Preliminary Report on the Sperm Whale Data Collected During the *Voyage of the Odyssey*

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Abstract

Ocean Alliance (OA) launched the *Voyage of the Odyssey* (VOO) in response to growing concern about the impact of chemical pollution in the marine environment. The Voyage of the Odyssey is a five-year global effort designed to gather a baseline dataset on levels and potential effects of synthetic contaminants in all of the world's oceans. The sperm whale (*Physeter macrocephalus*) was chosen as a bio-indicator species for the program. Lipophilic contaminants are likely to accumulate and biomagnify in sperm whales due to their high body fat content, their relatively long life span and their high trophic position within marine food webs. Moreover, because sperm whales have a global geographical distribution, a worldwide dataset can be collected from this one species. In addition to our toxicological studies, we are also collecting material and data for sperm whale genetics and acoustic analyses. The specific aims of the VOO program and the relevance of these aims to the International Whaling Commission (IWC) Scientific Committee's efforts at making an in-depth assessment of sperm whales are summarized. OA methodologies for biopsy collection and sub-sampling and the current status of our data collection and analyses are presented.

Introduction

The oceans are considered the final sink for many toxicants and there is a growing concern about the impact of chemical pollution in the marine environment and its potential effects on the health of animals, plants and humans. Lipophilic contaminants, such as dichlorodiphenyltrichloroethanes (DDTs), polychlorinated biphenyls (PCBs) and other organochlorines (OCs), are known to accumulate in animal species including fish and marine mammals (Colborn and Smolen, 1996; Jorgenson, 2001). Many of these compounds have been shown to adversely effect laboratory animals and wildlife (Safe, 1984; Colborn *et al.*, 1993). Much research effort is dedicated to understanding the potential links between chemical exposure and altered immune and reproductive systems, impaired physiological and endocrine functions as well as neurobehavioural disorders in traditional animal models (rodents) and certain wildlife species (Fry and Toone, 1981; Beard and Rawlings, 1998; Hany *et al.*, 1999; Guillette *et al.*, 2000; Fox, 2001). However, scientific understanding of the effects of environmental pollution in marine mammals remains limited (Reijnders, 1986; Beland *et al.*, 1993; Ross *et al.*, 1996; Ross, 2000; Martineau *et al.*, 2002).

Ocean Alliance launched the *Voyage of the Odyssey* (VOO) to address the need for a globally integrated dataset allowing a consistent analysis of exposure to, and potential effects of, persistent organochlorines in marine life worldwide. Many marine mammals harbour large fatty reserves in their body where high levels of organochlorines and other lipophilic contaminants can accumulate (Aguilar and Borrell, 1994; Colborn and Smolen, 1996; Ross *et al.*, 2000). Marine mammals are also subject to bioaccumulation and biomagnification of these fat soluble contaminants due to their relatively long life span and their high trophic position within marine food chains (Boon *et al.*, 1992). Marine mammals therefore can be considered environmentally relevant candidates for use as sentinel species when assessing marine pollution (Ross, 2000). Sperm whales were selected as the study species for the VOO program due to their high trophic position and their widespread geographical distribution and because they can be acoustically tracked.

Over 700 sperm whale skin and blubber biopsies have already been collected during the Voyage of the Odyssey. Analyses of concentration burdens, exposure and the molecular effects of bio-persistent toxicants in these biopsies are presently underway. Induction of the cytochrome P4501A1 enzyme (CYP1A1), which plays a critical role in the metabolism of planar halogenated and polycyclic aromatic hydrocarbons, is widely used as a biomarker of exposure to contaminants in many animal species (Stegeman *et al.*, 1992). The toxicological component of the VOO program is designed to investigate CYP1A1 (and other CYP1) protein and gene expression in sperm whales using immunohistochemistry, enzyme, and gene expression assays. For each biopsy sample, additional analyses including genetic and stable isotopes studies will provide a comprehensive framework for the interpretation of the toxicology data.

Specific Aims of the VOO Program and Relevance to Sperm Whale Assessment:

1. Provide samples to current researchers and establish an archive of samples from whales, squid, and pelagic fish living in the major ocean basins of the world. As new techniques or scientific questions arise, material from this archive will be made available to outside researchers.
2. Establish a biopsy sub-sampling protocol providing concurrent collection and storage of sub-samples for each biopsy, thereby maximizing the magnitude of information and data collected and potentially reducing the need for future re-sampling.
3. Conduct contaminant burden analyses of sperm whale samples with an emphasis on persistent organohalogens that are implicated in immunosuppression, altered endocrine and reproductive systems, cancer, and various pathologies in laboratory animals.
4. Conduct contaminant burden analyses of other biological samples (squid, fish) to examine biomagnification processes of other species in the food pyramids in which sperm whales feed.
5. Conduct analyses of biopsy samples for levels of CYP1A1. CYP1A1 can be used as a biomarker of exposure to environmental contaminants, such as persistent planar halogenated aromatic hydrocarbons and non-persistent polycyclic aromatic hydrocarbons.
6. Adapt toxicological assays and biomarkers developed in laboratory animals to marine mammal species. Examples include specific gene cloning, quantitative RT-PCR of CYP1A1 gene expression and assessment of cytochrome P450 1B1 (CYP1B1).
7. Compare data obtained with contaminant and biomarker analyses in order to investigate and potentially characterize the direct links between the two approaches and to validate and refine such techniques.

8. Broaden the fundamental understanding of cetacean, and particularly sperm whale, toxicology by:
    a. Analyzing VOO toxicological data (both chemical and mechanistic data) in the context of the additional information collected for each biopsy (genetic analyses, stable isotopes analyses).
    b. Comparing VOO toxicological data with other available marine mammal toxicology data and with laboratory animal studies.
9. Study the identity of sperm whale prey by comparative analyses of prey samples regurgitated by whales, potential prey species collected at sites of sperm whale sightings, and squid beaks collected in whale feces.
10. Investigate trophic relationships between sperm whales and potential prey species using stable isotope analyses.
11. Collect and analyze sighting data from the Odyssey to broaden the knowledge of sperm whale zoogeography and habitat use in tropical waters.
12. Generate a global data set of sperm whale communication sounds, so-called codas, to test theories of dialects and acoustically mediated culture in sperm whales.
13. Quantify and map anthropogenic noise sources in the world's oceans to form the basis for mitigation of human activities in critical habitats for marine mammals.
14. Record samples of sound from different tropical sperm whale populations to assess the size compositions of different stocks from the interpulse intervals in the multipulse structure of sperm whale clicks.
15. Quantify source parameters of sperm whale sounds in order to assess the communicative space and echolocation potential of different click types and to study potential effects of man made noise on such sounds.
16. Study sperm whale sound production with ultrasound-time-depth recording tags.
17. Use genetics and photo-identification analyses to investigate population and stock structure, short-term and long range movements of sperm whales, and habitat use.
18. Design and validate of new methodologies for toxicological, genetic and acoustic studies of sperm whales.
19. Design a comprehensive and collaborative interpretation of all VOO data (toxicology, genetics, acoustics) to benefit management strategies

Existing Methodologies and Current Status of Analyses

Biopsy Collection

In order to minimize potential disturbance to the whales due to close approach of the vessel, the *Odyssey* has a platform near the bow that projects laterally for eight meters (to starboard). Biopsy arrows are deployed from this platform and the minimum distance required between vessel and animal for sample collection is therefore achieved. Effort is made to sample whales when they are arching their backs for deep dives—a time when a greater proportion of the animal's flank clears the water. The biopsy darts used are 40mm long, 8mm in diameter and fitted with three internal prongs to retain the tissue plug. The stainless steel cylindrical punch is washed in soapy water, sterilized in alcohol and rinsed in de-ionized water before use. Arrows fitted with a compressed foam stopper (designed by Ceta-Dart, Dr. F. Larsen, Copenhagen, Denmark) are fired at a range of 10 to 20 meters from a 68kg pull, compound crossbow (Barnett RC 150). Biopsy samples are usually collected from the flank of the animal below the dorsal fin, and the region of each dart strike is recorded. The floating dart is recovered with a dip net and the biopsy tissue obtained is processed immediately. Over 700 biopsy samples have now been collected from the gulf of California, the Galapagos Islands, several locations across the Pacific Ocean, Papua New Guinea, Australia, the Chagos

Archipelago, the Seychelles, the Maldives, Sri Lanka and other locations from the Indian ocean.

Biopsy Tissue Sub-sampling
A detailed protocol for sub-sampling along with a review of the current data obtained for each type of sub-sample is in progress by the authors and will be the subject of a later communication.

Briefly, each biopsy sample is divided immediately after collection into the following sub-samples in order to maximize the data and information that can be gathered from each animal.

1. Biomarker analyses for cytochrome P4501A analyses: sub-samples of the epidermis/dermis interface layer are collected and stored in liquid nitrogen, RNAlater (Ambion) or in 10% neutral buffered formalin in accordance with the different storage requirements for future expression studies at the enzymatic, mRNA, or protein levels.
2. Contaminant burden analyses: blubber sub-samples are collected and stored at -20ºC in decontaminated glass vials for chemical analyses.
3. Genetics: subsamples of the epidermis are stored at room temperature in dimethyl sulphoxide saturated with sodium chloride as previously described (Amos and Hoelzel, 1991).
4. Stable isotope analyses: sub-samples of epidermis and/or dermis are allocated for stable isotope analyses and frozen at –20ºC.
5. Fatty acid analyses: whenever possible, sub-samples of dermis are also collected for fatty acid analyses and stored at -20ºC.

Biomarker Analyses
Sections of biopsy sub-samples stored in neutral buffered formalin are embedded in paraffin and prepared for immunohistochemical staining of cytochrome P4501A1 as previously described (Smolowitz *et al.*, 1991; Woodin *et al.*, 1997). Staining is achieved using the monoclonal antibody Mab 1-12-3 which is known to be highly specific to CYP1A1 in fish and mammals (Park *et al.*, 1986; Kloepper-Sams *et al.*, 1987; Drahushuk *et al.*, 1998). CYP1A1 protein expression was examined in sperm whale biopsies that we collected in the Sea of Cortez (Mexico), the Galapagos Islands, several locations across the Pacific, Papua New Guinea, and Australia. In each of these regions we detected such expression. Quantitative analyses using a modification of the procedure of Woodin et al. (1997) is underway and will allow comparisons among these geographical areas.
Skin samples, of similar sizes than the biopsies collected by VOO, have been collected from stranded whales and have been prepared and analyzed for CYP1A1 enzymatic activity in order to refine protocols. Samples from both stranded and biopsied cetaceans have also been used for the partial cloning of CYP1A1 and CYP1B1 in preparation for the development of a quantitative RT-PCR protocol for these two genes in the sperm whale (Godard, 2000; Godard *et al.*, 2000).

Contaminant Burden Analyses
A subset of 30 biopsy samples representative of the Sea of Cortez, Galapagos and the first VOO Pacific crossing leg were analyzed in collaboration with Dr. Kannan and Dr. Giesy, National Food Safety and Toxicology Center, Michigan State University. Tissue samples were pooled due to their small sample size (<1g), according to region and day to help validate interpretation. The samples were fibrous and had a lower lipid content (mean: 6.2%) than expected. DDTs (338 to 7942 ng/g lipid weight) were the leading compounds in most pooled

sperm whale samples, with p,p'-pDDE being the most predominant metabolite of DDT. Among organochlorine pesticides, Chlordane compounds were next in abundance to DDT. PCBs were found in all pooled samples with total concentrations between 166 and 3966 ng/g lipid weight. DDTs and PCBs levels do not appear to be correlated and vary significantly among pooled samples within a same region.

Genetics

Samples collected in the Sea of Cortez have been analyzed for gender in collaboration with Dr. S. Mesnick from the Southwest Fisheries Science Center (San Diego, CA), Dr. R. Vázquez-Juárez from the Centro de Investigaciones Biológicas del Noroeste (CIBNOR, Mexico) and Nadia Rubio (CIBNOR). DNA extraction and molecular sexing were determined using modifications of Aljanabi and Martinez (1997) and Richard *et al.* (1994), respectively. A complete report on the temporal and spatial distribution of female and male sperm whales in the Sea of Cortez is in preparation.

Photo-identification

Analog and digital photographs of the dorsal area and flukes of sperm whales are collected during each approach. When possible the flukes are photographed when the ventral surface is raised and is at a 90º angle to the camera's line of sight. The time, latitude and longitude, film roll, and frame numbers of the photographs are recorded on hard copy field forms and later entered into a computerized database. Analog photographs of sperm whales are taken using either a Minolta 9X1 or Minolta 7001 camera with Minolta 75 to 300 mm lenses and 400 ASA color print film. Digital images are taken using a Nikon Coolpix E950 with a wide angle lens. Digital videos of sperm whale approaches are taken opportunistically using a Canon XL-1 DV video camera. Digital photos and video images are used in short-term identification of individuals in the field to help eliminate multiple sampling of a single animal. All photos and videos are archived for future analyses of whale population stocks and structure.

Acoustics

Acoustic detections of marine mammals are made onboard the R/V Odyssey in real time, using a 100 m or 300 m towed acoustic array, each consisting of two PVC-encased hydrophone units (Benthos AQ4, with Benthos AQ201 pre-amplifiers). The effective listening range is 3 to 6 nautical miles depending on ocean conditions, and on whether the vessel is motoring or sailing. The output signal is monitored 24 hr a day using speakers located in the pilothouse as well as during a stop every half hour (when not on biopsy effort) using high-quality headphones. All acoustic contacts with marine mammals are entered in Logger 2000 v. 2.20 (International Fund for Animal Welfare, IFAW). The number of sperm whales clicking within the detection range of the hydrophone is calculated by Rainbow Click v. 1.03 (IFAW) for each encounter. Spatial bearing of each animal is automatically calculated by Rainbow Click using the time of arrival differences between receptions of clicks on two hydrophone channels. In addition, source parameters of marine mammal phonations are estimated with a state of the art, wide band, calibrated recording system consisting of three Reson TC4032 hydrophones that relay signals, via an amplifier/filter unit, to a 12 bit digital recorder (Wavebook 512) with a recording bandwidth of 160 kHz. This system sheds light on cetacean sound production, communication and echolocation in off-shore marine hanitats. All marine mammal acoustic detections as well as recordings of other relevant sounds (seismic signals, wind noise, ship traffic, etc.) have been archived on CDs. The sperm whale acoustic data have also been extracted from the raw data and archived separately for further analyses.

Additional Biological Sampling
Sloughed skin: Naturally sloughed sperm whale skin is opportunistically collected and used for genetic analysis.

Squid and squid beaks: Whenever possible, squid are collected at locations where sperm whales have been successfully biopsied. Squid beaks are collected opportunistically when a sperm whale is observed defecating at the surface. Beaks will serve for identification of prey species while squid samples are preserved for stable isotope and chemical analyses.

Fish*:* Samples from fish species that are of commercial or ecological importance are collected opportunistically and preserved for stable isotope and chemical analyses. The dorado *Coryphaena spp.* and tuna *Thunnus spp*. have been the most prevalent species collected to date.

Development of New Methodologies

Dosing protocol
We have designed a non-lethal dosing protocol using skin biopsy slices in order to investigate the inducibility of cetacean cytochrome P450 1A1 (Godard *et al.* 2003). The results of this protocol demonstrated a direct relationship between chemical concentrations and specific effects in P450 expression in sperm whales and therefore validated the use of CYP1A1 as a biomarker of contaminant exposure in cetaceans. This type of study would have been, until now, dependent on lethal or invasive sampling.

More specifically, a full dosing study using this new protocol was successfully completed on 50 sperm whales sampled in the Sea of Cortez and established that

1. CYP1A1 in cetaceans is inducible by betanaphthoflavone, a prototypical CYP1A1 inducer in laboratory animals and wildlife.
2. CYP1A induction in cetaceans occurs in three different cell types: endothelial cells, smooth muscle cells and fibroblasts.
3. CYP1A1 induction in smooth muscle and endothelial cells appears to be dependent on contaminant concentration.

This dosing protocol is currently being used to investigate the effect of other chemicals on sperm whales. We have recently completed the field component of a dosing study using the PCB 3,3',4,4' tetrachlorobiphenyl. Analyses of the samples are in progress. The protocol has a wide applicability and can be used for the whale species and chemicals or mixture of chemicals of interest. It may also prove very useful in studying the effects of chemicals on other endangered species for which common invasive toxicology protocols are not permitted.

Acoustic Tagging
A novel acoustic datalogger has been developed and deployed on sperm whales with a large suction cup to study their sound production at great depths. This datalogger contains an analog-to-digital converter that stores sound up to 30 kHz on a memory flash card along with UTC time and depth information. The housing of the tag is pressure resistant to a depth of 1500 meters and contains a VHF transmitter to locate the tag after it is released from the animal. Data from this study undertaken during VOO has led to publication of the first paper describing onboard sound recordings from a free ranging cetacean (Madsen *et al.*, 2002). We have demonstrated that the air driven sound production of sperm whales is unaffected by hydrostatic reduction of the air volumes contained in the nasal complex and in the lungs during deep dives. Furthermore it has been shown that sperm whales can regulate the acoustic

output and frequency content of clicks during dives, and generate click types with significantly different properties suited for echolocation and communication respectively. These findings have shed light on the biomechanics of sperm whale sound production and yielded information on the acoustic ecophysiology of sperm whales with implications for management and conservation. The technical gains from this study will hopefully allow Ocean Alliance to develop more sophisticated tags with cameras that may prove useful in understanding how sperm whales navigate and catch their prey at great depths.

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