

**Genetic Analysis of Stock Structure, Species Identification and Sex
Determination for Marine Mammal Biopsies and Strandings**

Prepared By:
Dr. Patricia Rosel
National Marine Fisheries Service
Southeast Fisheries Science Center
Patricia.rosel@noaa.gov

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Approval of this work plan is for the purposes of obtaining data for the Natural Resource Damage Assessment. Each party reserves its right to produce its own independent interpretation and analysis of any data collected pursuant to this work plan.

The trustees have developed a preliminary conceptual model of the DWH release, potential pathways and routes of exposure, and potential receptors. This preliminary model has informed the trustees' decision to pursue the studies outlined in the work plan. By signing this work plan and agreeing to fund the work outlined, BP is not endorsing the model articulated in the work plan.

Robert W. Ficker July 26, 2011
Department of Commerce Trustee Representative Date

[Signature] 8/11/2011
Louisiana Trustee Representative Date

[Signature] 7/29/2011
BP Representative Date

Background

The northern Gulf of Mexico, including estuarine, coastal and offshore waters, is a high-use habitat for many marine mammal species. Common bottlenose dolphins, *Tursiops truncatus*, inhabit the bays, sounds and estuaries, nearshore coastal waters, continental shelf waters and waters over the continental slope. Within the western North Atlantic and Gulf of Mexico there are two recognized ecotypes (also often called morphotypes) of bottlenose dolphins (Mead and Potter 1995) – a larger “offshore” type that inhabits deeper shelf and continental waters and a smaller “coastal” type that inhabits shelf, coastal and bay sound and estuary waters. Within the Gulf of Mexico, there are 37 stocks of bottlenose dolphins – 1 oceanic, 1 continental shelf, 3 coastal and 32 bay sound and estuary stocks (Waring et al. 2009). The oceanic stock consists of animals of the “offshore” ecotype, while the coastal (between the beach and the 20 m isobath) and bay, sound, and estuary stocks are of the “coastal” ecotype. A mix of both ecotypes is present in deeper waters of the continental shelf. The 32 bay, sound and estuary stocks are generally comprised of small numbers of individuals that show strong site fidelity to particular estuarine areas (Waring et al. 2009). In addition to bottlenose dolphins, the shelf and offshore waters each harbor their own unique suite of cetacean species that are adapted to the different ecological and oceanographic conditions of these regions.

Three Natural Resources Damage Assessment (NRDA) work plans address potential exposure and injury to cetaceans resulting from discharges from the Deepwater Horizon (DWH) event in the Gulf of Mexico:

- 1) a large-vessel oceanic cruise to examine potential impacts on endangered sperm whales, Bryde’s whales and other oceanic species took place from June to August 2010 and a second large-vessel cruise in October-November 2010 focused on Bryde’s whales and sperm whale prey (“Work Plan for the Collection of Data to Determine Impacts of the Deepwater Horizon Mississippi Canyon 252 Incident on Endangered and Protected Marine Mammals in the Northern Gulf”);
- 2) small-boat based photo-identification and biopsy efforts to assess possible injury to estuarine populations of bottlenose dolphins in Barataria Bay, LA, Chandeleur Sound, LA, Mississippi Sound, MS and St. Joseph Bay, FL started in May 2010 and are still on-going (“Proposed Data Collection Plan to Assess Injury to Louisiana and Mississippi Estuarine Dolphin Stocks”);
- 3) a dolphin health assessment plan “Assessing Sublethal and Chronic Health Impacts from the Mississippi Canyon 252 Oil Spill on Coastal and Estuarine Bottlenose Dolphins” which will collect tissue biopsy samples from live bottlenose dolphins in August 2011¹.

In addition, approximately 119 cetaceans have stranded dead within the spill area between April 20, 2010 and November 2, 2010. This proposed plan will cover genetic analyses of both the tissue samples collected under the NRDA work plans in the Gulf of Mexico and samples from

¹ This study includes two sites – Barataria Bay, LA and Sarasota, FL; however, biopsy samples from the Sarasota, FL site will not be included in this plan because stock structure analysis has already been performed at this site (see Sellas et. al., 2005).

cetacean strandings that have been collected in the event area between April 20, 2010 and November 2, 2010, as well as additional samples collected from neonates (which for bottlenose dolphins refers to animals < 115cm) and stillborn cetaceans and any visibly oiled cetaceans or pregnant females that have stranded between November 2, 2010 and May 30, 2011.

Project Description

This plan addresses molecular genetic analysis of cetacean samples collected during remote biopsy and capture-release projects as described in the three NRDA work plans described above, and from stranded animals. Specifically, stock structure analysis will be conducted for biopsy samples (remote and capture-release) collected from live common bottlenose dolphins, *Tursiops truncatus*, in coastal/estuarine waters of Barataria Bay, LA, Chandeleur Sound, LA, Mississippi Sound, MS, and St. Joseph Bay, FL. Inclusion of all four of these sites will improve assessment of overall stock structure in the northern Gulf of Mexico, and will thereby improve ability to assess potential injury to local populations. This work will entail DNA sequencing of a portion of the mitochondrial DNA (mtDNA) control region, collection of data from nuclear microsatellite loci, and molecular sexing. Biopsies collected from live cetaceans on the large-vessel cruises in summer and fall 2010 will also be sexed and sequenced. Finally, the cetaceans that have stranded in the spill zone between April 20, 2010 and November 2, 2010 (n≈119) as well as stranded neonates and stillborn calves and any pregnant females and visibly oiled animals that have been sampled between November 2, 2010 and May 30, 2011 (~96) will also be analyzed for species/ecotype verification/identification (see below for details). The information gained by this work will help delineate the stocks potentially exposed, confirm species identifications for sampled animals, and provide sex identification for biopsy samples.

Objectives

The objectives of the proposed study are to:

- Use molecular techniques to determine the sex of all remotely biopsied animals and of stranded animals that were not sexed in the field via morphological methods.
- Use mitochondrial DNA control region sequence data to verify species for remote biopsies collected during large vessel surveys and for stranded cetaceans and, for bottlenose dolphins, determine whether they are of the offshore or coastal ecotype.
- Collect mtDNA control region sequence data and microsatellite data to examine stock structure of estuarine bottlenose dolphins sampled in Barataria Bay, LA, Chandeleur Sound, LA, Mississippi Sound, MS, and St. Joseph Bay, FL.

Table 1. Summary of planned genetic analyses for cetacean samples collected during large vessel surveys, strandings, and for bottlenose dolphin samples collected from small-boat biopsy effort and health assessment in estuarine stock areas (Barataria Bay, Chandeleur Sound, Mississippi Sound, St. Joseph Bay)

Analytical Test	Biopsies collected on Large Vessel Surveys	Strandings	Bottlenose dolphin biopsies from health assessment and small-boat work
Sex	X	X (if needed)	X (except live capture samples)
Species Identification	X	X	
If <i>Tursiops</i> , ecotype identification	X	X	X
Population Structure			X

Methods

Sexing:

Unless accompanied by a calf, it is difficult to determine the sex of free-ranging cetaceans in the field. DNA will be extracted from biopsy samples using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), and used to determine the sex using molecular sexing techniques (Rosel 2003). Knowledge of the sex of each sample is integrated into the genetic analyses to test for evidence of sex-based dispersal rates among populations. The sex of biopsied animals will also be important to interpretation of data collected under other work plans. For example, interpretation of movements of whales is enhanced by knowledge of the sex of the tagged animals. Where sex of a stranded animal is not determined during recovery, molecular sexing will be performed.

Species identification:

Identification in the field of some cetacean species can be quite challenging, but genetic markers can be used to identify the species of an animal based on the sample collected. Species identifications of biopsy (remote) and stranded samples will be verified through sequencing of a portion of the mtDNA control region. This gene region is commonly used for species identification in cetaceans (Ross et al. 2003) and can also discriminate between the offshore and coastal ecotypes of bottlenose dolphins in the Gulf of Mexico. Genetic identification of all biopsy samples collected during the large vessel cruises will provide an accurate accounting of the species encountered during these cruises. The majority of stranding samples are bottlenose dolphins but it is difficult to determine from external features whether they are of the coastal or offshore ecotype (see Waring et al. 2009 for description of ecotypes). Analysis of the stranded bottlenose dolphin samples is aimed primarily at determining the ecotype of each animal. Knowing whether the stranded bottlenose dolphins came from nearshore waters or waters further offshore will be important to assessing which populations may have been impacted. All non-bottlenose dolphin stranded samples will also have species identification tests run.

Stock structure of estuarine populations of bottlenose dolphins:

For marine mammals in US waters, stocks are defined by the Marine Mammal Protection Act of 1972 as "a group of marine mammals of the same species or smaller taxa in a common spatial arrangement that interbreed when mature." To evaluate the degree of reproductive isolation that exists between stocks, an understanding of the degree of genetic exchange (gene flow or interbreeding) is required. Tagging and photo-identification studies inform about movements of individuals. They do not, however, provide information on breeding and genetic interchange, the cornerstones of stock delineation for cetaceans. Mitochondrial DNA (mtDNA) has proven an effective marker for stock discrimination in marine mammals. Analysis of mtDNA data provides an estimate of the degree of movement and genetic exchange of females, an important measurement for species such as bottlenose dolphins that exhibit a high degree of female site fidelity. To examine the contribution of both males and females to genetic exchange rates, we will also analyze 19 independent nuclear microsatellite loci tested and optimized for a wide range of common bottlenose dolphin populations in the Gulf of Mexico and western North Atlantic (see Rosel et al. 2009, Sellas et al. 2005). The microsatellite data can also be used for individual identification of dolphins, which will support the photo-identification and tagging work. Analysis of both mitochondrial and nuclear markers will provide a measure of the degree of interbreeding among these populations. Analysis of mitochondrial DNA and nuclear microsatellite markers will use standard methodologies (Rosel et al. 2009; Sellas et al. 2005) and will utilize samples collected from the four estuarine sites targeted by the NRDA photo-ID and biopsy and health assessment plans.

Understanding stock structure in this region is useful for assessing potential individual and population-level impacts of the DWH spill. To assess possible population-level impacts of any injury, it is useful to know the population structure of the animals in the area. In addition, this information provides the genetic underpinnings for interpreting other studies at both the individual and population level; for example, health indicators may be influenced by or associated with genetic profiles.

Data Handling

MC 252 NRDA chain-of-custody procedures will be observed for all samples. All samples will be transferred with appropriate chain-of-custody forms. All laboratory data will be collected, managed and stored in a secure facility under trustee control in accordance with written SOPs.

All materials associated with the collection or analysis of samples under these protocols or pursuant to any approved work plan, except those consumed as a consequence of the applicable sampling or analytical process, must be retained unless and until approval is given for their disposal in accordance with the retention requirements set forth in paragraph 14 of Pretrial Order # 1 (issued August 10, 2010) and any other applicable Court Orders governing tangible items that are or may be issued in MDL No. 2179 IN RE: Oil Spill by the Oil Rig "DEEPWATER HORIZON" (E.D. LA 2010). Such approval to dispose must be given in writing and by a person authorized to direct such action on behalf of the state or federal agency whose employees or contractors are in possession or control of such materials.

Data Sharing²

All sample analysis will be performed at NOAA's Southeast Fisheries Science Center (Lafayette Laboratory) in Lafayette, LA. Analysis will be completed on samples described in Table 2.

Table 2. Estimated Number of Samples for Each Analytical Category³

Source	Number of samples
Photo-ID biopsies	196
Live-capture health assessment biopsies	30
Oceanic cruises biopsies	97
Strandings ⁴	215

The first data report will provide:

1) sex determination of biopsied animals from photo-identification/biopsy studies described in the dolphin photo-identification/biopsy plan ("Proposed Data Collection Plan to Assess Injury to Louisiana and Mississippi Estuarine Dolphin Stocks"), 2) sex determination of stranded animals for which sex determination could not be performed in the field, and 3) species identification of stranded animals (including morphotype of stranded bottlenose dolphins). The first data report is targeted for transmission by December 30, 2011. Sex, morphotype and/or species identification, where appropriate and feasible, will be provided to the Trustees and to BP/Cardno/ENTRIX in a spreadsheet and posted to noaanrda.org with information keyed to each sample by the sample field identification number. Analysis of severely degraded stranded carcasses may require additional time and the results of that analysis may be provided in the second preliminary data report.

A second preliminary data report providing the results of the remaining analysis under this Plan is targeted for transmission by April 1, 2012. The second preliminary data report will include: 1) the results of sex determination and species identification (including morphotype determination of biopsied bottlenose dolphins) for biopsies collected during the oceanic cruises ("Collection of Data to Determine Impacts of the Deepwater Horizon Mississippi Canyon 252 Incident on Endangered and Protected Marine Mammals in the Northern Gulf of Mexico"); 2) the sex

² Sample inventory will be available on noaanrda.org upon signature of this plan, and stranding numbers are available on NOAA's 2010 Gulf of Mexico UME website: http://www.nmfs.noaa.gov/pr/health/mmume/cetacean_gulfofmexico2010.htm

³ These estimates are contingent on staff's capacity. Start date of no later than August 15, 2011 was used in the estimations of what analyses could be completed by the end of 2011.

⁴ This number includes 119 cetaceans stranded in the response area between April 20, 2010 and November 2, 2010, plus 86 neonates stranded since November 2, 2010, 9 oiled or suspected oiled animals, and at least 1 pregnant female. Cetaceans stranded in the spill zone between April 20, 2010 and November 2, 2010 (n=119), neonates, stillborn calves, pregnant females and visibly oiled animals that stranded and been sampled between November 2, 2010 and May 30, 2011 (~96) will be analyzed for species/ecotype verification/identification. Plans for analysis of additional stranded animals will be considered in a potential future Amendment to this Plan.

determination, if possible, of severely degraded stranded animals, and 3) the stock structure analysis of samples from live-capture health assessment (“Assessing Sublethal and Chronic Health Impacts from the Mississippi Canyon 252 Incident on Coastal and Estuarine Bottlenose Dolphins”) and photo-id/biopsy (“Proposed Data Collection Plan to Assess Injury to Louisiana and Mississippi Estuarine Dolphin Stocks”) Plans. The stock structure report will provide sample sizes for each location, a description of the statistical tests applied to the DNA sequence data and the microsatellite data, and the calculated estimates of population differentiation based on analogs of Wright’s F-statistic for the sequence data and the microsatellite data. This second preliminary report will also be posted to NOAA NRDA.

Project Budget

The budget provided is for a 12 month period. The budget includes the time and costs necessary for processing biopsies and stranding samples described, including DNA extraction, molecular sexing, mitochondrial DNA control region amplification and sequencing, and genotyping at 19 nuclear microsatellite loci, and including QA/QC of all data. The Genetics Plan as written covers samples collected from strandings or under the NRDA work plans described above. Additional analysis of biopsy samples or additional samples from stranded animals that may need analyzing may be addressed in an addendum to this plan.

**FOR ACTIVITIES RELATED TO OIL SPILL IN THE GULF OF MEXICO
RE: DEEPWATER HORIZON MC 252
FISCAL YEAR (FY) 2011**

Activity	Year 1 Cost Estimate(\$K)
Contractor labor	
Genetic Technician I. (40hours/week; 12 months)	
Genetic Technician II (40hours/week; 12 months)	
Supplies	
General reagents, consumables	\$4.6K
Sequencing reagents, consumables	\$12K
Genotyping reagents, consumables	\$11.5K
Subtotal	
Overhead	
TOTAL	\$193.25K

The Parties acknowledge that this budget is an estimate, and that actual costs may prove to be higher. BP's commitment to fund the costs of this work includes any additional reasonable costs within the scope of this approved work plan that may arise. The trustees will make a good faith effort to notify BP in advance of any such increased costs.

Literature Cited

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