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16

Multi-Trial Ecotoxicological Diagnostic Tool in Cetacean Skin Biopsies

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1. Introduction

In the last 20 years there has been growing concern about potential hazard to cetaceans occasioned by multiple stress factors due to bioaccumulation of anthropogenic contaminants combined with infectious diseases, food depletion and climate change. In this context, the main aim of this chapter is to present the development and the application of a non-lethal multi trial biomarker tool applied to cetacean skin biopsies to diagnose "toxicological stress syndromes" related to multiple human pressures. Here we present a multidisciplinary approach to detect presence and effects of anthropogenic contaminants combined with general stressors using a wide range of diagnostic markers (molecular and gene expression biomarkers, immunological and nutritional status) with contaminant analysis, for a complete assessment of health status of cetaceans. The chapter is subdivided in three sections: in section one we describe the main threats faced by cetaceans focusing on the case study of Mediterranean Sea; in the second part we will describe the sensitivity of the multi trial biomarker diagnostic tool in skin biopsy cetacean samples; and in the third part we will describe the application of the proposed multidisciplinary approach to explore the effects of multiple human pressures on cetacean health status (field applications), comparing fin whale (*Balaenoptera physalus*) (mysticete) population of Mediterranean compared to the one of Sea of Cortez (Mexico). Final development of this interdisciplinary diagnostic methodology, embedded in a statistical Expert System, will provide exhaustive information about "toxicological status" of cetaceans as a powerful frontier approach for the conservation of relevant species of sea biodiversity.

2. Section one - Threats faced by cetaceans: The case study of Mediterranean sea

Threats facing cetaceans have changed through time. While overkill from hunting was the most obvious and immediate threat to some species and populations during much of the 20th century, the relative importance of other threats, particularly bycatch in fisheries and contamination, has increased dramatically during the last few decades. It is difficult to distinguish the effects of one threat from those of another when multiple threats are acting simultaneously; this is particularly evident in the Mediterranean area. Exposure to contaminants combined with other human impacts can affect survivorship, recruitment,

reproductive success, mutation rates and migration and may play a significant role in the partitioning of genetic variation among high stress exposed and less stress exposed populations (Whitehead et al., 2003). Taking into account this complex pattern, international institutions like IWC and ACCOBAMS have recently encouraged research for development of suites of sensitive non-lethal tools in skin biopsies of free-ranging animals, to define the "health status" of threatened cetacean species (Pollution 2000+, IWC Project). Threats faced by cetaceans can be schematized below.

2.1 Pollution: Bioaccumulation and effects of anthropogenic contaminants

Mediterranean top predators, and particularly cetacean odontocetes, accumulate high concentrations of anthropogenic contaminants such as organochlorine compounds (OCs), polycyclic aromatic hydrocarbons (PAHs) and trace elements. The pollutant burden is lower at the lowest levels of the food chain and becomes higher as the position in the food web increases. Important factors for bioaccumulation are also metabolic rates, gender, age and adipose tissue concentration. The ecotoxicological risk of some species is also related to their "biochemical vulnerability" to lipophilic xenobiotics due to the low capacity of their detoxifying enzymatic systems (Tanabe & Tatsukawa, 1992; Fossi et al., 1997). Since the incidence of pathology in these species is closely related to the level of pollution in their environments, bacterial and viral infections and contaminants must be considered from a holistic point of view. Mass mortalities of dolphins and seals have occurred in particularly polluted areas (such as the Mediterranean Sea), when levels of OCs, PAHs and heavy metals reached very high levels. It is reported that lipophilic contaminants cause immune and reproductive dysfunctions (Hammond et al., 2005). Some contaminants, such as OCs are also known as endocrine disrupting chemicals (EDCs) (Fossi et al., 2003). In fact several examples suggest that exposure to levels of OC insecticides and PCBs commonly detected in Mediterranean odontocetes has affected endocrine function and reproduction in other marine mammal species (Fossi & Marsili, 2003). Moreover there is growing concern about accumulation and effects of emerging contaminants and polybrominated diphenyl ethers (PBDEs, a major family of flame retardants) in the food chain. PBDEs are lipophilic, persistent and toxic to fauna and humans (Alaee et al., 2003). The highest levels of PBDEs have been found in top marine predators, including Mediterranean odontocetes. Also phthalates (found in numerous commercial products as additives and used primarily to soften polyvinyl chloride - PVC) are a relevant and emerging cause of concern for these species, considered as potential EDCs. Some phthalates have been linked to reproductive effects and altered hormone levels, however the effects and concentrations of these compounds have not been assessed yet. Another important class of contaminants for cetaceans is PAHs, derived from both natural (e.g., oil spill, forest fires, natural petroleum seeps) and anthropogenic sources (e.g., combustion of fossil fuels, use of oil for cooking and heating, coal burning). Studies have shown that PAHs with four or more rings can induce dioxin-like activity and weak estrogenic responses (Villeneuve et al., 2002). As pointed out by the 2010 scientific expedition undertaken by the program Mediterranean Endangered, new priorities in marine environment contamination are the so-called microplastics (usually defined as plastic particles smaller than 5 mm) (Barnes et al., 2009). Micro debris floating on the Mediterranean Sea have reached 115,000 particles per km^2 with a maximum of 892,000 particles. Impacts of microplastics to organisms and the environment are largely unknown. More than 180 species have been documented to absorb plastic debris including planktophagous species. For example, laboratory study has shown that krill species *Euphasia*

pacifica ingested 20µm polyethylene fragments. Clearly, considerably reducing microplastic inputs must be a priority of this decade for the biodiversity conservation in the EU strategy.

2.2 Diseases: Immunosuppression and infectious diseases

Cetaceans are normally susceptible to a large plethora of pathogenic organisms. For example, the family *Cyamidae* comprises 28 species of parasitic crustaceans, all of which live exclusively on the cetaceans skin (Haney et al., 2004). The majority of these infective organisms are repelled by innate host defences that include nonimmunological anatomical and physiological barriers (e.g. mucociliary blanket), antimicrobial factors (e.g. lactoferrin, defensins, immuno-lysozyme) and immunological effector cells (e.g. neutrophils, eosinophyls, macrophages and natural killer cells). Several studies have suggested that contaminants could have immunosuppressive effects on marine mammals (Beckmen et al., 2003). Since 1987, at least 8 epidemics of *Morbillivirus* infection (MI) have caused mass mortality of various free-living pinniped and cetacean populations worldwide. Along the coast of Spain and Italy new cases of MI were reported in 2007 and 2008. Another intriguing issue regards the synergic effects, associated with chronic exposure to a number of environmental pollutants, such as OCs and trace elements, as previously mentioned. In fact, it is also unknown whether and how these chemicals contribute towards modulating the pathogenic and pathogenetic activity primarily displayed by sea mammal MI (Di Guardo et al., 2005).

2.3 Bycatch

Several species of marine mammals are at risk of extinction from being captured as bycatch in commercial fisheries. In the Mediterranean Sea sperm whales (*Physter macrocephalus*), striped dolphins (*Stenella coeruleoalba*), and short-beaked common dolphins (*Delphinus delphis*) are affected by pelagic driftnets and gillnets. The only mitigation measure applied today is the ban of swordfish driftnet fishery (Young & Iudicello, 2007). Other approaches that have been developed and implemented to address this problem, include devices and gear changes, time and area closures and moratoria. The scientific committee of ACCOBAMS and the IWC indicate the bycatch as one of the major threats to marine mammals. Between 1999 and 2004, SOS Grand Bleu counted 50,000 deaths due to sword fish "spadare".

2.4 Shipping, collision and noise pollution

All cetacean species, from fin whales to bottlenose dolphins (*Tursiops truncatus*) and from pelagic to coastal species are potentially subjected to collisions with boats, even if most collisions go unreported. Several cases of collisions between fin whales and large ships are reported from stranding cetaceans and from photo-identification data (Panigada et al., 2006). Cetaceans need acoustic signals to navigate, find and capture prey, and to locate mates, social partners and predators. Heavy vessel traffic, seismic testing, dredging, and drilling for oil and gas cause noise travelling thousands of km underwater. There is no doubt that cetaceans react to noise, but it is extremely difficult to quantify the effects and establish thresholds of disturbance. It is reported that use of mid-frequency long-range active sonar by navy caused the stranding of 11 Cuvier's beaked whales (*Ziphius cavirostris*) on the coastline of Greece (Frantzis, 1998).

2.5 Food depletion

Fisheries can have a major impact upon the status and distribution of various cetaceans. In the Mediterranean Sea, the decline in high-order marine predators feeding on epipelagic

prey (cetaceans, large pelagic fish) is consistent with the hypothesis of prey depletion, presumably due to intensive exploitation of fish stocks (over-fishing). In fact the combination of some 50,000–100,000 small gillnet fishing boats, plus large bottom trawlers, has depleted numerous fish, crustacean, and mollusc populations. In eastern Ionian Sea, the short-beaked common dolphin shows evidence of decline correlated with food depletion (Bearzi et al., 2006).

2.6 Climate change

It is generally agreed that the Mediterranean region is an area sensitive to global climate change, due to its position on the edge of the climatologically determined Hadley cell, which makes it a transition area between two very different climate regimes to the North and the South (Li et al., 2006). Many global and regional models simulate several degrees of warming (from 3 to 7°C) in the Mediterranean by the end of the 21st century. The potential impacts of climate change include drought, decline of water quality, floods, and change in seawater temperature/salinity, the rising of sea levels and reduction of biodiversity. This would pose several threats to cetaceans, mainly linked to changes in the distribution of sufficient quality and quantity prey of marine mammals (Simmonds & Nunny, 2002). Climate-related effects may combine with other factors such as pollution and disease to have significant impacts on cetacean populations.

2.7 Genetic erosion

Studies to date have indicated a complex pattern of population genetic structure for most cetacean species investigated. Seasonal patterns of movement and the possibility of extremely large scale dispersal, or local isolation (sympatric or parapratic) between populations, generate a mosaic of genetic diversity that cannot easily be determined by an intuitive assessment of geography. Although the Mediterranean basin is closed and has the heavily industrialized coasts, it hosts several cetacean species biologically differentiated between pelagic (*Stenella coeruleoalba*) and coastal (*Tursiops truncatus*) odontocetes and the resident mysticete (*Balaenoptera physalus*). Previous studies showed evidence of clear genetic differentiation between Atlantic and Mediterranean populations of striped dolphin and found much lower genetic variation of Mediterranean specimens (Garcia-Martinez et al., 1999). Gene flow through the Gibraltar Strait appears to be limited and easily prevents the genetic exchange between the Atlantic and Mediterranean cetacean populations. For the determination of population structure of wild species, genetic information about population subdivision has practical applications for short- and long-term strategies for animal management, conservation and protection (Hoelzel, 1998) and genetic diversity appears to be a major factor determining the success of a species in harsh environments. High genetic diversity within a population increases the chance for survival of at least some individuals, and possible changes in the environment, due to natural causes or anthropogenic pressures, can be better tolerated. Exposure to contaminants combined with other human impacts can affect survivorship, recruitment, reproductive success, mutation rates and migration, and may play a significant role in partitioning genetic variation among high stress exposed and less stress exposed populations (Whitehead et al., 2003). This is particularly true for populations inhabiting a closed basin characterized by considerable concentrations of environmental contaminants (OCs), orders of magnitude higher than those detected in oceanic environments. Bioaccumulation of pollutants, combined with other factors, such as

immunosuppression, infectious diseases, climate change, and food depletion could soon lead to a further additional decrease in total genetic variability of Mediterranean cetacean populations, posing alarming threat to these species.

3. Section two - Skin biopsy for diagnosis of anthropogenic threats

Cetacean skin biopsies are biological material suitable for the hazard assessment of free-ranging cetaceans (Fossi et al., 2010). Our research team firmly believe that the use of cetacean skin biopsies, as non-lethal biological material for the hazard assessment, is a powerful procedure to screen a large number of samples, with a minimal disturb to animals. In this chapter integument biopsy will be described as diagnostic tool for the comprehensive diagnosis of multiple stress factors, health status and genetic population variability. The diagnostic set developed in our lab and described in this chapter will enable to detect: i) presence of contaminants, ii) exposure to contaminants, iii) reproductive alteration, iv) genotoxicity, v) immunosuppression, vi) general stress, vii) feeding ecology (Fig.1).

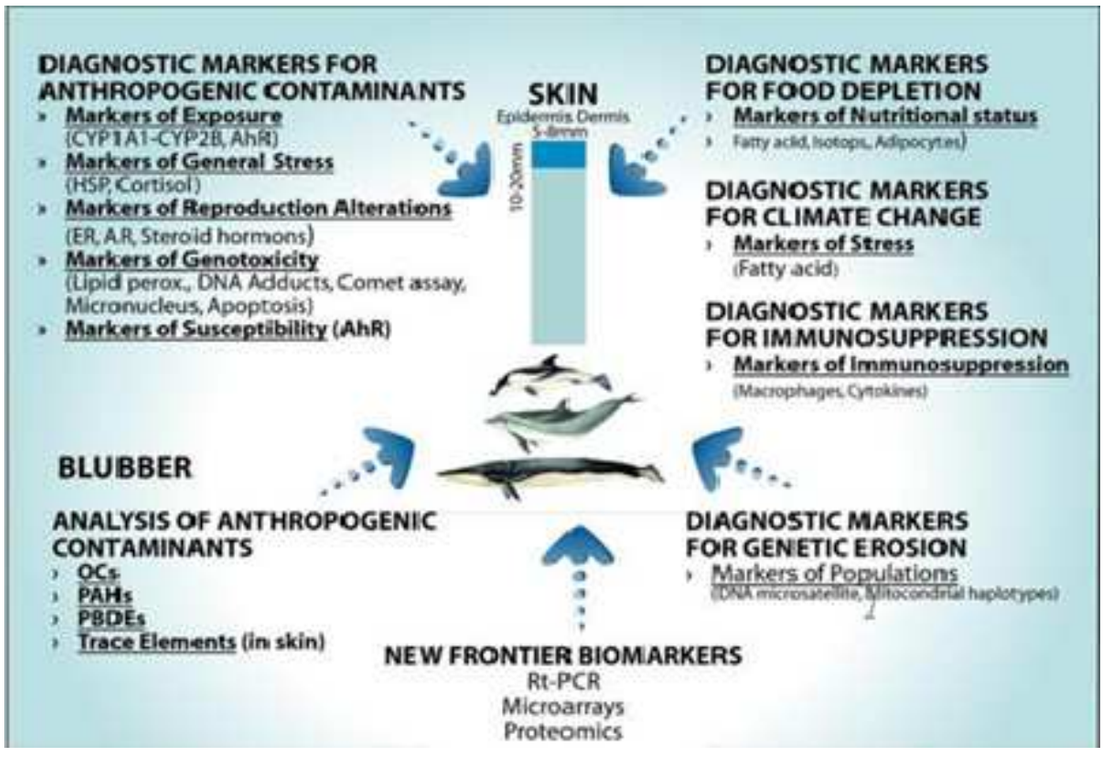

Fig. 1. Skin biopsy for diagnosis of anthropogenic threats

3.1 Sampling of integument biopsies

Samples of skin biopsy or integument biopsy (epidermis, dermis and blubber) can be obtained from free-ranging dolphin (es. *Tursiops truncatus, Stenella coeruleoalba)* from different study areas using an aluminium pole armed with biopsy tips (0.7 cm ø, 3.0 cm length) or with a crossbow and darts. Integument biopsies from large odontocete (es. *Physeter macrocephalus*) or mysticete *(Balaenoptera physalus)* can be obtained using a crossbow

and darts armed with tips (0.9 cm ø, 4.0 cm length). A Barnett Wildcat II crossbow with a 150-pound test bow, using a biopsy dart with modified stainless steel collecting tip and floater, can be used to collect biopsies. To avoid the possibility of infection, the bolt tip need to be sterilised before shooting. Biopsy samples can be taken between the dorsal fin and the upper part of the caudal peduncle. The procedure consists in approaching the specimen at low-to-moderate speed and shooting the dart at a range of 20-50 m. The skin biopsy will be immediately placed in liquid nitrogen or stored in cell medium (CITES Nat. IT025IS, Int. CITES IT 007).

3.2 Detection of anthropogenic contaminants

Analysis of contaminant levels provide important data about exposure of cetaceans, that can be correlated with the biomarker data for a complete evaluation of their toxicological stress. The blubber layer of cetacean integument biopsy is the target tissue for the analysis of lipophilic and non-lipophilic contaminants in free-ranging populations (Fossi et al., 2000). A set of major POPs, trace elements and emerging contaminants can be analysed in skin biopsies of different cetacean species. The methods are reported below.

Trace elements: For trace elements analysis, lyophilised and homogenised samples can be acid-digested and analysed for trace elements using Atomic Absorption Spectrometry and Emission Spectrometry with FIMS-AAS (Flow Injection Mercury System) for determining Hg, THGF-AAS (Transversely Heated Graphite Furnace) and ICP-AES (Inductively Coupled Plasma).

Organochlorines: The analytical method used for quantitative and qualitative analysis of HCB, DDTs, PCDDs, PCDFs and PCBs will be High Resolution Capillary Gas chromatograph equipped with an electron capture detector (63Ni ECD)(AGILENT 6890/N), according to the U.S. Environmental Protection Agency (EPA) 8081/8082 modified (Marsili & Focardi, 1996). The GC has a SPB-5 bonded phase in a fused silica capillary column, 30 m long.

PAHs: Levels of PAHs and PAH fingerprint will be evaluated by High Performance Liquid Chromatography (Waters 600 HPLC) with Fluorescence Detector (Waters 474 Scanning Fluorescence Detector) and UV Detector (Waters 2487 Dual ë Absorbance Detector); PAH separation will be performed using a reversed phase column with an acetonitrile/water gradient (Marsili et al., 1997).

PBDEs: To detect PBDE, sample extracts will be analyzed on a GC/MS system (HP 6890 gas chromatograph coupled to an HP 5973 low-resolution mass spectrometer) using both EI and negative-chemical ionization (NCI) on an HP-5MS (5% phenyl methyl siloxane) capillary column, according to Pettersson et al. (2004).

Emerging contaminants (Pthalates): Several analytical methods have been developed for determining phthalates by GC (Feng et al., 2005), and HPLC (De Orsi et al., 2006) using different preconcentration techniques (Li et al., 2008).

3.3 Markers of exposure

A set of biomarkers of exposure to anthropogenic contaminants can be investigated in cetaceans skin biopsies (Fig.1). Particular attention will be paid to:

Cytochrome P450 (CYP) - Cytochrome P450 is the most important metabolic/detoxifying enzyme system in mammals. It is substrate-inducible and substrate-specific. CYP1A and CYP2B have been detected in cetacean skin and the induction of these isoforms was found after exposure to lipophilic contaminants both *in vitro* and in field studies (Fossi et al., 2006;

Montie et al., 2008; Hooker et al., 2008). Induction of CYP isoforms can thus be considered as a powerful biomarker of exposure. The induction of different CYP enzymes can be investigated by western blot (WB), immunohistochemistry and quantitative real-time PCR (qRT-PCR) (Spinsanti et al., 2006, 2008).

Nuclear receptors (AhR) - The cytosolic aryl hydrocarbon receptor (AhR) is a ligand inducible nuclear transcription factor which can induces the transcription of xenobiotic metabolizing enzymes such as CYP1A. AhR gene expression was found to be significantly induced in skin of chloracne patients subject to long-term exposure to dioxins and dibenzofuranes (Tang et al., 2008). Presence and modulation of AhR in cetacean skin samples will be here investigated by qRT-PCR.

3.4 Markers of reproductive alterations

Endocrine Disrupting Chemicals (EDCs) are a structurally diverse group of compounds that may adversely affect the health of humans and wildlife, or their progenies, by interaction with the endocrine system (Colborn, 1998). Many of the known EDCs are estrogenic, affecting particularly reproductive functions. A main mechanism of endocrine disruption occurs through receptor-mediated mechanisms or the cross-talk of these receptors with the AHR. In skin biopsy we can investigate the gene expression of steroid hormone receptors and any possible alterations due to contaminants.

Steroid hormone receptors. Estrogen receptor(s) (*ER)*, androgen receptor(s) (*AR*) and progesteron receptor(s) (*PR*) presence can be investigated in biopsy samples of cetaceans by using WB and immunohistochemistry techniques. Gene expression will also be investigated by qRT-PCR. Steroid hormone receptors are known to be induced in various species and tissues by specific contaminants.

Steroid hormones and steroidogenic enzymes. A set of key genes (Hilscherova et al., 2004) involved in steroid hormone synthesis in humans can be investigated in cell cultures and skin biopsy samples by qRT-PCR and WB, to explore the expression and its potential alterations due to toxicants. We will explore the up/down-regulation of some genes including: CYP11A (cholesterol side-chain cleavage), CYP11B1 (steroid 11β-hydroxylase), CYP11B2 (aldosterone synthetase), CYP17 (steroid 17αHSD1-hydroxylase), CYP19 (aromatase) and CYP21B2 (steroid 21-hydroxylase).

3.5 Markers of genotoxicity

DNA integrity is essential for the correct transmission of genetic information to the next generation. Chemicals and/or physical agents that have the potential to cause loss of the DNA's structural and functional integrity are defined genotoxics (Shugart, 2000). Following failing of the repair mechanism, structural changes in DNA become irreversible (apoptosis, chromosome aberrations) and the cells may exhibit altered functions, from uncontrolled proliferation cells to carcinogenesis (Lee & Steinert, 2003). Finally the damage caused by genotoxic compounds leads to DNA mutations and possible alterations in subsequent generations (Gil & Pla, 2001). In this chapter we will present how to evaluate the presence of DNA damage and the efficiency of cells repair mechanism by comet assay. Diffusion assay will be used to evaluate apoptosis in cells.

The Comet Assay or single cell gel electrophoresis (SCGE) is a sensitive, rapid and inexpensive method for measuring DNA damage in individual cells. As reported by Frenzilli et al. (1999) the single cells are embedded in agarose on microscope slides, lysed to

remove the majority of the proteins, electrophoresed, then stained with Sybr Safe in order to visualize the DNA. As result, the DNA of undamaged cells appears as a spherical mass occupying the cavity formed by the lysed cell. On the contrary, cells with DNA damage form a comet image, due to the amount of DNA fragments: smaller is the fragment size, greater is their number and the percentage of DNA that migrates in an electric field. The assay can be performed to examine DNA single strand breaks or to measure double strand breaks in individual cells.

Markers associated with apoptosis: to detect the apoptotic cells, a comet assay modified protocol will be used (Singh et al., 1988). This test, as the comet assay, is relatively easy to apply to most eukaryotic cell types, it is sensitive for detecting low levels of DNA damage or multiple classes of DNA damage and require a small cells number (Dhawan et al., 2009).

Lipid peroxidation: it involves a set of chain reactions by ROS because of their double bonds. Lipid peroxidation is the result of interactions of lipidic radicals and/or formation of non radical species by lipid proxy radicals. The resulting LOOH (lipid hydroperoxide) can easily decomposes into several reactive species, including lipid alkoxyl radicals (LO), aldehydes, lipid epoxides and alcohols. Most of these products are toxic and active mutagens. Peroxidised membranes become rigid and lose permeability and integrity (Valavanidis et al., 2006). Lipid peroxidation will be investigated in skin biopsies by measuring levels of MDA using a spectrophotometric test.

3.6 Diagnostic markers of immunosuppression

Mammal skin is an important immune organ, displaying various defence mechanisms which can be divided into three major functional compartments: epithelial defence, innate-inflammatory immunity and adaptive immunity (Meyer et al., 2007). IIn this biological material we will develop and validate a set of biomarkers in cultured fibroblasts and skin biopsies to monitor the immunological status of cetaceans. The Major Histocompatibility Complex (MHC) is a set of molecules displayed on cell surfaces that are responsible for lymphocyte recognition and "antigen presentation". These molecules control the immune response through recognition of "self" and "non-self" and are of two types: Class I and Class II molecules. In the Class I molecules there is a family of polymorphic genes, named MIC genes, where MICA and MICB are functional. MIC genes are mainly expressed in endothelial cells and fibroblasts. MIC molecules are considered to be stress-induced antigens that are recognized by cytotoxic T cells and natural killer (NK) cells. The evaluation of MICA protein expression in cetaceans can be used to evaluate the status of the immune system of the different species and will be investigated by WB, immunohistochemistry, qRT-PCR and indirect immunofluorescence (Marsili et al., 2008).

3.7 Diagnostic markers of general stress

Cetaceans are exposed to a diverse array of multiple stressors and here we propose a suite of skin biomarkers to assess general stress.

Heat-shock proteins (HSPs): A number of environmentally and chemical agents are known to induce a set of cellular stress proteins, the heat-shock proteins (HSPs) (Nover, 1991; Nover & Scharf, 1991). Their amount increases rapidly when cells are subjected or exposed to a wide variety of stresses such as heat, xenobiotics or drugs; pathological stimuli such as viral, bacterial and parasitic infections, inflammation and autoimmunity (Wu & Tanguay, 2006). In skin biopsy we can explore the expression of HSPs by WB and qRT-PCR (Cao et al., 1999; Rossner et al., 2003).

Cortisol alteration: Cortisol is mainly involved in carbohydrate, lipid, and protein metabolism and serves as an indicator of an animal's state of well-being, as its levels increase during times of distress ("stress hormone"). We can measure levels of cortisol in the blubber biopsies of cetaceans by enzyme immuno-assays. In fact adipocytes are equipped with a functional G6PT-hexose-6-phosphate dehydrogenase-11ß-hydroxysteroid dehydrogenase type 1 system that is a potential target for modulating local glucocorticoid activation (Marcolongo et al., 2007).

3.8 Diagnostic markers of feeding ecology

Assessing the diet and trophic position of cetaceans is important for understanding the ecology of marine food webs. Decreased quantity and quality of prey, due mainly to over-fishing, have been cited as factors promoting population decline of Mediterranean cetaceans (Bearzi et al., 2006). Feeding ecology is a fundamental aspect in the understanding, management and conservation of free-ranging marine mammals. Nutritional status of marine mammals is a factor that can limit reproductive output and thus population growth. However, foraging of large cetaceans can be difficult to assess when direct observation is not possible, and the use of chemical feeding ecology (i.e., stable isotope and fatty acid analysis) has become increasingly important (Dehn et al., 2006). Stable isotopes of carbon and nitrogen, evaluated using Isotope Ratio Mass Spectrometer (Herman et al., 2005), have been established as powerful tools in animal ecology. They occur naturally, and nitrogen isotope ratios of prey are reflected in tissues of the consumer, with slight enrichment occurring at each trophic step (Kelly, 2000). Stable carbon isotopes are generally used to provide information on spatial habitat use and carbon sources rather than trophic relationships as they enrich in consumer tissues only to a minor degree (Burton & Koch, 1999). An increase of $\delta^{15}N$ values has been shown in specimens in particular conditions such as nutritional stress (Fuller et al., 2005) or disease (Katzenberg & Lovell 1999). Thus, measurements of the fatty acid composition of the blubber of marine mammals, evaluated using quadruple gas chromatography/mass spectrometry (Krahn, 2004), allow the identification of the diet of these animals. The differences either between individuals or between populations (Møller et al., 2003) or between species (Borobia et al., 1995), are a direct function of their prey fatty acid composition.

3.9 Diagnostic markers for genetic erosion and population studies

Accurate screening of current overall genetic variability of Mediterranean cetaceans and its constant monitoring over the years would identify potential environmental threats and interspecies differences in susceptibility to contaminants.

DNA microsatellites: DNA microsatellites are short (10-50 copies) tandem repeats of mono- to hexa-nucleotide units, assumed to be randomly distributed throughout nDNA, cpDNA and mtDNA. SSRs can appear as simple, interrupted and compound repeats, and are usually distributed across non-coding (and, less often, coding) regions of the genome. Irrespective of hypotheses about their evolutionary mechanisms (slipped-strand mispairing (slippage) error during DNA replication of a single DNA double helix or recombination), SSRs have been appliedas a highly variable and powerful genetic marker in areas ranging from ancient and forensic DNA studies to population and conservation genetics (Jarne and Lagoda, 1996). Nucleotide sequence analysis of the genes encoded in the mitochondrial genome (mtDNA) constitutes an additional powerful tool of modern phylogeography and molecular population genetics (Bickham et al., 2000; Gaspari et al., 2007). mtDNA is located

outside the nucleus and it encodes for a subset of the proteins necessary for the mitochondrion itself. The number of copies of mitochondrial genes greatly outnumbers that of nuclear encoded genes. The mitochondrial genome is inherited solely through the maternal line in a clonal fashion and usually does not undergo recombination. Moreover, its rapid rate of base substitution makes the mtDNA genome an ideal marker for establishing levels of variability between and among populations or species (Natoli et al., 2004, 2005).

4. Section three - The case study of the multi-trial diagnostic tool in fin whale *(Balaenoptera physalus)* skin biopsies of the Pelagos Sanctuary (Mediterranean Sea) and the Gulf of California (Mexico)

In this section we describe the application of the proposed multidisciplinary approach to explore the effects of multiple human pressures on cetacean health status (field applications), comparing *Balaenoptera physalus* cetacean population of Mediterranean with the Sea of Cortez population. As previously mentioned (section one) over the past decades, there has been a growing concern regarding the potential threat to Mediterranean cetaceans from persistent organic pollutants such as organochlorine compounds (OCs) (Fossi et al., 2006) and polybrominated diphenyl ethers (PBDEs). Cetaceans of the Gulf of California (Sea of Cortez – Mexico) are reputed to be less exposed to anthropogenic pressure. To date, OC concentrations have been investigated in only three marine mammal species from the Gulf of California (Niño-Torres et al., 2009). The main objective of this case study (Fossi et al. 2010) was to develop and apply a set of sensitive non-lethal diagnostic biomarkers to skin biopsies of fin whales to evaluate the toxicological status of this mysticete in the Pelagos Sanctuary (Ligurian, Corsica and North Tyrrhenian Seas) and in the Gulf of California. We propose a ''multi-trial diagnostic tool", combining molecular biomarkers and gene expression with the analysis of OCs, PAHs and PBDEs.

Mediterranean fin whale – The fin whale is the only mysticete that is regularly found in the Mediterranean Sea (Bérubé et al., 1998), facing a number of anthropogenic threats, such as chemical and acoustic pollution, entanglement in fishing gear and disturbance and collisions from commercial and pleasure boats.

Gulf of California fin whale – Fin whales are permanent residents of the Gulf of California. This population of approximately 610 animals (Urbán-Ramírez et al., 2005) is considered one of the most isolated in the world (Bérubé et al., 1998), and it constitutes a unique and separate conservation unit vulnerable to anthropogenic effects. Although the Gulf of California is considered one of the most pristine and bio-diverse areas of the world (hosting 36 species of marine mammals), increasing human activity is beginning to affect it.

4.1 Experimental design

4.1.1 Sampling

Integument biopsies were obtained from free-ranging fin whales in the Pelagos Sanctuary (n = 12, 6 males and 6 females) and the Gulf of California (n = 5, 3 males and 2 females) during the summer of 2008, using biopsy darts launched with a crossbow (CITES Nat. IT 025IS, Int. CITES IT 007). Sex was determined according to Bérubé and Palsbøll (1996).

4.1.2 Skin biopsy as diagnostic tool

To validate this ''multi-trial diagnostic tool", a two-phase experimental protocol was followed (Fig. 2). In the first phase of the project (field studies), we applied a multi-

disciplinary methodology to explore the effects of the exposure of Mediterranean and Mexican fin whales to anthropogenic contaminants, using skin biopsies as a diagnostic tool and combining the analysis of molecular biomarkers (Western Blot (WB) of CYP1A1 and CYP2B) and gene expression (qRT-PCR of HSP70, ERa, AHR, E2F-1) with the analysis of OC, PAH and PBDE residues in subcutaneous blubber. In the second phase (in vitro experiments), whale biopsy slices were treated with mixtures of OCs, as an innovative tool for the study of intra-species sensitivity to various classes of environmental contaminants.

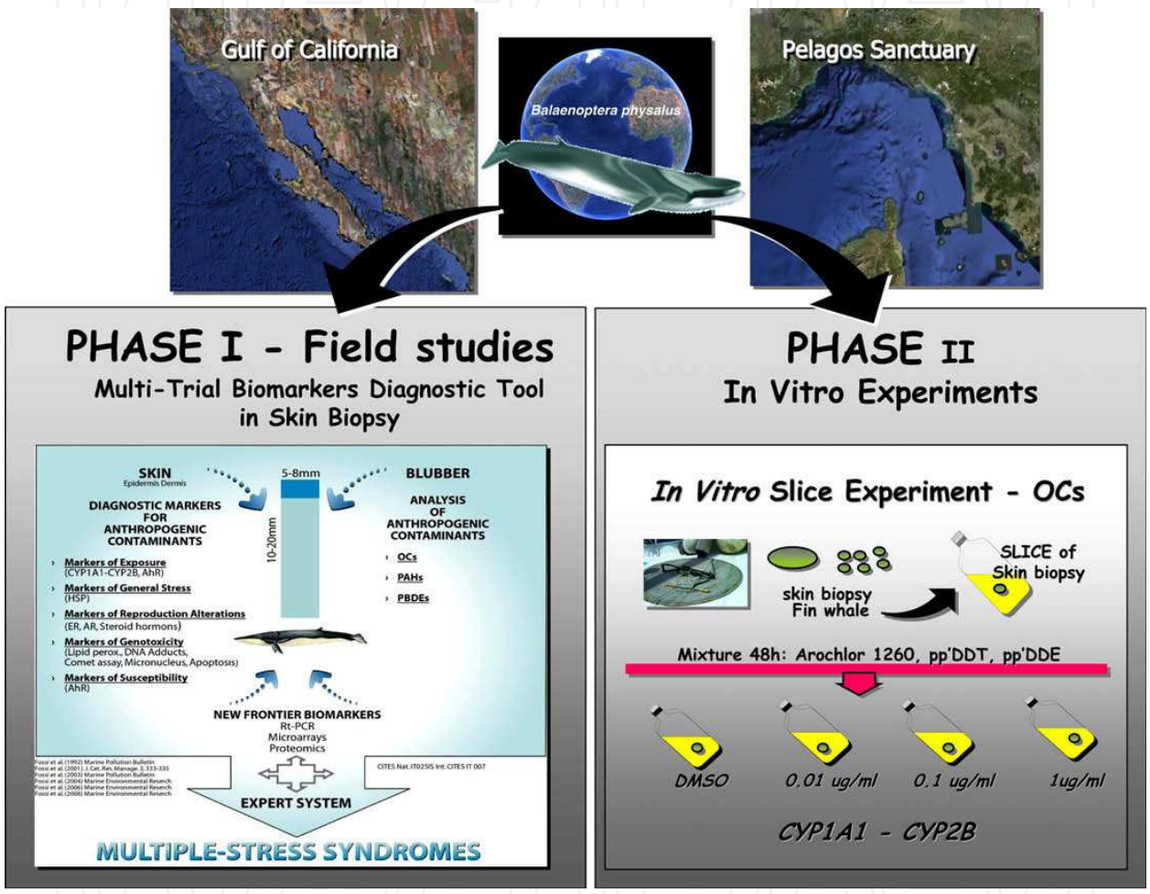

Fig. 2. Experimental design and sampling areas – validation of a ''multi-trial diagnostic tool" to evaluate the toxicological status of fin whales in the Pelagos Sanctuary (Mediterranean Sea) and the Gulf of California (Sea of Cortez – Mexico). A two-phase experimental protocol was followed: first phase: field studies; second phase: in vitro experiments (Fossi et al, 2010).

4.1.3 Results

The two populations of fin whales (first phase results) showed differences in contaminant levels and biomarker responses.

Higher levels of PCBs, DDTs, OCs-EDCs and PAHs were found in both male and female (PAHs $p < 0.1$) Mediterranean fin whales in comparison to the Cortez specimens (Fig. 3a–d), confirming the high toxicological stress to which the fin whale population in the Pelagos Sanctuary is exposed.

Levels of low-brominated PBDEs were higher in samples from the Sea of Cortez (Fig. 3e), ranging from 282 to 30,506 ng/g dw, while samples from the Mediterranean sea showed lower average levels. The most abundant congener was PBDE 47. In general, samples from the Sea of Cortez had a major number of detected congeners, such as 47, 100, 99, 154 and 153.

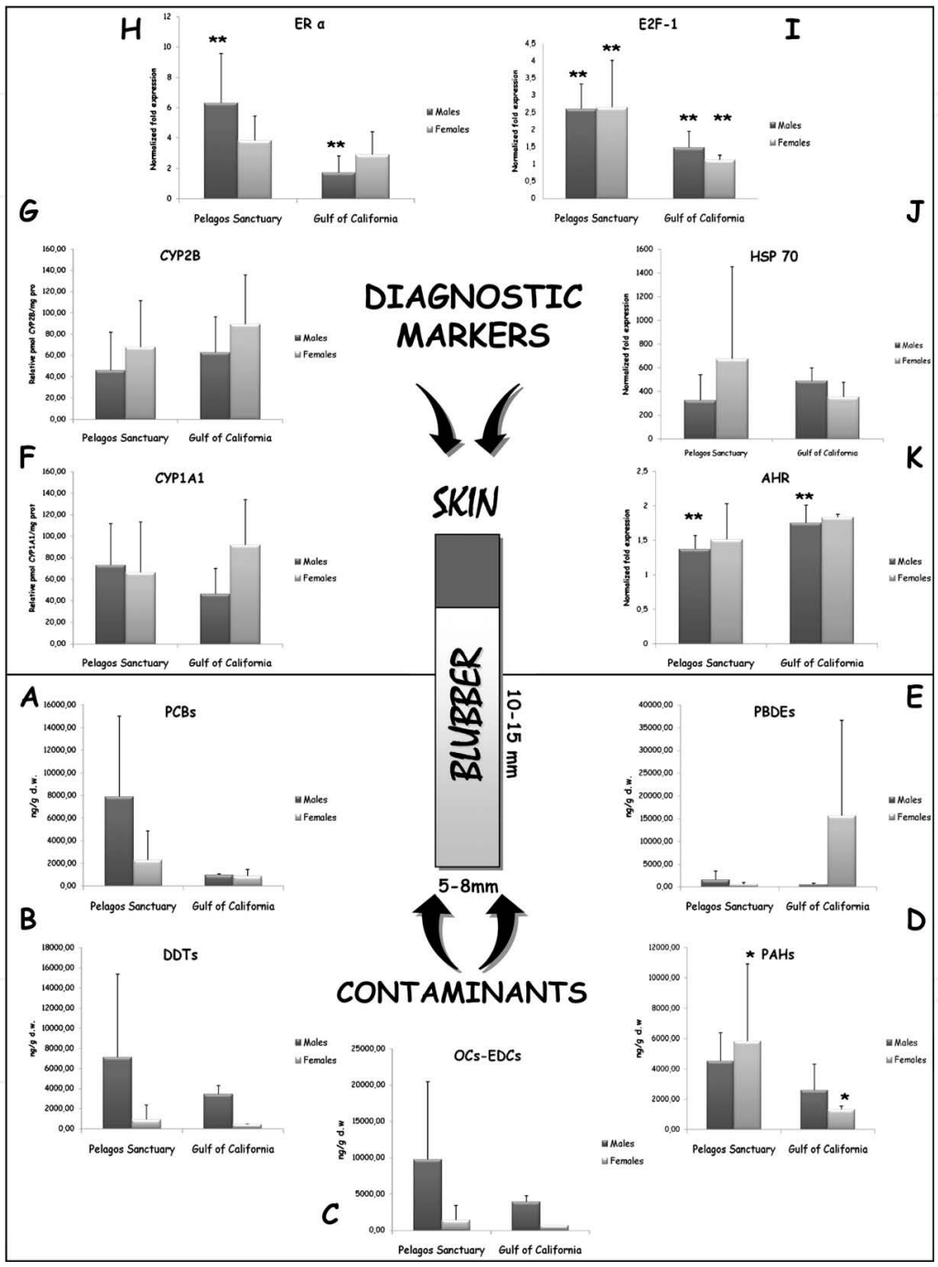

Fig. 3. First phase results – contaminant levels (PCBs (A), DDTs (B), OCs-EDCs (C), PAHs (D), PBDEs (E)) and biomarker responses WB of CYP1A1 (F), CYP2B (G), gene expression (qRT-PCR) of ERa (H), E2F-1 (I), HSP70 (J), AHR (K) in skin biopsies of specimens from the two populations of fin whales (*= $p < 0.1$; ** = $p < 0.05$). Pelagos Sanctuary (n = 12,males = 6; females = 6); Sea of Cortez (n = 5, males = 3; females = 2). (Fossi et al, 2010).

Exploring molecular biomarker responses, the induction of CYP1A1 in Mediterranean male whales, if compared to males from the Gulf of California (Fig. 3f), can be related to the presence of high levels of planar compounds, such as coplanar PCBs, and PAHs (Fig. 3a and 3d). A statistically significant positive correlation (rho Spearman = 0.73, p = 0.003) was found between total PAHs and CYP1A1 induction in male (n = 11) specimens.

A lack of CYP2B induction, despite high levels of lipophilic contaminants, was evident in both male and female Mediterranean whales, even though the differences between the Mediterranean and Mexican specimens are not statistically significant (Fig. 3g). On the other hand, a preliminary warning signal is represented by the high induction of CYP2B in the Mexican fin whales (Fig. 2g), but further investigations are needed.

Exploring gene expression biomarker responses (Fig. 2h–2k), the ERa and E2F-1 genes were up-regulated in the specimens from the Pelagos Sanctuary with respect to those from Mexico for both males (ERa: 3.6_-fold; E2F-1: 1.7_-fold) and females (ERa: 1.3_-fold; E2F-1: 2.4_-fold) ($p < 0.05$). These data suggest,in the first case (ERa), high exposure to EDC compounds such as OCs-EDCs and, in the second case (E2F-1), the presence of apoptosis processes as a sign of toxicological stress in the Mediterranean population. In contrast, the expression of HSP70 is higher in the Mexican male specimens than that of the Pelagos Sanctuary (1.5_-fold), whereas the Pelagos females exhibit an overexpression of the HSP70 gene with respect to the Mexican specimens (1.9_-fold). The AHR gene is slightly up-regulated in the Mexican specimens ($p < 0.05$ in males).

The results of the second phase (in vitro tests) show marked differences in CYP1A1 and CYP2B induction by OCs in the whale biopsy slices of the two populations (male specimens), with higher sensitivity responses in the Mexican mysticetes. A dose-dependent induction of CYP1A1 was detected only in biopsy slices from Cortez specimens (0.01 μg/ml = 2.6-fold, 0.1 μg/ml = 3.6-fold and 1 μg/ml = 4.4-fold with respect to the control). The in vitro tests showed no induction of CYP1A1 and CYP2B for the male Mediterranean whales (slices).

In conclusion, this ‘‘multi-trial diagnostic tool”, applied to skin biopsies, underlined differences in OC, OC-EDC, PBDE and PAH levels and molecular and gene expression biomarker responses between the two populations. The presence of a higher ‘‘toxicological stress” in the Pelagos population is highlighted by warning signals such as CYP1A1 induction and the up-regulation of ERa and E2F-1 genes, combined with a lack of CYP2B induction in both field and in vitro experiments. Moreover, particular concern arises from the high levels of low-brominated PBDEs found in the Mexican whale specimens. Future development of this methodology could provide a statistical system for obtaining more complete information about the ‘‘toxicological stress syndrome” in cetaceans, providing a predictive model for hazards in susceptible areas targeted by increasing tourism, such as the Gulf of California (Fossi et al, 2010).

5. Conclusion

The data reported in this chapter confirm that cetacean skin biopsies are a biological material suitable for the hazard assessment of free-ranging cetaceans. This chapter shows that the use of cetacean skin biopsies, as non-lethal biological material for the hazard assessment, is a powerful procedure to screen a large number of samples, with a minimal disturb to animals. Here integument biopsy is proposed as diagnostic tool for the

comprehensive diagnosis of multiple stress factors, health status and genetic population variability. The methods proposed could be used to:

- support in ranking the threatened levels for cetaceans populations/species;
- as an operative tool, suitable for both scientific and decision making purposes supporting in identification of proper mitigation measures;
- find application on two main EU areas of interest: Convention on Biological Diversity (CBD) (2010-2020) - EU Marine Strategy.

The final outcome of these studies will be the development of a prototype DSS - Decision Supporting System for ranking toxicological risk of cetacean populations/species based on integration and analysis of data from the above diagnostic approach. Such a scheme is currently not available to the scientific community nor to policy makers worldwide, neither in the terrestrial nor marine environment. In case of success these achievements are susceptible to be applied on a wider-scale to other marine threatened species.

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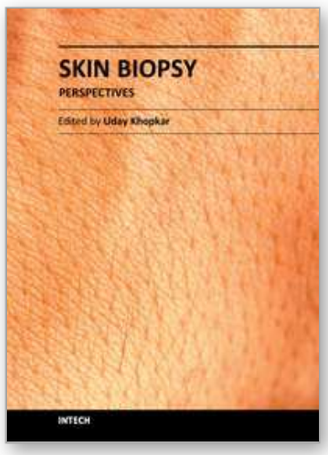

Skin Biopsy - Perspectives

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Skin Biopsy - Perspectives is a comprehensive compilation of articles that relate to the technique and applications of skin biopsy in diagnosing skin diseases. While there have been numerous treatises to date on the interpretation or description of skin biopsy findings in various skin diseases, books dedicated entirely to perfecting the technique of skin biopsy have been few and far between. This book is an attempt to bridge this gap. Though the emphasis of this book is on use of this technique in skin diseases in humans, a few articles on skin biopsy in animals have been included to acquaint the reader to the interrelationship of various scientific disciplines. All aspects of the procedure of skin biopsy have been adequately dealt with so as to improve biopsy outcomes for patients, which is the ultimate goal of this work.

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