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A multi-trial diagnostic tool in fin whale (*Balaenoptera physalus*) skin biopsies of the Pelagos Sanctuary (Mediterranean Sea) and the Gulf of California (Mexico)

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ABSTRACT

The main objective of this study was to apply a set of sensitive non-lethal biomarkers in skin biopsies of fin whales (*Balaenoptera physalus*) to evaluate the toxicological status of this mysticete in the Pelagos Sanctuary (Mediterranean Sea) and in the Gulf of California (Sea of Cortez-Mexico). We developed a "multi-trial diagnostic tool" (based on field and *in vitro* studies), combining molecular biomarkers (western blot of CYP1A1, CYP2B) and gene expression (qRT-PCR of HSP70, ERα, AHR, E2F-1) with the analysis of OCs, PAHs and PBDEs. The study revealed a higher level of toxicological stress in the Mediterranean fin whales.

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 study was to develop and apply a set of sensitive non-lethal diagnostic biomarkers to skin biopsies of fin whales (*Balaenoptera physalus*, Linnaeus, 1758) 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.

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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 Palsbøl (1996).

Experimental design – To validate this "multi-trial diagnostic tool", a two-phase experimental protocol was followed (Fig. 1). 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, ERα, 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.

Slices experiment – Slices of skin biopsies were incubated for 24 h in cell culture media with different mixtures of OCs (Arochlor 1260, pp′DDT and pp′DDE in DMSO) at three doses: 0.01 μg/ml, 0.1 μg/ml and 1 μg/ml, plus a DMSO (0.05%) control. CYP1A1 and CYP2B were detected by WB (Fossi et al., 2008).

Organochlorine compounds (HCB, DDTs and PCBs) were analysed according to the US EPA 8081/8082 method. The "EDC-OCs" consist of analysed OCs known as endocrine disruptors.

Low-brominated (tri- to hexa-) BDEs were analysed using GC-LRMS-ITD in the MS/MS operating mode, using the isotope dilution technique as described elsewhere (Gómara et al., 2006).

Polycyclic aromatic hydrocarbons levels were quantified with high performance liquid chromatography (Marsili et al., 1997).

CYP1A1–CYP2B western blot: CYP1A and CYP2B have been detected in cetacean skin, and the induction of these isoforms has been found after exposure to lipophilic contaminants such as OCs, PAHs and PBDEs, in both *in vitro* and field studies (Fossi et al., 1992, 2006; Godard et al., 2004). Analysis of CYP1A and CYP2B was performed in integument biopsies by WB in duplicate for each sample, using goat anti-rabbit CYP1A1 and CYP2B4 (Oxford MI, USA). Semi-quantitative analysis was performed with Quantity One software (BioRad) (Fossi et al., 2008).

E2F-1–HSP70–ERα–AHR: Quantitative real time PCR assay: the heat shock protein 70 (HSP70) is a stress-related protein belonging to a multi-gene family. It has been found to be induced by multiple stimuli, including PAHs and heavy metals.

The aryl hydrocarbon receptor (AHR) is a soluble ligand-activated transcription factor, a member of the basic-helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) gene superfamily. It is involved in processes that activate CYP1A and CYP1B.

Oestrogen receptors (ERs) are ligand-inducible transcription factors. Compounds such as PCBs and PBDEs can bind oestrogen receptors and interfere with signalling pathways.

The E2F-1 transcription factor (E2F-1) controls some genes involved in DNA synthesis, and its over-expression seems to up-regulate several genes involved in the activation of apoptosis.

Subsamples of integument biopsies were used for gene expression analyses by qRT-PCR. Each biopsy was homogenised, and the RNA was isolated using an Aurum Total RNA Fatty and Fibrous Tissue Kit (Biorad). One microgram of RNA was retro-transcribed with an iScript cDNA Synthesis Kit (Biorad). The real time PCR reaction conditions were set as described in Spinsanti et al. (2006). The primers were designed for the specific sequence of *B. physalus* by using Beacon Designer Software (Premier Biosoft International). The primer sequences used are as

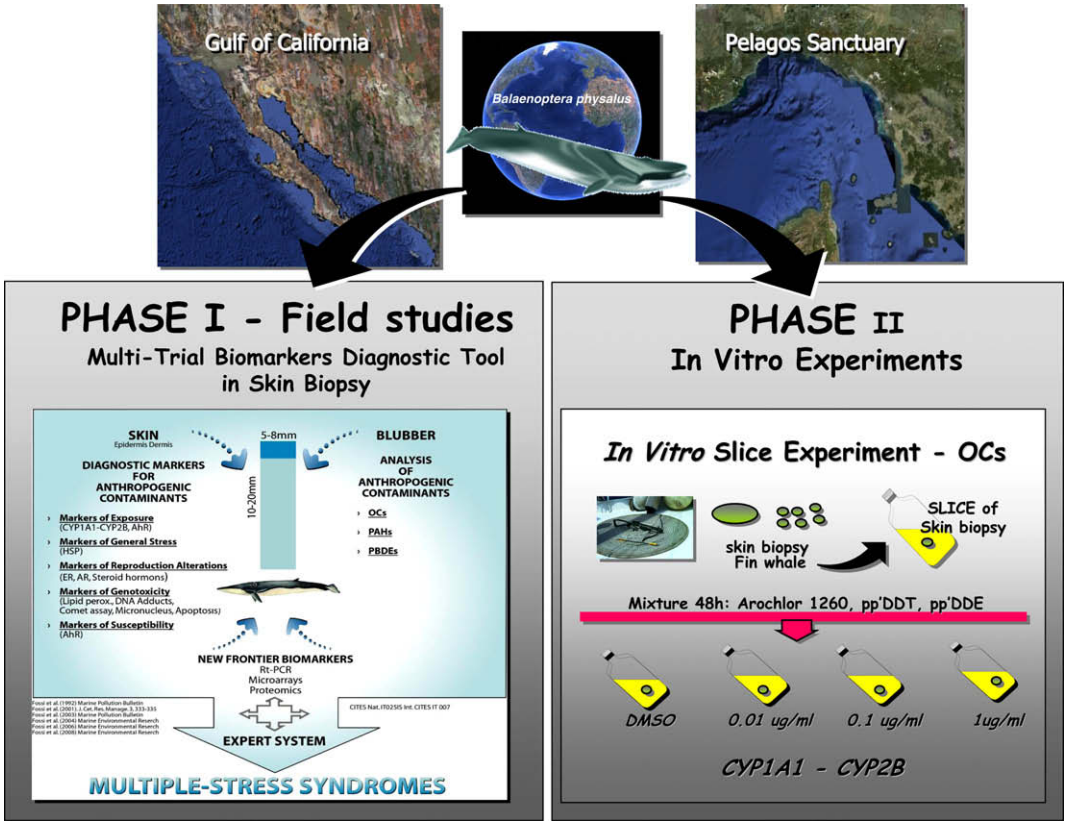

Fig. 1. *Experimental design and sampling areas* – validation of a "multi-trial diagnostic tool" to evaluate the toxicological status of fin whales *(Balaenoptera physalus)* in the Pelagos Sanctuary (Mediterranean Sea) and the Gulf of California (Sea of Cortez – Mexico). A two-phase experimental protocol was followed: first phase: field studies; second phase: *in vitro* experiments.

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follows: E2F-1 Fw 5′-TGCCACCACCACCATCATCTC-3′ E2F-1 Rv 5′-CGAGTCAGCCGCCACCAG-3′; HSP70 Fw 5′- AAGGGTCGTCTGA GCAAGG-3′, HSP70 Rv 5′- TTCTCG TCTTCCACCGTCTG-3′; ERα Fw 5′-GGAGACTCGCTACTGTGC-3′, ERα Rv 5′- CTCCTCTGCGGTCTT TCC-3′; AHR Fw 5′- CTTGTGGCACCACCGTAGC-3′, AHR Rv 5′- GTC CACCATACGTACAGACCG-3′. Each sample was run in triplicate, and the expression of the four genes of interest was normalised with the control genes GAPDH and YWHAZ using the ΔΔCt method. The gene expression was calculated using GenEx software v. 4.3.8 (MultiD Analyses AB).

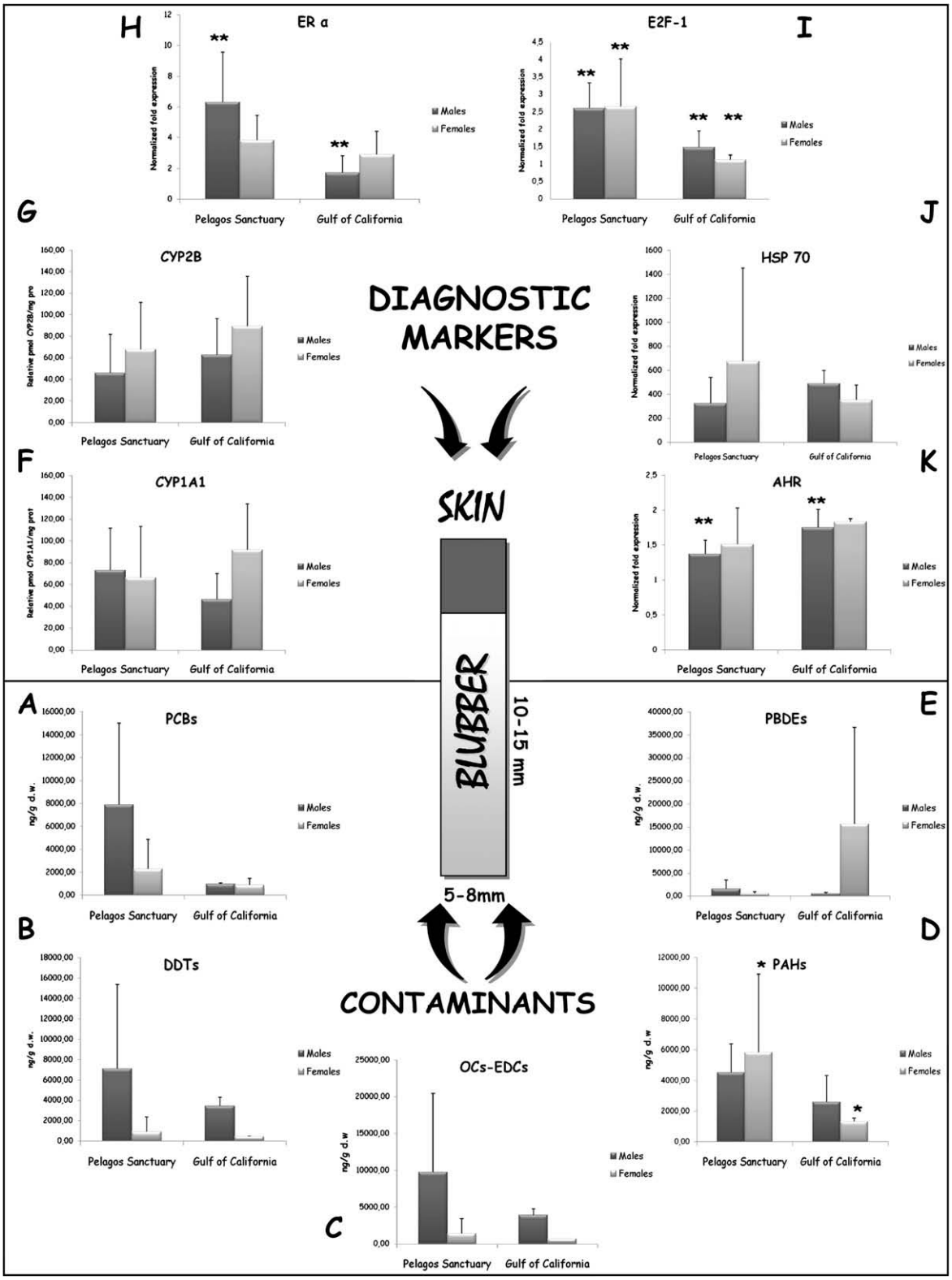

Fig. 2. *First phase results* – contaminant levels (PCBs (A), DDTs (B), OCs-EDCs (C), PAHs (D), PBDEs (E)) and biomarker responses WB of CYP1A1 (F), CYP2B (G), gene expression (qRT-PCR) of ERα (H), E2F-1 (I), HSP70 (J), AHR (K) in skin biopsies of specimens from the two populations of fin whales (* =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

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Statistical analysis – The data were processed using non-parametric tests. Differences among groups of data were tested by the Kruskal–Wallis test and the Mann–Whitney test. The Spearman rank order correlations were used as a non-parametric test to estimate possible correlations between variables.

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. 2a–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. 2e), 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.
- Exploring molecular biomarker responses, the induction of CYP1A1 in Mediterranean male whales, if compared to males from the Gulf of California (Fig. 2f), can be related to the presence of high levels of planar compounds, such as coplanar PCBs, and PAHs (Fig. 2a and d). 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. 2g). 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–k), the ERα and E2F-1 genes were up-regulated in the specimens from the Pelagos Sanctuary with respect to those from Mexico for both males (ERα: 3.6×-fold; E2F-1: 1.7×-fold) and females (ERα: 1.3×-fold; E2F-1: 2.4×-fold) ($p < 0.05$). These data suggest, in the first case (ERα), 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, OCs-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 ERα 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.

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