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Ecotoxicological diagnosis of striped dolphin (*Stenella coeruleoalba*) from the Mediterranean basin by skin biopsy and gene expression approach

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Abstract Mediterranean cetacean odontocetes are exposed to environmental stress, in particular to persistent organic pollutants, polycyclic aromatic hydrocarbons and trace elements. In the present study, the response of "gene-expression biomarkers" was evaluated in Mediterranean striped dolphin (*Stenella coeruleoalba*) skin biopsies collected in three sampling areas: Pelagos sanctuary (Ligurian sea), Ionian sea, and Strait of Gibraltar. The mRNA levels of five putative biomarker genes (aryl hydrocarbon receptor, E2F-1 transcription factor, cytochrome P450 1A, estrogen receptor 1, and heat shock protein 70) were measured for the first time by quantitative real-time PCR in cetacean skin biopsies. The different responses of most of the genes reflected contamination levels in the three sampling areas. Pelagos sanctuary dolphins appeared to be the most exposed to toxicological stress, having the highest up-regulation of CYP1A and AHR. Moreover, a cluster analysis distinguished the populations on the basis of the gene expression biomarker used in our study, showing different pattern between Mediterranean sea and Strait of Gibraltar. Our results suggest that this molecular approach applied to non-destructive biopsy material is a powerful diagnostic tool for evaluating ecotoxicological impact on cetacean populations.

Cristina Panti and Giacomo Spinsanti contributed equally to this work.

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Keywords Gene expression · Mediterranean basin · Biomarkers · Cetacean · *Stenella coeruleoalba*

Abbreviations

POPs	Persistent organic pollutants
PAHs	Polycyclic aromatic hydrocarbons
OCs	Organochlorines compounds
EDCs	Endocrine disrupting compounds.
PBDEs	Polybrominated diphenylethers
AHR	Aryl hydrocarbon receptor
E2F-1	E2F-1 transcription factor
CYP1A	Cytochrome P450 1A
CYP2B	Cytochrome P450 2B
ESR1	Estrogen receptor 1
HSP70	Heat shock protein 70
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
qRT-PCR	Quantitative real time PCR
IUCN	International union for conservation of nature
MPA	Marine protected area
CITES	Convention on international trade in endangered species

Introduction

Marine top predators, especially odontocete cetaceans, are threatened by complex interactions between different human activities. The main threats to cetaceans on a global scale are habitat loss and degradation, by-catch events, prey depletion, maritime traffic, epizootic events, and direct killing. Stress due to chemical compounds in Mediterranean cetaceans is higher than in the same cetacean

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species that live in other marine environments (Aguilar et al. 2002).

Since the Mediterranean sea is a semi-enclosed basin with limited exchange of water with the Atlantic ocean and surrounded by heavily industrialized countries, the anthropogenic pressure on long-living and top predator species, such as cetacean odontocetes, is elevated. Xenobiotic compounds, such as organochlorines (OCs) and polybrominated diphenylethers (PBDEs), are widespread in the environment and can affect animal health at different levels of biological organization because they are resistant to environmental and biological degradation. Polycyclic aromatic hydrocarbons (PAHs) are abundant and ubiquitous in the Mediterranean basin. Because of their lipophilic and persistent nature, several of these compounds and their metabolites bioaccumulate and biomagnify. Top predators are threatened by both processes (Corsolini et al. 2008; Fossi and Marsili 2003; Leonards et al. 2008). The high levels of OCs and PBDEs in cetaceans (Aguilar and Borrel 2005; Petterson et al. 2004) also suggest that top predators are at risk of endocrine disruption (Porte et al. 2006).

Since striped dolphins (*Stenella coeruleoalba*) have a pelagic distribution throughout the basin, feed on pelagic and bathypelagic species of teleosts and cephalopods (Aguilar 2000), have abundant fatty tissue and a limited capacity to metabolize certain PCB congeners (Norstrom et al. 1992; Tanabe et al. 1988), they and other small odontocetes show the highest levels of OCs of all marine mammals sharing the same habitat (Aguilar and Borrel 2005; Fossi et al. 2004; Storelli and Marcotrigiano 2003). A geographical trend of OC contamination in the Mediterranean sea was observed by measuring their accumulation in striped dolphin skin biopsy and CYP1A and CYP2B induction (Fossi et al. 2003). Little is known about the effects of PBDE exposure on Mediterranean cetaceans. PBDEs analyzed in five species of Mediterranean cetaceans showed the same congener pattern recorded in the Atlantic ocean and other seas (Boon et al. 2002; Isobe et al. 2009; Petterson et al. 2004).

The striped dolphins (classified as Least Concern by the IUCN Red List of Threatened Species) have an estimated number of about 117,880 individuals (line-transect survey of 1991 and 1992) measured after the massive die-off caused by Morbillivirus infection in the early 1990s (Forcada et al. 1994). The infection affected this species throughout the basin. Biomonitoring of the health status of the Mediterranean striped dolphin is therefore warranted. In the assessment of ecotoxicological hazard and stress exposure of animal species, biomarkers are powerful tools in the prognostic and diagnostic phases. Biomarkers at the molecular level indicate any variation linked to chemical, ecotoxicological or other environmental stresses at an early stage, increasing and integrating the specificity and sensitivity of conventional biomarker responses.

Non-lethal tools are of course mandatory for protected species or species "at risk" and skin biopsies from free-ranging animals are a validated non-destructive method of sampling (Fossi et al. 2000). Besides the conventional biomarkers (e.g. protein induction, enzymatic activity), variations at gene-expression level can be used in skin biopsies, because they require only a small amount of biological material, the tissue is of good quality, and a large number of samples can be analyzed. The high sensitivity of quantitative real-time PCR (qRT-PCR) makes molecular-level investigation possible, providing early warning of toxic stress or detoxification processes (Forbes et al. 2006; Piña et al. 2007). It also enables quantification of mRNA from genes transcribed at very low levels, however, an accurate experimental procedure is required. Two main strategies are used: relative quantification (with endogenous control genes) and absolute quantification (with an external standard) (Huggett et al. 2005; Kubista et al. 2006; Vandesompele et al. 2002). Endogenous control genes are assumed not to be modulated if exposed to the same experimental conditions as the target gene. The reliability of this strategy depends on choosing stable reference genes for normalization of gene-expression levels.

Three sampling areas across Mediterranean basin were selected to have an overview of the ecotoxicological status of striped dolphin populations in the western part of the basin, including the contiguous area of the Strait of Gibraltar (Fig. 1). They are geographically distinct with different geographical characteristics, levels and classes of contaminants, and types of anthropogenic pressure. The Pelagos sanctuary has been a Marine Protected Area (MPA) since 2002 and extends from southeastern France to northwestern Italy (Notarbartolo di Sciara et al. 2008). It is the largest European pelagic protected area and contains an abundance of cetaceans, however they are exposed to high anthropogenic pressure due to maritime traffic, high levels of POPs and trace elements, and heavy exploitation of the

Fig. 1 The Mediterranean basin showing the three sampling areas: *A* Pelagos sanctuary, *B* Strait of Gibraltar, and *C* Ionian sea

coasts. The Ionian sea sampling area lies between eastern Sicily and southwestern Calabria. In this area the levels of POPs and PAHs due to human activities are lower than in Pelagos sanctuary (Fossi et al. 2004). The Strait of Gibraltar sampling area includes Spanish and Moroccan waters where the Mediterranean meets the Atlantic Ocean. Human activities in the area are mainly due to its strategic position and include maritime traffic (tankers, containers and ferries) which also produce noise and collisions.

In this study we tested five putative "gene-expression biomarkers" for the first time in cetacean skin biopsies. Each biomarker is involved in responses to different environmental stresses (biomarkers of exposure), providing a broad spectrum of toxicological health status of the species. Two genes, heat shock protein 70 (HSP70) and E2F-1 transcription factor (E2F-1), are involved in responses to "generic stress"; two other genes, cytochrome P450 1A (CYP1A) and aryl hydrocarbon receptor (AHR), are involved in more specific pathways such as activating metabolism of planar fat-soluble compounds (e.g. PAHs and planar halogenated compounds, PHAHs); the fifth gene, estrogen receptor 1 (ESR1), is involved in some regulation processes of the reproductive system.

HSP70 is a stress-related protein belonging to a multi-gene family, induced by a variety of agents and conditions which can either directly damage proteins or indirectly cause production of abnormal proteins in cells (Nollen and Moromoto 2002). HSP70 family proteins are ubiquitous, underlying their fundamental protective role in cell response to stress. Among HSP families, HSP70 is often used as an early biomarker in environmental stress assessment (Aït-Aïssa et al. 2000; Varò et 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 Thangue 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. Inhibiting the expression of AHR increases oxidative stress and DNA damage and induces apoptosis modulated by E2F-1; on the contrary, activating AHR leads to formation of the AHR-E2F-1 complex, inhibiting expression of E2F-1-dependent genes and apoptosis (Marlowe et al. 2008).

CYP1A 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 AHR pathway which is activated by PAHs and PHAHs; CYP1A is 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), though few studies are available on gene expression in cetaceans.

AHR is a soluble ligand-activated transcription factor involved in processes that regulated Phase I enzymes as well as in cell cycle control and cell physiology, suggesting its importance as a fundamental component of cell defense against external toxicants or endogenous substances (Hahn 2002; Phelan et al. 1998). Although the physiological function of AHR is not yet clear, an endogenous role in physiological signaling pathways is suggested (Puga et al. 2009) by the receptor's ability to control the expression of drug-metabolism enzymes (Beischlag et al. 2008). AHR shows high affinity for PHAHs and PAHs, though there is evidence of species-specific variation in affinity and response (Hahn 2001).

ESRs are members of the nuclear receptor superfamily. They are ligand-inducible transcription factors and activate transcription of estrogen target genes which contain estrogen response elements (EREs), located within the promoter region. However, estrogen receptors can regulate gene expression activating estrogen responsive genes without EREs (Björnström and Sjöberg 2005). 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 et al. 2002). Activation of ESRs can affect AHR-regulated genes because the ESR1-AHR crosstalk seems to inhibit induction of genes regulated by AHR (Matthews and Gustafsson 2006). Various AHR ligands bind or activate ESR, suggesting competitive binding between the two receptors (Ohtake et al. 2008).

The aim of this study was to investigate gene expression by qRT-PCR in cetacean skin biopsy in order to obtain early warning of the toxicological hazard to which Mediterranean striped dolphins are exposed, the most abundant cetacean species in Mediterranean sea. These diagnostic signals were used to identify hot spots of contamination stress across the basin. Differences in gender response to stress were also investigated.

Materials and methods

Sampling area and biopsy procedure

Skin biopsies (skin and blubber) from free-ranging striped dolphin were obtained in the three areas: Pelagos sanctuary

($F = 8$, $M = 6$), Ionian sea ($F = 5$, $M = 8$), and Strait of Gibraltar ($F = 8$, $M = 7$) on several sampling efforts (summer 2006–2007). Striped dolphin were sampled using an aluminium pole as previously described (Fossi et al. 2000) (International CITES permit IT007, national CITES permit IT025IS). To avoid transmitting infections, the tip of the pole was sterilised each time with alcohol before sampling. Samples were immediately plunged into RNA later (Ambion), then stored in liquid nitrogen. The gender of the biopsied dolphins was determined according to Bérubé and Palsbøl (1996).

Total RNA and genomic DNA isolation, and cDNA synthesis

Sub-samples of the 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. Total RNA isolations were stored at −80°C. From the same samples, genomic DNA was isolated using the Wizard® SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions and subsequently quantified and used in PCR reactions.

DNA and RNA were 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. This kit enables an initial step at 42°C for 2 min with a wipeout buffer, aimed at eliminating genomic DNA. The amount of initial total retrotranscribed RNA was 500 ng.

Target gene sequencing and qRT-PCR primer design

Due to lack of information in sequence databases on our species of interest, PCR reactions were carried out using cDNA isolated from the *S. coeruleoalba* skin biopsies as template for coding sequences of the genes. Primers were designed by aligning sequences of the phylogenetically closest species of mammals retrieved from GenBank. The selected regions were amplified by standard PCR reactions. Amplification products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced. Sequences were corrected manually using Sequencer 4.2.2 software (Gene Codes) and the specificity of the products was checked using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Partial sequences of cDNAs coding for the selected genes (except CYP1A) were deposited in GenBank under the Accession Numbers shown in Table 1. Exon/intron localizations for each gene were deducted by alignment with homologous genes of *Homo sapiens* and confirmed by PCR using striped dolphin genomic DNA as template.

Particular attention was paid to qRT-PCR primer design. Specific primer pairs for each gene of interest were designed using Beacon Designer 2.06 (Premier Biosoft International). Primer length, annealing temperature, base composition, primer dimer artefacts, secondary structure and 3′-end stability were accurately considered. Amplicon lengths ranged from 111 to 234 bp to guarantee high efficiency during the reaction. Most primer pairs used in the study were designed on different exons or spanning exon–exon junctions to avoid any genomic DNA co-amplification. Amplification efficiency (E), slope (s) and correlation

Table 1 Details on qRT-PCR primer pairs and sequences

Gene	Sequence (5′ → 3′)	Position cDNA	Amplicon length (bp)	E%	R^2	Genbank accession number
AHR	Fw GTTCAGGTTACCATCAGCAACAGTC	9th exon	204	98.6	0.997	GU147939
	Rv AAGGCACGGATTGGTTCAAGTTC	10th exon				
CYP1A	Fw AAACGTTTGAGAAGGGCACATTC	5th exon	148	97.9	0.996	AF235141
	Rv TCAAACCCAGCTCCAAAGAGGT	6th exon				
E2F-1	Fw TGCCACCACCACCATCATCTC	6th exon	154	98.2	0.998	FJ748584
	Rv CGAGTCAGCCGCCACCAG	7th exon				
ESR1	Fw GGAGACTCGCTACTGTGC	2nd exon	234	96.4	0.997	GU147940
	Rv CTCCTCTGCGGTCTTTCC	4th exon				
HSP70	Fw AAGGGTCGTCTGAGCAAGG	5th exon	147	99.1	0.998	GU147941
	Rv TTCTCGTCTTCCACCGTCTG	6th exon				

For each gene is reported: primer sequences, position on the coding sequence, amplicon length, amplification efficiency (E%), correlation coefficient and GenBank accession numbers

coefficient (R^2) of each primer pair in the qRT-PCR were calculated using 1:5 serial dilutions of cDNA as template on a iQ5 machine (Bio-Rad) (Table 1). Products were checked for specificity on 2% agarose gel and sequenced.

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 2× (Bio-Rad) and 8 µl DNase/RNase-free sterile water.

The five genes of interest (GOI) and two housekeeping genes (HKGs) for the normalization procedure were amplified for each of the 42 skin biopsies. The housekeeping genes were selected in a previous study of *S. coeruleoalba* skin biopsies (Spinsanti et al. 2006). Each reaction was run in triplicate, as well as the no-template control. Amplification conditions were as 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 Schmittgen 2001).

Statistical data analysis

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 preprocessed by efficiency correction to indicate technical repeats normalization to reference genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) and to sample amount were applied. Normal distribution of the data was checked by the one-sample Kolmogorov and Smirnov test. For variables not normally distributed the data was expressed as natural logarithm. Two-way analysis of variance was then performed to verify whether sampling area and sex significantly affected expression of the selected genes and whether any significant effect was due to interaction of experimental factors. Multiple post-hoc analysis of variance was also used to consider all possible comparisons between areas. Specifically, Dunnett's T3 test was applied when variances were not homogeneous and the Student–Newman–Keuls (S–N–K) test was used when variances were homogeneous. Comparison of males and females within each sampling area was verified by Student's unpaired *t* test.

Hierarchical cluster analysis by the minimum energy (E) distance method was used to define clusters on the basis of areas 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

qRT-PCR and skin biopsies

The need to develop powerful non-destructive tools to evaluate the ecotoxicological status of Mediterranean cetaceans led us to investigate biomarker responses to stress and toxic compounds in the most abundant dolphin of the Mediterranean basin, *S. coeruleoalba*. The principal aim of this work was to develop new "gene-biomarkers" using qRT-PCR and assess their responses in Mediterranean striped dolphins representing a gradient of exposure to contaminants. This was done by analyzing skin biopsy samples collected in three areas of the basin (Pelagos sanctuary, Ionian sea and Strait of Gibraltar).

Detection of an early warning signal using a small amount of tissue sampled in a non-destructive way was perfectly coherent with the choice of validating biomarkers considering variation in mRNA levels. Furthermore, detection of variations in mRNA levels can be integrated with protein expression responses to obtain insights into the mechanisms of action of mixtures of known and unknown contaminants in organisms and enables a wide range of simultaneous analyses, integrating the responses of several genes involved in different physiological and metabolic pathways, from specific to generic stress.

Gene expression as a diagnostic signal

Five genes of interest were selected, partially sequenced and tested as biomarker responses in the 42 biopsies quantifying the mRNA expression levels of the target genes (AHR, CYP1A, E2F-1, HSP70, ESR1). The expression levels of the five genes were compared among areas and between males and females ($F = 22$, $M = 20$). The mRNA expression levels were normalized to GAPDH and YWHAZ reference genes.

Levels of mRNA expression for the genes AHR, CYP1A and E2F-1 reflected a similar trend in the three areas, suggesting exposure to different toxicological stressors. Gene expression levels were highest in specimens from the Pelagos sanctuary and lowest in those from Gibraltar Strait (Fig. 2a–f). The responses of the other two genes, ESR1 and HSP70, did not reflect the same trend but the Ionian samples showed the highest levels of mRNA

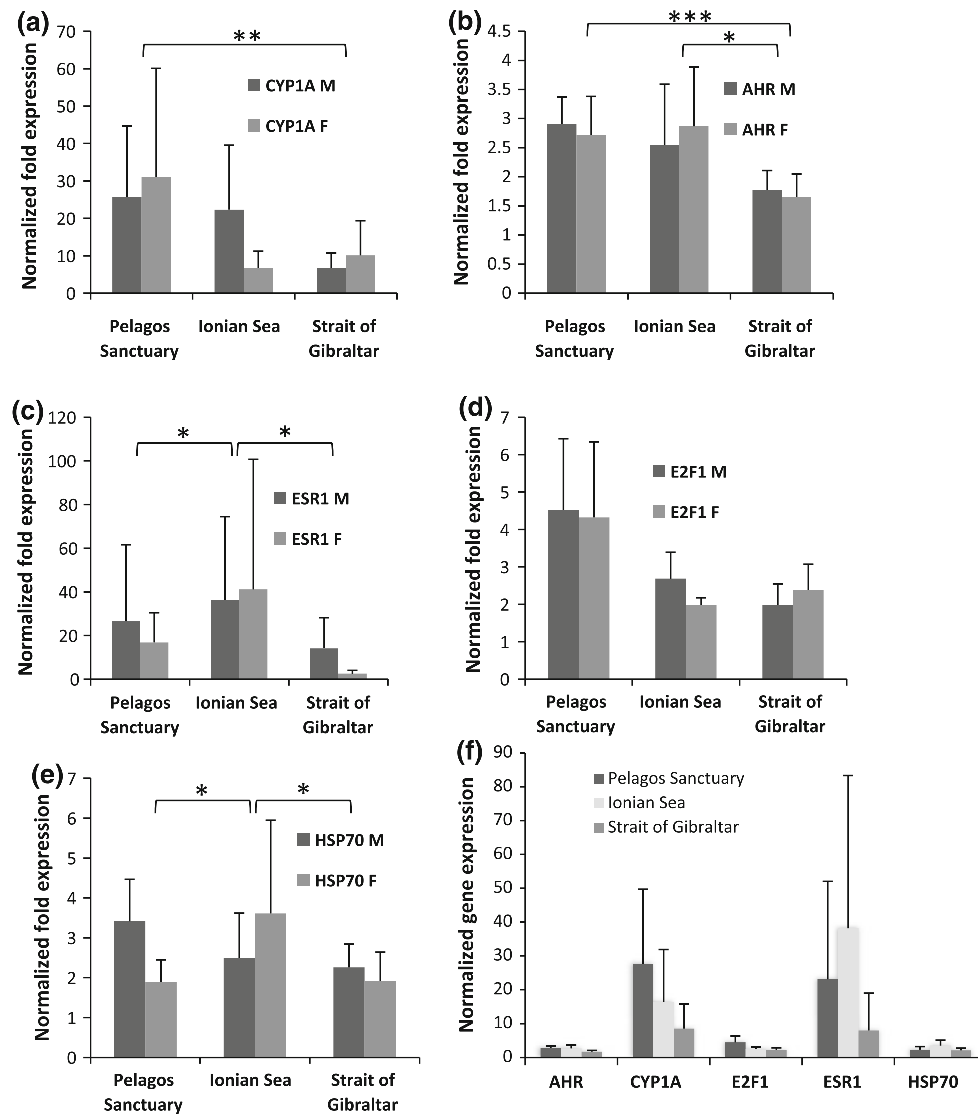

Fig. 2 Gene expression levels in 42 biopsies of striped dolphins from the three sampling areas. **a–e** The expression levels of males and females of the five genes (**a** CYP1A, **b** AHR, **c** ESR1, **d** E2F1, **e** HSP70), each bar correspond to the mean ± SD of the mRNA expression; **f** each *bar* corresponds to the mean expression of all samples coming from the same area ± SD. *Brackets* show the statistically significant comparisons (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Gene expression was normalized to GAPDH and YWHAZ genes

expression, followed by the Pelagos sanctuary and finally the Gibraltar Strait specimens (Fig. 2a–f).

Statistical analysis showed normal distributions for the genes AHR (K–S $Z = 0.060$, two tailed *t*-test $P = 0.857$), CYP1A (K–S $Z = 1.260$, two tailed *t*-test $P = 0.084$), E2F1 (K–S $Z = 1.167$, two tailed *t*-test $P = 0.131$) and HSP70 (K–S $Z = 0.997$, two tailed *t*-test $P = 0.273$), whereas ESR1 showed a normal distribution (K–S $Z = 0.815$, two tailed *t*-test $P = 0.520$) after log-transformation.

Analysis of variance between areas showed a statistically significant “area effect”, whereas the “sex effect” was not significant between specimens and areas, and was statistically homogeneous for all five genes.

CYP1A was used as biomarker of exposure to lipophilic and planar contaminants, allowing discrimination of areas/sub-populations exposed to different levels of lipophilic contaminants. Expression of the CYP1A gene in skin biopsies collected in the three areas showed differences, indicating that the striped dolphins were exposed to potential different toxicological risk. Comparison of the presumably most polluted (Pelagos sanctuary) and least polluted areas (Strait of Gibraltar) showed 3.24-fold induction ($P < 0.01$) of mRNA levels of CYP1A (Fig. 2a, f). It is well known

that Pelagos sanctuary is broadly contaminated by lipophilic and compounds, such as PAHs and OCs (Fossi et al. 2004).

Interaction of AHR with PHAHs and dioxins is widely documented, as is its role in the activation of CYP1A transcription. Gene expression values of AHR in our data set again reflected a regional response trend. Since males and females were homogeneously distributed, a post-hoc analysis of variance was applied, independent of sex (Levene test $F(2, 39) = 8.31$, $P = 0.001$) and Dunnett's T3 test underlined a significant difference between specimens from the Strait of Gibraltar and the Ionian sea ($P = 0.016$) and the Strait of Gibraltar and Pelagos sanctuary ($P < 0.001$, Fig. 2b, f). On the contrary, expression of ESR1 (applying post-hoc comparison of variance, independent of sex: Levene test $F(2, 39) = 0.81$, not significant) did not follow the same geographical trend, but individuals from the Ionian sea showed higher levels of mRNA than those from Pelagos sanctuary (Test S–N-K $P < 0.05$, 1.65-fold) and the Strait of Gibraltar (Test S–N-K $P < 0.05$; 4.75-fold, Fig. 2c, f). This probably indicates higher exposure of the Pelagos and especially Ionian populations to xeno-estrogens than dolphins from Gibraltar and suggests the hypothetical presence of different EDCs in different areas. However, since the estrogen receptor signaling pathway is complex, a more detailed functional assessment is warranted. The ligand (for instance dioxin-like compounds) that activates ESR1 seems to activate AHR as well, suggesting competitive binding (Ohtake et al. 2008) and inhibition of AHR induction. These findings may explain the low levels of induction of AHR compared to ESR1, but further investigation of this mechanism in our species is necessary.

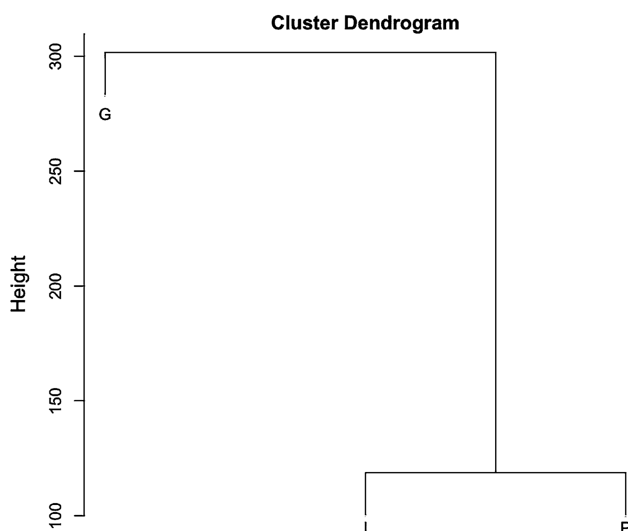

Fig. 3 Dendogram of the cluster analysis for the three areas (Pelagos Sanctuary = P, Ionian Sea = I, Strait of Gibraltar = G)

With regard to the E2F-1 gene, little is known about the effects of contaminants on its expression. Its role in regulation of the cell cycle and apoptosis and its response to stress led us to propose it as a possible biomarker of exposure to general stress. The formation of complexes composed of AHR/ARNT and E2F-1 have been demonstrated, indicating that AHR ligands, such as dioxins, are involved in activation of E2F-1 and therefore in induction of apoptosis (Watabe et al. 2010). The response in skin biopsies showed higher induction of mRNA levels in specimens from Pelagos sanctuary than in striped dolphins from the Strait of Gibraltar. Comparing the Pelagos sanctuary with Ionian sea and Strait of Gibraltar specimens, the gene is slightly modulated (1.85-fold and 2.00-fold, respectively) but the differences did not appear to be statistically significant (Fig. 2d, f).

Finally, the stress-related HSP70 gene showed greater up-regulation of expression in Ionian sea specimens than in those from the other two sites: 1.55 and 1.68-fold versus Pelagos sanctuary and Strait of Gibraltar, respectively (Dunnett's T3 test, $P = 0.027$). The ability of HSP70 to respond to multiple stressors, does not give a clear and specific cause-effect response, but underlines the exposure of dolphins to general stress that may be chemical or otherwise (Fig. 2e, f).

Effects of area on gene expression responses

The geographically different response exhibited by at least two genes (AHR and CYP1A) is a clue that dolphins from Pelagos sanctuary and the Ionian sea are more exposed to toxicological hazard than those inhabiting the Strait of Gibraltar. Since no clear genetic distinction exists between these three populations (also demonstrated in Gaspari et al. 2007), the responses to exposure to a wide range of other toxic compounds did not depend on intra-species variability but on the different levels of contamination of geographical area where the animals live, breed and feed, even if the striped dolphin is known to range widely. On this point, further analysis was performed to verify whether the differences among the proposed suite of gene-expression biomarkers could help distinguish responses on the basis of the geographical distribution of populations and which parameter (gene) contributed most to separation by areas. Cluster analysis and discriminant analysis of the PCA factors was performed. Cluster analysis indicated that specimens sampled in the Strait of Gibraltar area were significantly distinct from those from Pelagos sanctuary and the Ionian sea (Fig. 3), allowing the populations to be clearly distinguished by our variables. This revealed the greater ecotoxicological risk of the two Mediterranean sub-populations (Pelagos sanctuary and Ionian sea) compared

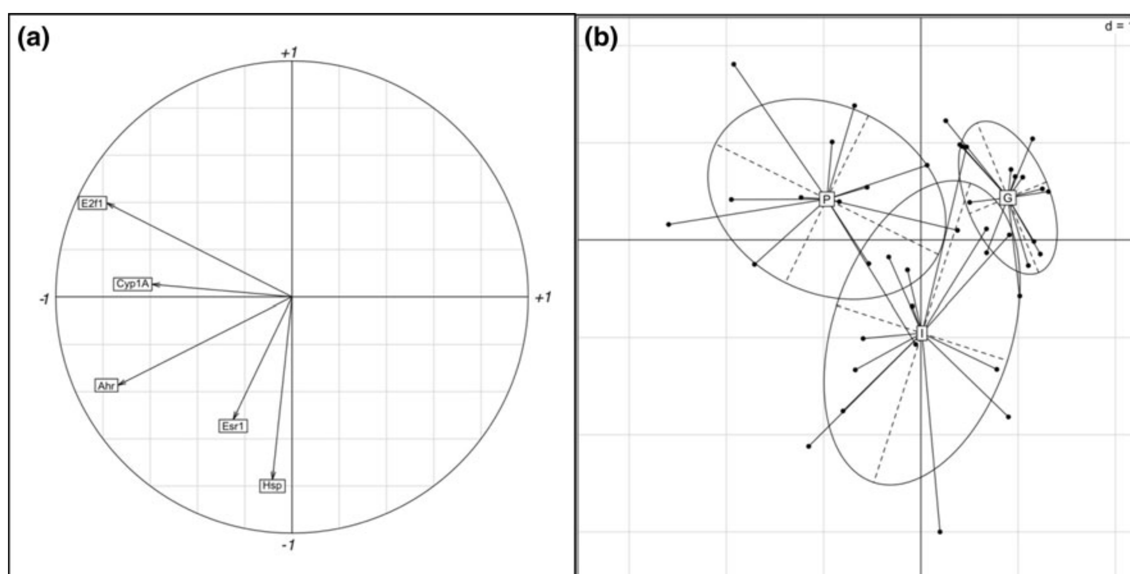

Fig. 4 Discriminant analysis applied on the Principal Components Analyisis (PCA). **a** Plot of the canonical weights for the five variables (genes). **b** Correlations between the discriminant variables and the discriminant functions plotted on the correlation circle. The three areas (P, I, G) are clearly distinguished by the variables in the *circles*

to dolphins living in the contiguous Mediterranean area (Gibraltar).

Comparison of the correlation between the discriminant variable plot, the discriminant function and the ellipsoid plot showed that specimens from the Ionian sea had high values in terms of canonical weights of HSP70 and ESR1, while those from Pelagos sanctuary had high levels of CYP1A, E2F1 and AHR. This evidence suggests the exposure to planar lipophilic compounds and compounds with dioxin-like activity. Specimens sampled in the Strait of Gibraltar had low canonical weights of the genes E2F1, CYP1A and AHR (Fig. 4). Discriminant analysis (Monte Carlo Test based on 999 permutations $RV = 0.201$, $P = 0.001$) confirmed that the three group-areas were significantly distinct.

Conclusions

In conclusion, clear evidence of geographical variability in the responses of the diagnostic biomarkers suggests different exposure to mixture of various classes of contaminants and varying levels of hazard in different areas of the Mediterranean basin. All five genes proved to be modulated in the skin biopsies and they can therefore be proposed as biomarkers for assessing the toxicological status of Mediterranean striped dolphins and other cetacean species and areas. The simultaneous analysis of genes involved in different signaling pathways, combined with proper multivariate statistical analysis, makes it possible to assess whether animals are exposed to stress, and is a more powerful tool than analysis of single biomarkers and/or contamination levels.

Finally, striped dolphins from the northwestern Tyrrhenian sea (Pelagos sanctuary) are evidently more exposed to ecotoxicological hazard than those inhabiting the Ionian sea and the Strait of Gibraltar. This evidence focuses attention on the potential risk to cetaceans inhabiting the largest pelagic MPA in Europe and underlines the importance of farsighted management of protected areas in order to preserve species in their habitats.

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References

Aguilar A (2000) Population biology, conservation threats, and status of Mediterranean striped dolphins (*Stenella coeruleoalba*). J Cetac Res Manag 2:17–26

Aguilar A, Borrel A (2005) DDT and PCB reduction in the western Mediterranean from 1987 to 2002, as shown by levels in striped dolphin (*Stenella coeruleoalba*). Mar Environ Res 59:391–404

Aguilar A, Borrel A, Reijnders PJH (2002) Geographical an temporal variation in levels of organochlorine contaminants in marine mammals. Mar Environ Res 53:425–452

Aït-Aïssa S, Porcher J, Arrigo A, Lambré C (2000) Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. Toxicology 14:147–157

Attwool C, Lazzerini Denchi E, Helin K (2004) The E2F family: specific functions and overlapping interests. EMBO J 23:4709–4716

Beischlag TV, Morales JL, Hollingshead BD, Perdew GH (2008) The Aryl Hydrocarbon Receptor complex and the control of gene expression. Crit Rev Eukaryot Gene Expr 18:207–250

Bérubé M, Palsbøl P (1996) Identification of sex in cetaceans by multiplexing with ZFX and ZFY specific primers. Mol Ecol 5:283–287

Björnoström L, Sjöberg M (2005) Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19:833–842

Boon JP, Lewis WE, Then-A-Choy MR, Allichin CR, Law RJ, De Boer J, Ten Hallers-Tjabbes CC, Zegers BN (2002) Levels of polybrominated diphenyl ether (PBDE) flame retardants in animal representing different trophic levels of the north sea food web. Environ Sci Technol 36:4025–4032

Carpenter DO, Arcaro K, Spink DC (2002) Understanding the human health effects of chemical mixture. Environ Health Perspect 110:25–42

Corsolini S, Guerranti C, Perra G, Focardi S (2008) Polybrominated diphenyl ethers, perfluorinated compounds and pesticides in swordfish (*Xiphias gladius*) from the Mediterranean sea. Environ Sci Technol 42:4344–4349

Forbes VE, Palmqvist A, Bach L (2006) The use and misuse of biomarkers in ecotoxicology. Environ Toxicol Chem 25:272–280

Forcada J, Aguilar A, Hammond P, Pastor X, Aguilar R (1994) Distribution and numbers of striped dolphin in the western Mediterranean sea after the 1990 epizootic outbreak. Mar Mam Sci 10:137–150

Fossi MC, Marsili L (2003) Effects of endocrine disruptors in aquatic mammals. Pure Appl Chem 75:2235–2247

Fossi MC, Marsili L, Neri G, Casini S, Bearzi G, Politi E, Zanardelli M, Panigada S (2000) Skin biopsy of Mediterranean cetaceans for the investigation of interspecies susceptibility to xenobiotic contaminants. Mar Environ Res 52:477–483

Fossi MC, Marsili L, Neri G, Natoli A, Politi E, Panigada S (2003) The use of non-lethal tool for evaluating toxicological hazard of organochlorine contaminants in Mediterranean cetaceans: new data 10 years after the first paper published in MPB. Mar Pollut Bull 46:972–982

Fossi MC, Marsili L, Lauriano G, Fortuna C, Canese S, Ancora S, Leonzio C, Romeo T, Merino R, Abad E, Jimenez B (2004) Assessment of toxicological status of a SW Mediterranean segment population of striped dolphin (*Stenella coeruleoalba*) using skin biopsy. Mar Environ Res 58:269–274

Fossi MC, Casini S, Bucalossi D, Marsili L (2008) First detection of CYP1A1 and CYP2B induction in Mediterranean cetacean skin biopsies and cultured fibroblast by Western Blot analysis. Mar Environ Res 66:3–6

Gaspari S, Azzellino A, Airoldi S, Hoelzel AR (2007) Social kin association and genetic structuring of striped dolphin populations (*Stenella coeruleoalba*) in the Mediterranean sea. Mol Ecol 16:2922–2933

Godard CAJ, Smolowitz RM, Wilson JY, Payne RS, Stegeman JJ (2004) Induction of cetacean cytochrome P4501A1 by β-naphtoflavone exposure of skin biopsy slices. Toxicol Sci 80:268–275

Hahn ME (2001) Dioxin toxicology and the aryl hydrocarbon receptor: insight from fish and other non-traditional models. Mar Biotechnol 3:224–238

Hahn ME (2002) Aryl hydrocarbon receptors: diversity and evolution. Chem Biol Interact 141:131–160

Hirakawa S, Iwata H, Takeshita Y, Kim E-Y, Sakamoto T, Okajima Y, Amano M, Miyazaki N, Petrov EA, Tanabe S (2007) Molecular characterization of cytochrome P450 1A1, 1A2, and 1B1, and effects of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners on their hepatic expression in Baikal seal (*Pusa sibirica*). Toxicol Sci 97:318–335

Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalization; strategies and considerations. Genes Immun 6: 279–284

Isobe T, Ochi Y, Ramu K, Yamamoto T, Tajima Y, Yamada TK, Amano M, Miyazaki N, Takahashi S, Tanabe S (2009) Organohalogen contaminants in striped dolphins (*Stenella coeruleoalba*) from Japan: present contamination status, body distribution and temporal trends (1978–2003). Mar Pollut Bull 58:396–401

Kubista M, Andrade JM, Bengtsson M, Forootan A, JonáK J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) The real-time polymerase chain reaction. Mol Aspects Med 27:95–125

La Thangue NB (2003) The yin and yang of E2F–1: balancing life and death. Nat Cell Biol 5:587–589

Leonards PE, van Hattum B, Leslie H (2008) Assessing the risk of persistent organic pollutants to top predators: a review of approaches. Integr Environ Assess Manag 4:386–398

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408

Marlowe JL, Fan Y, Chang X, Peng L, Knudsen ES, Xia Y, Puga A (2008) The Aryl hydrocarbon receptor binds to E2F1 and inhibits E2F1-induced apoptosis. Mol Biol Cell 19:3263–3271

Matthews J, Gustafsson JA (2006) Estrogen receptor and aryl hydrocarbon receptor signaling pathways. Nucl Recept Signal 4:e016

Montie EW, Fair PA, Bossart GD, Mitchum GB, Houde M, Muir DCG, Letcher RJ, McFee WE, Starczak VR, Stegeman JJ, Hahn ME (2008) Cytochrome P4501A1 expression, polychlorinated biphenyls and hydroxylated metabolites, and adipocyte size of bottlenose dolphins from southeast United States. Aquat Toxicol 86:397–412

Niimi S, Watanabe MX, Kim E-Y, Iwata H, Yasunaga G, Fujise Y, Tanabe S (2005) Molecular cloning and mRNA expression of cytochrome P4501A1 and 1A2 in the liver of common mike whales (*Balaenoptera acutorostrata*). Mar Pollut Bull 51:784–793

Nollen EAA, Moromoto R (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing “heat shock” proteins. J Cell Sci 115:2809–2816

Norstro R, Muir DCG, Ford CA, Simon M, Macdonald CR, Béland P (1992) Indications of P450 monooxygenase activities in beluga (*Delphinapterus leucas*) and narwal (*Monodon monoceros*) from patterns of PCBs, PCDD and PCDF accumulation. Mar Environ Res 34:267–272

Notarbartolo di Sciara G, Agardy T, Hyrenbach D, Scovazzi T, Van Klaveren P (2008) The Pelagos sanctuary for Mediterranean marine mammals. Aq Cons Mar Freshwat Ecosyst 18:367–391

Ohtake F, Baba A, Fujii-Kuriyama Y, Kato S (2008) Intrinsic AhR function underlines cross-talk of dioxins with sex hormone signaling. Biochem Biophys Res Commun 370:541–546

Petterson A, van Bavel B, Engwall M, Jimenez B (2004) Polybrominated diphenylethers and metoxylated tetrabromodiphenylethers on cetaceans from the Mediterranean sea. Arch Environ Cont Tox 47:542–550

Phelan D, Winter GM, Rogers WJ, Lam JC, Denison MS (1998) Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. Arch Biochem Biophys 357:155–163

Piña B, Casado M, Quirós L (2007) Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. Trends Anal Chem 26:1145–1154

Porte C, Janer G, Lorusso LC, Ortiz-Zarragoita M, Cajaraville MP, Fossi MC, Canesi L (2006) Endocrine disruptors in marine organisms: approaches and perspectives. Comp Biochem Physiol Part C 143:303–315

Puga A, Ma C, Marlowe JL (2009) The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem Pharmacol 77:713–722

Spinsanti G, Panti C, Lazzeri E, Marsili L, Casini S, Frati F, Fossi MC (2006) Selection of reference genes for quantitative RT-PCR in striped dolphin (*Stenella coeruleoalba*) skin biopsies. BMC Mol Biol 7:32

Storelli MM, Marcotrigiano GO (2003) Levels and congener pattern of polychlorinated biphenyls in the blubber of the Mediterranean bottlenose dolphin *Tursiops truncatus*. Environ Internat 28:559–565

Tanabe S, Watanabe S, Kan H, Tatsukawa R (1988) Capacity and mode of PCBs metabolism in small cetaceans. Mar Mam Sci 4:103–124

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:0034.1–0034.11

Varò I, Serrano R, Pitarch E, Amat F, López FJ, Navarro JC (2002) Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish. Arch Environ Contam Toxicol 42:229–235

Watabe Y, Nazuka N, Tezuka M, Shimba S (2010) Aryl hydrocrabon receptor functions as a potent coactivator of E2F1-dependent transcription activity. Biol Pharm Bull 33:389–397

Wilson JY, Wells R, Aguilar A, Borrel A, Tornero V, Reijnders P, Moore M, Stegeman JJ (2007) Correlates of Cytochrome P450 1A1 expression in bottlenose dolphin (*Tursiops truncatus*) integument biopsies. Toxicol Sci 97:111–119

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