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RESEARCH ARTICLE

Biomarkers assessment in the peacock blenny *Salaria pavo* exposed to cadmium

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Abstract Cadmium (Cd) is one of the most toxic metals and is widely distributed in freshwater and marine environments. It has received much attention from a toxicological perspective. The aim of this study was to assess the effect of Cd in the peacock blenny *Salaria pavo*, a species of the family of blennies that was used as bioindicator of water pollution. We performed a sublethal contamination of fish to 2 mg $CdCl_2$ L^{-1} during 1, 4, 10, and 15 days. Cd accumulation was measured in gills and liver and displayed a significant increase of its concentration throughout the experiment, with slightly higher levels in the liver, except after 4 days. Partial-length cDNA of *mt1*, *mt2*, *mnsod*, *cuznsod*, *cat*, and *gpx* were characterized. Results from mRNA expression levels displayed an up-regulation of *mt2* and *mnsod*. Biomarker activities were determined in gills and liver. In gills, data displayed an inhibition of EROD and GST activities. Cd exposure significantly increased GPx activities but did not affect CAT levels throughout the experiment. No LPO induction was observed in gills of exposed fish. Regarding the liver, the activity of all enzymes and MDA levels increased significantly from the beginning of the experiment except EROD that increased after 15 days of contamination only. At the histological level, fish exhibited pathological symptoms in gills and liver with a predominance of circulatory disturbances in gills and regressive changes in the liver. Our results displayed that peacock blennies are able to survive Cd toxicity due to various physiological adaptation mechanisms.

Keywords $CdCl_2$ · Peacock blenny · *Salaria pavo* · Cd accumulation · Biomarkers · Transcript levels · Enzyme activity · Histopathology

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Introduction

In the earth's crust, Cd is naturally found at 0.1 to 5 ppm. According to the World Health Organization, production of Cd is estimated to be around 18,000 T $year^{-1}$ which means 90 % of Cd-total emissions in the environment. Cd is a cumulative poison: humans absorb 5 % of the total ingested metal, and one third of its total concentration is accumulated in kidneys. In 1912, Cd had been responsible for the Itaï-Itaï disease characterized by extremely painful fractures of bones (Kjellstrom 1986). The release of Cd in the environment via various industrial production processes has resulted in a strong accumulation of Cd in the aquatic environment over the last century. In the marine environment, the most prevalent chemical form found is the chlorocomplex $CdCl_2$ (Miramand et al.

2002). Cd is also able to accumulate in sediments reaching up to 4 mg kg^{-1} (MPMMG 1998). Previous studies performed on common carp *Cyprinus carpio* exposed to 0.1 mg $CdCl_2$ L^{-1} reported that 75 % of the Cd were deposited and accumulated in liver and kidneys (Kraal et al. 1995), but it can also be present in gills and other tissues (Randi et al. 1996).

Accumulation of Cd in fish could stimulate the production of reactive oxygen species (ROS) (Cao et al. 2010). ROS are able to react with macromolecules resulting in oxidative stress that causes damages to organs, cells, proteins, and DNA in exposed fish (Farombi et al. 2007; Sassi et al. 2013). To overcome the effect of Cd, resistance mechanisms are implemented in order to protect fish against stress. These mechanisms include the regulation of the expression and activity of key enzymes involved in detoxification (Modesto and Martinez 2010). The primary defense against oxidative damages consists of some low molecular weight compounds like metallothionein (MT) and major enzymatic biomarkers such as GST, EROD, CAT, GPx, and SOD. MT, as a metal-binding protein, is considered as a defense mechanism that increases the resistance of organisms to Cd exposure (Tiwari et al. 2011). Several studies have investigated the induction of MT in Cd-exposed fish, such as the Antarctic ice fish *Chionodraco hamatus* (Carginale et al. 1998), the zebrafish *Danio rerio* (Gonzalez et al. 2006), the goldfish *Carassius auratus* (Choi et al. 2007), and the sea bream larvae *Sparus aurata* (Sassi et al. 2013).

Antioxidant enzymes play an important role to remove ROS (Cao et al. 2010). Among various studies are those of the antioxidant system in relation to Cd contamination in a variety of fish species (Souid et al. 2013; Blickley et al. 2014; Nunes et al. 2014). In particular, specific changes in gene expression with exposure to pollutants are indicative of the activation of reaction mechanisms to protect the body against the impact of stressors (Wangsongsak et al. 2007).

Histopathological analyses have been considered as biomarkers of Cd exposure (Au 2004). It has been shown that fish exposed to Cd exhibited structural damages in gill and liver tissues such as hypertrophy and hyperplasia of primary and secondary lamellae as well as hepatocyte vacuolations (Wangsongsak et al. 2007). Almost all studies of Cd-induced alterations in organ morphology to date have been purely qualitative without considering alteration prevalence and general health status of the fish body.

In this paper, we explored possible effects of Cd on a non-target species widely distributed in the Mediterranean coasts, the peacock blenny. This fish was selected as a test organism due to some of its biological characteristics making it of potential interest as a sentinel species. Blennies are very common benthic fish in Mediterranean coasts (Gharred et al. 1998; Barhoumi et al. 2009). They are relatively stationary in their natural environment allowing to consider that each response detected in the fish will directly reflect the state of environmental conditions (Asker et al. 2013). Gharred et al. (2015) displayed severe biochemical and histological alterations in blennies *Parablennius incognitus* collected from the Mediterranean coast of Tunisia impacted by different anthropogenic activities. A previous study of Barhoumi et al. (2009) reported that basil blennies *Salaria basilisca* liver accumulated high concentrations of Cd (1.8 μg g^{-1}) when fish were sampled from polluted seawater close to industrial activities and phosphogypsum stock in Tunisian coasts. La Mesa et al. (2006) and Tigano et al. (2009) tested different biomarkers of genotoxicity in blennies *Parablennius sanguinolentus* collected along the coasts of Sicily. They classified this species as a valid bioindicator of environmental quality in the Mediterranean Sea. Many other reports suggested blennies and especially the viviparous blenny *Zoarces viviparous* as a suitable species for environmental monitoring programs since it is responsive to many ranges of pollutants (Fricke et al. 2012; Asker et al. 2013; Brande-Lavridsen et al. 2013). All these authors pointed out that blennies are interesting sentinel organisms for contaminant exposure because of their widespread occurrence, their ubiquity, and also their position in the trophic chain that makes them particularly exposed to sediment-associated contamination.

The objective of this study was to investigate potential consequences of Cd exposure and the degree to which it accumulates in different tissues. We focused on molecular, physiological, and histopathological responses of peacock blennies experimentally submitted to an exposure of 15 days to chloride cadmium ($CdCl_2$) at a sublethal concentration (2 mg L^{-1}). This concentration is one tenth of the 96 h LC_{50} value from the acute toxicity test (20 mg L^{-1}) based on the data published by Messaoudi et al. (2009) in the basil blenny.

Materials and methods

Animals and exposure conditions

Forty-eight adult peacock blennies, weighing 23.2 ± 3.7 g and measuring 12.7 ± 1.3 cm, were sampled along the coast of Monastir (Tunisia). Fish were directly transferred to the National Institute of Science and Technology of the Sea (INSTM, Monastir/Tunisia) and acclimated to the laboratory for 2 weeks in a large rearing tank (1000 L) in which natural seawater was aerated, filtered, and maintained at 24 °C. Fish were fed daily with fresh shrimps until 24 h prior to their transfer to the experimental tanks. Tank maintenance was ensured manually during the period of acclimation, and uneaten

food and feces were collected daily by siphoning. Similarly, dead fish were immediately removed.

In order to respect the ethical statement regarding animals' care and use, fish were maintained according to the EEC 609/86 Directives. After acclimation, the experiment was conducted over a period of 15 days during which samples were taken at four time points (1, 4, 10, and 15 days). Fish were equally divided into two groups and transferred in the experimental tanks filled with 60 L of seawater. For each group, four tanks were prepared, each one containing six fish. The first group of peacock blennies, representing control fish, was maintained in Cd-free water. The second group was represented by exposed fish and maintained in natural seawater supplemented with 2 mg L^{-1} of $CdCl_2$ (Merck, Darmstadt, Germany). Throughout the experimental period, 50 % of the water was changed every 3 days in order to maintain stable water physico-chemical parameters and concentration of $CdCl_2$. Because peacock blennies do not swim, experimental tanks contained accessories (clay pots) where fish can hide. After each exposure time, fish were euthanized with an excess dose of tricaine methanesulfonate (MS-222) and tissues were dissected and stored depending on their use.

Cd analysis

Total Cd concentrations were measured using an ICP-OES spectrophotometer (Perkin Elmer, Optima 7000 DV spectrometer) in the water used for experimentations at each sampling time in triplicates. The limit of detection was 0.2 μg Cd L^{-1}. The blank was 2 % of HNO_3 and its impurity was less than 0.01 μg L^{-1}. This analysis was performed by "Eco2lab" (private laboratory in Sousse, Tunisia). For organs, gills and liver of six individuals (control and exposed fish at each sampling time) were dried for 3 days at 60 °C. Dried samples were weighed and digested overnight by adding 69 % HNO_3. Samples were then heated to 110 °C for 30 min. Five hundred microliters of H_2O_2 30 % was added to cooled samples and 30 min of heating at 110 °C was then applied. The measurement was performed using a high-resolution ICP-MS spectrometer (Element XR; Thermofisher Scientific, Bremen, Germany). Variation coefficient was inferior to 10 % and LOD (in 1 % nitric acid blank) was equal to 10 ppq Cd. The method quantification limit was 0.001 ppb and blank equivalent concentrations were inferior to 0.001 ppb. Tissue Cd concentrations were expressed as micrograms per gram dry weight (dw).

mRNA expression analysis

Transcriptional analysis was conducted in gills of peacock blennies because gills are in permanent contact with water and thus are directly affected by water pollution. It is also a metabolically active organ that is able to accumulate metals at higher concentrations (Allen 1994).

RNA extraction and cDNA synthesis

mRNA analyses were established in gills of six individuals at each sampling time. This organ was dissected, conserved in RNA Later (Sigma-Aldrich), and stored at −20 °C. Using Tri-reagent (Invitrogen), total RNA was isolated following the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm and RNA quality was checked using the Experion system (BioRad). To avoid genomic DNA contamination, the prepared RNA was treated with DNase I (Promega) at 37 °C for 30 min. Total RNA was then added to a mix of OligodT, random primers, and nuclease free water and denatured for 5 min in 70 °C. cDNAs were then synthesized with dNTPs, MMLV reverse transcriptase (Promega), RNAsin (Promega), and nuclease free water for 90 min at 42 °C.

Real-time quantitative PCR

Using a step One plus apparatus (Applied Biosystems), real-time PCR reactions were carried out on each of the six individual cDNA using Fast SYBR Green Master Mix (Applied Biosystems) and specific primers designed on fragments amplified and sequenced for each biomarker (*mt1*, *mt2*, *cat*, *gpx*, *cuznsod*, and *mnsod*) (see Supplementary data for accession numbers and primers). Four housekeeping genes (*EF1a*, *actin*, *tubulin*, and *18s*) were tested and the most stable ones (*EF1a* and *β-actin*) were selected using Bestkeeper (Pfaffl et al. 2004). The relative expression was determined by the comparative Ct method (Livak and Schmittgen 2001) using controls at time 0 (T_0) as calibrator (see Supplementary data).

Enzyme analysis and MDA levels

Sample preparation and protein assays

Gills and liver were homogenized in (1:5 *w*/*v*) potassium phosphate buffer (50 mM; pH 7.4) containing 0.25 % of complete protease inhibitor cocktail (Roche, Switzerland). For malondialdehyde (MDA) levels, a volume of total homogenate was directly stored at −80 °C. For enzyme activities, homogenates were centrifuged at 12,000*g* for 10 min at 4 °C to obtain the supernatant. Homogenates and supernatants were stored at −80 °C until enzymatic assays. Protein concentrations were evaluated according to the Pierce BCA method (Krieg et al. 2005). For enzymatic and MDA analysis, a microplate reader (FLUOstar Omega; BMG LABTECH, Germany) was used.

Enzyme activities

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured using the method of Kennedy and Jones (1994) with

some modifications. The enzyme activity was calculated and expressed as picomoles of resorufin per minute per milligram of protein. Glutathione-*S*-transferase (GST) activity was measured using the method of Habig et al. (1974) with some modifications. The enzyme activity was calculated as micromoles of CDNB conjugates per minute per milligram of protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. Glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine (1967). The enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram of protein. Catalase (CAT) activity was measured by the method of Baudhuin et al. (1964). The enzyme activity was calculated as nanomoles of H_2O_2 consumed per minute per milligram of protein.

Determination of the MDA activity

Lipid peroxidation (LPO) levels were determined by measuring the thiobarbituric acid reactive substances (TBARS) following the method of Fatima et al. (2000). Values for TBARS were reported as malondialdehyde (MDA) and expressed as micromoles of MDA per milligram of protein.

Histopathological study

Histopathological analyses were performed in gill and liver tissues of peacock blennies. Samples were fixed in paraformaldehyde 4 % for a few hours. The fixative was then removed by washing samples in phosphate-buffered saline (PBS). Samples were then fixed in ethanol 80 % until further analysis and dehydrated in successive baths of ethyl alcohol 95 and 100 % and finally embedded in paraffin. On average, 18 longitudinal sections of 5 μm were examined from each fish, and 6 fish were considered for each treatment. Slides were stained with Masson's trichrome for gills and hematoxylin and eosin for the liver (Woods and Ellis 1994) and observed under an optical microscope (LEICA DM 750) fitted with a digital camera (LEICA ICC50 HD). A comparison was made between control and exposed fish in order to distinguish pathological conditions. Based on the method of Bernet et al. (1999), a semi-quantitative analysis was performed and three reaction patterns were identified: circulatory disturbances (C), regressive changes (R), and progressive changes (P). When alterations were identified, an importance factor (*w*) was attributed indicating the potential of the alteration to affect organ function: (1) reversible lesion, (2) reversible lesion if stressor is neutralized, and (3) irreversible lesion. A score range corresponding to the degree and the extent of the alteration was assessed: (0) unchanged, (2) mild occurrence, (4) moderate occurrence, and (6) severe occurrence.

Organ indices (I_G and I_L), representing the degree of gills (G) and liver (L) damage in peacock blennies exposed to Cd at all sampling times, were calculated as follows:

$$I_{org} = \sum_{rp} \sum_{alt} \left(a_{orgrpalt} \times w_{orgrpalt} \right)$$

where org, organ; rp, reaction pattern; alt, alteration; *a*, score value; and *w*, importance factor.

According to Zimmerli et al. (2007) and Agbohessi et al. (2015a, b), I_G and I_L were classified as follows:

Class 1 (index <10): normal/healthy structure.
Class 2 (index 11–20): slight modifications of normal tissue architecture and morphology.
Class 3 (index 21–30): moderate modifications of normal tissue architecture and morphology.
Class 4 (index 31–40): pronounced modifications of normal tissue architecture and morphology.
Class 5 (index >40): severe alterations of normal tissue architecture and morphology.

For each fish, a measurement of the overall health status (total index I_T) was calculated by adding all organ indices (I_G and I_L).

Moreover, a prevalence test was carried for each alteration indicating the percentage of occurrence of an alteration within all fish, with prevalence of histopathological alterations = (number of fish that displays this alteration/total number of fish) × 100.

Statistics

All statistical analyses were performed using the statistical software package STATISTICA version 8. Factorial ANOVA analysis was used to assess Cd effects compared to control groups and within exposed groups. The significance of effects among means ($p < 0.05$) was tested using Scheffé's test. For histopathological data, the description of tissues was qualitative. The comparison was thus performed according to the semi-quantitative analysis of Bernet et al. (1999) and Agbohessi et al. (2015a, b).

Results

In the present work, peacock blennies were exposed to a sublethal concentration of $CdCl_2$ (2 mg L^{-1}) during 1, 4, 10, and 15 days. To assess the effect of this metal, selected molecular, physiological, and histopathological biomarkers were analyzed at each exposure time. Before starting our experiment, seawater from which peacock blennies were collected was analyzed and displayed low

concentrations of heavy metals (Cd 3.8, Pb 27.7, and Hg <0.1 μg L^{-1}). These concentrations did not exceed Tunisian norms (NT 106.002).

Cadmium concentrations

Average Cd concentrations in water, gills, and liver of peacock blennies are summarized in Table 1. Measured concentrations of Cd in contaminated water were lower than the nominal concentration (−11-fold) over the experiment. Cd accumulation in both tissues was proportional to the exposure time and displayed a significant increase over time ($p < 0.001$). In gills, Cd concentration significantly increased after 1 day of exposure and reached 15.6 μg g^{-1} dw at the end of the experiment. In liver, results displayed a significant increase in Cd concentrations ($p < 0.001$) throughout the experiment with a maximal uptake of 18.6 μg g^{-1} dw after 15 days of exposure. Regarding the distribution of Cd in gills and liver of peacock blennies, Cd was slightly higher in liver except on day 4.

Relative gene expression analysis

Figure 1 showed the relative expression results of selected genes (*mt1*, *mt2*, *mnsod*, *cuznsod*, *cat*, and *gpx*) in gills of peacock blennies exposed to Cd-contaminated water. For all target genes, control fish displayed a lower variability among samples over time. Moreover, exposure to Cd significantly induced the expression of both *mt2* and *mnsod* mRNA ($p < 0.05$; Fig. 1b, c). *mt2* mRNA transcription displayed a significant increase throughout the experiment with up to 20-fold the expression of controls after 10 days of exposure to Cd ($p < 0.01$; Fig. 1b). On the contrary, no significant difference was observed in *mt1* gene expression (Fig. 1a). For oxidative stress, *mnsod* mRNA expression displayed a significant increase only after 1 and 4 days of exposure to Cd and reached its highest value (6-fold the expression of controls) after 4 days ($p < 0.05$; Fig. 1c). For other genes of oxidative stress (*cuznsod*, *cat*, and *gpx*), expression was not significantly affected by Cd exposure at any exposure time (Fig. 1d–f).

Biomarker activities

Gills

Data on the profile of biomarkers in gill tissue after Cd exposure are shown in Fig. 2. In control fish, activities were stable over the whole experiment without any fluctuations between sampling times ($p < 0.01$). Decrease in EROD levels was significant only after 4 and 10 days of exposure to Cd ($p < 0.01$; Fig. 2a), whereas a significant decrease of GST activity was observed throughout the experiment (Fig. 2b). Cd exposure affected GPx activity, with a highly significant increase over time ($p < 0.01$) (Fig. 2c), while CAT activity did not differ between groups (Fig. 2d). No significant difference in MDA levels was recorded between control and Cd-exposed fish (Fig. 2e).

Liver

Cd induced variations in all enzymatic biomarkers of liver. For EROD (Fig. 3a), a highly significant difference was observed after 15 days of exposure to Cd ($p < 0.01$) only. On the other hand, hepatic GST was significantly ($p < 0.01$) increased in exposed fish at all sampling times (Fig. 3b). GPx activity significantly increased in Cd exposed fish throughout the experiment, with a maximal response observed after 1 and 4 days of exposure (Fig. 3c). CAT activity also displayed a significant increase from day 1 ($p < 0.01$) and reached its highest value after 15 days of contamination ($p < 0.001$; Fig. 3d). Despite such increase in enzyme activities, MDA was significantly induced by Cd exposure at all sampling times (Fig. 3e).

Histopathological observations

Gill histopathology

In control peacock blennies, gills displayed a homogenous structure in which primary (PL) and secondary lamellae (SL) were observed. A cartilaginous support, composed of chondrocytes, is located in the center of PL. The SL

Table 1 Cd concentrations in contaminated water, gills, and liver of peacock blennies exposed to $CdCl_2$ at different exposure times (1, 4, 10, and 15 days)

Exposure time (days)	Gills (μg g^{-1} dw)	Liver (μg g^{-1} dw)	Contaminated water (mg L^{-1})
Control	0.03 ± 0.003a	0.03 ± 0.004a	<LD
1	1.7 ± 0.3b	2.5 ± 0.3b	0.18 ± 0.0015
4	7.3 ± 1.1b	2.7 ± 0.5b	0.13 ± 0.001
10	10.4 ± 0.1b	12.9 ± 0.5c	0.12 ± 0.0016
15	15.7 ± 0.4b	18.7 ± 1.5c	0.12 ± 0.0015

Values are mean ± SD of six specimens. Different letters denote significant differences ($p < 0.05$). For water analysis, LD = 0.2 μg L^{-1}; for organs, LD = 0.01 μg g^{-1}

LD limit of detection

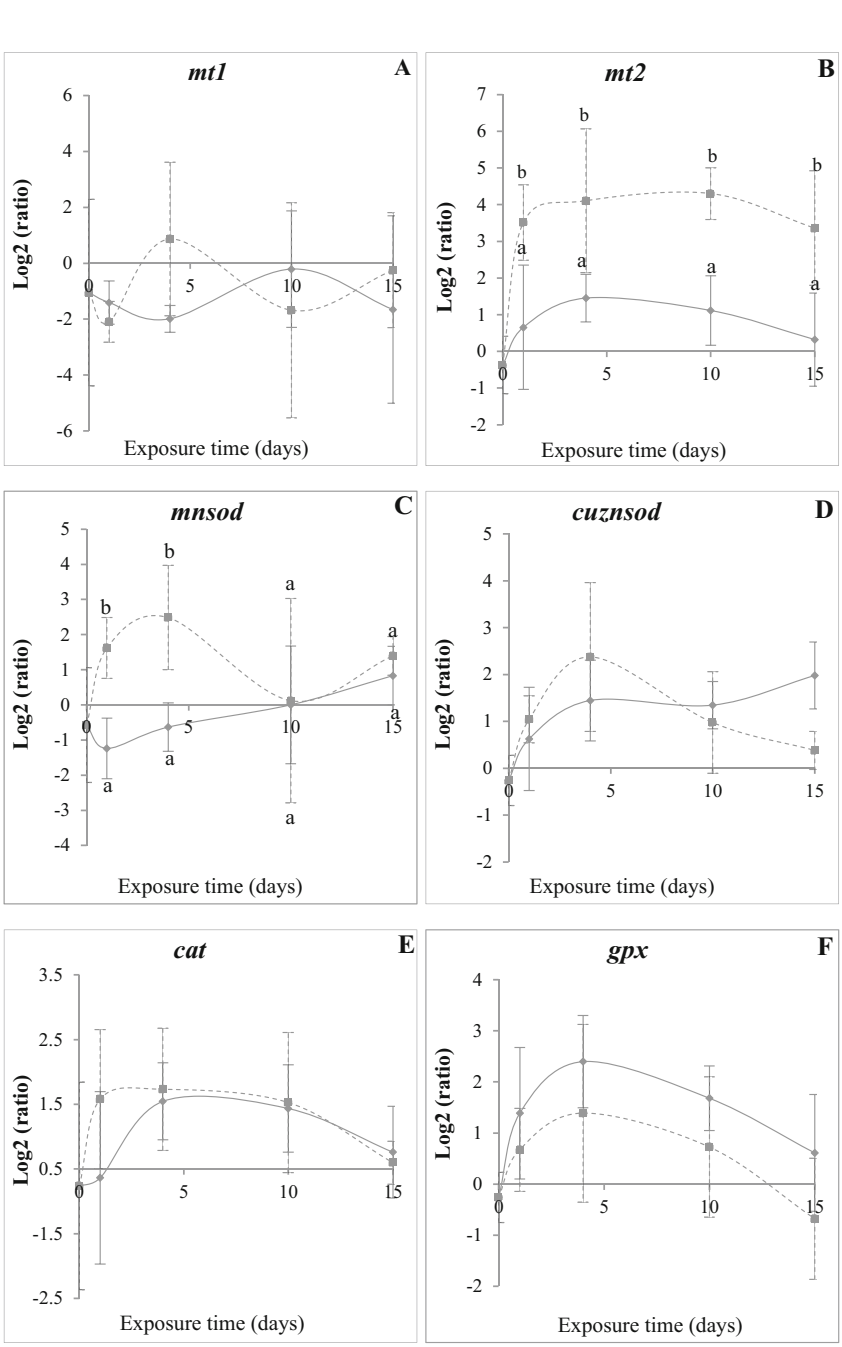

Fig. 1 Changes of gene mRNA expression in gills of peacock blennies exposed to $CdCl_2$ at different exposure times (1, 4, 10, and 15 days). Each *bar* represents mean ± SD of six specimens. Different *letters* denote significant differences ($p < 0.05$)

contains erythrocytes and pillar cells. Both PL and SL are recovered with epithelial cells. However, chloride cells are distributed at the bases of SL (Fig. 4a, b). After 1 day of Cd exposure, gills displayed damages including lamellar vacuolation, enlargement and dilatation of PL, telangiectasia, bi-layered and curved SL followed by epithelial lifting, swollen chloride and pillar cells, and necrotic cells. Hyperplasia, fusion, and congestion of SL were also observed in gills of exposed fish (Fig. 4c–e). The entire gill filament and interlamellar spaces appeared as solid masses of cells (Fig. 4c). Almost the same abnormalities were observed after 4 days of Cd exposure (Fig. 4f–h) with the appearance of short and long SL in the same PL (Fig. 4h). After 10 days of Cd exposure, injuries were more severe than after the other exposure times and revealed alterations in gill epithelium and lamellar breakage (Fig. 4i–k) as well as the detachment of gill epithelium after 15 days of Cd contamination (Fig. 4l, m).

The prevalence test of gill alterations displayed that circulatory disturbances prevailed over time compared to other reaction patterns (Table 2). Based on the semi-

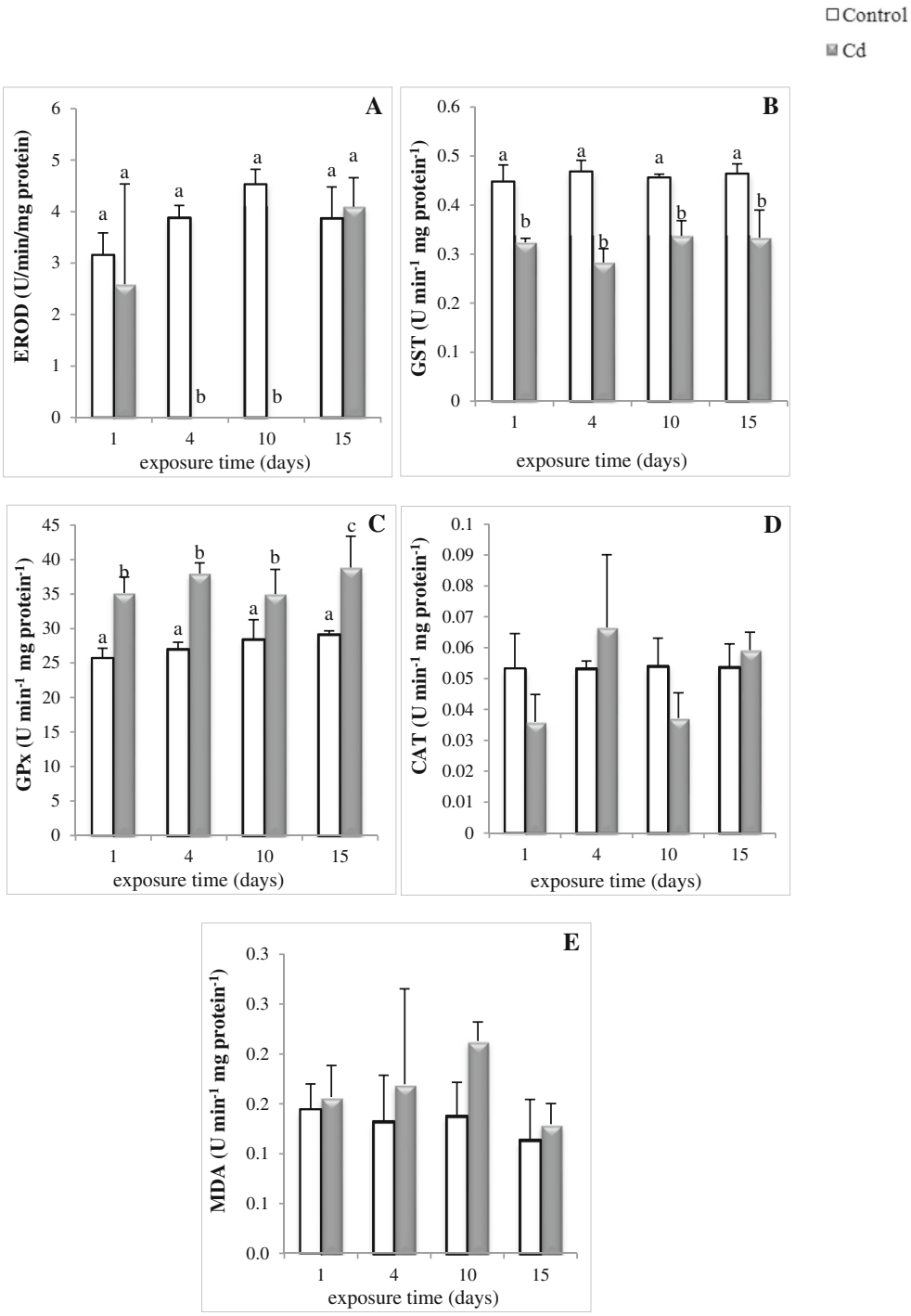

Fig. 2 Changes of enzyme activities in gills of peacock blennies exposed to $CdCl_2$ at different exposure times (1, 4, 10, and 15 days). Each *bar* represents mean ± SD of six specimens. Different *letters* denote significant differences ($p < 0.05$)

quantitative analysis, gill index (I_G) belonged to the class 3 ($21 < I_G < 30$) during the whole experiment (Table 4).

Liver histopathology

No histopathological alterations were observed in the liver of control fish. This organ is composed of central vein, sinusoids, and polygonal hepatocytes (Fig. 5a, b). After 1 day of Cd exposure, fish exhibited different pathological signs such as congestion and dilatation of the central vein, hemorrhage, and steatosis (Fig. 5c, d). After 4 days of exposure, lesions were similar to those observed after 1 day supplemented by congestion and dilatation of sinusoids and necrotic cells (Fig. 5e, f). Liver injuries were more pronounced after 10 days of Cd exposure with the presence of hepatitis foci (Fig. 5g, h). Added to the previous injuries

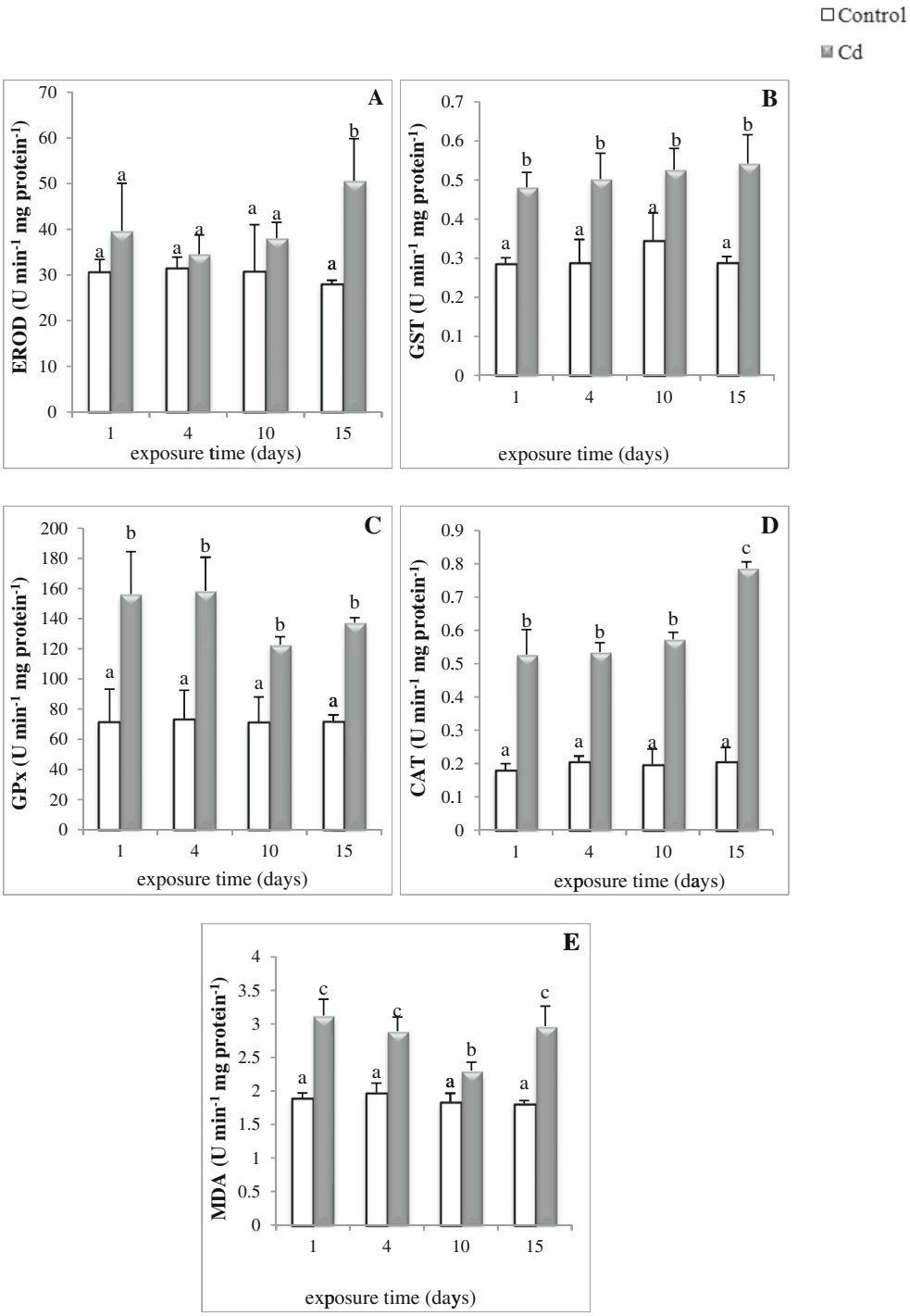

Fig. 3 Changes of enzyme activities in the liver of peacock blennies exposed to $CdCl_2$ at different exposure times (1, 4, 10, and 15 days). Each *bar* represents mean ± SD of six specimens. Different *letters* denote significant differences ($p < 0.05$)

noted at the other exposure times, severe steatosis was observed at the end of the experiment (Fig. 5i).

The prevalence test of liver alterations indicates that regressive changes were predominant over time except after 1 day of exposure where circulatory disturbances were found to be more prevalent (Table 3).

Based on the semi-quantitative analysis, the highest level of liver index (I_L) was observed after 4 days of exposure to Cd and reached the class 4 ($31 < I_L < 40$). A decrease in I_L was observed after 10 and 15 days in exposed fish (Table 4).

Total index (I_T) was calculated and summarized in Table 4 in order to evaluate the general health status of peacock blennies. I_T significantly increased until 4 days of exposure to Cd. A significant decrease of I_T was observed after 10 and 15 days indicating that fish were responding to the stress condition.

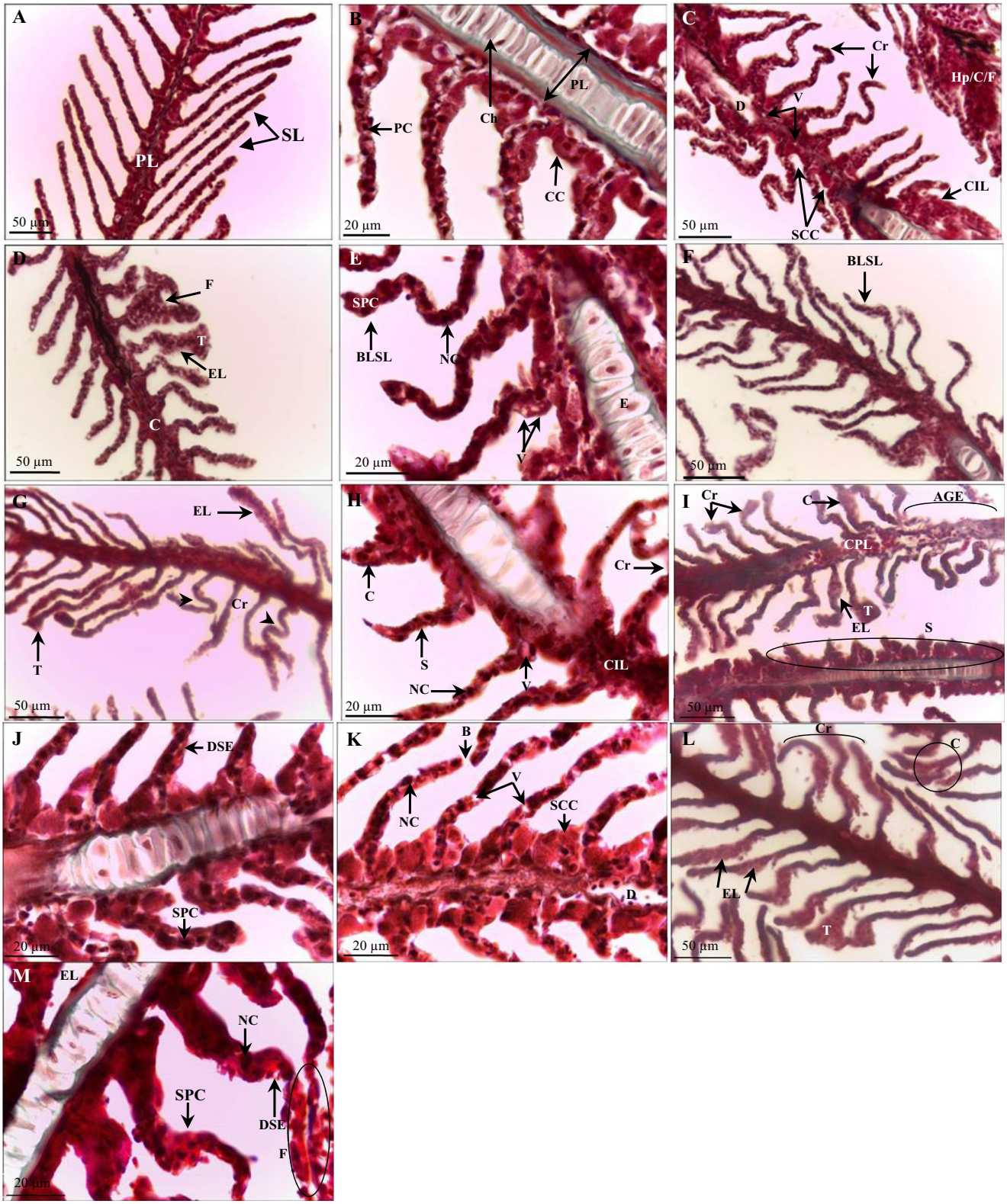

Fig. 4 Gill section micrography in control (**a**, **b**) and exposed (**c**–**m**) peacock blennies ($N = 6$). The gills of control fish displayed a normal histology with primary lamellae (PL), secondary lamellae (SL), pillar cells (PC), chondrocytes (Ch), and chloride cells (CC) (**a**, **b**). All treatments (**c**–**m**) displayed injuries including lamellar vacuolation (V), dilatation (D), congestion (C), curved SL (Cr), hyperplasia (Hp), telangiectasis (T), lamellar fusion (F), bi-layered SL (BLSL), epithelium lifting (EL), lamellar enlargement (E), swollen chloride cells (SCC), swollen pillar cells (SPC), necrotic cells (NC), congestion of interlamellar space (CIL), short SL (S), altered gill epithelium (AGE), and breakage of SL (B) (see text for details). All images were stained with Masson's trichrome

Table 2 Prevalence of histopathological alterations (%) identified in gill samples of peacock blennies at each exposure time

		Exposure time (days)				
Reaction patterns	Alterations	Controls	1	4	10	15
C	Congestion (1)	5.5	100	90	90.9	100
	Telangiectasis (1)	–	45.4	50	63.6	57.1
R	Vacuolation (1)	22	81.8	50	45.4	14.2
	Necrosis (3)	–	36.3	30	27.2	28.5
	Curved lamellae (1)	16.5	54.5	90	63.6	85.7
	Lamellar fusion (1)	5.5	45.4	14.3	36.6	42.8
	Short lamellae (1)	–	–	20	45.4	57.1
	Lamellar breakage (1)	–	–	–	45.4	28.5
	Epithelium alteration (1)	–	–	–	18.1	14.2
	Bi-layered SL (1)	27.5	72.7	50	36.3	28.5
	Epithelium lifting (1)	5.5	36.3	14.2	–	42.8
P	Dilatation (2)	–	36.3	–	27.2	14.2
	Hyperplasia (2)	11	42.6	10	9.09	–
	Lamellar enlargement (2)	–	45.4	40	–	14.2
	Swollen chloride cells (1)	11	27.2	42	54.5	–
	Swollen pillar cells (1)	5.5	36.3	40	27.2	14.2

The importance factor (*w*) is indicated in brackets for each alteration

C circulatory disturbances, *R* regressive changes, *P* progressive changes

Discussion

Cd is known to exert a wide range of pathological effects on fish (Vergauwen et al. 2013). The aim of the present study was to assess the effect of a sublethal concentration of $CdCl_2$ (2 mg L^{-1}) in peacock blennies using selected molecular, physiological, and histopathological biomarkers.

Cd concentration in water and accumulation in tissues

In its natural environment, the peacock blenny is not adapted to swim. This small fish lives under rocks, gravels, seagrasses, mangroves, and even empty shells. In the present study, the lower concentrations of Cd found in contaminated water throughout the experiment (0.12–0.18 mg L^{-1}) may be due to the adsorption of the metal on tank walls, clay pots, and filters. According to Chiffoleau et al. (1999), Cd adsorption on substrates like clay and organic particles could be considered as the dominant mechanism. The lower concentration of Cd in contaminated water may also be explained by the complexation of Cd in seawater especially with organic matter such as humic substances. Degradation products and exudates containing humic substances associated with Cd can be released to the surface water (Lara et al. 1989), thus decreasing Cd toxicity to fish (Brown et al. 1974). With a nominal concentration of $CdCl_2$ close to the one of our experiment, Messaoudi et al. (2009) displayed that the Cd concentration in contaminated water was 1041-fold lower than the nominal concentration when basil blennies were exposed to Cd over periods of 14 and 28 days. Defo et al. (2014) demonstrated that half of the nominal concentration of Cd was found in their experimental water. These authors also suggested that Cd may be adsorbed on filters and aquarium glass.

Cd enters gills through calcium channels and then alters gill functions (Farag et al. 1994). In the present work, a significant increase in Cd concentration was observed in gills over time, reaching up to 15.7 μg g^{-1} dw after 15 days of exposure. With a 10-fold higher concentration compared to our experiment, Mani et al. (2014) displayed that gills of marine catfish *Arius arius* accumulated 17.2 ± 0.3 μg Cd g^{-1} (wet weight) when fish were exposed to $CdCl_2$ over a period of 3 days.

The liver displayed a significant increase in Cd levels at all sampling times and reached its maximum concentration after 15 days of exposure with 18.7 μg g^{-1} dw. As for gills, our results displayed that liver of exposed fish are able to accumulate large amounts of Cd. Messaoudi et al. (2009) also demonstrated that liver of basil blennies exposed to 2 mg $CdCl_2$ L^{-1} were able to concentrate Cd up to 17.8 and 42.7 μg g^{-1} dw after 14 and 28 days, respectively. Overall, Cd burdens indicate that gills and liver of peacock blennies are considered as important organs for Cd accumulation and storage. This metal is capable to cross gill barriers and then enter the liver via blood transport where various mechanisms will occur to neutralize the metal toxicity.

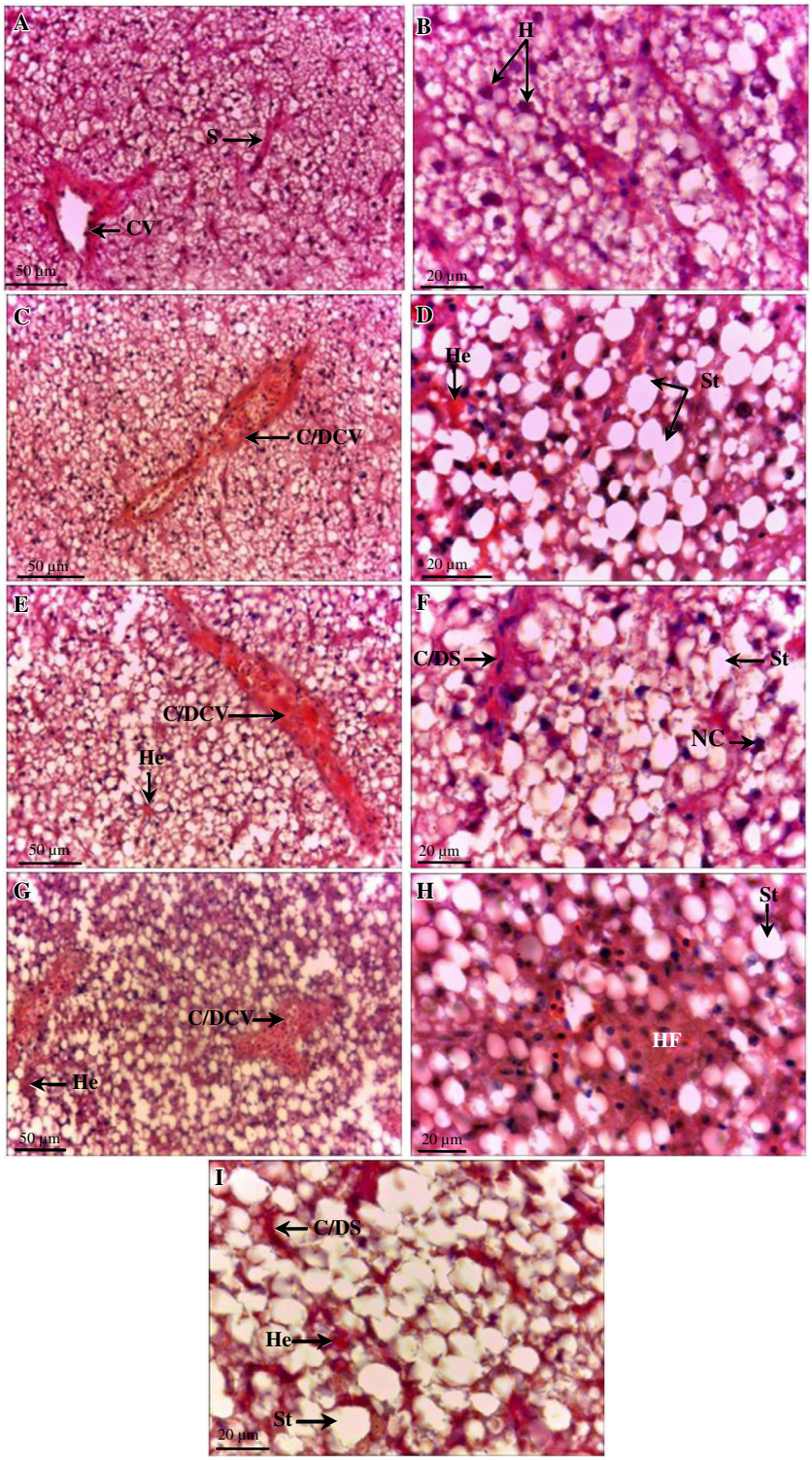

Fig. 5 Liver section micrography in control (**a**, **b**) and exposed (**c**–**i**) peacock blennies ($N = 6$). The liver of control fish displayed a normal histology with sinusoids (S), central vein (CV), and hepatocytes (H) (**a**, **b**). All treatments (**c**–**i**) displayed injuries including congested and dilated central vein (C/DCV), hemorrhage (He), steatosis (St), congested and dilated sinusoids (C/DS), necrotic cells (NC), and hepatitis foci (HF) (see text for details). All images were stained with hematoxylin and eosin

Cd exposure affects transcriptional levels of *mt2* and *mnsod* in gills

To our knowledge, this is the first study in which cDNA coding *Mts* (*mt1* and *mt2*) and antioxidant biomarkers (*mnsod*, *cuznsod*, *gpx*, and *cat*) have been characterized in peacock blennies (accession numbers in Supplementary data). Response of peacock blennies at the transcriptional level was variable. Genes coding *mt2* and *mnsod* were significantly up-regulated, while those coding *mt1*, *cuznsod*, *gpx*, and *cat* did not change over time when compared to control groups.

Table 3 Prevalence of histopathological alterations (%) identified in liver samples of peacock blennies at each exposure time

		Exposure time (days)				
Reaction patterns	Alterations	Controls	1	4	10	15
C	Congestion (1)	11	55.5	85.7	28.5	100
	Hemorrhage (1)	–	77.7	57.1	14.2	33.3
R	Steatosis (1)	33.2	100	100	85.7	100
	Necrosis (3)	–	–	85.7	85.7	16.6
P	Dilatation (1)	11	55.5	71.4	14.2	16.6
	Hepatitis foci (2)	–	–	–	42.8	16.6

The importance factor (*w*) is indicated under brackets for each alteration
C circulatory disturbances, *R* regressive changes, *P* progressive changes

MTs play different functions including the maintenance of the homeostatic metabolism of metals as well as their accumulation, detoxification, and transport (Li et al. 2016). In the present study, *mt2* mRNA expression dramatically increased over time up to 20-fold the one of controls after 10 days of exposure. On the contrary, no variation in *mt1* mRNA transcript was observed between control and exposed groups throughout the experiment. Thus, *mt2* seemed more effectively induced by $CdCl_2$ as reported by Wu et al. (2012) in zebrafish treated with different concentrations of Cd^{2+}. Airaksinen et al. (2003) reported a significant increase of *mt* expression in zebrafish exposed to Cd for 4 h and Van Cleef-Toedt et al. (2001) revealed the induction of *mt* mRNA expression in killifish exposed to 6 ppb of $CdCl_2$.

Besides sequestration by MT proteins, a series of antioxidant enzymes are also involved in detoxification processes by eliminating ROS (Cong et al. 2012). SODs are activated in order to scavenge ROS and maintain the redox balance. In the present study, *mnsod* was up-regulated up to 4 days after the beginning of the exposure to $CdCl_2$ (6-fold the expression of controls), while *cuznsod* did not change throughout the experiment. Our results indicate that *sod* expression may be acute at the beginning of the exposure to Cd. This scenario agrees with the results of Hansen et al. (2006) in brown trout during waterborne Cd exposure. It can be hypothesized that expression of *mnsod* mRNA could contribute to the synthesis of SOD proteins that might be sufficient to protect gills against Cd from the beginning of the exposure. To confirm this hypothesis, changes in enzyme activity should be followed.

On the other hand, there was no significant regulation in both *cat* and *gpx* mRNA transcriptions over time which could be explained by (1) the low concentration of Cd measured in contaminated water and/or (2) exposure times of this study were not long enough to induce the transcriptional modulation of these two genes and/or (3) *cat* and *gpx* expressions may have no real role in gills protection for short-term exposures. Our results corroborated the finding of Sassi et al. (2013) who did not find a significant regulation in *gpx* transcripts and Hansen et al. (2007) who did not report any change in *cat* mRNA expression levels in gills of brown trout after Cd exposure.

Table 4 Gills, liver, and total index (I_G, I_L, and I_T) of peacock blennies exposed to Cd

Exposure time (days)	I_G	I_L	I_T
Controls	5.3 ± 3a	3.16 ± 1a	8.5 ± 3.44a
1	30.4 ± 6.2b	14.4 ± 5.9b	45.1 ± 8.9b
4	25.4 ± 8.8b	33.1 ± 7.9b	59.1 ± 10.3b
10	27.1 ± 8.5b	29.7 ± 4.9b	56.9 ± 9.4b
15	24 ± 5.7b	14.7 ± 3.5b	41.6 ± 5.1b

Values are mean ± SD. Different letters denote significant differences ($p < 0.05$)

Cd exposure affects enzyme activity profiles in gills

Cd is also known to induce toxic effects on both enzyme activities involved in the oxidative stress and LPO (Liu et al. 2009). Our data showed that exposure to Cd induced the inhibition of phase I (EROD) and phase II (GST) enzyme activities in gills of peacock blennies exposed to Cd. As reported by previous studies, Cd has inhibitory effects on CYP, GST, and GSH (Iscan et al. 1995; Viarengo et al. 1997; Oliveira et al. 2004).

Our results displayed that exposure to Cd completely inhibited EROD activity after 4 and 10 days of exposure. Several hypotheses may explain EROD depletions: (1) Cd exposure tends to increase heme oxygenase activity, which breaks down heme groups. Because CYP enzymes need an intact heme group to function properly, induction of heme oxygenase will decrease CYP activity (Yoshida et al. 1979). According to Lai and Loo (2011), an induction of heme oxygenase is part of a defensive mechanism of cells to protect themselves against Cd toxicity; (2) Cd can inhibit CYP activity by binding to its sulfhydryl sites (Chandrasekera et al. 2008); (3) Cd can alter CYP activity by displacing the metal associated with the enzyme (Oliveira and Santos 2003); and (4) overproduction of ROS may cause a specific EROD inhibition through the activation of peroxisome proliferation activated receptors (Laville et al. 2004). Our results corroborated the finding of Oliveira et al. (2004) in the sea bass exposed to heavy metals. The authors displayed that high heavy metal concentrations exhibited deleterious effects on EROD activity up to 100 % of inhibition. The study of Chandrasekera et al. (2008) reported that exposure of tilapia *Oreochromis niloticus* to high concentrations of Cd (0.1 and 1 mg L^{-1}) for 14 days led to the inhibition of EROD activity. Likewise, Bozcaarmutlu and Arinc (2004) showed that 5 μM of $CdCl_2$ reduced EROD activity up to 95 % in exposed fish.

In the literature, Stacey and Klaassen (1981) showed that metals led to the depletion of GSH concentrations, and Gate

et al. (1999) suggested that contaminants directly bind to GST proteins. In the present study, exposed peacock blennies to Cd displayed a significant decrease of GST activities throughout the experiment. A similar result was found by Farombi et al. (2007) in African catfish sampled from polluted river containing Cd. In mice, GST activity was shown to decrease after Cd treatment (Iscan et al. 1995). Karmakar et al. (1998) noted that prolonged exposure to Cd resulted in a decrease of GST activity in the animal after 15 days of treatment.

CAT and GPx are key enzymes for the detoxification of hydrogen peroxide (H_2O_2) (Borkovic et al. 2008). CAT activity did not change significantly throughout the experiment. As mentioned earlier, *cat* mRNA transcription in exposed fish did not exhibit any changes throughout the experiment when compared to control groups indicating that transcriptional analysis of *cat* provides a useful supplement to the enzyme activity. With a 10-fold higher nominal concentration compared to our experiment, $CdCl_2$ did not induce any effect on CAT activities in goldfish (Zikic et al. 2001). On the contrary, GPx activity significantly increased over time reaching its maximum value on day 15 when compared to the control group. This result indicated that only GPx responded to the formation of H_2O_2 reflecting its effectiveness to protect gills against oxidative stress. When regarding GPx, discrepancies between mRNA expression and protein levels were observed. Even Cho et al. (2006) and Hansen et al. (2006, 2007) suggested that the transcript level might not always be related to the protein synthesis; more attention should thus be given to analyze the relation between mRNA transcription and protein accumulation of GPx in peacock blenny gills. Our results displayed an absence of changes in MDA levels in gills of peacock blennies over time indicating that gill mechanisms were efficient against Cd toxicity. These mechanisms include the increase of *mt2* and *mnsod* mRNA expressions as well as elevated GPx activity throughout the experiment.

Cd exposure affects enzyme activity profiles and lipid peroxidation in the liver

In the present study, the activity of all enzymes and MDA levels in the liver increased significantly immediately at 1 day of Cd exposure except for EROD activity that increased after 15 days only (1.8-fold the activity of controls). EROD is located in the endoplasmic reticulum, and its activation could be due to the direct effect of Cd on membranes as suggested by Bouraoui et al. (2008) in the sea bream exposed to Cd for 6 h. Increased EROD activity was also found with a 400-fold lower concentration when eels were exposed to Cd for a longer period (24 days) (Lemaire-Gony and Lemaire 1992).

GST activity can be significantly increased by exposure to many ranges of contaminants because it represents an adaptive response to stress (Sen and Kirikbakan 2004). According to Canesi et al. (1999), a high increase in GST activity may protect organs from LPO. Throughout the present experiment, our results showed a significant increase in GST levels up to 1.8-fold compared to the values measured in the control group. Our results confirmed the work of Chandrasekera et al. (2008) in tilapia *Oreochromis mossambicus* exposed to 5 mg Cd L^{-1} for 30 days. These authors observed an increase of the GST activity nearly by 80 % after 15 days of exposure to the metal. In gambusis, *Gambusia affinis*, Souissi et al. (2008) observed a significant increase of GST activities when fish were exposed to different concentrations of Cd. Overall, increased activities of both EROD and GST may strengthen the conjugation and the elimination of Cd from the liver.

In the current study, exposure to Cd resulted in a significant increase in CAT and GPx activities from the beginning of the experiment. These changes reflected the effect of Cd on the generation of oxygen free radical production in the liver of peacock blennies that stimulated the detoxification process. Contamination of basil blennies with the same nominal concentration of $CdCl_2$ displayed an increase in CAT levels after 14 and 28 days of exposure (Messaoudi et al. 2009).

MDA was significantly increased after 1 day under Cd exposure. Increased MDA reflected the elevation of oxidative stress in the liver of peacock blennies and thus the increase of free radicals. Despite the important stimulation of biomarker activities, our results displayed the failure of these enzymatic biomarkers to protect the liver against ROS generation and LPO. According to Sakuragui et al. (2013), higher levels of MDA may contribute to lipid alterations of cellular membrane leading to protein alterations and dysfunctions. Our results corroborated many other studies that highlighted the pro-oxidant activity of Cd (Sakuragui et al. 2013; Souid et al. 2013; Qu et al. 2014).

When comparing enzymatic biomarker responses in gills and liver, it seems that antioxidant defense were more effective against oxidative stress and LPO generation in gills.

Histopathological alterations in gills

When an imbalance between ROS and antioxidant systems occurs, the normal architecture and morphology of the tissue can be modified.

In the present experiment, three reaction patterns were identified in gills, namely, circulatory disturbances, regressive changes, and progressive changes. The first one that occurred during the whole experiment was represented by lamellar congestion and telangiectasia. According to Alazemi et al. (1996), telangiectasia results from the collapse of the pillar cell system and the breakdown of vascular integrity leading to hypoxia. Giari et al. (2007) reported similar alterations in European sea bass *Dicentrarchus labrax* exposed to 4.5-fold higher nominal concentrations of Cd compared to our study. Histopathological causes of gill regression identified in the present study were vacuolation, necrosis, curved lamellae,

lamellar fusion, short lamellae, lamellar breakage, epithelium alteration, bi-layered SL, and epithelium lifting. According to Wilson and Taylor (1993), hypoxia may result in epithelium lifting which contributed to increase the diffusion distance. Furthermore, shrinkage and lamellar fusion would reduce the flow oxygen-enriched water to the lamellar tissue (Cladwell 1997). Koca et al. (2008) demonstrated that the appearance of bi-layered SL might lead to the weakening of the capillary circulation due to the erythrocyte expansion. With a 200-fold lower nominal concentration of $CdCl_2$ compared to our experiment, Costa et al. (2013) observed vacuolation, epithelium lifting, desquamation of lamellar epithelium, and deformation of lamellae in rock sole *Solea senegalensis*. Lamellar dilatation, hyperplasia, lamellar enlargement, and swollen pillar and chloride cells were of a progressive nature and were less prevalent than the earlier reaction patterns. Since Cd enters gills through calcium channels, chloride cell hypertrophy occurred in order to eject Cd from gills (Thophon et al. 2003). This injury, leading to reduce the inter-lamellar space, would be considered as a resistance response of peacock blennies against Cd as hypothesized by Pandey et al. (2008) in spotted snakehead. Our results are similar to those of Thophon et al. (2003) who reported in white sea bass *Lates calcarifer* the prevalence of breakdown of pillar cell system, swollen chloride cells, and lamellar hyperplasia.

The gill index (I_G) displayed a significant increase from the beginning of the experiment up to 30.4 while a slight decrease was observed at the end of the experiment showing moderate modifications in gills. This decrease indicates that gills are challenged and respond to Cd toxicity in an effort to restore the normal architecture and functioning of the tissue. Among all biomarkers analyzed in gills of Cd-exposed fish, increased *mt2* and *mnsod* expressions and GPx activities may be considered as the most important responses allowing the protection of gills. In the same context, Atli et al. (2006) suggested that increased GPx activity might be due to a higher repair mechanism of gill epithelium.

Histopathological alterations in the liver

As for gills, three reaction patterns were identified in the liver of peacock blennies. The most prevalent reaction pattern was of a regressive nature (71.7 %), followed by circulatory disturbances (56.5 %) and then progressive changes (27.1 %). Steatosis and necrosis were observed as regressive changes in the liver of peacock blennies. Hughes (1979) noted that necrosis is closely related to the direct effect of the pollutant, and Arellano et al. (1999) displayed that steatosis may indicate an alteration of the lipid metabolism. Our results support the last hypothesis since LPO, which is a marker of lipid alteration, was induced in the liver of peacock blennies throughout the experiment. Hibiya (1982) classified steatosis as an irreversible alteration because hypertrophied vacuole forces nuclei to migrate towards the cellular membrane leading to the nuclear atrophy. Our results corroborate the study of Patnaik et al. (2011) who reported the prevalence of vacuolation and necrosis in mirror carp *Cyprinus carpio* exposed to a sublethal concentration of $CdCl_2$ over a period of 28 days. In the current study, circulatory disturbances were represented by hemorrhage and congestion of both sinusoids and central vein. Nevertheless, circulatory disturbances, mainly the congestion of blood vessels, may be associated to the response of hepatocytes to Cd (Hinton and Lauren 1990). The occurrence of this reaction pattern was previously reported in milkfish (Rajeshkumar and Munuswamy 2011), mirror carp (Van Dyk et al. 2007), goby *Synechogobius hasta* (Liu et al. 2011), and white seabass (Thophon et al. 2003). The less prevalent reaction pattern identified in the present work was of a progressive nature and was represented by dilatation and hepatitis foci. Vascular dilatation may contribute to the cellular degeneration and necrosis in the liver (Braunbeck et al. 1990). Costa et al. (2013) reported alterations of sinusoids and central vein in Senegalese sole exposed to much lower concentrations of Cd compared to our experiment.

Liver index (I_L) displayed a significant increase after 4 days and belonged to class 4 according to Zimmerli et al. (2007). At the end of the experiment, a significant decrease of I_L was noted with slight modifications in liver architecture and morphology. Despite the increased levels of Cd and LPO in the liver of peacock blennies, decreased I_L suggests that liver tissue developed resistance mechanisms against Cd exposure. Total index (I_T) indicated that the general health status of the peacock blenny was affected by Cd exposure mainly after 4 days. A significant decrease of the I_T value was observed after 15 days of Cd exposure indicating that fish may be able to restore the normal architecture of their tissues.

Conclusions

In conclusion, molecular, physiological, and histopathological alterations detected in peacock blennies exposed to 2 mg $CdCl_2$ L^{-1} demonstrated that fish responded to the contamination by developing various mechanisms. Cd concentrations measured in contaminated water at each sampling time showed that peacock blennies were actually exposed to lower concentrations of Cd (6 to 9 % of the $CdCl_2$ nominal concentration). Gills of peacock blennies accumulated large amounts of Cd that were sufficient to trigger the antioxidant response. Responses of enzymatic biomarkers in gills were successfully implemented and prevented oxidative stress and LPO generation. The histopathological analysis displayed that I_G belonged to the class 3 over the whole experiment, indicating moderate modifications of the normal gill architecture and morphology. Overall, all alterations identified in gills of Cd-

exposed fish may be considered as reversible lesions. Thus, the efficiency of all analyzed biomarkers could prevent the emergence of irreversible alterations whose prevalence may cause the dysfunction of gills and thus fish asphyxiation and death. On the other hand, we demonstrated that the liver was an important organ for Cd accumulation. Enzyme activities increased in exposed fish but were not enough to prevent LPO generation despite the fact that peacock blennies were exposed to lower concentrations of Cd compared to the nominal concentration of $CdCl_2$. The histopathological analysis displayed that I_L decreased from 10 days of exposure to Cd and belonged to the class 2 at the end of the experiment, indicating slight modifications of normal liver architecture and morphology. Certainly, increased activities of enzymatic biomarkers did not prevent oxidative stress but may allow the liver to restore its normal structure. In conclusion, the general health status of peacock blennies assessed throughout histopathological analysis indicated that fish might be able to restore their normal tissues structure and function.

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