Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas

Rosalind M. Rolland, D.V.M. (Lead PI)
Kathleen E. Hunt, Ph.D. (co-PI), Scott D. Kraus, Ph.D. (co-PI)
John H. Prescott Marine Laboratory
New England Aquarium
Central Wharf
Boston, MA 02110
phone: (617) 973-6587 fax: (617) 973-0242 email: rrolland@neaq.org

Diane E. Claridge, M.Sc., Charlotte A. Dunn, M.Res.
Bahamas Marine Mammal Research Organization
P.O. Box AB-20714
Marsh Harbour
Abaco, Bahamas
phone: (242) 366-4155 fax: (242) 366-4155 email: dclaridge@bahamaswhales.org

Award Number: N000141110540
http://tinyurl.com/MarineStress
http://tinyurl.com/RightWhaleConservationMedicine
http://www.bahamaswhales.org

LONG-TERM GOALS

The long-term goal of this project is to develop fecal hormone assays to assess stress responses in Blainville's beaked whales (*Mesoplodon densirostris*) and sperm whales (*Physeter macrocephalus*) inhabiting the northern Bahamas. These species were chosen to include a particularly acoustically-sensitive cetacean (beaked whales) and a co-occurring species (sperm whales) for comparison. The immediate goals are to determine baseline fecal hormone levels for un-disturbed reference populations of these two deep-diving whale species, and characterize the natural variations in stress-related hormones according to life history stage (age, sex, reproductive status). The results of this project will provide baseline levels of these hormones in beaked and sperm whales for comparison with those inhabiting the nearby U.S. Navy Atlantic Undersea Test and Evaluation Center (AUTEC), or other habitats with known acoustic exposures from man-made sounds.

OBJECTIVES

The objectives of this research project are to:

(1) In FY 2011 and 2013, conduct dedicated fecal sampling surveys for populations of beaked and sperm whales off southwest Great Abaco Island and refine methods to maximize sampling rates. Surveys are accompanied by concurrent photo-identification to identify individual, catalogued whales.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2012	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Assessing Stress Responses in Beaked and Sperm Whales in the Bahamas	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **John H. Prescott Marine Laboratory New England Aquarium Central Wharf Boston, MA 02110**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

(2) In FY 2012, validate immunoassays for fecal metabolites of reproductive hormones (estrogens, progestins, androgens), adrenal glucocorticoids (GCs) and thyroid hormone (tri-iodothyronine, T3) for beaked and sperm whales.

(3) In FY 2014, characterize baseline levels of five fecal hormones in beaked whales and sperm whales, and describe the natural variation according to life-history state (sex, age, reproductive state).

APPROACH

Our approach is to characterize baseline variation in stress-related fecal hormones in reference populations of beaked and sperm whales, as a method to ultimately determine whether anthropogenic noise exposures cause measurable physiological changes that can potentially lead to biologically significant effects on individuals and populations. This project is a collaboration between the New England Aquarium (NEAq) and the Bahamas Marine Mammal Research Organization (BMMRO). Year 1 (FY 2011) included one month of dedicated fecal sample collection from Blainville's beaked whales and sperm whales off Great Abaco Island in the Bahamas. Fecal sample collection was conducted by BMMRO scientists (D. Claridge, C. Dunn) with assistance from NEAq scientists (R.Rolland, K. Hunt, S. Kraus). In FY 2012, fecal samples from both species were used to develop methods for sample preparation and hormone extraction, and to conduct immunoassay validation studies for five hormones (estrogen, progesterone, testosterone, corticosterone and thyroid hormone). Samples from both species were assayed for all hormones, and the results will be analyzed in relationship to life history information (sex, age, reproductive state). This work was conducted in the Marine Stress Laboratory at the New England Aquarium (R. Rolland, K. Hunt, S. Kraus).

WORK COMPLETED

Task 1. Sample Collection from Beaked and Sperm Whales

Field work conducted in FY 2011 resulted in the collection of 10 samples from beaked whales and 9 samples from sperm whales off Great Abaco Island, Bahamas. In FY 2013, there will be an additional 30 days of dedicated fecal sample collection from beaked whales. In addition, we obtained archived (-80 °C) fecal samples (n=39) previously collected from both species in the same location (stored at BMMRO and at Woods Hole Oceanographic Institution), and evaluated them for appropriate preservation methods (i.e., no preservative) for fecal hormone assays.

Task 2. Radio-immunoassays for Fecal Hormones

A combination of newly collected (n=19) and archived fecal samples were used for these studies. Sixteen of the archived samples were appropriately stored, and were used for hormone assays including 3 from beaked whales, and 13 from sperm whales. Including both the newly collected and archived fecal samples, the total available for hormone analyses was 13 beaked whale and 22 sperm whale samples (n = 35).

Task 2a. Hormone Assay Validations

Fecal sample processing:

Two main issues were encountered related to fecal sample processing. In beaked whales, because feces were collected sub-surface in the water column, they were diffuse and sample mass was relatively low. In both species, samples contained a high volume (~100ml or more) of seawater relative to fecal weight (typically <1g). We therefore explored alternative extraction techniques and dilutions to

maximize hormone yield from small samples, and conducted a series of pilot tests to determine the best approaches to remove seawater, concentrate and extract the samples to prepare them for hormone assays.

1) Pilot test on filtration. Shortly after collection, a 60ml subsample of one beaked whale sample was poured through a quadruple layer of paper coffee filters. However, the filter did not retain any particles, and filtering was not pursued further.

2) *Pilot test on seawater removal.* Our original plan was to freeze-dry entire samples, but there was concern that the large volume of salt water collected with the fecal material might contribute a relatively large mass of dried salts and reduce apparent hormone concentrations. To test this possibility, subsamples from both beaked and sperm whales were fully dried down with the seawater, and the hormone content was compared to duplicate subsamples in which excess salt water was pipetted and/or centrifuged off the sample prior to freeze-drying. In both species fully dried samples (containing salt) had 23-31% lower apparent hormone content (for GCs and estrone-1-glucuronide) vs. spun/pipetted samples. Therefore, as much water as possible was removed from samples by pipetting and centrifugation prior to freeze-drying (Fig. 1).

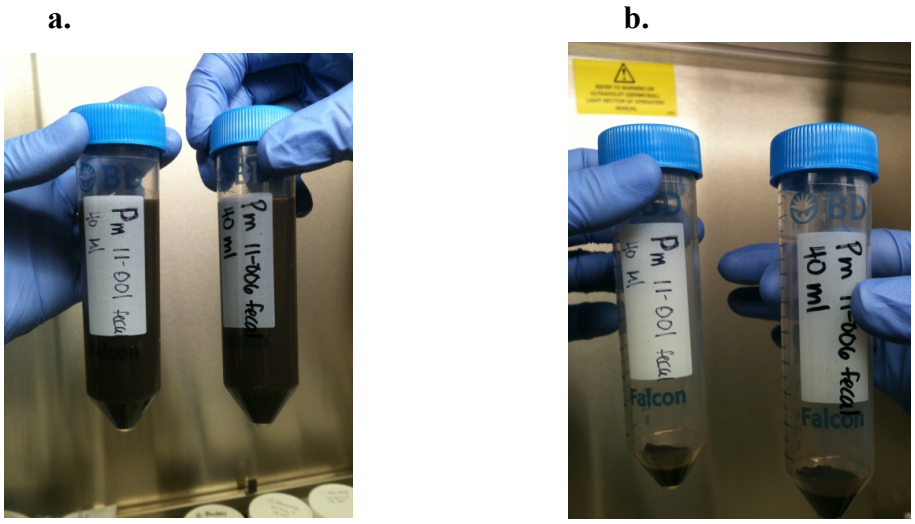

Figure 1. Processing of fecal samples for hormone assays. Samples collected from sperm whales before (a) and after centrifugation and decanting of water showing the concentrated fecal pellet used for hormone assays (b).

3) *Pilot test on extraction methods.* Median sample weight for beaked whales was just 0.0288g (range= 0.006-0.102g). In other vertebrates using a standard volume of solvent for both small (≤0.02g) and larger samples can cause variable hormone extraction efficiency, resulting in artificial inflation of hormone concentrations in smaller samples (Ayres et al. 2012; Hayward et al. 2010). To compensate for this small sample effect, we aimed for 100% extraction efficiency in all beaked whale samples, by using multiple rounds of extraction with 70% EtOH. This was based on reports that this solvent achieves higher extraction efficiency for thyroid hormone (T3, triiodothyronine), and acceptable extraction efficiency for steroids (Hayward et al. 2010; Wasser et al. 2010). The"small sample size" effect on hormone concentration was assessed for all hormones by checking for correlations of extraction weight with final hormone concentration (ng/g).

Sperm whale fecal samples were larger (median mass= 0.0845g) and did not present the small sample size issues. These samples were extracted with 90% methanol using a *consistent 20:1 ratio* of solvent (ml):dried feces (g) to adjust for variation in sample mass (rather than using a set volume of solvent for all samples).This ratio was greater than that used with right whale samples (10:1), because recent data indicates that higher solvent:fecal ratios have greater extraction efficiency (Wasser pers. comm., Hunt et al. unpub. data).

4) Pilot test of hormone content in seawater removed from samples. The large volume of salt water collected with samples raised the issue of possible migration of hormones from fecal material into water. Therefore, aliquots of seawater from all samples were assayed for all hormones: progestins, androgens, estrogens, glucocorticoids, and thyroid hormone. We tested parallelism for two hormone assays in water subsamples (glucocorticoids and estrone-1-glucuronide, E1G) to determine if detected immunoreactivity was due to hormone metabolites or to other compounds in the water.

Hormone Validation Studies:
Radio-immunoassays (RIA; 125-I and 3-H) for total-estrogens, progesterone, testosterone, corticosterone and T3 were validated for beaked and sperm whales using standard parallelism and accuracy studies (Diamandis and Christopoulos 1996). In addition, we conducted validations on two enzyme immunoassays (EIA; estrone-1-glucuronide and testosterone, R156/7). In total, including both species and water samples, 33 different assay validation tests were done.

Briefly, parallelism was tested by comparing slopes of a serial dilution of a fecal pool to the slope of the standard curve (log(dose) vs percent-bound (F test, Prism 5.0b for Macintosh). Good parallelism indicates that the fecal hormone is binding well to the antibody and that the assay can detect the hormone across a wide range of dilutions. Accuracy ("matrix test") was then tested by spiking a standard curve with equal volumes of a fecal pool. Acceptable results indicate that the assay correctly distinguishes low from high doses at a given dilution, and that there is no interference from other components in the fecal extract.

Task 2b. Hormone assays
Following validation studies, fecal samples (n=35) were assayed for all six hormones by immunoassay: total-estrogens, progesterone, testosterone, corticosterone, T3, and estrone-1-glucuronide (E1G). Radio-immunoassay methods are detailed in Rolland et al. (2005) and Hunt et al. (2006), and Wasser et al. (2010), with extraction modifications discussed above.

RESULTS

Sample processing
Using a consistent fecal:solvent extraction ratio eliminated the effect of smaller sample mass on hormone concentrations in sperm whales. Likewise, in beaked whales, there was no correlation of hormone level with extraction weight for progestins, androgens, glucocorticoids, or E1G. However, based on limited data, there was a significant trend towards ***lower*** fecal T3 levels in ***smaller*** samples. This is the reverse of the relationship noted by other researchers, who found ***higher*** hormone levels in small samples (Hayward et al. 2010). Additionally, progestins and androgens were usually non-detectable in samples weighing < 0.02g. Therefore, future field efforts should try to maximize the amount of fecal material collected to keep the sample mass ≥ 0.02g.

Migration of hormone metabolites from feces into seawater is occurring after sample collection. It is likely that water hormone represents polar metabolites, while nonpolar metabolites presumably stay in the fecal pellet. The important question was whether migration of fecal hormone to water affected observed patterns of fecal hormone concentrations. In sperm whales, the vast majority of the hormone (hundreds to thousands-fold more) stays in the fecal pellet, and water hormone content was low and insignificant. In beaked whales, a greater proportion of hormone appears to move into the water, but the fecal hormone concentrations are still much higher than the water concentrations, and there is a significant correlation between most fecal and water hormone concentrations, so relative hormone patterns appear unchanged. Nevertheless, these findings suggest that future sample collection should include the removal of as much excess water as quickly as possible following collection.

Validation studies

Validation studies were successful for the following five hormones in both species: progesterone, testosterone, total-estrogens, corticosterone, and T3. For example, Figure 2 shows the results of the corticosterone validations in beaked whales. Of the two novel EIAs tested, the E1G assay was validated successfully for both species, but detected much lower hormone concentrations (ng/g) than the total-estrogens assay, so we used both types of estrogen assays. The testosterone EIA failed parallelism for beaked whales, and based on this the testosterone 3H RIA was used for both species.

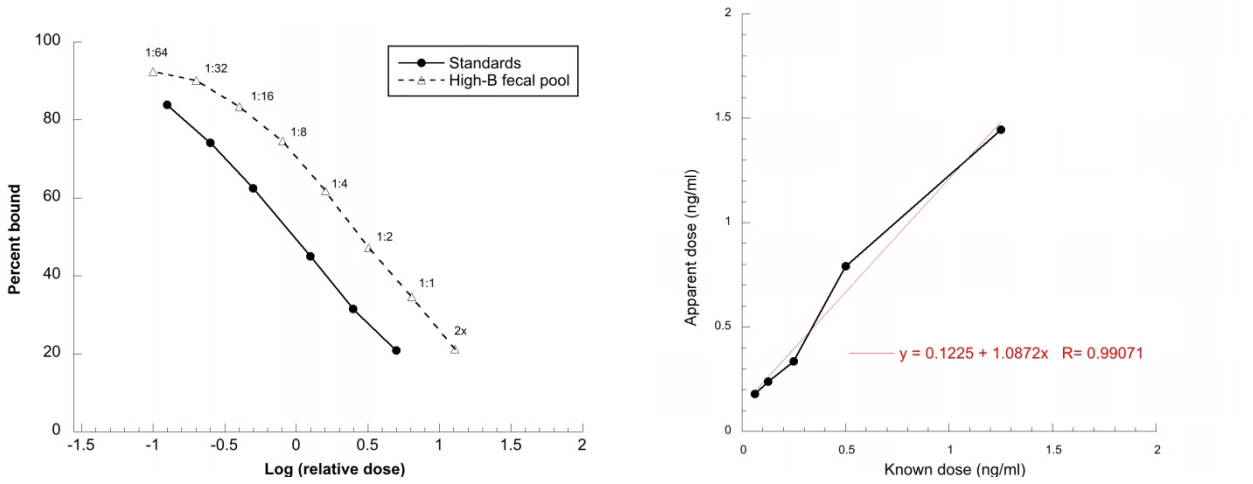

Figure 2. Successful validation studies for the corticosterone 125-I radioimmunoassay in beaked whales. The parallelism test shows that the fecal hormone is binding well to the antibody, and that the assay detected the hormone across a wide range of dilutions. The accuracy test indicates that there is no interference from other components in the fecal extract.

Hormone assays

We measured five different hormones (using six types of immunoassays) in fecal samples from both beaked and sperm whales. The biological relevance of these hormone assays can be inferred if hormone variation in different life history states concurs with known features of mammalian physiology, as has been described for North Atlantic right whales (Rolland et al. 2005; Hunt et al. 2006). While sample sizes are too small thus far for statistical characterization of hormone variation by life history stage, a few expected trends are already apparent. For example, in beaked whales, the highest fecal androgen level was in a mature male and the highest fecal progestin was in a mature

female. Although not many sampled sperm whales have been matched to known individuals at this time, the highest fecal progestin was also from a known mature female, and the variation in this hormone suggests that females in different reproductive states were sampled. Glucocorticoids were measurable in all samples from both species, and thyroid hormone was detectable in most samples with both hormones showing variability (Table 1). In beaked whales, GCs from an adult male and two lactating females were elevated relative to other whales, as has been described in N. Atlantic right whales (Hunt et al. 2006). A sample collected from a beaked whale carrying a LIMPET tag for over 1 month (Md 139) did not show an appreciable increase in GCs (18.4 ng/g) above the median (see Table 1). Further demographic characterization and interpretation of these preliminary hormone results requires larger sample sizes from whales of different sexes and life history stage.

Table 1. Fecal glucocorticoids and thyroid hormones (T3) in Blainville's beaked whales and sperm whales sampled in the northern Bahamas.

	Glucocorticoids (ng/g)			Thyroid hormones, T3 (ng/g)		
	n	median	range	n	median	range
Beaked whale	13	15.1	2-53	10	17.9	8-118
Sperm whale	22	32.1	8-78	19	9.9	3-30

IMPACT/APPLICATIONS

The second year of this project demonstrates that multiple hormones can be successfully measured in fecal samples from both Blainville's beaked whales and sperm whales. The preliminary results support the use of these hormone assays to monitor physiological status and assess relative levels of stress-related hormones in these species. These analyses can provide information on how acoustic (and other) stressors affect these species, which will be critically important for evaluating operational and management options in different habitats.

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, expanded our panel of stress-related hormones to include thyroid, and developed an alternative PCAD approach in right whales (Scott Kraus and Rosalind Rolland, PIs: ONR Award# N000141010614). A second project conducted preliminary studies on the detection and use of hormone data from right whale respiratory exudate (Kathleen Hunt, PI; ONR Award# N000141110435) .

REFERENCES

Ayres K.L., Booth, R.K., Hempelmann, J.A., Koski, K.L., Emmons, C.K., Baird, R.W., Balcomb-Bartok, K., Hanson, M.B., Ford, M.J., Wasser, S.K. 2012. Distinguishing the impacts of inadequate prey and vessel traffic on an endangered killer whale (*Orcinus orca*) population. *PLoS One* 7(6), e36842.

Diamandis, E.P., and Christopoulos, T.K. (Eds). 1996. Immunoassay. Academic Press, New York, NY.

Hayward, L.S., Booth, R.K., and Wasser, S. K. 2010. Eliminating the artificial effect of sample mass on avian fecal hormone metabolite concentration. *General and Comparative Endocrinology* 169:117-122.

Hunt K.E., Rolland R.M., Kraus S.D., and Wasser S.K. 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.

Rolland, R.M., Hunt, K.E., Kraus, S.D. and Wasser, S.K. 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. *General and Comparative Endocrinology* 142:308-317.

Wasser SK, Hunt KE, Brown JL, Cooper K, Crockett CM, Bechert U, Millspaugh JJ, Larson S, and Monfort SL. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of non-domestic mammalian and avian species. *General and Comparative Endocrinology* 120:260-275.

Wasser, S.K., Azkarate, J.C., Booth, R.K., Hayward, L., Hunt, K., Ayres, K., Vynne, C., Gobush, K., Canales-Espinosa, D., and Rodriguez-Luna, E. 2010. Non-invasive measurement of thyroid hormone in feces of a diverse array of avian and mammalian species. *General and Comparative Endocrinology* 168:1-7.