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Relationships Between Blubber and Respiratory Vapour Steroid Hormone Concentrations in Humpback Whales (*Megaptera novaeangliae*)

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Abstract

Respiratory vapour ("blow") has been successfully used as a sample matrix for reproductive and adrenal endocrine assessments of some captive cetacean species. Given that the accuracy of blow hormone measurements can be affected by seawater contamination, variable sample volumes, and respiratory water dilution, it is a much more difficult technique to use for studies on wild cetaceans. Herein, we aimed to determine if blow sampling was a viable technique to measure reproductive and adrenal hormone concentrations in humpback whales (*Megaptera novaeangliae*). To achieve this, concentrations of progesterone, testosterone, oestradiol, and cortisol were measured in paired blow and blubber samples collected from free-swimming whales (n = 48). Hormone concentrations were compared between sample types and examined in relation to a whale's demographic class and the season when it was sampled. Testosterone concentrations were poorly correlated between sample types. In contrast, blubber concentration had a significant impact on the fit of the blow progesterone, oestradiol, and cortisol models (χ^2 = [11.52, 22.57, 6.12], p = [< 0.001, < 0.001, 0.013], respectively). There was little further evidence that blow hormone concentrations were driven by the physiological condition of a whale. Overall, the strongest evidence came from one adult female who was presumed to be pregnant based on her blubber progesterone concentration (9.97 ng/g). This adult female displayed a blow progesterone concentration of 0.94 ng/mL, which was markedly higher than all other adult females and most other whales with the exception of two. Currently, however, blubber sampling is the preferred option for studying the endocrinology of humpback whales, primarily due to the ease of sample collection and robustness of hormone analyses. Further refinements and validation tests are required before blow hormone monitoring can be used as a non-invasive alternative.

Key Words: blow, blubber, hormone, reproductive assessment, humpback whale, *Megaptera novaeangliae*

Introduction

Hormones released into circulation enter and accumulate in a range of tissues and fluids. The most appropriate sample type to use for an endocrine study depends on which hormone or metabolite is of interest, the required resolution/sensitivity of measurements, and the anatomical and ecological characteristics of the target species (Ganswindt et al., 2012). Minimally invasive biological sampling methods are the preferred option when conducting endocrine studies on wild populations. Yet, for wild cetaceans, the most widely used sampling technique remains remote tissue biopsying (Hunt et al., 2013), which is considered invasive as it leaves a small wound on the animal. Faecal hormone monitoring, which is the standard option for evaluating the endocrine condition of terrestrial vertebrates (Goymann, 2005; Schwarzenberger, 2007; Narayan, 2013), has been achieved for several wild cetaceans (Rolland et al., 2005; Hunt et al., 2006; Ayres et al., 2012). However, faecal sampling remains problematic

for many cetacean species, which feed and defecate in spatially and temporally unpredictable areas. The remaining non-invasive alternative to remote tissue biopsying, when studying the endocrinology of wild cetaceans, is respiratory vapour ("blow") sampling.

Remote tissue biopsying and blubber hormone monitoring are now widely used techniques for evaluating the endocrine condition of wild cetaceans. Blubber has an affinity for lipophilic molecules, such as steroid hormones, which accumulate from peripheral circulation. A range of steroids has now been measured in blubber to monitor key reproductive parameters for several species, including some baleen whales. Pregnancy has been diagnosed in minke (*Balaenoptera acutorostrata*), bowhead (*Balaena mysticetus*), and humpback (*Megaptera novaeangliae*) whales using blubber progesterone measurements (Mansour et al., 2002; Kellar et al., 2013; Clark et al., 2016; Pallin et al., 2018). Further, for male humpback whales, blubber testosterone concentrations have been found to vary seasonally (Vu et al., 2015) in a similar manner to testicular measures (Matthews, 1937; Chittleborough, 1955). Overall, there appears to be a lag time between blood and blubber steroid hormone concentrations (Schwacke & Wells, 2015; Champagne et al., 2017), which means that other less sustained endocrine events (e.g., oestrus) might be more difficult to detect in this sample type.

Blow hormone concentrations appear to mirror patterns in blood hormone concentrations (Tizzi et al., 2010; Thompson et al., 2014; Richard et al., 2017), which should permit the detection of acute endocrine events. However, there still remain several issues with using blow for endocrine studies on cetaceans. In particular, blow hormone measurements are influenced by sample dilution from respiratory water, seawater contamination, and interference from some sampling materials (Hunt et al., 2014; Burgess et al., 2016; Richard et al., 2017; Mingramm et al., 2019). Many of these issues have been mitigated in studies on captive belugas (*Delphinapterus leucas*) by discarding the first exhalation after an animal surfaces, cleaning the blowhole prior to sampling, collecting multiple exhalations, and by placing a protective barrier around the blowhole during sample collection (Thompson et al., 2014; Richard et al., 2017). Under these conditions, the reproductive and adrenal condition of individual animals has been evaluated with moderate success (Thompson et al., 2014; Richard et al., 2017). Unfortunately, these types of blow-sampling modifications are impractical for studies on most free-swimming cetaceans.

Blow samples collected from baleen whales are likely to be of a higher volume, with a greater hormone signal, than many of the smaller odontocetes studied to date. As a result, blow hormone measurements from baleen whales might be slightly less susceptible to the impacts of sample material interference and seawater contamination. One study on North Atlantic right whales (*Eubalaena glacialis*; Burgess et al., 2018) has already had success with blow hormone monitoring, though, in part, this is likely due to the use of urea nitrogen as an endogenous standard to correct for sample dilution. For all other baleen whale species, it is unknown whether key endocrine trends that can be detected in blubber, such as pregnancy-related increases in progesterone concentrations, can also be detected in blow. Humpback whales are an ideal species to study next as their distribution, abundance, and behaviour have been well-described (Chittleborough, 1965; Clapham, 1996; Noad et al., 2019), and there is already some understanding of their endocrinology (Hogg et al., 2009; Mello et al., 2017; Pallin et al., 2018; Cates et al., in press). In light of this, the aims of this study were (1) to compare steroid hormone concentrations in paired blow and blubber samples from humpback whales, and (2) to examine whether patterns in blow and blubber hormone concentrations were similar when contrasted between whales of different age class and sex, and between animals sampled at different times in the breeding season.

Methods

Study Animals

Humpback whales from the East Australian population (Breeding stock E1; see Schmitt et al., 2014) were sampled in 2014 and 2015 (Table 1). Sampling was conducted under permits from The University of Queensland (QLD) Animal Ethics Committee (SVS/080/15/CEAL, CURTIN/SVS/152/14), the State Government of Queensland (WISP15866815, QS2015/MAN307), and the Commonwealth of Australia (2014-0002, AU-COM2014-246, AU-COM2015-273). Whales were sampled during the northern migration towards the Great Barrier Reef calving grounds (in 2015 only) and during the southern migration towards the Antarctic feeding grounds (in 2014 and 2015). Sampling was conducted off two locations: (1) Sunshine Coast, QLD (~26° 23' 33" S, 153° 08' 52" E), and (2) North Stradbroke Island, QLD (~27° 20' 43" S, 153° 33' 23" E). Individual whales were identified through photographs of the shape and markings of their dorsal fin and fluke. Both adult and juvenile whales were sampled. Age class was determined using visual estimates of body length, with the sampling vessel (~7 m long with bowsprit attached) used as a size reference: Whales longer than 12 m were considered adults, and those between 7 to 12 m in length were considered to be

Table 1. Number of adult female, adult male, juvenile female, and juvenile male humpback whales (*Megaptera novaeangliae*) sampled for both blow and blubber during the winter/northern and spring/southern migrations in 2014 and 2015

	2014	2015	
	Southern migration	Northern migration	Southern migration
Adult female	2	2	6
Adult male	2	14	6
Juvenile female	--	7	--
Juvenile male	--	6	3

juveniles (Coughran & Gales, 2010). The sex of a whale was determined through the genetic analysis of skin biopsies (as per Morin et al., 2005), which were performed by the Animal Genetics Lab at The University of Queensland.

Paired Blow and Biopsy Sample Collection

Humpback whales were encountered opportunistically on-water from a small research vessel. Groups of whales were then followed for a short duration, at a distance greater than 50 m, to monitor behavioural data for a concurrent study (swim speed, dive times, and travel direction) prior to sampling. Tissue biopsies were collected from humpback whales using a remote biopsy system as described elsewhere (PAXARMs New Zealand Ltd, Cheviot, New Zealand; Krützen et al., 2002). The protocol for blow sampling whales was adapted from previous studies (Hogg et al., 2009; Hunt et al., 2014), with slight modifications in the collection device to increase sample volume and in the preservation method to improve steroid extraction. Blow sampling kits were made by attaching a 30-cm^2 piece of nylon tulle (100% Nylon, White 12"X100YD; Wholesale Wedding Supplies, Woombye, Australia) to the rim of a 10-inch-diameter polypropylene microwave plate cover (d line, Albertslund, Denmark), using an elastic band. The nylon mesh and the dish had been cleaned and sterilised by alternating rinses of absolute ethanol (> 99%; Merck Millipore, Bayswater, Victoria, Australia) and distilled water. Kits were stored in new, plastic zip-loc bags until sampling commenced.

In 2014, paired biopsy and blow samples were collected from individual humpback whales during different surfacing events separated by 5 to 20 min. First, a target animal was approached to within 10 to 25 m so that the sampler could launch a biopsy dart at the flank of the animal. The vessel then ceased following the whale and retrieved the dart. Samples were removed from the cutting head of the biopsy dart, rinsed with distilled water, and stored on ice for 1 to 8 h until the end of the day. After 5 to 20 min, the vessel then headed back towards the target animal to collect a blow sample. The zip-loc bag was removed from the blow sampling kit, and the dish was attached using garden hose connectors and hose clamps to the end of a 6-m carbon-fibre pole. When the target animal surfaced, the vessel was manoeuvred alongside the whale (5 to 10 m), and the sampling kit was positioned into the plume of exhaled blow (1 to 5 m from the whale). After collection, the sampling kit was brought onboard the vessel, and the nylon tulle was removed from the collection dish using gloves and then placed into a 50-mL polypropylene conical tube (BD Falcon, North Ryde, New South Wales, Australia). All blow samples were stored on ice for 1 to 8 h until the end of the day.

In 2015, our methods were refined so that blow and biopsies could be collected without leaving the animal for an extended period of time in-between samples. First, the biopsy sampler would launch a dart during a humpback whale's initial surfacing after a dive from at least 20 m away to minimise behavioural disturbance. The dart was immediately retrieved and placed into a zip-loc bag after the whale had cleared the area (i.e., moved > 50 m away). Following retrieval, the vessel re-approached the target whale so that a blow sample could be collected during its next breath. If the whale was still underwater, the vessel was manoeuvred ahead of the whale's "footprint" to the predicted area of the next surfacing (based on estimated swim speed, heading, and dive times). A blow sample was then collected as the target animal surfaced again. All samples were then processed in the same manner as in 2014.

During both sampling years, blow sample quality ("high" or "low") was subjectively assessed after sample collection. Sample quality was assessed based on the strength of the exhalation, the position of the sampling kit relative to the respiratory plume, and the perceived presence/absence of any seawater contamination. There were three sampling kits that contained an extraordinarily large number of droplets. These

samples had been collected from humpback whales that remained stationary at the surface for multiple breaths or surfaced immediately next to the vessel. Given the high volume of these samples, fractions of "pure" blow droplets (0.6 to 3.5 mL) were aspirated directly into 2 mL micro-tubes (Sarstedt, Nümbrecht, Germany) using a plastic pipette; the associated nylon tulle was treated the same as all other samples. Seawater samples were collected in 5 mL polystyrene vials (LBSSP2002; ThermoFisher Scientific, Scoresby, Victoria, Australia) at the end of each day to act as a blank control for blow sampling.

At the end of each sampling day, seawater samples, biopsy samples, and micro-tubes containing pure blow droplets were transferred into a -20°C freezer. All 50 mL conical tubes that contained nylon tulle samples were partially filled with 15 mL of absolute ethanol, shaken for 1 min, and then stored at -20°C (adapted from Hunt et al., 2014). Given this treatment, the original blow sample volumes could not be calculated. After the completion of a field season (3 to 6 wks duration), all samples were then transferred into -80°C until analysis.

Steroid Hormone Extraction

The nylon material and ethanol fractions of blow samples were separated after storage. The blow hormone-ethanol solution was left in the original tube, whereas the nylon tulle was transferred into an empty 50 mL conical tube. Nylon tulle samples were centrifuged for 5 min at 3,000 x*g* to separate all remaining fluid. The tulle was prevented from settling on the bottom of the tube by placing it above an additional piece of nylon tulle that was secured in place with the screw-down cap. Extracted fluid was aspirated with a pipette and recombined with the solution in the original conical tube. The final solution was then dried under compressed air at 37°C (DBH10D; Ratek, Boronia, Australia), and the resulting residue reconstituted in 1.5 mL of assay buffer (NaH_2PO_4 [5.42 g], Na_2HPO_4 [8.66 g], NaCl [8.70 g], BSA [1.00 g], ProClin 150 [1 mL], H_2O Mili-Q [1,000 mL], and pH [7.0]). All samples were vortexed at high speed for 1 min to ensure even mixing. Blow hormone extracts were stored at -80°C until analysis.

Steroid hormones were isolated from blubber samples using an organic solvent extraction described elsewhere (Mingramm et al., 2019). In brief, 0.1 ± 0.005 g of blubber was dissected into small pieces (to ~ 2 mm^3) and then mechanically homogenised in 1 mL of phosphate buffered saline (PBS) (D8662; Sigma-Aldrich Ltd, Sydney, Australia) for three 5-min cycles (Mini-Beadbeater-16; BioSpec Products, Bartlesville, OK, USA). The homogenate was transferred into 5 mL polypropylene tubes (LBS504N; ThermoFisher, Australia) that had been filled with 1 mL of EtOH:acetone (4:1). Each empty homogenisation tube was then rinsed with an additional 1 mL of EtOH:acetone, with this liquid being combined with the contents of the polypropylene tube. Following this, all tubes were dried under compressed air at 37°C (DBH10D, Ratek). The resulting residue was first treated with 2 mL of diethyl ether (> 99%; Sigma-Aldrich Ltd) and then with 1 mL volume of acetonitrile (> 99%; Sigma-Aldrich Ltd). Between these two steps, the samples had been vortexed (for 1 min), centrifuged (for 1 min at 3,000 x*g*), and dried. A 1 mL volume of hexane (> 99%; Sigma-Aldrich) was then added to each tube, and the vortex and centrifuge steps repeated. The hexane layer was discarded from each tube, and a new 1 mL of hexane was added. The vortex and centrifuge steps were again repeated, and the hexane layer was discarded. The final acetonitrile-lipid mixture was dried under compressed air at 37°C, with the steroid residue then reconstituted in 0.5 mL of assay buffer. Samples were frozen at -80°C until analysis.

Hormone Analysis

Seawater samples, pure blow samples, undiluted blow steroid hormone extracts, and undiluted blubber steroid hormone extracts were analysed for progesterone (P4), testosterone (T), oestradiol (E2), and cortisol (F). Concentrations were measured using enzyme-immunoassays (EIAs) that have already been used, and for several sample types (e.g., blood and urine) validated, in studies on a range of cetacean species (O'Brien & Robeck, 2012; Steinman et al., 2016; Mingramm et al., 2019). Each EIA was performed as originally described (see Munro & Stabenfeldt, 1984; Munro & Lasley, 1988; Munro et al., 1991) with only minor modifications (see Mingramm et al., 2019). All EIAs were evaluated for colour development using a Biotek Reader ELx808 (*Gen5*™ software; Biotek, Winooski, VT, USA; with read and reference wavelengths of 405 and 540 nm [P4, T] or 630 nm [E2, F]). Individual results were accepted if the intra-assay (duplicate well) coefficient of variation (CV) was < 10%. Blow hormone concentrations were expressed as nanogram of hormone per millilitre of extract. Blubber concentrations were expressed as nanogram of hormone per gram of blubber (wet weight). The antibodies used in each assay (CL425 [P4], R156/7 [T], R9472 [E2], and R4866 [F]; Coralie Munro, University of California at Davis) are known to cross-react with parent hormones and structurally similar derivatives (see Young et al., 2004; Thompson et al., 2012; Knott et al., 2013). Consequently, in this study, any mention of measured steroids or hormones is a reference to both the parent hormone and its metabolites.

Each EIA was biochemically validated for use via parallelism and hormone recovery tests. Inter-assay CVs were also monitored for high and low concentration control samples (P4: [6.8%, 17%]; T: [9.6%, 16%]; E2: [7.2%, 15%]; and F: [9.3%, 17%], respectively) to ensure that EIA results were repeatable over time. Parallel displacement of serially diluted blubber and blow hormone extracts, relative to the standard curves, occurred for all hormones (P4: R^2 = [0.96, 0.97]; T: R^2 = [0.97, 0.98]; E2: R^2 = [0.97, 0.92]; and F: R^2 = [0.98, 0.93], respectively). For hormone recovery tests, pooled samples of humpback whale blubber and blow hormone extracts were prepared by combining samples from 13 individuals (100 µl each). Test samples were then prepared (different sets for each hormone and sample type) by spiking EIA hormone standards with an equal volume of pooled sample. Concentrations of test and pooled samples were then evaluated as previously described. Expected values (EIA standard concentration divided by 2) and observed values (test sample concentration minus pooled sample concentration) that fell within the linear range of the assay (~20 to 80% binding) were then modelled using linear regressions. Significant relationships were found between observed and expected parent hormone concentrations for all blow (P4: $F_{(1, 3)}$ = 275.08; T: $F_{(1,3)}$ = 10,840.31; E2: $F_{(1,2)}$ = 754.92; and $F_{(1,3)}$ = 974.96; all $p < 0.001$) and blubber hormones (P4: $F_{(1, 3)}$ = 357.05; T: $F_{(1,3)}$ = 6,640.56; E2: $F_{(1, 2)}$ = 3,626.46; and F: $F_{(1, 3)}$ = 1,371.09; all $p < 0.001$). Blow P4 and E2 slope coefficients (95% CIs) did not differ from 1, suggesting accurate parent hormone measurements. Slope coefficients for blow T (0.84, 0.89), blow F (0.69, 0.84), and blubber P4 (0.69, 0.92) were < 1, indicating an underestimation of true parent hormone concentrations. Slope coefficients for the remaining blubber hormones (T: [1.09, 1.15]; E2: [1.16, 1.26]; and F: [1.09, 1.29]) were > 1, which indicates an overestimation of parent hormone concentration in these assays.

The precision of hormone extraction and analysis procedures was also assessed for blubber (the reference sample). Spike-recovery tests were used to evaluate whether there was any loss of parent hormone concentration as a result of the extraction process and how much this varied between different samples. Eight blubber samples (two for each hormone test) were dissected into triplicate portions (0.1 g). One portion was extracted and analysed to determine background concentration levels ("unspiked values"). The remaining two portions were used to generate "spiked values." Herein, a 0.15 mL volume of a high concentration standard (P4: 4 ng/mL; T: 20 ng/mL; E2: 25 ng/mL; and F: 20 ng/mL) was added to the homogenisation tubes prior to pulverising the tissue sample. Spiked samples were extracted as previously described and then analysed at a 1:2 dilution. Extraction efficiency was calculated using the subtraction method (see Marcelletti et al., 2015). Parent hormone recovery values varied (mean recovery: 95% [P4], 126% [T], 72% [E2], and 104% [F]) but were highly repeatable for duplicate samples (CVs: 8.2% [P4], 3.1% [T], 4.2% [E2], and 0.92% [F]).

Statistical Analyses

Statistical analyses were undertaken using *R*, Version 3.4.0 (R Development Core Team, 2017), with the α value set at 0.05. Summary statistics for hormone concentrations in groups of interest (age class, sex, and winter/northern migration vs spring/southern migration) were reported as mean ± standard error (SE). Blow samples that had been visibly contaminated with a high level of seawater (i.e., animal exhaled while underwater; $n = 4$) were excluded from all statistical models.

Existing literature on humpback whales suggests that this species demonstrates similar endocrine patterns to many other seasonal breeding mammals (see Vu et al., 2015; Clark et al., 2016; Mello et al., 2017; Pallin et al., 2018; Cates et al., in press). Given this, we would expect to see higher and more variable P4 concentrations in adult females than in other demographic groups. In contrast, T should be highest in adult males, and their concentrations should also vary seasonally. Finally, we would expect to see the lowest concentrations of reproductive hormones in juvenile whales. An essential first step in this study was to ensure that our blubber hormone extraction and analysis procedures were capable of detecting these patterns. A permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was therefore used to test whether P4, T, and E2 concentrations differed, on whole (i.e., multivariate response variable), between demographic groups. This model was run using type II tests (unbalanced design) via the 'RVAideMemoire' package (Hervé, 2018), with Euclidean distances as the dissimilarity metric. Homogeneity of multivariate dispersions was assessed using the distance-based tests described by Anderson (2006) via the 'vegan' package (Oksanen et al., 2018). A generalised linear model (GLM) was then used to test whether blubber T levels varied seasonally in adult males, using "migration direction" (i.e., northern or southern) as a predictor variable. As blubber T data were positive and right-skewed, the model was fitted with a Gamma distribution and log-link function. Model residuals were examined for evidence of heterogeneity, autocorrelation, and overdispersion.

The next step was to determine whether blow hormone concentrations were affected by "sample

quality" ("high" or "low"), correlated with blubber hormone concentrations, and whether relevant biological patterns (e.g., male seasonality) could be detected. Given that measurement accuracy could differ for P4, T, E2, and F (see Mingramm et al., 2019), separate tests were conducted for each hormone. GLMs were constructed using "blow hormone concentration" as a response variable and "sample quality," "demographic group" (adult male, adult female, juvenile male, and juvenile female), and "blubber concentration" as predictors. Alpha values were adjusted using Holm-Bonferroni corrections to account for multiple testing. As hormone data were positive and right-skewed, all GLMs were fitted with a Gamma distribution and log-link function. Generalised variance inflation factors were calculated using the 'car' package (Fox & Weisberg, 2011) to assess multicollinearity between predictor variables. Residuals were then examined for evidence of heterogeneity, autocorrelation, and overdispersion. Coefficient estimates with SEs, Wald t values, and associated p values were calculated for each model parameter. Likelihood-ratio (LR) tests were then used to assess the impact of a predictor on the model fit. For instance, if removing "blubber concentration" decreased a model's performance, it would suggest that there is a significant relationship between blow and blubber concentrations for that hormone.

Results

Blubber Samples

Steroid hormones were found at detectable concentrations in all blubber samples. Blubber hormone concentrations were highly variable (P4: 0.34 to 9.97 ng/g; T: 0.16 to 2.77 ng/g; E2: 0.43 to 3.07 ng/g; and F: 0.32 to 2.86 ng/g) both within and between demographic groups (Figure 1). Despite this, there were significant differences between the reproductive hormone profiles of adult females, adult males, juvenile females, and juvenile males (PERMANOVA: $F_{3,44} = 2.91$, $p = 0.030$). As expected, adult females possessed the highest P4 concentrations (5.03 ± 2.50 ng/g), whereas adult males possessed the highest T concentrations (1.34 ± 0.14 ng/g). The lowest concentrations of all measured reproductive hormones were found in juvenile whales (Figure 1). For adult males, season was found to have had a significant impact on blubber T concentrations (GLM, LR test: $\chi^2 = 6.44$, $p < 0.001$). T concentrations were higher in adult males sampled en route to the breeding grounds in winter (i.e., the northern migration; 1.68 ± 0.15 ng/g) when compared to those sampled during the southern migration in spring (0.75 ± 0.13 ng/g; Figure 2). In contrast to the reproductive hormones, F concentrations were relatively similar across all demographic classes (Figure 1).

Blow Samples and Controls

Steroid hormones were detected in blow extracts but not in "pure" blow droplets aspirated directly from the sampling kit (i.e., unextracted; $n = 3$) or in raw, unextracted seawater. Overall, there were no consistent differences in blow hormone concentrations between demographic groups (Figure 1). For P4, E2, and F, the only factor found to have significant impact on the model fit was blubber concentration (GLM, LR test: $\chi^2 = [11.52, 22.57, 6.12]$, $p = [< 0.001, < 0.001, 0.013]$, respectively). In all three of these models, there appeared to be a positive relationship in blow and blubber hormone concentrations (GLM: Wald t = [2.86, 4.68, 2.43], $p = [0.0069, < 0.001, 0.020]$, respectively). Further, when blow and blubber hormone data were standardised (i.e., converted to z-scores) there were positive correlations observed between each sample type (Figure 3), though, for P4, this correlation appeared largely driven by one adult female. This adult female displayed a blubber P4 concentration of 9.97 ng/g, which was around three times higher than the next highest P4 measurement. In addition, this individual displayed a blow P4 concentration of 0.94 ng/mL—the third highest blow P4 concentration measured in this study. To see if this individual was biasing the results of the analysis, it was removed and the analysis repeated. "Blubber concentration" was still found to have a significant impact on the blow P4 model (GLM, LR test: $\chi^2 = 7.03$, $p < 0.001$).

The results for T differed slightly from the results of all other measured hormones. There was a weak, positive correlation between blow and blubber T concentrations (Figure 3); however, blubber concentration failed to have a significant impact on the fit of the blow T model ($p = 0.21$). Further, in contrast to the patterns observed for blubber T concentrations, there was no clear seasonal variation in adult male blow T concentrations (northern migration: 0.40 ± 0.08 ng/mL; southern migration: 0.36 ± 0.08 ng/mL; Figure 2).

"Sample quality" failed to have a significant impact on the concentration of any measured hormone in blow; concentrations were similar for "low-" and "high-quality" samples (P4: [0.36 ± 0.04, 0.47 ± 0.06 ng/mL]; T: [0.33 ± 0.05, 0.33 ± 0.06 ng/mL]; E2: [0.54 ± 0.06, 0.61 ± 0.07 ng/mL]; and F: [0.31 ± 0.03, 0.34 ± 0.03 ng/mL], respectively), though the results of four samples that were highly contaminated with seawater (collected from humpback whales that had started their exhalation while underwater) were excluded from these analyses. These particular samples

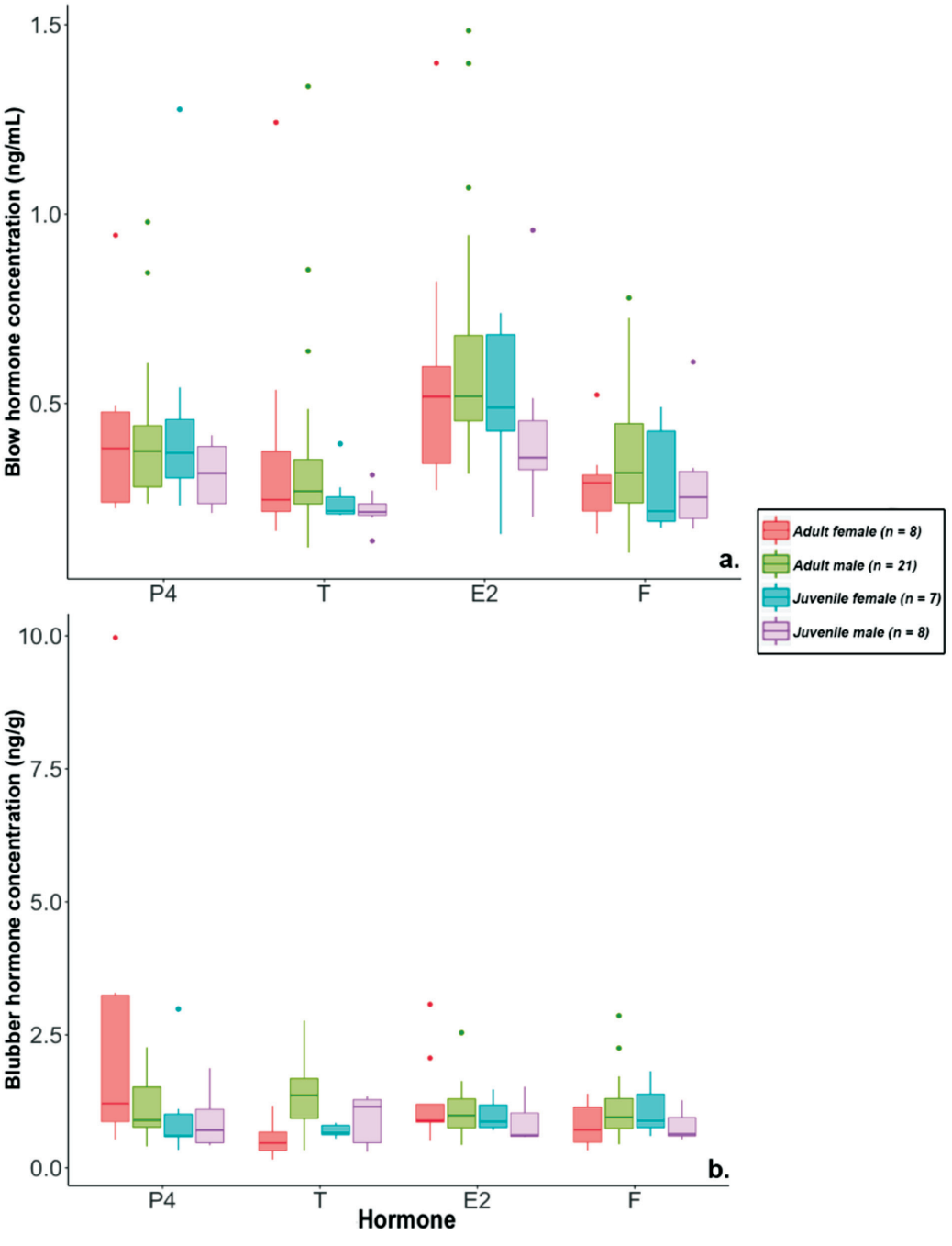

Figure 1. Steroid hormone levels (P4: progesterone, T: testosterone, E2: oestradiol, and F: cortisol) in paired blow (a) and blubber (b) samples from adult female ($n = 8$), adult male ($n = 21$), juvenile female ($n = 7$), and juvenile male ($n = 8$) humpback whales (*Megaptera novaeangliae*). Boxplot displays median value, with lower and upper hinges representing the interquartile range (25th and 75th percentiles, respectively). Whiskers represent the highest and lowest values within the 1.5× interquartile range. Values beyond this range are plotted as individual points.

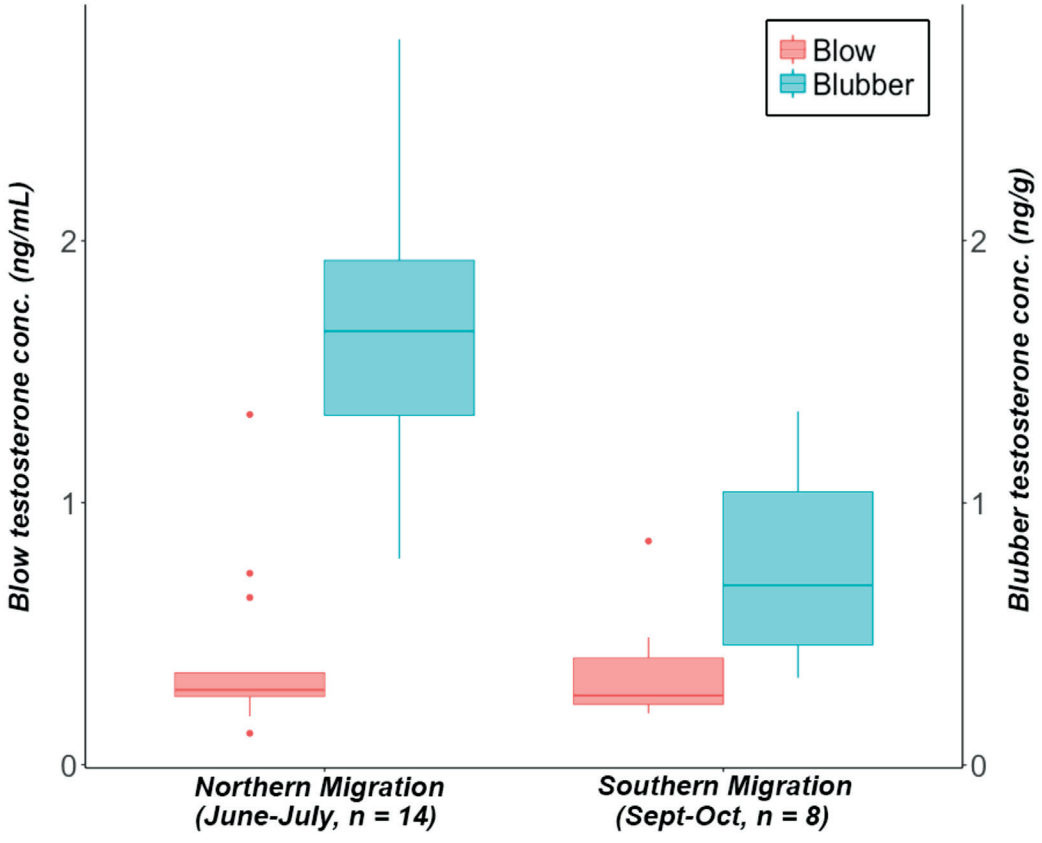

Figure 2. Testosterone levels in blow and blubber samples collected from adult male humpback whales during the winter northern migration in 2015 and spring southern migrations in 2014 and 2015. Boxplot displays median value, with lower and upper hinges representing the interquartile range (25th and 75th percentiles, respectively). Whiskers represent the highest and lowest values within the 1.5× interquartile range. Values beyond this range are plotted as individual points. "Season" had a significant impact on the blubber T model ($\chi^2 = 6.44$, $p < 0.001$) but not the blow T model ($p > 0.05$).

displayed concentrations (P4: 1.42 ± 0.27 ng/mL; T: 1.45 ± 0.29 ng/mL; E2: 1.79 ± 0.24 ng/mL; and F: 0.71 ± 0.27 ng/mL) roughly three to four times higher than mean steroid hormone concentrations for all other blow samples (P4: 0.41 ± 0.03 ng/mL; T: 0.33 ± 0.04 ng/mL; E2: 0.57 ± 0.05 ng/mL; and F: 0.32 ± 0.02 ng/mL). Therefore, seawater contamination—one factor that contributed to the classification of sample quality—was likely to have had a marked impact on blow hormone measurements.

There were a further two humpback whales—an adult male and juvenile female—that displayed blow hormone concentrations markedly higher than expected based on their blubber hormone concentrations (Figure 3). The samples collected from these whales had not been recorded as having suspected high levels of seawater contamination, nor were they biased towards a particular "sample quality." These samples had not been treated, stored, or processed in any abnormal way. The only common characteristic of these samples is that both were collected during the northern migration in 2015.

Discussion

This study assessed blow samples as a potential alternative to blubber samples for examining gross endocrine patterns in humpback whales. Hormone concentrations in both samples were weakly positively correlated with each other. Yet, in contrast to blubber hormone concentrations, blow concentrations did not show any consistent seasonal or demographic variation. Blow testosterone concentrations, for instance, were not higher in adult males when compared to females, and concentrations did not vary seasonally. This

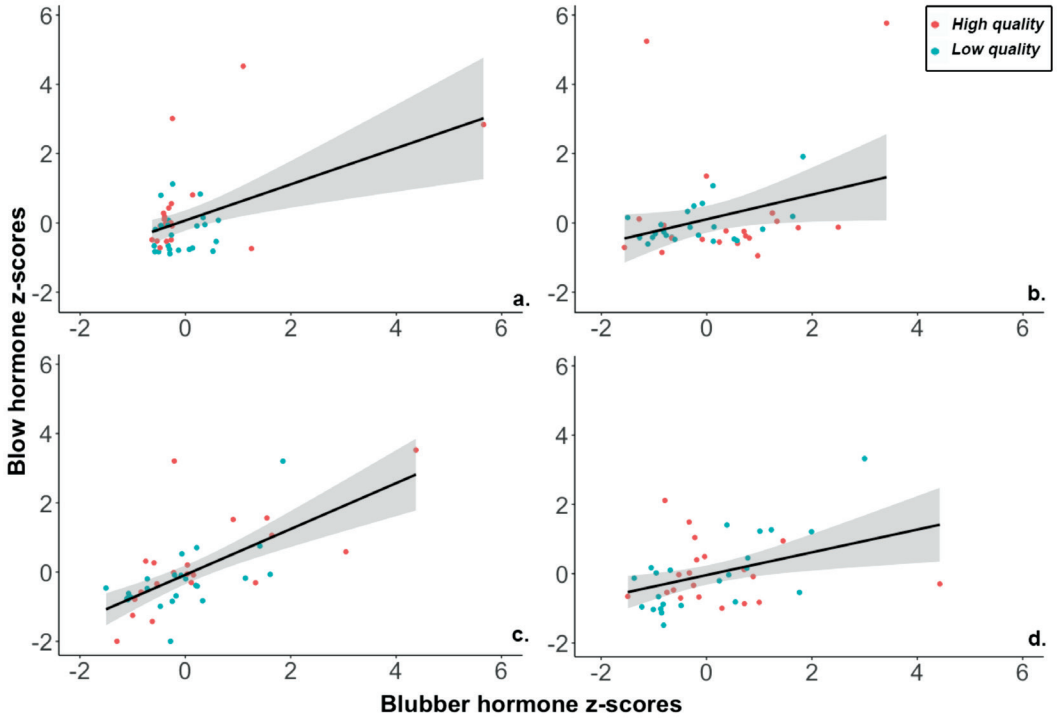

Figure 3. Relationship between blow and blubber levels of progesterone (a), testosterone (b), oestradiol (c), and cortisol (d) in humpback whales. Hormone concentrations are presented as standardised variables (z-scores) to control for different units of measure in blow and blubber. Line represents a linear approximation to the relationship between samples, with 95% CIs. Red symbols represent "high-quality" blow samples; green symbols represent "low-quality" blow samples.

was despite there being pronounced seasonal and sex-related differences in blubber concentrations. There was one sign that blow hormone concentrations were driven, to some extent, by the physiological condition of an animal, with one presumed pregnant adult female (possessing an elevated blubber progesterone level of 9.97 ng/g; see Clark et al., 2016) displaying a higher blow progesterone level. However, the progesterone value from this animal was still lower than two other measurements obtained from non-pregnant whales. Therefore, with these current collection and handling methods, blow hormone monitoring cannot be used to characterise even large-scale physiological changes, such as pregnancy, for this species. Future blow hormone studies should primarily focus on improving collection and analytical procedures before testing for biological relationships in hormone concentrations.

Sampling-related effects remain the largest and most obvious problem with blow hormone monitoring. In particular, the nylon sampling materials used here, and elsewhere, can lead to falsely elevated measurements when EIAs are used for hormone quantification (Burgess et al., 2016; Mingramm et al., 2019). This is unlikely to be the sole cause of outliers in this study as equal masses of nylon were used for each sampling kit. Additionally, it has yet to be determined whether storage times in ethanol influence the level of interference caused by nylon sampling materials. Conducting these storage tests is a priority for any future study that seeks to use an organic solvent extraction on nylon blow sampling kits. Further control studies are also warranted to determine if different collection materials (e.g., polystyrene dishes; Burgess et al., 2016) and other extraction and analysis techniques (e.g., chromatography coupled with mass spectrometry) can mitigate some sources of interference. Quantifying the volume of blow that was initially collected should also be considered as an essential first step for future blow hormone analyses.

In addition to material interference, seawater contamination remains a major problem for blow hormone studies. Seawater dilutes a sample at the time of collection and then directly interferes with EIA measurements after samples are treated with an ethanol-drying extraction (Mingramm et al.,

2019). Seawater contamination has not previously been highlighted as such a major issue for blow hormone measurements; however, many previous studies have employed techniques to actively reduce contamination such as cleaning an animal's blowhole prior to sampling and sampling stationary, captive, or wild-caught animals (see Hogg et al., 2005; Tizzi et al., 2010; Thompson et al., 2014; Richard et al., 2017). In this study, seawater was often observed resting in the depression of a humpback whale's blowhole. This seawater typically drained away within seconds after an animal surfaced and before it exhaled. However, in some instances, this seawater was forced up by a whale either beginning its exhalation while underwater or immediately upon reaching the surface. These last two situations usually occurred when sampling whales in fast-moving, competitive groups (Tyack & Whitehead, 1983) or animals that were attempting to flee the sampling vessel. Therefore, future studies on free-swimming cetaceans might benefit from collecting blow samples using unmanned aerial vehicles, which seem to cause fewer disruptions to an animal's behavior (Fiori et al., 2017; Pirotta et al., 2017).

Given the known sensitivity of blow testosterone measurements to both the seawater and nylon sampling effects (see Mingramm et al., 2019), it is not unexpected that this hormone was the most poorly correlated between blow and blubber, though, for all hormones, there were other factors that may have contributed to weaker than expected correlations between sample types. First, it is likely that each sample type accumulates and expresses circulating hormone concentrations over different timeframes. Previous studies on odontocetes suggest that blow most likely reflects circulating hormone concentrations over a period of tens of minutes (Thompson et al., 2014), with blubber incorporating hormones from circulation over a period of several hours to days (Schwacke & Wells, 2015; Champagne et al., 2017, 2018; Kellar et al., 2017). It is unknown whether hormone integration rates in baleen whales are similar to these timeframes. The second contributing factor is that the measurement accuracy and recovery rates for parent hormones, and likely their metabolites, differed for blow and blubber. Finally, the relative proportions of these parent hormones and metabolites—both of which are detected, to a different extent, by the antibodies used in each assay—probably differ in each sample type. Consequently, using direct methods of hormone quantification (e.g., chromatography coupled with mass spectrometry) might be more suitable when comparing hormone concentrations between different sample types.

In this study, we developed an improved method to collect paired blow and biopsy samples from humpback whales in quick succession. Once implemented, this method increased the success rate for paired sampling from ~17% in 2014 to ~43% in 2015. The new method also reduced the number of sampling attempts on each animal and the encounter times, facilitating the sampling of more whales each day with less disturbance to their behaviour. The skin samples collected during paired sampling attempts were used to undertake genetic tests, which, for sexually monomorphic humpback whales, was essential to classify the sex of an individual. In theory, these tests could have been conducted using DNA extracted from cetacean blow (see Frere et al., 2010); however, the small sample volumes collected here, and our treatment of these samples, prevented us from conducting both endocrine and genetic tests. Given that we did require skin samples and that no extra physiological information was gained from our blow hormone analyses, it raises the question of whether there is any added value to paired sampling over remote biopsying alone. Paired sampling likely caused slightly more disruption to a whale's behavior and increased the cost and time for analyses. Thus, remote biopsying should be considered a simpler, more economical, and ethical approach to studying the endocrinology of humpback whales.

The blow hormone measurement methods used here are not yet adequate to assess the physiological condition of humpback whales. There was some evidence that blow progesterone could be used as a marker for humpback whale pregnancy once certain issues, such as seawater contamination, have been resolved. The testing of new collection materials, endogenous standards (to assess sample dilution), and alternative blow hormone extraction and analysis techniques will be essential steps in resolving these sampling-related issues. Overall, remote biopsying remains the preferred sampling method for endocrine studies on humpback whales due to the ease of sample collection, accuracy of blubber hormone measurements, and the ability to conduct other analyses (e.g., genetics and toxicology) using other portions of the sample.

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