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Blubber and serum cortisol concentrations as indicators of the stress response and overall health status in striped dolphins

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ABSTRACT

The impacts of environmental changes and anthropogenic threats in marine mammals are a growing concern for their conservation. In recent years, efforts have been directed to understand how marine mammals cope with stressors and to assess and validate stress biomarkers, mainly levels of glucocorticoid hormones (e.g. cortisol) in certain body tissues. The aims of this study were to assess the impact of different causes of stranding (chronically affected and bycaught striped dolphins) on cortisol concentrations in serum and in blubber; and to evaluate the association between cortisol levels in these tissues. Blubber and blood samples were collected from striped dolphins ($n = 42$) stranded on the Mediterranean coast between 2012 and 2018. Cortisol concentrations were measured by using enzyme immunoassay. A high correlation was found between circulating and blubber cortisol concentrations ($R^2 = 0.85$, $p < 0.01$). Necropsies and pathological studies concluded that a third of the dolphins were bycaught in fishing nets and released by fishermen (Bycaught animals group), while the other two thirds were euthanized, or died, due to a disease or chronic condition (e.g. calves separated from the mother or animals infected with dolphin morbillivirus or *Brucella ceti*) that impeded survival (Chronically affected animals group). Cortisol concentrations (mean ± SD) were six times higher in chronically affected animals (35.3 ± 23 ng cortisol/g blubber and 6.63 ± 3.22 μg cortisol/dl serum) compared to those bycaught in fishing nets (6.2 ± 4.3 ng cortisol/g blubber and 1.15 ± 1.51 μg cortisol/dl serum). Results suggests that serum and blubber cortisol concentrations can contribute in inferring the overall health and welfare of free-ranging cetaceans. However, further research is required to understand better the kinetics of blubber cortisol incorporation and removal, the factors involved in these processes, and the local conversion of cortisol in the blubber.

1. Introduction

As inhabitants of a changing environment, wild animals are exposed to physical or psychological events that can disrupt their homeostasis. The stress response occurs when the central nervous system perceives these events as a threat, developing a variety of behavioural and physiological responses that allow the animal to cope with a dynamic environment (Moberg and Mench, 2000). Anthropogenic stressors in marine ecosystems, such as boat traffic, fisheries interactions, noise or pollution, are increasing and could be affecting the ability of marine mammals to cope with stress and maintain populations' sustainability (Fair and Becker, 2000). Marine mammal communities have been described to be at high-risk in 47% of coastal waters worldwide (Avila et al., 2018). In this context, understanding the interactions between stressors and marine mammal welfare, fitness and survival is necessary to guide effective conservation efforts (Papastavrou et al., 2017).

The hypothalamic-pituitary-adrenal (HPA) axis is a crucial element of the stress response, releasing glucocorticoids into the bloodstream when activated (Sapolsky et al., 2000). When released after an exposure to short-term stressors they are considered beneficial and indicative of

Abbreviations: HPA, hypothalamic-pituitary-adrenal; BCC, blubber cortisol concentrations; SCC, serum cortisol concentrations; EIA, enzyme immunoassay; CBG, cortisol binding globulin.

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resilience (Mostl and Palme, 2002), but the continued releasing due to persistent stressors can have a direct negative effect on fitness (Romero et al., 2009). Glucocorticoids are also mediators of essential metabolic processes such as fat metabolism or energy regulation (Sapolsky et al., 2000). Cortisol is one of the main glucocorticoid hormones secreted in marine mammals and there is an increasing interest in its assessment as a biomarker for stress, health and physiological state (Atkinson et al., 2015; Kellar et al., 2015; Kershaw et al., 2017; St. Aubin et al., 2001; Trana et al., 2016).

Cortisol concentrations in cetaceans can be measured in multiple matrices such as serum or plasma, saliva, respiratory vapour ('blow'), urine, faeces, blubber or skin (Bechshoft et al., 2015; Champagne et al., 2018; Champagne et al., 2017; Fair et al., 2014; Hart et al., 2015; Mingramm et al., 2019; Pedernera-Romano et al., 2006; Suzuki et al., 1998). Alternatives to the use of blood present advantages as most are less invasive and facilitate hormonal assessments in free-ranging individuals. Moreover, alternative matrices can offer different insights of cortisol levels over time, and are less affected by the time of the day, the individual differences in circadian rhythms, and the transient exposure to acute daily stressors or capture stress (Lightman et al., 2008).

Blubber is the specialized hypodermic adipose tissue found in marine mammals and circulating cortisol, as a lipophilic compound, can diffuse to fatty tissues (Hunt et al., 2013). Blubber cortisol concentrations (BCC) have been measured in a variety of marine mammals in recent years (Atkinson et al., 2019; Galligan et al., 2019; Kellar et al., 2015; Kershaw et al., 2017).

For instance, beach-stranded short-beaked common dolphins (*Delphinus Delphis)* showed sixfold higher BCC than bycaught individuals (Kellar et al., 2015) while ice-entrapped beluga whales (*Delphinapterus leucas*) presented a sevenfold increase of BCC when compared with harvested ones (Trana et al., 2016; Trana et al., 2015). Moreover, elevated BCC have been related with higher calf mortality and disease in indo-pacific humpback dolphins (*Sousa chinensis*) (Guo et al., 2022). Overall, these studies revealed blubber as an alternative matrix to blood useful for assessing the stress response in cetaceans. Moreover, blubber cortisol concentrations increased during fasting season in wild harbour seals (*Phoca vitulina*) (Kershaw and Hall, 2016) and during substantial nutritional deficits in short-beaked common dolphins (Kellar et al., 2015) and harbour porpoises (Kershaw et al., 2017), evidencing the role of cortisol during highly energetically demanding periods in these species.

Interestingly, elevations in BCC in association with serum cortisol concentrations (SCC) have been observed in samples collected simultaneously (Champagne et al., 2018; Champagne et al., 2017). This relationship was detected after performing hormonal challenges with hydrocortisone (Champagne et al., 2017) and out-of-water stress tests (Champagne et al., 2018) in captive bottlenose dolphins (*Tursiops truncatus*). Recently, Mingramm et al. (2019) and Sherman et al. (2021) also found that patterns in blubber and serum steroid hormones levels of bottlenose dolphins were positively correlated. Overall, previous work highlights that BCC determination can be a good tool to assess cortisol levels in free-ranging animals.

The striped dolphin (*Stenella coeruleoalba*) is the most abundant cetacean in the Mediterranean Sea. It is considered 'Vulnerable' by the IUCN (Aguilar and Gaspari, 2012), with incidental captures in fishing nets listed as one of the main threats. In the Catalan coast, the most frequent cause of death in stranded striped dolphins between 2012 and 2019 was bycatch (Cuvertoret-Sanz et al., 2020), which emphasizes an existent need to study and address better this threat in the western Mediterranean Sea (Aguilar and Gaspari, 2012; Bearzi, 2002).

The aims of this study were to assess the impact of different causes of stranding on dolphins. We assessed serum and blubber cortisol concentrations in two groups: dolphins that died during bycatch events and dolphins that stranded as a result of chronic effects (bycatch and chronically affected, respectively). Furthermore, we evaluated the association between cortisol levels in serum and blubber.

2. Materials and methods

2.1. Ethics statement

All samples for this study were collected post-mortem after carcasses were recovered by the Marine Fauna Stranding Network of the Catalan Government. No animals were directly targeted for this or any other associated study. Handling of carcasses was done with the official governmental permits AG-2015-474, PTOP-2016-663 and PTOP-2021-14,

2.2. Study area and animals

Samples were collected from 42 stranded striped dolphins along the North-western Mediterranean Sea, between June 2012 and February 2018. All carcasses were necropsied at the Veterinary School of the Universitat Autònoma de Barcelona (UAB) by ECVP-certified pathologists, according to established procedures (Kuiken, 1991). Details about the subsequent pathological investigation can be found at (Cuvertoret-Sanz et al., 2020). The preservation status of the carcasses was classified on a scale from 1 (very fresh) to 5 (very autolytic), according to (Jauniaux et al., 2005). The percentages of carcasses in the preservation states 1, 2, 3 and 4 were 14%, 86%, 10% and 7%, respectively.

Individuals included were 19 females and 23 males and were classified into four life history categories following guidelines of previous authors (Calzada et al., 1997; Gómez-Campos et al., 2011): (1) calves, including neonates and individuals up to 1 year of age; (2) juvenile animals, (considering juvenile animals as those which measure <187 cm for females and <190 cm for males); (3) mature females, including resting, lactating and pregnant females; and (4) mature males.

The nutritional status was classified as 'non-emaciated' or 'emaciated' with reference to anatomical parameters such as the evidence of certain prominent bones, the dorso-axial muscular mass, and the absence or limited presence of fat, taking account the age of the animal. Blubber thickness was assessed during necropsy from the medium lateral region behind the flipper. Lastly, bycaught animals were diagnosed following the criteria described by (Vázquez et al., 2016).

Quetelet's index (mass/length^2; Kershaw et al., 2017) was used for assessing the correlation of BCC and SCC with body condition index in striped dolphins. Prior to relate Quetelet's index with BCC and following the recommendations from Kershaw et al. (2017) to validate a body condition index, we checked if Quetelet's index was able to differentiate between emaciated and non-emaciated individuals (classified by visual inspection), death causes, age classes, and life-history stages.

2.3. Causes of death

Thirteen individuals were included in the Bycaught group (6 females and 7 males). General findings for these animals were absence of disease, good body condition, full stomach, net marks, visceral congestion and marked muscular exertion.

Twenty-nine individuals were included in the Chronically affected group (13 females and 16 males). The cause of chronic stranding was attributed to six contributing factors characterized as: calves separated from the mother ($n = 6$); animals infected with dolphin morbillivirus (DMV) (n = 6); animals infected with *Brucella ceti* ($n = 5$); animals with septicaemia of unknown bacteria ($n = 3$); extensive parasitism and emaciation ($n = 1$); and unknown ($n = 8$).

2.4. Sample collection

Full depth integument samples ($N = 42$) were collected from the left lateral flank and stored at −20C°. Cardiac blood ($N = 28$) was collected through a cardiac puncture at the central point of a coronal line from sternum to axillar fold, using a 20 ml syringe and a 16 G, 1.7 × 133 mm catheter needle (AngiocathTM). Samples were placed in sterile serum

collector tubes and centrifuged (1372 *g* 6 min). Then, serum was aliquoted in 1 ml cryotubes and frozen at −80 °C analysis.

2.5. Sample preparation and hormone extraction

Epidermis and outer layer of the dermis, mostly composed of connective tissue, were removed from all samples leaving only fat-filled *panniculus adiposus*, which is going to be referred to as blubber. Each sample was then divided in two equal subsamples: the first one was kept frozen at −20C° until hormone extraction, whereas the second one was freeze-dried (Kinetic Thermal System: condenser Dura-Dry Model FD2055D0TOO, US) for 96 h as suggested by (Dunkin, 2005) before cortisol extraction. Water content was determined by weighing subsamples before and after freeze-drying.

Blubber hormone extractions were based on the methods described by (Kellar et al., 2015) with some modifications. Outer edges of the subsamples were removed to avoid potential contamination or changes in the tissue by direct contact with air. Prior to the extraction, 0.1 ± 0.002 g of all blubber subsamples (fresh and freeze-dried) were transferred to a 2 ml eppendorf tube (T1). The contents of T1 were pre-grinded using small scissors for one minute and fixed inside a 10 ml stainless-steel grinding jar to homogenize the contents using a ball mill for 15 min at 25 Hz (MM200, Retsch, Haan, Germany). Grinding media was separated and transferred to a new eppendorf tube (T2) that was rinsed with 0.5 ml of ethanol, and the washed contents were transferred into a falcon tube (T3). The homogenization tube (T1) was then rinsed with 0.5 ml of ethanol and all content was transferred into T3. This step was repeated once more, resulting in a total of 1.5 ml of ethanol with the homogenate inside T3. Two ml of a 4:1 ethanol:acetone solution were added to T3 and the resulting solution was vortexed and centrifuged at 2800 *g* for 15 min. The supernatant was transferred into a new weighted falcon tube (T4) and evaporated at 37 °C. After evaporation all T4 were weighted to obtain the amount of lipid extracted by subtraction of the pre-weighed T4 from the T4 after evaporation (Mello et al., 2017). Two ml of diethyl ether were added to T4, vortexed and centrifuged. The supernatant collected was transferred into a 10 ml-glass vial and introduced into the oven (Heraeus model T6; Kendro Laboratory Products, Langenselbold, Germany) with continuous venting at 30 °C. The boiling point of diethyl ether is at 34.6 °C, therefore, this step should always be performed with caution. The residue was resuspended in 1.5 ml of acetonitrile, vortexed and 1.5 ml of hexane were added to the acetonitrile-lipid mixture. After vortexing and centrifuging, 1.5 ml of the acetonitrile lower layer were transferred into a new glass vial (T6) and the process was repeated once more. The final portion of acetonitrile was transferred into a falcon tube (T7) and evaporated. The evaporated contents were resuspended in 0.25 ml of 1 M phosphate buffered saline, vortexed and stored at −20 °C until analysis.

2.6. Hormone detection and assay validation tests

Cortisol concentrations and validation tests were determined by using competitive EIA kits (Neogen® Corporation Europe, Ayr, UK). Following the essential criteria for immunological validation (Reimers and Lamb, 1991), the accuracy, precision, specificity and sensitivity of the assays were determined. Extracts from fresh blubber, freeze-dried blubber and blood serum were separately pooled for assay validation. Specificity was given by the dilution test, determined by using 1:1, 1:2, 1:5 and 1:10 dilutions of pools with EIA buffer. Accuracy was assessed through the spike-and-recovery test ($N = 7$). Precision was evaluated by calculating intra-assay ($N = 6$ in each assay) and inter-assay ($N = 12$) coefficients of variation (CV). Finally, sensitivity was given by the smallest amount of hormone concentration detected.

2.7. Statistical analyses

Striped dolphins were divided into two main groups according to the cause of death: (1) Bycaught, that included stranded animals suspected to have been captured in fishing nets and released after incidental capture; and (2) Chronically affected animals, that included alive and dead stranded animals which were euthanized or died due to a disease or chronic condition that impeded survival.

Data were processed and analysed using SPSS software package version 17.0 (SPSS, Chicago, IL). All the values are presented as mean ± SD. A *P*-value <0.05 was considered for significance. Prior to analyses, a Shapiro–Wilk test was performed to check normality of all the data. Data with non-normality distribution were square root and logarithmic (log 10) transformed and normality was evaluated again. Data in which an improvement in normality was not achieved were analysed by non-parametric tests.

One-way analyses of variances (ANOVA) ($p = 0.05$) were performed to determine if there were significant differences between striped dolphins life history categories in blubber thickness, amount of lipid extracted, and water content (blubber composition parameters). Independent sample *t*-tests were used to determine significant differences between non-emaciated and emaciated dolphins in blubber thickness, amount of lipid extracted and water content. Spearman's correlation test was used to examine the association between cortisol values in fresh and freeze-dried blubber samples, in serum and blubber and between BCC using grams of blubber and grams of lipid as denominator units. Pearson's correlation test was used to determine the co-linearity between the amount of lipid extracted from wet blubber and the water content. Correlations of blubber thickness and amount of lipid extracted were performed with a simple linear regression.

Ten factors were assessed as potential correlates with BCC and SCC: 1) cause of death (Bycaught or Chronically affected animals), 2) stranded death or alive, 3) sex, 4) emaciation 5) life history category, 6) stranding season, 7) carcass state of preservation, 8) blubber thickness, 9) amount of lipid extracted in fresh samples, and 10) Quetelet's morphometric index (mass/length^2). The factors 1–4 were assessed through an independent sample t-test, the factors 5–7 using ANOVA and the factors 8–10 using Pearson's correlation test. Tests including BCC as the dependent variable were run twice using grams of wet blubber and grams of lipid extracted as denominator units.

A generalised linear model (GLM) using a normal distribution and identity link function was conducted for Quetelet's body condition index ($N = 42$) to test the significance of the explanatory variables death cause, age class and life-history stage. Backwards model selection using the stepwise function was used to identify the variables that best explain the variation in Quetelet's body condition index and include it in the final model based on the smallest AICc. The model fit was assessed using the adjusted R^2.

3. Results

3.1. Assay and method validation

Mean intra- and inter-assay coefficients of variation were 3.56% and 4.92% respectively, indicating high repeatability of the test. The linearity of dilution presented a $R^2 = 0.99\%$ and a mean percentage error of −0.92 showing a high correlation between the obtained and the theoretical cortisol values. The average recovery percentage from the spike recovery test was 107.5 ± 7.65%. The sensitivity of the assay was 0.071 μg of cortisol/dl of serum (μg/dl), 2.28 ng cortisol/g of blubber and 5.98 ng cortisol/g of freeze-dried blubber.

A significant correlation was detected between cortisol values (mean ± SD) of fresh (26.26 ± 23.55 ng/g of wet blubber, range 2.28 to 105.79) and freeze-dried (31.42 ± 25.39 ng/g of dry blubber, range to 5.98 to 96.3) blubber samples (Pearson: $r(31) = 0.92$, $p < 0.01$). When cortisol concentration was measured as cortisol per gram of lipid extracted (ng cortisol/g lipid), a high correlation was also found between fresh (mean ± SD) (48.68 ± 47.81 ng/g of lipid extracted from wet blubber, range 4.18 to 165.87) and freeze-dried (36.8 ± 30.83 ng/g

of lipid extracted from dry blubber, range to 7.26 to 122.22) blubber samples (Pearson: r(31) = 0.94, p < 0.01). The percentages of extracted lipids in 0.1 g of blubber were (mean ± SD) 86.93 ± 5.65% (range 71 to 95) in freeze-dried samples and 59.17 ± 13.08% (range 33 to 81) in fresh samples.

From these results, values of cortisol in blubber were assessed only in fresh tissue (referred as ng/g of blubber) without the freeze-drying procedure throughout the study due to the higher simplicity of the method (Palme et al., 2013). Cortisol concentrations in fresh blubber were expressed using two denominators: ng of cortisol/g of blubber (wet) and ng of cortisol/g of lipid extracted (from wet blubber). As we suspected that the denominator unit used (mass of blubber vs mass of lipid) is a potential confounding factor for interpreting hormone levels, and research lacks for determining which one is the preferred, we decided to use both throughout the study to enable future interpretations and comparisons.

3.2. Blubber thickness and composition

Significant differences in blubber thickness were found among the life history categories considered (ANOVA: F(3, 37) = 2.81, $p = 0.05$; Table 1) as calves had significantly thinner blubber than other groups (juveniles, mature males and females). No significant differences were detected across life history categories in the amount of lipid extracted from wet blubber (ANOVA: F(3, 37) = 2.19, $p = 0.11$; Table 1), neither in the percentage of water (ANOVA: F(3, 27) = 2.27, $p = 0.10$; Table 1).

Mean blubber thickness of emaciated mature dolphins was not significantly different from the average of those non-emaciated (*t*-test: t(16) = −0.99, $p = 0.119$; Table 1). Conversely, there were significant differences in the amount of lipid extracted from wet blubber and the percentage of water between emaciated and non-emaciated mature dolphins (t-test: t(17) = 3.51, $p < 0.01$ and t(11) = −5.8, p < 0.01 respectively; Table 1).

A significant negative relationship was detected between amount of lipid extracted from wet blubber (59.17 ± 13.08% lipid extracted mass/wet mass, range from 33 to 81) and percentage of water (22.82 ± 13.05% water mass/wet mass, range 7.56% to 59.91%; Pearson: r(31) = 0.92, $p < 0.01$).

3.3. Correlation between cortisol levels in blubber and serum

Blubber cortisol concentrations significantly correlated with SCC when BBC were measured as a function of blubber tissue (Pearson: r(26) = 0.89, p < 0.01) and when BCC were measured as cortisol per gram of lipid extracted (Pearson: r(26) = 0.8, $p < 0.01$; Fig. 1). When the Bycaugh group was analysed separately a high correlation was found between BCC and SCC for both BCC measured as ng/g of blubber (Pearson: r(5) = 0.86, $p = 0.01$) and measured as ng/g of lipid extracted (Pearson: r(5) = 0.91, $p < 0.01$). Similarly, a high correlation was found between BCC and SCC in the Chronically affected group for both BCC measured as ng/g of blubber (Pearson: r(19) = 0.79, $p \leq 0.01$) and measured as ng/g of lipid extracted (Pearson: r(19) 0.72, p < 0.01).

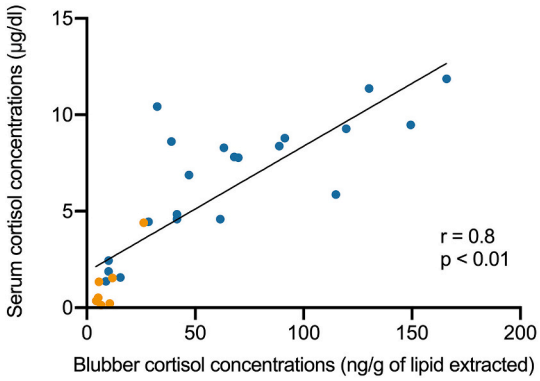

Fig. 1. Blubber cortisol concentrations were associated with serum cortisol concentrations. There was a positive correlation between blubber and serum cortisol concentrations (Pearson: r(26) = 0.89, $p < 0.01$). Yellow dots represent individuals of the Bycaugh group and blue dots individuals of the Chronically affected group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Cortisol levels in relation to the type of death

Serum cortisol concentrations ranged between 0.071 and 11.8 µg/dl. Blubber cortisol concentrations in fresh samples ranged from 2.28 to 105.79 ng/g of blubber and from 1.03 to 74.05 ng/g of lipid extracted.

Blubber cortisol concentrations (mean ± SD) were up to 6 orders of magnitude significantly higher in individuals of the Chronically affected group (35.3 ± 23 ng/g of blubber and 66.24 ± 47.93 ng/g of lipid extracted) compared to those from the Bycaught group (6.18 ± 4.35 ng/g of blubber and 6.18 ± 4.35 ng/g of lipid extracted; *t*-test: t(32.15) = −6.53, $p < 0.01$ and t(30.13) = −6.51, p < 0.01 respectively; Fig. 2A).. Mean SCC values of the Chronically affected group (6.63 ± 3.22 µg/dl) were also 6-fold higher compared to values of the Bycaught group (1.15 ± 1.51 µg/dl; *t*-test: t(22.44) = −6.06, $p < 0.01$; Fig. 2B).

3.5. Cortisol levels as a function of biological features

No significant differences were detected between males and females in SCC (*t*-test: t(26) = 1.613, $p = 0.119$), neither in BCC measured as ng/g of blubber (*t*-test: t(40) = −0.177, $p = 0.86$) nor measured as ng/g of lipid extracted (t-test: t(40) = −0.99, $p = 0.922$). Similarly, no significant differences were found across life history categories in SCC (ANOVA: F(3, 24) = 1.489, $p = 0.243$), neither in BCC measured as ng/g of blubber (ANOVA: F(3, 38) = 0.558, $p = 0.646$) nor measured as ng/g of lipid extracted (ANOVA: F(3, 38) = 0.635, $p = 0.597$).

Significant difference in BCC was found between stranded alive (36.2 ± 21.9 ng/g of blubber and 72.2 ± 49.9 ng/g of lipid extracted) and stranded dead animals (20.16 ± 22.8 ng/g of blubber and 34.2 ± 41 ng/g of lipid extracted; t-test: t(40) = −3.361, $p < 0.01$ and t(40) = −2.687, $p = 0.01$ respectively). This difference was not found for SCC concentrations (stranded alive: 7.21 ± 3.02 µg/dl; stranded dead: 5.7 ± 3.5 µg/dl).

Table 1
Striped dolphins (*Stenella coeruleoalba*) blubber composition data for each life history category and body condition.

	Calves	Juveniles	Mature females	Mature males	*p* value	Non-emaciated mature individuals	Emaciated mature individuals	p value
Blubber thickness (mm)	8.9 ± 3.1[a]	12.5 ± 3.1 [b]	10.6 ± 3.3[a,b]	10.4 ± 2.2[a,b]	0.05	10.9 ± 2.6	8.3 ± 1.5	0.119
Lipid extracted mass/wet mass (%)	66.5 ± 5.7	61.2 ± 13.2	59.8 ± 14.6	51.9 ± 13.4	0.10	59.8. ± 12	38 ± 4.5	< 0.01
Water mass/wet mass (%)	14.8 ± 4.5	22.6 ± 13.5	17.9 ± 7.2	30.6 ± 15.0	0.10	20.3 ± 8.1	48.2 ± 3.3	< 0.01

Values are expressed by mean ± S.D.
For all measurements, categories with the same letter are not significantly different (p > 0.05)

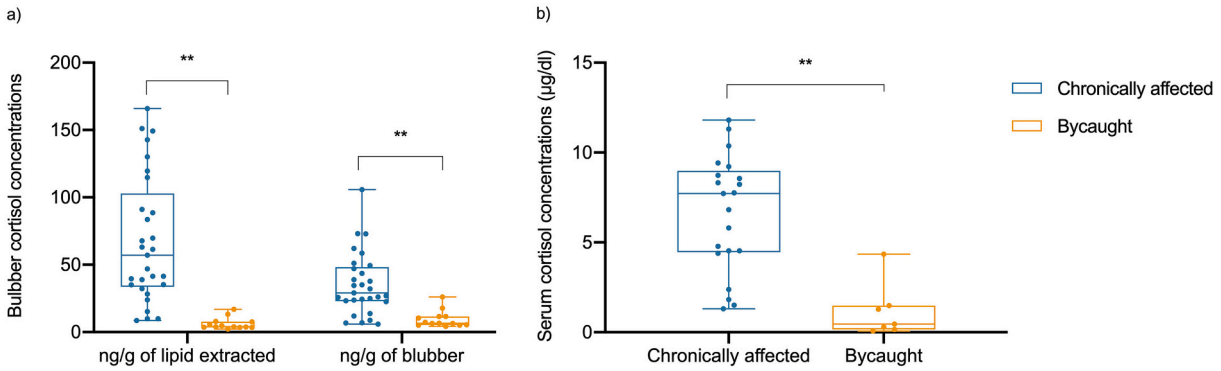

Fig. 2. (A) Blubber cortisol concentrations in bycaught and stranded striped dolphins (*Stenella coeruleoalba)*. Horizontal box lines represent the lower quartile, median and upper quartile values. Whiskers lines indicate range of concentrations. Chronically affected individuals (35.3 ± 23 ng/g of blubber and 66.24 ± 47.93 ng/g of lipid extracted) had on average 6 times more blubber cortisol concentrations than bycaught individuals (6.18 ± 4.35 ng/g of blubber and 6.18 ± 4.35 ng/g of lipid extracted; t-test: $t(32.15) = -6.53$, $p < 0.01$ and $t(30.13) = -6.51$, $p < 0.01$ respectively). (B) Serum cortisol concentrations in bycaught and stranded striped dolphins (*Stenella coeruleoalba)*. Chronically affected individuals (6.63 ± 3.22 µg/dl) had on average 6 times more serum cortisol than bycaught individuals (1.15 ± 1.51 µg/dl; t-test: $t(22.44) = -6.06$, $p < 0.01$).

No significant differences were detected across stranding seasons (autumn, spring, summer and winter) in SCC (ANOVA: $F(3, 24) = 1.138$, $p = 0.354$), neither in BCC measured as ng/g of blubber (ANOVA: $F(4, 37) = 1.24$, $p = 0.311$) nor measured as ng/g of lipid extracted (ANOVA: $F(4, 37) = 1.039$, $p = 0.4$). No significant differences were detected across carcass states of preservation (1 to 4) in SCC (ANOVA: $F(2, 25) = 0.572$, $p = 0.572$), neither in BCC measured as ng/g of blubber (ANOVA: $F(3, 38) = 0.253$, $p = 0.859$) nor measured as ng/g of lipid extracted (ANOVA: $F(3, 38) = 0.651$, $p = 0.587$).Emaciated individuals presented significantly higher BCC (mean ± SD) (45.8 ± 26.8 ng/g of blubber and 95.5 ± 48.6 ng/g of lipid extracted) than those non-emaciated (20.15 ± 19 ng/g of blubber and 34 ± 37.5 ng/g of lipid extracted; t-test: $t(40) = -3.361$, $p < 0.01$ and $t(40) = -4.207$, $p < 0.01$ respectively). No significant differences were detected between emaciated and non-emaciated individuals in SCC (t-test: $t(19.93) = -1.796$, $p = 0.088$). A negative correlation was found between BCC and blubber thickness for both BCC expressed as ng/g of blubber and ng/g of lipid extracted (Pearson: $r(40) = -0.41$, $p = 0.08$ and $r(40) = -0.39$, $p = 0.01$ respectively).

There was a significant effect of death cause on Quetelet's body condition index, which showed that the Chronically affected animals were in poorer condition than Bycaught ones ($p < 0.01$). The index also suggested that mature animals were in better condition than calves and juveniles ($p < 0.01$), but age was not retained in the best-fitting model ($p = 0.74$). The model for mass/length^2 fitted well to the data with an adjusted R^2 value of 0.49. Moreover, mean mass/length^2 of the animals classified as non-emaciated by visual inspection (0.0019 ± 0.0005) was higher than in emaciated animals (0.0014 ± 0.0004; $p < 0.05$).

Quetelet's index (mass / length^2) significantly correlated with BCC when measured as a function of blubber tissue ($r = -0.34$, $p < 0.05$) but not when measured as cortisol per gram of lipid extracted ($p = 0.19$). No correlation was found between Quetelet's index (mass / length^2) and SCC ($p = 0.548$).

No significant differences or correlations were found when analysing BCC and SCC with stranding season, carcass state of preservation and amount of lipid extracted in fresh samples ($p = 0.311$, $p = 0.4$, $p = 0.354$ respectively).

4. Discussion

Here, blubber cortisol concentrations were examined as a potential supplement to serum cortisol concentrations for assessing the physiological stress response of cetaceans. Concentrations in both matrices were markedly higher in individuals stranded with a disease or chronic condition that impeded survival than in stranded animals that were diagnosed as bycaught in fishing nets. This study used a modified method for steroid hormone extraction based on that from (Kellar et al., 2015) and an enzyme immunoassay (EIA) to measure BCC and SCC in striped dolphin (*Stenella coeruleoalba*). Serum cortisol concentrations and BCC were associated and were influenced by the cause of death and by some biological features like the body condition.

4.1. Assay and method validation

Assay results indicate that blubber cortisol detection by EIA with the methodology presented here is successful, even using an EIA kit not designed specifically for blubber.

The excellent correlation obtained in BCC between fresh and freeze-dried blubber samples suggests that both preparation techniques present similar extraction efficiencies. In addition, these results suggest that the elimination of water by freeze-drying the samples is increasing the amount of cortisol detected but in a proportional way.

4.2. Blubber thickness and composition

Calves presented the thinner blubber thickness compared to the other life history categories evaluated (Table 1). This result was expected given that blubber thickness has been related to body morphometries (Gómez-Campos et al., 2011). Blubber thickness of emaciated individuals did not vary compared to those animals not emaciated (Table 1) and a negative relationship was found between blubber lipid and water content of all individuals. Blubber thickness may not significantly diminish during catabolism of fat, as blubber contains many collagen bundles and elastic fibbers in which adipocytes are interspersed (Rommel and Lowenstine, 2001). In emaciated adults, where lipid content was clearly reduced, water content increased. These results may indicate a replacement of lipid with water, which is thought to be a mechanism for maintaining the structural integrity of the tissue to preserve blubber's other functions (Dunkin, 2005). Therefore, blubber thickness seems an inadequate tool for assessing nutritional condition of dolphins, coinciding with other authors (Caon et al., 2007; Gómez-Campos et al., 2011; Joblon et al., 2014). Conversely, blubber lipid content seems to be a better indicator of nutritional status (Gómez-Campos et al., 2011).

The lack of differences between life history categories in the blubber lipid and water contents (Table 1) may be due to the limited number of individuals since significant differences have been shown with a larger sample size (Gómez-Campos et al., 2011).

4.3. Correlation between blubber and serum cortisol concentrations

There is very little published data about corticosteroid levels in marine mammals in simultaneously collected tissue samples. Champagne et al. (2018 and 2017) results indicate an increase in BCC concomitant with increased SCC within 2 h of the onset of stress. Time elapsed since the capture of free-ranging bottlenose dolphins was a significant predictor of BCC, thus occurring an increase in BCC due to capture stress between 53 min and 215 min post capture (Galligan et al., 2020). Meanwhile, a high positive relationship of testosterone concentrations has been described in bottlenose dolphin serum and blubber paired samples ($r^2 = 0.932$; Sherman et al., 2021). As it is thought that free cortisol diffuses into adipose tissue (Breuner et al., 2013), BCC can somehow reflect circulating cortisol concentrations that are integrated over a short time period (less than ~2 h). Nevertheless, Galligan et al. (2020) did not found this correlation between the two matrices probably because samples were not collected simultaneously and the intervals between blood and blubber collection were different across individuals.

Overall, our results support previous findings and suggest that this association occurs during endogenous cortisol release in free-ranging animals. However, in the case of acute stress responses, the reflection in blubber would depend on the delay between adrenal cortisol secretion and its integration in blubber, as well as the clearance rate and the sampling moment (discussed in 4.4). Moreover, blubber has been recognized as a site of active steroid metabolism and it should be considered when quantitatively linking circulating cortisol concentrations with BCC (Galligan et al., 2018).

4.4. Cortisol levels in relation to the cause of death

Blubber cortisol concentrations and SCC values from the stranded animals were higher than those detected among bycaught animals. This result was indeed not unexpected as cortisol concentrations can be influenced by many factors such as duration of stress, changes in cortisol binding globulin (CBG) concentrations and degree of perfusion in the case of blubber.

The increased serum cortisol concentrations in the Chronically affected group (6.63 ± 3.22 μg/dl) are comparable and slightly higher to what has been reported during stressful events in other species: ~4.54 μg/dl (Fair et al., 2014) and ~ 4.27 μg/dl (Hart et al., 2015) after capture and handling in free-ranging bottlenose dolphins; ~5.04 μg/dl after chase and encirclement by a tuna purse seiner in free-ranging pantropical spotted dolphins (*Stenella attenuate*; St. Aubin et al., 2013); and 3.62 μg/dl (Champagne et al., 2018).

Meanwhile, serum cortisol concentrations in the Bycaught group (1.15 ± 1.51 μg/dl) are marginally higher than some baseline concentrations reported from captive animals: 0.87 μg/dl (Champagne et al., 2018) and 0.725 μg/dl (Champagne et al., 2017) in bottlenose dolphins and 0.50 μg/dl in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*). Although data is difficult to compare and cortisol levels may vary across species and due to captivity condition, low levels in comparison to the Chronically affected animals and similarity to basal levels in other species suggest that SCC in bycaught animals are unexpectedly around baseline levels of striped dolphins.

Pathological studies have concluded that hypoxia and subsequent asphyxiation is the main cause of mortality of bycaught cetaceans in bottom-set nets (Ijsseldijk et al., 2021; Soulsbury et al., 2008). Animals may not be able to rise to the surface and thus the period between capture and death may be in the order of minutes, leaving limited time for animals to overstrain (Ijsseldijk et al., 2021; Soulsbury et al., 2008). Marked muscular exertion findings in some bycaught dolphins could be related to a certain degree of physical struggle (Moore et al., 2013). If the animal struggled to free itself, this would require a greater delivery of oxygen, which, added to the physiological acute stress-response, could very quickly lead to asphyxiation. The maximum dive duration of striped dolphins may be similar to that described for common dolphins (*Delphinus capensis* and *Delphinus delphis*) and spotted dolphins (*Stenella attenuata*): 5 and 4.7 min respectively (Evans, 1971; Heyning and Perrin, 1994; Scott et al., 1993).

The mammalian stress response is characterized by two temporal waves of stress mediated actions. The first one occurs in seconds and includes noradrenaline and corticotropin-releasing hormone (CRH). Then, with the HPA pathway activated, adrenocorticotropin hormone (ACTH) is released from the anterior pituitary into the bloodstream stimulating glucocorticoid production in the adrenal cortex. Mammalian endocrinological research shows that generally an increase in circulating glucocorticoids above baseline levels can be measurable within 3–5 min. Glucocorticoid concentrations continue to rise to peak levels after 15–30 min and then decline to baseline levels within 60–90 min (Ron de Kloet et al., 2005; Sapolsky et al., 2000). A death within a few minutes of entrapment is likely and could explain the low levels of SCC of the Bycaught group. If bycaught dolphins had died in a hyper-acute way, i.e., in <3–5 min by asphyxia, this would explain both SCC and BCC low levels. Further, variability in times between entrapment and death may have had influence on SCC.

Mean BCC values obtained in relation to the type of death (Bycaught group: 6.18 ± 4.35 ng/g of blubber and 6.18 ± 4.35 ng/g of lipid extracted; Chronically affected group: 35.3 ± 23 ng/g of blubber and 66.24 ± 47.93 ng/g of lipid extracted) are close to those obtained by Kellar et al. (2015), who reported mean values of 3.99 ng/g of blubber and 24.3 ng/g of blubber in bycaught and stranded short beaked common dolphins, respectively. Trana et al. (2016) reported mean BCC values of 0.25 and 1.79 ng/g of blubber in blubber from subsistence harvest and entrapped beluga whales respectively. It is noteworthy that although the magnitude of the values differs, the differences observed between groups are very similar in the three species: a sevenfold increase in entrapped beluga whales (Trana et al., 2016) and a sixfold increase in stranded short beaked common dolphins (Kellar et al., 2015) and the striped dolphins of this study. Moreover, Champagne et al. (2017) reported baseline BCC of 1.4 ng/g of lipid in captive bottlenose dolphins and in the same animals, after the onset of a stress test, mean blubber cortisol concentrations raised from 3.4 to 16.9 ng/g of lipid at sample times of 0 and 120 min respectively. Therefore, blubber cortisol seems to increase in a similar way during chronic stress and shortly after acute stress scenarios.

The time course of cortisol aggregation into blubber may vary as a function of environmental conditions. Ambient temperature and diving-surfacing periods are factors that are thought to affect blubber cortisol integration. Exposure of blubber to circulating cortisol (and therefore the subsequent hormonal quantification) could increase due to heart rate increase and peripheral vasodilation. This happens during surface intervals, temperature increases and after exercise (Champagne et al., 2018; Noren et al., 1999, 2012; Williams, 2015). Therefore, stranded animals that are unconditioned to being out of the water might accumulate cortisol faster than bycaught ones. Further, illness could lead to foraging decline and therefore to a nutritional deficit that causes prolonged elevated cortisol levels (Kellar et al., 2015). Prolonged perception of threat, persistent pain and group separation could also elevate glucocorticoid levels in a permanent way.

Cortisol synthesis occurs in pulsatile events in the adrenal gland followed by infusion into the bloodstream (Chrousos, 1995). From blood, free cortisol reaches target tissues through capillaries. Blubber accumulates contaminants and hormones by passively diffusing from the capillaries found throughout the lipid (Deslypere et al., 1985; Mead et al., 1986). To our knowledge, cortisol uptake into mammalian adipose tissue cells occurs by passive diffusion through the phospholipid bilayer because of its lipophilic character (Deslypere et al., 1985; Mead et al., 1986). Nevertheless, it is not clear whether active transport through carriers also occurs in this process (Lee et al., 2016; Rao, 1981). In human adipose intracellular compartment, individual cortisol pulses can be distinguished, and cortisol peaks seems to occur with a delay of ~3 min in adipose capillary bead and of ~35 min. in interstitial and

intracellular space (Lee et al., 2016). In addition, intraadipose cortisol also comes from the local conversion of cortisone into cortisol by the enzyme 11βhydroxysteroid dehydrogenase type 1 (Stimson et al., 2009). Thus, BCC measured here may reflect a combination of both circulating concentrations of cortisol and the local transformation of the hormone in the tissue itself (Kershaw et al., 2017).

The uptake of steroid hormones by the total adipose mass has been described as dependent of fat content, being proportionally higher in obese rats (Zomzely et al., 1959) and humans (Bleau et al., 1974). Bleau et al. (1974) suggested that the uptake of cortisol by adipose tissue is much less than that of other steroid hormones and that cortisol is not stored in this tissue for prolonged periods as occur with progesterone.

Thus, we suggest that blubber cortisol concentrations are not useful to distinguish between short (acute) and long-term (chronic) stress. However, BCC have been signalled as an indicator of chronic activation of the HPA axis in different species of cetaceans (Loseto et al., 2017; Mingramm et al., 2020; Teerlink et al., 2018; Trana et al., 2016). Blubber cortisol concentrations units vary among reports making interpretations and direct comparisons difficult. Here we propose that the unit 'ng of cortisol/g of lipid extracted' may be more suitable that 'ng of cortisol/g of blubber' as a large part of the measured cortisol is probably dependent of fat content as discussed before. Either way, it is necessary to reach a consensus or until then authors should present the results in both units or justify the use of either. However, further research comparing BCC in both scenarios (acute vs. chronic stress), revealing hormone integration and clearance rates in blubber and determining how much of the cortisol pool in cetacean blubber is derived from local production is necessary to confirm these affirmations.

Baseline BCC of healthy populations can be obtained through remote biopsy sampling while baseline SCC are unlikely to obtain from samples of alive free-ranging marine mammals due to capture stress alteration. So far, circulating hormone concentrations from free-ranging cetaceans have been measured from blood obtained by capturing live animals, a process that is not easy and implies an invasive procedure (Atkinson et al., 2015; Fair et al., 2014; Hart et al., 2015). We suggest that the use of blood collected from dead stranded animals for hormone analysis is possible, but the effect of haemolysis and post-mortem changes in carcasses on different preservation states should be assessed accurately.

4.5. Cortisol as a function of biological features

No differences in BCC nor in SCC among sexes, ages or seasons were found coinciding with St. Aubin et al. (1996) and Kellar et al. (2015). Conversely, other authors detected seasonal variations in SCC (Funasaka et al., 2011; Houser et al., 2021; Mingramm et al., 2019; Suzuki et al., 2003) and BCC (Mingramm et al., 2019) as well as sex-related variations in SCC (Houser et al., 2021; Mingramm et al., 2019). To date, these relationships are not clear since research on cetacean stress physiology has been carried out from animals under pathological processes, using low sample sizes or isolated samples. This also occurs in the present study, where the specific differences in age, sex, or seasonality, if any, would be masked by type of death. Considering that many studies across vertebrate taxa have found a variation in cortisol levels with respect to sex, age, reproductive state, and season, more studies are needed to address if this also occurs in cetaceans. Healthy animals together with larger sample sizes and long-term serial sampling would help to solve these issues.

Mass/length^2 appears to be an appropriate morphometric index for inferencing about the body condition of striped dolphins. The index was consistent with the hypothesized relationships between type of death, life history classes and emaciation classified by visual inspection. Bycaught animals were in better body condition as they died in a sudden event in comparison with the Chronically affected animals where a general debilitation and deterioration in health occurred due to disease or starvation, as described on harbour porpoises by (Kershaw et al., 2017). As expected, adults were in significantly better condition than calves. Nevertheless, we did not assess other potential indices that might fit better to de data and our sample was not large enough to represent all reproductive classes. Moreover, we were not able to assess blubber trunk lipid mass (BTLM), which was signalled as the best nutritional condition index for striped dolphins (Gómez-Campos et al., 2011).

Analyses showed that BCC when measured as a function of blubber tissue were weakly negatively correlated with body condition index, as described on harbour porpoises in Kershaw et al. (2017). Importantly, this relationship was not found when BCC was measured as cortisol per gram of lipid extracted, highlighting the significance of the choice of one or other unit when interpreting data. Long-term periods of reduced food intake or fasting are associated with an increase in the circulating concentrations of cortisol in pinnipeds (Champagne et al., 2012). During these periods cortisol increases gluconeogenesis (Exton et al., 1972) and is involved in lipolysis to provide energy (Bergendahl et al., 1996). Therefore, increased cortisol concentrations in the blubber of striped dolphins in poorer body condition may also be related with increased mobilization of fat reserves (Kershaw et al., 2017).

Blubber cortisol concentrations should therefore not only be interpreted based on the psychological stress but considering the other roles of cortisol in the regulation of lipolysis and overall energy balance in mammals. In turn, the analysis of cortisol concentrations in remotely obtained blubber biopsies may be also informative of the body condition of free-ranging cetaceans for which morphometric measurements cannot be obtained. However, further research is needed to differentiate the effects of emaciation, acute and chronic stress on blubber cortisol concentrations.

5. Conclusions

Cortisol concentrations in blubber and blood collected from dead stranded animals were successfully measured by using EIA. Blubber cortisol concentrations and SCC were on average of six to seven times higher in stranded dolphins compared to those bycaught. Moreover, the two matrices showed a high correlation, suggesting that in this case BCC reflected circulating cortisol concentrations. The results obtained show that both BCC and SCC can help to infer in the physiological state and overall health status in wild cetaceans. Additionally, mass/length^2 ratio appeared to be an appropriate morphometric index for striped dolphins that correlated negatively with BCC, highlighting the role of cortisol in the regulation of overall energy balance in mammals. Further, BCC added to other indicators could provide valuable information about the physiological state before death in stranded cetaceans and therefore about the potential causes of death. However, further research is required to understand better the kinetics of blubber cortisol incorporation and removal, the factors involved in these processes, and the potential local conversion of cortisol in the blubber.

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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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References

Aguilar, A., Gaspari, S., 2012. Stenella coeruleoalba. The IUCN red list of threatened species 2012: e.T20731A2773889.

Atkinson, S., Crocker, D., Houser, D., Mashburn, K., 2015. Stress physiology in marine mammals: how well do they fit the terrestrial model? J. Comp. Physiol. B. 463–486 https://doi.org/10.1007/s00360-015-0901-0.

Atkinson, S., Gendron, D., Branch, T.A., Mashburn, K.L., Melica, V., Enriquez-Paredes, L. E., Brownell, R.L., 2019. Pregnancy rate and biomarker validations from the blubber of eastern North Pacific blue whales. Marine Mammal Science 000, 1–23. https://doi.org/10.1111/mms.12616.

Avila, I.C., Kaschner, K., Dormann, C.F., 2018. Current global risks to marine mammals: taking stock of the threats. Biol. Conserv. 221, 44–58. https://doi.org/10.1016/j.biocon.2018.02.021.

Bearzi, G., 2002. Interactions between cetaceans and fisheries in the Mediterranean Sea. Section 9. In: Cetaceans of the Mediterranean and Black Seas: state of knowledge and conservation strategies. A report to the ACCOBAMS Secretariat, Monaco, February 2002.

Bechshoft, T., Wright, A.J., Weisser, J.J., Teilmann, J., Dietz, R., Hansen, M., 2015. Developing a new research tool for use in free-ranging cetaceans : recovering cortisol from harbour porpoise skin. Conservation Physiology 3, 1–9. https://doi.org/10.1093/conphys/cov016.

Bergendahl, M., Lee Vance, M., Iranmanesh, A., Thorner, M.O., Veldhuis, J.D., 1996. Fasting as a metabolic stress paradigm selectively amplifies cortisol secretory burst mass and delays the time of maximal nyctohemeral cortisol concentration in healthy men. J. Clin. Endocrinol. Metab. 81, 692–699. https://doi.org/10.1210/jcem.81.2.8636290.

Bleau, G., Roberts, K.D., Chapdelaine, A., 1974. The in vitro and in vivo uptake and metabolism of steroids in human adipose tissue 1. J. Clin. Endocrinol. Metab. 39, 236–246. https://doi.org/10.1210/jcem-39-2-236.

Breuner, C.W., Delehanty, B., Boonstra, R., 2013. Evaluating stress in natural populations of vertebrates: Total CORT is not good enough. Funct. Ecol. 27, 24–36. https://doi.org/10.1111/1365-2435.12016.

Calzada, N., Aguilar, A., Lockyer, C., Grau, E., 1997. Patterns of growth and physical maturity in the western Mediterranean striped dolphin, Stenella coeruleoalba (Cetacea: Odontoceti). Can. J. Zool. Rev. Can. Zool. 75, 632–637. https://doi.org/10.1139/z97-078.

Caon, G., Fialho, C.B., Danilewicz, D., 2007. Body fat condition in franciscanas (Pontoporia blainvillei) in Rio Grande do Sul, southern Brazil. J. Mammal. 88, 1335–1341. https://doi.org/10.1644/06-MAMM-A-364R.1.

Champagne, C.D., Houser, D.S., Costa, D.P., Crocker, D.E., 2012. The effects of handling and anesthetic agents on the stress response and carbohydrate metabolism in northern elephant seals, 7, e38442. https://doi.org/10.1371/journal.pone.0038442.

Champagne, C.D., Kellar, N.M., Crocker, D.E., Wasser, S.K., Booth, R.K., Trego, M.L., Houser, D.S., 2017. Blubber cortisol qualitatively reflects circulating cortisol concentrations in bottlenose dolphins. Marine Mammal Science 33, 134–153. https://doi.org/10.1111/mms.12352.

Champagne, C.D., Kellar, N.M., Trego, M.L., Delehanty, B., Boonstra, R., Wasser, S.K., Booth, R.K., Crocker, D.E., Houser, D.S., 2018. Comprehensive endocrine response to acute stress in the bottlenose dolphin from serum, blubber, and feces. Gen. Comp. Endocrinol. 178–193 https://doi.org/10.1016/j.ygcen.2018.05.015.

Chrousos, G.P., 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N. Engl. J. Med. 332, 1351–1362. https://doi.org/10.1056/NEJM199505183322008.

Cuvertoret-Sanz, M., López-Figueroa, C., O'Byrne, A., Canturri, A., Martí-Garcia, B., Pintado, E., Pérez, L., Ganges, L., Cobos, A., Abarca, M.L., Raga, J.A., Van Bressem, M.F., Domingo, M., 2020. Causes of cetacean stranding and death on the Catalonian coast (western Mediterranean Sea), 2012-2019. Dis. Aquat. Org. 142, 239–253. https://doi.org/10.3354/DAO03550.

Deslypere, J.P., Verdonck, L., Vermeulen, A., 1985. Fat tissue: a steroid reservoir and site of steroid metabolism. J. Clin. Endocrinol. Metab. 61, 564–570. https://doi.org/10.1210/jcem-61-3-564.

Dunkin, R.C., 2005. The ontogenetic changes in the thermal properties of blubber from Atlantic bottlenose dolphin Tursiops truncatus. J. Exp. Biol. 208, 1469–1480. https://doi.org/10.1242/jeb.01559.

Evans, W.E., 1971. Orientation behavior of delphinids: radio telemetric studies. Ann. N. Y. Acad. Sci. 188, 142–160. https://doi.org/10.1111/j.1749-6632.1971.tb13094.x.

Exton, J.H., Friedmann, N., Wong, E.H., Brineaux, J.P., Corbin, J.D., Park, C.R., 1972. Interaction of glucocorticoids with glucagon and epinephrine in the control of gluconeogenesis and glycogenolysis in liver and of lipolysis in adipose tissue. J. Biol. Chem. 247, 3579–3588. https://doi.org/10.1016/s0021-9258(19)45180-6.

Fair, P.A., Becker, P.R., 2000. Review of stress in marine mammals. J. Aquat. Ecosyst. Stress. Recover. 7, 335–354. https://doi.org/10.1023/A:1009968113079.

Fair, P.A., Schaefer, A.M., Romano, T.A., Bossart, G.D., Lamb, S.V., Reif, J.S., 2014. Stress response of wild bottlenose dolphins (Tursiops truncatus) during capture-release health assessment studies. Gen. Comp. Endocrinol. 206, 203–212. https://doi.org/10.1016/j.ygcen.2014.07.002.

Funasaka, N., Yoshioka, M., Suzuki, M., Ueda, K., Miyahara, H., Uchida, S., 2011. Seasonal difference of diurnal variations in serum melatonin, cortisol, testosterone, and rectal temperature in indo-pacific bottlenose dolphins (*Tursiops aduncus*), 37, pp. 433–442. https://doi.org/10.1578/AM.37.4.2011.433.

Galligan, T.M., Schwacke, L.H., Mcfee, W.E., Boggs, A.S.P., 2018. Evidence for cortisol – cortisone metabolism by marine mammal blubber. Mar. Biol. 165, 1–8. https://doi.org/10.1007/s00227-018-3373-4.

Galligan, T.M., Balmer, B.C., Schwacke, L.H., Bolton, J.L., Quigley, B.M., Rosel, P.E., Ylitalo, G.M., Boggs, A.S.P., 2019. Examining the relationships between blubber steroid hormones and persistent organic pollutants in common bottlenose dolphins. Environ. Pollut. 249, 982–991. https://doi.org/10.1016/j.envpol.2019.03.083.

Galligan, T.M., Boggs, A.S.P., Balmer, B.C., Rowles, T., Smith, C.R., Townsend, F., Wells, R.S., Kellar, N.M., Zolman, E.S., Schwacke, L.H., 2020. Blubber steroid hormone profiles as indicators of physiological state in free-ranging common bottlenose dolphins (Tursiops truncatus). Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology 239, 110583. https://doi.org/10.1016/j.cbpa.2019.110583.

Gómez-Campos, E., Borrell, A., Aguilar, A., 2011. Assessment of nutritional condition indices across reproductive states in the striped dolphin (Stenella coeruleoalba). J. Exp. Mar. Biol. Ecol. 405, 18–24. https://doi.org/10.1016/j.jembe.2011.05.013.

Guo, Y., Gui, D., Zhang, X., Liu, W., Xie, Q., Yu, X., Wu, Y., 2022. Blubber cortisol-based approach to explore the endocrine responses of indo-pacific humpback dolphins (Sousa chinensis) to diet shifts and contaminant exposure. Environ. Sci. Technol. 56, 1069–1080. https://doi.org/10.1021/acs.est.1c04550.

Hart, L.B., Wells, R.S., Kellar, N., Balmer, B.C., Hohn, A.A., Lamb, S.V., Rowles, T., Zolman, E.S., Schwacke, L.H., 2015. Adrenal hormones in common bottlenose dolphins (Tursiops truncatus): influential factors and reference intervals. PLoS One 10, 1–16. https://doi.org/10.1371/journal.pone.0127432.

Heyning, J.E., Perrin, W.F., 1994. Evidence for two species of common dolphins (genus Delphinus) from the eastern North Pacific. Contrib. Sci. 442, 1–35. https://doi.org/10.5962/p.226804.

Houser, D.S., Champagne, C.D., Wasser, S.K., Booth, R.K., Romano, T., Crocker, D.E., 2021. Influence of season, age, sex, and time of day on the endocrine profile of the common bottlenose dolphin (Tursiops truncatus). Gen. Comp. Endocrinol. 313, 113889 https://doi.org/10.1016/j.ygcen.2021.113889.

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

Ijsseldijk, L.L., Scheidat, M., Siemensma, M.L., Couperus, B., Leopold, M.F., Morell, M., Gröne, A., Kik, M.J.L., 2021. Challenges in the assessment of bycatch: postmortem findings in harbor porpoises (Phocoena phocoena) retrieved from gillnets. Vet. Pathol. 58, 405–415. https://doi.org/10.1177/0300985820972454.

Jauniaux, T., Beans, C., Dabin, W., 2005. Stranding, necropsy and sampling: collection data, sampling level & techniques. In: European Cetacean Society Student ECS Workshop La Rochelle, France, 7 April.

Joblon, M.J., Pokras, M.A., Morse, B., Harry, C.T., Rose, K.S., Sharp, S.M., Niemeyer, M. E., Patchett, K.M., Sharp, W.B., Moore, M.J., 2014. Body condition scoring system for delphinids based on short-beaked common dolphins (Delphinus delphis). Journal of Marine Animals and Their Ecology 7, 5–13.

Kellar, N.M., Catelani, K.N., Robbins, M.N., Trego, M.L., 2015. Blubber cortisol: a potential tool for assessing stress response in free-ranging dolphins without effects due to sampling. PLoS One 10, 1–17. https://doi.org/10.1371/journal.pone.0115257.

Kershaw, J.L., Hall, A.J., 2016. Seasonal variation in harbour seal (Phoca vitulina) blubber cortisol - a novel indicator of physiological state? Sci. Rep. 6, 1–9. https://doi.org/10.1038/srep21889.

Kershaw, J.L., Sherrill, M., Davison, N., Brownlow, A., 2017. Evaluating morphometric and metabolic markers of body condition in a small cetacean, the harbor porpoise (Phocoena phocoena). Ecology and Evolution 7, 3494–3506. https://doi.org/10.1002/ece3.2891.

Kuiken, T., 1991. Proc first ECS workshop. (Special Publication Series Newsletter 17). ECS.

Lee, M.A., Bakh, N., Bisker, G., Brown, E.N., Strano, M.S., 2016. A pharmacokinetic model of a tissue implantable cortisol sensor. Advanced Healthcare Materials 5, 3004–3015. https://doi.org/10.1002/adhm.201600650.

Lightman, S.L., Wiles, C.C., Atkinson, H.C., Henley, D.E., Russell, G.M., Leendertz, J.A., McKenna, M.A., Spiga, F., Wood, S.A., Conway-Campbell, B.L., 2008. The significance of glucocorticoid pulsatility. Eur. J. Pharmacol. 583, 255–262. https://doi.org/10.1016/j.ejphar.2007.11.073.

Loseto, L.L., Pleskach, K., Hoover, C., Tomy, G.T., Desforges, J.-P., Halldorson, T., Ross, P.S., 2017. Cortisol levels in beluga whales (Delphinapterus leucas): setting a benchmark for marine protected area monitoring. Arctic Science 372, 358–372. https://doi.org/10.1139/as-2017-0020.

Mead, J.F., Alfin-Slater, R.B., Howton, D., Popjak, G., 1986. Lipids: Chemistry, Biochemistry and Nutrition. Plenum Press, New York.

Mello, D., Colosio, A., Marcondes, M., Viau, P., Oliveira, C., 2017. Feasibility of using humpback whale blubber to measure sex hormones. J. Exp. Mar. Biol. Ecol. 486, 32–41. https://doi.org/10.1016/j.jembe.2016.09.010.

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

Mingramm, F.M.J., Keeley, T., Whitworth, D.J., Dunlop, R.A., 2020. Blubber cortisol levels in humpback whales (Megaptera novaeangliae): a measure of physiological stress without effects from sampling. Gen. Comp. Endocrinol. 291, 113436 https://doi.org/10.1016/j.ygcen.2020.113436.

Moberg, G.P., Mench, J.A., 2000. The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI.

Moore, M.J., Van Der Hoop, J., Barco, S.G., Costidis, A.M., Gulland, F.M., Jepson, P.D., Moore, K.T., Raverty, S., McLellan, W.A., 2013. Criteria and case definitions for serious injury and death of pinnipeds and cetaceans caused by anthropogenic trauma. Dis. Aquat. Org. 103, 229–264. https://doi.org/10.3354/dao02566.

Mostl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74. https://doi.org/10.1016/s0739-7240(02)00146-7.

Noren, D.P., Williams, T.M., Berry, P., Butler, E., 1999. Thermoregulation during Swimming and Diving in Bottlenose Dolphins, *Tursiops truncatus*. University of California-Santa Cruz, Center for Ocean Health-Long Marine Lab, Department of Ecology and Evolutionary Biology. https://doi.org/10.1007/s003600050198.

Noren, S.R., Kendall, T., Cuccurullo, V., Williams, T.M., 2012. The dive response redefined: underwater behavior influences cardiac variability in freely diving dolphins. J. Exp. Biol. 215, 2735–2741. https://doi.org/10.1242/jeb.069583.

Palme, R., Touma, C., Arias, N., Dominchin, M.F., Lepschy, M., 2013. Steroid extraction: get the best out of faecal samples. Wiener tierärztliche Monatsschrift 100, 238–246.

Papastavrou, V., Leaper, R., Lavigne, D., 2017. Why management decisions involving marine mammals should include animal welfare. Mar. Policy 79, 19–24. https://doi.org/10.1016/j.marpol.2017.02.001.

Pedernera-Romano, C., Valdez, R., Singh, S., Chiappa, X., Romano, M., Galindo, F., 2006. Salivary cortisol in captive dolphins (Tursiops truncatus): a non-invasive technique. Anim. Welf. 15, 359–362.

Rao, G.S., 1981. Mode of entry of steroid and thyroid hormones into cells. Mol. Cell. Endocrinol. 21, 97–108. https://doi.org/10.1016/0303-7207(81)90047-2.

Reimers, T.J., Lamb, S.V., 1991. Radioimmunoassay of hormones in laboratory animals for diagnostics & research. Lab Animal 20, 32–38.

Romero, L.M., Dickens, M.J., Cyr, N.E., 2009. The reactive scope model - a new model integrating homeostasis, allostasis, and stress. Horm. Behav. 55, 375–389. https://doi.org/10.1016/j.yhbeh.2008.12.009.

Rommel, S.A., Lowenstine, L.J., 2001. Gross and microscopic anatomy. In: Gulland, L.A., Dierauf, F.M.D. (Eds.), Marine Mammal Medicine. CRC press, Sokolov, pp. 129–164. https://doi.org/10.1201/9781420041637.sec2.

Ron de Kloet, E., Joëls, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nature Reviews 6, 463–475. https://doi.org/10.1038/nrn1683.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, supressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89. https://doi.org/10.1210/er.21.1.55.

Scott, M.D., Chivers, S.J., Olson, R.J., Lindsay, R.J., 1993. Radiotracking of spotted dolphins associated with tuna in the eastern tropical Pacific. In: Abstracts of the Tenth Biennial Conference on the Biology of Marine Mammals. Society of Marine Mammalogy, Galveston, Tex. Nov. 11–15.

Sherman, K.K., Beaulieu-McCoy, N.E., Wurster, E.L., Wells, R.S., Smith, C.R., Barleycorn, A.A., Allen, J.B., Kellar, N.M., 2021. Serum correlation, demographic differentiation, and seasonality of blubber testosterone in common bottlenose dolphins, Tursiops truncatus, in Sarasota Bay. FL. Scientific Reports 11, 8941. https://doi.org/10.1038/s41598-021-88602-z.

Soulsbury, C., Iossa, G., Harris, S., 2008. The animal welfare implications of cetacean deaths in fisheries, Shool of biological sciences University of Bristol. Whale and dolphin conservation society (2008) (October), pp. 1–54.

St. Aubin, D.J., Deguise, S., Richard, P.R., Smith, T.G., Geraci, J.R., 2001. Hematology and plasma chemistry as indicators of health and ecological status in beluga whales, Delphinapterus leucas. Arctic 54, 317–331. https://doi.org/10.14430/arctic791.

St. Aubin, D.J., Forney, K.A., Chivers, S.J., Scott, M.D., Danil, K., Romano, T.A., Wells, R. S., Gulland, F.M.D., 2013. Hematological, serum, and plasma chemical constituents in pantropical spotted dolphins (Stenella attenuata) following chase, encirclement, and tagging. Marine Mammal Science 29, 14–35. https://doi.org/10.1111/j.1748-7692.2011.00536.x.

St. Aubin, D.J., Ridgway, S.H., Wells, R.S., Rhinehart, H., 1996. Dolphin thyroid and adrenal hormones: circulating levels in wild and semidomesticated *Tursiops truncatus*, and influence of sex, age, and season. Mar. Mammal Sci. 12 (1), 1–13. https://doi.org/10.1111/j.1748-7692.1996.tb00301.x.

Stimson, R.H., Andersson, J., Andrew, R., Redhead, D.N., Karpe, F., Hayes, P.C., Olsson, T., Walker, B.R., 2009. Cortisol release from adipose tissue by 11 β-hydroxysteroid dehydrogenase type 1 in humans. Diabetes 58, 46–53. https://doi.org/10.2337/db08-0969.

Suzuki, M., Tobayama, T., Katsumata, E., Yoshioka, M., Katsumi, A., 1998. Serum cortisol levels in captive killer whale and bottlenose dolphin. Fish. Sci. 64, 643–647. https://doi.org/10.2331/fishsci.64.643.

Suzuki, M., Uchida, S., Ueda, K., Tobayama, T., Katsumata, E., 2003. Diurnal and annual changes in serum cortisol concentrations in Indo-Pacific bottlenose dolphins *Tursiops aduncus* and killer whales *Orcinus orca*, 132, pp. 427–433. https://doi.org/10.1016/S0016-6480(03)00100-X.

Teerlink, S., Horstmann, L., Witteveen, B., 2018. Humpback whale (Megaptera novaeangliae) blubber steroid hormone concentration to evaluate chronic stress response from whale-watching vessels. Aquat. Mamm. 44, 411–425. https://doi.org/10.1578/AM.44.4.2018.411.

Trana, M.R., Roth, J.D., Tomy, G.T., Anderson, W.G., Ferguson, S.H., 2015. Influence of sample degradation and tissue depth on blubber cortisol in beluga whales. J. Exp. Mar. Biol. Ecol. 462, 8–13. https://doi.org/10.1016/j.jembe.2014.10.010.

Trana, M.R., Roth, J.D., Tomy, G.T., Anderson, W.G., Ferguson, S.H., Roth, J.D., Anderson, W.G., Ferguson, S.H., 2016. Increased blubber cortisol in ice-entrapped beluga whales (Delphinapterus leucas). Polar Biol. 39, 1563–1569. https://doi.org/10.1007/s00300-015-1881-y.

Vázquez, J.A., De la Fuente, J., Martínez- Cedeira, J.A., Fernández, C., Gonzalves, P., López, A., Arbelo, A.M., 2016. Documento técnico sobre protocolo nacional de actuación para varamientos de cetáceos. Informe realizado para el Ministerio de Agricultura, Alimentación y Medio Ambiente. Imagen.

Williams, T., 2015. High risk behaviors in marine mammals: Linking behavioral responses to anthropogenic disturbance to biological consequences. In: University of California-Santa Cruz, Center for Ocean Health-Long Marine lab. Department of Ecology and Evolutionary Biology.

Zomzely, C., Asti, R., Mayer, J., 1959. Storage of steroid hormones by adipose tissue in two experimental obesities. Science. 129, 1546–1547. https://doi.org/10.1126/science.129.3362.1546.