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The Chronicles of the Contaminated Mediterranean Seas: A Story Told by the Cetaceans' Skin Genes.

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Keywords

Cetaceans, stranding, contaminants, skin, gene expression, biomarkers.

Abstract

Wild animals in their natural environment could provide a big source of information, but sampling can be very challenging, above all for protected species. Significant data can be obtained sampling stranded animals right after their death. Minute skin samples tell the story of the specific geographic location where the marine mammal spent its life, thanks to the different impact on gene expression exerted by different contamination levels. However, the time and the method of sampling are crucial.

1. Introduction

Marine mammals are top predators that are essential for the health and function of the oceans, too often affected by various detrimental factors. Fourty-four percent of stranded marine mammals die from unknown causes (Gulland and Hall, 2007), while disease is a major cause of population decline, augmented by the immune suppression and dysfunction caused by anthropogenic compounds released in the environment (Gulland and Hall, 2007; Van Bressem et al., 2009). Contaminants of emerging concern (CEC), e.g. chemicals, pharmaceuticals, personal care products, nanomaterials and plastics, are increasingly being detected in surface water. Several are classified as endocrine disruptors compounds (EDCs), for they can alter the normal hormone functions affecting reproduction, development and metabolism (Casals-Casas and Desvergne, 2011). EDCs pose the entire marine ecosystem at risk, at every trophic food levels, with the top predators, such as cetaceans, being the most vulnerable.

This study aimed at identifying gene markers specific for different CEC exposure in the skin of cetaceans. Accordingly, we analyzed skin biopsies of stranded specimens from geographic locations known to be differentially contaminated. Few skin samples from dart biopsies of free-ranging animals were included in the study to support the findings. Samples were collected from three principal basins (Ionian, Adriatic and Tyrrhenian) in the Mediterranean Sea, for a total of 33 skin biopsies of about 1 cm^3 collected from the side of the animal, close to the dorsal fin. Thirty-two biopsies were from toothed whales (Odontoceti) and precisely 2 from the *Tursiops truncatus* species, 29 from *Stenella coereouloalba* and 1 from *Grampus griseus;* one sample was from the baleen whale (Mysticete) *Balaenoptera physalus*. Timing and methods of sampling are crucial to obtain good quality RNA, therefore we considered only a subset of samples (N=12) suitable for quantitative gene expression analysis.

2. Methods

A total of 30 skin samples were collected between 2014 and 2016 from stranded *T. truncatus* and *S. coeruleoalba* along the Italian coasts. Three more skin biopsies from free-ranging cetaceans, *S. coeruleoalba*, *G. griseus* and *B. physalus*, were collected by dart biopsy in the Italian marine protected area Santuario Pelagos for Mediterranean Marine Mammals located in the Ligurian basin of the Mediterranean Sea (Prot. Num: 0017889/PM).

For all skin biopsies, total RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) and cDNA was obtained using iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) both according to manufacturer's instructions. Relative RNA expression levels of 4 genes were determined through quantitative real time PCR (qPCR) analysis on CFX Connect (Biorad, Hercules, California, USA) with species-specific primers designed and optimized for efficiency and specificity by running standard curves. qPCR efficiencies were calculated using the equation from Dhar et al., (2009). Optimized qPCR parameters for each gene were determined using diluted (1:10) cDNA reverse transcribed from 1 µg of total RNA using SsoFast™ EvaGreen® Supermix (Bio-Rad) in a total volume of 10 µl of a reaction mix containing 10 ng cDNA, 0.3 µM of each primer **(Table 1)**, 2X Evagreen enzyme and DNase-free sterile water.qPCR reactions were run as follows: 1 cycle of 98 °C for 30 min, 49 cycles of 95 °C for 5 min, 60 °C for 10 min; melting curve 65 °C - 95 °C: increment 0.5 °C every 5 min. Each reaction was run in triplicate, together with a triplicate of no-template controls. The average Ct values were normalized to the values of the housekeeping genes *GAPDH* and *YWHAZ*. Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control and treated samples on CFX connect manager software 3.1 (Bio-Rad). Two-tailed, un-paired one-way ANOVA and Tukey post hoc test were performed used GraphPad Prism 5 (https://www.graphpad.com/scientific-software/prism/).

3. Results

The RNA samples used to test the 4 potential gene markers were extracted from 12 individuals. Although the skin RNA recovery was successful from 14 individuals (**Figure 1, Table 2**), 2 samples were excluded from further analysis, based on RNA contamination (sample Tt02IZS) or pronounced differences in gene expression compared with the other cetacean species (sample Bp01PB). In this latter sample from *B. physalus* quantitative PCR showed level of expression about 10 times higher when compared to the level of expression in the other samples for all genes but *AHR* (data not shown). *B. physalus* is a migrating cetacean feeding mostly on plankton, while *T. truncatus*, *S. coereuleoalba* and *G. griseus* are mostly resident, ordinarily feeding on fish and cephalopods. This diversity of diet and migration behaviors may likely account also for differences in the exposition level and molecular response to contaminants.

Three out of the 4 genes analyzed were selected within those differentially expressed after exposition of dolphin skin cultures to environmentally relevant concentrations of two CEC (Lunardi et al., 2016). Cell division control protein 42 homolog (*CDC42*) is differentially regulated by perfluorooctanoic acid (PFOA), while metastasis suppressor 1 (*MTSS1*) and B-cell receptor 31 (*BCAP31*) are differentially regulated by bisphenol A (BPA).

CDC42 is a small GTPase of the Rho-subfamily, regulating signaling pathways that control diverse cellular functions including morphology, migration, endocytosis and cycle progression. The role of CDC42 is important for normal cell function and its failure has been associated with a number of pathological conditions associated with several human disease states and/or developmental disorders (Melendez et al., 2011). MTSS1 was first identified as a suppressor of metastasis while accumulating evidences support the concept that as a scaffold protein would interact with multiple partners to regulate actin dynamics and is involved in the Shh signaling pathway in the developing hair follicle and in basal cell carcinomas of the skin. MTSS1 is a multiple functional molecular player and has an important role in development, carcinogenesis and metastasis (Xie et al., 2011). BCAP31 is a multi-pass transmembrane protein of the endoplasmic reticulum involved in the

anterograde transport and also in caspases mediated apoptosis. The increased expression of BCAP31 protein activated by DNA demethylation is associated to psoriasis, a human disease of abnormal keratinocyte differentiation and apoptosis (Ruchusatsawatet al., 2016). Moreover, recent findings showed a protective role of BCAP31 in keratinocytes during UVB-induced skin cancer development through the induction of caspase-1 apoptosis (Sollberger et al., 2015). It has been recently showed that BCAP31 may play an important role in T cell activation by regulating TCR signaling (Niu et al., 2017).

An additional fourth gene, aryl hydrocarbon receptor (*AHR*), was also analyzed in this study due to its well-known role in the xenobiotic metabolism of mammals (Hahn, 1998). AHR is a ligand-activated transcription factor abundantly expressed in epidermal keratinocytes with the function of chemical sensor mediating the production of reactive oxygen species. AHR interacts with polycyclic and planar halogenated aromatic hydrocarbons (PAH or PHAH) and dioxins, activating the cytochrome P4501A, a member of the superfamily of enzymes involved in Phase I of the oxidative metabolism of exogenous compounds, with a key role in the biotransformation of contaminants (Hahn, 1998). Endocrine disruptors, such as BPA, have diverse endocrine effects on mammalian and non-mammalian systems because they can bind the AHR, promoting both antagonist and agonist activities (Kharrazian, 2014).

The expression levels of the four selected genes were quantified and compared across geographic areas of sampling and genders.

Different trends in the three different Mediterranean Sea basins are reported. *AHR* and *BCAP31* transcripts were significantly higher in the samples from the Adriatic Sea than those from the Tyrrhenian Sea. *BCAP31* mRNA levels were also significantly higher in Adriatic samples than in the Ionian ones (**Figure 2**). *CDC42* had the higher level of expression in the animals sampled in the Adriatic Sea and the lower in those sampled in the Tyrrhenian Sea; conversely, *MTSS1* had higher levels of expression in the animals from the Ionian Sea, but in both instances the differences were not statistically significant with the available sample set (**Figure 2**).

Gender-related differences were not significant, with only 2 specimens in the experimental male group (data not shown).

4. Discussion

This study reports about different levels of expression of some selected genes that may indicate different environmental quality in the sampling sites. Indeed, samples from the Adriatic Sea, a well-known contaminated area, are those showing the most significant variations. The higher expression of *AHR* in the skin of the animals inhabitants of the Adriatic Sea may result from the higher exposure to PAHs, dioxins, and anthropogenic contaminants that have been previously described in this basin (Garritano et al., 2005; Perra et al., 2011). Likewise, *BCAP31*, chosen for its upregulation in dolphin skin cultures following BPA exposure, had higher expression in the samples from the Adriatic Sea when compared to those from Tyrrhenian and Ionic Sea, and may be indicative of the presence of the BPA chemical (or any other CEC with a BPA-like mechanism of action) in the Adriatic basin as well.

The higher concentration of the contaminants in the Adriatic Sea is probably related to its geographical characteristics. This northernmost basin of the Mediterranean Sea is a semi-enclosed sea, surrounded by the Italian Peninsula in the W and NW part, and by Slovenia and Greece in its NE part, with only one connection to the Ionian Sea through the Strait of Otranto. In the Adriatic Sea, currents run counter-clockwise from the Strait of Otranto, making water exchange difficult thus allowing accumulation of contaminants, especially in the northern part. Conversely, the samples from the Tyrrhenian Sea were collected from the Pelagos Sanctuary, a more pristine marine area, protected since 1992, characterized by a management plan focused on the reduction of human activities and pollution (http://www.sanctuaire-pelagos.org/en/).

The collection and testing of more samples are needed to establish these preliminary indications, as well as the targeting of the mechanism of action of the most prevalent contaminant (or mixture of contaminants) in the different locations. The work presented here was not meant to show the effect of the contaminant on the cetaceans, as we know that chemicals can bioaccumulate and biomagnify, above all in these mammals with a natural lipophilic repository (the blubber). Rather, we wanted to show that the genes of recently deceased animals carry memories of what the animal was exposed

to during its life and have the potential to tell the story of the specific environment. Key factors for successful outcome are 1) timing: to be on site at the right moment, when the carcass is still fresh (it is usually a matter of few hours *post-mortem,* depending on the tissue sampled with 8 hrs being the maximum); 2) sampling method: follow the protocol, avoid contamination, collect only the amount in the ratio required with the stabilization buffer, resuspending the sample properly; 3) cleanliness: avoid contamination of any source. The method is very simple and non-invasive (dealing with dead animals) but could inform about the animal life history but also that of the seas and how the presence and concentration of contaminants in the Mediterranean basin can impact on top predators health and survival.

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Figure legends

Figure 1. Location of the samples from the Mediterranean basin used in this study.

Symbols: square, *B.physalus*; triangle, *G.griseus*; star, *T.truncatus*; circle, *S.coereoulalba*.

Figure 2. Gene expression levels of AHR, BCAP31, CDC42 and MTSS1 in 12 skin biopsies.

Each bar represents the mean expression of all samples from the same geographical location ± SD. Brackets show statistically significant comparison after Tukey test (*p-value < 0.05). (One-way ANOVA p-value: AHR: 0.0183; BCAP31: 0.0106).

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Tables.

Contents:

Table 1. Sequence of primers used in quantitative real time PCR.
Table 2. Sample origin for RNA samples used in quantitative gene expression analysis.

Primer ID	Sequence (5' -> 3')
GAPDH-F	CGACCACTTTGTCAAGCTCA
GAPDH-R	CGGAGGACCTCTCTCTTCCT
YWHAZ-F	AGACGGAAGGTGCTGAGAAA
YWAHZ-R	TTTCTTGTCGTCACCAGCAG
BCAP31-F	GGTCGTACTCCTTGGTCAGG
BCAP31-R	GCCGTCAACAAGCAAAAACT.
CDC42-F	AGTTGCTGGCCTTCTGAATC
CDC42-R	TCCAAGAGAGAAGGAATACATGC
MTSS1-F	AACCCACTTCAAGAGCAGATG
MTSS1-R	AGCTGGTTGGCCACTTTCT
AHR-F	AAGTCCATCCCAGGTGACAG
AHR-R	GCAAGTTCAGGCCTTCTCTG

Table 1. Sequence of primers used in quantitative real time PCR.
Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*; tyrosine 3-monooxygenase/tryptophan 5- monooxygenase activation protein zeta, *YWHAZ*; B-cell receptor 31, *BCAP31*; cell division control protein 42 homolog *CDC42*; metastasis suppressor 1, *MTSS1*; aryl hydrocarbon receptor, *AHR*. F, forward primer; R, reverse primer.

Sample ID	Species	Sex	Sampling site	RNA (ng/µl)	RNA (OD 260/280)	RNA (OD 260/230)
Sc17IZSS	*S. coeruleoalba*	female	Ionian	173.1	2.1	2.0
Sc19IZSS	*S. coeruleoalba*	male	Ionian	105.8	2.0	1.4
Sc20IZSS	*S. coeruleoalba*	female	Ionian	74.8	1.8	1.1
Sc07IZSS	*S. coeruleoalba*	female	Ionian	70.5	2.4	1.9
Sc26IZSS	*S. coeruleoalba*	male	Ionian	301.6	2.1	1.8
Sc32IZSS	*S. coeruleoalba*	female	Ionian	263.7	2.1	1.9
Sc01IZSLER	*S. coeruleoalba*	female	Adriatic	696.8	2.2	2.2
Sc34IZSLER	*S. coeruleoalba*	female	Adriatic	411.4	2.1	2.5
Sc01PB	*S. coeruleoalba*	female	Tyrrhenian	117.0	2.2	1.9
Sc06IZSPLVA	*S. coeruleoalba*	female	Tyrrhenian	100.4	2.1	1.8
Bp01PB	*B. physalus*	male	Tyrrhenian	263.1	2.2	1.3
Tt01LT	*T. truncatus*	female	Tyrrhenian	1072.2	2.5	NA
Gg01PB	*G. griseus*	male	Tyrrhenian	612.0	2.2	2.2

Table 2. Sample origin for RNA samples used in quantitative gene expression analysis.
The table shows animal ID, gender, geographic location of sampling, concentration and absorbance of RNA successfully extracted from skin biopsy of 13 stranded cetaceans. The protocol and primers for sex determination of the 3 free-ranging animals sampled (Sc01PB, Bp01PB, Gg01PB) were obtained from Bérubé et al., (1996). Sc, *S. coeruleoalba*, Tt, *T. truncatus*, Bp, *B. physalus*, Gg, *G. griseus*. IZSS, Istituto Zoo Profilattico della Sicilia; IZSLER, Istituto Zoo Profilattico della Lombardia ed Emilia Romagna, IZSPLVA, Istituto Zoo Profilattico del Piemonte, Liguria e Valle D'Aosta; PB, Plastic Buster Santuario Pelagos of Ligurian basin.

Figure(s)

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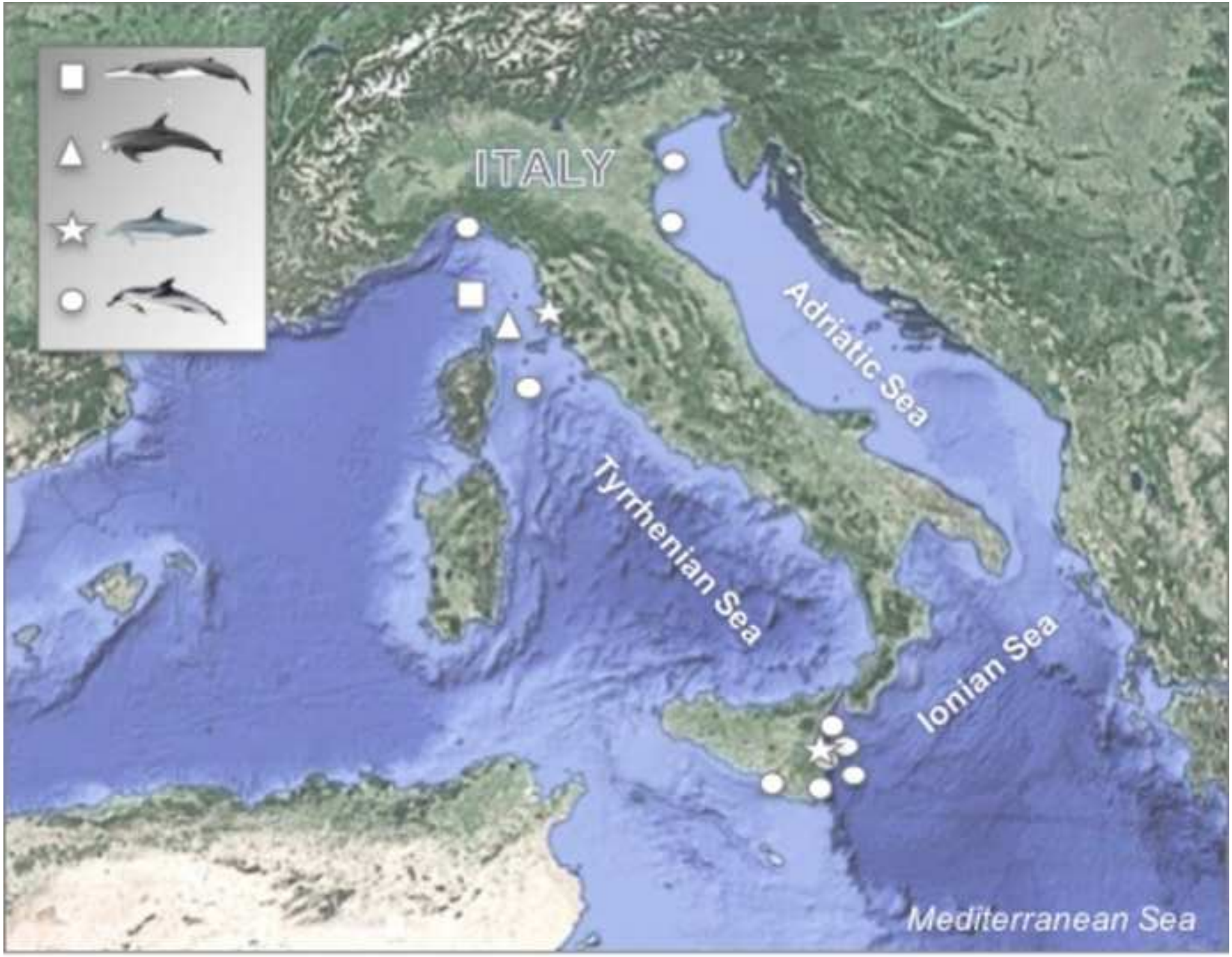
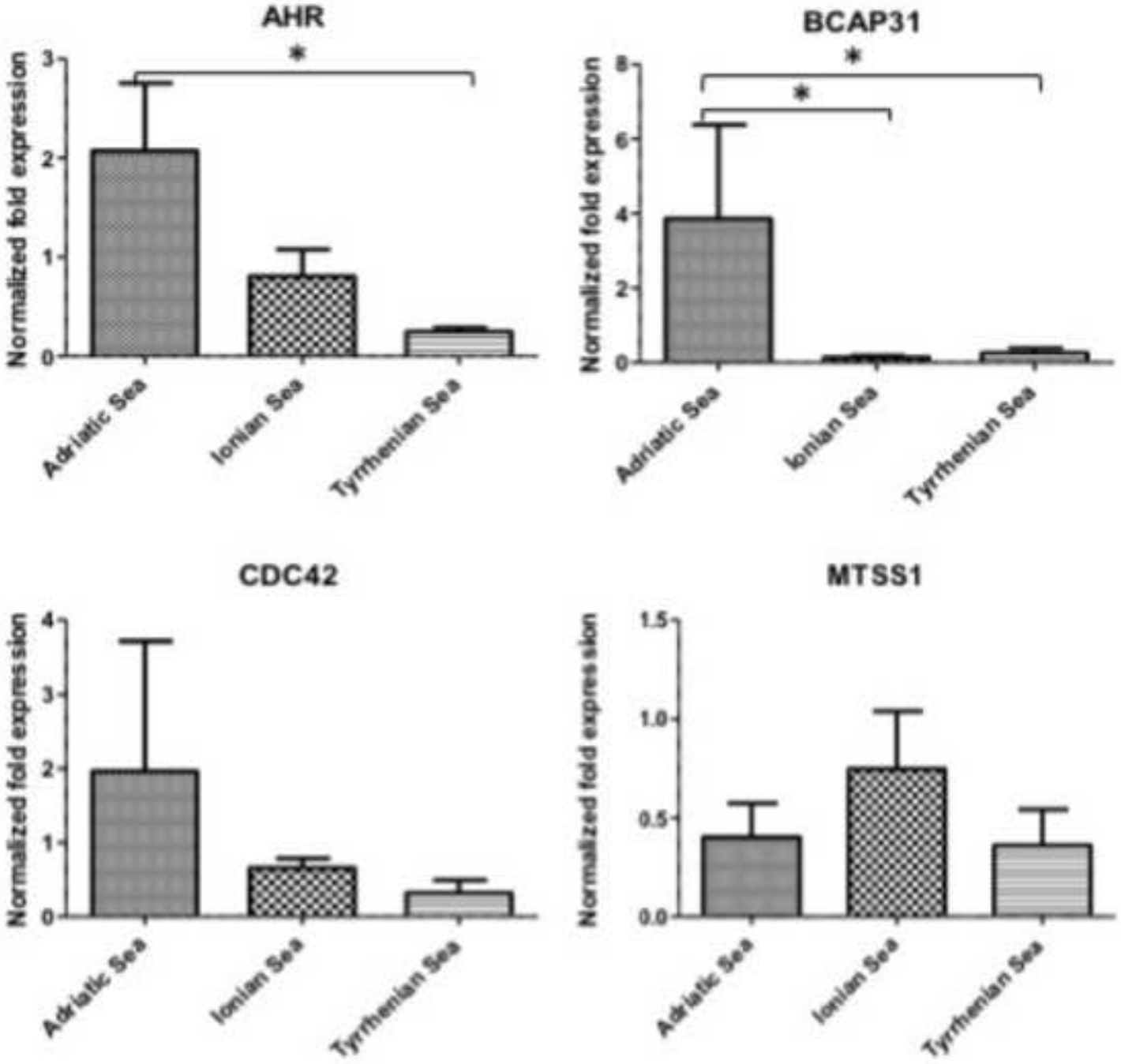
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