# Development of Respiratory Sampling to Assess Stress Responses in North Atlantic Right Whales

Kathleen E. Hunt (Ph.D.), Rosalind M. Rolland (D.V.M.), & Scott D. Kraus (Ph.D.)
New England Aquarium
Central Wharf
Boston MA 02110
phone: (617) 226-2175 fax: (617) 973-0242 email: huntk@neaq.org

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## LONG-TERM GOALS

Our main long-term goal is to assess whether respiratory sample ("blow") analysis can be developed as a practical method of physiological stress assessment in free-living cetaceans, particularly baleen whales. Few methods exist for assessment of physiological stress levels of baleen whales (Amaral 2010, Office of Naval Research 2010). Blow samples can be collected from targeted individuals and from large numbers of whales, and the method is non-invasive and can be employed multiple times on known individuals. Blow analysis may offer a novel method for assessment of short-term stress (minutes/hours) in cetaceans, and could complement existing fecal-sampling methods (Rolland et al. 2005, Hunt et al. 2006).

## OBJECTIVES

Our objective in FY2011 was to develop and test a practical field methodology for collecting respiratory samples from large baleen whales. In FY2012 our primary objective was to develop and test laboratory techniques appropriate for blow sample hormone analysis, focusing on the following questions: 1) do blow samples contain detectable levels of the primary hormones of interest for stress assessment studies (e.g., glucocorticoids, thyroid hormones, androgens, estrogens, and progestins), 2) can low-cost immunoassay techniques be validated for use with blow samples, and 3) does blow hormone content accurately reflect the internal physiological state of the whale.

## APPROACH

Our approach in FY2011 involved construction of a carbon-fiber pole and associated boat mount; design, testing and construction of sample collectors; and field testing with a well-studied population of North Atlantic Right Whales (NARW) in the Bay of Fundy during August and September, 2011. In FY 2012, the blow samples collected in 2011 were used to test laboratory analytic techniques, including: (1) sample processing and concentration methods; (2) investigation of sample storage temperature and storage time; (3) validation of immunoassays for cortisol, corticosterone, thyroxine (T4), tri-iodothyronine (T3), testosterone, progesterone, estrone, and estradiol; (4) using the best-performing of the tested assays, analysis of all samples for up to five hormones; and (5) preliminary

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analyses of resulting endocrine data for correlations with known state of individual NARW. This work was conducted at the Marine Stress Laboratory of the New England Aquarium with the involvement of PI Kathleen Hunt, Ph.D., Co-PI Rosalind Rolland, D.V.M., and Co-PI Scott Kraus, Ph.D.

## WORK COMPLETED

### *Task 1: Testing Field Collection Techniques for Respiratory Sampling*

In FY2011 we designed and constructed a 32' carbon-fiber pole and boat mount for our 7.6m research vessel *Callisto*, and developed a sampler consisting of $2m^2$ of nylon veil wrapped around a plastic bottle at the end of the pole. We extensively tested this system with NARW in the Bay of Fundy during August-September 2011. We also briefly tested an alternative system of a plexiglass plate sampler. (See FY2011 Annual Report for details.) All samples received a "boat quality score" from 0-3 (0=poor, 3=excellent) based on apparent proximity of the sampler to the blowholes, and also a "lab quality score", also from 0-3, based on volume of pipettable sample obtained the evening of collection.

### *Task 2a: Laboratory Validations - Concentration & Recovery of Sample*

*Sample processing and concentration.* Many samples produced two types of droplets: "fresh droplets" that were visible the evening of collection, and additional "spun droplets" recovered later by centrifuging the nylon veil in the lab. In FY2012 we devised a novel method of obtaining additional sample by rinsing every veil (after "spun droplets" were recovered) with 100mL ethanol, drying down the ethanol, and resuspending in 1.0mL of distilled water. This is referred to as the "rinse". Many samples produced all three types of subsamples: fresh droplets, spun droplets and rinses. Where sample volume was large enough, these subsamples were retained separately for later comparison of cortisol content, in order to identify the best method of sample processing for blow hormone analysis. We also created a pool from the ten largest "fresh droplet" samples to test whether water can be removed from samples by freeze-drying or centrifugation (e.g. separation of a lipid or mucoid layer).

*Recovery of known added hormone.* To test whether rinsing nylon veils with ethanol is an effective method of recovering small volumes of hormone, we sprayed 1.0mL of three different hormone solutions (containing different ratios of cortisol and testosterone) onto six nylon veils, rinsed the veils as described above, and assayed the rinse for cortisol and testosterone. Results were analyzed for (1) percentage of hormone recovered, and (2) observed vs. expected cortisol:testosterone ratio. We also tested two veils that received zero doses (distilled water) as negative controls, to test whether the nylon fabric affects the hormone assays. We did not test percentage-recovery of extraction methods as originally planned, since it became apparent that assays were performing well with unextracted samples (see Results). Therefore all subsequent assays used unextracted samples.

*Validating hormone assays.* Using a pool created from the ten largest "fresh droplet" samples, we first tested parallelism (of serially diluted sample to the standard curve; Diamandis & Christopoulos 1996) for three different glucocorticoid assays: a cortisol enzyme immunoassay (EIA), a cortisol radioimmunoassay (RIA), and a corticosterone RIA. The best-performing of these three assays was later tested for parallelism with rinse samples as well. Upon discovering that rinse samples have much higher hormone content than droplet samples (see Results), subsequent parallelism tests used rinse samples exclusively. We tested parallelism with rinse samples in a further seven assays, including: a testosterone EIA, two progesterone assays (a 3H RIA and an EIA), an estrone EIA, and estradiol EIA, and two thyroid-hormone assays (total-T4 and T3, both RIAs). In total we were able to perform 11 parallelism tests, six more than we originally planned and covering all major hormones of interest and several types of immunoassay. Using the remaining rinse sample, we then tested assay accuracy (e.g.

spiking a set of known-dose standards with pooled sample; Diamandis & Christopoulos 1996). Since accuracy tests require a great deal of sample, we tested accuracy for the cortisol, progesterone, and testosterone EIAs only.

***Task 2b: Testing Effects of Storage Temperature and Storage Time***
Five samples had sufficient volume of "fresh droplets" to allow subdivision between -20°C and -80°C storage temperatures, followed by analysis for cortisol after 6 mos in storage. Only two samples had sufficient volume of fresh droplets to perform additional tests for storage time (since most sample was used for the assay validations described in Task 2a). Therefore the storage time test was limited to comparing these two samples at 6 mos and again at 9 mos; this should be regarded as a pilot test only.

***Task 2c: Assaying Individual Blow Samples for Stress and Reproductive Hormones***
After analyzing parallelism results and selecting the most appropriate assays, we assayed all rinse samples for cortisol, T3, testosterone, estrone, and progesterone. The largest droplet samples were also assayed for cortisol and for T3.

***Task 3: Data Analysis and Reporting***
During FY2012, photo-identification was completed for all NARW from which we obtained samples. Data analyses for all methodological comparisons described above have been completed. Final analysis of endocrine hormone content in relation to known state of individual NARW will focus on analysis of hormone data from samples that received high quality scores of 2 or 3; these analyses are underway and will be completed in FY2013 under a no-cost extension. Manuscript preparation and presentation at a national scientific meeting will also occur in FY2013 under the no-cost extension.

**RESULTS**

***Task 1: Testing Field Collection Techniques for Respiratory Sampling***
In FY2011 we successfully collected 55 samples during 7 days at sea. (For details, see our Annual Report for FY2011). Boat quality scores were significantly correlated with lab quality scores (Pearson correlation, P=0.0051), and thus in subsequent analyses involving sample quality, we used only the lab quality scores. 100% of our samples proved to be from individually known whales that could be linked to the North Atlantic Right Whale catalog: 23 samples were from adult males, 9 from lactating females, 5 from "resting" (adult, not pregnant, not lactating) females, 1 from an adult of unknown sex, 16 from juveniles, and 1 from a calf. We did not sample any pregnant females in 2011. Eleven individuals were sampled multiple times, though in only in one case (a lactating female) were both samples of high quality (lab quality score of 2 or 3). Overall, these results indicate that our sample collection technique is practical and feasible. The fact that 100% of our samples were from known, photo-identified individuals is notable, and underscores the fact that NARWs are an ideal population with which to develop novel techniques that require identification of known individual cetaceans.

***Task 2a: Laboratory Validations***
*Sample processing and concentration.* The majority of "fresh droplet" and "spun droplet" samples were between 10-200uL in volume, with a few samples over 1000uL. In FY2011 we determined that samples are primarily aqueous, and that neither freeze-drying nor centrifugation produces any visible pellet and or lipid/mucoid layer. It was also apparent from hormone concentrations and sample volume that samples vary widely in water content, presumably due to variable water vapor content of the blow (strongly affected by ambient air temperature) and seawater contamination. It is our opinion that

development of an appropriate "internal control" to correct for variable water content (e.g. a substance secreted at constant rate into lung exudate) should be a high priority for future study.

All three types of samples - fresh droplets, spun droplets, and rinses - contained detectable hormone. However, the concentration of hormone varied markedly in these three types of samples. In cases where subsamples could be assayed separately, fresh droplets always had the lowest cortisol content, spun droplets had higher cortisol content (median=85% higher than fresh droplets), and rinses always had by far the greatest cortisol content (median = 4.6x higher than fresh droplets). Rinse samples were more likely to have detectable hormone, and also produced the largest sample volume (1.0mL, sufficient for testing of least 10 analytes). We interpret these patterns to indicate that the oily, surfactant-rich portion of whale blow (presumably containing the steroid and thyroid hormones) adheres to the sampler fabric and is best recovered with a rinse with an appropriate solvent. Based on these results, subsequent analyses concentrated on the "rinse" samples.

*Recovery of added hormone.*
Testing of ethanol rinses of hormone-spiked veils indicated that an average of 65% of added testosterone and 89% of added cortisol is recovered in the veil rinse. The observed ratios of cortisol:testosterone matched the expected ratios closely. This indicates that the rinse method is an effective means of recovering and concentrating hormone for analysis. Furthermore, control veils had no detectable cortisol or testosterone, indicating that nylon fabric does not interfere with the assays. We recommend that similar validations be conducted with other hormones of interest.

*Validating hormone assays.*
We discovered that most immunoassays that have sensitivity into the pg/mL range are suitable for use with blow samples. For example, one cortisol assay with a sensitivity limit of approximately 1.0 ng/mL did not detect any hormone and showed no parallelism, but a different cortisol assay with a reported sensitivity of 17.3 pg/mL (0.0173 ng/mL) detected hormone in almost all samples and also passed the parallelism validation test. Parallelism graphs for this latter assay, for both droplet samples and rinse samples, are presented in Fig. 1 as a typical example of our parallelism results.

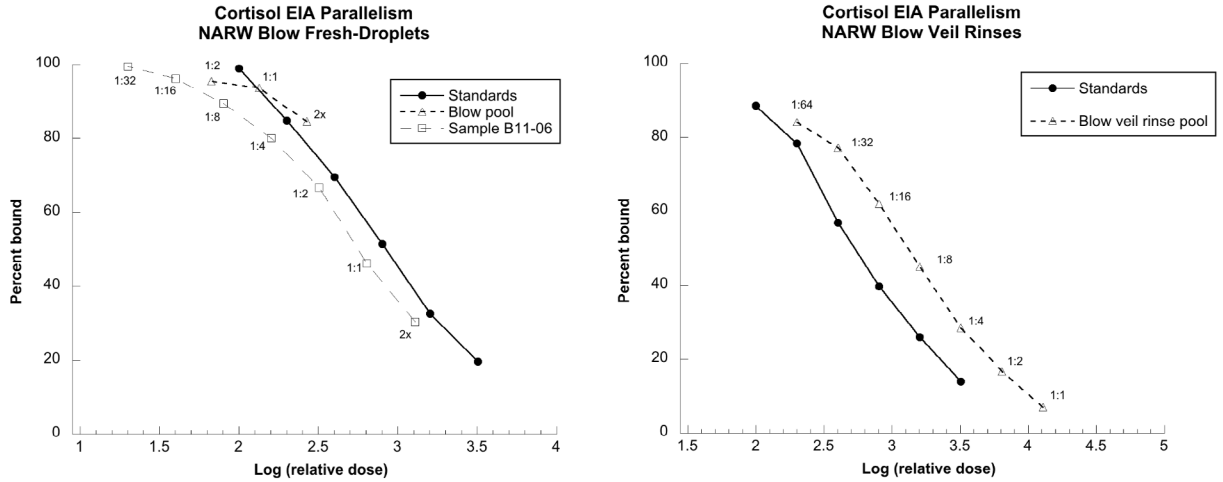


***Figure 1. Parallelism results for a cortisol EIA with "fresh droplet" samples (left) and veil rinses (right) from respiratory samples of North Atlantic Right Whales. Note close parallelism of the serially diluted samples to the standard curves; this indicates that the sample contains cortisol.***

In all, the following assays passed the parallelism validation test (indicating that the substance being measured is most likely the hormone of interest): cortisol (EIA), T3, T4, testosterone, progesterone (both assays), and estrone. (The corticosterone RIA and estradiol EIA failed parallelism, possibly due simply to lack of hormone, e.g. both these hormones are often low in mammalian plasma.) Finally, all three accuracy validations (testosterone, progesterone, and cortisol EIAs) were successful as well. The few previously published studies on blow hormones have used either liquid chromatography/mass spectroscopy (LC/MS; Hogg et al. 2005, 2009) or ultra-performance liquid chromatography (UPLC; Dunstan et al. 2012). These are both expensive methods that are out of reach for many marine-mammal investigators. Our results indicate that immunoassays are a viable, low-cost, alternative.

***Task 2b: Testing Effects of Storage Temperature and Storage Time***

There was no discernable effect of storage temperature or storage time on hormone content, though sample sizes were quite small for these tests. The five pairs of matched subsamples stored at -20°C and -80°C had very similar cortisol content after 6 mos in storage (paired t-test, P=0.9504). There was also no discernable change in cortisol content over time; of the two samples tested both at 6mo and 9mo, one exhibited a slight increase in cortisol content and the other a decrease, and in both cases the change was within the range of normal assay variation. We recommend these questions be further pursued with greater sample sizes in future studies, but the patterns seen so far are reassuring.

***Task 2c: Assaying Individual Blow Samples for Stress and Reproductive Hormones***

All rinse samples have been assayed for cortisol, T3, progesterone, testosterone, and estrone. Preliminary analyses of hormone data in relation to reproductive state indicate likely differences between the two reproductive categories for which we have the best sample size, adult males and lactating females. Final analyses will be completed in FY2013.

As previously noted, it was apparent from hormone assay results that samples vary widely in water content, as well as in how much blow vapor was captured by the sampler in the first place. For example, some samples had elevated levels of all hormones, while others had low levels of all hormones. The same phenomenon has been reported in analyses of human breath samples (e.g. exhaled breath condensate) for asthma research (Effros 2010). Asthma researchers are investigating several possible "internal controls" that could be used to correct for variable water content, e.g. substances that are present at relatively constant concentration in mammalian plasma and hence in mammalian breath vapor (Kazani and Israel 2010; Davis et al. 2012). As stated previously, we recommend that this approach should be a priority for future research on cetacean blow.

## IMPACT/APPLICATIONS

The second year of this project demonstrated that all hormones of interest for stress assessment are present and detectable in NARW blow samples, that these hormones can be successfully recovered and concentrated for analysis, and that low-cost immunoassays are a feasible technique for blow hormone analysis. Though many validations remain to be done, these preliminary results strongly support continued development of this technique as a novel method for obtaining physiological information from targeted, known individual whales. The work described here may add to the tools needed to evaluate the physiological consequences of different disturbance levels not only in right whales, but in many other large whale species as well.

## RELATED PROJECTS

As part of the New England Aquarium's Marine Stress Program, under separate ONR funding we now have a fully equipped endocrinology laboratory, we have expanded our panel of stress-related hormones to include thyroid hormones, and we have developed an alternative PCAD approach in right whales (Scott Kraus and Rosalind Rolland, PIs: ONR Award #N000141010614). A second project developing analytic techniques for stress-related hormones in fecal samples from sperm whales and Blainville's beaked whales is underway (Rosalind Rolland, PI; ONR Award #N000141110540).

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