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NOTE

Marine Mammal Science

Influence of sample location on blubber cortisol concentration in striped dolphins (*Stenella coeruleoalba*): The importance of the reference denominator

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The measurement of glucocorticoid hormones, such as cortisol, is a technique being increasingly used as a welfare or stress indicator (Mormède et al., 2007; Palme, 2019). In response to perceived stressors, these hormones are released into the bloodstream through activation of the hypothalamic–pituitary–adrenal (HPA) axis. The release of cortisol, a principal glucocorticoid in many mammals, enables the body to cope with threatening or demanding situations. Although this adaptive response is usually beneficial for the animal, chronic activation of the HPA axis and long-term cortisol elevations can reduce growth and reproduction, compromise the immune system, and impair the individual's ability to respond to subsequent stressors (Moberg & Mench, 2000).

Blubber, a specialized hypodermic adipose tissue found in most marine mammals, has emerged as a practical alternative tissue wherein steroid hormones can be measured. The blubber tissue is thought to accumulate cortisol over hours to days that passively diffuse from blood with a delay of one to several hours after the onset of the stressor (Champagne et al., 2017; Kellar et al., 2015). These attributes of hormone integration offer a potentially new method of studying baseline cortisol levels that otherwise might be difficult to obtain. The growing number of endocrine studies using blubber suggests that this tissue may become the preferred method of hormone analysis in wild cetaceans (Beaulieu-McCoy et al., 2017; Kellar et al., 2015; Trana et al., 2015).

Despite its popularity, few studies have addressed whether collection protocols can influence steroid hormone levels present in blubber. For instance, the state of sample decomposition could be a source of variability of these

levels in the blubber of humpback (*Megaptera novaeangliae*) and beluga (*Delphinapterus leucas*) whales (Mello et al., 2017; Trana et al., 2015). As demonstrated in belugas and harbor porpoises (*Phocoena phocoena*), cortisol is stratified through the blubber, thus caution should also be taken when different sample depths are collected (Kershaw et al., 2017; Trana et al., 2015). Similarly, the body location of blubber collection has been shown to influence sex hormone levels in some species (Kellar et al., 2006; Mello et al., 2017). Regarding blubber cortisol, Kershaw et al. (2017) did not detect differences in blubber cortisol among three body regions in samples collected from stranded harbor porpoise. As observed in terrestrial mammals, where hair hormone levels can be influenced by body location in some species (Ashley et al., 2011; Macbeth et al., 2010; Terwissen et al., 2013) but not in others (Carlsson et al., 2016; Macbeth et al., 2012; Tallo-Parra et al., 2017), species-specific patterns could also be possible in marine mammals (Trana et al., 2015). Revealing whether and how cortisol levels vary with blubber location will help interpret physiological metrics and improve future collection protocols.

To improve accuracy and interpretation of hormone measurements in this sample matrix, the present study was designed to evaluate whether sample location within the body influences blubber cortisol concentration in striped dolphins (*Stenella coeruleoalba*). As reviewed elsewhere, two different reference denominators have been used to estimate blubber cortisol levels (Champagne et al., 2018); blubber tissue mass and amount of lipid extracted. Therefore, the present study also aims to highlight the influence that the reference denominator can have on blubber cortisol concentrations.

A total of 10 striped dolphins (Table 1), stranded along the Catalan coast (northwestern Mediterranean Sea) and in a well-preserved state according to classification of Jauniaux et al. (2002), were transported to Veterinary School of the Autonomous University of Barcelona for necropsy. Blubber samples were collected from five different locations: cranial and caudal areas in relation to the dorsal fin, and dorsal, medial, and ventral areas in relation to the pectoral flipper (Figure 1). Samples were surgically excised before necropsy using a scalpel. To avoid potential effects of cortisol stratification (Kershaw et al., 2017; Trana et al., 2015), full-depth blubber samples were uniformly collected. The epidermis and the outer layer of the dermis, mostly composed of connective tissue, were discarded leaving only fat-filled *panniculus adiposus*, referred to as blubber. Then, a full-depth subsample of 100 mg from the original block was taken for hormone extraction.

Cortisol was extracted from blubber as described by Kellar et al. (2015) with some modifications. In brief, 100 mg of each blubber sample were placed in a homogenization microtube with five 0.7 mm balls and ground using a ball mill for 15 min at 25 Hz (MM200; Retsch, Haan, Germany). The homogenized tissue was first separated, rinsed with ethanol, and the washed contents transferred to a new 15 ml polypropylene tube. Ethanol was also added to the homogenization microtube and all contents transferred to the polypropylene tube. The rinse step was repeated once more obtaining a tube with the sample soaked with a total of 1.5 ml of ethanol. Afterwards, we added 2 ml of a 4:1 ethanol:

TABLE 1 Individual ID, sex, cause of death, and body condition of all subjects sampled in the study.

ID	Sex	Cause of death	Body condition[a]
SC1	Male	Chronic disease	17.86
SC2	Male	Accidental capture	25.98
SC3	Female	Unknown	22.19
SC4	Male	Accidental capture	24.35
SC5	Male	Unknown	19.65
SC6	Female	Chronic disease	17.00
SC7	Male	Accidental capture	22.04
SC8	Male	Accidental capture	19.57
SC9	Female	Accidental capture	22.40
SC10	Male	Unknown	20.14

[a]Body condition was calculated based on the Quetelet's index (mass/length^2 in kg/m^2).

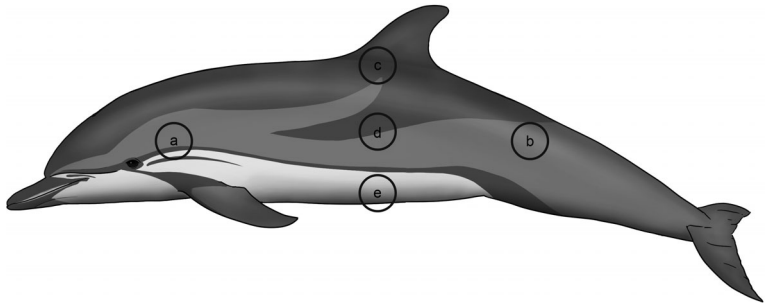

FIGURE 1 Picture illustrating the five different sampling locations of blubber in the striped dolphins: cranial (a) and caudal (b) areas in relation to the dorsal fin, and dorsal (c), medial (d) and ventral (e) areas in relation to the pectoral flipper. Photo credit: Emma Abad García, 2020.

acetone solution, vortexed for 5 min and centrifuged at 3,000 g for 15 min. The supernatant was transferred into a new weighed 15 ml tube and was incubated in an oven at 37°C. After evaporation the entire polypropylene tube with residue was weighted to obtain the amount of lipid extracted by subtraction of the first weight (before evaporation) from the second weight obtained after evaporation of the contents (Mello et al., 2017). We measured the amount of lipid extracted in each individual sample and calculated the proportion of extracted lipid by: proportion extracted lipid = blubber lipid weight (grams)/total blubber weight (0.1 g). Then, 2 ml of diethyl ether were added to the tube, vortexed for 5 min, and centrifuged at 3,000 × *g* for 15 min. 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. Once completely evaporated, the residue was resuspended in 1.5 ml of acetonitrile and lightly vortexed for 5 min. A total of 1.5 ml of hexane was then added to the acetonitrile-lipid mixture, vortexed for 5 min and centrifuged (1,500 × *g*, 15 min). After centrifugation, 1.5 ml of the acetonitrile present in the lower layer was transferred into a new 10 ml glass vial and the process was repeated once more. The final volume of acetonitrile (3 ml) was transferred into a 15 ml polypropylene tube and introduced into the oven at 37°C. The evaporated contents were resuspended in 0.25 ml of enzyme immunoassay (EIA) buffer provided by the EIA kit and stored at −20°C until analysis.

Cortisol concentrations from blubber extracts were measured by EIA (Cortisol EIA KIT; Neogen Corporation, Ayr, UK). The assay was validated for the species and sample of interest (Buchanan & Goldsmith, 2004; Reimers & Lamb, 1991) following the criteria for an immunological validation (Midgley et al., 1969; Reimers & Lamb, 1991). Blubber extracts from 20 different samples were first pooled for the assay validation. Intra- and interassay coefficients of variation (*CV*) from duplicated samples analyzed (intra-assay *CV*, $n = 11$ samples; interassay *CV*, $n = 3$ samples) were calculated for precision assessment. The specificity was tested with the linearity of dilution, determined by using 1:1, 1:2, 1:5, and 1:10 dilutions of the pool with EIA buffer. Through the spike-and-recovery test we assessed the accuracy of the EIA; different volumes of the pool were spiked with different volumes of hormone standard of known concentrations provided by the EIA kit (0.19, 0.45, and 1.05 ng cortisol/ml). Finally, we measured the sensitivity of the test, which was given by the smallest amount of cortisol that the assay can distinguish and measure from the blubber extracts previously diluted. Mean intra- and interassay coefficients of variation were 4.15% and 4.92%, respectively, indicating high repeatability of the test. The dilution test showed an $R^2 = 99.82\%$ and a mean percentage error (± *SD*) of 0.92% ± 12.07%, demonstrating a high correlation between obtained and theoretical cortisol values (Figure 2). The spike-and-recovery values from the spike recovery test ranged from 0.61 ng/ml to 1.17 ng/ml ($M = 0.9$ ng/ml) and the average recovery percentage was 107.5% ± 7.65%, suggesting that other potential compounds present in the tissue may not interfere with the analytical assay. Finally, the sensitivity of the test was 0.124 ng cortisol/g blubber. These results indicate that cortisol detection in striped dolphin blubber by the EIA kit used and with the methodology presented is precise, specific, accurate, and sensitive.

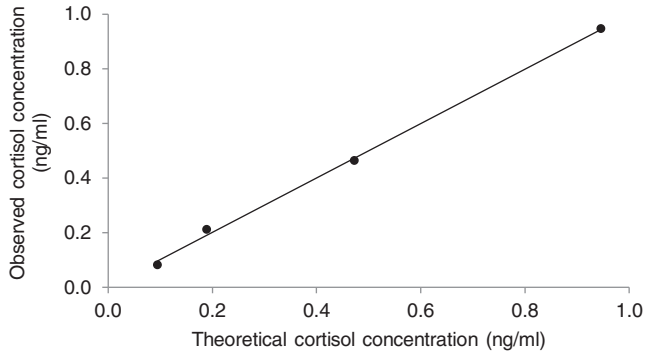

FIGURE 2 Correlation between observed and theoretical cortisol concentrations obtained in the dilution test.

TABLE 2 Model selection and final model output for generalized linear mixed models studying the effect of the body region and the influence of the covariate body condition (BC) in blubber cortisol concentration estimated using wet tissue mass (Model 1, ng cortisol/g blubber) and using lipid content (Model 2, ng cortisol/g lipid) in striped dolphin (*Stenella coeruleoalba*). Individual was included as a random effect in each model.

Model	*df*	AICc	ΔAICc	Wi
Model 1 Wet tissue mass (ng cortisol/g blubber)				
Concentration ~ Region + BC	8	239	0.00	0.731
Concentration ~ Region	7	241	2.03	0.266
Concentration ~ Region + BC + Region*BC	12	250	10.97	0.003
Model 2 Lipid content (ng cortisol/g lipid)				
Concentration ~ Region + BC	8	344.5	0.00	0.835
Concentration ~ Region	7	347.8	3.29	0.162
Concentration ~ Region + BC + Region*BC	12	355.8	11.3	0.003

Abbreviations: *df* are the degrees of freedom; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; Wi are the model weights.

R software (Version 3.5.3; R Core Team, 2016) was used to analyze the data, with a *p*-value below .05 as a criterion for significance. First, normality of the data distribution was tested by applying the Shapiro–Wilk test. Generalized linear mixed models (GLMMs) were used to investigate the effect of body location on cortisol concentrations. The GLMMs was applied since this model allows considering for potential variation among individuals. Individual was accordingly treated as a random effect. To account for the skewed and continuous positive data of hormone concentrations, the GLMMs followed a gamma distribution with a log link function. First, one GLMM (*glmer* function in the R package *lme4*) was applied with concentration measured as ng of cortisol per gram of blubber tissue (ng cortisol/g blubber) and a second GLMM was applied with concentration measured as ng cortisol per gram of lipid extracted (ng cortisol/g lipid). Because body condition can influence cortisol concentrations (Kershaw et al., 2017), this variable was introduced as a covariate in both models. Body condition was calculated following the Quetelet's index (mass/length^2) as this formula has been demonstrated to be the most appropriate for small cetaceans (Kershaw et al., 2017). The interaction between body condition and body location was also added in the models. In both cases, variables were eliminated in a backward stepwise procedure, and selection of the best fit model was performed based upon Akaike's information criterion corrected for small sample size (AICc), ΔAICc (difference between each model's AICc and that of the lowest model) and Akaike weight (Wi). Candidate set models were chosen for which ΔAIC ≤2 (Table 2). When significant, a multiple comparison post hoc test (*glht* function from the R package

multcomp) was applied to determine which body location differed. Finally, the correlation between blubber cortisol concentration and proportion of lipid extracted was studied with a GLMM in which individual was also treated as a random effect. Hormone levels obtained from all five body locations and estimated using blubber tissue mass were used to study the correlation.

While the measurement of steroid hormones in blubber samples is becoming increasingly popular, only one study to date has focused on elucidating whether the body region from which samples are collected influences blubber cortisol levels (Kershaw et al., 2017). In the present study, we compared blubber cortisol levels across different body sites in order to assess the influence of sample location on hormone concentrations in striped dolphins. To our knowledge, this is the first time that levels of blubber cortisol are reported in this species (Tables 3 and S1). Importantly, results demonstrated that the chosen denominator used to estimate hormone levels could potentially influence the interpretation of hormone concentration.

The best fit model based on AIC selection for the blubber cortisol concentrations measured as a function of blubber wet tissue (ng cortisol/g blubber) failed to find differences across the five body regions evaluated in striped dolphins ($p > .05$; Figure 3a), similarly to results reported previously in harbor porpoises (Kershaw et al., 2017). However, differences between regions were detected when cortisol assessments were made using the total amount of lipid as the denominator (ng cortisol/g lipid; Figure 3b). Under this scenario, the dorsal area to the pectoral flipper presented higher cortisol levels when compared to the caudal ($p = .002$), cranial ($p < .001$) and medial ($p < .001$) areas, and concentrations of the ventral area differed significantly to levels of medial samples ($p < .05$). In both cases, the AIC selection indicated that the best fit model included body condition as a covariate. The GLMM in turn revealed that this covariate was negatively related to both estimates of blubber cortisol concentrations ($p < .01$).

The layer from which samples are collected and the sample mass itself can both be a source of variation in the extraction efficiency and consequently, in the levels of cortisol measured (Mello et al., 2017; Trana et al., 2015). To avoid the potential influence of these intrinsic factors, full-depth blubber was used in the present study and the mass was kept constant across all samples processed. Differences between hormone estimates could therefore be driven by blubber lipid differences.

The blubber tissue has different functions across the body (i.e., insulator, buoyancy, and energy storage), and as such, the lipid content can vary depending on the anatomical region (Gómez-Campos et al., 2015; Tornero et al., 2004). The ventral region in striped dolphins, similar to harbor porpoises and common dolphins (Koopman et al., 2002; Tornero

TABLE 3 Blubber cortisol concentrations in five different regions of the body of 10 striped dolphins (*Stenella coeruleoalba*) estimated by wet blubber mass (ng/g blubber) and by amount of lipid extracted (ng/g lipid) as denominators.

	Caudal		Cranial		Dorsal		Medial		Ventral	
ID	blubber	lipid	blubber	lipid	blubber	lipid	blubber	lipid	blubber	lipid
SC1	15.9	49.2	14.2	41.5	24.0	55.5	14.4	32.0	21.1	68.4
SC2	2.7	5.2	2.0	3.2	2.8	8.3	3.0	5.8	2.8	5.2
SC3	6.4	10.2	5.3	8.9	9.2	72.8	7.8	12.5	7.3	28.9
SC4	3.8	9.4	3.5	5.9	4.4	14.7	5.2	7.2	4.0	NA[a]
SC5	15.0	64.0	18.1	31.9	6.8	60.7	23.5	38.0	27.0	49.4
SC6	15.3	43.5	16.1	45.1	18.6	98.0	17.6	36.5	19.6	88.6
SC7	3.0	5.1	3.4	11.1	5.5	18.8	2.1	3.4	2.7	5.2
SC8	2.2	5.5	2.1	2.9	6.0	20.3	2.0	9.0	2.8	9.9
SC9	3.6	NA[a]	2.6	4.1	2.2	4.9	2.7	3.9	3.1	7.5
SC10	5.9	16.2	7.4	20.1	2.7	7.3	7.1	11.9	12.8	22.7

[a]Samples in which the amount of lipid extracted could not be measured.

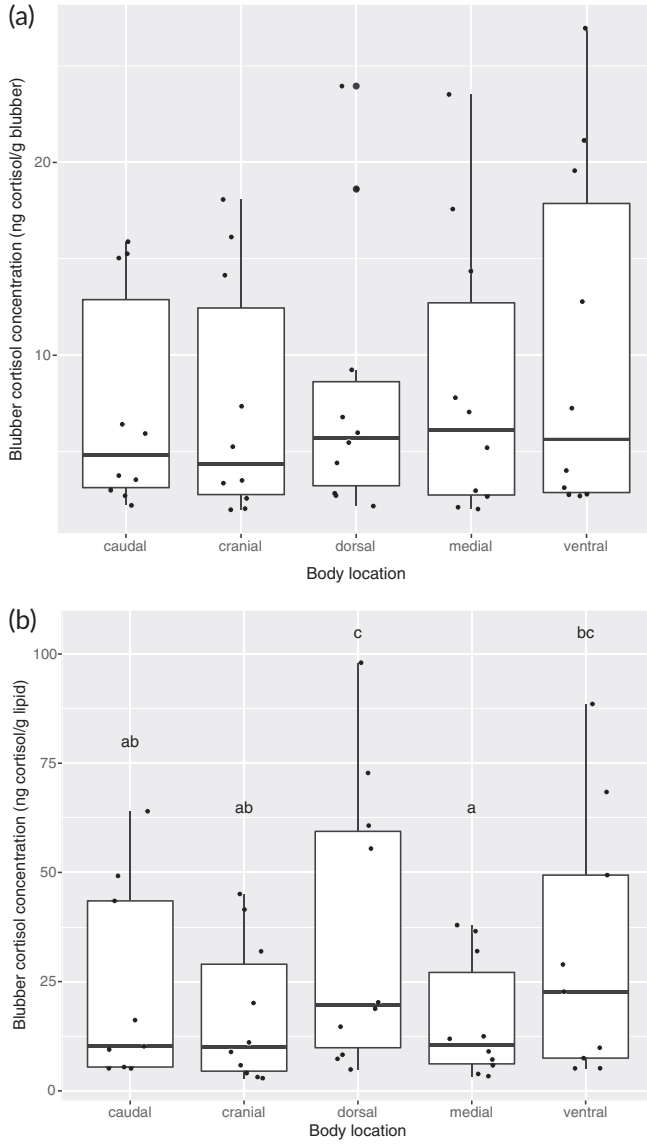

FIGURE 3 (a) Blubber cortisol concentration measured as cortisol per gram of blubber sample (ng cortisol/g blubber) and (b) blubber cortisol concentration measured as cortisol per gram of lipid extracted (ng cortisol/g lipid) across five body sites in 10 striped dolphins (*Stenella coeruleoalba*): cranial and caudal areas in relation to the dorsal fin, and dorsal, medial, and ventral areas in relation to the pectoral flipper. Letters above the bars denote significant differences between body regions in cortisol levels estimated with gram of lipid extracted as denominator (ng cortisol/g lipid).

et al., 2004), presents higher lipid concentration and as such, this location has been suggested to have the function of energy storage (Gómez-Campos et al., 2015). Higher number of adipocytes and lipid concentration could explain the higher blubber cortisol concentrations detected in the ventral region. The reason why the dorsal area also presented higher hormone levels measured as ng cortisol/g lipid is unclear. In striped dolphins, the dorsal area presents the lower adipocyte number and lipid content compared to the ventral locations (Gómez-Campos et al., 2015), further hindering interpretation. Blubber has been recognized as a site of active steroid metabolism (Galligan et al., 2018) and evidence in other mammals suggests that the hormonal activity of the adipose tissue differs across body regions (Lindsay

et al., 2003). Therefore, results could reflect a different diffusion and local transformation of the hormone across the body blubber. Nevertheless, although we collected fresh tissue whenever possible, we must recall that samples were taken from stranded animals and cortisol values may not represent the healthy population.

The units used to reflect blubber cortisol concentrations vary among reports, making interpretations and direct comparisons difficult. If blubber cortisol is predominantly dependent on fat content, then "ng cortisol/g lipid extracted" is the appropriate unit to use. Accordingly, our results here could reflect a different rate of cortisol deposition across the body and anatomical variation should be considered to ensure consistent sampling. Conversely, if cortisol incorporation into blubber is not only dependent on fat content, using the amount of lipid extracted as the reference denominator would be acting as a confounding factor. In this context, our results would suggest a homogeneous distribution of cortisol across the blubber.

Given that the use of one denominator over the other remains unclear, and results can vary depending on this factor (Mello et al., 2017), defining the anatomical sampling location on the body should be a relevant prerequisite for the appropriate physiological interpretation of blubber analyses. To date, the dorsal area has been the preferred body location sampled as it is easily accessible, especially in wild living animals biopsied by remote methods. We therefore suggest caution when making straightforward equivalences between the dorsal samples and the whole-body regions and recommend studying different sites simultaneously until more validation work is available.

Finally, body condition, a measure that reflects the energy stores of an individual (Aguilar & Borrell, 1990), was negatively related to both estimates of blubber cortisol concentrations. In general, individuals sampled with a chronic disease (SC1 and SC6) presented worst body condition indices compared to those accidentally captured, and accordingly, higher cortisol levels. Certain pathogens and diseases can increase cortisol concentrations (Cook, 2012; Hunt et al., 2018). This hormone in turn may increase lipolysis to provide energy resources (Galligan et al., 2019; Khudyakov et al., 2017; Reynaert et al., 1976), as supported by the significant relationship detected between blubber cortisol levels and proportion of lipid extracted (Figure 4; $p < .001$).

Overall, although the present study was not specifically designed to evaluate which reference denominator best fits cortisol blubber assessments, these findings are indeed biologically relevant because they highlight that results depend on which of the two measurements is assigned to the denominator.

Given that standardization of data is a key element for proper comparison between studies, future effort should integrate results using both wet-tissue mass and lipid content to estimate hormone concentrations in order to gain further consensus into this important issue. We encourage forthcoming studies to use biopsy samples from live free-ranging striped dolphins and to evaluate whether the active steroid metabolism takes place homogeneously within the whole-body blubber tissue to better understand how cortisol varies across anatomical sites.

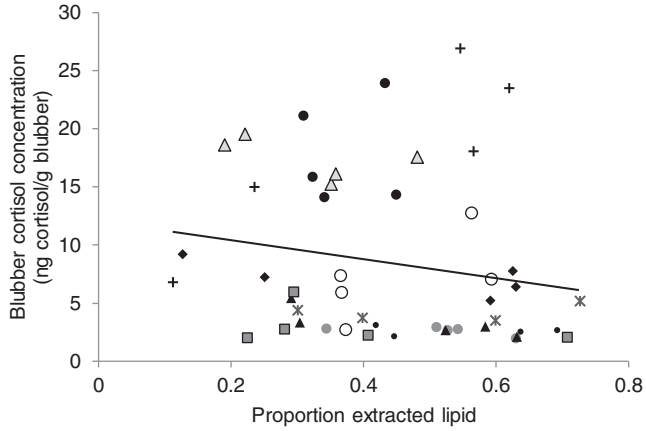

FIGURE 4 Relationship between blubber cortisol concentration estimated using blubber tissue mass (ng cortisol/g blubber) and proportion of lipid extracted. Each individual is represented by a different shape.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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