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Feasibility of using humpback whale blubber to measure sex hormones

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ABSTRACT

This study encompasses a series of interrelated experiments regarding the appropriate handling of samples and the interpretation of measurements of estradiol (described here for the first time in cetacean blubber), progesterone, and testosterone from both live and dead humpback whales. The experiments determined the effects on hormone levels of the following parameters: the state of decomposition of the blubber, the location on the body, the depth of the blubber layer, and the mass of the analyzed sample. The decomposition of carcasses for up to six days (144 h) after death of the animal under natural conditions increased the levels of all three hormones. The dorsal fin presented higher levels of testosterone than other locations. The outer layer of blubber in decomposing samples exhibited higher values of progesterone and estradiol than the middle and inner layers and also exhibited a greater amount of extracted lipids. A lack of adjustment for relative volumes of extract and solvent led to an inverse relationship between hormone level and sample mass; smaller samples (25–50 mg) exhibited higher levels of hormones than did larger ones (50–300 mg). Certain data adjustments are proposed to minimize the effect of sample mass on hormone measurement, including the use of an alternative mass unit (amount of extracted lipid). The methodological approaches presented here contribute to the better standardization of this emerging technique and thereby facilitate the comparison of hormone levels among different cetacean populations and species.

1. Introduction

Although the humpback whale is currently one of the most studied cetacean species worldwide, few studies have examined the reproductive endocrinology of this species. The collection of biological matrices commonly used to measure sex hormones in small cetaceans, such as blood (Robeck et al., 2005, 2009) urine (Steinman et al., 2012), and feces (Biancani et al., 2009; Kusuda et al., 2011), becomes logistically very difficult in larger whales. Measurements of the fecal metabolites cortisol and progesterone (Rolland et al., 2005; Hunt et al., 2006) have been performed in northern right whales (*Eubalaena glacialis*), although this technique is restricted to feeding areas, and the populations that have been photographically identified are well known. Subcutaneous blubber may be used for sampling and can be collected from both live animals and carcasses. Recent studies have shown the viability of subcutaneous blubber for measuring progesterone in dolphin carcasses (Kellar et al., 2006; Trego et al., 2013) and whale carcasses (Mansour et al., 2002; Kellar et al., 2013), for measuring testosterone in dolphin carcasses and biopsies (Kellar et al., 2009) and, more recently, for measuring cortisol in beluga whale (*Delphinapterus leucas*) carcasses (Trana et al., 2015). Very few studies have addressed the blubber hormone concentration of living great whales. To date, there is only one published study on seasonal variation on blubber testosterone concentration of 32 humpback whales from the North Pacific (Vu et al., 2015). In addition, estradiol, an important indicator of ovarian activity in female cetaceans (Fragalà et al., 2015), has not been measured in the blubber of any species of aquatic mammal.

Every year, dozens of humpback whale carcasses wash up on the Brazilian coast (Marcondes, M.C.C., pers. comm.; Groch et al., 2012; Moura et al., 2013). These carcasses represent a potentially enormous source of information regarding the reproductive status of whales. Most of these whale carcasses beach in a state of advanced decomposition, and the extent to which the state of decomposition of these samples affects the extraction of lipids and the accuracy of hormone measurements derived from carcass samples is currently unknown. Geraci and Lounsbury (2005) created body-condition codes for beached marine mammals ranging from 1 (indicating live individuals) to 5 (indicating mummified or skeletal remains only). These body-condition codes were used to classify the carcasses used in this study.

The collection of blubber from live animals is usually performed remotely through biopsy, with the point at which the dart lands on the whale being potentially quite variable (Noren and Mocklin, 2012). Typically, the region struck is in the latero-superior portion of the body from the dorsal fin nearly up to the peduncle (Gauthier and Sears, 1999). Blubber, an adipose tissue characteristic of marine mammals, differs from the adipose tissue of other classes of mammals mainly in that it is rich in collagen and connective tissue (Iverson, 2002). The amount

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of lipid present in blubber adipocytes is known to vary among the outer, middle, and inner layers in several cetacean species (Iverson, 2002), including humpback whales (Waugh et al., 2014). In addition, blubber samples of the same volume can contain different amounts of lipid depending on the nutritional or reproductive status of the whale (Miller et al., 2011).

The average amount of blubber obtained using a small biopsy dart is 150 mg (Kellar et al., 2006). Nevertheless, the size of the blubber sample collected using larger stainless steel collection tips (9 × 50 mm) is extremely variable; the angle, speed and distance at which the dart strikes the whale are some of the variables that affect the amount of material collected (Noren and Mocklin, 2012). In addition, a single sample can be used in multiple areas of inquiry, such as research on pollutants (Waugh et al., 2014), diet (Borobia et al., 1995), and trophic ecology (Budge et al., 2006). In such studies, knowledge of whether sample mass can affect the final results is essential for correct physiological interpretation.

When dealing with non-conventional matrices, a series of tests should be conducted to eliminate bias and "noise" in hormone extraction and measurement. Not only storage time and the type of solution used for extraction but also the initial treatment and handling of the samples may affect the final results (Kalbitzer and Heistermann, 2013; Khan et al., 2002). For example, fecal samples that are freeze-dried at −20 °C prior to lyophilization yield better results than those subjected to other treatments (Lynch et al., 2003; Terio et al., 2002). Regarding blubber samples, the mass unit used, i.e., g of raw sample or g of extracted lipid, may affect the final results and the interpretation of the hormone measurements.

Based on the above information, this study sought to evaluate whether humpback whale blubber from carcasses and live animals is viable for the measurement of three sex steroids (progesterone, estradiol, and testosterone), whether the state of decomposition of the carcass affects hormone levels, whether different regions of the body contain different concentrations of hormones, whether the depth of blubber collection affects the measured hormone levels, and whether the mass of the sample affects the final result of a measurement.

2. Materials and methods

Four different interrelated pilot experiments were conducted to examine potential trends regarding whether the following conditions can affect the measured concentrations of progesterone, estradiol, and testosterone per g of (raw) blubber: 1 - the state of decomposition of the sample (carcass samples); 2 - the region of the body from which the sample was collected (carcass samples); 3 - the depth of sample collection (carcass samples); and 4 - the mass of the sample (carcass and biopsy samples).

2.1. Animals

In experiment 1, blubber samples from a recently (~2 h) dead male humpback whale calf were used. In experiments 2 and 3, samples from two humpback whale male calves, also recently dead, were taken. The samples for experiment 4 were five samples from carcasses in different states of decomposition and biopsies from live humpback whales, as described below. The animal carcasses included two male calves, an adult female, and two adult males classified according to the decomposition scale of Geraci and Lounsbury (2005) as 2 (freshly dead), 4, 4, 4 (advanced decomposition), and 2 (freshly dead), respectively (see decomposition effect).

The carcasses were recovered on the coast of Brazil in the region south of Bahia and north of Espírito Santo between coordinates 16°09′S, 38°56′W and 19°51′S, 40°04′W in July and September of 2011 and 2013 (Fig. 1). In total, eight biopsies were obtained from six adult males, a juvenile female and a juvenile male and were collected in October 2012 near the Abrolhos Marine National Park (17°49′S, 38°49′W), the site with the highest concentration of humpback whales in the western South Atlantic Ocean (Zerbini et al., 2004) (Fig. 1).

All samples were kept frozen at −20 °C from the time of collection until extraction. All of the samples weighed 100–150 mg, with the exception of those used in the sample-mass experiment. Epidermal tissue was removed before weighing.

2.1.1. Decomposition effect

The objective of the first experiment was to investigate the extent to which it is possible to collect and measure samples from a whale when the carcass has already begun to decompose. Thus, the exposure of a whale carcass to environmental weathering over a six-day period was simulated, while the air temperature was continuously monitored with a digital thermometer. A piece of blubber (approx. 30 × 30 cm) from a recently dead male calf beached in Prado, Bahia on September 30, 2013, was collected and placed in an empty, upside-down, ventilated plastic box such that the piece of blubber was in direct contact with the sand and protected against scavengers. Sub-samples were collected from the original sample throughout the six-day period (day zero to day six), and their physical attributes, including color, smell, and thickness, were measured and recorded.

Sample-state descriptions and sample classifications according to Geraci and Lounsbury (2005) can be found in Table 1. The classification metric is as follows: 1 - alive; 2 - freshly dead; 3 - decomposed but with organs basically intact; 4 - advanced decomposition (i.e., organs not recognizable, carcass intact); and 5 - mummified or skeletal remains only. The colors described in the table represent the shallowest layer of the sample. When cut more deeply, the blubber layer had a singular light-pink tint, regardless of the day of collection (Figs. 2A, 3B and 3C). The outer, middle and inner layers were not macroscopically distinguishable.

Given the limited number of fresh carcasses used in this experiment, the statistical analysis may have low power, although some trends could be clearly observed through graphical representation.

2.1.2. Body location effect

In the second experiment, samples were collected from eight different regions of the dorsum of two recently dead male calves to simulate collections performed using a crossbow in biopsies of live animals. The objective was to test whether significant differences in concentrations of progesterone, estradiol, and T are observed depending on the region of the body from which the sample was collected. One of the animals beached at Barra do Riacho, Espírito Santo (ES) on September 8, 2013. The second animal used in this experiment is the same calf used in experiment 1 (decomposition effect). Each sample location was identified by color; at each body location, the outer, middle and inner layers were collected.

2.1.3. Layer effect

Samples from the animals in experiments 1 and 2 were used to test whether there is variation in hormone concentrations depending on the depth of blubber collection. Sixteen pieces of blubber from different body locations (as in experiment 1) were cross-sectioned such that the layers were proportionally spaced between skin and muscle. A total of 48 samples were collected, 16 from the outer layer (close to the skin), 16 from the middle layer, and 16 from the inner layer (close to the muscle layer), each weighing approximately 150 mg. Each sample was sectioned into small pieces (approximately 25 mg) to facilitate homogenization.

Comparisons among the layers were performed in two distinct ways. First, the outer, middle, and inner layers were compared across days. Second, the outer, middle, and inner layers were compared among the different regions of the body. Again, the statistical power of these results may be low due to the small number of samples analyzed.

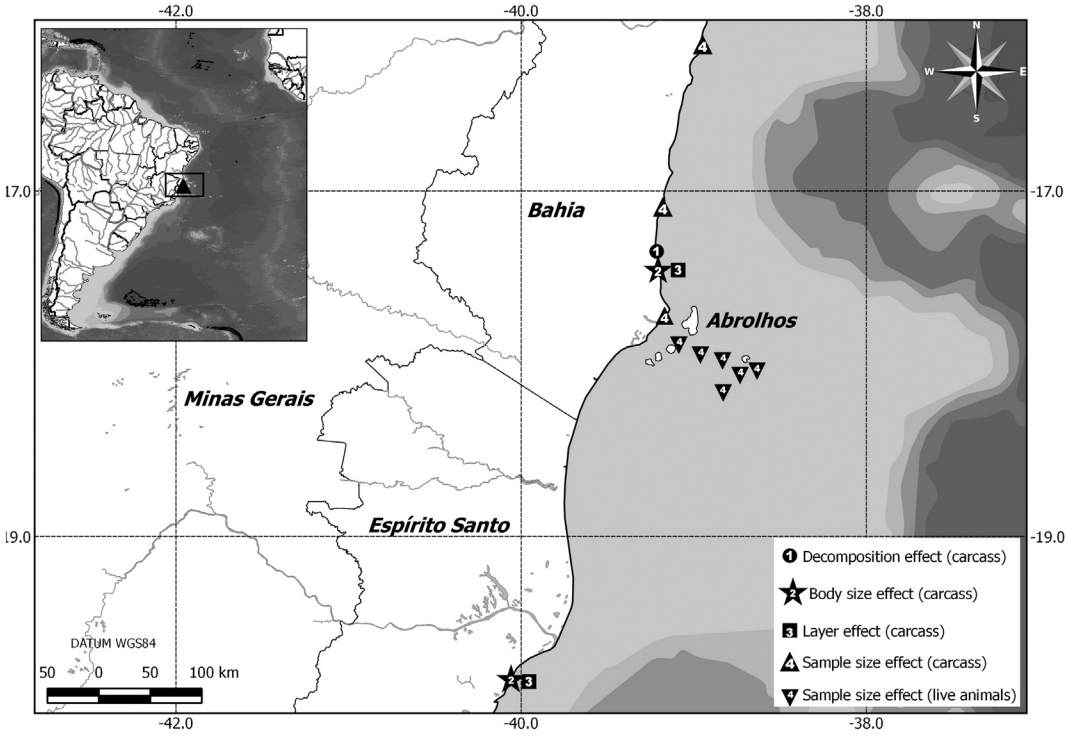

Fig. 1. Geographic distribution of the blubber samples collected from carcasses and live humpback whales in experiments 1, 2, 3, and 4: 2011–2013, Brazil.

2.1.4. Sample-mass effect

Samples of carcasses and biopsies were sectioned into progressively smaller pieces and grouped by size class: 25–50 mg, 50–100 mg, 100–150 mg, and 150–300 mg for carcasses; and 25–50 mg, 50–100 mg, 100–150 mg, and 150–200 mg for biopsies. The samples collected via biopsy contained approximately 1 cm of skin and 3 cm of adipose tissue. The carcass samples were sectioned to approximate this size, i.e., so that they included tissue up to 4 cm below the skin. After separation of the samples into groups according to mass, all of the samples were sectioned, while still frozen, into small pieces of approximately 25 mg to optimize the homogenization process and the subsequent extraction of hormones.

It has been hypothesized that homogenization might be more efficient in smaller samples and therefore that extraction from smaller samples may yield proportionally more hormone. Therefore, the correlation between the mass of the biopsy sample and the mass of extracted lipids was assessed.

All of the samples in this experiment are derived from the outer layer of an adult whale because the mean thickness of the blubber layer in the dorsal region of an adult humpback whale is approximately 15 cm, whereas that of a juvenile whale is approximately 9 cm (Ackman et al., 1975).

Table 1
Gross morphological changes in humpback whale blubber during six days of decomposition.

Day	Time after death (h)	Thickness (cm)	Color	Skin	Temp min-max (°C)	Code*
0	2	3.2	Light yellow	Attached	26.6	2
1	26	3	Light yellow	Attached	19.8–31.8	2
2	50	2.6	Yellow	Attached	21.4–33.9	3
3	74	2.5	Dark yellow	Attached	22.8–36.2	3
4	98	2.5	Yellow/orange	Attached	23.8–35.1	3
5	122	1.8	Orange	Separated	23.4–35.1	4
6	146	1.8	Orange/brown	Separated	21.8–40.4	4

* Decomposition scale described by Geraci and Lounsbury (2005).

2.1.5. Hormonal extraction

Hormonal extraction was based on the method described by Kellar et al. (2006), with some modifications. The samples were homogenized five times in an Omni Bead Ruptor (Omni International, Kennesaw, GA, USA) device at a speed of 5.5 m/s at intervals of 45 s in 1 mL of ethanol and with 0.7 mm Garnet Beads Bulk (Omni International, Kennesaw, GA, USA). After this solution was transferred to a new tube without beads, 2 mL of 4:1 ethanol:acetone solution was added to the tube, and the sample was vortexed for 7 min at 2200 rpm. The solution was then centrifuged for 15 min, and the supernatant was transferred to a new tube. An important modification to the Kellar et al. (2006) protocol was weighing of the empty tubes to which the previously described solution was transferred. After they were dried by airflow in a hot water bath (45 °C), the tubes were weighed again, and the amount of lipid extracted was calculated by subtraction of the first weight from the second weight. At this time, a drop of lipid could be observed at the bottom of the tube. After this stage, 2 mL of ether was added to the tube, and the tube was then vortexed for 7 min at 2500 rpm. The solution was centrifuged for 15 min, and the supernatant was transferred to a new tube. After the supernatant was dried by airflow, the residue was resuspended in 1 mL of acetonitrile and vortexed for 7 min at 2500 rpm. Next, 1 mL of hexane was added to the tube, which was again vortexed for 7 min at 2000 rpm and centrifuged for 15 min. The acetonitrile layer was aspirated into a new tube, and the vortexing and centrifugation steps were repeated with an additional 1 mL of hexane. The final portion of acetonitrile was transferred to a new tube and evaporated by airflow in a hot water bath (45 °C). The final extract was frozen at −20 °C until hormone measurement.

2.1.6. Hormone analysis

For the measurement of testosterone, extracts of the samples were suspended in 500 μL of PBS one hour prior to beginning the assay. After vortexing for 30 min, 250 μL of this volume was added to an additional 250 μL of PBS for the measurement of estradiol and progesterone. Some samples required further dilution in 2000 μL of PBS to be within the range of the standard curve provided with the progesterone kit.

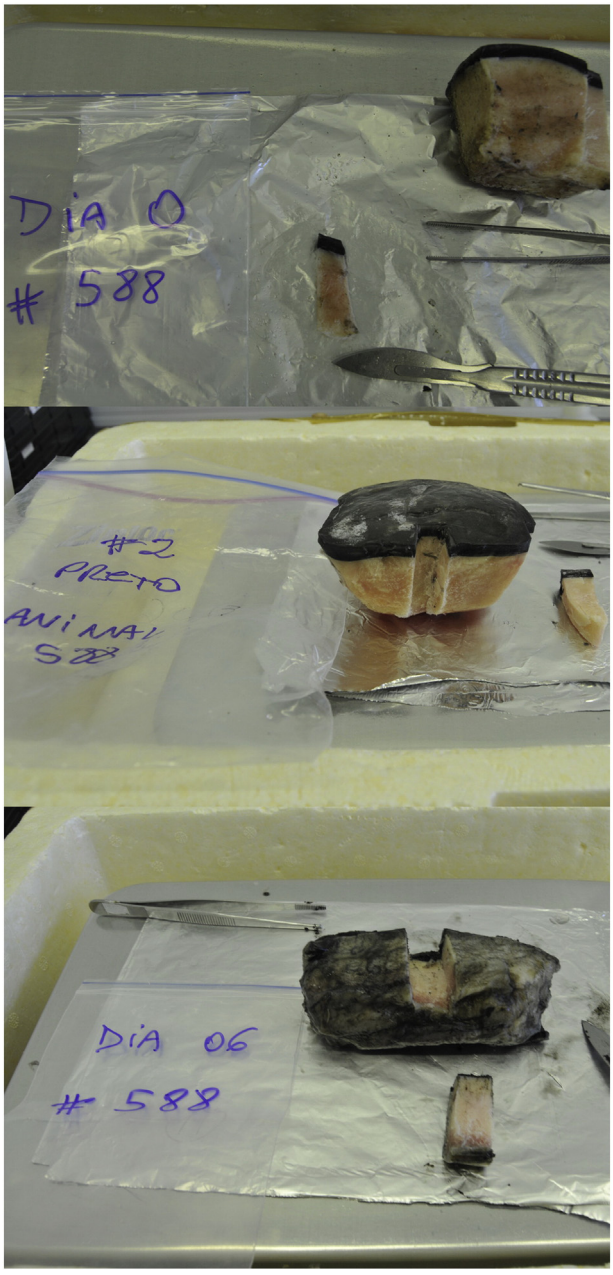

Fig. 2. Blubber sub-samples collected on day 0, 2 and 6, respectively, from a carcass of a recently dead male calf beached in Prado, Bahia (17° 20′ S; 39° 13′ W) – 2013.

The levels of the three hormones were measured using commercially available enzyme immunoassay kits from Enzo Life Sciences (Farmingdale, NY, USA). These kits were selected because, among the commercially available kits, they performed best when tested for use in hormone measurements in dolphins and whales (Kellar et al., 2013; Trego et al., 2013). The manufacturer's protocols provided with the kits were followed without modification. Total activity, zero samples and blank samples were included in all plates. All samples, standards and controls were assayed in duplicate. Samples that exhibited results with CV > 12% were extracted and measured again. A pool of samples with low hormone concentrations was measured at the end of all of the plates. Samples that fell outside the range of the standard curve were diluted appropriately – up to eight times for progesterone (1:8) and two times for testosterone (1:2) – and assayed again.

According to the manufacturer, the sensitivities of the assays for progesterone, testosterone and estradiol are 8.57 pg/mL, 2.6 pg/mL and 14.0 pg/mL, respectively, and the limits of the detection curves are 15.62–500 pg/mL for progesterone, 3.9–1000 pg/mL for testosterone and 15.6–1000 pg/mL for estradiol. The cross-reactions of the progesterone kit are given as 100% for progesterone and 5α-pregnane-3,20-dione, 3.46% for 17-OH-progesterone, and 1.43% for 5-pregnen-3β-ol-20-one. The cross-reactions of the testosterone kit are given as 16.4% for androstenedione, 7.6% for 19-hydroxytestosterone, and 2.7% for dihydrotestosterone, whereas for the estradiol kit, these values are 17.8% for estrone and 0.9% for estriol. The intra-assay coefficients of variation were 7.6–4.9% for progesterone, 2.3–4.1% for testosterone, and 2.1–5.7% for estradiol. The inter-assay coefficients of variation were 2.7–6.8% for progesterone, 7.4–10.2% for testosterone, and 8.3–14.2% for estradiol.

2.1.7. Parallelism, controls and efficiency of extraction

The quality and accuracy of the hormone assays were tested using a series of analyses, as described below.

A parallelism test was performed by progressively diluting two pooled samples in the same proportion as the dilution of the points of the standard curves of the kits (a factor of two for progesterone and estradiol and a factor of four for testosterone). The F test was employed to evaluate the parallelism between the kit's standard curve and the data obtained using the diluted pools in assays for the three hormones (progesterone, testosterone and estradiol). The accuracy of the assay was tested by using the pooled samples to spike a set of standards at known concentrations.

The efficiency of extraction, which tested the effect of the sample matrix on hormone measurement, was measured by adding known quantities of hormones obtained by diluting the kit standards to subsamples obtained from the single samples. Immediately prior to extraction, 0, 25, or 50 ng of progesterone, 0, 12.5, or 25 ng of testosterone, or 0, 12.5 or 25 ng of estradiol was added to each subsample. After the plates were read, the efficiency of extraction was determined by subtracting the results obtained from unspiked samples from those obtained from spiked samples and dividing by the initial amount of hormone added. Although this method of testing hormone extraction efficiency is not as accurate as the use of radiolabeled hormones, it may provide reasonable results when good assay accuracy is achieved.

2.1.8. Statistical analysis

The Kolmogorov-Smirnov test was used to assess deviations from normality, and Levene's test was used to assess the homoscedasticity of the sample variances. Analyses that involved the comparison of three or more means were performed using one-way ANOVA in the case of homoscedastic samples and using the Kruskal-Wallis test for non-homoscedastic samples. The ANOVA or Kruskal-Wallis test was followed by a post-hoc Tukey test. Repeated-measures ANOVA was used to compare hormone measurement over days. An F-test tested the parallelism among diluted sample pools and the standard curves of the kits, and accuracy was checked by regression analysis, comparing the slope of the expected and observed doses.

All statistical comparisons in this study were considered significant at $P \leq 0.05$. Dispersion plots and some simple regression analyses were prepared using Microsoft Excel®. All statistical analyses were performed using Statistica version 7.0 (StatSoft®, Tulsa, OK, USA).

3. Results

Estradiol was successfully measured in samples from the blubber layer of humpback whales.

3.1. Parallelism, controls and efficiency of extraction

The inter-assay %CV of the pools was 7.01% for progesterone, 6.4% for testosterone and 7.27% for estradiol. The average intra-assay %CV was 4.4% for progesterone, 3.53% for estradiol, and 2.2% for testosterone.

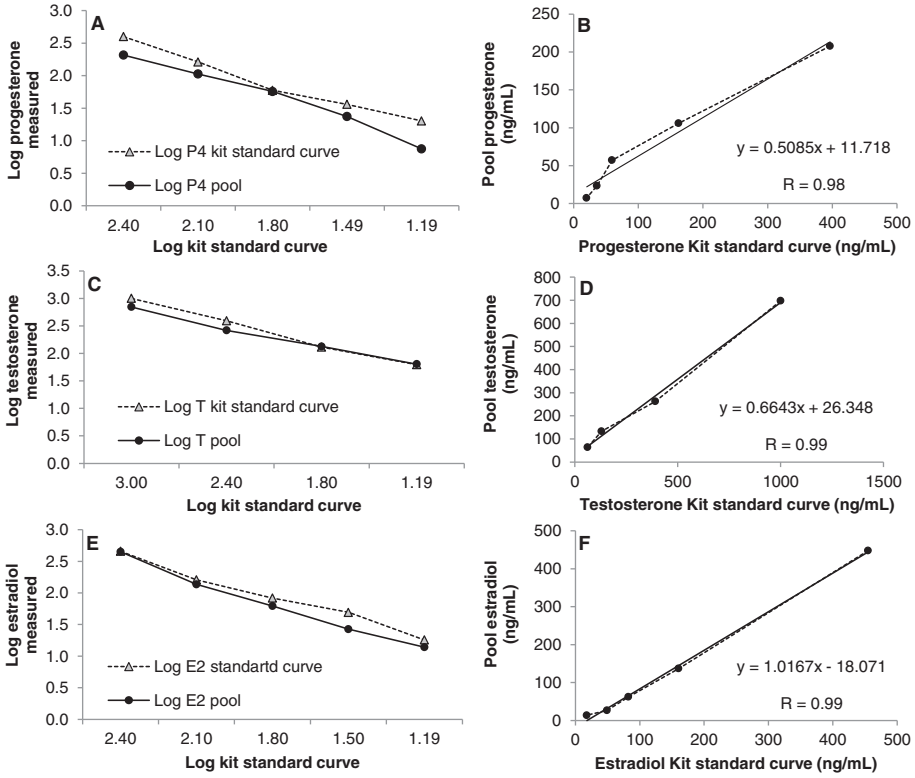

Fig. 3. Validation results for progesterone, testosterone and estradiol for blubber samples from humpback whales. Parallelism tests for progesterone, testosterone and estradiol (A, C and E); and accuracy tests for progesterone, testosterone and estradiol (B, D and F).

The estradiol assay presented excellent parallelism ($F_{1,5} = 0.96$; $p = 0.49$); the progesterone and testosterone assays yielded slightly imperfect parallel curves, but their parallelism was still good ($F_{1,5} = 3.76$, $p = 0.12$ and $F_{1,5} = 2.26$, $p = 0.26$, respectively). These results indicated that the substances measured were most likely the hormones of interest (Fig. 3).

The accuracy test showed a positive linear relationship between the observed and expected doses for estradiol; a slope of approximately 1.0 was observed. Acceptable accuracy was found for testosterone (slope, 0.79), and poor accuracy was found for progesterone (slope, 0.51) (Fig. 3). The progesterone and testosterone accuracy graphs showed a non-linear pattern in which the slope was greater/better at low hormone concentrations. Most of the samples had low progesterone and testosterone levels and fell in the concentration range in which accuracy is good. Of the tested samples, 71% contained ≤200 ng/mL of progesterone, and 88% contained ≤150 ng/mL of testosterone.

The mean efficiency of extraction was 76% for progesterone, 91% for testosterone, and 61% for estradiol. The low accuracy of the progesterone assay might have interfered with calculation of the extraction efficiency.

3.2. Decomposition effect

Each of the three hormones shows a distinct trend of increasing over time (Fig. 5B), although statistical significance was not reached ($F(6,12) = 1.58$, $p = 0.24$ for progesterone, $F(6,12) = 2.91$, $p = 0.05$ for testosterone, and $F(6,12) = 1.41$, $p = 0.29$ for estradiol).

3.3. Body location

The dorsal fin exhibited higher concentrations of testosterone than the other locations tested ($F = 2.47$; $p = 0.04$) (Fig. 4). The concentrations of progesterone and estradiol did not differ significantly among the regions sampled (data not shown).

3.4. Layer effect

3.4.1. Layer effect in decomposing samples

When comparing the hormone concentrations measured in the outer, middle, and inner layers of the samples on days 0 to 6, the outer layer exhibited significantly higher concentrations of progesterone ($F(2,18) = 17.25$, $p = 0.00007$) and estradiol ($F(2,18) = 3.8816$, $p = 0.04$) than the other layers (Fig. 5A). To consider a possible effect of the efficiency of lipid extraction on this result (that is, whether more complete extraction of lipids contributed directly to the measured hormone levels), an analysis of variance comparing the amount of lipid extracted from the outer, middle and inner layers was performed. The outermost layer yielded the greatest amount of extracted lipid ($p = 0.04$), with the difference particularly evident from day two onward (Fig. 5B). No statistical analysis involving days and layers together was performed, although each of the three hormones showed a distinct trend of increasing over time (Fig. 5B).

3.4.2. Layer effect in fresh carcasses

When the outer, middle, and inner layers of blubber from the two recently beached calves were compared, no differences among the layers were observed for any of the hormones evaluated or for any of the samples utilized: progesterone, ng/g: $F(2,41) = 1.69$, $p = 0.19$; testosterone, ng/g: $F(2,33) = 2.81$, $p = 0.07$; estradiol, ng/g: $F(2,27) = 1.29$, $p = 0.29$.

3.5. Sample mass

3.5.1. Sample mass in decomposing carcasses

The measured levels of the hormones per g of blubber increased significantly as the sizes of the samples from the carcasses decreased (Fig. 6).

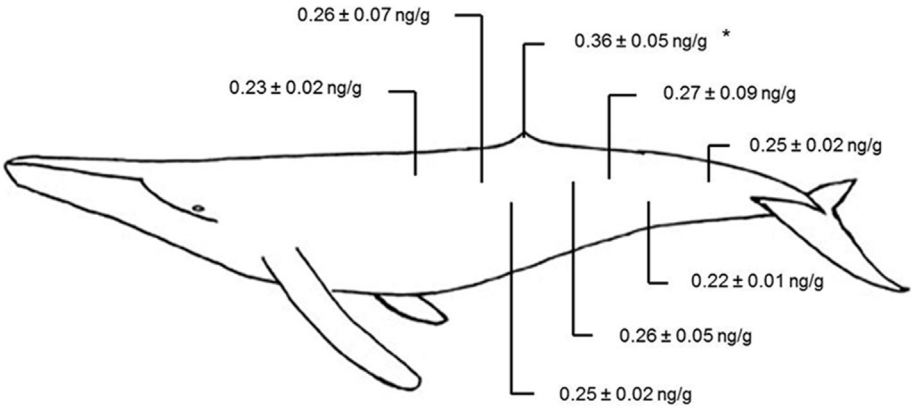

Fig. 4. Testosterone concentration in eight different sites of the body of two male calves. Three samples from the outer, middle, and inner layers at each point were used. * Mean significantly higher than other locations.

3.5.2. Sample mass in fresh carcasses

In the carcass samples, as well, the concentration of hormones increased significantly as the size of the biopsy sample decreased (Fig. 7A). Although Fig. 7A shows a clear trend toward higher values of progesterone in smaller samples, no significant difference among the sample-mass classes was observed. For estradiol and testosterone, the size class 25–50 mg yielded a significantly higher mean than the others did: testosterone, ng/g: $F(3,20) = 4.02$, $p = 0.02$; estradiol, ng/g: $F(3,20) = 26.07$, $p = 0.0000004$ (Fig. 7).

Fig. 8 shows a direct and significant relationship between sample mass and the mass of extracted lipids. That is, smaller samples yielded equivalently less extracted lipid and vice versa, whereas the variation in the amount of extracted lipid was greater in samples weighing 100 mg or more. Most of the values obtained from samples of 70 mg or less are within the confidence interval, whereas for larger samples, the opposite is true.

Further, proportionally more lipid was extracted from smaller samples. The mean percentage of extracted lipid per blubber mass in each sample class size was as follows: 25–50 mg: 32.84%; 50–100 mg: 31.54%; 100–150 mg: 27.20%; 150–200 mg: 24.50%.

When the amount of lipid was used as the mass unit, the hormone concentration no longer differed among sample-mass classes:

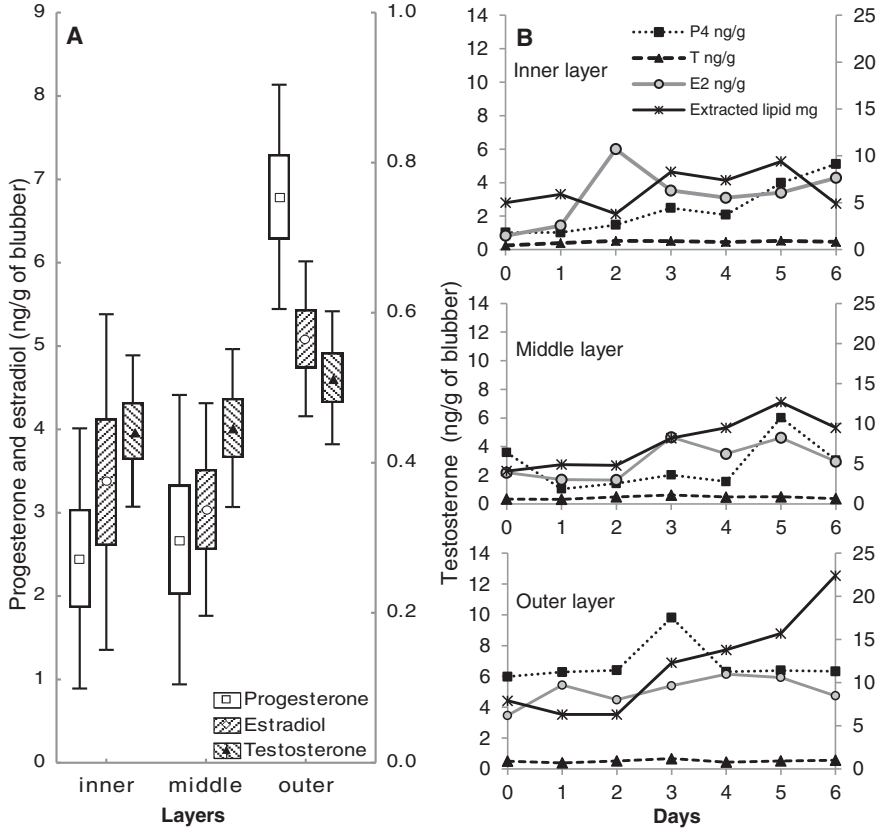

Fig. 5. Mean concentrations of progesterone, testosterone and estradiol in ng/g of blubber of the outer, middle, and inner blubber layers of a male humpback whale calf over six days. The boxes represent the means ± the standard error of the mean (SE), and the whiskers represent the means ± the mean standard deviation (SD). * P4 and E2 mean significantly higher than other layers (A). Concentrations of progesterone, testosterone, and estradiol of a male humpback whale calf over six days (B).

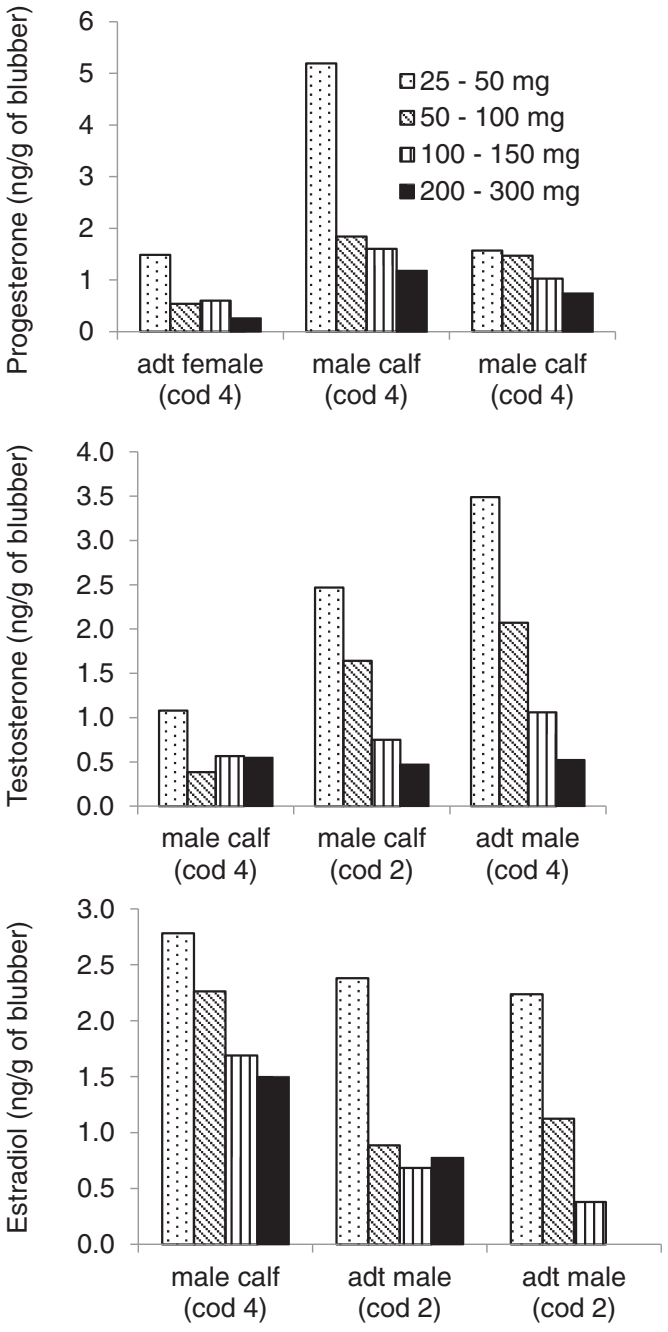

Fig. 6. Concentration of progesterone, testosterone and estradiol in carcasses of an adult female, two male calves and two adult male's humpback whale separated into four distinct sample-mass classes. *Measurements below the limit of the kit's detection curve.

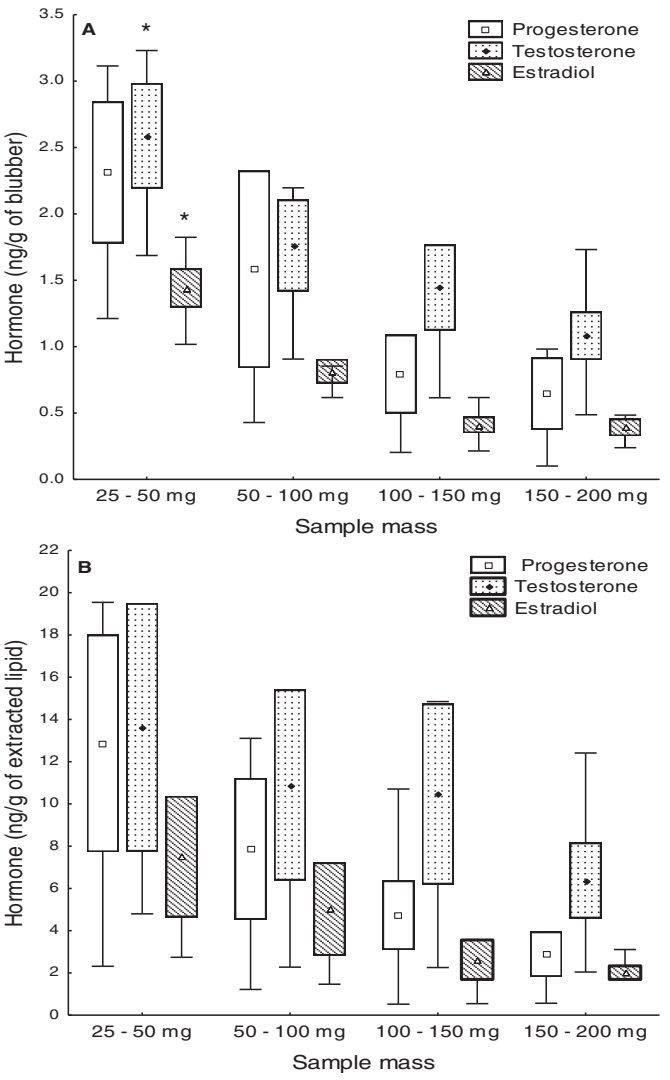

Fig. 7. Mean concentrations of progesterone, testosterone and estradiol in ng/g of blubber and ng/g of extracted lipid, respectively, in biopsy samples from of an adult female, four adult males, and a male juvenile (progesterone and estradiol); and an adult female, six adult males, and a juvenile male (testosterone). The boxes represent the means ± the standard error of the mean (SE), and the whiskers represent the non-outlier range. * Means of T and E2 significantly higher than the other size classes.

progesterone ng/g lipid: $F(3,20) = 0.883$, $p = 0.47$; testosterone ng/g lipid: $F(3,28) = 0.84$, $p = 0.49$; estradiol ng/g lipid: $F(3,20) = 1.80$, $p = 0.18$ (Fig. 7B).

4. Discussion

The determination of the reproductive status of cetaceans can provide valuable information regarding the reproductive parameters of populations. The collection and examination of the gonads of beached animals is an efficient method of studying reproductive biology, especially in small cetaceans (Urian et al., 1996; Westgate and Read, 2006). Still, the size of the animal, its state of decomposition, and the beaching site do not always permit a complete necropsy during which such material can be collected or observed. Blubber proved to be a reliable

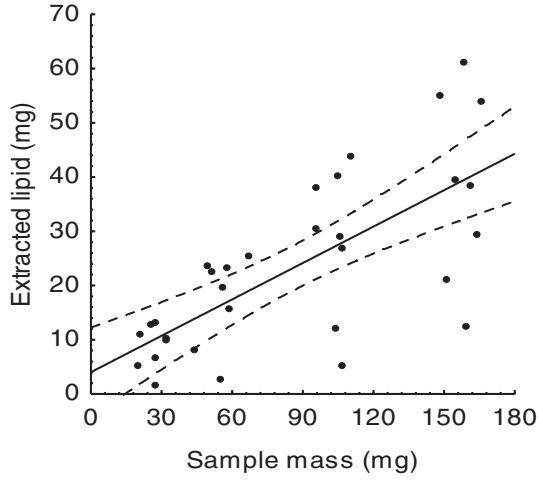

Fig. 8. Increase in the amount of extracted lipids as a function of increase in sample mass ($p = 0.0005$; $r = 0.71$; $r^2 = 0.50$).

matrix for use in the measurement of reproductive steroid hormones from carcasses and free-ranging humpback whales, although some adjustments may be necessary to properly measure and interpret the results. Care should be taken to consider the body site of blubber collection, the blubber depth and the mass of the sample. Decomposed samples of carcasses may yield higher hormone levels after three days of stranding at tropical sites (temperature 19.8–40.4 °C).

Estradiol is an important indicator of ovarian activity and sexual maturity in female mammals (Senger, 2003). This is the first report of the measurement of estradiol from the blubber of aquatic mammals. Seasonal variations, such as an increase in the serum concentration of this hormone, have been correlated with estrus and with implantation in California sea lions (*Zalophus californianus*) (Greig et al., 2007). Seasonal oscillations in conjugated urinary estrogens are also found in belugas (Robeck et al., 2010) and Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) in captivity (Robeck et al., 2009). In this study, estradiol was measured in humpback whale blubber extracts using an enzyme immunoassay technique for both carcass and biopsy samples, demonstrating the feasibility of extracting and measuring this hormone as a potential tool in the endocrine monitoring of humpback whales.

The best assay validation was found for measurements of estradiol in blubber, followed by testosterone and then progesterone. Higher accuracy was found at lower concentrations of the three hormones; this finding was particularly important for progesterone validation because the overall accuracy of progesterone measurement was poor. Although a non-parametric test is commonly used when these conditions apply, here parametric tests were used for this measurement because most of the progesterone measurements yielded values of <200 ng/g.

Every year, hundreds of whale carcasses in various states of decomposition wash up on beaches (Cole et al., 2005; Moore et al., 2005; Wiley et al., 1995). The blubber layer, which is almost always present, is potentially rich in information, although the maximal amount of decomposition that still permits the use of this material for various analyses, including hormone measurements, remains unknown. Kellar et al. (2006) reported that for up to 52 h at 22 °C, the state of decomposition did not significantly affect the progesterone concentration in a *Lissodelphis borealis* female. When the fat (blubber) of beluga whales in several states of decomposition was analyzed, increasing decomposition was observed to decrease the cortisol concentration (Trana et al., 2015). The preliminary experiments in this study demonstrated that up to 146 h postmortem, after exposure to temperatures between 19.8 and 40.4 °C, decomposing blubber of humpback whale carcasses (code 4 according to Geraci and Lounsbury, 2005) showed apparent increases in the levels of progesterone, testosterone and estradiol. Under some conditions, decomposing tissues might release more steroid hormones than fresh tissues, as has been observed for fecal metabolites in primates (Khan et al., 2002; Lynch et al., 2003) and cheetahs (Terio et al., 2002). Additionally, the loss of water from decomposing blubber, especially in the outer layer, may have increased the percentage of lipid by weight and consequently the amount of hormone (ng) per sample (g). It is important to note that the low statistical power of these preliminary results, due to the small number of samples analyzed, showed only a potential upward trend over time. In the future, the use of a more robust sample size is recommended to generate more accurate information.

Although the stranding of humpback whale carcasses on the Brazilian coast is quite common during the breeding period, a large majority of these carcasses are decomposed carcasses of calves (Moura et al., 2013). The absence of fresh adult carcasses stranded during the study period prevented the comparison of the hormone concentrations from different locations in the bodies of sexually mature individuals. As with some species of dolphins, the dorsal fin of the humpback whale exhibited testosterone concentrations that differed from those of adipose tissue. In contrast to *Delphinus delphis*, in which testosterone is less concentrated in the dorsal fin (Kellar et al., 2009), the dorsal fin of humpback whales exhibited higher concentrations of testosterone than did the rest of the body. The reasons for this difference are not clear. It may be that more blood is present in tissue that is still developing, leading to increased hormone levels; alternatively, there may be a direct effect of the biological matrix on the extraction process. In any case, the dorsal fin should be avoided when performing biopsies for the measurement of sex hormones in wild populations.

The blubber layer from which collection is performed may affect the final results of hormone measurements in humpback whale calves. This trend was most evident for samples in the most advanced state of decomposition. In recently dead animals, hormone concentrations did not differ significantly among the outer, middle, and inner layers of the blubber. Still, in decomposing animals, the outer layer of blubber showed higher concentrations of progesterone and estradiol, most likely due to greater efficiency of extraction of lipids from this layer in decomposed samples. It is possible that the collagen and elastic fibers of the decomposing blubber "release" adipocytes with greater ease during homogenization, thereby increasing the amount of available hormones. In addition, the loss of water due to sample dehydration might have an important effect on this phenomenon. Storage of samples at room temperature was found to increase fecal testosterone metabolite levels in the feces of giant pandas (*Ailuropoda melanoleuca*) after 36 h and to increase the levels of fecal progesterone, estradiol, cortisol metabolites, and progesterone after 24 h (Deng et al., 2014). In samples of baboon (*Papio cynocephalus*) feces stored in 95% ethanol at ambient temperature, estrogen and glucocorticoid metabolites increased by 122% and 92% after 90 and 120 days, respectively (Khan et al., 2002).

Because fresh blubber samples from mature individuals could not be accessed for comparison, the results presented here regarding blubber depth should be interpreted with caution. Little is known about how the incorporation and depletion rates of hormones are affected by blubber depth, and rates likely vary among life stages and seasons (Trana et al., 2015). Species-specific variation is also possible; Trana et al. (2015) found that the cortisol level in the inner blubber layer of belugas was higher than that in other species.

The mass of the sample significantly affected the hormone measurements. Contrary to expectation, there was an inverse relationship between sample weight and measured hormone level. Given the number of studies on cetaceans that use blubber as a data source (for example, studies on pollutants (Aguilar and Borrell, 1991), diet (Hooker et al., 2001), and morphology (Montie et al., 2008) and given the limited numbers of samples collected via biopsy, it is often necessary to split the sample among groups of researchers. Knowledge of the ideal sample mass and how different sizes can affect the results is fundamental to correctly interpreting the data.

In this study, samples of lower weight tended to exhibit higher hormone levels, indicating an inverse relationship between sample mass and hormone levels. Samples weighing 25–50 mg displayed considerably higher hormone concentrations than other sample-mass classes. A similar phenomenon was observed (Hayward et al., 2010) in the measurement of avian fecal hormone metabolites, i.e., the use of smaller sample masses resulted in higher apparent hormone concentrations. Apparently, hormone measurement varies according to the ratio of fecal sample mass to extraction-solvent volume (in this case, ethanol) (Wasser et al., 2010). Solvent saturation might explain why larger blubber samples yielded lower hormone concentrations. It is therefore recommended that further analysis using different blubber sample masses and different extraction-solvent volumes be performed. Further investigation is especially important because this phenomenon is likely to occur across multiple sample types and across many taxa.

Meanwhile, the percentage of lipid extracted from smaller samples was proportionally higher than the percentage extracted from larger samples. Importantly, the amount of lipid reported herein refers to the amount extracted following the protocol described by Kellar et al. (2006) with modifications and does not necessarily represent all of the lipid contained in the sample. The difference in lipid extraction not only showed that the homogenization process can be more or less effective depending on the mass of the sample when a fixed/non-variable

extraction-solvent volume is used but also indicated that, under the extraction conditions used, the mass of the sample has a direct influence on the hormone concentration results. Interestingly, when extracted lipid was used as the mass unit, there was no difference in hormone concentration with sample size. It is likely that the substantial variation in the extraction efficiency of lipids from larger samples lowered the statistical power of the analysis, and the absence of significant differences might not simply be a result of mitigation of the sample-mass effect. The correction of the ratio of extraction-solvent volume to the sample mass might decrease this variation. In fact, the measurement of steroid hormone per mass of extracted lipid proved to be reliable. The latter method might offer an alternative when samples of variable sizes must be used, as long as the correct proportion of sample mass to solvent volume is employed.

Whenever possible, all processed samples should be similar in size. Otherwise, statistical verification of potential differences in hormone measurement as a function of sample mass should be performed.

5. Conclusions

The results presented here not only show the feasibility of using humpback whale blubber for reproductive steroid measurements but also describe methodological approaches that can be combined to produce more uniform data collection and analysis among cetacean species. Estradiol can be successfully extracted and measured from humpback whale blubber. Hormone measurement from carcasses maintained under natural environmental conditions for up to six days showed increasing trends in the levels of all three hormones. The dorsal fin showed higher levels of testosterone than other body locations and should be avoided as a sampling site. The outer, middle and inner layers of blubber can yield different hormone concentrations, especially in decomposing animals. The outer layer showed significantly higher values of progesterone and estradiol and also yielded a greater amount of extracted lipids than the other two layers. An inverse relationship between hormone level and sample mass was observed, and lipids were extracted more efficiently from smaller samples. Apparently, homogenization was more efficient in smaller samples when the extraction-solvent volume was not adjusted according to the size of the sample. Future studies might be necessary to evaluate the effects on hormone measurement of increasing the number of rounds of homogenization for larger samples (>75 mg) and of adjusting the ratio of extraction-solvent volume to sample mass to avoid solvent saturation. Samples of similar size (preferably between 25 and 75 mg) should be collected from the same blubber layer to reduce the effects of mass and origin on hormone measurements. The collection of blubber for the determination of hormone levels in carcasses and live animals may be an effective method for determining the reproductive stage of whales using a relatively simple collection technique.

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