the release of lipolytic products into culture media to quantify release of fatty acids and glycerol, and the expression of *PPARG* in PCATS, as well

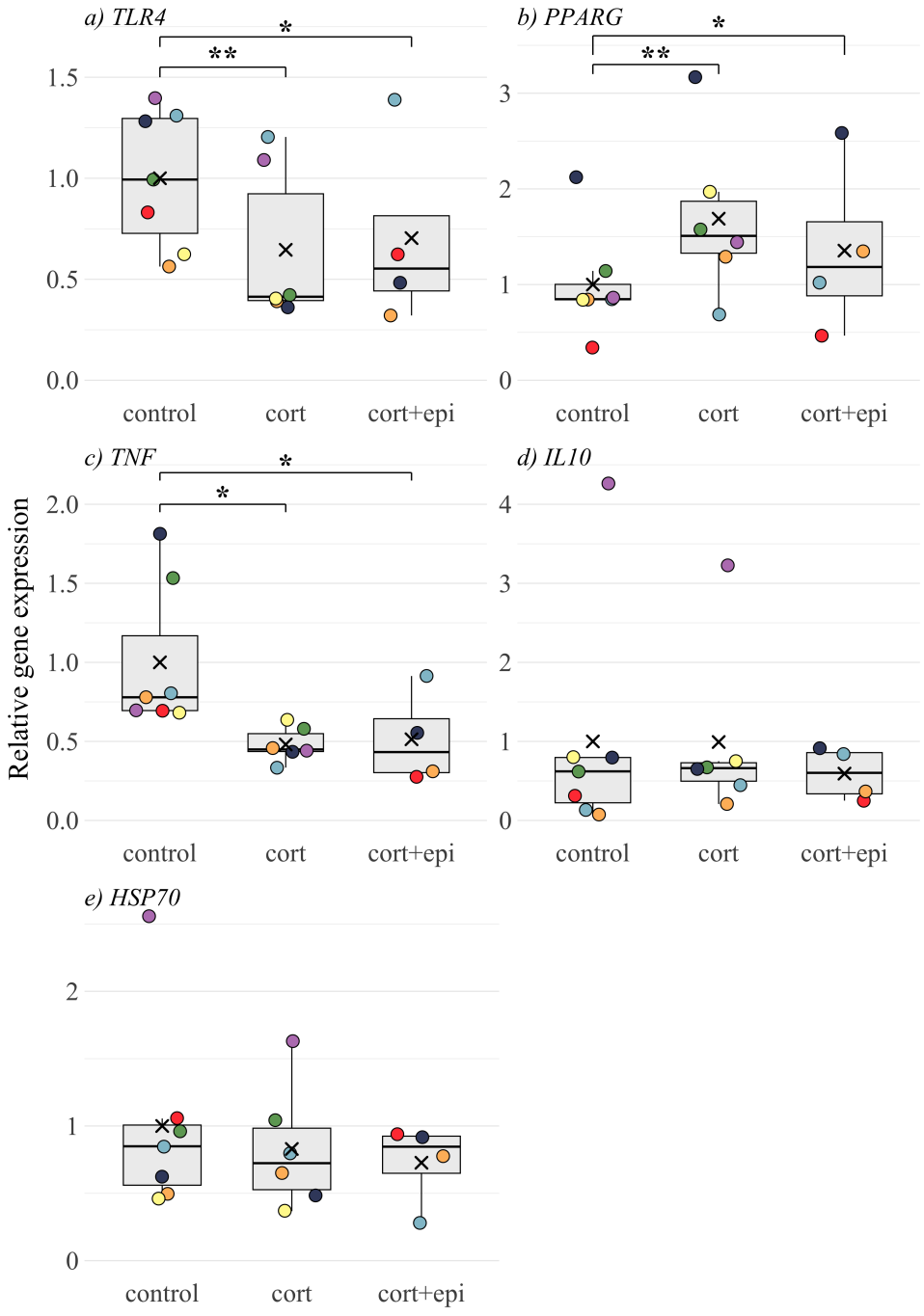

FIGURE 2
Comparisons of the relative mRNA expression of 5 genes relative to the control in precision-cut adipose tissue slices (PCATS) of humpback whales exposed to 400 nM cortisol ("cort") every 12th hour for 48 hours alone, or in combination with 10 μM epinephrine ("cort+epi") for the last 12 hours. **(a)** toll-like receptor 4 (*TLR4*), **(b)** peroxisome proliferator-activated receptor gamma (*PPARG*), **(c)** tumor necrosis factor alpha (*TNF*), **(d)** interleukin 10 (*IL10*) and **(e)** heat shock protein 70 (*HSP70*). The median is indicated by a solid black line, while the mean is marked with an "x" symbol. Each color represents the whale ID; control (N = 7), cort (N = 6), and cort+epi (N = 4). Statistical significance from linear mixed-effect model is indicated with asterisks (**: $p < 0.01$, *: $p < 0.05$).

as to assess the effect of epinephrine alone to describe the specific impact of an acute stress response on *PPARG* expression. This is particularly relevant given that humpback whales are capital breeders and rely on their fat reserves to survive during their seasonal fasts. The effect of stress hormones on energy homeostasis may therefore have important ecological implications, and future studies should investigate the connection between these feeding cycles and anthropogenic stressors.

The observed downregulation of *TLR4* in humpback whale PCATS following exposure to cortisol alone or in combination with epinephrine could have implications regarding the responsiveness to ligands such as lipopolysaccharides (LPS), which are major compounds of outer membranes in gram-negative bacteria (Takeda et al., 2003) that typically elicit a strong immune reaction. Although cetacean TLR4 has been described as less responsive to LPS induction compared to terrestrial species

(humans and bovines) (Tian et al., 2019), its expression was upregulated in the peripheral blood mononuclear cells of sick compared to healthy captive belugas, highlighting its link with immune responses in cetaceans (Hofstetter et al., 2020). The present result aligns with a correlative field study conducted on beluga whales showing that *TLR4* expression was negatively related to cortisol levels in blood and skin samples (Unal et al., 2018). Conversely, an *in vivo* study conducted on northern elephant seal showed that *TLR4* was upregulated in blubber 2 hours after ACTH injection and subsequent increase in circulating cortisol (Khudyakov et al., 2017). In the study of Kashiwabara et al. (2023) on northern elephant seal PCATS, *TLR4* expression was upregulated following combined exposure to cortisol and epinephrine, whereas no regulation was observed with cortisol alone (Kashiwabara et al., 2023). These differences highlight species- or tissue-dependent expression and suggest that exposure time or concentrations may also influence the expression of *TLR4*.

The downregulation of *TNF* in PCATS exposed to cortisol alone and combined with epinephrine corroborates findings from a correlative field study using blubber biopsies of humpback whale, showing that individuals with higher cortisol concentrations had decreased *TNF* expression (Linsky et al., 2024). Similarly, free-swimming harbor porpoises subjected to capture and release procedures, were found to have higher cortisol levels and a non-significant trend toward lower humoral *TNF* compared to captive individuals (Müller et al., 2013). The present results also align with well-established glucocorticoid-mediated regulation of *TNF* in human and rodent adipose tissue (da Cruz Nascimento et al., 2022), demonstrating a consistent response to glucocorticoid in fat tissues across different species. As observed for *TLR4, TNF* was also upregulated in the peripheral blood mononuclear cells of sick compared to healthy captive belugas (Hofstetter et al., 2020), highlighting its role in immune responses in cetaceans.

The current study did not reveal any regulation of the anti-inflammatory cytokine *IL10* or the chaperone protein *HSP70* following exposure to cortisol either alone or combined with epinephrine. Yet, as the approach captured the expression of mRNA of the target gene, the absence of expression changes for *IL10* and *HSP70* does not exclude indirect effects of cortisol on their regulators (e.g. receptors or chaperones). For instance, in PCATS of northern elephant seals exposed to 2 µM cortisol for 48 hours, the alpha receptor of *IL10* (*IL10RA)* was upregulated (Kashiwabara et al., 2023). In humans, IL10 is primarily known for its anti-inflammatory properties associated to humoral immunity (Fang and Zhu, 2020). Similarly, *IL10* expression was found to be downregulated in the peripheral blood mononuclear cells of sick captive beluga whales compared to healthy individuals (Hofstetter et al., 2020). A correlative study on free-ranging harbor porpoises showed a positive association between cortisol and *IL10* levels in blood (Müller et al., 2013). Yet, the inflammatory role of IL10 in adipose tissue remains poorly referenced. An *in vitro* study showed that human macrophages (THP-1) exposed to 0.3 µM of dexamethasone for 24 hours exhibited reduced *IL10* expression when incubated alone, whereas co-culture with preadipocytes (SGBS) increased its expression. As for *IL10*, the expression of *HSP70* was not regulated by stress hormones in humpback whale PCATS. Yet, the correlative study on blood from capture-release harbor porpoises found a positive relation between cortisol concentrations and *HSP70* expression (Müller et al., 2013). The present result aligns with correlative studies showing no link between anthropogenic stress (i.e., ship traffic and pollutants) and *HSP70* expression in the blubber of striped dolphins (Fossi et al., 2013), nor in cortisol concentration and *HSP70* in humpback whale blubber (Linsky et al., 2024). Further research is needed to investigate the impact of stress hormones, individually and in combination, on the regulation of IL10 and HSP70, ideally using transcriptomic approaches that allow for broad screening of genes encoding IL10 regulators, receptors and chaperones.

This study demonstrated a viable *ex vivo* PCATS model for cetaceans and demonstrated its utility for investigating the effects of anthropogenic stressors. Cortisol exposure induced changes in the expression of *PPARG, TLR4* as well as *TNF* suggesting that cortisol may trigger an anti-inflammatory state in humpback whale blubber. These findings align with known cortisol-mediated regulatory networks in humans and rodents (da Cruz Nascimento et al., 2022; Timmermans et al., 2019) and support the use of *PPARG, TLR4* and *TNF* as potential biomarkers of stress in cetaceans (da Cruz Nascimento et al., 2022; Timmermans et al., 2019). Notably, the downregulation of *TLR4* may have implications regarding the responsiveness to ligands such as *Brucella* spp., a pathogen of emerging concern for marine mammals (Mazzariol et al., 2018). These results also highlight the ecological implications of stress-induced immune modulation, particularly in individuals exposed to frequent anthropogenic activities (e.g., acoustic disturbances from boat traffic, whale watching touristic activities and non-lethal entanglements in fisheries) during seasonal feeding aggregations, as observed in northern Norway, which may contribute to the emergence of infectious hotspots (Bjørge et al., 2023; Hale, 2025). Furthermore, populations of cetaceans face additional challenges such as high contamination by persistent organic pollutants accumulating in blubber (Jepson and Law, 2016), underscoring the need to understand the combined effect of multiple stressors. While epinephrine is known to regulate a wide range of genes involved in key biological processes, such as energy homeostasis and inflammation, in humans, rodents and northern elephant seal PCATS (Barnes et al., 2015; Collins, 2022; Pirard et al., 2023), its specific effects on cetacean blubber remains underexplored. Investigating its role could provide valuable insight into the acute stress response in these fasting-adapted species (Bjørge et al., 2023).

Our study was conducted on a limited sample size, and we cannot rule out the possibility that inter-individual metabolic variations, in response to different nutritional states, might limit the possibility to demonstrate differential expression of *IL10* and *HSP70* between treatments. The use of 6 cm tips for biopsy sampling in large whales, such as humpback whales, enables the collection of blubber samples from the middle/outermost layers, known to be less affected by nutritional status as compared to the more metabolically active innermost section (Khudyakov et al., 2022; McClelland et al., 2012). Nevertheless, we encourage future studies to document the body condition of sampled whales, using photogrammetry, to implement the potential role of nutritional status and health conditions in shaping individual responses

(Napoli et al., 2024). More generally, we acknowledge the importance of assessing and disentangling the role of biological, ecological and life-history traits in individual variations of toxicological responses to stressors *in vitro/ex vivo*. Additional individual factors, such as sex, age, reproductive status, immune status (e.g. presence of injuries or infections) and endogenous levels of stress hormones may have driven the observed inter-individual variation. The *in vitro/ex vivo* approach enables the inclusion of each sampled individual within all conditions enhancing the ability to detect effects that might otherwise remain undetected or be concealed by individual variation. Finally, additional studies involving the application of in-depth transcriptomics analyses (RNAseq) are needed to gain insights into stress hormone-regulated immune and metabolic processes. The development and use of an *ex vivo* PCATS model is a promising approach to assess cumulative impacts of multiple stressors in cetaceans including e.g., direct disturbances, chemical pollution, nutritional stress, and pathogens. Such knowledge is ultimately crucial for the conservation of marine mammals.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Norwegian Food Safety Authority Permit FOTS ID 30924. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

VM: Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Investigation, Methodology. DO: Writing – review & editing, Investigation, Methodology. JN: Resources, Writing – review & editing. AR: Writing – review & editing, Resources. AW: Investigation, Writing – review & editing. CD: Writing – review & editing. KB: Writing – review & editing, Methodology. PB: Writing – review & editing, Funding acquisition, Conceptualization, Resources, Project administration, Supervision. LP: Supervision, Conceptualization, Writing – review & editing, Project administration.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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