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14. ABSTRACT

Project goals were to test new methods for assessing stress-related hormones in respiratory vapor (blow) and feces from North Atlantic right whales (Eubalaena glacialis). We tested and compared three blow sampling devices, and successfully performed blow collection at sea from live whales. Blow hormone content, when corrected with a suitable internal control (e.g. urea), may reflect physiological state of the whales. Fecal mineralocorticoids (fMC) were quantifiable in whale feces, and, complementing existing glucocorticoid techniques, could be used to assess adrenal activity. Together, respiratory and fecal analyses could help assess physiological stress in whales at different timescale after disturbance.

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Development of Novel Noninvasive Methods of Stress Assessment in Baleen Whales

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LONG-TERM GOALS

The long-term goal of this project was to broaden the existing panel of endocrine stress assessment techniques for large whales. Few methods exist for assessment of physiological stress levels of free-swimming cetaceans (Amaral 2010, ONR 2010, Hunt et al. 2013a). We previously demonstrated that respiratory vapor (blow) sampling is practical and feasible for large whales, and that blow samples contain detectable steroid and thyroid hormones (Hunt et al. 2013b). We also developed a suite of fecal hormone assays for reproductive and stress-related hormones in North Atlantic right whales (Rolland et al. 2005, Hunt et al. 2006). However, blow sampling needed further development before widespread use, and additional stress-related fecal hormones had not yet been tested, particularly fecal mineralocorticoids (aldosterone and related metabolites). Our aim in this project was to further develop both techniques — respiratory and fecal hormone analysis — for use in stress assessment of large whales.

OBJECTIVES

The two specific objectives of this project were: (1) further development of respiratory sampling methodology, via modifications to our sampling apparatus and testing of "internal controls" to standardize for variable sample water content; and (2) development of a fecal mineralocorticoid (fMC) assay as an additional measure of adrenal activation, complementing existing fecal glucocorticoid (fGC) assays.

APPROACH

Our general approach included: (1) fieldwork for collection of respiratory and fecal samples from individually-identifiable, free-swimming North Atlantic right whales (NARW); (2) methodological testing and validation of blow-collecting sampler devices ("sampler testing experiment") to compare accuracy, repeatability and extraction efficiency for hormones of interest; (3) investigation of alternative analytes in blow (e.g., urea) for potential use as an "internal control" that could improve predictive value of blow hormone concentrations; (4) validation of a fMC assay, followed by assay of archived NARW fecal extracts; (5) assay of all blow and fecal samples for steroid hormones, as well as the internal control for blow; (6) hormone data analysis (blow and fecal) comparing levels by age-

class, sex and reproductive state of photo-identified NARW, with the goal of evaluating whether both types of hormone data accurately reflect physiological state.

Key individuals involved in this project were: Post-doctoral researcher Elizabeth Burgess, Ph.D. (fieldwork, all laboratory analyses, R&D of novel lab analyses, data interpretation and analysis, and lead writer for manuscript preparation); PI Kathleen Hunt, Ph.D. (fieldwork, experimental design, data interpretation, reports, budgetary oversight); Co-PI Rosalind Rolland, D.V.M. (fieldwork, experimental design, data interpretation); Co-PI Scott Kraus, Ph.D. (pole design/construction, boat piloting and other field logistics, experimental design, data interpretation).

WORK COMPLETED

Task 1: Fieldwork for Blow and Fecal Sample Collection

Fieldwork was conducted from 2013-2015, resulting in successful collection of 100 respiratory blow samples from known individual NARW. Thirteen fecal samples were also collected to add to our NARW fecal sample archive.

Task 2a: Blow Sampler Testing

The goals of this experiment were to: (1) assess whether sampling materials cause interference with assay results; (2) examine whether typical storage conditions of samples in the field (e.g., 6 hr in a cooler with ice packs, followed by storage in a -20°C freezer) causes variation in hormone content; and (3) determine the most effective sampling material for accurate recovery of hormone concentrations, using known-dose hormone solutions.

Three materials were selected for testing: 1) commercial nylon veil; 2) Nitex mesh; and 3) polystyrene dishes (see Results for full description). We compared recovery of known doses of steroid and thyroid hormones from the three sampler types. Three different solutions were created by mixing six hormones (testosterone, 17β-estradiol, progesterone, cortisol, aldosterone, and tri-iodothyronine) at various doses (high 10 ng/mL, medium 1 ng/mL or low 0.1 ng/mL) in combinations designed to mimic physiological states of interest: pregnant female, adult male and "stressed whale". A control solution consisting of distilled water was also tested. Due to earlier findings (Hunt et al. 2013b) that blow hormones can adhere to plastic surfaces, dish samplers were further tested to compare alternative methods of recovering hormone: (a) pipetting droplets directly, vs. (b) rinsing of dishes with an appropriate solvent (e.g. 70% ethanol) followed by collection and dry-down of the solvent. We also tested whether hormones degrade during short-term storage at field stations, by storing dish samplers -20°C in a standard household freezer for two weeks vs. extracting immediately. Each treatment group (hormone solution x sampler type x extraction/storage method) had eight replicates.

Samples (n = 128) were analyzed with commercial immunoassays for progesterone, 17β-estradiol, testosterone, cortisol, aldosterone, and tri-iodothyronine (enzyme immunoassays [EIA] #K025-H1, K036-H1, K032-H1, K003-H1, K052-H1, and coated-tube ^{125}I radioimmunoassay [RIA] #06B-254215, respectively; all EIAs from Arbor Assays, Ann Arbor, MI; RIA from MP Biomedicals, Costa Mesa, CA). Data analysis compared known hormone concentrations to the apparent hormone concentration reported by the assays.

Task 2b: Development of an Internal Control for Blow Hormone Analysis

Background and rationale. Our pilot project revealed that respiratory vapor samples can vary dramatically in water content and in total volume, which confounds determination of hormone concentration. A comparable phenomenon occurs in urinary hormone analysis, in which creatinine is

widely used to control for variable water concentration of the sample. Using this approach as a model — expression of hormone concentration per unit of internal control — we tested several potential internal controls for respiratory vapor.

Experimental approach. Literature reviews revealed that albumin, total protein, and urea showed promise based on relatively consistent concentrations in mammalian blood and indications that these substances are likely excreted in mammalian respiratory vapor. Albumin and creatinine were tested, but neither compound was detectable in NARW blow. Urea, however, was detectable, so we focused on urea as the most feasible candidate for an internal control.

Validations for urea in whale respiratory vapor began with testing of two commercial urea assays that were reported to have good sensitivity (Arbor Assays urea BUN kit #K024-H1; Bioassay Systems QuantiChrom™ urea assay kit #50-489-225), using NARW blow samples (n = 19) archived from our previous study (ONR # N000141110435). The more sensitive of the two assays was then tested for parallelism and accuracy with fresh samples collected in FY2015. The assay protocol was refined several times to optimize detectability and accuracy for the low sample volumes and concentrations that proved to be characteristic of field-collected NARW blow samples (see Results). All samples were then assayed successfully using the final optimized protocol. Steroid hormone concentrations in blow were compared with vs. without urea as a correction factor, and final results were compared to the physiological state of the whale (i.e., sex, age class, and reproductive state).

Task 2c: Lab Validations - Development of a Fecal Aldosterone Assay

Background and Rationale. Physiological stress assessment in large whales has heretofore largely focused on fGCs (e.g. Hunt et al. 2006; Rolland et al. 2012; Rolland et al, in review). However, fGC concentrations can elevate in response to numerous other factors, and cross-reactivity of fGC assay antibodies can occur with other fecal hormone metabolites (Hunt et al. 2006), complicating interpretation. Therefore, we investigated an additional category of adrenal hormone, fMCs (i.e., aldosterone and related fecal metabolites).

Experimental approach. Three fMC kits were selected for testing: (1) Siemens Coat-a-Count aldosterone ^{125}I RIA (#TKAL1), an assay already proven to perform well with cetacean serum (D. Crocker, pers. comm.); (2) Alpco aldosterone EIA #11-ALDHU-E01, and (3) Creative Diagnostics aldosterone EIA #DEIA4474. All three kits were tested for parallelism with a pooled NARW fecal extract. The most sensitive assay with the best parallelism and accuracy was then used to quantify fMCs in 315 NARW fecal samples collected between 2000-2015. For examination of fMC patterns in relation to known age class, sex, and reproductive state, this complete dataset was screened to exclude samples from unknown whales, known whales of unknown sex or age class, repeated samples from the same individual, and samples from carcasses. The resulting dataset consisted of 82 fecal samples from 32 male and 50 female NARW, with age classes and reproductive categories represented as follows: 10 immature males, 12 immature females, 20 mature males, 15 resting females (parous, non-pregnant, non-lactating females), eight confirmed pregnant females (calf sighted in the following year), 14 lactating females (calf <1 yr old in close attendance) and three calves (two males and one female). fMC data were compared to existing fGC and reproductive hormone data from the same samples. Our prediction was that if fMC and fGC both reflect adrenal activity, (a) fMC and fGC should show a significant positive correlation, and (b) physiological stressors already known to elevate fGCs (such as pregnancy) should also result in elevations of fMCs.

Task 2d: Assaying New Blow and Fecal Samples for Stress and Reproductive Hormones

Blow sample analysis. Hormone assays of blow samples are 100% complete. Immediately after fieldwork, steroid and thyroid hormones were extracted from all blow samples using an ethanol-rinse method (see previous Annual Reports for technical details). Assays for cortisol, progesterone, and testosterone were prioritized, as these are the hormones most likely to provide key information on reproductive state and stress responses in whales (there was insufficient sample volume to assay estrogens or thyroid hormones). The progesterone (EIA kit #K025-H1, Arbor Assays), testosterone (EIA kit #K032-H1, Arbor Assay) and cortisol assays (EIA kit #K036-H1, Arbor Assays) had previously been validated for NARW blow samples (Hunt et al. 2013b), but low cortisol detectability prompted a switch to a new cortisol assay (EIA #ISWE, Arbor Assays; see Results). Urea was quantified using the assay validated in Task 2b.

Fecal sample analysis. Fecal sample analysis is also 100% complete. Fecal samples collected during 2012-2014 were processed in FY2015 and three samples collected during 2015 were processed in FY2016. All samples were assayed for progestins, androgens, estrogens, glucocorticoids, mineralocorticoids and thyroid hormones, using methods described in Rolland et al. (2005), Hunt et al. (2006), and Wasser et al. (2010).

Task 3: Data Analysis, Manuscript Preparation, and Reporting

Data analysis is fully complete for both respiratory vapor and fecal hormone results.

Manuscript preparation. Three manuscripts are published or in preparation: The sampler-testing study has been published (Burgess et al. 2016), a fecal aldosterone paper has been submitted to a peer-reviewed journal, and a manuscript on blow hormone analysis is in preparation.

Reporting. Annual reports were submitted in FY2013, FY2014, FY2015, and FY2016.

RESULTS

Task 1: Fieldwork for Blow and Fecal Sample Collection

Refining blow sampling collection methods. Blow samples were collected using a cantilevered carbon-fiber pole attached to the bow of R/V *Callisto*; in FY2013 we improved this apparatus with a longer pole mount to improve balance, and a redesigned pole handle. Multiple sampler types were compared (Fig. 1), including: (1) nylon veil samplers (Hunt et al. 2013b); (2) Nitex, a 110um mesh laboratory-grade nylon; (3) dish samplers (square laboratory-grade sterile polystyrene dishes) with hormones later recovered either by pipetting or by rinsing dishes with an appropriate solvent. Based on initial results in laboratory trials, only the latter two sampler types (Nitex and dish) were tested at sea.

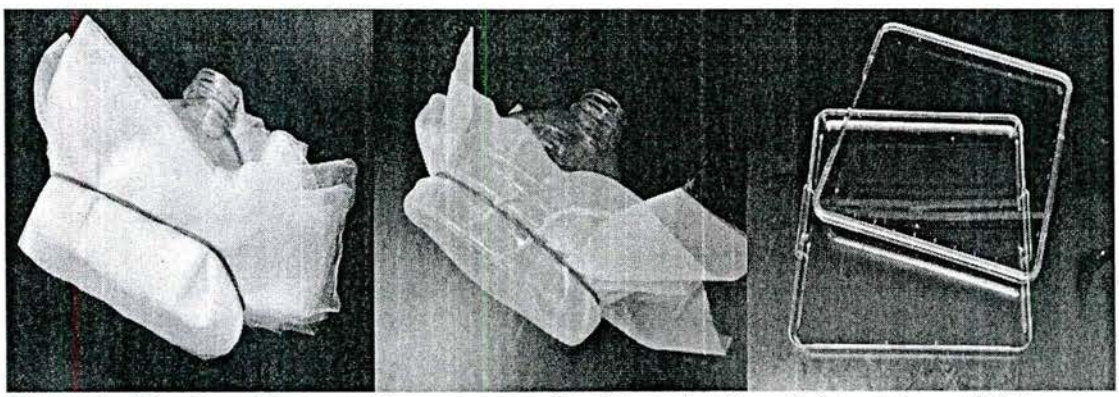

Figure 1 - The three blow-sampler types tested in this study. From left to right: veil, Nitex, and sterile polystyrene dish.

Blow sample collection. We collected 100 blow samples from NARW during eight days at sea (66 hours of survey effort), with 74 samples collected with dishes and 26 with Nitex (Fig. 2). Samples were collected by approaching whales very gradually, at an idle, from an oblique angle. Any whales that exhibited avoidance behavior were not pursued further. Overall, blow samples were collected from 65% of whales approached for blow sampling and from 51% of all whales photographed on survey.

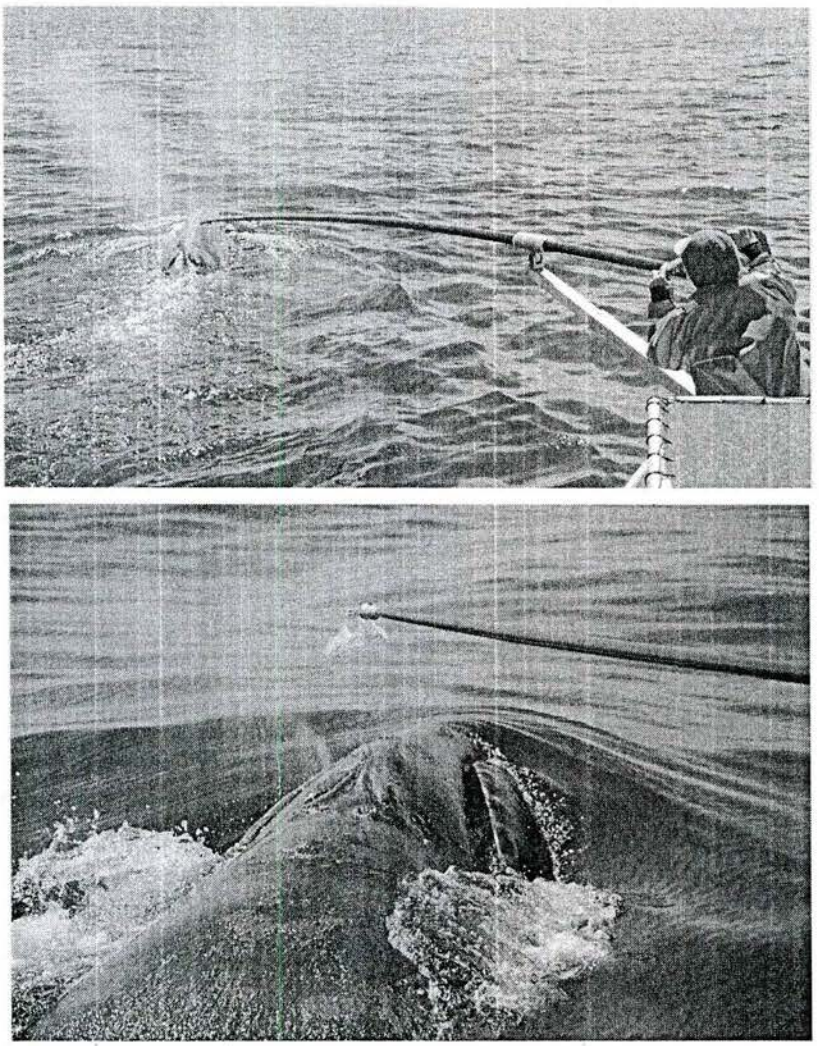

Figure 2. Collection of blow (respiratory vapor) samples from North Atlantic right whales with a polystyrene dish (top) and Nitex (bottom).

All blow samples were assigned quality scores of 0-3 (0 = poor, 3 = excellent) at the time of collection, based on proximity of sampler to the blowholes and visible blow droplets on the sampler (see Hunt et al. 2013b); 17% of samples were "fair" quality (n = 17); 17% of samples were "good" quality (n = 17); and 66% of samples were "excellent" quality (n = 66).

All whales sampled were photographed for subsequent identification through the North Atlantic Right Whale Sightings and Identification Database (Hamilton et al., 2007). All samples were matched to known whales representing 46 individuals (30 males, 13 females and 3 unknown sex < 3 y.o.).

Fecal sample collection. Thirteen NARW fecal samples were collected during 2012 through 2015. These sample sizes were lower than anticipated due to low NARW abundance, but when combined with our existing NARW fecal archive, the sample sizes are robust (total n = 315 fecal samples).

Task 2a: Sampler Testing for Blow

All sampler types yielded consistent and repeatable results when tested with known doses of hormones, as indicated by low standard deviations in recovered hormone (Burgess et al. 2016). The dish sampler required an ethanol rinse for efficient hormone extraction (i.e. direct pipetting off the dish was insufficient), particularly for the recovery of progesterone. The veil and Nitex mesh exhibited mild 'non-zero' background levels in some hormone assays, and additional analyses with high performance liquid chromatography (HPLC) confirmed that veil and nitex samplers produced substantial immunoreactive interference whereas dish samplers did not. Storing samples for 6 h in a cooler, and/or subsequent storage for 2 weeks at -20°C, did not affect any of the six hormones, which verifies that basic field storage conditions are adequate for this type of sample collection. Importantly, expected hormone ratios (i.e. low/high levels) could be correctly identified for all hormones measured in each sampler type — that is, relative patterns and ratios were consistent, even for those sampler types that produced some assay interference (Burgess et al. 2016). Overall, the results of our study demonstrate that (1) polystyrene dish samplers result in the highest accuracy and precision of hormone data; (2) field storage for 6h does not affect results; and (3) a solvent rinse of the dish sampler is advisable to ensure adequate recovery of hormone. See Burgess et al. (2016) for full results.

Task 2b: Development of an Internal Control for Blow

The final assay protocol (Arbor Assays urea BUN kit #K024-H1 with in-house adjustments to improve sensitivity and accuracy) achieved good precision for both inter- and intra-assay variation (Fig. 3). Detectability of urea was excellent, with 97% of field-collected NARW blow samples having detectable urea. See Task 2d for data analysis results.

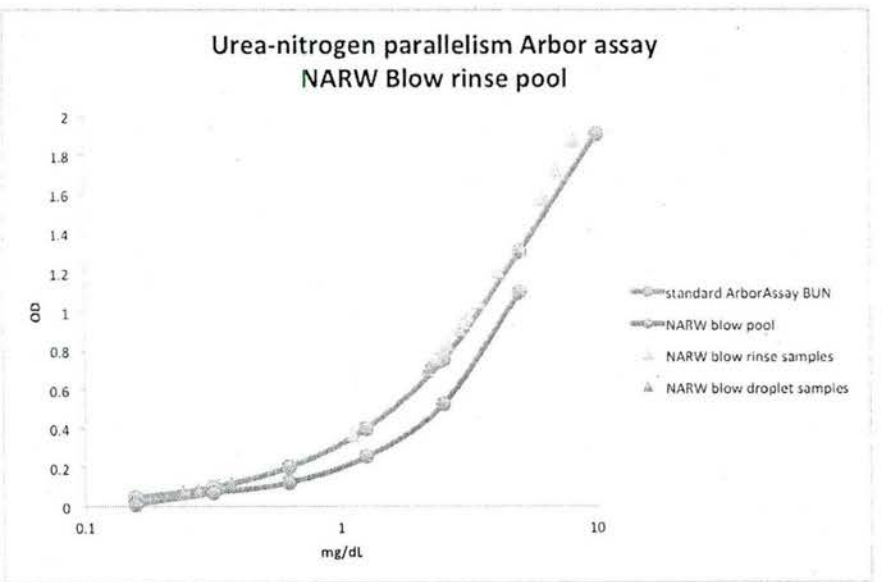

Figure 3. Parallelism results for the final optimized protocol for the Arbor Assays urea assay kit #K024-H1. Note parallelism of standards (blue lines) with serially diluted NARW blow extract (red lines).

Task 2c: Lab Validations - Development of a Fecal Mineralocorticoid (fMC) Assay
All three aldosterone assays demonstrated good parallelism with pooled NARW fecal extract, indicating that fMCs are present and detectable in NARW fecal extracts. The Siemens RIA had excellent parallelism (Fig. 4, left) and also had best sensitivity. We therefore selected this kit for further testing for accuracy, i.e., spiking a set of standards with NARW fecal extract (at a 1:16 dilution) and testing them alongside unspiked standards. Accuracy was very good (Fig 4, right). We then assayed 315 fecal extracts for fMCs with the Siemens aldosterone assay (see Task 3).

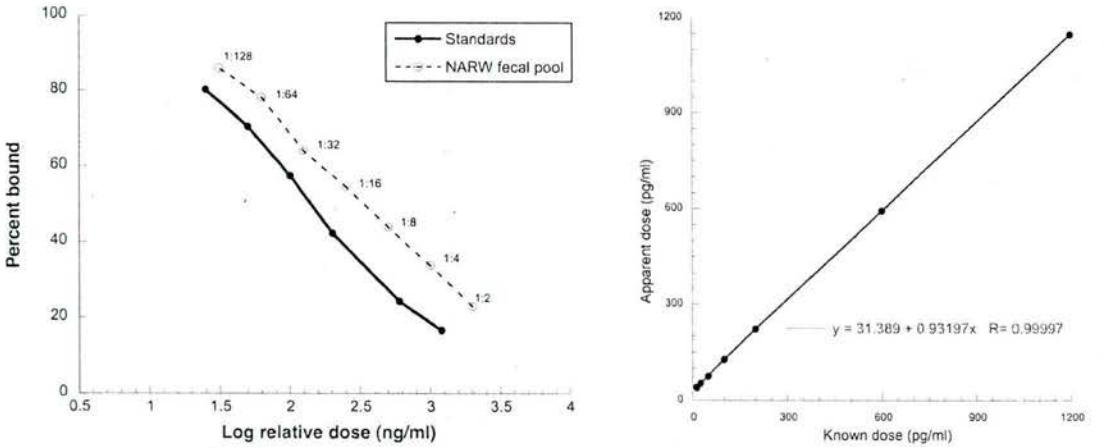

Figure 4. Validation results for a fecal mineralocorticoid assay (Siemens aldosterone RIA). Left, parallelism; note parallelism of standards (solid lines) compared to serially diluted NARW fecal extract (dashed lines). Right, accuracy with NARW blow diluted to 1:16; note straight line with slope close to 1.0.

Task 2d: Assaying New Blow and Fecal Samples for Stress and Reproductive Hormones
Blow assays. Steroid and thyroid hormones were extracted from all blow samples using the ethanol-rinse method that was previously tested in the sampler-testing experiment (see previous Annual Reports for extraction details). However, many blow samples had very low cortisol concentrations and fell below detectability in our existing cortisol EIA (kit #K036-H1, Arbor Assays). We investigated other cortisol assays, and ultimately, switched to a more sensitive kit using a newly available cortisol antibody (cortisol kit #ISWE, Arbor Assays). This new cortisol kit was successfully validated for NARW blow, and all blow samples were re-assayed with this kit. 100% of samples had detectable cortisol using the new kit. See Task 3 for analysis results.

Fecal assays. All fecal samples were extracted and analyzed for fecal progestins, androgens, estrogens, and glucocorticoids (Rolland et al. 2005, Hunt et al. 2006), fecal thyroid hormones (Wasser et al. 2010) and fecal mineralocorticoids (see below). See Task 2d for analysis results.

Task 3: Data Analysis, Manuscript Preparation, and Reporting
Blow data analysis. Urea concentrations in blow samples were highest in samples scored as 'excellent' quality (ANOVA, $P = 0.02$; Fig. 5), suggesting that urea may be a reliable indicator of collected sample concentration.

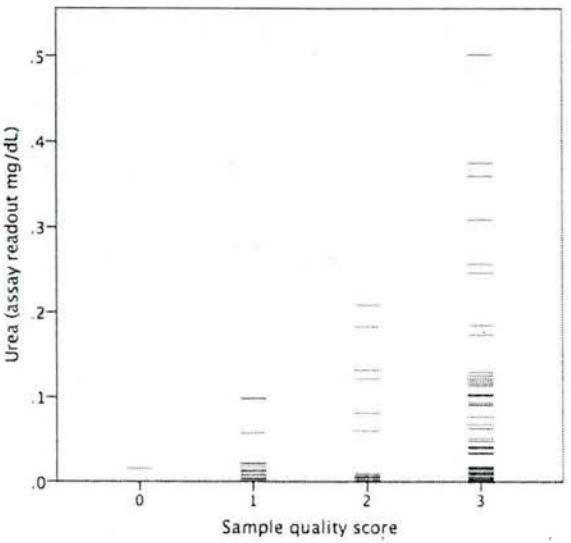

Figure 5. Amount of urea measured in blow samples (total n = 100) of each quality score (visually scored as, poor = 0, fair = 1, good = 2 or excellent = 3).

Final blow hormone concentrations were calculated with vs. without the internal control, i.e., ng hormone per mL of sample extract (Fig. 6A) vs. ng hormone per mg urea (Fig. 6B), with results compared for correspondence with physiological state of the sampled whale. Analyses initally focused on whether quantified blow progesterone was higher in pregnant than in non-pregnant females. Using the ratio of progesterone to urea in blow samples, four samples from three individual whales were identified as having extremely high progestin/urea ratios. These three NARW were all females, ages 6 y.o., 14 y.o. and 25 y.o. Two of the blow samples were from the 25 y.o. female, whale Eg #3101; both these blow samples were collected in 2015 and she was subsequently re-sighted in early 2016 on the NARW calving grounds with a live neonate calf. This independent confirmation of pregnancy provides a critical biological validation for the progestin/urea analysis. We also obtained a fecal sample from the same pregnant female (Eg #3101), concurrent with the blow sampling, which showed elevated fecal progestins indicative of pregnancy. The concordance of high progestin concentrations in a fecal sample and high progestin-to-urea ratio in blow samples from this female during a confirmed gestational period supports using an internal control to improve the predictive value of blow hormone data (e.g. hormone data when expressed per mg urea show an improved match to the animal's actual physiological state; Fig. 8). However, the high progesterone/urea ratio of blow from the other two females that were not subsequently sighted with a calf indicates variability in the data, and we recommend that pregnancy should not be diagnosed based on only a single blow sample. Calving rates have dropped significantly over the past 5 years in NARW, with only 14 calves born in the winter of 2015-2016. Therefore, one possibility is pregnancy loss in these two females, however, this is impossible to confirm. Further study with greater sample sizes will be necessary to determine the normal range of variation of progesterone/urea ratio across different categories of age, sex, and reproductive states.

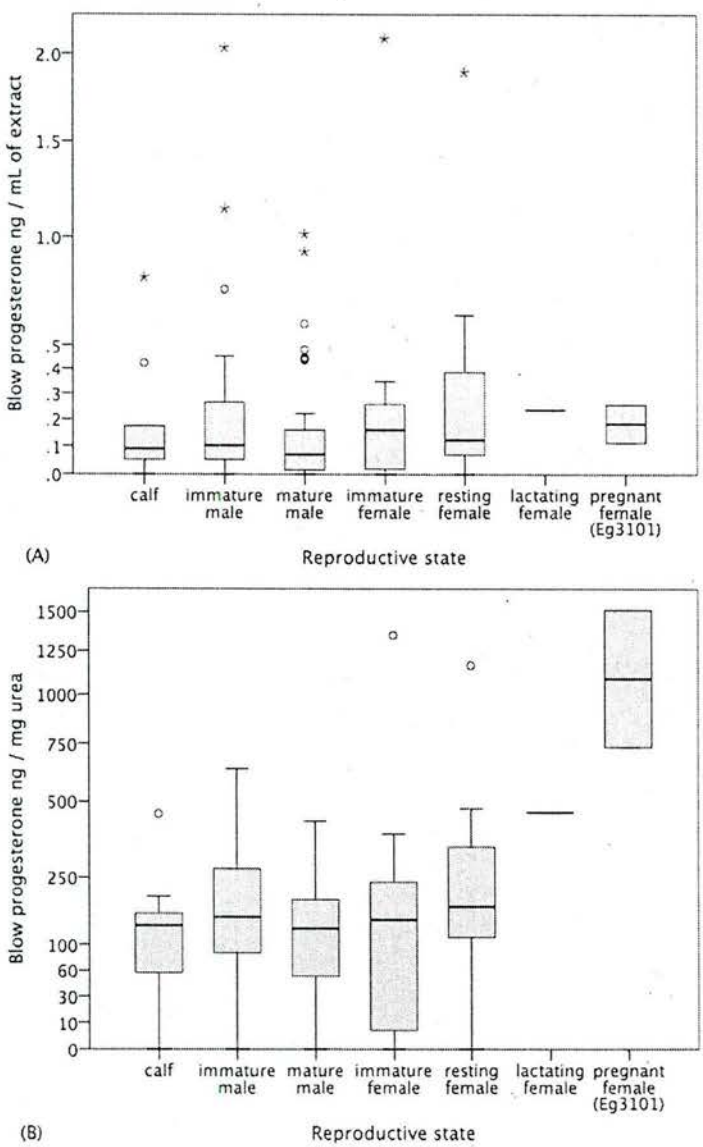

Figure 6. Blow progesterone of NARW by sex and reproductive state (total n = 100), using (A) no adjustment for sample dilution; and (B) concentration adjusted per mg of urea. Horizontal line indicates median, box height encompasses 25th-75th quartiles, whiskers delineate extreme observations. Outliers are marked with an open circle or asterisk (>1.5 or >3 × interquartile range). Note logarithmic scale of y-axes.

Concentrations of testosterone and cortisol in blow samples, expressed per mg of urea, also showed meaningful trends (although statistically non-significant; ANOVA, all $P > 0.05$) corresponding with known physiological state (Fig. 7A, 7B). In contrast, results for testosterone per mL of extract were contrary to expected patterns, i.e., the highest value of testosterone per mL was recorded in an adult female, and mature males had lower values compared to all other groups. Cortisol/urea concentrations were (on average) higher in the pregnant female (Fig. 7B), a trend similar to that seen from fecal data (Rolland et al. 2005, Hunt et al. 2006). Without the urea correction, the pregnant female had the lowest record cortisol per mL of extract, a pattern opposite to that found for fecal hormones (Hunt et al., 2006). Generally, when using the urea correction method, trends in blow hormone data across sex and reproductive states were similar to those reported for fecal hormones (Rolland et al., 2005; Hunt et al.,

2006). In sum, we found that expressing blow hormone concentrations per mg urea (rather than per mL of sample droplet) reduced variation in the data and produced more biologically meaningful patterns as related to life history state. Thus, urea correction may help improve the explanatory value of blow hormone data. Note, though, that the patterns seen to date were not statistically significant; however, low sample sizes restricted statistical power. We are continuing to investigate inter-sample variation of individual whales as well as quality control criteria for accepting sample data (i.e., assay limits of detection) in order to provide well-grounded recommendations for future research.

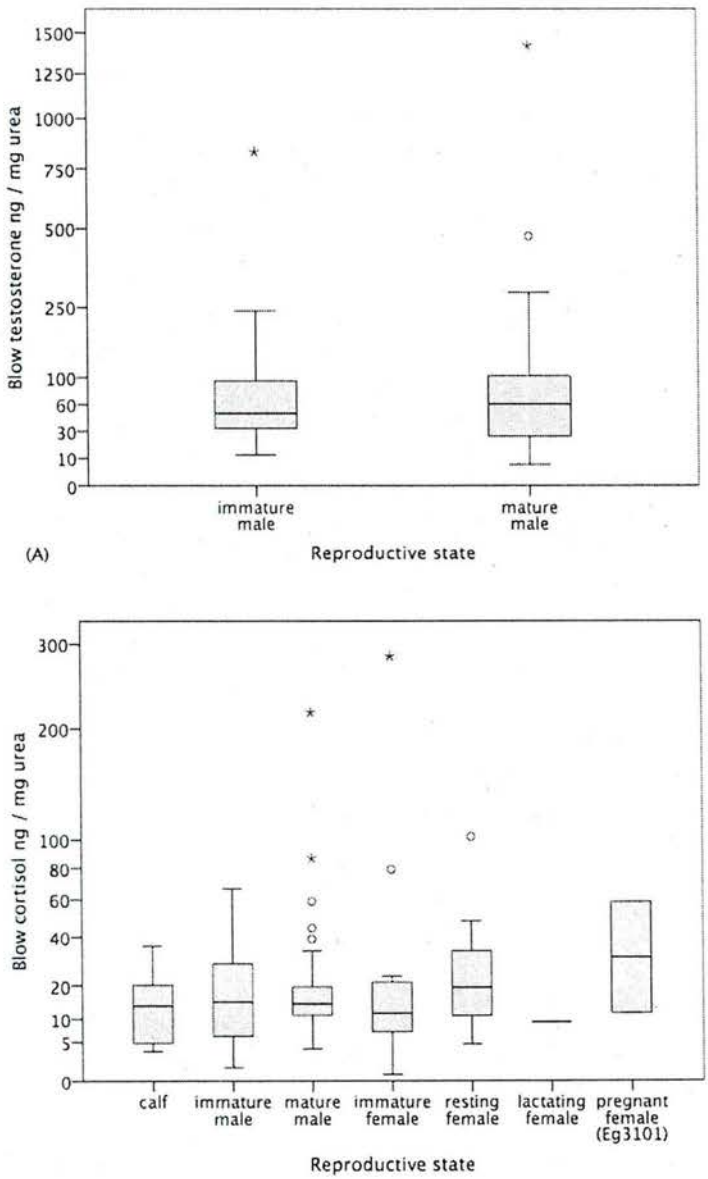

Figure 7. Blow testosterone (A) and cortisol (B) of NARW by sex and reproductive state (total n = 100), with concentration adjusted per mg of urea. Horizontal line indicates median, box height encompasses 25th-75th quartiles, whiskers delineate extreme observations. Outliers are marked with an open circle or asterisk (>1.5 or >3 × interquartile range). Note logarithmic scale of y-axes.

Fecal hormone data analysis.

Fecal MCs varied significantly with sex and reproductive state (Fig. 8). Pregnant females had the highest fMC concentrations, followed by reproductively mature males (ANOVA, $P < 0.001$). Individual sample variation in fMC levels was significantly influenced by concentration of fGCs (GLM fitted by maximal likelihood, $P < 0.001$) and fecal progestins ($P = 0.01$), but not by fecal androgens ($P = 0.33$) or fecal estrogens ($P = 0.05$). Fecal MC concentrations exhibited the strongest correlation with fGCs (linear regression, $r = 0.59$, $P < 0.001$), with levels of both these adrenal hormones increasing congruently (Fig. 9). These data suggest that a combination of fGCs and FMCs may work well to identify periods of heightened adrenal activity in whales.

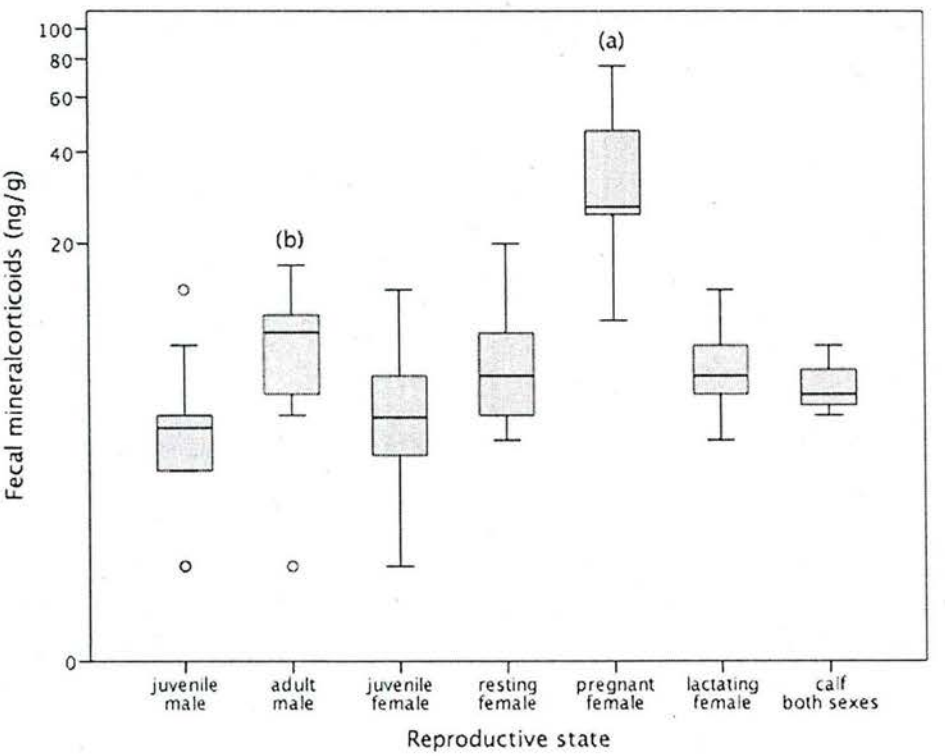

Figure 8. Fecal mineralocorticoid concentrations (ng/g, plotted on a logarithmic scale) in NARW, by sex and reproductive state. Horizontal line indicates median, box height encompasses 25th-75th quartiles, whiskers delineate extreme observations. Outliers are marked with an open circle (>1.5 × interquartile range). Different letters denote significant difference between groups at P < 0.05.

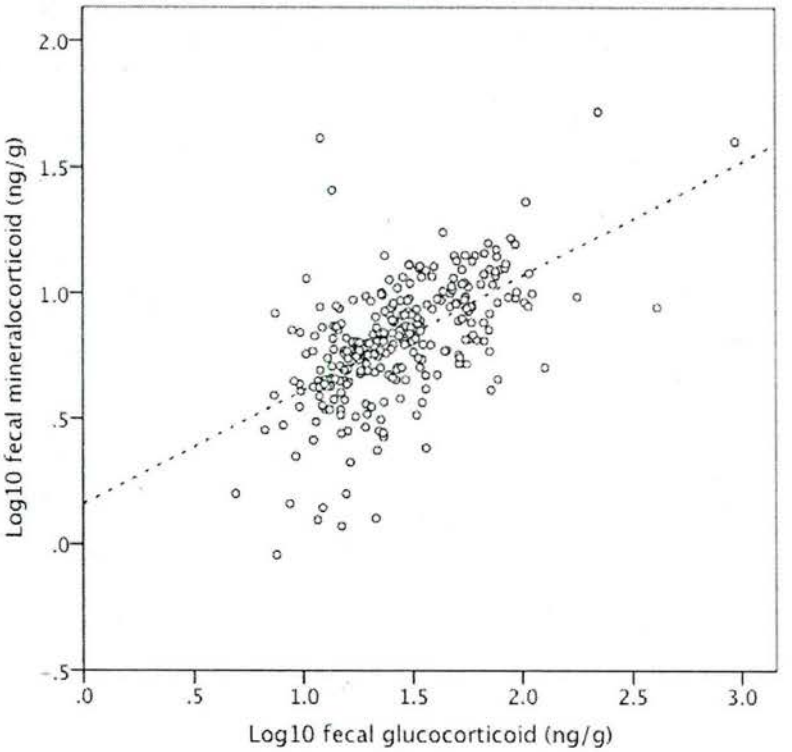

Figure 9. Significant correlation of mineralocorticoids and glucocorticoids in North Atlantic right whale fecal samples. Dashed trend line represents least-squares linear regression fit to the data set, y = 0.16 + 0.45x.

Manuscript Preparation

Data from this grant were organized into three manuscripts on the sampler-testing experiment, fecal aldosterone, and blow hormone analysis. The sampler-testing paper (Burgess et al. 2016a) is now published. The fecal aldosterone paper (Burgess et al. 2016b) was submitted to the journal *General And Comparative Endocrinology* in fall 2016, and the blow analysis manuscript (Burgess et al. 2016c) is in preparation with submission expected in late 2016.

Reporting and Presentations

Interim reports were submitted to ONR in FY2013, FY2014, FY2015, and FY2016. Annual presentations of results occurred at the ONR Stress Program Review Meetings. Multiple presentations at scientific conferences occurred throughout this grant at annual meetings of the Society for Integrative and Comparative Biology and the North Atlantic Right Whale Consortium, at biennial meetings of the International Society for Wildlife Endocrinology and the Society for Marine Mammalogy, and at a workshop on marine mammal stress organized by the National Marine Fisheries Service. Presentations, listed below in chronological order, included:

Hunt K, Rolland R, Robbins J, Kraus S. 2013. Detection of steroid and thyroid hormones in respiratory vapor of two baleen whale species via immunoassay. Biennial Meeting of the Society for Marine Mammalogy, Dunedin, New Zealand, 9-13 December 2013. Poster presentation.

Hunt K, Rolland R, Kraus S. 2013. Advances in right whale endocrinology and what it can tell us. Right Whale Workshop, Biennial Meeting of the Society for Marine Mammalogy, Dunedin, New Zealand, 7 December 2013. Oral presentation.

Hunt K, Rolland R, Kraus S, Burgess E. 2014. Development of novel methods of stress assessment in baleen whales. Annual Program Review, Office of Naval Research, 13 May 2014. Oral presentation.

Burgess EA, Hunt KE, Rolland RM, Kraus SD. 2014. Optimizing sampling methodology for collection of whale respiratory samples for endocrine analysis. Annual Program Review, Office of Naval Research, 13 May 2014. Poster presentation.

Hunt K, Rolland R, Burgess E, Kraus S. 2014. Development of a fecal aldosterone assay for North Atlantic right whales (*Eubalaena glacialis*): a potential additional measure of adrenal physiology. Annual Meeting of the North Atlantic Right Whale Consortium, 5-6 November 2014. Oral presentation.

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IMPACT/APPLICATIONS

This project achieved refinement and accuracy testing of sampling techniques for collection of respiratory vapor from NARW, and developed an assay for stress-related adrenal hormones (aldosterone and related mineralocorticoids) that had not been adequately studied in baleen whales. Respiratory sampling is a method of physiological assessment for large whales that may offer the ability to rapidly collect repeated, noninvasive samples from targeted individuals with minimal disturbance. Fecal mineralocorticoids may be a useful complement to existing glucocorticoid assays for assessing acute and chronic stress in whales. Furthermore, quantification of hormones in blow samples from large whales by using urea as a denominator helped to improve the explanatory value of blow hormone data. Together, the two analytic techniques — respiratory analyses and fecal analyses — could help assess physiological stress at different timescales after disturbance, potentially with respiratory analyses reflecting short-term disturbances and fecal analyses longer-term disturbances (Hunt et al. 2013a), better enabling identification of causes and consequences of physiological stress in whales. The work described here may add to the tools available to evaluate the physiologic consequences of noise on one species of baleen whale, and may be applicable to other whales and other marine mammals as well.

RELATED PROJECTS

This grant is a follow-up to a prior ONR project exploring feasibility of respiratory vapor sampling in whales (K. Hunt, PI; "*Development of Respiratory Sampling to Assess Stress Responses in North Atlantic Right Whales,*" ONR # N000141110435). A concurrent grant titled "*Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas*" (R. Rolland, PI; ONR # N000141110540) focused on development and validation of fecal hormone assays to assess stress responses in Blainville's beaked whales (*Mesoplodon densirostris*) and sperm whales (*Physeter macrocephalus*) inhabiting the northern Bahamas; that grant was completed in 2016.

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PUBLICATIONS

Published or in press:

Hunt KE, Moore MJ, Rolland RM, Kellar NM, Hall AJ, Kershaw J, Raverty SA, Davis CE, Yeates LC, Fauquier DA, Rowles TK, Kraus SD. 2013. Overcoming the challenges of studying conservation physiology in large whales: a review of available methods. *Conservation Physiology* 1:cot006.

Burgess EA, Hunt KE, Kraus SD, Rolland RM. 2016. Get the most out of blow hormones: validation of sampling materials, field storage, and extraction techniques for whale respiratory vapor samples. *Conservation Physiology* 4:cow024.

Submitted:

Burgess EA, Hunt KE, Kraus SD, Rolland RM. Adrenal responses of large whales: integrating fecal aldosterone as a complementary biomarker. Submitted to *General and Comparative Endocrinology*.

In preparation:

Burgess EA, Hunt KE, Kraus SD, Rolland RM. In prep. Quantification of hormones in whale blow: finding a denominator substance for concentration adjustment.