Liquid chromatography tandem mass spectrometry for the quantification of steroid hormone profiles in blubber from stranded humpback whales (*Megaptera novaeangliae*)

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Marine mammal blubber is known to have quantifiable concentrations of steroid hormones and is increasingly chosen as a matrix for the detection of these reproductive and stress biomarkers. Steroid hormones act through complex cascades, often in concert, yet studies conducted on cetaceans have rarely measured more than two steroids simultaneously. Due to the role of steroid hormones in multiple physiological processes, and variability in concentration among individuals, data on single compounds are often difficult to interpret. Here a liquid chromatography tandem mass spectrometry method for the simultaneous analyses of multiple steroid hormones in cetacean blubber was validated and applied to samples from 10 stranded humpback whales (*Megaptera novaeangliae*). Progesterone, 17α-hydroxyprogesterone, testosterone, androstenedione, oestrone, oestradiol, cortisone, cortisol, corticosterone and 11-deoxycorticosterone were reliably (relative standard deviation on six replicates <15%) and accurately (recovery of an amended sample between 70% and 120%) quantified, but not 11-deoxycortisol. With the exception of progesterone, testosterone, oestradiol and cortisol, these compounds were quantified for the first time in humpback whales. Given that blubber is frequently collected from free-swimming cetaceans in ongoing research programs, the technique developed here could substantially strengthen understanding and monitoring of the physiological condition of these species.

Key words: Baleen whale, cortisol, endocrinology, LC-MS/MS, marine mammal, stress response

Editor: Kathleen Hunt

Received 27 August 2018; Revised 12 April 2019; Editorial Decision 14 May 2019; Accepted 16 May 2019

Cite as: Dalle Luche G, Bengtson Nash S, Kucklick RJ, Mingramm FMJ, Boggs ASP (2019) Liquid chromatography tandem mass spectrometry for the quantification of steroid hormone profiles in blubber from stranded humpback whales (*Megaptera novaeangliae*). Conserv Physiol 7(1): coz030; doi:10.1093/conphys/coz030.
Introduction

Steroid hormones are now frequently used as biomarkers of stress, sexual maturity and reproductive state in cetaceans. After baseline hormone values have been established for each species and tissue type, steroids can be used for studying wild population dynamics and health parameters in cetaceans, including large whales (Rolland et al., 2012; Wasser et al., 2017; Pallin et al., 2018a; Trumble et al., 2018). Several remotely accessible biological sample types, such as blubber (Vu et al., 2015; Mello et al., 2017), feces (Rolland et al., 2005) and blow (Hogg et al., 2009), have been collected from live whales for hormone analysis. Non-lethal tissue sampling and non-invasive techniques, such as remote imaging (Cayler et al., 1992; Bradford et al., 2012; Miller et al., 2012; Seyboth et al., 2016), are viable alternatives to the practice of ‘scientific whaling’ when studying whale physiology (Hunt et al., 2013).

To date, only a limited selection of steroid hormones (e.g. progesterone, testosterone, cortisol and oestradiol) have been measured in free-roaming cetacean populations, and most studies have focused only on a single compound (Kellar et al., 2009, 2015; Pallin et al., 2018b). As a consequence, the narrative surrounding steroid hormones in cetaceans is mainly dictated by consideration of one or rarely two or three hormones. This translates to a knowledge gap in our understanding of marine mammal endocrinology. Some physiological states involve downstream endocrinological signals (e.g. spermatogenesis or pregnancy) that entail changes in the concentrations of multiple steroid hormones. Subsequently, information based upon multiple steroid hormones enhances the possibility of a more accurate physiological diagnosis.

Incomplete steroid pathway analysis in wildlife studies can be partially explained by the common application of radioimmunoassays (RIAs) and enzyme immunoassays (EIAs). Both EIA and RIA are based on a hormone-specific antigen–antibody reaction, targeting an individual parent hormone or its metabolites. In particular, EIA techniques are widely employed for their high degree of sensitivity and technical simplicity. However, while the analytical cost of EIAs can be considered modest, the cost and labour of the analysis increases with each additional hormone quantified. Moreover, as immunoassays rely on antigen–antibody interaction, their accuracy may be affected by interference from non-target chemicals (cross-reactivity) compared to direct measurement methods (Hansen et al., 2011). Recently, Boggs et al. (2017) developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) analytical method for the measurement of multiple hormones in dolphin blubber to overcome some of the above-named limitations of immunoassays. LC-MS/MS instrumentation has a high installation cost and requires larger tissue portions than EIAs (Boggs et al., 2017; Mello et al., 2017). However, the possibility of using LC-MS/MS for simultaneous measurement of multiple steroid hormones from a single sample extraction diminishes both cost and relative sample amount per hormone included in the analysis. More importantly, LC-MS/MS has the capacity to separately quantify metabolites with different masses but similar immuno-reactivity, which is pivotal for mapping of endocrinological pathways (Robeck et al., 2016; Galligan et al., 2018a).

Humpback whale blubber differs from dolphin blubber in thickness and composition and can dramatically change in volume and lipid content as a function of the animal’s energetic state (Parry, 1949; Lockyer, 1987; Bengtson Nash et al., 2013; Cropp et al., 2014; Castrillon et al., 2017). Steroid hormone concentrations are usually normalized by a biomarker (i.e. creatinine, dissolved proteins) (Atkinson et al., 1999; Robeck et al., 2005) when they are measured in biological matrices that have variable physical–chemical composition, such as saliva, urine and blow. By contrast, blubber hormonal concentrations are most commonly expressed in the literature on a wet weight basis (Kellar et al., 2006; Vu et al., 2015; Kershaw et al., 2017). Recently, lipid percentage has been proposed as a normalization measure for cortisol concentration in bottlenose dolphin blubber (Champagne et al., 2017, 2018). If variable proportions of water and oil in blubber influence diffusion and retention of the lipophilic steroid compounds in blubber (e.g. favour the passive transfer and retention of steroids from blood and result in an enhanced hormonal signal), expressing hormone concentrations on a lipid basis could help to reduce such bias.

This study specifically sought to (i) assess the performance (method linear range, repeatability, and limits of detection) of the previously detailed LC-MS/MS method developed in bottlenose dolphin (Boggs et al., 2017) on humpback whale blubber; (ii) field validate the method on humpback whale blubber from stranded individuals; and (iii) explore correlations between steroid concentrations and blubber lipid content.

Materials and methods

Samples

The blubber of 10 southern hemisphere (SH) humpback whales stranded along the east and west coasts of Australia [namely breeding stocks E1 and D, respectively, as identified by the International Whaling Commission, IWC (1999)] between 2006 and 2015 were used to evaluate the performance of the analytical method. Blubber from adult individuals was preferentially selected in this study when available among biobanked tissues of the Southern Ocean Persistent Organic Pollutants Program (SOPOPP). As the great majority of humpback whale stranding events involve single calves and juveniles (Meynecke and Meager, 2016), adult females were, however, unavailable. As such, blubber from immature female individuals was employed for validation instead. Stranding information accompanying these animals (males = M1 through M5; females = F1 through F5) is included in Table 1. Full-depth blubber samples
(skin to muscle) were excised within 3 days post-mortem from a standardized section of the ventral–dorsal region, slightly posterior to the dorsal fin after Lambertsen et al. (1994). All samples were stored at −20°C until subsampling. Due to the relatively long time in storage, any decomposition in our samples could be caused by the initial carcass exposure to the environment, as well as by the time in storage itself. For this reason, upon subsampling, a thin layer of blubber exposed to air was shaved off to avoid oxidized tissue. The colour of the samples were recorded as a proxy for the state of decomposition (Mello et al., 2017). White or pink samples should be considered well preserved, while yellow or beige blubber samples show signs of decomposition. Subsamples were obtained from the outer 4 cm of blubber (with the skin removed) to target a blubber region similar to that accessible by conventional biopsy darts in free-swimming animals. The majority of E1 population samples were obtained through the SOPOPP environmental permits (Scientific Purposes Permit WISP04862307, WISP10018311, WISP07789610 and Moreton Bay Marine Park Permit #QS2014/CVL1397). Three samples of individuals stranded in Tasmania were collected under permit by staff from the Tasmanian Department of Primary Industries, Parks, Water and Environment. West Australian samples were collected under Department of Environment Scientific Purposes Licenses SF000007, SC000619, SC000941 and SC001255.

| Code | Sex | Age       | Sample colour | Date of collection | Location of the stranding |
|------|-----|-----------|---------------|--------------------|---------------------------|
| F1   | Female | Juvenile | Light pink   | 16 October 2013    | Hillarys, WA              |
| F2   | Female | Neonate  | Light pink   | 17 August 2011     | Walpole, WA               |
| F3   | Female | Calf     | Pink         | 23 July 2015       | Currumbin, QLD            |
| F4   | Female | Juvenile | White        | 3 September 2014    | King Island, TAS          |
| F5   | Female | Calf     | Pink         | 2 September 2005    | Nelson Bay, TAS           |
| M1   | Male  | Adult    | Beige        | 2007*              | Southport, QLD            |
| M2   | Male  | Adult    | Pink         | 1 October 2011      | North Stradbroke Island, QLD |
| M3   | Male  | Adult    | White        | 2009^              | Moreton Island, QLD       |
| M4   | Male  | Adult    | White        | 3 August 2008       | Southport, QLD            |
| M5   | Male  | Juvenile | White        | 10 November 2012    | Tomahawk, TAS             |

* Exact date unknown.

Table 1: List of humpback whale blubber specimens collected from whales stranded on Australian coasts

LC-MS/MS analysis

IS and control material

Reference materials for hormones in whale blubber were not available. Therefore, National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1945 (Organics in Whale Blubber), a cryo-homogenized female pilot whale blubber sample certified for persistent organic pollutants, was used as a quality control material. Approximately 0.4 g of SRM 1945 was extracted in triplicate during each analysis to ensure repeatability (percent relative standard deviation, RSD% <15%). Isotopically labelled steroids were used as internal standards (ISs). Briefly, a weighed quantity (~80 mg) of a mixture in methanol was added to each sample prior to extraction. Assuming a similar recovery between the hormone analyte and its associated IS, the instrumental signal of each target steroid was adjusted by the recovery of the associated IS for each sample, thereby accounting for any loss of analyte during the extraction method. All IS were present in the mixture in similar concentrations (~60 μg/g each, <15% difference between compounds). Each steroid concentration was normalized according to their analogous IS (where commercially available) or according to the most similar IS for molecular mass and chromatographic elution time (Supplementary Table S1). All IS except for progesterone-$^{13}$C$_3$, were purchased from Cerilliant (Round Rock, TX) and had a declared purity of 99.99%. Progesterone-$^{13}$C$_3$ was purchased from Cambridge Isotopes (Tewksbury, MA) at a declared purity of 98%. All calibration steroid standards were purchased from Sigma-Aldrich (St. Louis, MO), except for 11-deoxycortisol and 11-deoxycorticosterone, which were manufactured by Steraloids (Newport, RI). Calibration standard purity was equal or superior to 98%, except for 17α-hydroxyprogesterone (≥95%).

Sample extraction and clean-up

The sample preparation followed the work of Boggs et al. (2017) on dolphin blubber. Briefly, blubber samples (0.3 – 0.8 g) were minced (<1 mm) with blade and forceps in a dry-ice-cooled beaker to provide as much homogeneity as possible without compressing the tissue, which can result in the loss of oil. The minced blubber was then added to a bead homogenizer vial (~2 ml) containing garnet beads (MO-BIO; Qiagen, Hilden, Germany) and homogenized (four times for 30 s each at 681 rad/s). Sample clean-up was conducted using the
Agilent (Santa Clara, CA) Bond Elut QuEChERS EN Extraction kit with a C18 dispersive solid phase (dSPE) extraction for lipid removal. Specifically, the entire contents of the bead homogenizer vial were transferred into a 50 ml Falcon tube. The bead vial was then rinsed twice with water (~2 ml each time) and three times with acetonitrile (ACN). The Falcon tube was vortexed for 10 s in between additions of the water and ACN rinses. The volume was brought up to 15 ml volume with ACN and shaken for 30 s. A QuEChERS EN extraction salt packet (4 g MgSO₄; 1 g NaCl; 1 g sodium citrate; 0.5 g disodium citrate sesquihydrate) was then added to the sample, and the Falcon tube was again shaken vigorously for 1 min. Samples were then centrifuged at 2900 g (4°C) for 5 min. About 7 ml of the supernatant was cleanly transferred to a C18 dSPE vial (Agilent Bond Elut QuEChERS dispersive-SPE 15 ml tube), vortexed for 1 min and then centrifuged at 20,000 g (4°C) for 3 min. The supernatant was transferred to a clean glass test tube and the solvent was exchanged to 80:20 H2O:ACN (v:v) by drying under a gentle stream of nitrogen (N₂), reconstituting in 2 ml of 80:20 H2O:ACN, vortexing 1 min and sonicating for 9 min. The solution was spin-filtered at 12,000 g for 1 min on a 0.22 μm cellulose acetate spin filter. Finally, the sample was evaporated under stream of N₂ and reconstituted in 200 μL of MeOH. The derivatization reagent dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride; Sigma Aldrich, St. Louis, MO] was used to treat from 50 μL to 75 μL of the final extract for oestrogen analysis as previously described by Boggs et al. (2016). Briefly, 50–75 μL of sample extract was aliquoted into a culture tube with 500 μL of acetone and 500 μL of 0.1 mol/L NaHCO₃ buffer. The contents of the culture tube were vortexed for 1 min before 500 μL of freshly prepared dansyl chloride/acetone solution (1.28 mg/g) was added. The tube was vortexed again and incubated at 60°C on a heating block for 3 min. Finally, the sample was evaporated at 40°C under a gentle flow of N₂, reconstituted in a volume of MeOH equivalent to that of the initial extract (50 μL to 75 μL) and transferred to a vial with insert avoiding undissolved salts.

**Instrumental method**

The analysis of 11 steroid compounds and of the relevant IS was undertaken on an Agilent 1200 Series LC system equipped with a binary pump and autosampler, coupled to an AB Sciex (Framingham, MA) API 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer. The high performance liquid chromatography (HPLC) system was interfaced to a triple quadrupole mass spectrometer through an electrospray ionization source operating in positive mode. The voltage at the source was 5500 V, and the temperature was 700°C. The curtain gas (N₂) was at a pressure of 207 kPa, and ion source gases 1 and 2 at 310 kPa and 414 kPa, respectively. The quantification of all steroids and IS was obtained in scheduled multiple-reaction monitoring (sMRM) acquisition mode. Data collection was conducted using Analyst (Version 1.5.1, AB Sciex, Framingham, MA).

Two different chromatographic separations were used in order to achieve the optimal detection for the full compound suite. Each sample was injected three times, with each injection targeting a specific steroid compound class (androgens and progestogens, oestrogens and corticosteroids). Androgens and progestogens (17α-hydroxyprogesterone, progesterone, testosterone and androstenedione), together with oestrogens (oestrone and oestradiol), were separated on a Restek (Bellefonte, Pennsylvania) Ultra Biphenyl column (5 μm, 250 mm × 4.6 mm) heated at 35°C using two separate injections (oestrogens post-derivatization) (Boggs et al., 2016). The extract for the analysis of corticosteroids (cortisone, cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone) was solvent exchanged to methanol: water, 50:50% (v:v) (both containing 0.1% acetic acid) prior to injection on an Agilent Eclipse Plus C18 column (5 μm, 150 mm × 21 mm). Chromatographic parameters and quantitative mass fragmentation patterns are summarized in the Supplementary Data (Tables S1 and S2). The optimization of these parameters can be found in Boggs et al. (2016) (oestrogens only) and Boggs et al. (2017) (all compounds except oestrogens).

**Hormone quantification and validation**

Quantification was performed using an extract calibration curve with isotopically labelled IS, i.e. by preparing and analysing the calibrants according to identical extraction protocols with an isotopically labelled IS mixture used for the samples, calibrants and blanks. Blanks consisted of 0.5 ml of deionized water and IS mixture prepared analogously to blubber samples. The calibration curves were built by selecting the best-fit linear regression of the calibrants, based on at least four calibration points. If necessary, different calibration curves were employed to optimize fitting of responses of the same analyte at different mass fractions. Peak areas were quantified manually using Analyst 1.6.2 (AB Sciex, Framingham, MA) or MultiQuant software (Version 3.0.2, AB Sciex, Framingham, MA). Repeatability of the measurement was evaluated by RSD% on six measurements of independently prepared samples analysed under a single operator and instrument. Initially, one female and one male whale sample (F1, M2) were randomly chosen and analysed in six replicates. After initial analysis, some steroids were not detected in these randomly selected samples. Therefore, an additional male sample (M3) was selected for repeatability of testosterone. The limit of detection (LOD) was defined as three times the standard deviation plus the mean of the extracted blanks for each analyte. The reporting limit (RL) was determined as the lowest calibration standard included within the regression with a minimal signal to noise ratio of three to one. These definitions have been used in previous studies (Ragland et al., 2011; Keller et al., 2012; Boggs et al., 2016, 2017; Galligan et al., 2018a). In these analyses the RL was higher than the LOD per each compound, therefore the RL was chosen as the reporting threshold. The method accuracy of the procedure was evaluated by measuring steroid retrieval in a fortified
Sample M2 was divided into eight homogenous sub-samples (~0.5 g each). Four subsamples were analysed to determine the endogenous concentration. The remaining four subsamples were spiked with known amounts of a calibration standard (between 2 ng and 17 ng of each steroid) before extraction.

**Lipid determination**

Lipid determination of the blubber tissue of each animal was performed gravimetrically. Each sample (0.500–1.00 g) was weighed, thawed at room temperature and homogenized with Na₂SO₄ by mortar and pestle. The sample was extracted on a cold column at atmospheric pressure with 100 ml of dichloromethane. The lipid content was determined as the mass of the desiccated oil extracted relative to the initial fresh sample mass.

**Statistical analysis**

Data were censored at the RL, and hormone concentrations below the RL were inputted with the RL, except for 17α-hydroxyprogesterone and oestrone, as the detection frequency of these two compounds in our samples was below 50%. After substitution, we used conservative non-parametric tests on ranks (\(r_s\), Spearman rho) to test for pairwise correlations among all hormones, as suggested by Helsel (2011). Principal component analysis (PCA) by covariance was used as a dimensionality reduction technique to visualize multivariate relationships among hormones. In this case, data for each sample were normalized by the sum of hormone concentrations (i.e. all compounds) and pareto-scaled. Data processing, PCA and correlation analysis were performed by MetaboAnalyst 3.5.

**Results**

**Peak quality**

Progesterone, 17α-hydroxyprogesterone, testosterone, androstenedione, oestrone, oestradiol, cortisone, cortisol, corticosterone, 11-deoxycortisol and 11-deoxycorticosterone were detected and quantified. Peaks were baseline resolved at different concentrations for all compounds except 11-deoxycorticosterone (Fig. 1). As a consequence, the limits of detection and quantification for 11-deoxycorticosterone were notably higher than those of the other steroids, although no negative effect was observed on the repeatability and accuracy of this compound.

**Method validation results**

The repeatability measured as RSD% between replicates ranged between 4.32% and 14.0%, with the exception of 11-deoxycortisol (RSD% for sample F1 = 17.4%), for

![Figure 1: Examples of sMRM chromatograms of detectable endogenous steroids in the blubber from stranded humpback whales: (A) corticosteroids in M1 adult male; (B) reproductive steroids in M4 adult male; (C) oestrogens in F3 female calf.](image)
which the repeatability exceeded the maximum acceptance bound of 15.0% (Fig. 2A). Reported concentrations for 11-deoxycortisol should therefore be considered semiquantitative. Replicates on the steroid residues in SRM 1945 yielded repeatability results similar to our samples (<15%), although testosterone, oestradiol and oestrone were not quantifiable in this sample. Coefficients of determination ($R^2$) of the calibration curves were all above 0.973. Different calibration curves were employed for cortisol (min = 0.614 ng/g; max = 85.1 ng/g) and progesterone (min = 0.159 ng/g; max = 35.4 ng/g) quantification and for measuring corticosteroids in spiked and unspiked samples (Supplementary Tables S3–S5). LODs and RLs were in the mid-to-high pg/g range for all hormones (Table 2). Method accuracy, measured as the recovery from a fortified sample, ranged from 83.8% (min recovery) to 112% (max recovery) (Fig. 2B). Testosterone and oestradiol endogenous concentrations were below the LOD in the sample selected for this assessment (M2) and therefore were set to the RL for purposes of spike retrieval calculations. Since the spike recoveries met the acceptance criteria (recovery bounds, 70.0–120%) (Boggs et al., 2017; Galligan et al., 2018a) we concluded that all steroid hormones analyses described in this manuscript, except for 11-deoxycortisol, were accurate and precise over the concentration ranges found in blubber.

**Table 2:** LODs (ng/g wet weight) and RLs (ng/g wet weight) of the LC-MS/MS method

| Steroid                      | LOD (ng/g) | RL (ng/g) |
|-----------------------------|------------|-----------|
| Progesterone                | 0.0282     | 0.0560    |
| Testosterone                | 0.0190     | 0.0627    |
| Androstenedione             | 0.00374    | 0.0482    |
| 17α-Hydroxyprogesterone     | 0.0166     | 0.565     |
| Corticosterone              | 0.0224     | 0.399     |
| 11-Deoxycortisone           | 0.00277    | 0.384     |
| 11-Deoxycorticosterone      | 0.513      | 0.515     |
| Cortisone                   | 0.00324    | 0.0435    |
| Cortisol                    | 0.00337    | 0.0470    |
| Oestrone                    | 0.00296    | 0.0136    |
| Oestradiol                  | 0.0769     | 0.339     |

**Steroid profiles**

Steroid profiles varied greatly among the whale samples, and not all steroids were detectable in every sample (Table 3). Cortisol was detected in 9 out of 10 samples. Cortisol was detected in 9 out of 10 samples.
Table 3: Blubber steroid hormone profiles (ng/g, wet and lipid weight) and lipid content (%) of 10 stranded Southern Hemisphere humpback whales

| Steroid            | F1       | F2       | F3       | F4       | F5       | M1       | M2       | M3       | M4       | M5       | Median |
|--------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|---------|
| Cortisone          | 1.69 ± 0.07 | 3.32     | 1.19     | 1.55 ± 0.00 | 17.7 ± 0.0 | 2.79 ± 0.01 | 1.71 ± 0.12 | <0.0435 | 2.30 ± 0.05 | 0.214 ± 0.00 | 1.70     |
|                    | (3.00 ± 0.13) | (7.67)   | (22.9)   | (2.15 ± 0.00) | (1060 ± 0) | (5.59 ± 0.02) | (2.25 ± 0.16) | (3.29 ± 0.08) | (0.285 ± 0.002) |             |
| Cortisol           | 4.05 ± 0.21 | 6.09     | 4.16     | 3.43 ± 0.02 | 85.1 ± 0.8 | 13.6 ± 0.0 | 6.80 ± 0.45 | <0.0470 | 6.53 ± 0.02 | 0.614 ± 0.036 | 5.12     |
|                    | (7.20 ± 0.38) | (14.1)   | (79.7)   | (4.74 ± 0.03) | (5120 ± 50) | (27.3 ± 0.1) | (8.94 ± 0.59) | (9.35 ± 0.02) | (0.817 ± 0.049) |             |
| Corticosterone     | 1.18 ± 0.10 | <0.399   | 0.93     | <0.399    | 6.14 ± 0.09 | 2.97 ± 0.07 | 0.894 ± 0.078 | <0.399  | <0.399     | <0.399     | 0.646    |
|                    | (2.09 ± 0.18) | (17.9)   | (369 ± 6) | (5.96 ± 0.15) | (1.17 ± 0.10) |             |             |             |             |             |
| 11-Deoxy cortisol  | 1.45 ± 0.21 | 1.14     | 1.13     | <0.384    | 4.13 ± 0.15 | 1.52 ± 0.06 | 2.18 ± 0.19 | <0.384  | 3.55 ± 0.07  | <0.384     | 1.29     |
|                    | (2.58 ± 0.37) | (2.63)   | (21.7)   | (249 ± 9) | (3.05 ± 0.11) | (2.86 ± 0.25) |             |             |             | (5.09 ± 0.11) |             |
| 11-Deoxy- cortisol | <0.515     | <0.515   | <0.515   | 2.27 ± 0.18 | 1.44 ± 0.02 | 1.42 ± 0.05 | 0.972 ± 0.109 | 1.10 ± 0.11 | 2.33 ± 0.30  | <0.515     | 1.03     |
|                    |           |          |          | (3.14 ± 0.25) | (86.9 ± 1.1) | (2.86 ± 0.10) | (1.28 ± 0.14) | (2.00 ± 0.20) | (3.34 ± 0.44) |             |
| 17α-Hydroxy progesterone | <0.565   | <0.565   | <0.565   | <0.565    | 0.917 ± 0.026 | 1.30 ± 0.12 | <0.565     | 1.87 ± 0.07  | <0.565     | 0.565     |
|                    |           |          |          |           | (1.84 ± 0.05) | (1.71 ± 0.16) |             |             |             | (2.68 ± 0.10) |             |
| Testosterone       | <0.0627   | <0.0627  | 0.591    | <0.0627   | 0.172 ± 0.006 | <0.0627     | 0.133 ± 0.009 | 1.19 ± 0.12 | 5.44 ± 0.00  | <0.0627    | 0.147    |
|                    |           |          |          |           | (10.4 ± 0.4) |             |             |             |             | (7.79 ± 0.01) |             |
| Androstenedione    | <0.0482   | <0.0482  | <0.0482  | 0.104 ± 0.001 | 0.495 ± 0.007 | 0.251 ± 0.002 | 0.475 ± 0.031 | 1.78 ± 0.06  | 12.7 ± 0.1   | 0.6097 ± 0.0013 | 0.177   |
|                    |           |          |          |           | (0.143 ± 0.002) | (29.8 ± 0.4) | (0.503 ± 0.003) | (0.625 ± 0.041) | (3.25 ± 0.12) | (18.2 ± 0.1) | (0.0927 ± 0.0018) |
| Progesterone       | 0.750 ± 0.081 | 2.38    | 0.208    | 0.500 ± 0.034 | 1.08 ± 0.01 | 3.54 ± 0.0 | 4.86 ± 0.28 | 0.159 ± 0.018 | 3.93 ± 0.08 | 0.392 ± 0.092 | 0.917   |
|                    | (1.33 ± 0.14) | (5.48)  | (3.99)   | (0.692 ± 0.047) | (65.3 ± 0.8) | (71.0 ± 0.1) | (6.39 ± 0.37) | (0.290 ± 0.033) | (5.63 ± 0.11) | (0.521 ± 0.121) |             |
| Oestradiol         | <0.339    | <0.339   | 0.704    | <0.339    | <0.339    | <0.339    | <0.339    | <0.339    | <0.339    | <0.339    | 0.339   |
|                    |           |          |          | (13.50)   |             |             |             |             |             |             |             |
| Oestrone           | <0.0136   | <0.0136  | 6.54     | <0.0136   | 1.29 ± 0.04 | 0.185 ± 0.010 | 0.172 ± 0.012 | <0.0136 | 0.359 ± 0.041 | <0.0136    | 0.0273   |
|                    |           |          |          | (125)     | (77.9 ± 2.3) | (0.372 ± 0.019) | (0.226 ± 0.016) |             |             | (0.514 ± 0.058) |             |
| Lipid (%)          | 56.2      | 43.3     | 5.21     | 7.22      | 1.7       | 49.8      | 76.1      | 54.9      | 69.8      | 75.2      |

Notes: when replicates were available, mass fraction values are expressed as confidence intervals: arithmetic mean ± (student t) × (standard deviation)/(number of replicates)^1/2. Values in parentheses, in italics, express the hormone concentrations as ng/g of lipid. Values below the RL are indicated as ‘<’-respective RL’.
Significant correlations (Spearman, \( r_s \); \( P < 0.05 \)) between steroid hormone concentration pairs are summarized in Table 4. Male and female clusters occupied two different regions of the PCA scoring plot, although they were not separated at 95% probability (Fig. 3).

**Lipid content**

No significant relationship (Spearman, \( P < 0.05 \)) was observed between the blubber lipid content and any of the individual steroid hormones quantified, nor with the sum of all steroid hormone concentrations in each sample.

**Discussion**

The LC-MS/MS method presented here provides the first simultaneous quantification of 11 steroid hormones (progesterone, 17α-hydroxyprogesterone, testosterone, androstenedione, cortisone, cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, oestrone, oestradiol) in humpback whale blubber. Compared to the commonly used EIA techniques, and to the recently published mass spectrometric method for steroids in blubber (Hayden et al., 2017), the number of analytes that can be measured, following a single extraction, is significantly increased by this method. We quantified two additional hormones, oestradiol and oestrone, to those measured in the first application of this technique in bottlenose dolphin blubber (Boggs et al., 2017). Androstenedione, 17α-hydroxyprogesterone, cortisone, corticosterone, 11-deoxycortisol and 11-deoxycorticosterone have not previously been measured in any baleen whale species. The method’s LODs and RLs were lower than the steroid hormone concentrations previously reported in the literature for stranded and free-swimming humpback whales (Vu et al., 2015; Clark et al., 2016; Mello et al., 2017) and adequate to quantify concentrations of compounds that had not been previously measured in humpback whales. The validation experiments described in this manuscript show that the method had good performance as evidenced by the recovery of amended steroids (spike recovery between 83.8% and 112%) and the repeatability of the measurements (RSD% <15% for all hormones but 11-deoxycortisol). Further optimization of the chromatographic separation of the corticosteroids is required so as to obtain better baseline separation of 11-deoxycorticosterone’s signal and a more reliable measurement of 11-deoxycortisol. The choice of using long (250 mm and 150 mm) HPLC columns was motivated by the challenge of separating structurally similar steroid congeners, present in the sample in trace concentrations. Unpublished efforts to adapt the procedure to shorter columns encountered difficulties in maintaining the same signal-to-noise ratio on concentrations (0.61–85 ng/g wet weight, ww) resulted in the widest range and highest median among the analysed steroid hormones in our dataset. Progesterone (0.159–35.4 ng/g ww) and cortisone (0.214–17.7 ng/g ww) possessed the next largest concentration ranges and were also frequently detected (in 10 and 9 out of 10 samples, respectively). Significant correlations (\( r_s \), \( P < 0.05 \)) between steroid hormone concentration pairs are summarized in Table 4.
Figure 3: PCA by covariance of the steroid hormones quantified in >50% of analysed humpback whale blubber samples (concentrations normalized by the sum of all hormones and concentration values pareto-scaled) (A); full red triangles denote female samples (F1–F5) while green crosses denote male samples (M1–M5), while purple and blue ellipses represent the confidence intervals (95%) of the score distribution for the male and female samples, respectively.

reduced injected volumes. Additionally, long runs were made necessary by the inclusion of a wash section in the elution, which avoided the build-up of a matrix effect.

We found that cortisol was the most abundant steroid in concentration among the profiles of the analysed animals. Cortisol is a non-specific marker of stress secreted by marine mammals in response to a variety of different stimuli, such as capture or examination (Ortiz and Worthy, 2000; Fair et al., 2014), cold exposure (Houser et al., 2011), beaching (Champagne et al., 2018) or nutritional stress (Kershaw and Hall, 2016). Currently, little research has been conducted on how degradation, and post- or ante-mortem hormone metabolism, might affect hormone measurements in stored biological samples. Previous studies that have measured cortisol and progesterone degradation in marine mammal blubber using EIAs have found the degradation rate of steroids in biological matrices to be moderate (Kellar et al., 2015). Cortisol in frozen (−40°C) archived samples is not substantially affected by the time in storage (Trana et al., 2015), while progesterone has been measured in samples stored (−20°C) for up to 17 years in concentrations consistent with those more recently collected (Pallin et al., 2018b). Therefore, the endocrine profiles found here feasibly reflect the endocrine milieu in blubber at the time of an animal’s death (Kellar et al., 2006; Beaulieu-McCoy et al., 2017).

Progesterone was measured in all individuals, of both sexes (maximum M1 = 35.4 ng/g ww, minimum F3 = 0.208 ng/g ww; Table 3). Elevated blubber progesterone is a pregnancy marker in humpback whales (Pallin et al., 2018b). As our sample set included males and immature females, we can
exclude the possibility that elevated progesterone levels were related to pregnancy for some individuals. Given the correlations between progesterone, cortisol ($P < 0.01$) and cortisone ($P < 0.01$), another possibility is that elevated progesterone in some whales might reflect severe adrenal stimulation prior to death (Kershaw and Hall, 2016; Boggs et al., 2017). Progesterone is a precursor in corticosterogenesis (Fig. 4). The secretion of this hormone could enhance the bioavailability of cortisol in circulation by competitively binding to corticosteroid binding protein (Brien, 1981). Interestingly, blubber cortisone and cortisol concentration were highly correlated ($P < 0.001$), which supports the hypothesis that conversion of these two steroids may in part occur peripherally, in marine mammal adipose tissue (Rask et al., 2002; Kershaw and Hall, 2016; Galligan et al., 2018b).

The testosterone, progesterone and cortisol concentrations found in this study were similar to those previously reported for humpback whale carcasses (Mello et al., 2017). By contrast, oestradiol, which Mello et al. (2017) measured as ranging from 1.5 ng/g to 2.5 ng/g ww in all age groups, was quantifiable in just one of our samples—F3, a female calf. Interestingly, F3 also featured the highest concentration of oestrone (6.54 ng/g ww). Oestrogens are known to have both anti-inflammatory and pro-inflammatory roles (Straub, 2007). Further, elevated oestrogen levels can occur when individuals experience tissue trauma or critical illness (Spratt et al., 2006). Together, this highlights the importance of including information on pathology when interpreting hormone levels in deceased animals. For example, accessing information on adrenal mass and histopathology appears crucial when interpreting steroid concentrations in stranded individuals and attributing them to chronic or acute stress.

Significant correlations between the concentrations of hormonal precursors and their derivatives were detected in humpback whale blubber samples (Table 4). In the absence of baseline data for these hormones in healthy whales, these relationships may represent a natural state of endocrine homeostasis or indicate activation of a certain biosynthetic pathways (Fig. 4). Similarly, correlations between ostensibly unrelated hormones (e.g. 11-deoxycorticosterone and androstenedione or corticosterone and oestrone) could represent homeostasis or result from simultaneous activation of multiple hormone biosynthetic routes.

Contrary to the expectation that blubber lipid content could influence the concentration of single or total steroid hormone concentrations in the samples, this study found no significant relationship between these variables. Beaulieu-McCoy et al. (2017) found that cortisol concentration and

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**Figure 4:** Steroid biosynthetic pathways (black arrows) in mammals. Steroid hormones detected in this study are in black font, while green boxes denote steroid hormones quantified in at least 50% of our samples.
blubber percent lipid were significantly and negatively correlated in the blubber of stranded California sea lions. Humpback whale stranding in Australia is highly biased towards calves and juveniles and often associated with malnutrition (Hollyoake et al., 2012), although other causes of death have been recognized (e.g. hypoxia, entanglement, ship strike, shark attack) (Wiley et al., 1995; Meynecke and Meager, 2016). Extremely low blubber lipid content in samples F5 and F3 (F3 = 5.21%, F5 = 1.66%) indicates poor nutritional condition or poor long-term health (Hollyoake et al., 2012). These conditions could explain the high concentrations of certain stress hormones, which are also involved in energy balance and lipid remobilization (Sapolsky et al., 2000). The removal of those samples did not produce any significant correlation between hormonal content and lipid percentage. Other factors that could have influenced our analysis are (i) the inclusion of samples that might have undergone degradation (e.g. loss of water) (Mello et al., 2017), (ii) changes in blubber blood vessel dilation (e.g. through exposure of the samples to different ambient temperatures) that could potentially affect steroid hormone diffusion prior to sampling (Champagne et al., 2018), as well as (iii) the presence of individuals of different ages. For example, cortisol concentration has been shown to increase with blubber depth in other cetacean species (Trana et al., 2015; Kershaw et al., 2017). Since calves are equipped with thinner and less lipid-rich blubber compared to adults (Miller et al., 2011), it is possible that the same blubber depth represents a more inner and possibly more metabolically active layer for calves. Therefore, although results suggest that lipid percentage does not influence steroid hormone content, further investigations with a larger sample set, particularly of live whales, is necessary before any potential advantage of normalizing hormone content on lipid percentage can be confirmed.

The sample mass used in this study can be systematically collected from free-swimming whales through standard biopsy darts. Hence, multiple hormones could be quantified reliably in live humpback whales to study their reproductive and adrenal physiology. The profile we collected from stranded whales demonstrate that a much larger suite of steroid hormones than those previously analysed (i.e., testosterone, progesterone, cortisol, and oestradiol) can be present in the blubber of humpback whales. Multiple hormones can potentially be used, through correlation and multivariate analysis as shown here, to aid the interpretation of endocrine status in whales in the wild. Consequently, this method constitutes an important contribution in furthering the understanding of humpback whale physiology and endocrinology.

Disclaimer

Certain commercial equipment, instruments or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Acknowledgements

The authors acknowledge Douglas Coughran and Carly Holyoake for the donation of Western Australian stranded animal tissue samples and the Tasmanian Department of Primary Industries, Parks, Water and Environment for the donation of the Tasmanian stranded animal tissues. The authors also acknowledge Teri Rowles, Amanda Moors and the whole NIST Marine Environmental Specimen Group for their help with shipping samples. G.D.L. acknowledges receipt of the Griffith University Postgraduate Research Scholarship and Griffith University International Postgraduate Research Scholarship. G.D.L., A.B., S.B.N. and J.K. conceived and designed the experiments. G.D.L. and A.B. performed the experiments. G.D.L. and A.B. analysed the data. J.K., A.B., S.B.N. and F.M. contributed reagents/materials/analysis tools. G.D.L., A.B., S.B.N., J.K. and F.M. wrote the paper.

Funding

This work was supported by the National Institute for Standard and Technology.

Supplementary material

Supplementary material is available at Conservation Physiology online.

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