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Research Perspectives for Dolphin Mortalities in North America

by

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PREFACE

The Environmental Protection Agency's Center for Marine & Estuarine Disease Research (CMED) was created in 1990 when the First Gulf Breeze Symposium on Marine and Estuarine Disease Research was convened. The symposium emphasized the need for scientific coordination and sound, comprehensive programs with consistent funding for aquatic disease research. Eighteen presentations during the symposium were published in a book *(Pathobiology of Marine and Estuarine Organisms)*, edited by Drs. J.A. Couch and J.W. Fournie (CRC Press *Advances in Fisheries Science Series, 1993).*

Since then, CMED has relied on internal, extramural and collaborative projects to address priority areas of aquatic animal disease. In most cases, these projects involve laboratory and field research, but CMED has also sponsored meetings and workshops to strengthen communication and collaboration among scientists.

The Second Gulf Breeze Symposium was co-sponsored by CMED and the National Marine Fisheries Service, Office of Protected Resources, in late 1992 to address questions of dolphin mortalities in North America. The symposium allowed more than 40 participants to focus on current scientific information, research strategies, and research needs related to deaths of bottlenose dolphins *Tursiops truncatus*. Presentations from the second symposium and several invited manuscripts will be featured in the publication *(Dolphins: Factors in Morbidity and Mortality)* to be edited by Drs. R. Haebler (EPA) and A. Hohn (NMFS).

During the second symposium, four study groups were formed to address research questions related to (1) pathology and disease, (2) pollution analyses and biomarkers of exposure, (3) physiology and biomarkers of effects and (4) stranding and sampling logistics for bottlenose dolphins. The responses and recommendations summarized by group leaders are presented in this document.

CONTENTS

Introduction

Dolphin strandings are not recent occurrences; they were recorded by Aristotle at least 2300 years ago. Public interest in dolphin and other marine mammal strandings has intensified during the past century and numerous theories have been generated concerning their causes. Recently there has been an increase in reported marine mammal mortality events throughout the world. A mortality event is generally considered to be an unusually large number of animals dying, beach-cast or floating, over a relatively short period of time in a restricted geographic area. Large-scale dolphin mortalities have included bottlenose dolphins (*Tursiops truncatus*) along the U.S. mid-Atlantic coast in 1987-1988 and two separate mortality events in the Gulf of Mexico in 1990 and 1992. Additionally, over 1000 dead striped dolphins (*Stenella coeruleoalba*) were recovered from the Mediterranean Sea in 1990-1991. Stranded dolphins may represent only a fraction of the population affected.

Possible causes for unusual dolphin mortalities include fishery-bycatch, pathogens and pollution. Pollution can be directly responsible for mortalities or can indirectly alter behavioral or immunological responses, increasing susceptibility to predators and infectious diseases.

Investigations to link dolphin mortality events with potential causes must include thorough examination of stranded dolphins and consideration of several interacting factors. Knowledge of life history data (age, reproductive status, etc.) can be important in interpreting clinical data. Gross and histological pathology initiated before deterioration of tissues can provide the best insight to potential trauma and disease. Microbial cultures can help to determine whether the microorganisms present caused the mortality, or merely represent an opportunistic infection of a dying individual. Tissue samples can be analyzed and, if properly interpreted, may indicate the type and degree of exposure to toxicants. Physiological and immunological evaluations may also indicate changes associated with disease or intoxication prior to death.

Post-mortem analyses must be supported by knowledge of the normal range of microbial, toxicant, physiological, and immunological characteristics of living dolphins. Due to the protection afforded dolphins as marine mammals, such knowledge can only be gained through non-lethal sampling of dolphins in aquaria or capture-release studies. Such studies not only support post-mortem analyses, but may also lead to prediction of compromised conditions that may lead to mortality in free-ranging populations.

Three groups of participants at the Dolphin Mortality Conference posed and addressed several questions related to investigations of disease and contaminant exposure and their physiological effects on dolphins. A fourth group examined the ability of stranding and sampling programs to respond to scientific needs.

Pathology and Disease

Group Leader: Gregory Bossart

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Jack Fournie	Robin Overstreet	Diane Sips
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Remarks

Largely because of dolphin mortality events along the mid-Atlantic coast of the U.S. and the Gulf of Mexico, a Federal marine mammal die-off response plan was instituted. This plan included an "unusual mortality" task group to investigate events where large numbers of mortalities occurred in a short period of time and where mortalities appeared unique or alarming. Improvements have also been made in regional marine mammal stranding networks. But despite organizational progress and advancement of the general biologic knowledge of marine mammals, there is a paucity of data on diseases and associated pathologic lesions in dolphins; consequently, understanding of the causes and mechanisms of diseases is very limited.

In considering diseases and pathology of free-ranging dolphins, it is important that each mortality event be approached as a unique incident to be accompanied by a systematic gathering of relevant environmental, biological, clinical, and pathological data. The time-honored systematic approach to the pathologic basis of disease must be continually emphasized in characterizing dolphin mortalities; the concept that should be held foremost is that pathologic investigations are studies in clinical medicine to address altered structure and function. This systematic approach must include a logical and stepwise plan of collection, documentation and storage of antemortem clinicopathologic data, must employ standardized gross necropsy and tissue collection procedures, and must provide for histopathologic evaluation of freshly-fixed tissues and subsequent studies in specialized disciplines such as immunology, virology, and toxicology.

The scope of these pathologic studies should be expanded to investigate the mechanisms of marine mammal diseases and to characterize distributions, origins, morphologic changes, and resultant clinical symptoms. Attention should be directed to the pathophysiologic mechanisms leading to morphologic changes and to the clinical implications of these cellular and organ changes. Causes of dolphin mortalities may be revealed only if diseases are recognized as dynamic processes influenced by abiotic and biotic environmental factors, individual susceptibility, and population interactions.

Question 1

Do gross, histological and clinical (hematological, serological, serum chemical, and microbiological) databases exist for "normal" dolphins?

Response

A need exists to widen the data base of gross, histologic, and clinical parameters for "normal" or "healthy" dolphins. Presently, clinical and histological data from normal dolphins are either inadequate or difficult to access and retrieve. Clinical data can vary tremendously among studies performed at oceanaria or by stranding personnel. For example, peripheral blood studies could range from standard blood cell counts to in-depth studies involving viral serologic or immunologic parameters. Gross necropsy quality (i.e., completeness, lesion description, reporting protocols, etc.) depends on the experience of the facility prosector and histopathologic evaluation may depend on the experience and commitment of the pathologist reading the tissues. Access and retrieval of these data may be problematic since each facility has its own program and procedures.

Recommendations

1. A focused project should be implemented through the Armed Forces Institute of Pathology (AFIP) to establish a complete histologic set of tissues from 12 "normal" Atlantic bottlenose dolphins (*Tursiops truncatus*). Code 1 (live) or Code 2 (freshly-dead) animals from incidental takes or human interactions are needed to ensure quality specimens and a "normal" status. This will require coordination with regional stranding coordinators, National Marine Fisheries Service (NMFS) observer programs, or other sources.

2. The AFIP would subsequently act as a central tissue repository for wet/paraffin-embedded tissues (for light microscopy) and resin-embedded tissues (for electron microscopy) from both normal and stranded dolphins.

3. A reference set of normal tissues should be incorporated into a histological atlas for this species.

4. Future reference tissues from other dolphin species should be organized and archived in a similar fashion.

Question 2

Are present descriptions of pathology well-defined for infectious, non-infectious and parasitic diseases?

Response

Investigations of disease and mortality in captive and stranded dolphins have resulted in an initial list of pathogenic microorganisms, parasitic infections, and some nutritional disorders. However, even though primary morphologic diagnoses of disease states have been defined, the etiologic diagnoses remain obscure (viruses, intoxications, etc.). It is not uncommon to describe histopathologic lesions for which the etiology cannot be

determined; hence, emphasis is placed on reporting morphologic diagnoses and the systematic classification of lesions. This is especially true for viral infections, due in large part to the lack of specific dolphin cell lines generally necessary for viral isolation. Similar problems also exist in suspected intoxications due to lack of baseline tissue concentration data and specific toxic effects data in the target species.

Recommendations

1. Provide a mechanism to advise the NMFS on the various elements that should be incorporated into the marine mammal data base. Implementation of such a data base could be aided by a computer network.

2. Initiate a multi-disciplinary approach that would widen the data base through correlation of clinical data (hematology, serology, serum chemical) with gross, histological and microbial data. This should be coordinated so that all regions use similar protocols.

Question 3

Can present pathology databases help in determining the causes of (free-ranging) dolphin mortalities?

Response

The present data bases, if consolidated into a usable format, could help in determining the causes of mortality. For example, hematologic, serum analyte, and microbiologic data already collected and recorded from "healthy" dolphins could be collated from various facilities to generate a baseline data bank. Likewise, pathologic lesions already described for individual cases might be categorized by specific morphologic diagnoses to give a better picture of disease incidence in the study population. Stockpiled serum samples at various oceanaria should be an invaluable source for determining seroprevalance of known infectious agents (e.g., viruses, bacteria, protozoans, fungi) in a population.

Recommendations

1. Current databases could be improved by (a) increasing the amount and type of data collected, (b) concentrating on live or freshly-dead animals, (c) incorporating concurrent comparative studies with "healthy" dolphins and (d) standardizing necropsy protocols and tissue submission forms.

2. Databases from different facilities should be consolidated and made more accessible to scientists.

3. Photography should be used more often for documentation.

Question 4

Are captive dolphin studies useful for understanding mortalities of free-ranging dolphins?

Response

Captive dolphins are a useful resource for understanding mortalities in free-ranging dolphins. Captive dolphins can

provide information essential to develop techniques and understand mechanisms of disease even though etiologies may differ from free-ranging dolphins. It is believed that abundant information is available (e.g., hematologic, serum chemical), but not in a standardized format or a central location. Sampling procedures used on captive animals should be adaptable for use on free-ranging dolphins.

Recommendations

1. Coordinate/standardize protocols and data collection with cooperating marine mammal facilities.

2. Establish blood serum banks for captive and stranded dolphins; this is a valuable resource for insight to mortalities of free-ranging dolphins.

3. Support collaborations with marine mammal facilities to use captive dolphins for developing new sampling techniques and procedures.

4. The condition of free-ranging dolphins could be better evaluated if NMFS observers on fishing vessels were trained to collect samples from dolphins caught in nets.

Question 5

Are logistical procedures (stranding networks, sampling protocols, tissue banks, etc.) adequate and realistic for successful pathologic evaluations?

Response

There appears to be a positive evolution toward optimizing data collection and improving logistical procedures for pathologic evaluations. This is partly due to the development of a national die-off response plan,improvement of the regional stranding networks, and development of a marine mammal tissue bank to evaluate anthropogenic toxins and biotoxins. For example, standard protocols have been or are being developed for performing gross necropsies and collecting tissue samples for histopathologic, microbiologic, and toxicologic evaluations. Also, pathologists are being consulted and are participating in necropsies more frequently.

To identify and investigate future mortality events more effectively, studies must be designed and conducted to advance the knowledge of the etiology of dolphin disease (i.e., infective pathogenic agents as well as metabolic, genetic, and neoplastic disease). These advances will also require use of code 1 or 2 animals and more complex sampling protocols necessary for proper specimen evaluation.

The concept of specimen retrieval from tissue banks is a potentially valuable tool for broadening the pathologic data base. Currently, tissue banks for histopathologic or serologic evaluation are either not developed or poorly organized for retrieval purposes.

Recommendations

1. Code 1 or 2 dolphins should be the highest priority and the scientific investigation should follow a tiered progression that incorporates the classical characterization of disease states: (a) clinical data collection, e.g., hematologic or serum analysis, (b) complete gross and histopatho-

logic evaluation, and (c) any evaluations for specialized disciplines (e.g., virology, toxicology, immunology).

2. When possible, veterinarians or other trained personnel should conduct necropsies for optimal quality of samples and reporting. This will require participation by laboratories and veterinarians in all regions that in contact with the stranding network.

3. Databases should be expanded through establishment and/or improvement of tissue banks that are accessible to scientists.

Question 6

How should epizootics, especially those related to unusually high dolphin mortalities, be tracked and investigated?

Response

Response to unusual dolphin mortality events is now reasonably well coordinated in the sense of a multi-disciplinary scientific response. For the response to be successful, the NMFS must be notified of the mortality event in a timely manner. Live "healthy" and "sick" dolphins should be sampled during an epizootic. This approach was applied recently after of the 1992 mortality in Texas.

Success of the 1992 Texas capture set a precedent in live dolphin captures, involving the live-capture and release of 36 bottlenose dolphins (*Tursiops truncatus*) from the region where mortality occurred. During the brief holding period, each dolphin was given a complete physical examination. Blood samples were withdrawn and later evaluated for standard hematologic and serum analyte determinations, as well as specialized serologic and immunologic studies. Sampling of free-ranging wildlife populations is frequently used with terrestrial wildlife species in a disease outbreak to monitor antemortem clinical data and obtain tissue samples not influenced by postmortem autolysis. This methodology can provide important data, including disease etiology, disease pathogenesis, disease incidence, and other epidemiologic characteristics. This is perhaps the first time capture/release techniques was used in U.S. dolphin mortality studies.

Recommendations

1. Time is a critical factor in an epizootic. Although live captures have been successfully implemented in an epizootic (see above), live capture/sampling techniques and permit mechanisms must be assessed continually for rapid response.

2. Physiological measurements of live dolphins should be improved and be comparable to measurements of stranded or moribund animals.

3. Euthanasia of "healthy" dolphins should be a last resort; current NMFS requirements for special authorization should be retained. Euthanasia should be performed by a veterinarian only when a consensus of experts agrees that all options have been exhausted.

Pollution Analyses/Biomarkers of Exposure

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Remarks

The organohalogens (OH), aromatic hydrocarbons (AH), polynuclear aromatic hydrocarbons (PAH), and toxic metals include several broad groups of chemicals extensively used in industrial processes or arising from the recombination of fossil fuel wastes. These compounds are widely disseminated as both terrestrial and aquatic pollutants and their environmental distribution has been clearly identified by sedimentation analysis to coincide with the worldwide growth and development of the chemical industry. The stability and lipophilicity of the organic chemicals and some metals (e.g., mercury) result in their tendency to be concentrated in fatty tissues, leading to their bioaccumulation up the food chain. Detectable concentrations of a variety of the OH/AH/PAH and metals have been identified in the tissues of both terrestrial and aquatic animals. Public awareness, of and concern for, the potentially adverse human health and environmental effects resulting from exposure to these chemicals has increased significantly with reports of agricultural and occupational exposures, a series of PCB poisonings in the Orient leading to the onset of immunodeficiency diseases, and the sequelae of immunotoxic effects of the PBB cattle feed contamination disaster in Michigan.

Shallow bays and estuaries in the vicinity of chemical plant discharge/ shipping sites and large urban areas are among the most heavily contaminated coastal regions and in some instances have been shown to constitute a potential threat to the safety and well-being of humans and animals in close proximity to the polluted sites. Animals taken directly from contaminated sites may have high tissue concentrations of lipophilic chemicals, as evidenced by PCB concentrations as high as 600 ppm in fat of beluga whales from the St. Lawrence estuary. Marine mammals sampled in virtually all oceans, including pristine waters generally considered to be uncontaminated, have alarmingly high, but perhaps not acutely toxic, tissue residues of organic and inorganic chemical contaminants.

Although physiological consequences of chronic cellular exposure to subacute levels of chemicals and resultant

bioaccumulation of contaminants relevant to metabolism are unknown, elevated PCB residues in tissues may initiate a state of immunosuppression, while elevated levels of some metals may contribute to neurological damage. Contaminant-initiated immunosuppression may have contributed to recent epizootic deaths of dolphins in U.S. and the western Mediterranean, where PCB levels as high as 3,000 ppm were detected.

Enormous volumes of data collected from almost all of the oceans show that organohalogen and, to a lesser degree, aromatic hydrocarbon residues are elevated in tissues of marine mammals. The fact that animals are exposed to these compounds is clear. The effects of such exposure and the synergism between classes of pollutants that may be found as tissue residues is unknown. Organohalogen pollutants may interact with a series of cellular receptors to (a) induce the synthesis of cytochrome P450 enzymes, (b) alter immune system function, (c) cause dermal lesions, (d) change hepatic function, (e) modify endocrine profiles, (f) decrease reproductive capacity, and (g) initiate embryotoxic phenomena. Recent data show that elevated levels of organohalogens such as PCBs and TCDD may predispose animals to increased DNA damage from subsequent exposure to hydrocarbon contaminants by increasing the induction of enzyme systems that biotransform procarcinogenic compounds. While data from these studies support the proposal that hydrocarbon induction of cytochrome P450 enzyme systems may serve as an early warning indicator for organic pollutants, the existence of pollutants and the exposure of animals to these pollutants is not the question. Rather, the question is whether a suite of biomarkers (physiological effects) can be developed to monitor the health of animals relative to their exposure states.

Examinations of subacute vs. acute levels of toxicity of organic and inorganic chemicals in mammals must consider the stress-induced mobilization of chemicals bioaccumulated in fat reserves and increased concentrations of lipophilic xenobiotic compounds in milk that would be ingested by nursing offspring.

Question 1

What dolphin populations should be evaluated to best understand pollution exposure?

Response

Due to existing ancillary information, the large volume of information available, and the infrastructure for continued collection of data, the Sarasota dolphin population is a leading choice for study. The Sarasota population resides in a unique area with both industrially polluted and reasonably clean regions; it has been continuously monitored and studied for many years.

At least one other large, well-established and relatively stable population should be studied. The Matagorda Bay population could provide comparative information, even though it is not yet well characterized. Matagorda Bay receives runoff from a highly cultivated area of Texas via the Colorado, Navidad and Lavaca Rivers, carrying a periodic heavy load of insecticides and herbicides to expose resident dolphins and their prey.

The Matagorda Bay area also receives industrially-derived pollutants from chemical and petrochemical plants. Identification and characterization efforts have been initiated on this population.

A population of bottlenose dolphins on the East Coast of Florida (Indian River Lagoon) has been monitored since 1974, with three mark and release studies performed. This population should also be considered for future study.

There was concern that a reference population in an unpolluted area should be identified. However, participants agreed that such a population was not known.

Recommendations

1. Efforts should be made to continue comprehensive studies on the Sarasota dolphin population while attempting to characterize the Matagorda Bay population for comparative purposes.

2. A dolphin population from an unpolluted site should be sought for similar study. However, it is possible that the Sarasota population may be from a sufficiently clean environment to be used as a reference site for comparison with Matagorda Bay dolphins.

Question 2

What dolphin tissues are best sampled for analysis of xenobiotics residues?

Response

Blubber was the first choice for xenobiotic tissue analysis. A baseline characterization of analytes in blubber should be completed for comparison with other tissues. However, sampling of blubber could provide highly variable aromatic hydrocarbon, organohalogen and polycyclic aromatic hydrocarbon (AH/OH/PAH) data unless the moisture content and lipid profile of the blubber are known. For live animals, the order of tissue of preference is (a) blubber, (b) milk in lactating females, (c) blood serum, and (d) cellular blood components. Necropsy samples of choice were (a) blubber, (b) kidney, (c) liver, and (d) a hematopoietic tissue, in order of preference. It is noted that the team of scientists for the National Marine Mammal Tissue Bank, preferred liver over kidney for contaminant analysis.

Recommendations

1. Establish baseline levels of analytes in blubber, milk of lactating females, blood serum and cellular blood components.

2. Develop methods to normalize blubber residues that take into consideration moisture content and lipid profiles.

3. Life history information needs to be collected whenever analyses of this nature are performed; results may be influenced by age, gender, reproductive status, nutritional status, etc.

Question 3

Which biological or chemical analyses are most relevant for determining xenobiotic exposures?

Response

There are no unconditional methods to quantify dolphin exposure to xenobiotics. At this time, no publications indicated a direct correlation between environmental exposure and tissue residue levels, much less a physiological change in the animals. Residue analysis needs to be as inclusive as possible, even though correlations between tissue residues and mortalities are limited to relatively few examples. These include (a) fish in Lake Michigan, (b) cormorants in Green Bay, and (c) pinnipeds in the Baltic. Analyses of organohalogen-induced cell function changes might be best achieved *in vitro*.

There are limited studies of biochemical markers of contaminant exposure in dolphins. Early studies have shown cytochrome P450 to be detectable in dolphins, but no information is available to indicate the relative degree of similarity among dolphin cytochrome P450 systems and other species. No existing data show correlations between xenobiotic load in dolphin tissues and the induction of cytochrome P450s. Preliminary data (unpublished, Busbee and Carvan) indicate that monoclonal antibodies against rat CYP1A1, which is induced by exposure to OH and PAH, do not react against dolphin cytochrome P450s, but that cDNA probes developed from rats hybridize with cetacean CYP1A1 mRNA.

Recommendations

1. Standardize the various analytical/chemical methods using reference standards obtained from the National institute of Standards and Technology.

2. Correlate the state of dolphin health with tissue residue levels of OH, AH, and PAH. The best measure of *in vivo* health may be immune system function.

3. Characterize the cytochrome P450 system in dolphins to better understand its capacity to biotransform contaminants: This would entail (a) development of *in vitro* cell lines, (b) development of monoclonal antibodies specific to dolphin cytochrome P450 isozymes, (c) development of dolphin-specific cDNA probes (or determination that rat cDNA probes hybridize with dolphin DNA or mRNA sequences), and (d) application of molecular detection techniques (e.g., cDNA probes, antisense riboprobes).

4. In addition to cytochrome P450 induction studies, resident DNA adduct levels and glutathione depletion should be measured and correlated with xenobiotic exposure.

Question 4

What are the preferred methods to ensure analytical quality?

Response

Sampling of tissues from live-capture and necropsied animals has been addressed (Question #2). For each tissue (blubber, milk, blood serum and cells), samples should be analyzed for organohalogens by gas chromatography of organic solvent-extracted analytes. Inadvertent bias in residue analyses should be rigorously avoided. To that end, both gel permeation and silica gel column separations should be used to obtain both hydrophilic and hydrophobic contaminants. Whatever methods of OH/AH/PAH residue analysis are used, the investigator needs to know the wet, dry, and lipid weight of the sample. This need stems from the fact that both the water and fat content and type will differ in blubber samples dependent on the health and relative hydration of the animal. Methods used for residue analysis must be validated through reference standards for OH/AH/PAH to ensure quality and comparability of residue quantitation.

Recommendations

1. Analysis of organohalogen residues in marine mammal tissues has been completed with techniques that provide excellent reproducibility. All analyses must include reference materials to assess the accuracy of the measurements and to allow comparisons among different studies. Marine mammal tissues are currently being prepared as control materials.

2. Analyses for organohalogens should allow for correlation among samples with different wet/dry/lipid content.

Question 5

What are the known effects of xenobiotics and heavy metals on other animals and can this knowledge be extrapolated to dolphins?

Response

The organohalogens (PCBs, TCDD, TCDF, etc.) are known to cause immune system dysfunction in rats, man, monkeys, cattle, and avian species, and the assumption is that they cause the same responses in marine mammals. There are some significant problems in interpretation of data derived from animals exposed to complex mixtures of pesticides, insecticides and herbicides. Some of the compounds showing up as environmental contaminants are known to bind to the Ah receptor (induction of cytochrome P450s), whereas others are known to act as xenobiotic steroids and apparently bind to the cortisone type and/or estrogen type receptors. Wasting syndrome, weight loss, immune dysfunction (including thymic atrophy), porphyrias, chloracne and dermal lesions and fetotoxicity are all associated with exposure to pollutant chemicals that bind to Ah and steroid receptors. The precise biochemical and physiological mechanisms by which ligand binding to receptors initiates toxic responses are not known. Some of the OH, AH and PAH have estrogenic activity and some have anti-estrogenic activity. Care must be taken in attempting to interpret data for animals exposed to mixtures of contaminants.

Toxic metals analysis in cetaceans, typically whales of a variety of species, has not led to an overt correlation of toxicity,

neoplasia, or death of animals with high residue levels. It is not known whether metal contamination is synergistic to OH/AH/PAH toxicity.

Recommendations

1. Different potential surrogate species should be evaluated in relation to their responses to OH/AH/PAH exposure. Mink and pinnipeds are known to react adversely to OH, but it is not known if these animals are appropriate surrogates for dolphins.

2. Research on surrogate species should include investigation of interactions between OH/AH/PAH and heavy metals.

Question 6

What in vitro methods are being applied or could be applied to dolphin mortality research?

Response

There is an immediate need for studies on dolphin cells to determine baseline data on cytochrome P450 induction and activity, DNA adduct levels correlated with OH/AH/PAH tissue residues, and glutathione depletion or enhancement analyses. Such studies would be best suited to a fetal derived cell line, preferably of hepatocytes or keratinocytes. A hepatocarcinoma line would be less useful but more likely to be obtained and more practical than a fetal hepatocyte line. Only one of these cell lines, a fetal keratinocyte line, is currently available for study (originated by Busbee and Carvan, Carvan Dolphin Kidney, CDK). A series of immortalized CDK derivatives have been initiated using pSV3.neo, an SV40-derived plasmid that expresses large T-cell antigen as the only virally encoded protein in the host cell. Additional immortal cell lines can be initiated from tumor tissue; however, tumor tissue from dolphins is not regularly available.

Recommendations

1. Continue development of *in vitro* fetal cell lines and/or immortalized cell lines for use in toxicological analyses. Cell lines of hepatocytes and epithelioid cells should be a high priority.

2. Initiate methods development for *in vitro* determination of cytochrome P450 induction, for evaluation of *in situ* DNA adduct levels in stranded animals or in capture-release dolphins, and for assessing *in situ* glutathione content.

3. Compare *in vitro* and *in situ* techniques, especially with respect to correlations with exposure to DNA damaging agents.

4. Institute a strategy for recovery of biopsied, rather than necropsied, dolphin tumor tissue from which immortal cell lines might be initiated.

Question 7

Can dolphins be used as a "pollution biomarker" with respect to potential environmental health hazards?

Response

Established biomarkers of pollutant exposures that are pre-clinical or pre-acute should be developed. Rather than trying to establish the cause of a complex series of morphological and physiological changes in a morbid animal, there is a need for identification of small, pre-morbid changes associated with exposure to organic and inorganic contaminants.

Recommendations

1. *In vitro* studies should be pursued to develop the means to utilize dolphins as biomarkers of pollution.

2. Contaminant residue analyses of tissues should be further evaluated with regard to physiological factors (e.g., lipid content and lipid profiles) and life history factors (e.g., age, gender, maturity, reproductive status) to improve the assessment of dolphin exposure to organic and inorganic contaminants.

3. *In vitro* analyses should be pursued to determine the cellular responses of dolphins to OH/AH/PAH exposure.

4. Develop a reliable mechanism (such as HLA typing for humans or PCR and DNA fingerprinting) for determining the genetic identity of animals.

Physiology/Biomarkers of Effects

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Remarks

It is possible to determine causes of dolphin die-offs only if the biological mechanisms that link cause and effect are understood. By conducting non-invasive evaluations of healthy dolphins and using the vast amount of data generated from studies of laboratory animals, causes of mortality can be better understood. This scientific approach requires expanded capture/ release programs to characterize the biology of living dolphins. Further, this approach requires public and scientific consensus that studies of laboratory models are adequate alternatives to invasive studies of dolphins to determine effects of stressors (e.g., pollutants, brevetoxins, viruses).

Three levels of investigation can link dolphin mortality with potential causes. Pathological studies define the final disease state of dead dolphins. Physiological dysfunctions responsible for the diseased state present a second tier of investigation. Competent pathological studies can provide insight into this tier. For example, studies of the pathology ofstranded dolphins have indicated that the diseased animals were heavily infected with opportunistic bacterial species, indicative of an underlying immune (physiological) dysfunction. A third tier of investigation involves determination of causes of the dysfunction witnessed at the physiological level. Possible causative agent(s) that would be investigated at the third level include pollutants, toxins, and viruses.

Question 1

Is there reasonable understanding of the biology of healthy dolphins at the cellular and molecular levels of organization?

Response

Very little research on dolphin physiology has incorporated modern techniques of cell and molecular biology. Since dolphins (and all marine mammals)

have become high-profile species, it is not possible to sacrifice animals for study. Consequently, research must be performed on stranded (morbid or dead) dolphins or on live dolphins using non-invasive techniques in capture-release programs or oceanaria. The value of studying morbid or dead animals is limited, since most if not all of the normal biological functions of these animals is minimal or non-existent. The understanding of most of the cellular biology of these animals is derived solely from capture-release specimens. Interpretation of the data gathered from such studies should also consider information gained from studies of non-marine laboratory mammals.

Recommendations

1. Strong support should be given to capture-release programs because these are the only source of relatively healthy free-swimming individuals for study.

2. Studies on captive dolphins should be initiated where non-invasive measurements are monitored for variations related to season, reproduction, feeding, and other controllable or measurable factors.

3. Extrapolation of data from non-marine laboratory mammals should be emphasized in toxicological studies, since dose-response studies cannot be conducted on dolphins.

4. Training of NMFS observers on fishing vessels to collect samples for cellular and molecular study could augment information collected from live healthy animals.

Question 2

Which are the most important physiological systems that could be linked to dolphin mortalities?

Response

Many physiological systems are important to understanding stress and mortality in bottlenose dolphins. The following were considered most sensitive to environmental stressors:

Reproduction
Neurology and Behavior
Endocrine physiology
Immunology
Metabolism (bioenergetics, nutrition)
Renal physiology
Gastro-intestinal physiology

These systems have been shown to be susceptible to stressors, such as pollutants, in studies of other mammal species. Polychlorinated biphenyls (PCBs), for example, have been shown to impair reproduction, behavior, endocrine physiology, and immune function in rats. Renal function can be severely impaired when rodents are exposed to methyl mercury. Lead has dramatic effects on behavior. Gastro-intestinal function can be demonstrably perturbed by entero-pathic bacterial species. Given the constraints imposed by non-invasive studies of dolphins and the high probability that immune dysfunction plays some role in unusual dolphin mortality

events, understanding the immune system and using it as an indicator of health should be a high priority.

Recommendations

1. Develop and standardize new non-invasive techniques to measure elements of each of the seven disciplines (above) considered most sensitive to environmental stressors.

2. Pursue a better understanding of immunological functions and dysfunctions of dolphins.

Question 3

For the most critical physiological systems, what technical hurdles limit their use in determining causes of dolphin mortalities?

Response

Most technical hurdles to investigating critical physiological systems stem from the necessity of obtaining samples using non-invasive techniques. Several non-invasive techniques are currently available or under development:

Reproduction: genital size via ultrasound; sperm viability from sperm capture techniques; blood hormone levels.

Neurology/Behavior: electro-encephalograms with dermal patches; neurotransmitters in serum; behavioral observations using ethograms.

Endocrine physiology: endocrine biochemicals from blood serum.

Immunology: B and T lymphocyte proliferation; cytotoxic T cell killer assay; antibody production; lymphocyte phenotype profiles; macrophage function.

Metabolism: lipid levels, retinoic acid, porphyria and fluid electrolytes from serum; DNA adducts from whole blood; blubber thickness using ultrasound.

Renal physiology: standard assays (BUN, creatinine) from blood and urine.

Gastro-intestinal physiology: digestive/absorptive efficiencies from the stool.

Recommendations

1. Continue the development and application of non-invasive techniques toward understanding dolphin immunological systems.

2. The continued development of non-invasive techniques for physiological measurements requires a coinciding progression in sampling methodology and quality assurance; quality methods should be generated and standardized for each new physiological measurement.

3. Liquid nitrogen storage of live samples should be included as an essential element of archiving, as it allows for supplementary analysis of cellular function.

Question 4

To what extent can we predict dolphin biology and responses to stress from existing laboratory models?

Response

Information derived from other biological systems, such as rodent models, should at least be used to prioritize areas of research. In Question 2, specific tissues were identified for study based on the knowledge that such tissues in other species were highly sensitive to certain xenobiotics. Additionally, hypotheses can be forwarded from existing laboratory models. For example, the adverse effects of 2,3,7,8-TCDD (dioxin) on immune function in rodents has been extensively documented. Therefore, similar studies on dolphins could be initiated to determine whether parallels exist. A prudent study design would involve measurement of immunological endpoints in dolphins which are the most sensitive endpoints in rodent laboratory models.

It is clear that while dolphins may be similar to rodent models or humans in general biological terms, reagents and assays need to be developed to accommodate inherent species-specific differences. For example, most mammalian proteins retain highly conserved cross-species functions, but they can exhibit significant epitopic variability. It is possible then, to use rodent data to predict the function of various dolphin proteins but ultimately species-specific monoclonal antibodies will be needed to identify them.

Recommendations

1. Rodent models should be emphasized as laboratory surrogates for prioritizing and guiding dolphin research; rodents are phylogenetically related and an enormous scientific foundation exists at the cellular and molecular levels for both normal and compromised animals.

2. Reagents (monoclonal antibodies) specific to dolphins must be developed for application to relevant investigations.

Question 5

Is there a research strategy that would enhance the use of cellular and molecular endpoints in determining the cause(s) of mortalities?

Response

A good understanding of dolphin physiology at the cellular and molecular level will require sampling of healthy, or living individuals. The simplest strategy is to relate xenobiotic or stressor dose with physiological dysfunction through animal sacrifice and analysis, i.e., establish a typical laboratory dose-response relationship. But since dolphins are a protected species and cannot be sacrificed, less direct means must be used to obtain the same information.

This is a situation similar to human health concerns. For human health issues,

medical science uses two major pathways to establish information on healthy and compromised individuals: laboratory models (surrogate species, in vitro cell culture), and non-invasive physiological measurements. The same must be followed for dolphins, as has been described in response to other questions above.

However, given the rapid decreases in dolphin populations around the world, the option of animal sacrifice may become more attractive as determination of cause becomes more pressing. The public and scientific community would like to avoid sacrifice of individuals, but when survival of an entire population is at stake, indirect methods may be insufficient.

Recommendations

1. Provide long-term support for capture-release programs to establish normal ranges of physiological values; the sampling font approach should be epidemiological rather than opportunistic to insure statistically relevant data.

2. Develop in vitro cell culture techniques using dolphin tissue to pursue xenobiotic dose-response relationships.

3. Investigate similarities and differences of surrogate species, including other marine mammals and rodents.

4. Establish criteria for limited sacrifice of individuals for scientific study.

Stranding and Sampling Logistics

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Remarks

This working group examined lgistics of stranding events and sampling required for a proper scientific response. This included consideration of the limitations of current stranding networks consisting primarily of lay volunteers. This working group addressed questions pertaining to sampling protocols and data forms, administrative channels, sampling techniques and volunteer and scientific response to strandings. A summary of recommendations follows:

1. Implement existing protocols and make any organizational changes that are needed for implementation.

2. Actively seek new participation by laboratories, universities, and government agencies to expand the capabilities of some regions and to initiate operations in others.

3. Pursue and develop alternate funding sources to supply networks.

4. Initiate a philosophical change from a "volunteer" to a more "professional" network and more active input of NMFS laboratories in terms of time and resources.

5. Develop a set of national priorities for study in order to concentrate efforts on certain questions such as the development of cell culture lines and standard histological sets or obtaining baseline data on the natural history of populations.

Question 1

How might current sampling protocols and data forms be improved to increase data collection and improve scientific response?

Response

In terms of available protocols, the recent release of a training handbook by Dr. S. J. Geraci and V. Lounesburg ("Marine Mammals Ashore," Texas A&M Sea Grant Publication) and the forensic manual being prepared by Dr. S. Galloway will more than adequately cover procedures required for a scientific response. Improvement is needed more in use of the protocols than the protocols themselves. An upgrade in the level of expertise of personnel in stranding networks required for proper implementation and quality assurance. The video produced by the

Southeastern U.S. Marine Mammal Stranding Network *Video Guide to Recording Marine Mammal Stranding Data* will aid immensely in training, but covers only measurements and data required for the basic stranding report and does not detail forensic procedures. Current NMFS scientific requirements are mirrored in existing protocols but expectations should be modified depending on the condition code of the stranded animal; maximal information can be derived from code 1 and 2 animals. These animals should be transported to a facility staffed by well-trained personnel. Quality control for all samples rests solely with individual researchers who request samples. They alone are responsible for training network personnel. Scientific needs will change over time; the system should be flexible to respond quickly to those needs.

Recommendations

1. Provide training to upgrade expertise in stranding networks.

2. Attract new laboratories and agencies into stranding network organizations to expand the capacity and upgrade scientific response.

Question 2

Can administrative channels be altered to foster a greater research effort?

Response

The changing focus toward better-trained personnel may require initiation of Letters of Authorization to denote different levels of training. Network personnel must agree to higher expectations regarding data and tissue collections or be denied a Letter of Authorization. A Chain of Custody form is not desired and should not be required under routine conditions. A Chain of Custody procedure should be implemented in unusual mortality events, especially if there is a possibility of future litigation. Transport of collected tissues is prohibited without authorization, which is generally granted by NMFS with few (if any) restric tions. Current NMFS policy that allows qualified individuals access to tissue bank samples for research or teaching is appropriate.

Recommendations

1. Strandings should be prioritized by condition code to maximize data collection for code 1 and 2 animals and reduce effort on animals of higher codes due to the progressive deterioration of tissues.

2. Letters of Authorization should reflect training of stranding network personnel and NMFS expectations. The more demanding procedures required for code 1 and 2 animals should be met by trained personnel only.

3. A policy pertaining to proper procedures for a Chain of Custody should be developed by the Unusual Mortality Event Working Group of NMFS. Simple criteria to determine cases that would benefit from a Chain of Custody procedure should also be generated.

4. A policy should be developed to require data-sharing from sample end users (scientists) relating to the

results of analyses. The data should be included in a federal repository and made available to other researchers. This requirement should be similar to the policy of the Marine Mammal Tissue Bank.

Question 3

How is the quality of sample collection and storage assured?

Response

The quality of sample collection and storage is unquestionably a direct function of proper training for stranding network volunteers and scientific personnel. All samples must be properly identified with a field number assigned by the network followed by proper curation and/or storage. Effectiveness of the procedures can be assessed only by end users, i.e., scientists who ultimately derive a product. At this time, end users have no formal means to evaluate sampling procedures. A feedback mechanism needs to be formalized to assure quality control and also provides a means to modify procedures.

Recommendations

1. Ensure adequate materials and training for stranding network volunteers to assure quality of samples.

2. Continue efforts to improve training materials and training programs.

3. When possible, the American Society of Mammalogists Curatorial Guidelines should be applied to storage of samples.

4. Develop mechanisms to solicit feedback from end-users on the quality of samples and incorporate this information into protocol improvements.

Question 4

Do sampling techniques provide the range and quality of materials required for current and emerging research efforts?

Response

There are increasing demands being placed on network volunteers. Researchers must take into account the realities of sampling and modify or simplify protocols to be functional under a variety of conditions. They should also be realistic about the amount of time required for their particular procedures in light of all of the other sampling that network personnel may be asked to perform. Foreknowledge of sampling priorities can alleviate many of the sampling problems.

Recommendations

1. Initiate pilot studies to assess the changes which take place over time with tissue deterioration and the impacts (if any) of this on the usability of tissues with changing condition code of an animal. This could be incorporated into studies designed to characterize "normal" dolphins.

2. Establish priorities for sampling so that network personnel can first satisfy those elements of highest priority.

3. The availability of material could be greatly increased if NMFS observers on fishing vessels were trained in collection of life-history information and specimens for analysis; free-ranging dolphins are accidentally caught in the fishing nets, but are otherwise healthy and could augment understanding of the "normal" condition.

Question 5

How can the volunteer response be improved to meet growing scientific and monitoring needs?

Response

The stranding networks have been relatively successful in reporting stranded marine mammals. There are geographical gaps in coverage of some segments of the U.S. coastline and these are regions where some investment of time and effort, particularly in developing new grass-root organizations of volunteers, may yield some positive results. Improvements in existing networks are also required as higher quality and different types of samples are needed for developing research areas. In terms of dealing with mass mortality events, the Southeast Region of NMFS is better equipped than a few years ago due to the development of the Task Force, the identification of key individuals with specialized expertise, and the placement of emergency response kits with both NMFS laboratories and stranding network personnel in all of the major stranding areas.

Recommendations

1. Organize new volunteers to cover gaps in the network reporting of marine mammal strandings.

2. Institute a philosophical shift in stranding network programs that requires a higher level of expertise for volunteers to enable higher quality samples for research. This should include the addition of better trained volunteers at high quality laboratory facilities, increased training for current olunteers, and movement toward a more professional network.

3. Implement a multi-tiered response approach to address the problem of coverage. Such a strategy could include key "index" areas where there is a very efficient coverage of the beaches, a high recovery rate of fresh specimens and high quality facilities and personnel to examine animals. Other areas will have a lower grade of response and be used to monitor trends in stranding rates and for possible occurrence of unusual mortality events.

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