BIOMARKER RESPONSES AND CONTAMINANT LEVELS IN FIN WHALE (*Balaenoptera physalus*) SKIN BIOPSIES OF THE PELAGOS SANCTUARY (MEDITERRANEAN SEA) AND OF THE GULF OF CALIFORNIA (MEXICO)

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ABSTRACT

The main objective of this study was to apply a suite of sensitive non-lethal biomarkers in skin biopsy 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-biomarker-tool", combining molecular biomarkers (western blot of CYP1A1, CYP2B) and gene expression (qRT-PCR of HSP70, ER, AhR, E2F-1) with analysis of OCs, PAHs and PBDEs. In the first phase we explored the level and the effects of OCs, PBDEs and PAHs in skin biopsies of fin whales of the two populations. In the second (in vitro) phase we applied this approach to whale biopsy slices treated with mixtures of OCs and PBDEs in order to explore the toxicological effects of these contaminants. The multi-trial biomarker tool applied to skin biopsies underlined differences in OCs, OCs-EDCs, PBDEs, PAH levels and molecular and gene expression biomarker responses between the two populations, revealing a higher toxicological stress in the Mediterranean fin whales. Of particular concern were the high levels of low brominated PBDEs found in the Mexican whale specimens.

KEYWORDS

Monitoring, *Balaenoptera physalus,* Mediterranean Sea, Gulf of California, pollutants, biomarkers.

INTRODUCTION

In the last decades there has been a growing concern regarding the potential threat to Mediterranean cetaceans by persistent organic pollutants such as organochlorine compounds (OCs) (Fossi *et al.*, 2002, 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, OCs concentration has been investigated in only three marine mammal species from the Gulf of California: blue whale (*Balaenoptera musculus)* (Valdez-Marquez *et al.,* 2004), California sea lion (*Zalophus californianus)* (Niño-Torres *et al.,* 2009), and fin whale (*Balaenoptera physalus)* (Niño-Torres *et al.,* in press).

The main objective of this study was to develop and apply a suite 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 *(*Fossi *et al*., 2009). In this paper we proposed the development of a diagnostic "multi-trial-biomarker-tool" to apply to skin biopsies, combining molecular biomarkers (western blot of CYP1A1 and CYP2B) and gene expression (qRT-PCR of HSP70, ER, AhR, E2F-1) with analysis of OCs, PAHs and PBDEs. The two studied areas and, presumably the two fin whale populations, are characterized by different anthropogenic impacts.

Mediterranean fin whale - The fin whale is the only mysticete that regularly occurs in the Mediterranean Sea. Genetic analyses performed on Mediterranean specimens revealed a limited gene flow with North Atlantic conspecifics, showing that this may be a diverging population (Bérubé *et al.*, 1998; Palsbøll *et al.,* 2004). During

the summer months, this species is known to concentrate in high numbers in the Pelagos Sanctuary (Notarbartolo di Sciara *et al.,* 2003). Line-transect surveys, conducted in the western Ligurian Sea in August 1992 yielded an estimate of 901 fin whales (% CV 21.77, 95% CI 591-1,374; Forcada *et al.,* 1995). Fin whales in the Mediterranean basin face a number of actual and potential anthropogenic threats, such as chemical and acoustic pollution, entanglement in fishing gear and disturbance from commercial and pleasure boats (Jahoda *et al.,* 2003). Moreover, ship strikes are common in Mediterranean waters and most likely represent the major cause of non-natural mortality for fin whales (Panigada *et al.*, 2006).

Gulf of California fin whale - Fin whales are permanent residents of the Gulf of California. This population is considered one of the most isolated in the world (Bérubé et al., 1998; Bérubé et al., 2002). With approximately 610 animals (SE=133, $CI_{95\%}$= 426-970) (Díaz-Guzmán, 2006; Urbán-Ramírez et al., 2005), this population constitutes a unique and separate conservation unit, vulnerable to anthropogenic and natural effects (Bérubé et al., 2002). Although the Gulf of California is considered one of the most pristine and bio-diverse areas of the world (hosts ~36 species of marine mammals), regrettably, the increasing human activity (pollution from industrial and human waste, aquaculture residues, and agricultural run-off) is beginning to affect it (Lluch-Cota *et al.,* 2007). Moreover, a foreseeable increase in naval traffic, both for leisure and commercial, will inevitably lead to higher numbers of fatal ship strikes with large cetaceans.

MATERIALS AND METHODS

Sampling - Integument biopsies (epidermis, dermis and blubber) were obtained from free-ranging fin whales in the Pelagos sanctuary (Ligurian, Corsica and North Tyrrhenian Sea) (spring-summer 2008, n = 12, males =6; females = 6) and Gulf of California (Sea of Cortez, n = 5, males = 3; females = 2) (summer 2008). Skin biopsy samples were obtained using biopsy darts launched with a crossbow (CITES Nat. IT025IS, Int. CITES IT 007). Samples were immediately placed in liquid nitrogen or stored in cell medium for the "slice" experiments.

Experimental design - To validate a diagnostic multi trial biomarker tool, a two-phase experimental protocol was followed (First Phase: field studies; Second Phase: *in vitro* experiments) (Fig.1). In the first phase of the project (field studies) we applied a multidisciplinary methodology to explore the effects of the exposure of Mediterranean and Mexican fin whales to anthropogenic contaminants, using skin biopsy as a diagnostic tool, and combining the analysis of molecular biomarkers (western blot of CYP1A1 and CYP2B) and gene expression (qRT-PCR of HSP70, ER, AhR, E2F-1) with the detection of OCs, PAHs and PBDEs residues. In the second phase (*in vitro* experiments), we explored the sensitivity of the multi-trial biomarker tool in slices of skin biopsies after experimentally exposing them to OCs and PBDEs. In this study *in vitro* tests were also used as an innovative tool for the study of inter- and intra-species sensitivity to various classes of contaminants present in the marine environment.

Contaminants analysis

Organochlorine Compounds (OCs): The analytical method used for quantitative and qualitative analysis of HCB, DDTs and PCBs was 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 by us (Marsili and Focardi, 1996). The gas chromatograph had a SPB-5 bonded phase in a 30 m long fused silica capillary column. The "EDC-OCs" consisted in analysed organochlorines known as endocrine disruptors such as: HCB, PCB95, PCB99, PCB101, PCB118, PCB153, pp'DDT, op'DDT, pp'DDE, op'DDE, pp'DDD, op'DDD.

Low-Brominated (Tri- to Hexa-) BDEs: GC-LRMS-ITD in theMS/MS operating mode, using the isotope dilution technique as described elsewhere (Gòmora *et al*., 2006) was used for analysis of tri- to hexa-BDEs. Analyses were performed on a CP-3800 GC (Varian Iberica, Spain) equipped with an ITD (Saturn 2000, Varian) and an 8200 CX autosampler (Varian).

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

Biomarkers analysis

CYP1A1 and CYP2B western blot - ***CYP1A*** and ***CYP2B*** have been detected in cetacean skin and induction of these isoforms was found after exposure to lipophylic contaminants such as OCs, PAHs and BFR both *in vitro* and in field studies (Fossi *et al*., 2006; Fossi *et al*., 2008). For WB analysis, S9 fractions of tissue homogenates (biopsy and slice biopsies, in duplicate for each sample) were separated by SDS-PAGE (10% polyacrylamide gels – Criterion XT Precast Gel - BioRad) and blotted onto nitrocellulose sheets for 1 hour at the constant

voltage of 200 V. The membranes were saturated by incubating them with a blocking solution (3% gelatin dissolved in Tris Buffered Saline containing 0.05% Tween-20, TTBS) for 1 hour at room temperature. Primary polyclonal rabbit antibodies from Oxford Biochemical Research were used (Oxford MI, USA). Goat anti-rabbit CYP1A1 and anti CYP2B4, diluted 1:5000 and 1:1000, respectively, in TTBS-1% gelatin, were incubated overnight at room temperature with cetacean proteins. Incubation with anti-rabbit HRP-labelled secondary antibody (1:3000 final dilution) was performed for 1.5 hours at room temperature and protein detection was done according to the BioRad Immun-Star HRP Chemiluminescent Kit booklet, using standardized times (Fossi *et al.*, 2008). Semi-quantitative analysis was performed for each WB (in triplicate) with Quantity One software (BioRad, 1-D Analysis Software) using the methods proposed by Fossi *et al.* (2008).

E2F-1 - HSP70 - ERα and AHR: Quantitative Real Time PCR assay (qRT-PCR)- The E2F-1 transcription factor is a member of the E2F family (***E2F1-6***) and it is important in regulating cell cycle. It has two roles: it controls some genes involved in DNA synthesis and it is involved in the apoptosis processes (La Thangue, 2003, Attwool *et al.*, 2004). E2F-1 over-expression seems to up-regulate several genes involved in the activation of apoptosis.

The heat shock protein 70 (***HSP70***) is a stress-related protein belonging to a multigene family. In environmental stress assessment, the HSP70 is often used as an early biomarker in a wide variety of organisms. The HSP70 was found to be induced by multiple stimuli including PAHs and heavy metals, and this protein has been used as biomarkers of environmental exposure in organisms that live in contaminated areas (Köhler *et al.*, 1999; Varó *et al.*, 2002).

Estrogen receptors (***ERs***) are members of the nuclear receptor superfamily and they are ligand-inducible transcription factors. The ligand-binding signaling is due to the binding of the estrogen (or a compound structurally similar to that, such as OCs or PBDEs). Subsequently, the specific transcriptional response is activated (Mueller, 2004). Compounds like PCBs and PBDEs, having dioxin-like property, can bind estrogen receptors and interfere with signaling pathways.

The aryl hydrocarbon receptor (***AHR***) is a soluble ligand-activated transcription factor, member of the basic-helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) gene superfamily. The AHR receptor is involved in processes that activate Phase I enzymes such as CYP1A and CYP1B (Whitlock, 1999), but it is also involved in cell physiology and in the control of the cell cycle. Because of its main role in the metabolism of xenobiotics, in particular of dibenzo-*p*-dioxins, AHR is used as a biomarker of exposure to dioxins and organochlorine compounds in general.

In this study, subsamples of skin biopsies were used for the gene expression analyses by qRT-PCR. Each biopsy was homogenized and the RNA isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Biorad). One µg of RNA was retrotranscribed with the iScript cDNA Synthesis Kit (Biorad). The Real Time PCR reaction conditions were set as described in Spinsanti *et al.* (2006). The expression of the four genes of interest (Heat Shock Protein 70, Estrogen Receptor α, E2F-1 Transcription Factor, Aryl Hydrocarbon Receptor) was normalized with the control genes GAPDH and YWHAZ. The gene expression was calculated using the GenEx software v. 4.3.8 (MultiD Analyses AB).

Slices experimental design - The need to develop *in vitro* model to explore the different susceptibilities of cetaceans to several classes of "old" and "emerging contaminants", led us to use tissue slices (skin biopsy) of different cetacean species as a new non-lethal tool of investigation (Fossi *et al.*, 2008). Slices of skin biopsy were subject to several different experimental tests, using mixtures of anthropogenic contaminants such as OCs and PBDEs (Fossi et al., 2006). We incubated the slices (treated slice) for 24 hr in cell culture media with different mixture of two classes of CYP1A1 and CYP2B inducers (Fig.1). The first mixture (OCs) was of Arochlor 1260, pp'DDT and pp'DDE in DMSO (0.05%) at three doses: 0.01 µg/ml, 0.1 µg/ml and 1 µg/ml, plus a DMSO (0.05%) control. The second mixture (PBDEs) contained 27 PBDEs, from mono- to deca-brominated, dissolved in nonane (0.01 µg/ml) at three doses: 0.01 µg/ml, 0.05 µg/ml and 0.1 µg/ml, plus a nonane (0.01 µg/ml) control. Several biomarker responses (CYP1A1 and CYP2B) were developed and evaluated using a multidisciplinary approach.

Statistical analysis - Data was processed using Statistica 5.0 (Statsoft). For the present data we chose the Shapiro-Wilks W test. If the W-test is significant ($p<0.05$), the distribution cannot be normal. The greater part of the variables had a non-normal distribution ($p<0.05$), therefore we have applied non-parametric tests. Differences among groups of data were tested by ANOVA (Kruskal-Wallis test; significance level: $p < 0.05$) and the Mann-Whitney test (significance level: $p < 0.1$). The Kruskal-Wallis test was applied to reveal any differences in variance. Because this test does not discriminate which groups differed or to what extent, the Mann-Whitney test was used on pairs of samples. The non parametric test used to estimate possible correlations between variables was the Spearman rank order correlations (significance level: $p < 0.1$) that assume that the variables under consideration were measured on at least an ordinal (rank order) scale, that is, that the individual

observations can be ranked in two ordered series. In other words this procedure uses the ranks of the data rather than the values themselves.

RESULTS AND DISCUSSIONS

First Phase - The two populations of fin whales showed differences in organochlorine, OCs-EDCs (organochlorines with Endocrine Disruption potential), PAHs, PBDEs 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 with the Cortez specimens (Fig.2 a,b,c,d), confirming the high toxicological stress to which the fin whale population in the Pelagos Sanctuary is exposed. The difference in bioaccumulation levels between males and females is likely related to the high capacity of females to excrete lipophylic contaminants during lactation.
- Levels of emerging contaminants, such as low brominated PBDEs are presented in Fig 2d. Total values, in average, were higher in samples from Cortez, ranging from 282 to 30506 ng/g dw, while samples from Mediterranean sea showed lower average levels, ranging from 31 to 5121 ng/g dw. The most abundant congener was PBDE 47, detected in all samples analyzed. In general, samples from Cortez Sea had a major number of detected congeners, such as 47, 100, 99, 154 and 153. It is remarkable that PBDE 183 was detected only in one sample from Mediterranean Sea .
- Exploring molecular biomarker responses, the high induction of CYP1A1 in Mediterranean male whales, if compared to males from the Gulf of California (Fig. 3a), can be related to the presence in the blubber of high levels of planar OCs, such as coplanar PCBs, and PAHs (Fig.2a,c). When data from male specimens of the two populations were pooled together a statistically significant positive correlation (rho Spearman = 0.73, p = 0.003) was found between total PAHs and CYP1A1 induction in male specimens.
- Moreover a lack in the CYP2B induction, despite the high levels of liphophylic contaminants, was evident in both male and female Mediterranean whales, suggesting a down-regulation dose-dependent effect on CYP2B and a potential high "*toxicological stress*" in this population (Fig.3b).
- On the other hand, high induction of CYP2B in Mexican fin whales (Fig.3b) could be related to the presence of high levels of low brominated PBDEs (Fig 2d) and also of OC globular insecticides such as DTTs and its metabolite pp'DDE in the blubber.
- Exploring gene expression biomarker responses (fig.3c,d,e,f), the levels of mRNA were calculated comparing 5 skin biopsies from the Sea of Cortez to 12 skin biopsies from the Pelagos Sanctuary. The gene expression of the ERα and E2F-1 genes was up-regulated in the specimens from Pelagos Sanctuary in respect with those from Mexico both in males (ERα: 3.6x fold; E2F-1: 1.7x fold) and females (ERα: 1.3x fold; E2F-1: 2.4x fold) ($p<0.05$). These data suggest, in the first case (ERα), the high exposure to EDCs compounds such as OCs-EDCs (Fig.2c), and in the second case (E2F-1) the presence of high apoptosis processes as a sign of toxicological stress in the Mediterranean population. On the contrary, the expression of the HSP70 is higher in the Mexican male specimens than in those coming from the Pelagos Sanctuary (1.5x fold) ($p<0.05$), whereas the Pelagos females exhibit an over-expression of HSP70 gene respect to the Mexican specimens (1.9x fold). The AHR gene is slightly up-regulated in the Mexican specimens with no differences between males and females.

Second Phase – In this study, *in vitro* tests were used as an innovative tool for the investigation of intra-species sensitivity to various classes of environmental contaminants present in the two different marine environments.

- Marked differences in CYP1A1 and CYP2B induction by OCs (Fig. 4) and PBDEs were detected in whale biopsy slices of the two populations (male specimens), with higher sensitivity responses in the Mexican mysticete (Fig. 4). A dose dependent (OCs treatment) induction of CYP1A1 was detected only in biopsy slices of Cortez specimens (0.01 µg/ml = 2.6 fold, 0.1 µg/ml = 3,6 fold and 1 µg/ml = 4.4 fold respect to the control) (Fig. 4).
- The *in vitro* tests showed no induction of the male Mediterranean whale (slice) CYP1A1 and CYP2B (Fig. 4).

CONCLUSIONS

In conclusions, the multi-trial biomarker tool applied to skin biopsies, underlined differences in OCs, OCs-EDCs, PBDEs, PAHs levels and molecular and gene expression biomarker responses between the two populations, revealing a higher toxicological stress in the Mediterranean fin whales. The presence of a "*toxicological stress*" in the Pelagos population is pointed out by warning signals such as the CYP1A1

induction, the up-regulation of ERα and E2F-1 genes, combined to the lack in the CYP2B induction in both field and *in vitro* experiments.

Moreover, particular concern is due to 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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CAPTIONS

Figure 1. ***Experimental design and sampling areas –*** Validatation of a diagnostic "multi trial biomarker" tool to evaluate the toxicological status of fin whales *(Balaenoptera physalus)* in the Pelagos sanctuary (Mediterranean Sea) and in the Gulf of California (Mexico). A two-phase experimental protocol was followed: First Phase: field studies; Second Phase: *in vitro* experiments.

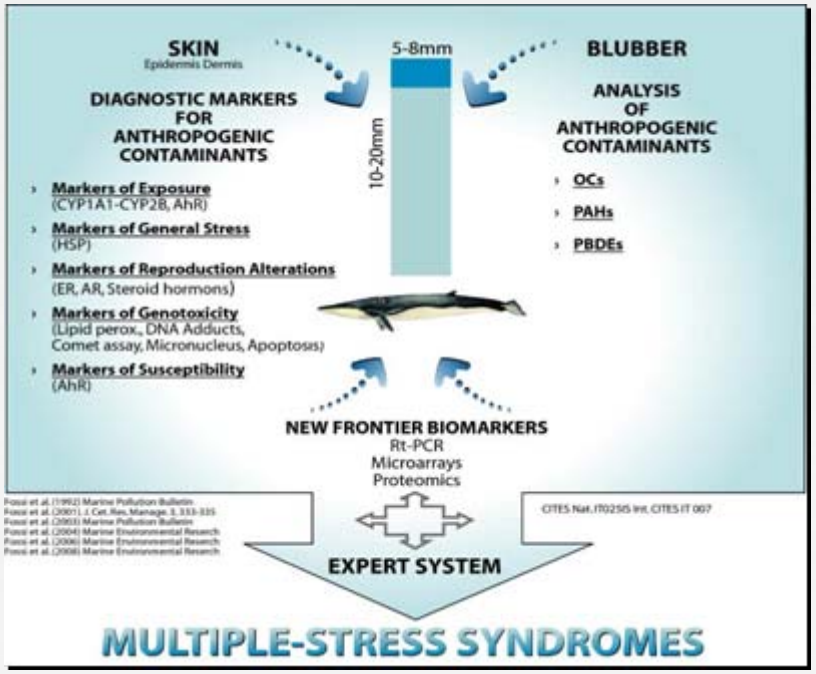
Figure 2. ***First Phase results: contaminant levels*** - Organochlorines (a), OCs-EDCs (b), PAHs (c), PBDEs (d) levels in skin biopsy of specimens of the two populations of fin whale (* = $p< 0.1$).

Figure 3. ***First Phase results: biomarker responses*** - Western blot of CYP1A1 (a), CYP2B(b), in skin biopsy of specimens of the two populations of fin whale. Gene expression (qRT-PCR) of ER (c), E2F-1 (d), HSP70 (e), AhR (f) in skin biopsy of specimens of the two populations of fin whale (** = $p< 0.05$).

Figure 4. ***Second Phase results***. WB analysis of CYP1A1 in slices of skin biopsy of male specimens of the two populations of fin whale treated with mixtures of OCs. Different steps of the methodology (WB, Quantity one analysis, and Relative pmol CYP1A1 and CYP2B) are reported. The results are expressed as relative pmol CYP/mg prot.

PHASE I - Field studies

Multi-Trial Biomarkers Diagnostic Tool in Skin Biopsy



PHASE II

In Vitro Experiments

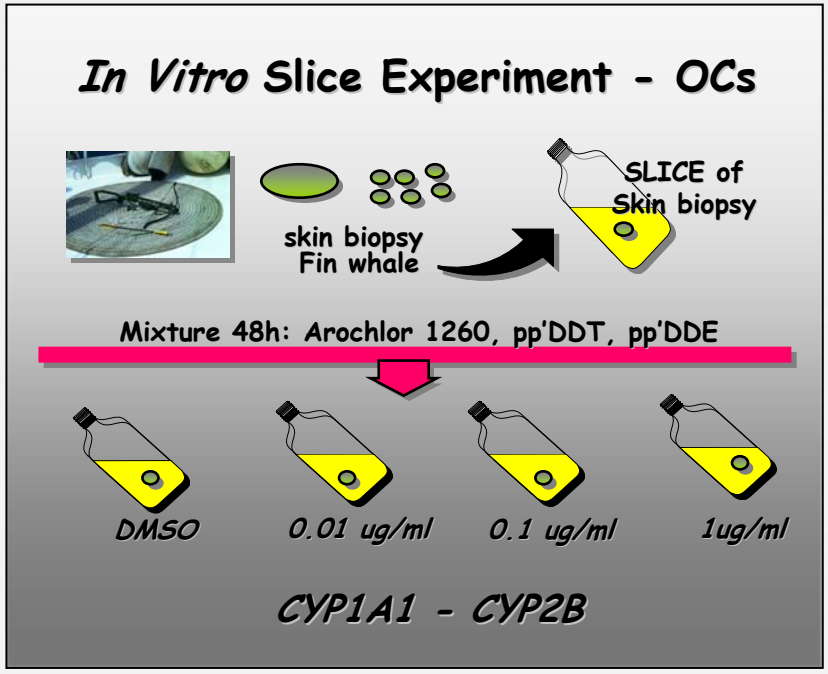
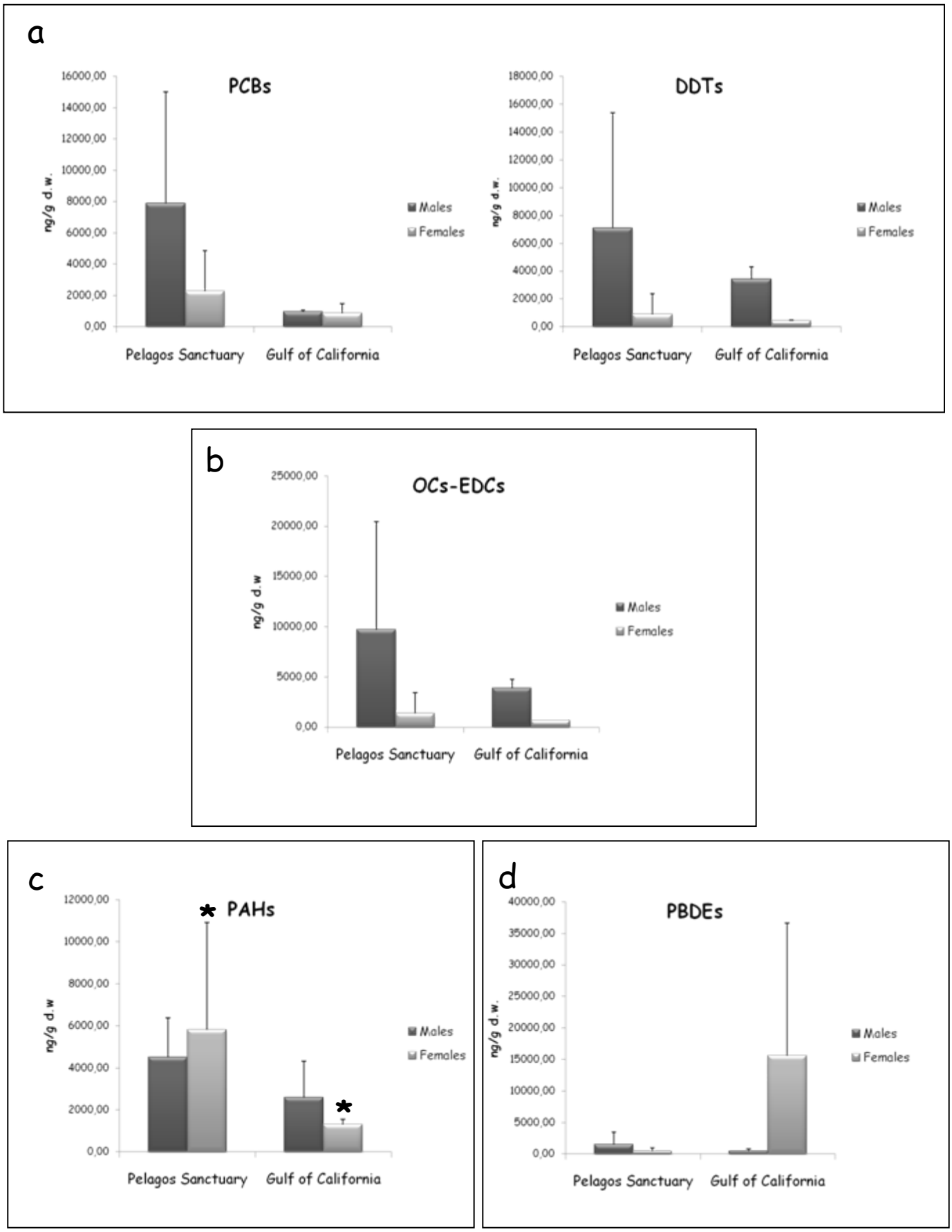


Fig.1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Males
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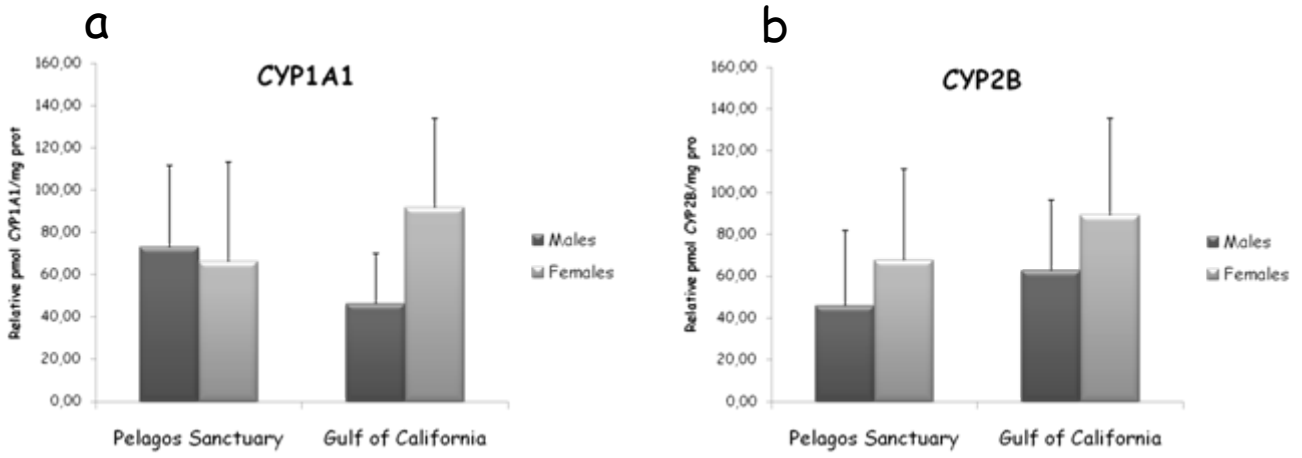
Pelagos Sanctuary
Gulf of California
DDTs
18000,00
16000,00
14000,00
12000,00
10000,00
8000,00
6000,00
4000,00
2000,00
0,00
ng/g d.w.
Males
Females
Pelagos Sanctuary
Gulf of California
b
OCs-EDCs
25000,00
20000,00
15000,00
10000,00
5000,00
0,00
ng/g d.w
Males
Females
Pelagos Sanctuary
Gulf of California
c
* PAHs
12000,00
10000,00
8000,00
6000,00
4000,00
2000,00
0,00
ng/g d.w
Males
Females
Pelagos Sanctuary
Gulf of California
d
PBDEs
40000,00
35000,00
30000,00
25000,00
20000,00
15000,00
10000,00
5000,00
0,00
ng/g d.w.
Males
Females
Pelagos Sanctuary
Gulf of California

Fig.2

Molecular Biomarkers



Gene Expression

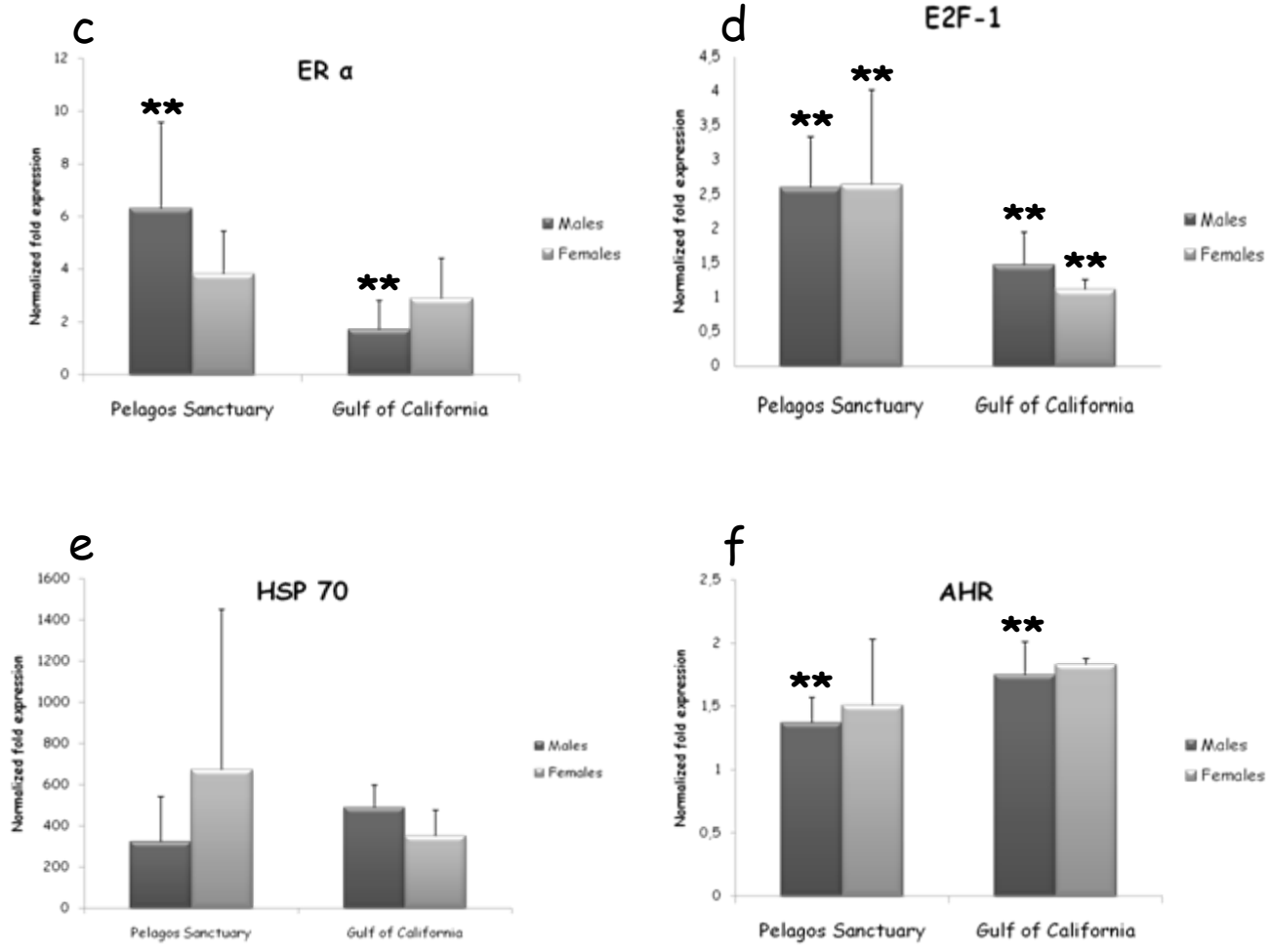
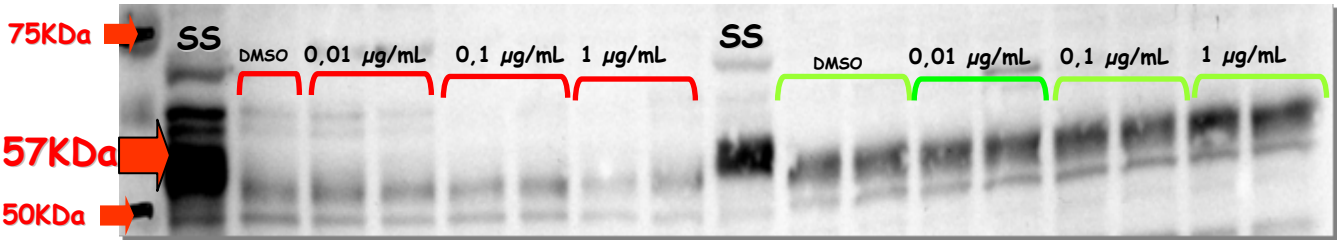


Fig. 3

CYP1A1: OCs treatment in skin biopsy slices of fin whale
(Pelagos Sanctuary – Gulf of California)



IB20 Pelagos Sanctuary **MBP1 Gulf of California**

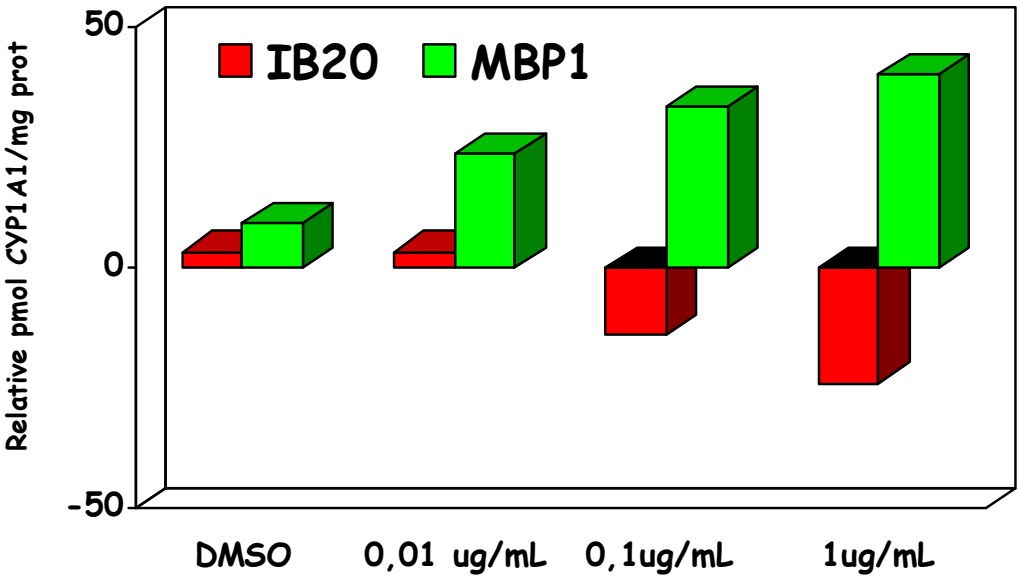


Fig.4