INTER-SPECIES DIFFERENCES IN BIOMARKER RESPONSES AND CONTAMINANT LEVELS IN THREE MYSTICETE SPECIES (*Balaenoptera musculus, Balaenoptera physalus* and *Balaenoptera edeni)* OF GULF OF CALIFORNIA (MEXICO)

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ABSTRACT

The main objective of this project was to investigate the inter-species differences in CYP1A1 and CYP2B expression, gene biomarkers expression (AHR, E2F1 and ER1) *and contaminant levels (OCs, PBDEs and PAHs) in three mysticete species, blue whale (Balaenoptera musculus), fin whale (Balaenoptera physalus) and Bryde's whale (Balaenoptera edeni) of Gulf of California (Mexico) using skin biopsy as diagnostic tools. A suite of sensitive non-lethal biomarkers was applied to the three mysticete species in order to evaluate the toxicological status of this cetacean species in the Gulf of California and also to explore the role of migratory/resident "behavior" and the feeding habits (zooplankton-eating species fin whale and blue whale, fish-eating species Bryde's whale) in the responses of the two isoforms of CYP. This "multi-trial diagnostic tool", applied to skin biopsies, underlined differences in POP levels and molecular biomarker responses between the three mysticete species of Gulf of California. Two main factors seem to regulate the expression of different CYP isoforms in the three species studied: the inductive ability of POPs and PAHs and the different evolution of the two cytochromes related to the different feeding habits of the mysticete species. With regard to the level of contaminants the highest levels of PAHs and PCBs were detected in blue whale and the highest levels of DDTs and PBDEs were detected in fin whale in comparison to the fish-eating species (Bryde's whale). Particular concern can be raised by the high levels of PCBs detected to in the migratory species blue whale, that could bioaccumulate POPs while moving along the Californian coast. This contamination phenomena can have generate induction of both CYP1A1 and CYP2B in this species and upregulation of estrogen receptor gene. On the opposite, extremely high level of both CYP1A1 and CYP2B were detected in the fish-eating species, showing similar level to odontocete species. Lower levels of OCs and high level of the CYP2B were detected in the Bryde's whale specimens, suggesting a higher detoxification ability in the fish-eating species. In conclusion, these data indicate that two main factors can regulate the expression of the two CYP proteins in the mysticete species of Gulf of California: a) the inductive phenomenon linked to the presence of both planar (CYP1A1) and globular (CYP2B) POPs of in the blubber of blue whale; b) the role of evolutionary pressures related to the different dietary habits of the species.*

KEYWORDS

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

INTRODUCTION

The main objective of this project was to investigate the inter-specific differences in cytochrome P450 1A1 (CYP1A1) and cytochrome P450 2B (CYP2B) expression, gene expression biomarkers (Aryl hydrocarbon

receptor - AHR, E2F1 transcription factor - E2F1, and Estrogen Receptor 1 - ER1) and contaminant levels (Organochlorine Compounds - OCs, Polybromodiphenyl ethers – PBDEs, and Polycyclic Aromatic Hydrocarbons - PAHs) in three mysticete species: blue whale (*Balaenoptera musculus),* fin whale (*Balaenoptera physalus*) and Bryde's whale (*Balaenoptera edeni*), sampled in the of Gulf of California (Mexico) using skin biopsy as ecotoxicological diagnostic tools. A suite of sensitive non-lethal biomarkers was applied to the three mysticete species in order to evaluate the toxicological status of this cetacean species in the Gulf of California and also to explore the role of migratory/resident "behavior" and the feeding habits (zooplankton-eating species fin whale and blue whale, fish-eating species Bryde's whale) in the responses of the two isoforms of CYP. CYP1A and CYP2B have been previously detected in cetacean skin and induction of these isoforms was measured after exposure to lipophilic contaminants such as OCs, PAHs and PBDEs, both *in vitro* and in field studies (Fossi *et al.*, 1992; Fossi *et al.*, 2006; Godard *et al.*, 2004).

Balaenoptera edeni (Anderson, 1879). Bryde´s whale; ballena de Bryde, rorcual tropical: Bryde´s whales are found worldwide in tropical to temperate waters, rarely above latitudes of about 35° (Reeves *et al.*, 2002). Bryde´s whales are frequently observed annually in the entire Gulf of California, particularly in coastal waters (Urbán and Flores, 1996). Molecular analyses suggest the possibility that two stocks may be present in the southern gulf, one resident and another from the eastern tropical Pacific (Dizon *et al.*, 1995). The Bryde´s whale abundance in the Gulf of California is estimated at 564 (453-2085) individuals, based on line transect methods (Gerrodette and Palacios, 1996).

Balaenoptera physalus (Linnaeus, 1758). Fin whale; ballena de aleta, rorcual común: The Fin whale is found in all oceans ranging from the Equator to Polar Regions with the largest concentrations located in temperate and cold waters. There is a resident population of fin whales in the Gulf of California (Berubé *et al.*, 2002, Urbán *et al.*, 2005). They present a general pattern of distribution; the west coast from the Canal de Ballenas to Bahía de La Paz in winter and spring and the midriff islands and northern gulf in summer and autumn. The Fin whale population size in the Gulf of California was estimated at 820 (594-3229) individuals, based on line transect methods (Gerrodette and Palacios, 1996), and 656 (374-938) based on mark-recapture methods Díaz-Guzmán (2006).

Balaenoptera musculus (Linnaeus, 1758). Blue whale; Ballena azul: The blue whale is a wide-ranging species that is distributed throughout all oceans and inhabit coastal, shelf and oceanic waters (Reeves *et al.*, 2002). Blue whales concentrate in the Gulf of California during winter and spring especially in the southwest coast, from Loreto to Los Cabos; but there are records all seasons of the year as north as the Midriff Islands. The blue whales from the Gulf of California expend the summer feeding in the waters off California. Gendron and Gerrodette (2003) estimate a population size of 362 (CV=47.5%) individuals based on aerial line transect methods.

In this study we tested the protein level of CYP1A1 and CYP2B and three "gene-expression biomarkers" (AHR, E2F1 and ER1). Each biomarker is involved in responses to different environmental stress, providing a broad spectrum of toxicological health status of the species.

Cytochrome P450 1A is a member of the superfamily of enzymes involved in Phase I oxidative metabolism of exogenous compounds, playing a key role in biotransformation of contaminants like dioxins, furans, PCBs and PAHs. Induction of CYP1A is mediated by the aryl hydrocarbon receptor (AHR) pathway which is activated by PAHs and planar halogenated compounds (PHAHs); CYP1A and AHR are therefore widely used as biomarker of exposure to these compounds, also in marine mammals (Hirakawa *et al.*, 2007; Godard *et al.*, 2004; Montie *et al.*, 2008; Niimi *et al.*, 2005; Wilson *et al.*, 2007), Estrogen receptors (ESRs) are members of the nuclear receptor superfamily. They are ligand-inducible transcription factors and activate transcription of estrogen target genes Ligand-binding signaling is due to binding of estrogen (or a structurally similar compound, such as an OC or PBDE) and consequent activation of the specific transcriptional response. Exposure to exogenous compounds (such as EDCs) with estrogenic or anti-estrogenic activity and with high affinity for ERs may therefore impair endocrine and sexual functions, enhancing the response of endogenous estrogens or agonistically binding receptors and inhibiting the physiological action of estrogens (Carpenter at al. 2002). The E2F transcription factor is a member of the E2F family (E2F1-8) which has a dual role in cell cycle regulation, controlling certain genes during DNA synthesis and apoptosis (Attwool *et al.*, 2004; La Tangue 2003). Over-expression of E2F-1 seems to up-regulate several genes involved in the activation of apoptosis and to interact with and be modulated by AHR.

METHODS

Sampling

Integument biopsies (epidermis, dermis and blubber) were collected from free-ranging Bryde's whales (n=4) and fin whales (n=5) in the Gulf of California during the summer 2008 and blue whales (n=4) during the

winter 2010 using biopsy darts launched with a crossbow (CITES Nat. IT 025IS, Int. CITES IT 007). 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.

Brominated (tri- to deca-) diphenyl ethers (PBDEs) were analyzed by high resolution gas chromatography low resolution mass spectrometry (HRGC-LRMS) using a 6890N gas chromatograph coupled with a 5975 quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA) operated in selected ion monitoring mode (SIM) with electron capture negative ionization (ECNI). A detailed description of the instrumental parameters involved in the analysis of PBDEs is published elsewhere (Muñoz-Arnanz *et al.*, 2011).

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 biopsy (Fossi *et al.*, 2006; Fossi *et al.*, 2008). For WB analysis, S9 fractions of tissue homogenates (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. 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).

Quantitative Real-Time PCR (qRT-PCR) analysis

Total RNA cDNA synthesis - Sub-samples of the skin biopsies (about 30 mg) were homogenized using a tissue lyser (Qiagen). Total RNA was extracted from homogenized material using the Aurum™ Total Fatty and Fibrous Tissue kit (Bio-Rad) following the manufacturer's instructions. Genomic DNA was eliminated by DNase-on-column treatment of each sample. RNA was quantified by Nano-Drop® ND-100 UV-vis spectrophotometer (NanoDrop Technologies). The integrity of RNA samples was assessed by denaturing agarose gel (1.2%) electrophoresis and ethidium bromide staining. Reverse transcription reactions were performed using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The amount of initial total retrotranscribed RNA was 500 ng.

qRT-PCR assays - The qRT-PCR assays were carried out in 96-well reaction plates with an iCycler iQ5 (Bio-Rad) using SYBR® Green detection chemistry. In a total volume of 20 µl, the reaction contained 0.8 µl cDNA, 0.6 µl of each primer (300 nM), 10 µl iQ™ SYBR® Green Supermix 2x (Bio-Rad) and 8 µl DNase/RNase-free sterile water. The three genes of interest (Aryl Hydrocarbon Receptor, Estrogen Receptor 1, E2F-1 transcription factor) and two reference genes (Glyceraldehyde 3-phosphate dehydrogenase - GAPDH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide - YWHAZ) for the normalization procedure were amplified for each of the 3 species skin biopsies. Each reaction was run in triplicate, as well as the no-template control. Amplification conditions were described in Spinsanti *et al.* (2006). To compare data from different experimental plates, threshold values were set manually to the arithmetic mean of the automatically determined values. Raw threshold cycles (Ct) were converted to quantities by the comparative ΔΔCt method (Livak and Schimttgen, 2001).

Gene expression levels in the skin biopsies were calculated using *GenEx* v. 4.3.8 Software (MultiD Analyses AB). Input Ct values (for reference and target genes) were pre-processed by efficiency correction to indicate technical repeats. Normalization to reference genes GAPDH and YWHAZ and to sample amount were applied.

Statistical data analysis

Hierarchical cluster analysis by the minimum energy (E) distance method was used to define clusters on the basis of species and canonical discriminant analysis on PCA factors was performed to reveal clustering variables. All statistical analysis was performed by SPSS 12.0 Software (IBM® SPSS® Statistics).

RESULTS AND DISCUSSION

This multi-trial diagnostic tool, applied to skin biopsies, underlined differences in POP levels, PAH levels and molecular biomarker responses between the three mysticete species of Gulf of California. Two main factors seem to regulate the expression of different CYP isoforms in the three species studied; the inductive power of POPs and PAHs and the different evolution process of the two cytochromes related to the different feeding habits of the mysticete species.

With regard to the level of contaminants, the highest levels of PAHs and PCBs were detected in the zooplankton-eating species (blue whale) and the highest levels of DDTs and PBDEs were detected in the zooplankton-eating species (fin whale) in comparison to the fish-eating species (Bryde's whale) (Fig.1). Particular concern can be generated from the high levels of PCBs and PAHs detected in the migratory species blue whale, that could have bioaccumulated POPs and PAHs during the migratory route along the Canada and United States coasts. This contamination input can have induced both CYP1A1 and CYP2B in this species and could be responsible of the up-regulation of the E2F1 and ER1 genes (Fig.2). Higher level of DDTs and PBDEs and intermediate CYP expressions are detected in the Sea of Cortez resident species fin whale (Fig.2). On the opposite, extremely high level of both CYP1A and CYP2B and AHR were detected in the fish-eating species, showing similar level of cytochromes to odontocete species resident in Sea of Cortez (Fossi, data not shown) (Fig.2) Lower levels of OCs and high level of the CYP2B were detected in the Bryde's whale specimens, suggesting a higher detoxification ability in the fish-eating species.

Hierarchical cluster analysis, by the minimum energy (E) distance method used to define clusters on the basis of species, show that the three species revealed a completely different distribution respect to contaminants, with the migratory species blue whale completely separated to the two resident specie fin whale and Bryde's whale (Fig.3).

The canonical discriminant analysis on PCA factors, performed to reveal clustering variables, confirmed the previous data, showing that blue whale are characterized by the highest levels of PAHs and PCBs, fin whale are characterized by the highest levels of DDTs and PBDEs and Bryde's whale is characterized by lowest levels of the all contaminants investigated (Fig.4).

The canonical discriminant analysis on PCA factors applied to the biomarkers data show a extremely interesting inter-species differences between the three species revealing high E2F1 and ER1 responses in the blue whale, highest CYP1A, CYP2B and AHR expression in the fish-eating species and lowest biomarker responses in fin whale.

CONCLUSIONS

In conclusion these data show that two main factor can regulate the expression of the two CYP proteins and gene expression biomarkers in the mysticete species of Gulf of California: a) the inductive phenomenon linked to the presence of planar (CYP1A1) and globular (CYP2B) halogenated compounds in the blubber of blue whale; b) the role of evolutionary pressure related to the different dietary habits of the species (zooplankton-eating species fin whale and blue whale, fish-eating species Bryde's whale). In particular, these preliminary evidences suggest a peculiar evolutionary process of the two isoforms of CYP in the fish-eating species (Bryde's whale), which showed levels of both cytochromes similar to the odontocete species.

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CAPTIONS

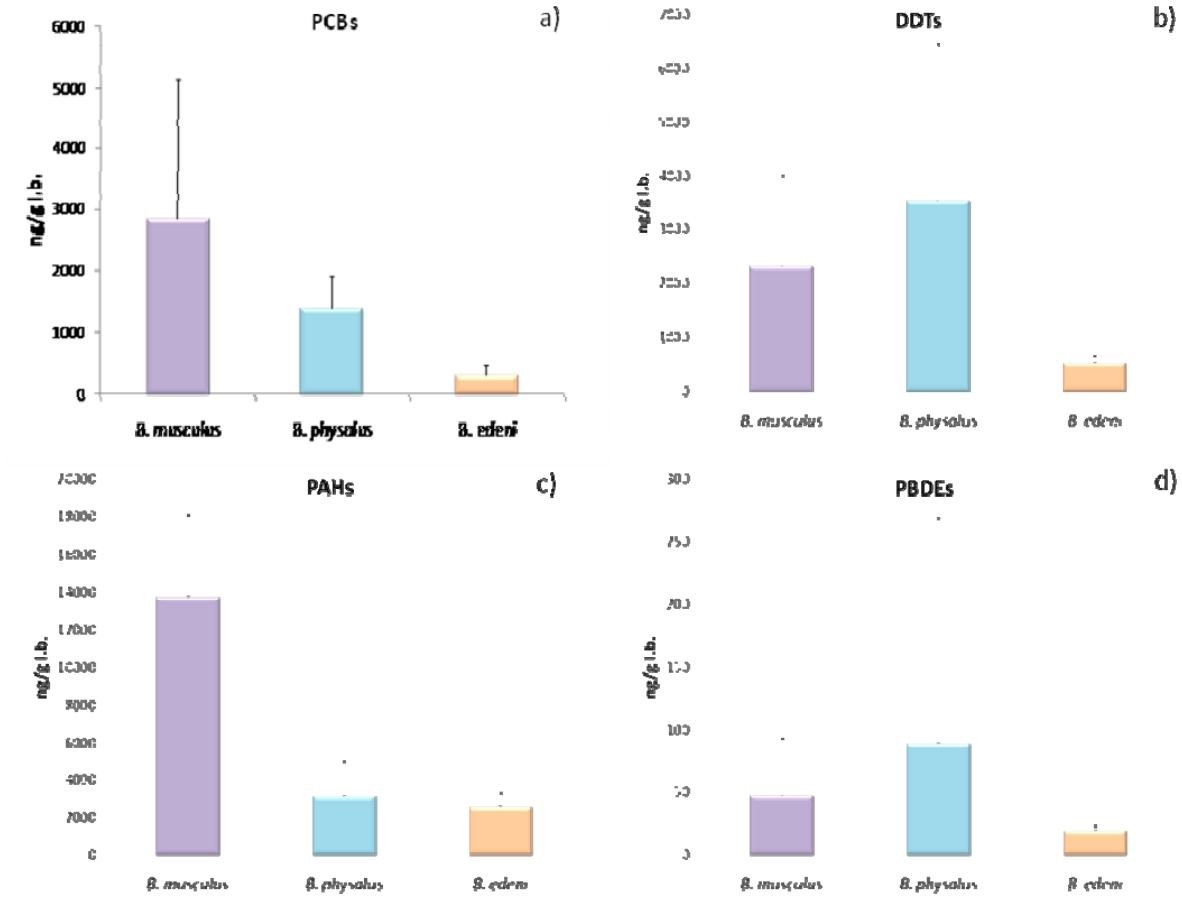
Figure 1 - PCBs levels (a), DDTs levels (b), PAHs levels (c) and PBDEs levels (b) in skin biopsy of specimens of *Balaenoptera musculus, Balaenoptera physalus* and *Balaenoptera edeni* of Gulf of California.

Figure 2 - Biomarker responses (CYP1A1 (a), CYP2B (b), AHR (c), EDF1 (d), ER1 (e)), in skin biopsy of specimens of *Balaenoptera musculus, Balaenoptera physalus* and *Balaenoptera edeni* of Gulf of California.

Figure 3– Dendograms of classification of the three species *(Balaenoptera musculus, Balaenoptera physalus* and *Balaenoptera edeni*) studied. Species grouped by contaminant (OCs, PAHs and PBDEs).

Figure 4 - A) Correlation between contaminant variables (OCs, PAHs and PBDEs) and discriminant functions. **B)** Plot of discriminant scores in the three species*(Balaenoptera musculus, Balaenoptera physalus* and *Balaenoptera edeni*).

Figure 5 - A) Correlation between biomarkers variables (CYP1A1, CYP2B, AHR, E2F1, ER1) and discriminant functions. **B)** Plot of discriminant scores in the three species *(Balaenoptera musculus, Balaenoptera physalus* and *Balaenoptera edeni*).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3000
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B. physalus
B. edeni
DDTs
b)
ng/g l.b.
B. musculus
B. physalus
B. edeni
PAHs
c)
ng/g l.b.
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PBDEs
d)
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B. musculus
B. physalus
B. edeni

Figure 1

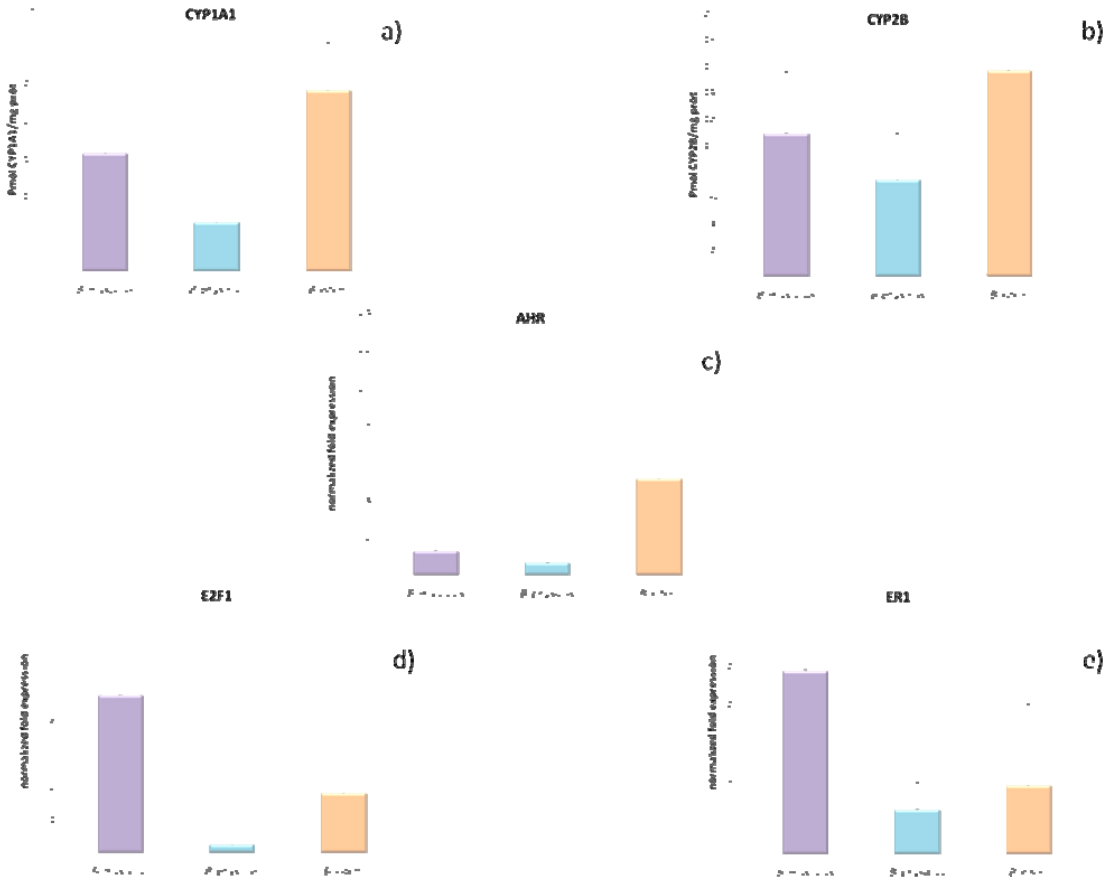
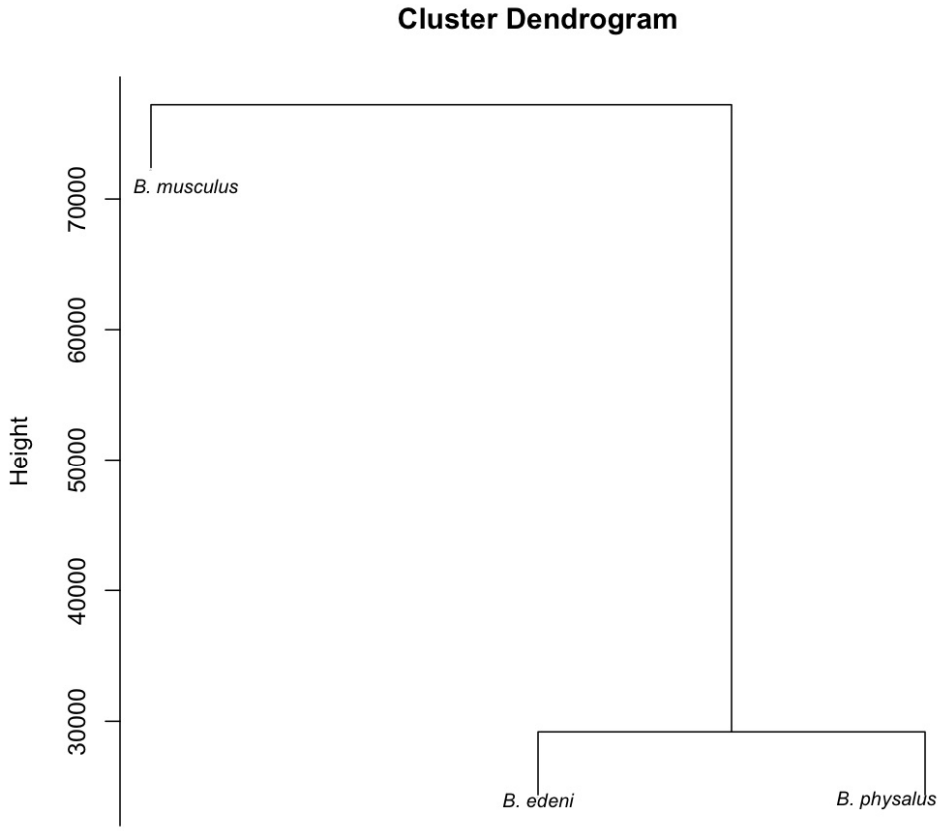


Figure 2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B. musculus
B. edeni
B. physalus

Figure 3

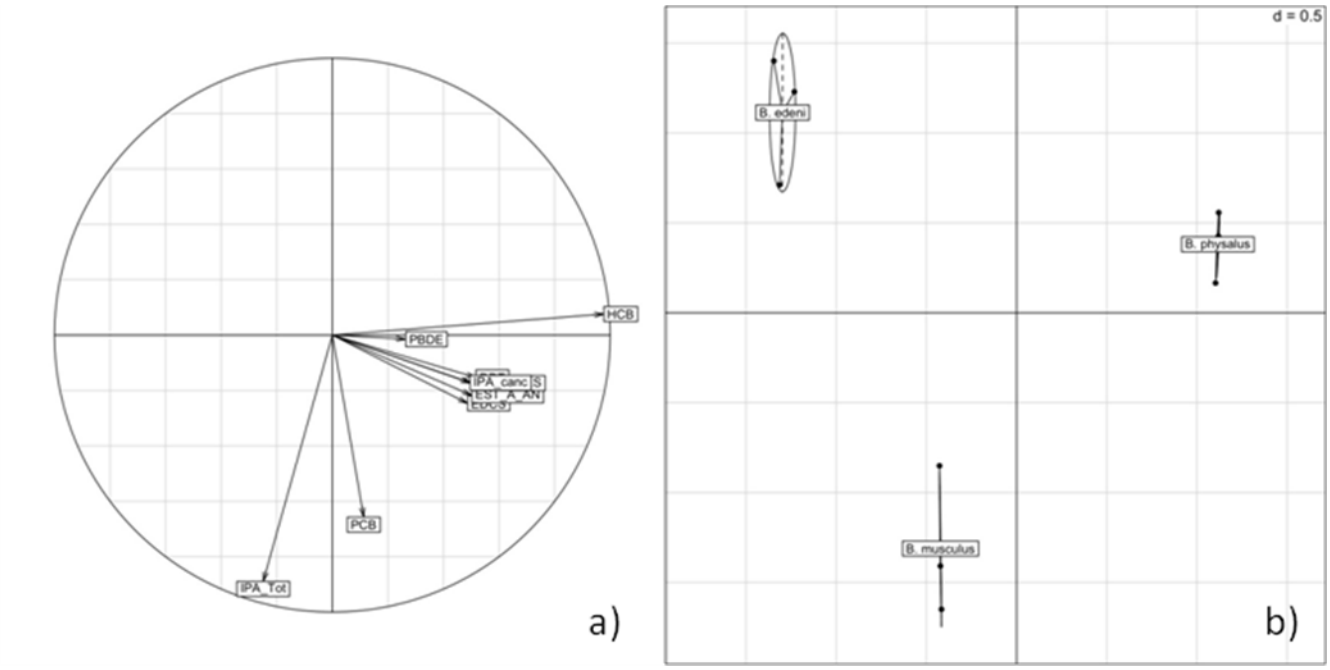

Figure 4

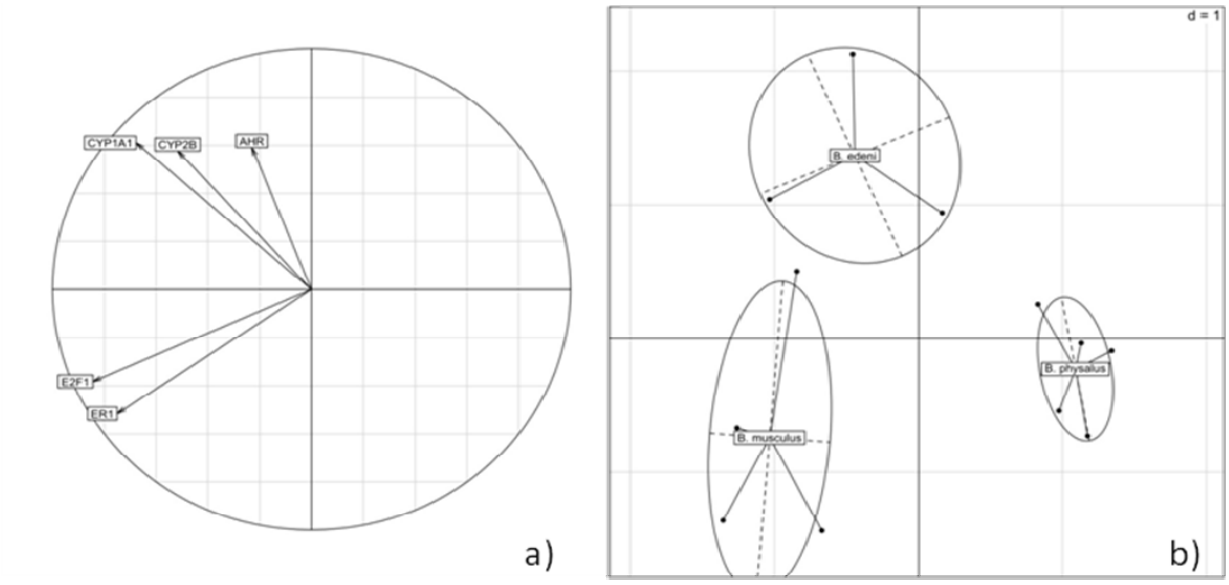

Figure 5