FIRST ASSESSMENT OF BIOMARKER RESPONSES AND CONTAMINANT LEVELS IN *Balaenoptera edeni* SKIN BIOPSIES OF GULF OF CALIFORNIA (MEXICO)

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ABSTRACT

The main objective of this project was to apply, for the first time, a suite of sensitive non-lethal biomarkers in skin biopsy of the mysticete unexplored species Bryde's whale (Balaenoptera edeni) to evaluate the toxicological status of this cetacean species in the Gulf of California. In this species we developed a "multi-trial-biomarker-tool", combining protein biomarkers (western blot of CYP1A1, CYP2B) with analysis of OCs and PAHs. In this paper we also explored the level and effects of OCs and PAHs in skin biopsies of fin whale (Balaenoptera physalus) populations of the Gulf of California and the heavy polluted areas of the Mediterranean Sea.

KEYWORDS

Balaenoptera edeni, Balaenoptera physalus, skin-biopsy, Biomarkers, OCs, PAHs, Gulf of California, Mediterranean Sea.

INTRODUCTION

The main objective of this project was to investigate the inter- and intra-species differences in biomarker responses and contaminant levels in two Mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California (Mexico) and Mediterranean Sea (*Balaenoptera physalus)* using skin biopsy as diagnostic tools. A suite of sensitive non-lethal biomarkers was applied, for the first time, to the mysticete unexplored species Bryde's whale to evaluate the toxicological status of this cetacean species in the Gulf of California. We developed a "multi-trial-biomarker-tool", combining protein biomarkers (western blot of CYP1A1, CYP2B) with analysis of OCs and PAHs. In this phase of the project we also explored the level and effects of OCs, PBDEs and PAHs in skin biopsies of the two mysticete species (*Balaenoptera physalus* and *Balaenoptera edeni)* of Gulf of California in comparison to fin whale of Mediterranean Sea.

Gulf of California Bryde's whale - With a maximum size of 14m, the Bryde's whale (*Balaenoptera edeni*) is the second smaller whale among Balaenopterid species (rorquals). Their color pattern is mainly dark black on the dorsal area and soft gray/white on ventral area. Some animals presents whitely spots over the animal flanks, probably caused by the cockiecutter shark (*Isistius brasiliensis*). An important distinctive characteristic (unique among rorquals), is that this species presents three longitudinal ridges on its rostrum (Guerrero Ruiz *et al.,* 2006). The Bryde's whale is probably the most common whale in the Gulf of California (Urban and Flores, 1996). The latest estimation indicates that approximately 950 animals inhabit the gulf (Gerrodette and Palacios, 2004). However, this number could be overestimated based on that genetics studies has suggested that in the gulf are presents two different populations of this species, one resident (year around) and the other related with the Northeast Pacific population that visit the Gulf of California seasonally (Dizon *et al.,* 1995). Numerous sightings of this species have been reported in Baja California (From San Felipe to Salsipuedes Channel), Baja California Sur (from Loreto to Los Cabos), and Sonora (Puerto Peñasco, Bahia de Kino, Guaymas) (Guerrero Ruiz *el al.,* 2006). Differently to other regions, the Gulf of California Bryde's whale looks prefer coastal than oceanic areas. Among the preys items reported for this species into the Gulf of California are: the pelagic red crab (*Pleuroncodes planipes),* anchovy *(Opisthonema libertate)* (Rice, 1977; Leatherwood *et al.,* 1982; Urbán and Flores, 1996), sardines

(Sardinops sagax), and eufasusids *(Nyctiphanes simplex)* (Tershy, 1992; Tershy *et al.,* 1993; Urbán and Flores, 1996).

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.

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 one of the major cause of non-natural mortality for fin whales (Panigada *et al.*, 2006).

METHODS

Sampling – Integument biopsies (epidermis, dermis and blubber) were obtained from free-ranging Bryde's whale in the Gulf of California during the summer of 2008 (n =4, 2 males and 2 females) using biopsy darts launched with a crossbow (CITES Nat. IT 025IS, Int. CITES IT 007). Integument biopsies were also 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). Sex was determined according to Bérubé and Palsbøll (1996).

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.

Polycyclic aromatic hydrocarbons (PAHs) **-** Levels of PAHs and 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, 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).

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

RESULTS AND DISCUSSION

This "multi-trial diagnostic tool", applied to skin biopsies, underlined differences in OC, PAH levels and molecular biomarker responses between the two mysticete species (Balaenoptera edeni and Balaenoptera physalus) of Gulf of California and also between the two fin whale populations(Gulf of California and Mediterranean Sea) .

- Higher levels of DDTs (Fig.1A), PCBs (Fig.2A), and PAHs (Fig.3A) were detected in the zooplankton-eating species fin whale in comparison to the fish-eating species Bride's whale; on the opposite extremely higher level of both CYP1A1 (Fig.4A), CYP2B (Fig.5A) are detected in the fish-eating species, showing similar level to odontocete species resident in Sea of Cortez (Fossi personal communication)(Fig. 7). Higher levels of OCs and low level of the CYP2B were detected in the fin whale specimens, on the opposite lower levels of OCs and high level of the CYP2B were detected in the Bride's whale specimens, suggesting a higher detoxification ability in the fish-eating species (Fig.6).

- The interspecies investigation shows the presence of a higher "toxicological stress" in the fin whale Pelagos population highlighted by warning signals such as higher levels of DDTs (Fig.1B), PCBs (Fig.2B), and PAHs (Fig.3A) high levels of CYP1A1 induction and, as previously reported by Fossi and collaborators (2010), the up-regulation of ERα and E2F-1 genes, combined with a lack of CYP2B induction in both field and *in vitro* experiments (Fossi et al, 2010).

CONCLUSIONS

In conclusions, the multi-trial biomarker tool applied, for the first time, to skin biopsies to the mysticete unexplored species Bryde's whale, underlined differences in OCs, PAHs levels and CYP1A1 and CYP2B responses in the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California. These data suggest a peculiar evolutionary process of the two isoforms of CYP in the fish-eating species Bride's whale, that showed levels of both Cytochromes similar to the other odontocete species resident in Sea of Cortez (Fig. 7).
The use of skin biopsy as diagnostic tools was confirmed in this project as a powerful method to investigate both the inter- and intra-species differences in biomarker responses and contaminant levels in two Mysticete species of Gulf of California (Mexico) in comparison to the *Balaenoptera physalus* of Mediterranean Sea. The presence of a "*toxicological stress*" in the Pelagos fin whale population is pointed out by warning signals using the "multi-trial-biomarker-tool". Moreover, particular concern is due to the high levels of low brominated PBDEs found in the Mexican whale specimens (Fossi et al, 2010).
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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References

Bérubé, M., Aguilar, A., Dendanto, D., Larsen, F., Notarbartolo di Sciara, G., Sears, R.,Sigurjónsson, J., Urban-R, J., Palsbøll, P.J., 1998. Population genatic structure of North Atlantic, Mediterranean Sea and Sea of Cortez fin whales, Balaenoptera physalus (Linnaeus 1758): analysis of mitochondrial and nuclear loci. Molecular Ecology 7, 585–599.

Dizon, A. E., C. A. Lux, R. G. Leduc, J. Urbán, M. Henshaew y R. L. Brownell. 1995. An interim phylogenetic analysis of sei and Bryde's whale mitochongdrial DNA control region sequences. Paper SC/47/NP23 presented to International Whaling Commission, Scientific Committee. May of 1995. 12 pp.

Fossi, M.C., Marsili, L., Leonzio, C., Notabartolo di Sciara, G., Zanardelli, M., Focardi, S., 1992. The use of non-destructive biomarker in Mediterranean cetaceans: preliminary data on MFO activity in skin biopsy. Marine Pollution Bulletin 24, 459–461.

Fossi, M.C., Marsili, L., Casini, S., Bucalossi, D., 2006. Development of new-tools to investigate toxicological hazard due to endocrine disruptor organochlorines and emerging contaminants in Mediterranean cetaceans. Marine Environmental Research 62, 200–204.

Fossi, M.C., Marsili, L., Casini, S., Bucalossi, D., 2008. First detection of CYP1A1 and CYP2B induction in Mediterranean cetacean skin biopsies and cultured fibroblasts by Western blot analysis. Marine Environmental
Research 66, 3–6.

Fossi, M.C., Urban, J., Casini, S., Maltese, S., Spinsanti, G., Panti, C., Porcelloni, S., Panigada, S., Lauriano, G., Niño-Torres, C., Rojas-Bracho, L., Jimenez, B., Muñoz-Arnanz, J., Marsili, L., 2010. A multi-trial diagnostic tool in fin whale (*Balaenoptera physalus*) skin biopsies of the Pelagos Sanctuary (Mediterranean Sea) and the Gulf of California (Mexico). *Mar. Environ. Res. In press.*

Gerrodette, T. y D. M. N. Palacios. 1996. Estimates of cetacean abundance in EEZ waters of the eastern Tropical Pacific. *Southwest Fisheries and Science Center*. Administrative Report LJ-96-10. 28 pp.

Godard, C.A.J., Smolowitz, R.M., Wilson, J.Y., Payne, R.S., Stegeman, J.J., 2004. Induction of cetacean cytochrome P4501A1 by ß-Naphthoflavone exposure of skin biopsy slices. Toxicological Sciences 80, 268–275.

Gómara, B., Herrero, L., Bordajandi, L.R., González, M.J., 2006. Quantitative analysis of polybrominated diphenyl ethers in adipose tissue, human serum and foodstuff samples by gas chromatography with ion trap tandem mass spectrometry and isotope dilution. Rapid Communications in Mass Spectrometry 20, 69–74.

Guerrero Ruiz, M., Urbán R. J.,y L Rojas Bracho.2006. Las Ballenas del Golfo de California. Secretaría de Medio Ambiente y Recursos Naturales (Semarnat), Instituto Nacional de Ecología (INE). Ciudad de México. 524pp.

Leatherwood, S., R. R. Reeves, W. F. Perrin y W. E. Evans. 1982. Whales, dolphins and porpoises of the eastern North Pacific and adjacent Arctic waters: A guide to their identification. NOAA Technical Report, NMFS. Circular 444. 245 pp.

Marsili, L., Fossi, M.C., Casini, S., Savelli, C., Jimenez, B., Junin, M., Castello, H., 1997. Fingerprint of polycyclic aromatic hydrocarbons in two populations of southern sea lions (Otaria flavescens). Chemosphere 34, 759–770.

Niño-Torres, C.A., Gardner, S.C., Zenteno-Savín, T., Ylitalo, G.M., 2009. Organochlorine pesticides and polychlorinated biphenyls in California Sea Lions (Zalophus californianus californianus) from the Gulf of California, México. Archives of Environmental Contamination and Toxicology 56, 350–359.

Urbán-Ramírez, J., Rojas-Bracho, L., Guerrero-Ruíz, M., Jaramillo-Legorreta, A., Findley, L.T., 2005. Identification of sex in Cetaceans by multiplexing with ZFX and ZFY specific primer. In: Cartron, J.E., Ceballos, G., Felger, R.S. (Eds.), Biodiversity Ecosystems and Conservation in Northern Mexico. Oxford University Press, New York, pp. 276–297.

Rice, D. W. 1977. A list of the marine mammals of the world. NOAA Tech. Rep. NMFS SSRF 771. 15 pp.

Tershy, B. R. 1992. Body size, diet, habitat use, and social behavior of *Balaenoptera* whales in the Gulf of California. *J. Mamm*. 73(3): 477-486.

Tershy, B. R., A. Acevedo, D. Breese y C. S. Strong. 1993. Diet and feeding behavior of fin and Bryde's whales in the central Gulf of California. *Rev. Inv. Cient.* Vol 1 (No.Especial. SOMEMMA): 69-72.

Urbán, J. y S. Flores R. 1996. A note on Bryde's whales *(Balaenoptera edeni)* in the Gulf of California, México. *Rep. Int. Whal. Commn.* 453-457.

CAPTIONS

Figure 1. ***Contaminant levels*** – Total DDTs (A) levels in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California (male and female). Total DDTs (B) levels in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California in comparison to the specimens of *Balaenoptera physalus* of Mediterranean Sea .

Figure 2. ***Contaminant levels*** – Total PCBs (A) levels in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California (male and female). Total PCBs (B) levels in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California in comparison to the specimens of *Balaenoptera physalus* of Mediterranean Sea.

Figure 3. ***Contaminant levels*** – Total PAHs (A) levels in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California (male and female). Total PAHs (B) levels in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California in comparison to the specimens of *Balaenoptera physalus* of Mediterranean Sea. (* = $p < 0.05$).

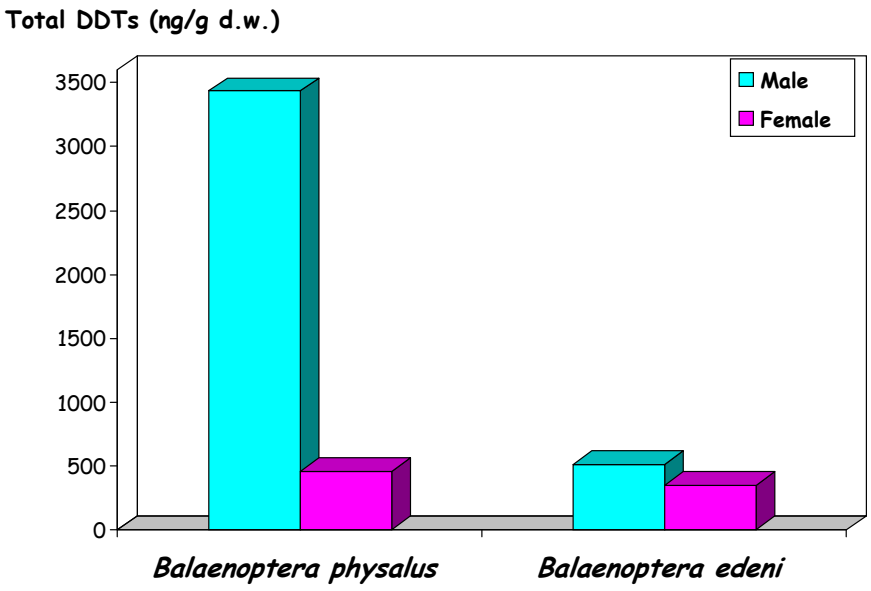
Figure 4. ***Biomarker responses*** - Western blot of CYP1A1 (A) in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California (male and female). Western blot of CYP1A1 (B) in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California in comparison to the specimens of *Balaenoptera physalus* of Mediterranean Sea.

Figure 5. ***Biomarker responses*** - Western blot of CY2B (A) in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California (male and female). Western blot of CYP2B (B) in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* and *Balaenoptera physalus)* of Gulf of California in comparison to the specimens of *Balaenoptera physalus* of Mediterranean Sea. (* = $p < 0.05$).

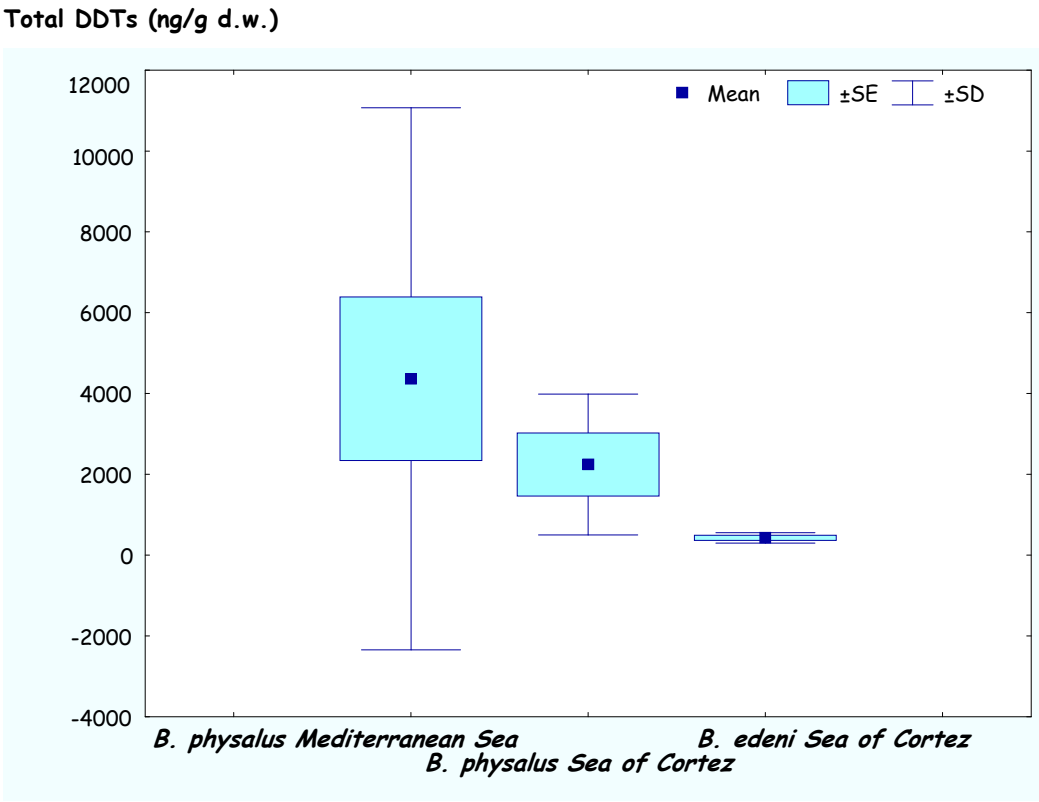
Figure 6. ***Biomarker responses*** - Scatterplots of CY2B and Total OCs in skin biopsy of specimens of the two mysticete species (*Balaenoptera edeni* (■) and *Balaenoptera physalus* (●) of Gulf of California .

Figure 7. ***Biomarker responses*** - Western blot of CY1A1 and CYP2B in mysticete and to odontocete species of Sea of Cortez. Line represent the average of CYP2B in *Balaenoptera edeni.*

Total DDTs (ng/g d.w.)



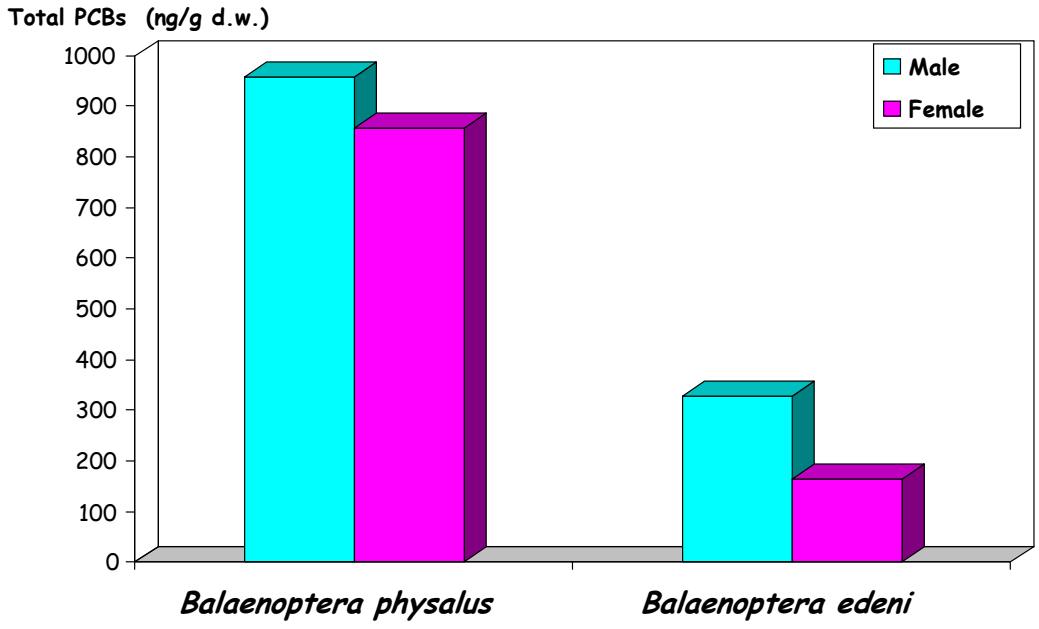
A



B

Fig.1 –

Total PCBs (ng/g d.w.)



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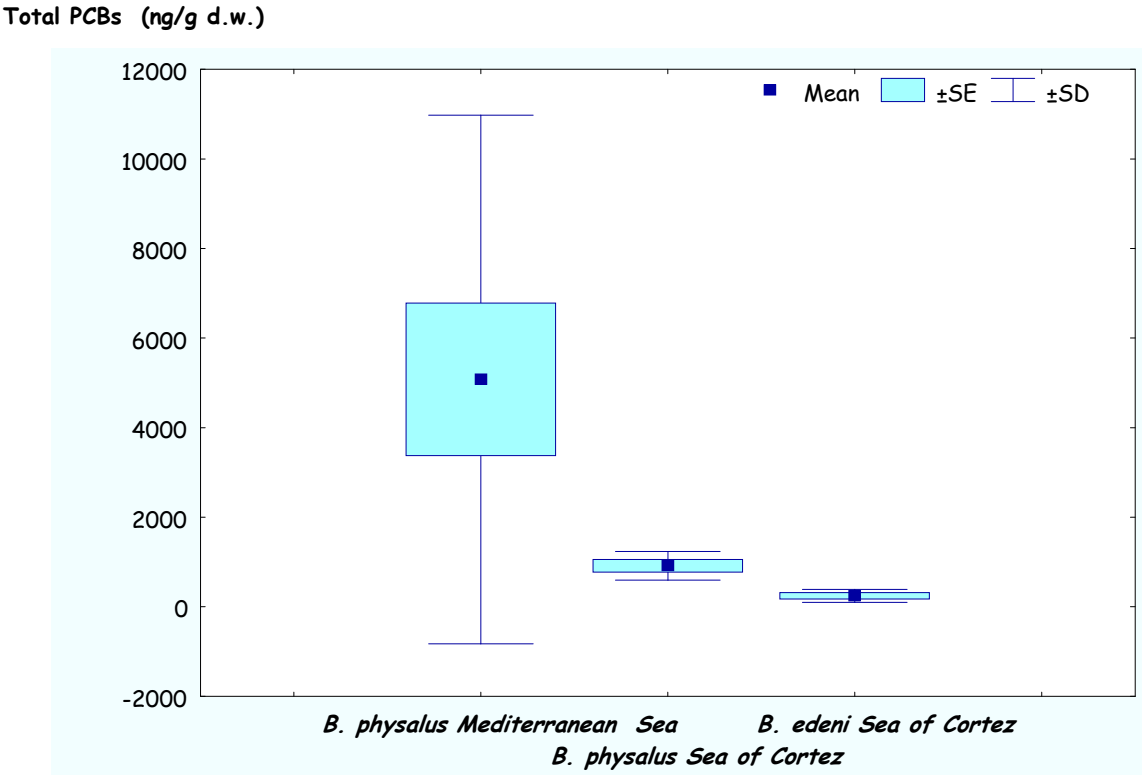
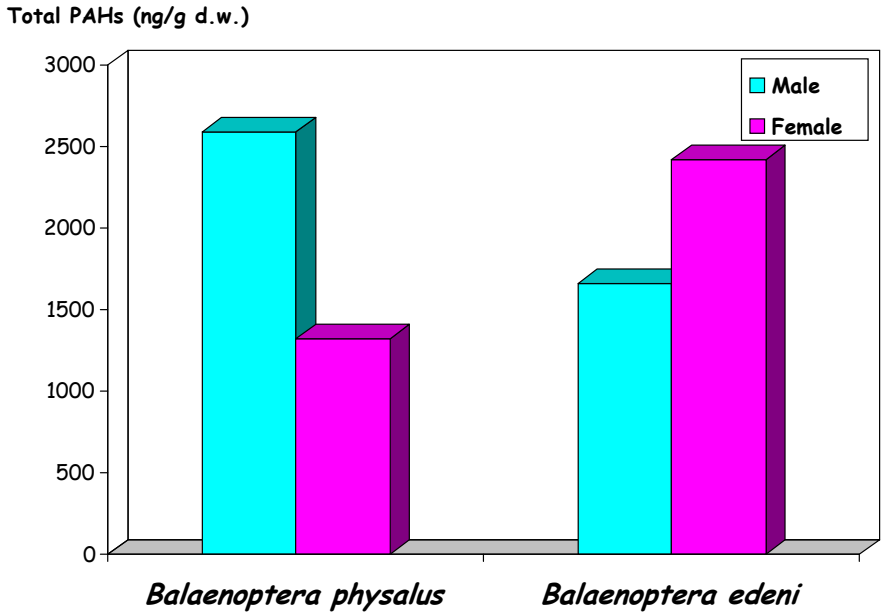


B

Fig.2 –

Total PAHs (ng/g d.w.)



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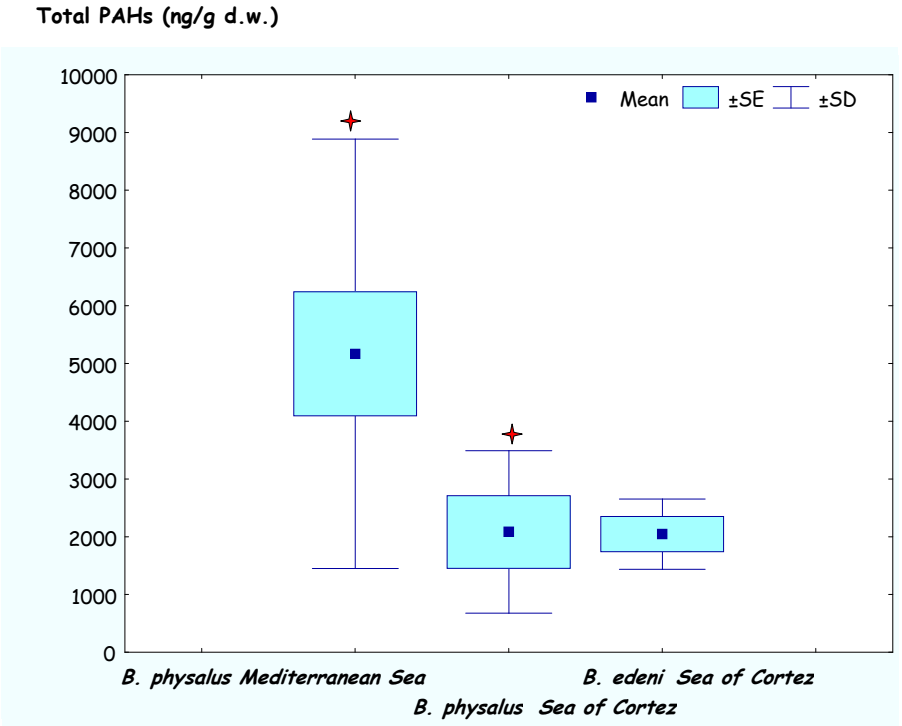
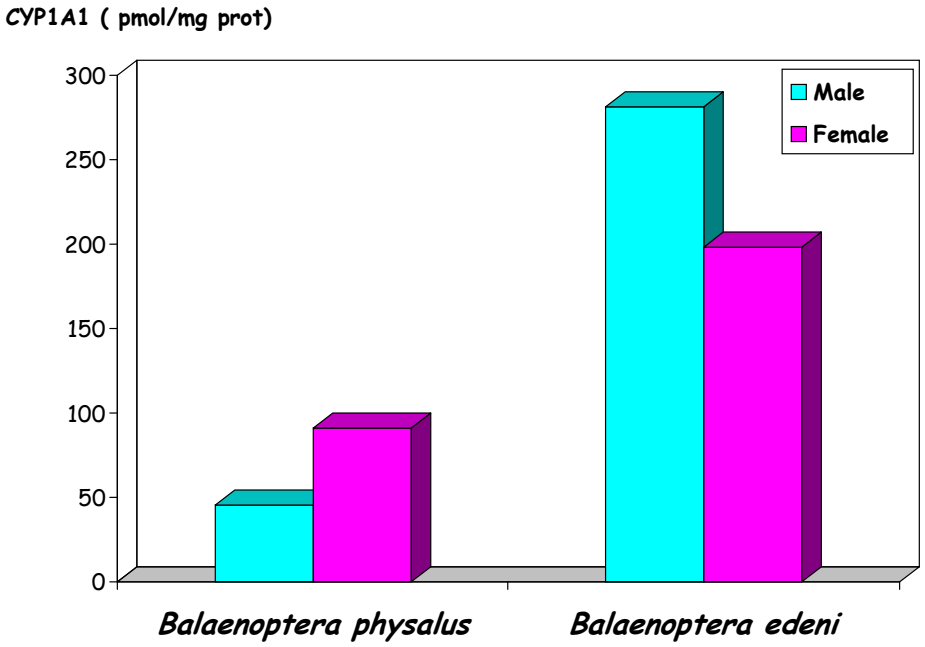


B

Fig.3 –

CYP1A1



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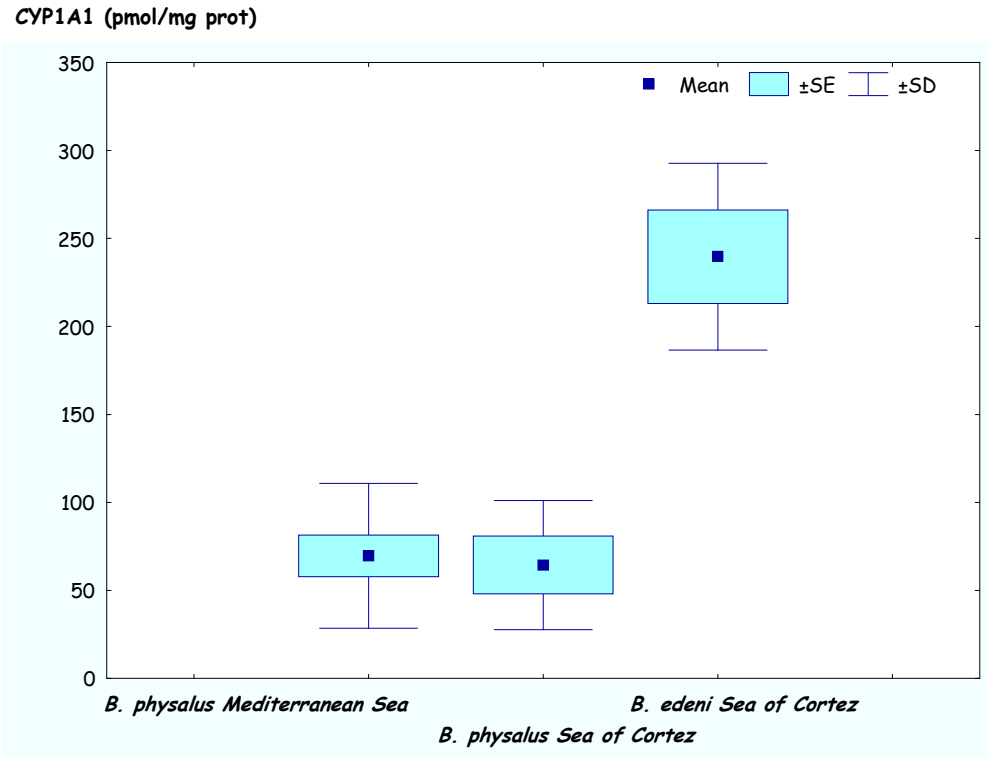
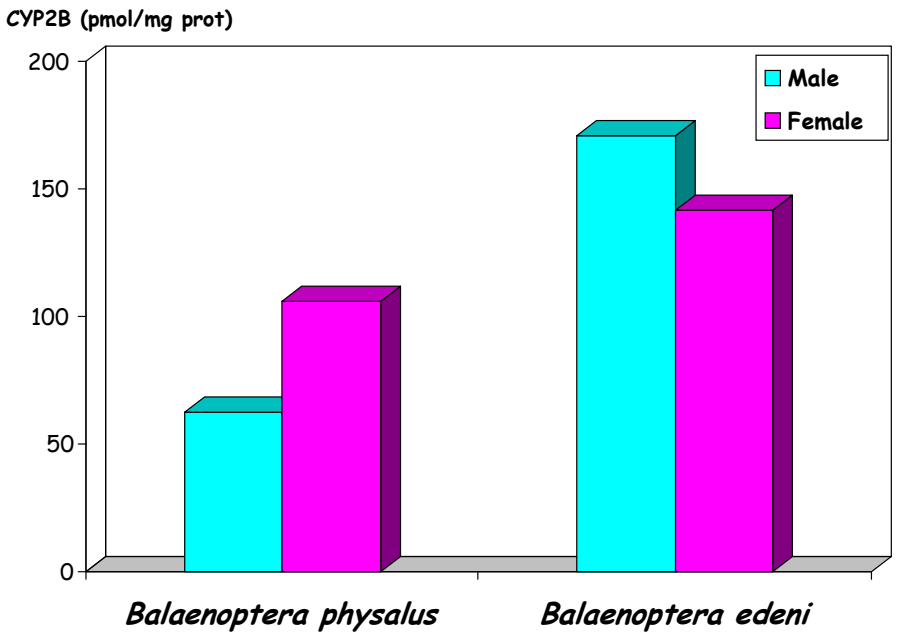


B

Fig.4 –

CYP2B



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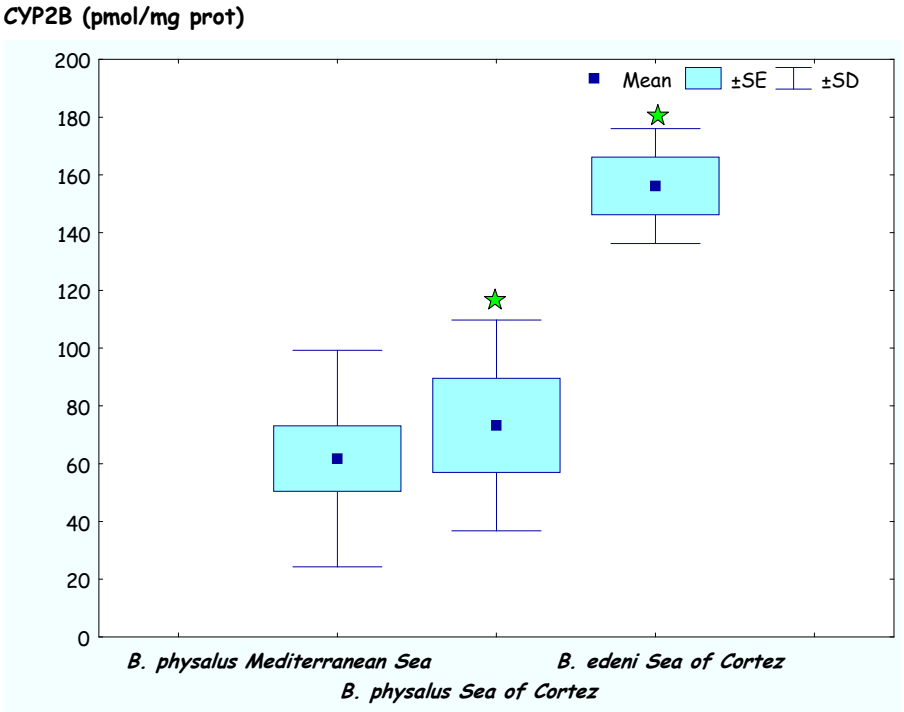

B

Fig.5 –

Scatterplots of CYP2B and OCs

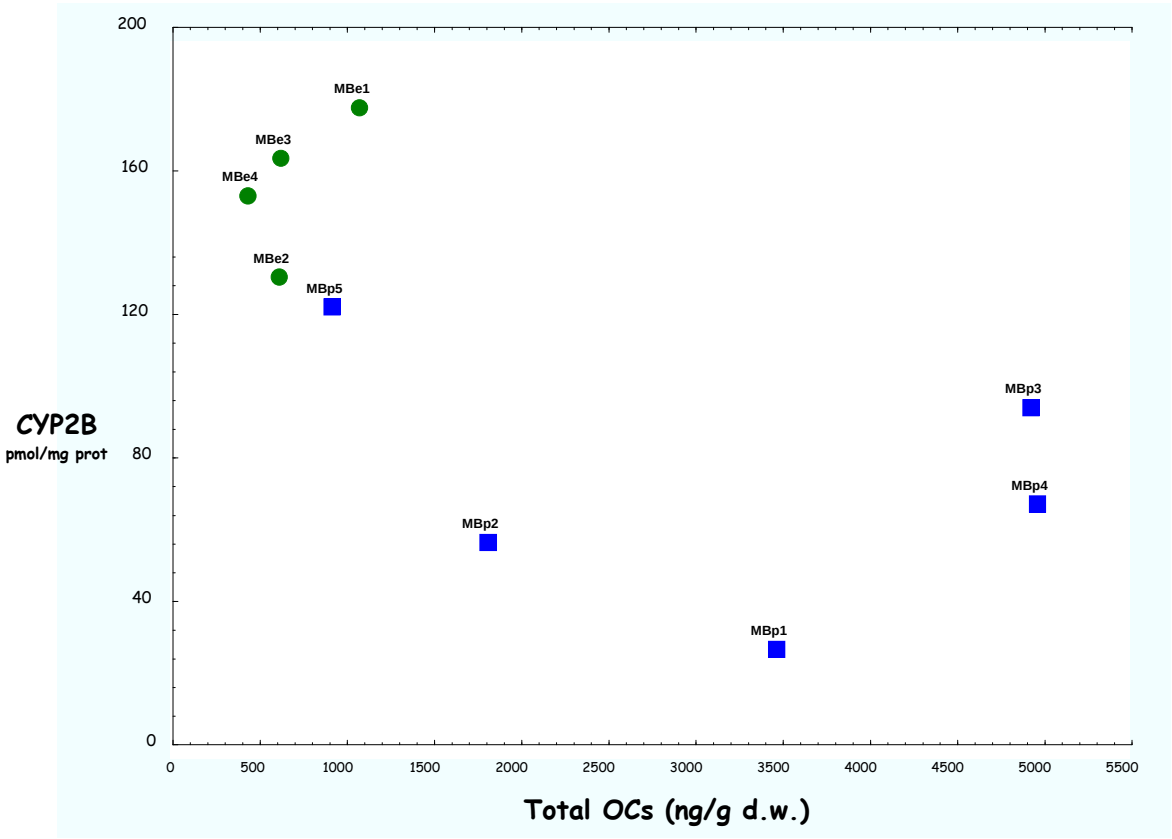

Fig.6 –

CYP1A1 and CYP2B in Cetaceans of Sea of Cortez

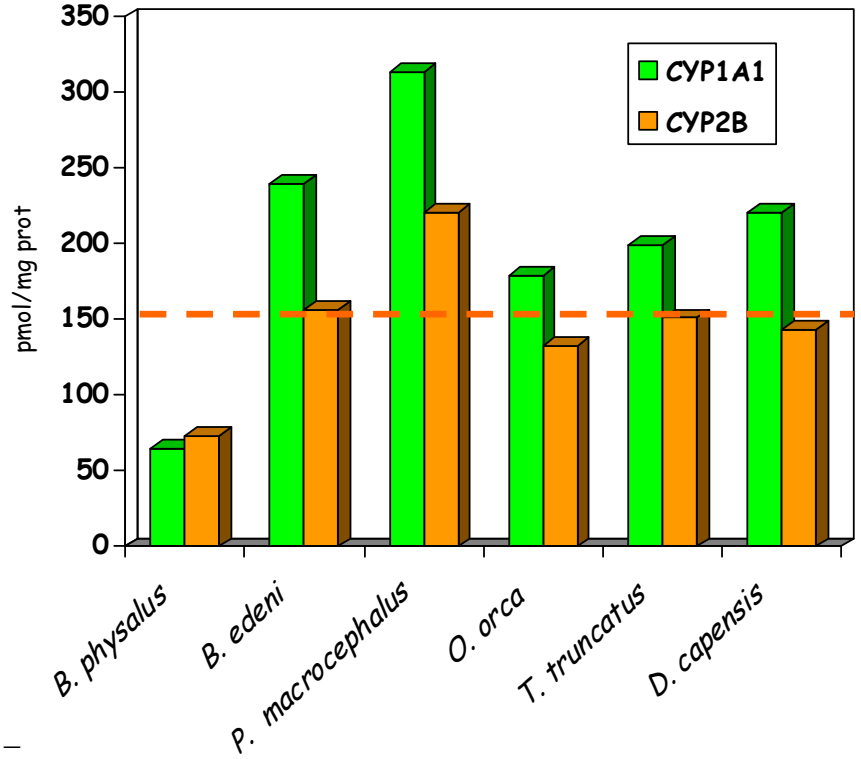

Fig.7 –