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Aberrant gene expression profiles in Mediterranean sea urchin reproductive tissues after metal exposures

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HIGHLIGHTS

- Non-lethal metal exposures do not modify sea urchin gonadosomatic indices.
- Metal exposures affect mRNA expression profile in sea urchin reproductive tissues.
- Coexposures attenuates transcriptional response.

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ABSTRACT

Marine organisms are simultaneously exposed to numerous pollutants, among which metals probably represent the most abundant in marine environments.

In order to evaluate the effects of metal exposure at molecular level in reproductive tissues, we profiled the sea urchin transcriptional response after non-lethal exposures using pathway-focused mRNA expression analyses.

Herein, we show that exposures to relatively high concentrations of both essential and toxic metals hugely affected the gonadic expression of several genes involved in stress-response, detoxification, transcriptional and post-transcriptional regulation, without significant changes in gonadosomatic indices.

Even though treatments did not result in reproductive tissues visible alterations, metal exposures negatively affected the main mechanisms of stress-response, detoxification and survival of adult *P. lividus*. Additionally, transcriptional changes observed in *P. lividus* gonads may cause altered gametogenesis and maintenance of heritable aberrant epigenetic effects.

This study leads to the conclusion that exposures to metals, as usually occurs in polluted coastal areas, may affect sea urchin gametogenesis, thus supporting the hypothesis that parental exposure to environmental stressors affects the phenotype of the offspring.

1. Introduction

Human activities in coastal areas are known to introduce significant amounts of pollutants. Among them, metals are among the most represented pollutants in marine environments (Wowk, 2013) and are considered hazardous substances of primary concern as they persist in the environment, undergo bioaccumulation and biomagnification, so that they can affect human health (Bonsignore et al., 2013; Baki et al., 2018).

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Marine organisms are simultaneously exposed to numerous chemicals, but effects of such mixed exposure may be unpredictable and not overlapping to the toxicity of each contaminant (Norwood et al., 2003; Ragusa et al., 2017a).

Therefore, combinatorial effects need to be assessed for reliable ecotoxicological assessment (Manzo et al., 2010; Ragusa et al., 2017a).

Echinoderms represent reliable model systems in several lines of investigations including developmental biology, molecular evolution and molecular ecology. Similarly, they have been used for ecotoxicological assessment of marine environment in light of increased anthropization, global warming, and ocean acidification (Todgham and Hofmann, 2009; Dupont et al., 2010; Matranga et al., 2012).

The sea urchin *Paracentrotus lividus,* one of the most widely distributed echinoderms in the Mediterranean Sea, is recognized as a key species for biomonitoring of marine environment (Warnau et al., 1997; Soualili et al., 2008).

Most toxicological studies focusing on environmental challenges exploited the morphological perturbations during *P. lividus* embryo development exerted by several pollutants (Warnau and Pagano, 1994; Ragusa et al., 2017a; b).

However, *P. lividus,* as sedentary inhabitant of coastal waters, is continuously exposed to anthropogenic activities (Boudouresque and Verlaque, 2001). Thus, adult sea urchins have been also used as bioindicator species for detecting marine metals pollution (Strogyloudi et al., 2014).

In addition, male and female specimens of *P. lividus* are often harvested for their gonads, which are considered for human consumption especially in the Mediterranean and Atlantic Europe (Lawrence, 2001; Bertocci et al., 2014). Thus, to avoid potential risk of human exposure, its safety as food is a matter of great interest.

Because of its specific habitat and ecology, the sea urchin *P. lividus* may experience polluted conditions during gametogenesis, which in turn may affect both gametes and offspring. Recently, several lines of evidence have provided insights into transgenerational effects in which parental sea urchin exposure to environmental stressors modelled the phenotype of the offspring. In this scenario, in *Strongylocentrotus intermedius* it has been shown that parental exposure to warming affected hatching and larval morphology (Zhao et al., 2018). Similarly, progeny of metal conditioned *P. lividus* showed fertilisation problems and development abnormalities (Migliaccio et al., 2014, 2015).

Efforts were also carried out to define, both at specific gene level and transcriptome, the transcriptional profile of the offspring and it has been defined that adult conditioning affects the gene expression patterns of the progeny during embryo development (Evans et al., 2013, 2017; Migliaccio et al., 2015; Wong et al., 2018). Noteworthy, the tissue specific response of adult member of Echinodermata has been addressed (Matranga et al., 2012) and a few reports characterised the transcriptome of different adult tissues including ovary and testis from the sea urchin species *Arbacia lixula* (Perez-Portela et al., 2016) and *Strongylocentrotus nudus* (Jia et al., 2017).

However, no data are currently available on differential gene expression profile of sea urchin reproductive tissues in response to environmental challenges.

In order to evaluate molecular changes in the dynamic tissues that support gametogenesis, we evaluated the sea urchin transcriptional response in testis and ovary after metal exposure. Male and female of *P. lividus* embryos were treated with both single metals and mixtures of them at different concentrations for 10 days. Modifications in the reproductive and health status were monitored assessing the gonadosomatic index (GSI) of ovary and testis.

The molecular effects were then evaluated by means of pathway-focused mRNA expression analysis. In this scenario, the transcription of stress response genes (*HSP90, HSP70, HSP60* and *HSP56*), metal scavenging (metallothioneins, *MT*s), detoxification and survival related genes (*MDR1* and *HIF1A*) and transcriptional and post-transcriptional gene regulator (*DNMT1, BLIMP-1* and *P38MAPK*) was profiled. Because their involvement in mechanisms of stress response and homeostasis maintenance, changes in their mRNA levels and related pathways could result in alteration of the gonads physiology thus affecting the gametogenesis. Therefore, a wide picture of the selected pathways is herein provided.

2. Materials and methods

2.1. Sampling and experimental design

Adult individuals of *P. lividus* were collected in the South-Western coast of Sicily, nearby Capo Granitola (37°34′19.8″N; 12°39′33.2″E) and transported to the laboratory within 1 h after collection. For each specimen the diameter of the animal was determined in millimeters by measuring perpendicularly to the oral-aboral axis. Only specimens with a diameter of at least 20 mm were considered. The animals were acclimated for 15 days in aquarium with artificial sea water (ASW) (Instant Ocean Aquarium System), salinity 35 ± 1‰; temperature 17.0 ± 1.0 °C; pH 7.80 ± 0.10, 12 h:12 h light:dark photoperiod and continuous aeration. During acclimation, animals were fed *ad libitum* by using dehydrated macroalgae (Sera Marin Gourmet Nori) every day and feeding was interrupted 2 days before experimental sampling. Moreover, health of the sea urchins was monitored through continuous observation and no mortality occurred during the acclimation period.

After acclimation, organisms (n = 3 male and 3 females per treatment) were maintained for 10 days in the presence of different metals at 3 theoretical concentrations as reported in Table 1; while animals maintained in ASW were used as controls. Each experiment was performed three times. Metal solutions were prepared using sulphate salts (Sigma-Aldrich).

During exposure, animals were fed with rations meal of dehydrated macroalgae, twice a week. After dissection, ovary and testis were weighed on an electronic balance and stored at −80 °C until used.

The reproductive state of the specimens was assessed by means of the gonadosomatic index (GSI) calculated for each urchin as the wet mass gonad/wet weight of whole animal x 100.

Statistical significance of differences between values of different treated groups and the reference control groups were determined by t Student's.

2.2. RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from control and exposed *P. lividus*

Table 1
Concentrations of considered metals.

Treatment	Low (μg/l) L	Intermediate (μg/l) I	High (μg/l) H
Cd^{2+}	1	10	100
Mn^{2+}	5	50	500
Ni^{2+}	1	10	50
Pb^{2+}	1	5	10
Zn^{2+}	4	40	200
Mix[a]	–	$\sum$[a] intermediate (Mix I)	$\sum$[a] high (Mix H)

[a] Mix (intermediate and high) consist of all the metal solutions at intermediate and high concentrations, respectively.

using Trizol Reagent (Invitrogen Corporation, Carlsbad, USA) and following the manufacturer's instructions. RNA concentrations were fluorometrically verified on Qubit® 2.0 Fluorometer, while RNA integrity was checked using a 1.5% agarose gel. RNA was stored at −80 °C for future use. An amount of total RNA corresponding to 2 μg was treated with Deoxyribonuclease I, Amplification Grade (Sigma-Aldrich) to remove any residual genomic DNA contamination, and DNAseI was inactivated by adding 50 mM EDTA. First-strand cDNA was synthesized from 1 μg DNAseI-treated total RNA samples using SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen), following the manufacturer's instructions. The cDNA mixture was tested by PCR using *18S* rRNA and *β-actin* primers (Table 2) and diluted 1:10 prior to use in Real Time qPCR experiments.

2.3. Gene expression by real-time quantitative polymerase chain reaction (qPCR)

The qPCRs were performed using the ABIPRISM 7500 System (Applied Biosystems, Forster City, USA) with Power Sybr Green as detection chemistry (Applied Biosystems, Forster City, USA).

The *18S* ribosomal RNA and *β-actin* were selected as control genes based on their expression stability in all tested conditions and the normalization factor was calculated using the GeNorm software (Vandesompele et al., 2002). Serial dilutions of pooled cDNAs from both control and treated samples were prepared to determine the PCR efficiency of the target and reference genes (data not shown) and amplification efficiency ranged from 1.8 to 2.1. Primer sequences used in this study are listed in Table 2. Some of them had been previously designed elsewhere (Ragusa et al., 2013, 2017a; 2017b). Quantitative real-time PCRs were conducted according to the manufacturer's recommended procedures, and each reaction was repeated in triplicate. The amplification conditions were the following: initial denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 60 °C for 50 s, followed by a melting curve from 60 to 95 °C. Amplicons were detected by agarose gel analysis after each PCR to confirm the amplification of the specific gene.

Gene expression results are presented as heat maps mRNA levels are represented as mean centered; while S.D. (n = 3) were below 0.5%. Significant differences between values of different treated groups and the reference control groups were determined by t Student's and provided as supplementary materials. All graphs and statistical analyses were performed by means of R software v. 3.5.0 (R Core Team, 2018).

3. Results and discussion

3.1. Gonad indices evaluation

In order to analyze the effects of 10-day metal exposure on *P. lividus* reproductive tissues, adult sea urchins were exposed to different metals: zinc (Zn) and manganese (Mn) which represent two essential metals, and cadmium (Cd), nickel (Ni) and lead (Pb) as metals with recognized toxicity.

It has been reported that female and male individuals belonging to the same species display different vulnerability to environmental stressors (Au et al., 2001a; Afonso, 2003). Therefore, to evaluate the possible occurrence of gender specific response, male and female adults of *P. lividus* were maintained for 10 days in ASW in the presence of three different nominal concentrations of each metal. For each treatment, we chose concentration levels near to those

Table 2
Genes and oligonucleotide primers used in this study.

Gene name	Gene symbol	Primer sequence (5'–3')
Heat shock protein 70	*HSP70*	GGGTACGACCTATTCCTGTGTTG [a] CTTAGCAGCATCTCCAATCAGTC
Heat shock protein 56	*HSP56*	AGACTTTCCCCCAAAACAACTG [a] AAACCCGCTGGATTCCTTTAG [b]
Heat shock protein 60	*HSP60*	GAATATCCAGTGTACTCCGAC [a] GCATCAGCTAAGAGGTCAACAC [b]
Heat shock protein 90	*HSP90*	TTGAACTCCCTGAGGATGAAGAG [a] AGACAAGACGGTTGGAAACTACC [b]
DNA-methyltransferase 1	*DNMT*	CAGCAGACTGGAAGGTCAATACC [a] TGTCAAACTCGGCAACTAGGATC [b]
P38 mitogen-activated protein kinase	*P38 MAPK*	GCTTGCTTGACTGCTTCACTCC [a] GTAGATGAGGAACTGGACGTG [b]
Multi drug resistance protein 1	*MDR1*	GTCAAGGTACTCAATGGGGTC [a] CGGATGTCAATGCCATCAATC [b]
Hypoxia inducible factor 1-alpha	*HIF1A*	GAGGAGGATGCTTTTGGATTG [a] TCACAGTCTGCGATGATGATG [b]
PR/SET Domain 1	*BLIMP1*	CTGTCTACTCCATGCCGTCC[a] GCCTCCTGCTTCAGATCAGC[b]
Metallothionein 4	*MT4*	GCTCAAAATCTTCAACATGGCTAATGA[a] AGCACTTTCCAGTTTCACAACAAGC[b]
Metallothionein 5	*MT5*	CGACTTTAGCTCAAATTCATCACCATG[a] TCCACAGCATTTACCATCCTTGC[b]
Metallothionein 6	*MT6*	CACGATTTGTGCTCAATCCTTCAT[a] TTTGTGCATGATGTTCCACAGC[b]
Metallothionein 7	*MT7*	CGTCAAGAGATCAAAATCATCAACCA[a] ACAGCACTCGCCAGTAATACAGCAC[b]
Metallothionein 8	*MT8*	GATGGTTGTCGTCGCTCCTAACA[a] TCAAGAAAGGCTGGTATCAAATCTGAC[b]
18S ribosomal RNA	*18S*	GAATGTCTGCCCTATCAACTTTCG[a] TTGGATGTGGTAGCCGTTTCTC[b]
β-actin	*ACT*	AGCGTGGCTACTCTTTCACC[a] CGTTGCCAATGGTGATGACC[b]

[a] Forward primer.
[b] Reverse primer.

found in marine environments with different degrees of pollution or, alternatively, those used in other studies (Varotto et al., 2013; Martinéz-Soto et al., 2016; Zhu et al., 2016; Baltas et al., 2017). Moreover, such concentrations were checked for the absence of any short-term lethal effect. Since organism exposure to different metal combinations may produce either neutralizing, additive or synergistic effects, thus resulting in variable levels of toxicity (Wah Chu and Chow, 2002; Kumar et al., 2015), two additional mixture (Mix I and Mix H), which includes all the metals at different concentrations, were used as well.

During experiments, no mortality occurred and specimens did not show dropped spines and reduced movement, which are known as indicators of sea urchin illness; while retraction and protraction of the tube feets was similar to that of control groups.

GSI index evidenced either no or little effects of metals on the gonads (Fig. 1) which maintained similar values ($p > 0.05$), compared with the related control group even after exposure to highest concentrations or mixtures.

Values herein observed were higher than GSI previously reported in ovary after 9 days of Cd and Mn exposures (Migliaccio et al., 2015). The different data herein obtained could be explained by the diverse culture conditions; indeed, in our experiments lower nominal concentrations were used.

The GSI has been often used as a proxy in analyses of growth, reproduction and health condition in different marine species including sea urchin. Even if it has been reported that GSI do not represent a sufficient tool for health assessment (Au et al., 2001b), these results together with the absence of illness indicators and mortality suggest that the adult sea urchins maintain at least a basic physiological state.

3.2. Pathway-focused gene expression analysis

Based on the recognized involvement of specific genes in mechanisms of homeostasis and gene regulation, we performed a pathway-focused gene expression analyses centered on genes involved in stress-response (*HSP90, HSP70, HSP60* and *HSP56*), metal scavenging (metallothioneins, *MTs*), detoxification and survival (*MDR1* and *HIF1A*), as well as transcriptional and post-transcriptional gene regulators (*DNMT1, BLIMP-1* and *P38MAPK*).

3.2.1. HSPs expression profiles

The heat shock proteins (HSPs) are recognized to exert a protective role as chaperones, by assisting protein folding and preventing their aggregation in response to stressors including metals (Clayton et al., 2000; Gupta et al., 2010). They have been extensively used as stress-response markers in different organisms belonging to evolutionary distant phyla and their pattern of mRNA expression has been shown to be altered by metals (Liu et al., 2014; Nicosia et al., 2014, 2015; Guo et al., 2018).

Because it is widely accepted that oxidative stress associated to metal exposures severely modifies canonical *HSP* transcriptional patterns, such effects were investigated in reproductive tissues of sea urchins and results are shown in Fig. 2.

The *HSPs* mRNA levels were found to be generally downregulated both in testis and ovary by metal exposures, but a strict rule for a dose-dependent relationship couldn't be found. However, a few exceptions emerged, as occurred for *HSP56* overexpression in testis after Mn (I) and Cd exposures or the ovary specific *HSP70* upregulation in response to the highest Zn dosage, Mn (I), Cd (I and L) and Pb (H and I). Interestingly, *HSP70* and *HSP90* mRNAs resulted downregulated at Zn H, Pb I and Zn H, respectively, in testis.

Moreover, some concentration-dependent variation in *HSPs* mRNA levels emerged in Mn exposed males. In particular, *HSP70*

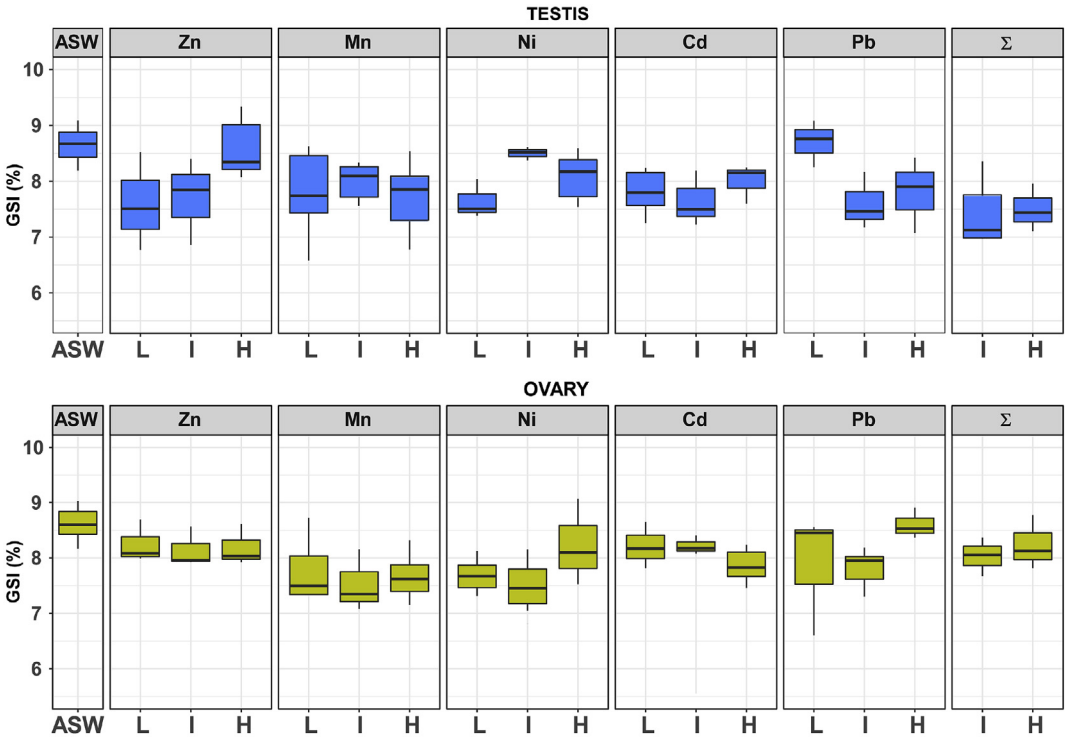

Fig. 1. Gonadosomatic index of *P. lividus* in response to different treatments. Sea urchins were separately exposed to different metal solutions at low (L), intermediate (I) and high (I) concentrations or applied in combination (Mix I or H) as reported in Table 1. Individuals maintained in ASW were used as controls. A box plot diagram representing median distribution of three experiments is shown.

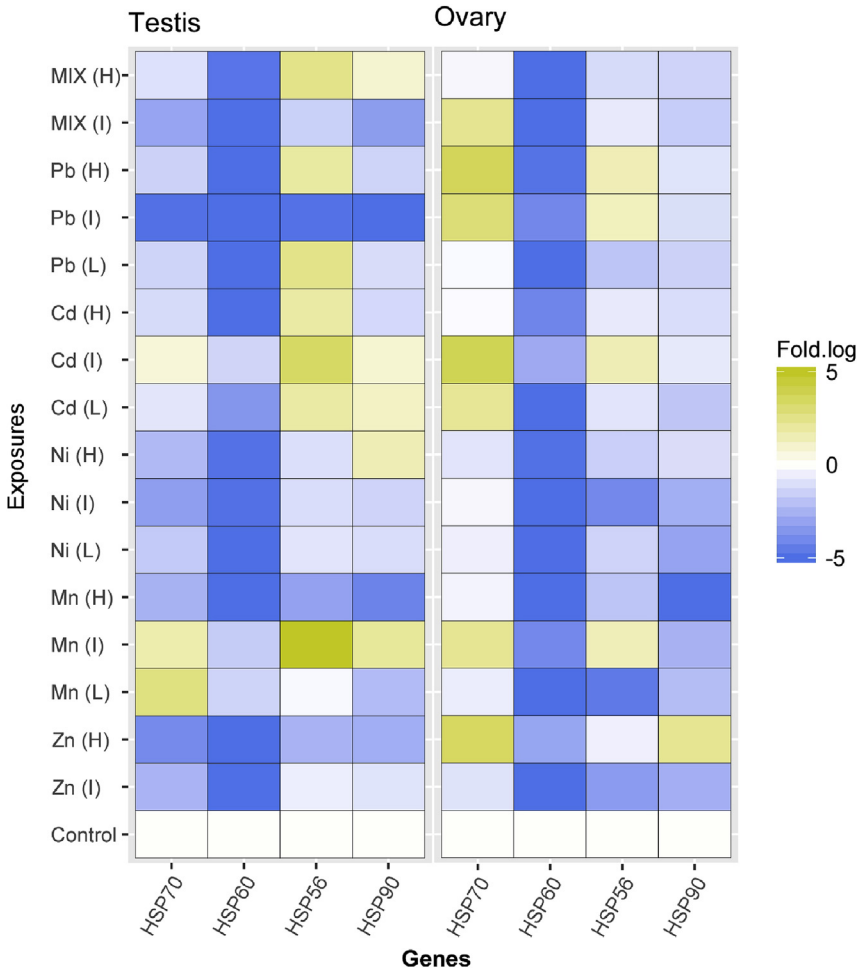

Fig. 2. Metal exposures induce alterations in mRNA expression of stress-response related genes. Heat map representation of the mean-centered data of RT-qPCR results showing *HSP70*, *HSP60*, *HSP56* and *HSP90* mRNA levels in *P. lividus* reproductive tissues, with respect to *18S* and *Act* after 10 days of treatment with low, intermediate or high metal concentrations. Gene expression values are colored from blue (low) to yellow (high). Fold-changes in log (e)-transformed values are represented. Statistical analyses by t Student's are reported in Supplementary materials. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was upregulated at low and intermediate Mn concentrations, whereas the highest Mn concentration downregulated *HSP70* transcript.

Evidences for gender-specific responses also emerged for *HSP56* and *HSP90,* whose transcripts were generally downregulated in ovary.

Exposure to Pb negatively affected the mRNA expression level of *HSPs* in testis with the exception of *HSP56,* which peaked at low Pb dosage. Different responses were detected in ovarian tissues, where intermediate and high Pb concentrations induced upregulation of both *HSP70* and *HSP56,* while *HSP60* remained downregulated.

Cd exposure differently affected the gonad *HSP70* mRNA expression profile; indeed, mRNA levels resulted unaffected or decreased at high Cd exposure in testis; while, they were upregulated in ovary at low and intermediate dosages. Similarly to what occurred with other metals, *HSP60* was once again downregulated in both male and female gonads. The transcript levels of *HSP56* were found increased in testis in all Cd exposure conditions while in ovary it was increased exclusively at the intermediate Cd concentration.

In order to investigate the effects of combined metal exposures, the expression of these genes was also analysed after sea urchin treatment with Mix H and Mix I (Fig. 2).

The qPCR analysis revealed that metals co-exposure negatively affected gene expression; all the transcripts were significantly downregulated regardless of the gender, except for the concentration-dependent upregulation observed for *HSP56* in testis and for *HSP70* in ovary after exposure to Mix H and Mix I, respectively.

Interestingly, both positive and negative fold changes in mRNA levels appeared mitigate. In particular, exposure to Mix I neutralize the transcriptional induction of *HSP90* mRNA in ovary, while the higher dose specifically abrogates the transcriptional down-regulation of *HSP90* in testis. Similarly, the *HSP56* upregulation associated to Mn (I) exposure is abrogated when combined metals were tested (Mix I); while the highest Mix dosage appears to neutralize the *HSP70* mRNA overexpression which has been observed in response to different metals.

These results suggest that the co-occurrence of different stressors, which simultaneously trigger different pathways, may result in gene expression profiles slightly related to those activated by a single contaminant. Moreover, it is likely to suppose the occurrence of regulatory mechanisms differentially acting in testis and ovary.

The *HSPs* mRNA have been usually found as induced after exposure to metal stressors (Navarro et al., 2011; Nicosia et al., 2014, Nicosia et al., 2015, Liu et al., 2014) and are considered as a part of an evolutionary maintained mechanism of stress response.

However, the down-regulation herein reported for certain conditions is consistent with findings reported for the oysters *Crassostrea gigas* and *C. hongkongensis* in response to long term exposure to metals (Boutet et al., 2003; Luo et al., 2014). Similar results were also obtained in the earthworm *Lumbricus terrestris* and the seabream *Sparus aurata* after prolonged metal contaminations (Nadeau et al., 2001). Reduced expression of the *HSP90* and *HSP70* genes were also observed in dinoflagellates of the genus *Symbiodinium* in response to thermal stress (Rosic et al., 2011).

Even if the transcriptional patterns of *HSPs* in response to metals are variable, relying on exposures (acute or prolonged) and used concentration, prolonged treatment may result in possible acclimation and tolerance to stress.

It could be also hypothesised that in response to long term exposures and different levels of generated toxicity, the stress response system may result suppressed in the sea urchin reproductive tissues, thus inhibiting the *HSPs* mRNA expression.

However, the existence of regulatory mechanisms involving a sufficient amount of HSP proteins, which can negatively modulate *HSP*s transcription (Morimoto et al., 1997), may likely act on such system.

3.2.2. Metallothioneins expression profiles

Metallothioneins (MTs) are efficiently induced by a mechanism involving the upstream antioxidant responsive element resulting in inhibition of the Fenton reaction and, in turn, in DNA protection from oxidative damage (Chiaverini and De Ley, 2010). Thus, the effects of single or combined metal exposures, were analysed on the expression of sea urchin metallothioneins (*MT4, MT5*, *MT6, MT7* and *MT8*).

Overall, a shared molecular response was observed after all challenges herein analysed (Fig. 3).

It has been reported that during sea urchin development, embryos express *MT7* and *MT8* transcripts at high levels. Conversely, *MT4*, *MT5* and *MT6* are expressed at low levels and were found induced by metal exposures (Ragusa et al., 2013, 2017b). Surprisingly, metal exposures did not activate the expression of the inducible metallothioneins, indeed the *MT4* mRNA expression level was negatively affected, while *MT5* and *MT6* levels were slightly reduced or unchanged in both gonads. Interestingly, constitutive *MT7* and *MT8* were faintly upregulated after metal exposures.

The RT-qPCR analysis revealed that combined exposures differently affected *MTs* transcriptional expression. Interestingly, MT4 mRNA levels were found increased in both tissues in response to MIXs while it was downregulated either in testis and ovary after all the individual exposures; while *MT7* and *MT8* maintained similar mRNA level compared to control groups.

Because combinatorial exposure may result in unpredictable

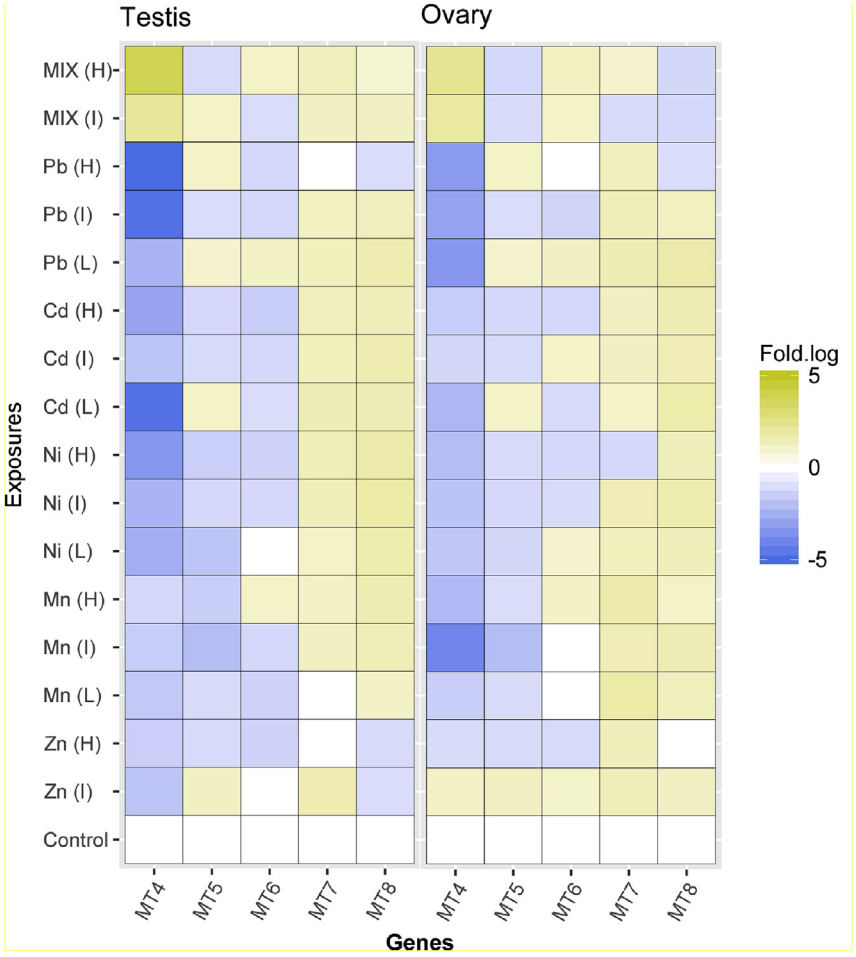

Fig. 3. Metal exposures induce alterations in metallothionein gene expression. Heat map representation of the mean-centered data of RTqPCR results showing *MT4, MT5, MT6, MT7* and *MT8* mRNA levels in *P. lividus* reproductive tissues, with respect to *18S* and *Act* after 10 days. Gene expression values are colored from blue (low) to yellow (high). Fold-changes in log (e)-transformed values are represented. Statistical analyses by t Student's are reported in Supplementary materials. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

toxicity effects (Wah Chu and Chow, 2002), it is reasonable to suppose that the co-occurrence of different stressors may abrogate the actions of specific members of the pathways that have acted to downregulate *MT4* mRNA expression in individual treatments.

Moreover, the presence of specific gene regulatory elements in the promoter of *MT4* could be responsible for the observed pattern. However, other efforts should be carried out to identify *cis* acting modules and regulatory mechanisms responsible for the *MT4* regulation.

Upregulation of *MTs* represents an ubiquitous system which is known to counteract the metal-induced cytotoxicity in different systems (Amiard et al., 2006; Arini et al., 2015; Chaâbene et al., 2018) including the sea urchin embryo exposed to metals (Ragusa et al., 2013, 2017b; Migliaccio et al., 2015). However, the lack of MTs overexpression has been also described elsewhere and our results appeared consistent with findings reported for *Ruditapes philippinarum* in which the increased temperature of summer reduced MT production in clams (Oaten et al., 2017). Similarly, *P. lividus* gonads from Amvrakikos gulf, characterised by moderate anthropogenic activity and metal contaminations, did not result in a marked increase of MT protein amount (Strogyloudi et al., 2014).

Moreover, it is known that metals induce *MTs* expression either after acute exposures or at high concentrations (Durnam and Palmiter, 1981; Shaw et al., 2007; Chen et al., 2014). Therefore, it is reasonable to suppose that sea urchin exposures at non-lethal conditions may result in tolerance to metal toxicity, thus attenuating the *MTs* expression in the stress response. Further researches are required to evaluate the *MTs* mRNA expression levels over the exposure time.

Although MTs are commonly included in biomonitoring studies, interferences related to the physiology of anatomical districts have been described (Van Cleef-Toedt et al., 2000; Oaten et al., 2017). Therefore, it could be hypothesised that in sea urchin gonads, the gametogenesis process may neutralize the induction events on *MTs* transcriptional rate.

3.2.3. Expression profiles of detoxification and survival related genes

A well-known response mechanism to xenobiotics is the activation of detoxification systems that includes, among other, the multi drug resistance protein 1 (*MDR1*). It represents a critical component of defense systems and is induced by various oxidative stresses thus protecting cellular components from increased Reactive oxygen species (ROS) levels (Goldstone et al., 2006).

As a pivot regulator of adaptive transcriptional responses, hypoxia inducible factor 1-alpha transcription factor (*HIF1A*) is known to bind to the hypoxia-response element (HRE) in the promoter region of several genes involved in stress response and cell death. Thus, *HIF1A* modulates directly and indirectly cell survival especially under prolonged or severe stress conditions (Wang and Semenza, 1993; Speer et al., 2013).

In order to evaluate the perturbation of detoxification system in response to metals exposure, transcriptional expression levels of *MDR1* and *HIF1A* genes were investigated (Fig. 4). The expression of both genes was affected in male and female gonads, and huge negative peaks were measured in response to different metal exposures. Nevertheless, up-regulation was also observed for both genes in testis in response to intermediate Mn and Cd or to low Pb exposures. Different responses in testis and ovary were also observed for *MDR1* mRNA at intermediate concentrations of Zn, Mn and Pb, low amount of Pb and Cd and to highest Zn dosage. A general down-regulation of *HIF1A* transcript was observed in ovary at different treatments and also in this case differential profiles were detected in testis and ovary as occurred for intermediate Pb,

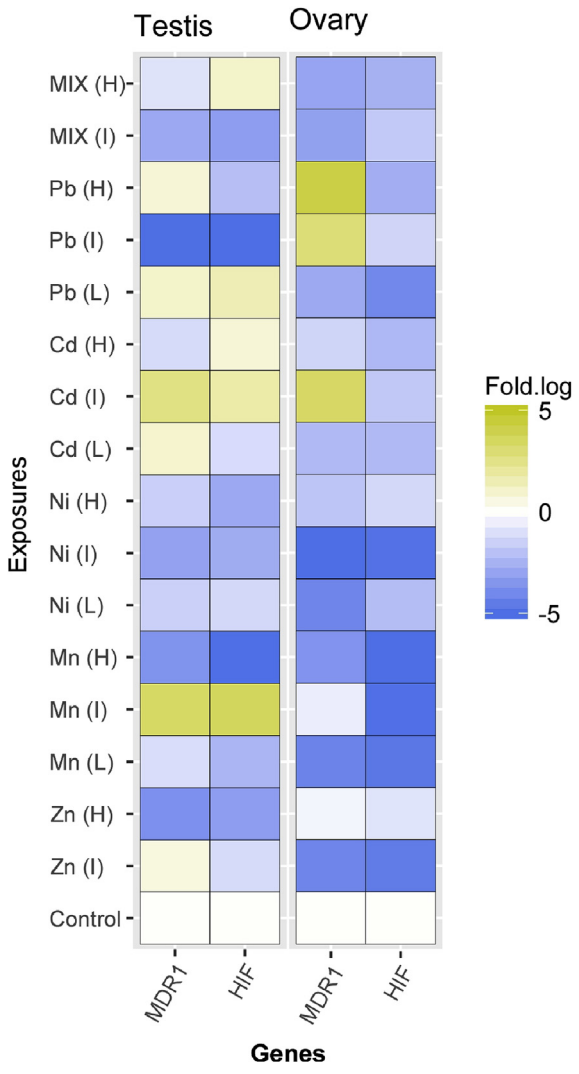

Fig. 4. Metal exposures induce alterations in mRNA expression of detoxification and survival pathways. Heat map representation of the mean-centered data of RT-qPCR results showing *MDR1* and *HIF1A* mRNA levels in *P. lividus* reproductive tissues, with respect to *18S* and *Act* after 10 days. Gene expression values are colored from blue (low) to yellow (high). Fold-changes in log (e)-transformed values are represented. Statistical analyses by t Student's are reported in Supplementary materials. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Cd and Mn treatments as well in response to high Zn and low Pb dosages.

Moreover, except for up-regulation of *HIF1A* mRNA in testis, no sex related-differences were measured after exposure to metal mixtures, which resulted in an overall downregulation.

Our data are in agreement with those reported in other works, where *MDR1* and *HIF1A* transcriptional changes have been reported in different systems in response to metals (Ke et al., 2005; Permenter et al., 2011; Migliaccio et al., 2014, 2015). Moreover, MDR1 downregulation was also recorded after long-term copper exposure in the HepG2 cell line (Groba et al., 2017). These results also suggest that MDR1, mainly reported to mediate drug resistance, could act to maintain metal homeostasis in sea urchin reproductive tissues. Additionally, it is reasonable to suppose that other systems may counteract damages induced by metal exposures differentially acting on testis and ovary maturation.

As probably occurred for other genes herein analysed, 10-day

metal exposure may be associated to adaptation mechanisms in response to specific threshold of toxicity leading to transcriptional inhibition of the profiled genes.

3.2.4. Expression profiles of transcriptional regulators

Metal exposure is usually associated to ROS production, which has been shown to activate p38 MAPK (Ushio-Fukai et al., 1998). p38MAPK is widely known as a stress responder (Casano et al., 2003; Bonaventura et al., 2018) and as key regulator of pro-inflammatory cytokines at transcriptional and translational levels (Han et al., 1997; Cuenda and Russeau, 2007). Moreover, the promoters of genes involved in the inflammatory response are known to show a p38 MAPK-dependent Ser 10 phosphorylation on histone H3 (Khan et al., 2016), thus indicating a p38 MAPKs involvement in mechanisms of chromatin dynamics.

Toxicant exposure has also been shown to affect the expression of DNA methyltransferases (*DNMTs*) thus altering the overall pattern of DNA methylation (Aluru et al., 2015; Dorts et al., 2016; Sanchez et al., 2017) which in turn may affect chromatin accessibility and gene expression.

Mechanisms of development, specification and differentiation are known to be affected by environmental stressors, which usually act altering the gene regulatory networks behind (Kobayashi and Okamura, 2005; Runcie et al., 2012). BLIMP-1 transcription factor is recognized as a pivot in the regulatory network leading to tissue specification and germ-line determination, also in distantly related organisms (Livi and Davidson, 2006; Saitou et al., 2005; Seervai and Wessel, 2013; Nakamura and Extavour, 2016); additionally, it is transcriptionally induced in response to diverse cellular stressors (Doody et al., 2006).

Thus, to evaluate changes involving key factors of the general gene regulation in response to metal exposures, the mRNA levels of *DNMT1, P38MAPK,* and *BLIMP1* genes were investigated (Fig. 5).

With a few exceptions, *DNMT* and *p38MAPK* mRNAs resulted generally downregulated both in testis and ovary in presence of different metals.

However, upregulation events were also reported since *DNMT* mRNAs peaked in ovary after exposure to higher Zn concentration.

The possible involvement of DNA methylation in mechanisms of stress response (Campos et al., 2007) was herein inferred due to the reduction in *DNMT* mRNA level after treatment with different metals. Modulation of *DNMT* transcript levels have been reported in diverse systems exposed to metals, including human specimens and cell lines (Eid et al., 2017; Benbrahim-Tallaa et al., 2007) zebrafish larvae (Dorts et al., 2016) and sea urchin embryos (Varrella et al., 2014, 2016). The definition of a specific epigenetic mark is usually associated to gene expression regulation. Strikingly, changes observed in *P. lividus* gonads may result in altered spermatogenesis and oogenesis, which in turn could lead to creation and maintenance of heritable epigenetic chromatin states or, at least, modifications of the expression of specific gene sets required for synthesis and accumulation of maternal determinants.

Transcriptional fluctuations in *P38MAPK* mRNA levels were also reported in sea urchin embryos exposed to toxic aldehydes from diatoms (Varrella et al., 2016). Similarly, a *P38MAPK* homolog from the midge *Chironomus riparius* showed both up- or down-regulation in response to dose and exposure duration of contaminants (Park and Choi, 2017). Thus, our results suggest that defence mechanisms associated to p38 MAPK against environmental stresses could act also in reproductive tissues of adult *P. lividus*.

Interestingly, the mRNA expression profile of BLIMP1 provided different results. The BLIMP1 mRNA was generally down-regulated in testis, with a few exceptions as occurred for sea urchin exposed to intermediate and high levels of Cd and intermediate level of Mn, while it peaked in ovary from sea urchin exposed to all conditions tested. Even if no reports are currently available on *BLIMP1* transcriptional changes in response to metal exposures, it has been shown that it regulates stress-specific developmental adaptations under stressful conditions in *Caenorhabditis elegans*, specifically interacting with LIN-40 (Hyun et al., 2016). Therefore, it could be hypothesised that *BLIMP1* upregulation in ovary may results in a gene expression reprogramming which in turn could affect sea urchin adaptation to stress conditions.

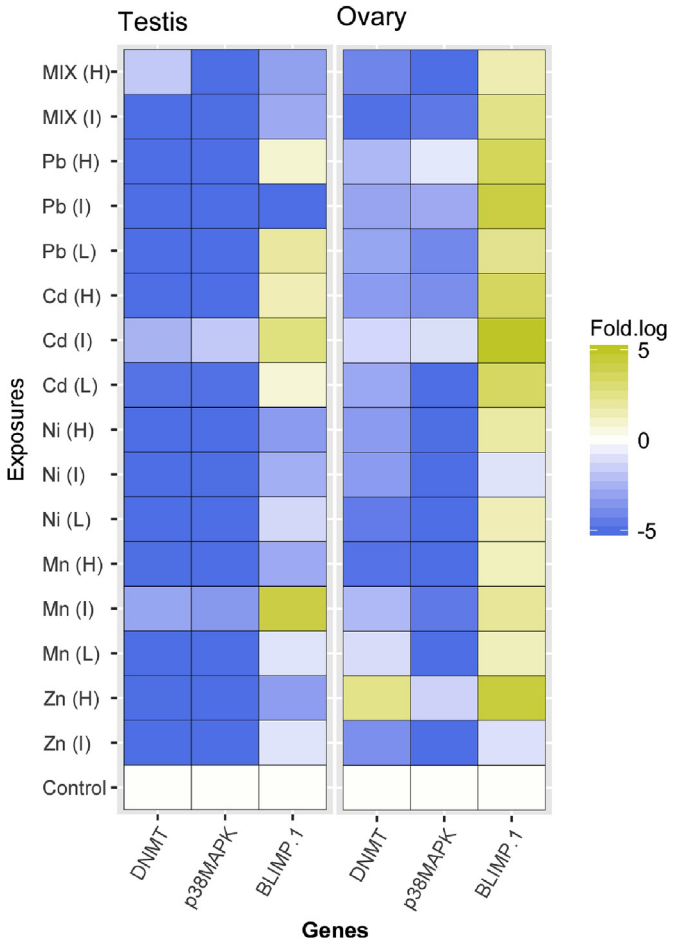

Fig. 5. Metal exposures induce alterations in mRNA expression of general gene regulators. Heat map representation of the mean-centered data of RT-qPCR results showing *DNMT, P38MAPK* and *BLIMP1* mRNA levels in *P. lividus* reproductive tissues, with respect to *18S* and *Act* after 10 days. Gene expression values are colored from blue (low) to yellow (high). Fold-changes in log (e)-transformed values are represented. Statistical analyses by t Student's are reported in Supplementary materials. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

In this work, we evaluated for the first time the effects of different metal exposures on *P. lividus* reproductive tissues in order to characterise the defence mechanisms activated in response to these pollutants.

We have chosen concentration levels measured in polluted marine environment or already used in other studies, checking for the absence of lethal effects. Despite we didn't observe effects of metals on the gonadosomatic index, the profiling of transcriptional response of ovary and testis frequently showed relevant impacts on gene expression, also suggesting the occurrence of sex specific regulatory mechanisms.

Differences in terms of susceptibility to stress and tolerance among sexes, as reported elsewhere (Afonso, 2003; Schäfer et al., 2011), often emerged. However, results herein presented do not solve unambiguously such issue because of the variable pattern of gene expression in response to metals with different toxicity degrees. This is consistent with findings reported for the sea urchin *Psammechinus miliaris* in which the profiling of different biochemical markers resulted in difficulties to identify sex-specific injury in reproductive tissue in response to phenanthrene (Schäfer et al., 2011). Therefore, it could be hypothesised that physiological processes associated to gonad maturation and gametogenesis may likely superpose to certain expression pattern thus overriding the effects of metal exposure in reproductive tissues.

During the last years there has been a great deal of interest in characterising the offspring phenotypic changes in response to the environmental stress experienced by parentals which can occur especially during gametogenesis (Hamdoun and Epel, 2007; Byrne, 2010). Our experiments showed that non-lethal metal exposures hugely affect the canonical transcriptional profiles in gonads. Since pathways herein analysed, including genes regulating transcriptional rates and epigenetic changes, undergo abrupt modifications in the reproductive tissues, the occurrence of altered gametogenesis, which may cause effects across a generation, could be hypothesised.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.10.137.

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