Development of Novel Noninvasive Methods of Stress Assessment in Baleen Whales

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LONG-TERM GOALS

Our long-term goal is 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. 2013). Prior to this grant, we 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. 2014). We had also developed a suite of fecal hormone assays for reproductive and stress-related hormones in North Atlantic right whales (NARW; Hunt et al. 2006, 2015; Rolland et al. 2005, 2012). However, blow sampling needs further testing before it can enter widespread use, and some additional stress-related hormones have not yet been tested in either feces or blow, particularly the adrenal hormone aldosterone. Our aim in this project is to further develop both techniques - respiratory hormone analysis and fecal hormone analysis - for use in stress assessment of large whales.

OBJECTIVES

We have two overall objectives in this project: (1) further development of respiratory sampling methodology, via modifications to our sampling apparatus and testing of "internal controls" to control for water content; and (2) development of a noninvasive aldosterone assay (for both feces and blow) that can be used as an alternative measure of adrenal gland activation relative to stress responses, to complement existing glucocorticoid assays.

APPROACH

Our general approach for FY2015 included: (1) fieldwork in Cape Cod Bay, a new fieldsite not originally part of the grant, for blow and fecal sample collection from free-swimming NARW; (2) beginning of labwork (extractions and 5 hormone assays) on all blow samples collected in the field; (3) continued testing of urea assays and other alternative analytes for potential use as "internal controls" for blow samples, first using archived NARW blow samples and next using the field-collected samples; (4) data analysis and manuscript writing of the "sampler testing experiment" completed in

FY2014 (comparison of performance of three types of respiratory samplers using simulated blow samples in the lab) and the aldosterone experiment of FY2013.

Key individuals in this project are: Post-doctoral researcher Elizabeth Burgess, Ph.D. (fieldwork, laboratory analyses, R&D of novel lab analyses, data interpretation and analysis); lead PI Kathleen Hunt, Ph.D. (fieldwork, analyses, reports and general oversight); Co-PI Rosalind Rolland, D.V.M. (fieldwork, experimental design and data interpretation); and Co-PI Scott Kraus, PhD. (right whale biologist, pole design/construction, boat piloting and field logistics).

WORK COMPLETED

Task 1: Fieldwork for Blow Sample Collection
Blow sample collection from photo-identified NARW was our top priority for this final year of fieldwork. A secondary priority was to collect any fecal samples that might be observed opportunistically. This grant was originally planned with fieldwork in Aug-Sept each year in the Bay of Fundy (BOF), based on historic patterns of NARW abundance. However, low NARW abundance in the BOF during FY2013 and FY2014 necessitated adaptation of these plans. After evaluation of changing patterns of NARW abundance and distribution, we decided to shift our field location from BOF to Cape Cod Bay (CCB). NARW are usually present in CCB during spring migration northward, typically in April. To conduct this work in an untried location, we arranged boat slip rental and fieldhouse rental in Sandwich, MA for the month of April, and relocated field crew and R/V *Callisto* to this location. Beginning on April 1, 2015, we surveyed CCB for NARW on all days with suitable conditions for respiratory-vapor sampling (sea state <3, good visibility, no precipitation). NARW were located both by sightings from our own vessel R/V *Callisto*, as well as by colleagues on other vessels (R/V *Selkie*, R/V *Stellwagen*) and aerial surveys. We attempted respiratory-vapor sampling on all days when NARW were sighted.

Blow samples were collected with two different sampler types for comparison, mesh fabric on a plastic frame and square polystyrene dishes. We had previously determined that these two sampler types showed promise for routine use in fieldwork, based on accurate recovery of known doses of steroids in the FY2014 sampler-testing experiment, as well as small size and simple extraction protocols for labwork. The mesh fabric samplers are similar to the "brideshead" type used in our 2011 pilot study but with a different type of nylon, a square of 110µm mesh fabric (“Nitex”) that can fit in a 50ml polypropylene tube (thus simplifying extraction of hormones from the nylon). The "dishes" are square sterile polystyrene plates that can be velcroed to the end of the sampling pole and can later be stored in a Ziploc bag compactly in a boat cooler. For preparation, nitex fabric was washed by soaking in warm soapy water, rinsing with tap water, rinsing twice with distilled water, submerging and agitating in 70% ethanol for 10 min, and air-drying. Dishes had a sterilized surface and lid that did not require any washing before experimental treatment. Forty-eight of these samplers (twenty-four bridesheads and twenty-four dishes) had been previously prepared in FY2014, stored at the end of that season and saved for re-use; an additional 80 were constructed new during the FY2015 field season.

Task 2: Labwork
2a. *Sampler testing for blow collection.* This experiment was fully completed in FY2014; see Task 3 for progress on the associated manuscript.

2b. *Development of an internal control for blow.* We had already performed preliminary testing of two urea assays in FY2014. In FY2015 we tested the most promising of these assays further. We completed the following tasks:

- Confirmed parallelism of urea assay (urea BUN kit #K024-H1, Arbor Assays) with a pool of NARW blow samples collected in 2015. (Urea parallelism had been assessed in the previous year of this grant, in a pilot trial with archived older samples, but needed to be checked with fresh samples as well.)
- Conducted tests to determine whether the urea assay would perform at a reduced volume (i.e., 60% volume) to allow more sample to be preserved.
- Discovered and solved a potential problem caused by sample turbidity that causes distorted results when using the urea assay.
- Tested parallelism of an albumin assay (kit #30-6330, Alpco) with a pool of NARW blow samples collected in 2015.

2c. *Development of a fecal aldosterone assay.* This experiment was fully completed in FY2014; see Task 3 for progress on the associated manuscript.

2d. *Assaying new blow and fecal samples for stress and reproductive hormones*.
Hormone assay of blow samples are approximately 70% complete. Immediately after the conclusion of our Cape Cod Bay fieldwork in early May, steroid and thyroid hormones were extracted from all 100 blow samples using an ethanol-rinse method that had been previously tested in FY2014 (see FY2014 Annual Report for technical details). By late May all extractions had been completed and we then shifted focus to dilutions and hormone assays. Assays for progesterone, testosterone, estradiol, cortisol, aldosterone and tri-iodothyronine began in June and are still underway. We prioritised progesterone, testosterone and cortisol for immediate analysis, as these key hormones are most likely to provide information on reproductive state and stress responses in whales. Progesterone (EIA kit #K025-H1, Arbor Assays) and testosterone (EIA kit #K032-H1, Arbor Assay) have been validated for NARW blow samples, and all collected blow samples (n = 100) have been analysed for these hormones (8 assays conducted for progesterone and 5 assays conducted for testosterone in FY2015). The analysis of cortisol (EIA kit #K036-H1, Arbor Assays) in currently in progress, although sensivitity of the chosen assay has been an issue (i.e., many samples are below detectability). In FY2016 we may consider testing a different cortisol assay with improved sensitivity at low concentrations. Remaining hormones will be analyzed stepwise in a priority order (estradiol, then T3, then aldosterone) to ensure that there is enough sample remaining to conduct the assays.

Hormone assays of fecal samples are approximately 80% complete. In FY2015, all fecal samples collected and archived during earlier year were freeze-dried, sifted, extracted and assayed; these include three samples collected in 2012, four from 2013, and three from 2014. These ten samples were freeze-dried, extracted and assayed for six hormones: progestins, androgens, estrogens, glucocorticoids, mineralocorticoids (e.g. aldosterone and its fecal metabolites) and thyroid hormones. Fecal samples collected in FY2015 (to date, n = 3) are presently freeze-drying and will be assayed in early FY2016.

Task 3: Data Analysis, Manuscript Preparation, and Reporting
We are presently focusing on two papers on the work completed in FY2014, as well as ongoing data analysis of the hormone assay data generated in FY2015; see Results for details.

RESULTS

Task 1: Fieldwork for Blow Sample Collection
Our field effort in Cape Cod Bay was greatly successful. We collected 100 blow samples and three fecal samples from NARW during eight days at sea (66 hours of survey effort). Both sampler types were used, with 74 samples collected with dishes and 26 with nitex. Several individual NARW were sampled multiple times, which will allow assessment of inter-individual hormone variation in samples collected on different dates or with different sampler types. Sample collection rates are given in Table 1.

Table 1. Numbers of blow and fecal samples collected in Cape Cod Bay in 2015 by R/V Callisto.

Date (2015)	Blow samples	Fecal samples
April 6	0	
April 12	1	
April 16	13	
April 18	13	2
April 26	23	
April 27	9	
April 30	30	1
May 02	11	
Totals	100	3

Overall, blow samples were collected from 65% of approached whales for blow sampling and 51% of all whales photographed on survey. This is a relatively high rate that indicates that there is a good probability of obtaining a blow sample from any given whale. Photographs for individual identification were also taken for every whale sampled for blow; and catalogue matching to obtain information on the sex, age and life history of the whale is underway. Preliminary match results were obtained in September 2015; at least 79 of our 100 samples are from known individual NARW and at least 36 separate individuals are represented, including both sexes and a variety of age classes.

All 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. 2014). 17% of samples were given a "fair" quality score of 1 (n = 17); 17% of samples were given a "good" quality score of 2 (n = 17); and 66% of samples were given the "excellent" quality score of 3 (n = 66). No samples were given a quality score of zero.

The blow-sampling success in Cape Cod Bay is particularly notable not only for high sampling rate, but also because many samples were collected when NARW were skim-feeding or sub-surface feeding. Blow sampling has not been attempted before when NARW are behaving in this way.

Task 2: Labwork

2a. *Sampler testing for blow collection.* This experiment was fully completed in FY2014; see Task 3 for progress on the associated manuscript.

2b. *Development of an internal control for blow.*

As review, in FY2014 we conducted initial testing of urea as a potential "internal control" for blow samples, with successful testing of two urea kits (urea BUN kit #K024-H1, Arbor Assays; and QuantiChrom™ urea assay kit #50-489-225, Bioassay Systems). The Arbor Assays kit was further tested by assaying 19 individual archived NARW blow samples (n = 15 rinse samples and 4 droplet samples. Parallelism was good and urea was detected in all samples, providing evidence that urea may hold promise as an internal control (see FY2014 Annual Report for details).

In FY2015 we focused on further testing of urea as a possible internal control. To date, results show that urea is detectable in NARW blow samples collected using either dish and nitex mesh samplers. However, we found that samples need to be filtered prior to conducting the urea assay to remove turbidity that will distort assay results. We also tested whether it was feasible to perform the urea assay at 60% volume; thereby, allowing a more conservative use of sample (i.e., 60 ul of sample used rather than 100 ul); this approach was successful and detectability was good. Furthermore, we added an extra standard to the protocol in order to increase the assay accuracy at low-volume concentrations. We now have a rigorous protocol for analyzing urea, and have proceeded to assayed NARW blow samples for urea. Thirty-nine of the 100 blow extracts from the 2015 field season have so far been analyzed, with this task continuing through September and into early FY2016.

We also tested another potential control, albumin, with kit #30-6330, Alpco. Unfortunately, albumin was not detected in NARW blow samples and failed parallelism. Due to the promising successful of urea as an internal control, we have not continued any further testing with albumin.

2c. *Development of a fecal aldosterone assay.*

This experiment was successfully completed in FY2014.

Task 3: Data Analysis, Manuscript Preparation, and Reporting

Review of noninvasive physiological research on North Atlantic right whale. A review paper on development of noninvasive methodology for NARW, including blow sampling and fecal sampling, was published in *Integrative & Comparative Biology* in FY2015. A talk on the same topic was presented by the lead PI (Dr. Hunt) at the Annual Meeting of the Society for Integrative and Comparative Biology (January 3-7, 2016, West Palm Beach, FL).

Sampler-testing paper. A detailed manuscript on this experiment has been completed by lead author Dr. Burgess and was submitted to *Conservation Physiology*, a peer-reviewed scientific journal, in September 2015 (Burgess et al., 2015). During FY2014 abstracts on this experiment were also submitted, and accepted, for both the 5th Conference of the International Society of Wildlife Endocrinology (October 2015, Berlin, Germany), and the 21st Biennial Conference of the Society for Marine Mammalogy (December 13-18, 2015, San Francisco, CA).

Fecal aldosterone paper. This paper is approximately 50% completed and will be submitted to a peer-reviewed scientific journal in FY2016. Results have also been reported at the North Atlantic Right Whale Consortium meeting in November 2014, and an abstract has been submitted for the Annual Meeting of the Society for Integrative and Comparative Biology (January 3-7, 2016, Portland OR).

Blow sample analysis from free-swimming whales. Hormone and urea analyses on field-collected blow samples is expected to result in a fourth paper in FY2016.

Work to be completed in FY2016
In the final year of this grant, we will complete the last labwork (all remaining hormone assays and urea assays on all blow samples and the last fecal samples), and will perform final data analysis of blow hormones, with particular attention to the potential use of urea as an internal control. Data analysis will therefore focus on the question of whether using urea as a denominator (i.e., expressing hormone concentrations as ng hormone per mg urea) results in improved correspondence of hormone patterns to the known age, sex and reproductive state of photo-identified NARW. Our final goal by the end of this grant is to complete drafts of the peer-reviewed manuscripts described above.

IMPACT/APPLICATIONS

During earlier years, we completed the first rigorous, controlled analysis of hormone recovery from respiratory sampling devices; to our knowledge nobody has performed these validations before for any hormone for any respiratory sampling device in whales. We have also demonstrated that fecal mineralocorticoid (e.g. aldosterone) is present and measurable in right whale feces, and that it shows significant differences between different ages, sex, and reproductive states. Aldosterone may therefore be a useful complement to existing measures for assessing acute and chronic stress in whales. We are now writing and disseminating this data. During FY2015 we have demonstrated that blow-collecting at sea is feasible in a different field site and with different whale behaviors than previously attempted (skim-feeding, subsurface feeding). We have also discovered that urea is detectable in whale blow and may be provide an analytic method to control for the highly variable water content of blow samples. The work described here may add to the tools needed to evaluate the physiologic consequences of different noise levels on one species of baleen whale, and the hormone assays and field methodology tested here are likely to be useful with other whales and other marine species as well.

RELATED PROJECTS

The New England Aquarium's Ocean Health and Marine Stress Program includes a related ONR-funded project titled "*Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas"* (R. Rolland, PI; ONR # N000141110540). The goal of that project is to develop and validate fecal hormone assays to assess stress responses in Blainville's beaked whales (*Mesoplodon densirostris*) and sperm whales (*Physeter macrocephalus*) inhabiting the northern Bahamas.

REFERENCES

Amaral RS. 2010. Use of alternative matrices to monitor steroid hormones in aquatic mammals: a review. *Aquatic Mammals* 36:162-171.

Burgess EA, Hunt KE, Kraus SD, Rolland RM. Get the most out of blow hormones: validation of sampling materials, field storage, and extraction techniques for whale respiratory vapor samples. *Conservation Physiology,* submitted 28 September 2015.

Hunt KE, Rolland RM, Kraus SD, Wasser SK. 2006. Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*) using high-performance liquid chromatography (HPLC). *General and Comparative Endocrinology* 148:260-272.

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.

Hunt KE, Rolland RM, Kraus SD. 2014. Detection of steroid and thyroid hormones via immunoassay of North Atlantic right whale (*Eubalaena glacialis*) respiratory vapor. *Marine Mammal Science* 30(2):796-809.

Hunt KE, Rolland RM, Kraus SD. 2015. Conservation physiology of an uncatchable animal: the North Atlantic right whale (*Eubalaena glacialis*). Integrative and Comparative Biology. doi: 10.1093/icb/icv071.

Office of Naval Research. 2010. Final Workshop Proceedings, "Effects of Stress of Marine Mammals Exposed To Sound", Arlington, VA; meeting dates 4-5 November 2009.

Rolland RM, Hunt KE, Kraus SD,Wasser SK. 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. *General and Comparative Endocrinology* 142:308-317.

Rolland RM, Parks SE, Hunt KE, Castellote M, Corkeron PJ, Nowacek DP, Wasser SK, Kraus SD. 2012. Evidence that ship noise increases stress in right whales. *Proceedings of the Royal Society B* 279:2363-2368.

PUBLICATIONS

Burgess EA, Hunt KE, Kraus SD, Rolland RM. Get the most out of blow hormones: validation of sampling materials, field storage, and extraction techniques for whale respiratory vapor samples. *Conservation Physiology,* submitted 28 September 2015. [refereed]

Hunt KE, Rolland RM, Kraus SD. 2015. Conservation physiology of an uncatchable animal: the North Atlantic right whale (*Eubalaena glacialis*). Integrative and Comparative Biology. doi: 10.1093/icb/icv071. [published, refereed]