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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.

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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.

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