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# HSP90 and pCREB alterations are linked to mancozeb-dependent behavioral and neurodegenerative effects in a marine teleost

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ABSTRACT

The pesticide mancozeb (mz) is recognized as a potent inducer of oxidative stress due to its ability to catalyze the production of reactive oxygen species plus inhibiting mitochondrial respiration thus becoming an environmental risk for neurodegenerative diseases. Despite numerous toxicological studies on mz have been directed to mammals, attention on marine fish is still lacking. Thus, it was our intention to evaluate neurobehavioral activities of ornate wrasses (*Thalassoma pavo*) exposed to 0.2 mg/l of mz after a preliminary screening test (0.07–0.3 mg/l). Treated fish exhibited an evident ($p < 0.001$) latency to reach T-maze arms (>1000%) while exploratory attitudes (total arm entries) diminished (−50%; $p < 0.05$) versus controls during spontaneous exploration tests. Moreover, they showed evident enhancements (+111%) of immobility in the cylinder test. Contextually, strong (−88%; $p < 0.01$) reductions of permanence in light zone of the Light/Dark apparatus along with diminished crossings (−65%) were also detected. Conversely, wrasses displayed evident enhancements (160%) of risk assessment consisting of fast entries in the dark side of this apparatus. From a molecular point of view, a notable activation ($p < 0.005$) of the brain transcription factor pCREB occurred during mz-exposure. Similarly, in situ hybridization supplied increased HSP90 mRNAs in most brain areas such as the lateral part of the dorsal telencephalon (Dl; +68%) and valvula of the cerebellum (VCe; +35%) that also revealed evident argyrophilic signals. Overall, these first indications suggest a possible protective role of the early biomarkers pCREB and HSP90 against fish toxicity.



## 1. Introduction

Water pollution represents a serious threat to aquatic organisms and especially fish. Pesticide runoff from agricultural lands are one of the main sources of water contamination, which induces dangerous disturbances to the different physiological facets of fish such as swimming and feeding behaviors (Ullah et al., 2014). Pesticides may easily enter in the fish body through gills, skin and via the food-chain thereby reaching target organs like the brain (Atamaniuk et al., 2014). At this regard, deleterious effects of pesticides are often related to neuronal dysfunctions in both mammals (Lee et al., 2015) and fish (Renick et al., 2016). Consequently the dithiocarbamate fungicides, widely used for preserving different agricultural yields, are considered hazardous toxicants (Rath et al., 2011) due to the alteration of key enzymes such as α-carbonic anhydrase thus accounting for the failure of pH homeostasis, respiration and electrolyte secretion (Kolayli et al., 2011). Among dithiocarbamates, mancozeb (manganese (Mn)/zinc (Zn)-ethylene-bis-dithiocarbamate; mz) is composed of different sub-compounds (Mn, Zn plus ethylene thiourea) that together account for multiple toxic mechanisms operating simultaneously during exposure to this fungicide (Geissen et al., 2010). For this reason, we purposely decided to focus on the effects of the integral compound, which causes wide neuronal damages (Harrison Brody et al., 2013). Indeed, the presence of this molecular complex permits it to catalyze the production of reactive oxygen species (ROS) as well as inhibiting mitochondrial respiration at the brain level (Todt et al., 2016) thus proposing it as an environmental risk for neurodegenerative diseases such as Parkinson's (Pezzoli and Cereda,

*Abbreviations:* ACS, amino cupric silver stain; CCe, corpus of the cerebellum; DIG, digoxigenin-11-dUTP; Dl, lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; HSP90, heat shock protein 90; mz, mancozeb; NDLI, diffuse nucleus of the inferior lobe; NG, nucleus glomerulosus; nIV, trochlear nerve nucleus; OT, optic tectum; pCREB, cAMP response element-binding protein; RS, superior reticular nucleus; TLo, torus longitudinalis; VCe, valvula of the cerebellum; Vl, lateral part of the ventral telencephalon; VTel, ventral telencephalon; Vv, ventral part of the ventral telencephalon.

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2013). At present, indications are beginning to consider mz as an important endocrine disruptor (Thienpont et al., 2011), an oxidative stressor of gills and blood (Kubrak et al., 2012), plus being an inductor of oxidative damage to lipids and proteins in brain, liver and kidney of fish (Atamaniuk et al., 2014).

Based on the above considerations, together with the lack of toxicological studies on marine fish contaminated by mz, the present study aimed to investigate neuronal and behavioral effects of this fungicide on the marine teleost ornate wrasse (*Thalassoma pavo*) exposed to 0.2 mg/l of mz. This concentration was chosen on the basis of a preliminary screening test of sublethal concentrations (0.07, 0.14, 0.2, 0.3 mg/l) handled in our laboratory. These doses are in line with those used in other studies (Jarrard et al., 2004) along with environmentally relevant concentrations detected in waterbodies near agricultural fields (Shenoy et al., 2009) and in view of the recommended mz concentrations being a thousand times higher for crop treatment (Atamaniuk et al., 2014). Despite the frequent application of mz throughout the world, indications regarding its environmentally relevant concentrations in seawater are still lacking, perhaps because this fungicide is often considered a compound with a low toxicity even if recent evidences are beginning to indicate a high risk imposed to fish due to water contamination (Marques et al., 2016). In any case, it is considered a marine pollutant as reported by the Pesticide Properties Database of the University of Hertfordshire (http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/424.htm) and it is known that the runoff of mz from the river or soil near coasts can easily reach the sea especially since Italy is geographically surrounded by the Mediterranean sea.

Hence, the different behavioral parameters (Light/Dark Test, Spontaneous Exploration Test in a T-maze and Cylinder Test) along with specific molecular markers were investigated during cellular impairments following exposure to this fungicide. For the present work, the phosphorylated and active form of CREB (cAMP response element-binding protein, pCREB) was preferred since it is an important transcription linker between a number of neurotransmitters and downstream gene expression thus favoring neuronal survival and proliferation (Dworkin et al., 2007) together with antianxiety-like conditions (Chakravarty et al., 2013). At the same time, HSP90 was also evaluated due to its well-known role on environmental stress (Wang et al., 2016; Zizza et al., 2016) in which they mitigate deleterious effects of protein misfolding in a similar manner to neurodegenerative diseases (Marino Gammazza et al., 2016). Indications deriving from the present study may provide novel bearings concerning the activation of protective mechanisms against mz-dependent toxicity on fish neurobiological activities.

## 2. Materials and methods

Before treating fish, it was necessary to determine the solubility parameter of mz due to the lack of indications in seawater. For this part, experiments were conducted according to previous procedures (Furia et al., 2011; Naccarato et al., 2016).

### 2.1. Analytical procedure

Mz was not quantified directly but the samples were treated to quantify Mn as previously reported (Pariseau et al., 2009). With this aim, mz (1.5 mg) was dissolved in 1.5 l of seawater; the mixture was vortexed at least for 2 h and then filtered with an highly retentive filter paper (Whatman 42). The filtrate was diluted (1:100) with filtered seawater. This solution was then analyzed by adding the reagent $HNO_3$ (65%; Suprapur; Merck, Darmstadt, Germany) via Elan DRC-e ICP-MS instrument (Perkin-Elmer SCIEX, Canada) using 55 Mn isotope and seawater as a blank. Quantitative analysis was performed designing an eight-point calibration curve (calibration range: 0.1–1000 μg/l) in which the calibration solutions were prepared by diluting Merck XXI and Perkin Elmer 2 multi-element standards solutions to 10 mg/l (Ultrapure water; Milli-Q plus system, Millipore, Bedford, MA). Total Mn concentration (519 μg/l) corresponds to the highest solubility of mz in seawater. The quantification limit of the analyte is 0.03 μg/l. We had a satisfactory quantitative recovery for Mn and thus we avoided using isotope dilution (Mn does not have stable isotopes to use for isotope dilution).

### 2.2. Animals and treatments

Adult female specimens of *Thalassoma pavo* (7–13 g body weight; 8–11 cm body length: n = 30), which was already used for the evaluation of neurotoxic effects (Zizza et al., 2013, 2014), were obtained from a local supplier. They were acclimated in our laboratory for at least 1 week in 80 l tanks under a natural photoperiod in aerated and filtered seawater. During acclimation, fish were fed once a day with small pieces of frozen prawns corresponding to 2% of the biomass in the tank (Facciolo et al., 2010). Water quality parameters i.e. salinity (35%), density (1.027–1.028 g/cm$^3$), hardness (100 mg $CaCO_3$/l) and dissolved oxygen (8–8.6 mg/l) as well as temperature (20–22 °C) plus pH (7.5–8.0) were daily checked to assure that they remained within these ranges. Animal maintenance and experimental procedures complied with the legislative law n°116 (27-01-1992) and with European Directive (2010/63/EU) for the correct use of laboratory animals. Efforts were made to minimize animal suffering and reduce number of fish used.

After acclimation, fish were exposed for 96 h to mz (Sigma, Milan-Italy) dissolved in seawater to reach the nominal sub-lethal concentration of 0.2 mg/l (n = 15) and compared to controls (n = 15), which were not exposed to the pesticide. This concentration fell within the solubility parameters of mz in seawater according to indications obtained by a ICP-MS analytical procedure. It resulted to be the most effective non-lethal dose capable of inducing behavioral effects (data not shown) among the different mz concentrations (0.07, 0.14, 0.2, 0.3 mg/l) as well as falling within the same range used by others (Jarrard et al., 2004). A static renewal exposure system was applied, with the pesticide concentration being renewed in seawater every 24 h, according to standard procedure guidelines (American Society for Testing Material, 2014). This type of exposure system, together with a basic pH and a relatively high temperature, guarantees a constant pesticide concentration within 24 h since degradation of mz occurs after a much later time, i.e. 39 h (López-Fernández et al., 2017). Tanks were only equipped with an aerator without any chemical filters to avoid modifications of the pesticide concentration. Water parameters were constantly checked to ensure that they remained within the ranges reported above. During exposure, fish were fed as above according to our previous toxicological studies (Giusi et al., 2008; Zizza et al., 2014).

### 2.3. Behavioral assessment

*2.3.1. Spontaneous exploration test in a T-maze.* A T-shaped glass tank consisting of a start compartment of 40 × 40 cm, a passage lane (40 × 20 cm) and two arms of the same length (20 × 20 cm) was used to assess the effects of mz on spontaneous exploration. In the present study, this type of maze represents an novel arena to assess fish exploration according to a previous study (Grossman et al., 2010). Following a 96 h exposure to the fungicide, all fish were individually tested. Each fish was placed in the start compartment and observed for 6 min using a digital camera positioned at the top. The following parameters were evaluated:

- *latency time to reach arms*: the time (s) to reach arms from the start compartment;
- *total arm entries*: the total number of entries in the maze arms;
- *time spent in arms*: the total time (s) spent in the maze arms.

*2.3.2. Cylinder test.* Each animal was introduced alone in a cylinder apparatus, which consisted of a 6 l glass cylinder (16 cm diameter and 20 cm height) maximally filled with seawater and divided in two

equal virtual portions as reported by Grossman et al. (2010). Tests (6 min) were recorded by two digital cameras, one at the top and the second one opposite to the cylinder. Such an apparatus was used as a novel tank to examine effects of mz on the following end-points: time (s) spent at the bottom of the tank, time (s) spent along the cylinder walls, time (s) spent in a immobile state and time (s) spent in different movements (motor activity).

*2.3.3. Light/dark preference test.* Light/Dark preference test was carried out at the end of the exposure session, in order to assess anxiety-like behaviors (Maximino et al., 2011). The Light/Dark apparatus used for the test consisted of a rectangular glass tank (25 × 20 × 40 cm h × d × l) subdivided in two equal compartments without any physical barrier between them, as previously reported (Zizza et al., 2016). The light part of the apparatus consisted of the transparent walls of the aquarium with a diffused light located above. The dark compartment was shielded from the light source with an opaque black lid along with opaque black walls and bottom. Also in this case, all animals were individually introduced into the Light/Dark apparatus. Each behavioral test, which lasted 6 min, was filmed and the following end-points were evaluated:

- *light permanence*: the total time (s) spent in the light compartment;
- *crossings*: the number of transitions between the two compartments;
- *risk assessment*: the number of fast entries in the dark side of the apparatus or a partial entry within the white compartment (Maximino et al., 2011).

All behavioral data were analyzed using the software EthoLog 2.2.5 (Visual Basic; Brazil) and values were reported as mean activity ± standard error of mean (SEM).

At the end of the behavioral session and before molecular procedures, fish were checked for sexual identification by morphological observations of the ovary that did not show any sign of ovarian atresia that could have indicated an initial transition to the testicular growth (Liu et al., 2017).

### 2.4. Neurodegenerative analysis

A neurodegenerative analysis was carried out by applying Amino Cupric Silver (ACS) technique to verify neuronal damages caused by mz. This technique has been widely used for the detection of both necrotic and apoptotic events in fish via the formation of silver precipitated granules (argyrophilic reaction) in neuronal fields where neurodegeneration occurred (Zizza et al., 2016). With this purpose, fish (n = 5) exposed to the fungicide for 96 h plus controls (n = 5) were first anesthetized in ice water and then sacrificed by spinal transection. Brains were removed, frozen on dried ice and stored at −20 °C. Subsequently, they were mounted on a freezing stage of a sliding cryostat (Microm-HM505E; Zeiss, Wallford, Germany) to obtain a series of representative sections (30 μm) for ACS protocol as previously described (Zizza et al., 2014). Afterwards, sections were kept in a rapid fixer solution for 5 min and counterstained with 0.5% neutral red solution (Carlo Erba, Milan-Italy) for 25 min, dehydrated in ethanol (50–100%) plus xylene, and mounted with DPX (*p*-xylene-bis[*N*-pyridinium bromide]; Sigma) for observations at a bright-field Dialux EB 20 microscope (Leitz, Stuttgart, Germany).

### 2.5. Western blot

Brain tissues of exposed-fish (n = 5) plus controls (n = 5) were homogenized and lysed on ice with RIPA lysis buffer containing protease and a mixture of phosphatase inhibitors (Santa Cruz, Biotechnology, Milan-Italy) for 30 min. Homogenates were centrifuged at 12000 rpm for 20 min at 4 °C. Total protein quantities were determined by using BCA protein assay kit (PIERCE, Milan-Italy). 50 μg of proteins of each sample were boiled for 5 min in SDS buffer [50 mM Tris-HCl (pH 6.8), 2 g/100 ml SDS, 10% (v/v) glycerol, 0.1 g/100 ml Bromophenolblue], separated on 10% SDS-PAGE and transferred to a PVDF membrane for blotting (Trans-Blot® Semi-Dry Transfer Cell, Biorad). Membranes were incubated for 1 h at room temperature with a blocking buffer (TBS, 0.05% Tween-20 and 5% BSA). After blocking, membranes were incubated overnight at 4 °C with Rabbit anti-pCREB antibody or rabbit anti-β-actin antibody (Santa Cruz Biotechnology) diluted 1:200 in TBS-T containing 2% BSA. The membranes were washed four times for 5 min in TBS, 0.05% Tween-20 before a 1 h incubation in a buffer (TBS, 0.05% Tween-20 and 2% BSA) containing horseradish peroxidase-linked anti-rabbit IgG (Santa Cruz Biotechnology) at 1:4000 dilution. The membranes were washed four times and specific protein bands were detected with chemiluminescence (ECL, Santa Cruz, Milan-Italy) using C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). Western blots were analyzed using Image Studio Software to determine optical densities (OD) of the bands. OD readings were normalized to β-actin values to account for variations in loading.

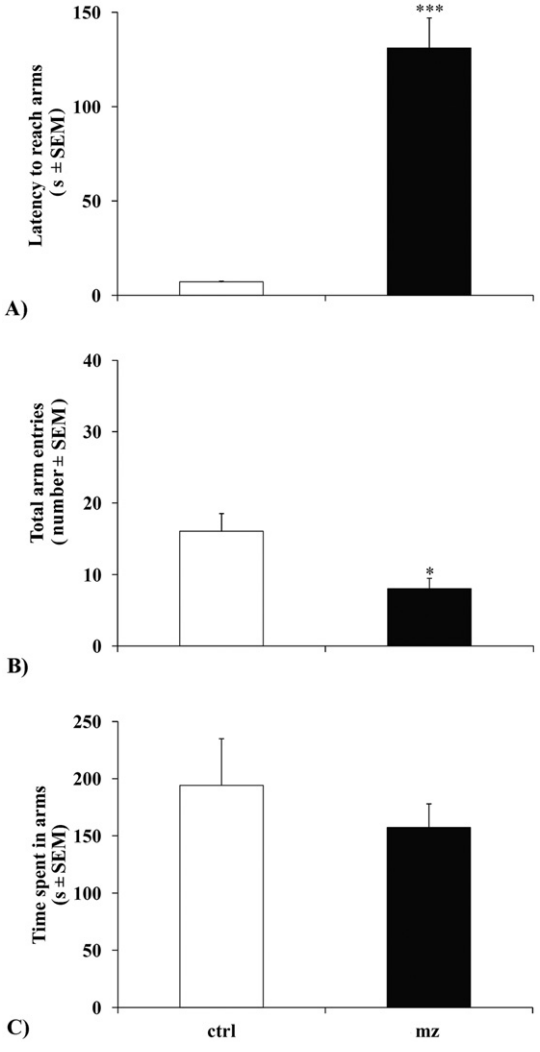



**Fig. 1.** The effects of mz on spontaneous exploration of *Thalassoma pavo* in a T-shaped apparatus. Latency time to reach arms (s ± SEM; A), total arm entries (number ± SEM; B) and time spent in arms (s ± SEM; C) were tested during a 6 min behavioral session. The above behavioral activities were evaluated in fish exposed to mz (0.2 mg/l) for 96 h (n = 15) and compared to controls (ctrl; n = 15) by using unpaired Student's *t*-test. $^{*}p < 0.05$, $^{***}p < 0.001$.

### 2.6. Transcriptional analysis

To evaluate the influence of mz on brain HSP90 transcription, an in situ hybridization analysis was performed by using a specific antisense oligonucleotide DNA probe. Such a probe of 43 nucleotides was 5′-CACAAAGAGGGTGATGGGGTATCCGATGAACTGAGAGTGCTTT-3′ previously designed (Giusi et al., 2008) on the basis of HSP90 partial nucleotide sequence of *Thalassoma pavo* (GenBank cod. EF392848) labeled at the 3′-tailing with digoxigenin-11-dUTP (DIG, Roche Diagnostics, Monza-Italy) and compared to a sense oligonucleotide probe. With this intention, fish exposed for 96 h with mz (n = 5) and controls (n = 5) were sacrificed as described above. Subsequently, brains were rapidly removed, stored at −40 °C and mounted on a cryostat freezing stage (Microm-HM505E; Zeiss) to obtain a series of coronal sections (14 μm) that were incubated with a 100 ng of HSP90 antisense probe overnight at 50 °C in a humidified chamber as described in another study (Facciolo et al., 2012). Immunological detection using an anti-digoxigenin antibody (1:100) was obtained as previously reported (Zizza et al., 2014). Hybridization signals (expressed as OD ± SEM), observed at a bright-field Dialux EB 20 microscope (Leitz) were determined in duplicates on each brain antimere of 6 brain sections for anterior plus posterior brain slides. Expression levels of HSP90 mRNA were obtained by using an Image Software of the National Institutes of Health (Scion Image 2.0), in which an internal standard was used for OD calibration. Background level was estimated and included in all final calculations. The different encephalic nuclei were identified using perciformes atlases (Cerdá-Reverter et al., 2001a,b, 2008).

### 2.7. Statistical analysis

Statistical differences between mz-exposed fish with respect to controls were evaluated for all experimental data by using an unpaired Student *t*-test with a significant level of $p < 0.05$. The determination of number of animals of the present study was carried out using a free online statistical program (http://stat.ubc.ca/~rollin/stats/ssize/n2.html; Department of Statistics of the University of British Columbia-Canada) in which a 95% power corresponded to a sample size of at least 5 and 15 individuals for molecular/neurodegenerative and behavioral analyses, respectively, when a 2-sided 5% level of significance was used.

## 3. Results

### 3.1. Effects of mancozeb on behavior

*3.1.1. Spontaneous exploration test.* Fish treated with mz exhibited significantly differentiated exploration activities as indicated by their very strong ($p < 0.001$) increased latency to reach arms (>1000%) with respect to controls that instead demonstrated immediate and rapid movements from the start compartment (Fig. 1A). Contextually, exploratory attitudes in mz-exposed fish diminished as pointed out by a moderate reduction of total arm entries (−50%; $p < 0.05$) while controls displayed a more consistent number of arm entries (Fig. 1B). Conversely, the total time spent in arms did not significantly change with respect to un-treated animals (Fig. 1C).

*3.1.2. Cylinder test.* During the cylinder test, neither mz-exposed fish nor controls exhibited any type of transition toward the upper portion of the apparatus (Fig. 2A). Indeed, the two experimental groups spent the entire time at the bottom of the tank without reaching the upper zone. However, it was worthy to note that fish exposed to mz spent a conspicuous part of the test along the walls of the cylinder as demonstrated by the strong increase ($p < 0.01$; +76%) of such an endpoint with respect to controls (Fig. 2B). Accordingly, an evident enhancement (+111%) of immobility was observed after mz exposure with respect to controls (Fig. 2C). At the same time, the fungicide induced a moderate reduction (−56%) of motor activity compared to untreated fish (Fig. 2D).

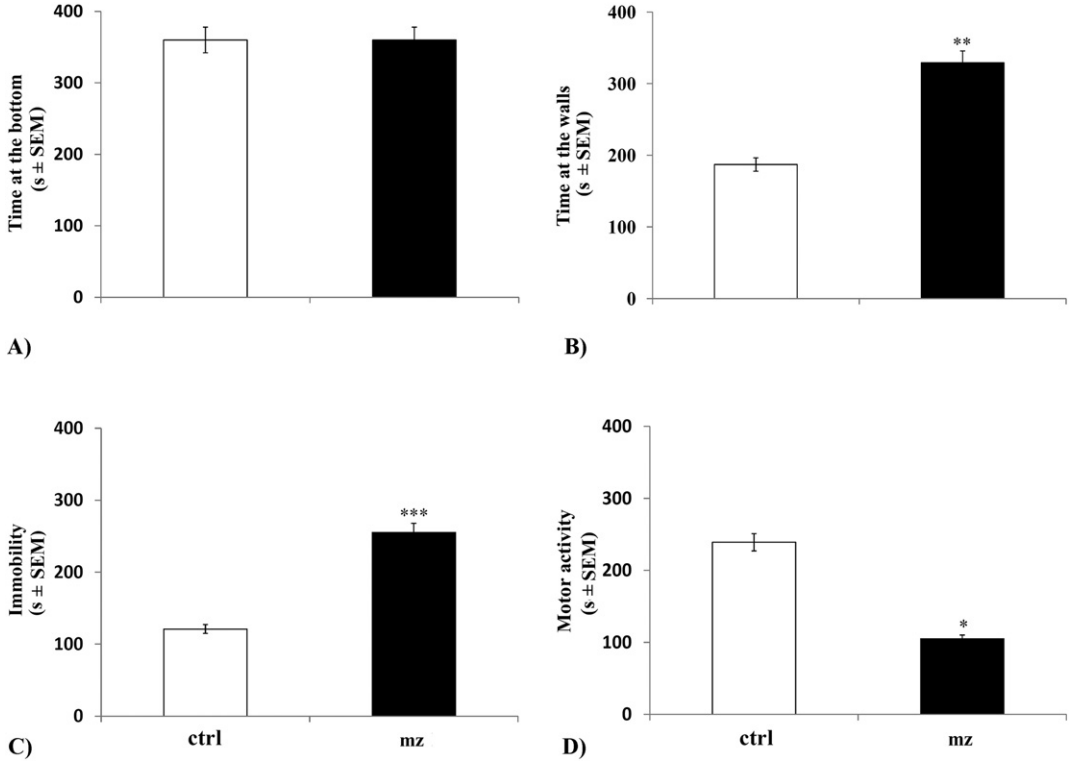



**Fig. 2.** The effects of mz during a cylinder test in the ornate wrasses. Data were expressed as total time spent (s ± SEM) at the bottom (A), along the cylinder wall (B), in an immobility state (C) and during motor activity (D) by fish exposed to 0.2 mg/l of mz (n = 15) with respect to controls (ctrl; n = 15) for a 6 min behavioral session in a cylinder tank. Statistical differences were evaluated by using unpaired Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

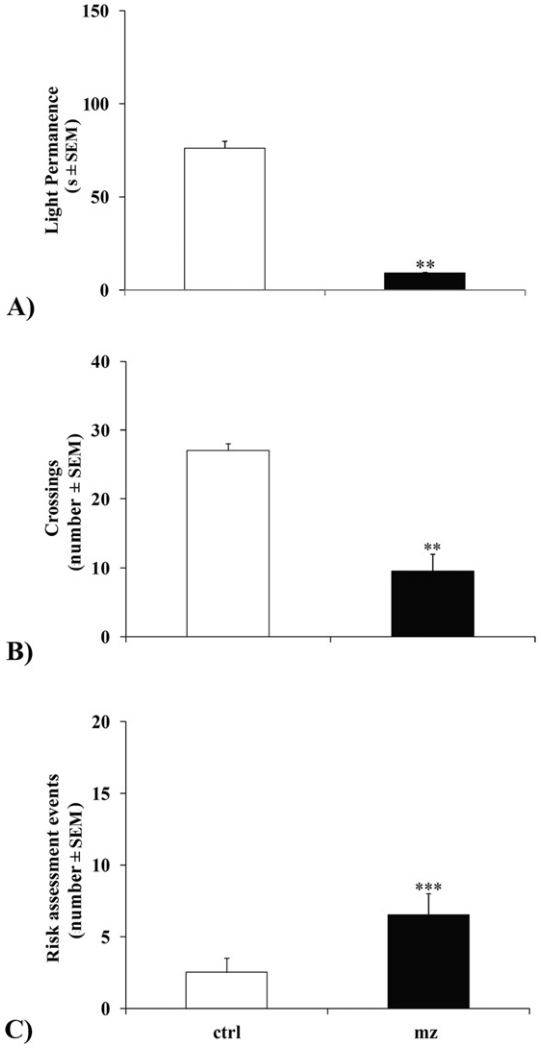



**Fig. 3.** The effects of mz on anxiety-like behaviors of *Thalassoma pavo*. Light permanence (s ± SEM; A), the number of crossings (number ± SEM; B) and of risk assessment events (number ± SEM; C) were tested during a 6 min behavioral session in a Light/Dark apparatus. The above behavioral activities were evaluated in fish exposed to mz (0.2 mg/l) for 96 h (n = 15) and compared to controls (ctrl; n = 15) by using unpaired Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

*3.1.3. Light/dark preference test.* Light/dark test highlighted an anxiety-like behavior in fish exposed to the pesticide. Indeed, treated fish displayed a strong (−88%) reduction of permanence in the light zone of the apparatus compared to controls (Fig. 3A). Moreover, the number of crossings strongly diminished (−65%) in fish exposed to mz thus indicating reduced explorative attitudes with respect to controls (Fig. 3B). Conversely, a very strong enhancement (160%) of the number of risk assessment events occurred in the pesticide-treated group versus controls (Fig. 3C).

### 3.2. Neurodegenerative effects of mancozeb

The behavioral impairments observed in treated fish coincided with several damaged cells displaying heavily stained granules within the numerous neuronal fields following exposure to mz as revealed by ACS analysis of the anterior and medio-posterior areas (check schemes). The argyrophilic reaction was evident in the lateral part of the dorsal telencephalon (Dl; Fig. 4A), in the medial part of the dorsal telencephalon (Dm; Fig. 4B) and in the ventral telencephalon (VTel; Fig. 4C) of mz-exposed specimens with respect to few rare dark granules of controls as shown in the respective brain areas (Fig. 4A′–C′). As far as diencephalon was concerned, the diffuse nucleus of the inferior lobe (NDLI; Fig. 5A) also resulted to be heavily damaged. Similarly, an evident argyrophilic signal was reported in the different cellular layers of the optic tectum (OT; Fig. 5B), in the valvula of the cerebellum (VCe; Fig. 5C) and in the superior reticular nucleus (RS Fig. 5D) compared with the respective brain areas (Fig. 5A′–D′) of controls.

### 3.3. Neuromolecular effects of mancozeb

From a molecular point of view, a notable activation of the transcription factor pCREB occurred during mz-exposure. Indeed, OD levels of pCREB band (Fig. 6A), which were normalized by using values deriving from densitometric evaluation of β-actin bands (Fig. 6B), were very significantly ($p < 0.005$) enhanced in treated fish rather than the respective pCREB band of controls. At the same time, in situ hybridization revealed an activation of HSP90 transcriptional responses as displayed by very dense antisense signals in the representative brain section of exposed-fish (Fig. 7A) and controls (Fig. 7B) compared with sense signals (Fig. 7C). In particular, a notable increase of mRNA levels was found in Dl (+68%), whereas in other telencephalic fields such as Dm (+30%), the ventral part of the ventral telencephalon (Vv; +33%) and the lateral part of ventral telencephalon (Vl; +41%), only moderate transcript enhancements were observed (Fig. 7D). Similarly, moderate HSP90 up-regulation events occurred in some extra-telencephalic sites such as NG (+30%), OT (+30%), VCe (+35%), the trochlear nerve nucleus (nIV; + 31%) and RS (+30%).

## 4. Discussion

This work provided first evidences about behavioral and neuronal effects of the dithiocarbamate mz in a marine fish, in which the molecular elements pCREB and HSP90 constitute crucial factors for the activation of neuroprotective measures against pesticides. At the behavioral level, motor performances were notably impaired by mz since spontaneous exploration activity revealed significant alterations of almost all parameters and namely the latency to reach arms or total arm entries. Such explorative alterations occurred together with other motor deficits, which included increase of fish immobility and reduction of time spent moving when fish were tested in the cylinder apparatus. Similarly, these behavioral disturbances were also detected in *Caenorhabditis elegans* after exposure to mz as suggested by this fungicide disrupting its swim to crawl locomotor transition (Harrison Brody et al., 2013), which may very likely be linked to mitochondrial dysfunctions and increased production of ROS (Todt et al., 2016). In addition, motor impairments related to an altered synaptic transmission in the developing cerebellar cortex were also observed in mice prenatally exposed to mz (Miranda-Contreras et al., 2005). In line with the above findings, recent works have reported the ability of Maneb to account for failure of motor activity and motor coordination in rats (Tinakoua et al., 2015) thus suggesting such behaviors as a major target of Mn-containing dithiocarbamates. Among the behavioral difficulties reported in the present study, it was interesting to note the onset of anxiety-like behaviors in mz-treated wrasses. Indeed, the Light/Dark test revealed not only a great reduction of the permanence in the light compartment of the apparatus but also an evident enhancement of risk assessments, which corroborate an additional element of anxiogenic performances as previously indicated in fish exposed to copper and mercury (Maximino et al., 2011; Zizza et al., 2016).

It is known that exposure of fish to pesticides is often related to severe behavioral deficits (Bonansea et al., 2016) deriving in many cases from damages of specific brain areas (Pereira and de Campos Júnior, 2015). Even for this fungicide, its elevated toxic potentiality seems to be directed at the neuronal level especially since Mn and Zn, which are part of the molecular complex, are neurotoxic themselves (Apaydin et al., 2016; Eom et al., 2016). Indeed, the behavioral

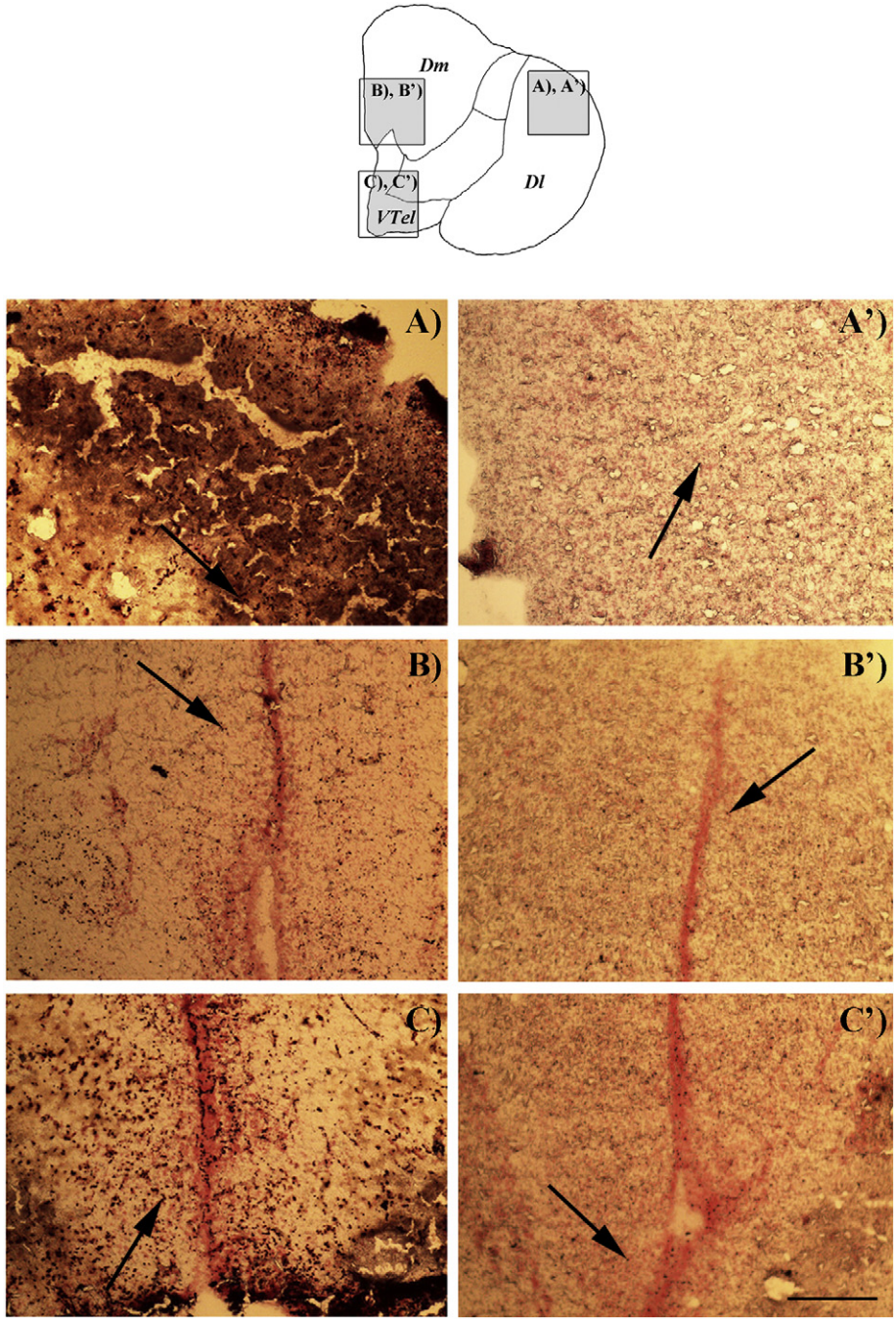
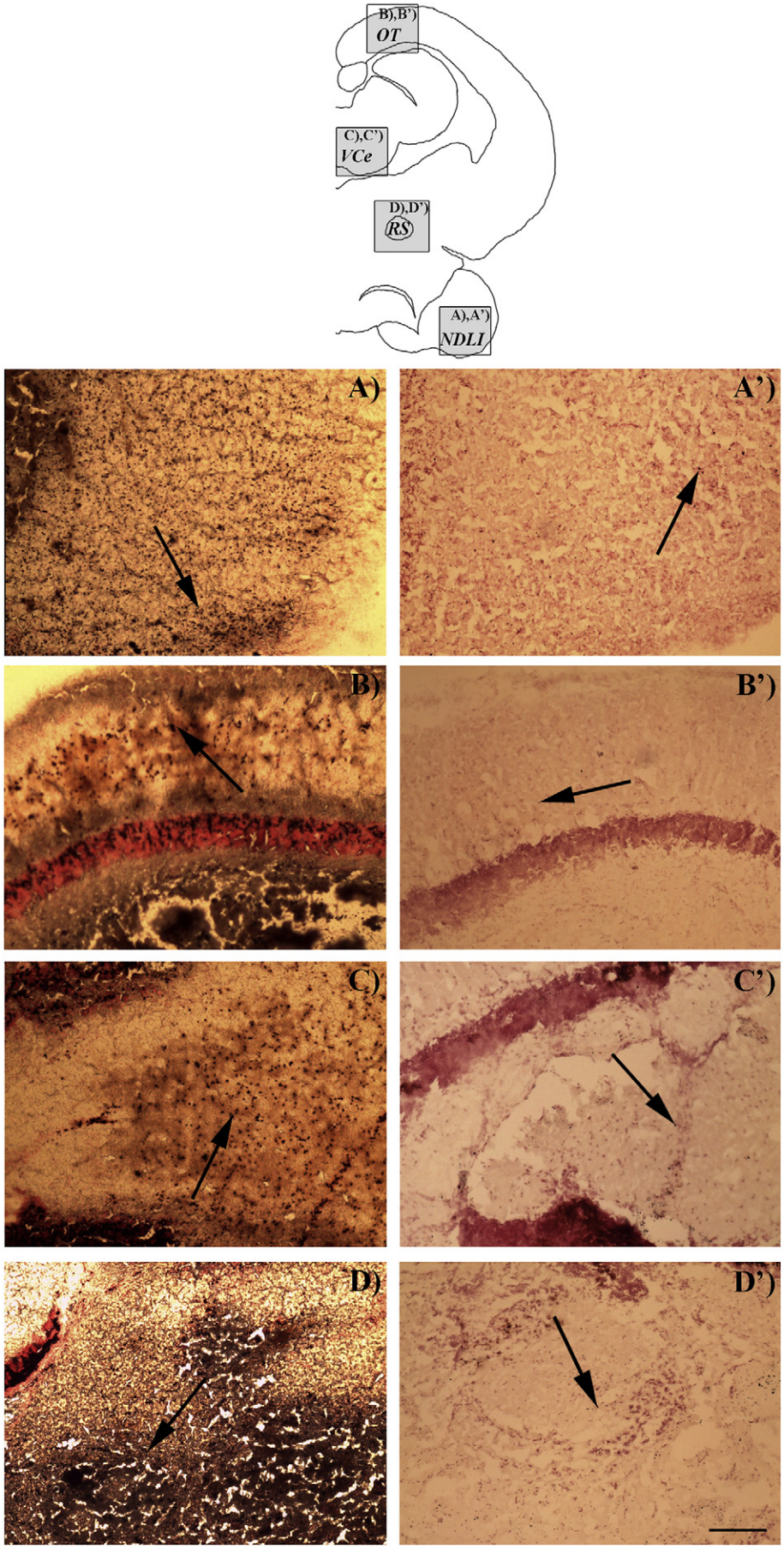



**Fig. 4.** Neurodegeneration in the telencephalic areas of *Thalassoma pavo* following mz exposure. ACS photomicrographs (and the relative scheme) of some brain sections of fish exposed to 0.2 mg/l of mz (n = 5) displayed argyrophilic signals (arrows) consisting of dark granules in Dl (A), Dm (B), VTel (C) with respect to only few granules observed in the respective areas (A′, B′ C′) of controls (n = 5). Scale bar: 100 μm.

alterations of our wrasses tend to preferentially point to encephalic damages in motor-related regions such as telencephalon, OT, cerebellum and RS as shown by their intense argyrophilic reaction after mz exposure. In particular, the latter nucleus belonging to the reticular formation is the largest source of descending signals to the spinal cord that are involved in initiation and directional control over the fast escape behavior in teleosts (Babin et al., 2014). Following this line, it has been also demonstrated that motor disturbances may be due to degeneration of certain categories of cells such as the astrocytes of the nigro-striatal circuit known to actively control motor functions (Tatsumi et al., 2016). In this context, it may very well be that astrocytes play a major role during toxic reactions especially if we consider their protective role against neurotoxicants via a notable release of ATP in age-related neurodegeneration (Kubik and Philbert, 2015). Furthermore, it appears that mz and other Mn-containing dithiocarbamates (Maneb) may induce additive toxic effects on enhanced nuclear factor

**Fig. 5.** Neurodegeneration in the medio-posterior brain areas of *Thalassoma pavo* following mz exposure. ACS photomicrographs (and the relative scheme) of some brain sections of fish exposed to 0.2 mg/l of mz (n = 5) displayed argyrophilic signals (arrows) consisting of dark granules in NDLI (A), OT (B), VCe (C) and RS (D) with respect to only few granules observed in the respective areas (A′, B′, C′, D′) of controls (n = 5). Scale bar: 100 μm.


B),B')
OT
C),C')
VCe
D),D')
RS
A),A')
NDLI
A)
A')
B)
B')
C)
C')
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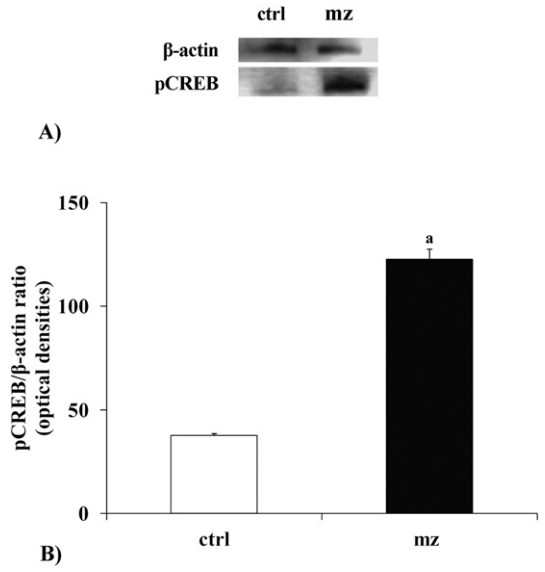


**Fig. 6.** The effects of mz on pCREB expression in *Thalassoma pavo* brain. Data were reported as optical densities of pCREB bands that were normalized by using β-actin values (A) in fish exposed to 0.2 mg/l of mz (n = 5) and compared to controls (ctrl; n = 5; B). Statistical differences were evaluated by using unpaired Student *t*-test. $^{a}p < 0.005$.

(NF-κB)-dependent dopaminergic cell damages triggered by sub-toxic doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPP(+), an active metabolite of the Parkinsonian mimetic MPTP (Williams et al., 2013).

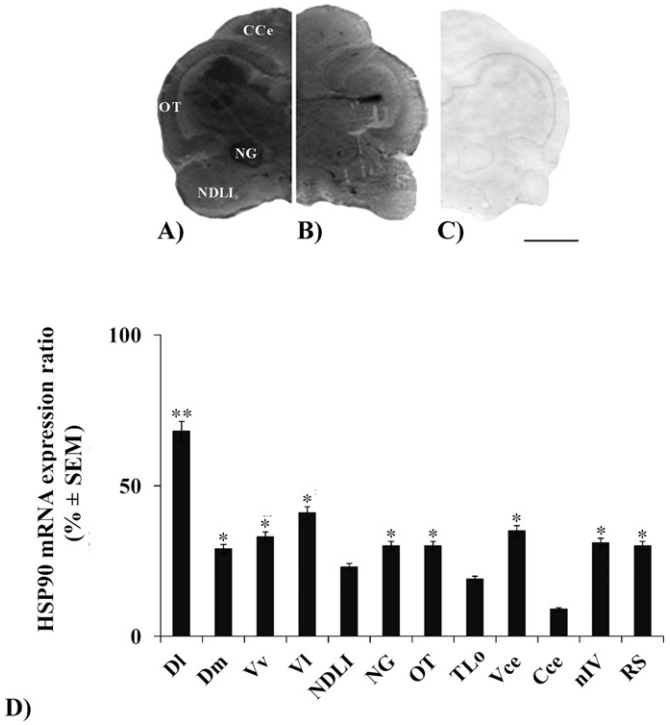


**Fig. 7.** HSP90 transcriptional changes in some encephalic nuclei of *Thalassoma pavo* following exposure to mz. Representative medio-posterior brain sections labeled with 100 ng of HSP90 antisense probes of treated (A) and control (B) fish were compared to the sense probe (C). Scale bar: 1.25 mm. Data were reported as expression ratio (% ± SEM) of HSP90 mRNAs in some brain sites of fish exposed to 0.2 mg/l of mz for 96 h (n = 5) with respect to controls (ctrl; n = 5). Statistical differences were evaluated by using unpaired Student *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$. Abbreviations: CCe, corpus of the cerebellum; Dl, lateral part of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; NDLI, diffuse nucleus of the inferior lobe; NG, nucleus glomerulosus; nIV, trochlear nerve nucleus; OT, optic tectum; RS, superior reticular nucleus; TLo, torus longitudinalis; VCe, valvula of the cerebellum; Vl, lateral part of the ventral telencephalon; Vv, ventral part of the ventral telencephalon.

It was noteworthy that a marked activation of pCREB was reported during exposure to this fungicide, which may underlie a neuroprotective role in the fish brain exposed to contaminants. Such a feature tends to go in a similar direction of the abundant expression of this transcription factor being responsible for the activation of synaptic plasticity together with neuronal survival (Kitagawa et al., 2012). Moreover, and of greater importance is that its activation following the phosphorylation of serine 133 induces gene expression of survival factors thus yielding neurons resistant to subsequent severe ischemia. Recently, increased expression levels of such a protein have been associated with enhanced hippocampal neurogenesis induced by environmental enrichment in adult rats (Zhang et al., 2016). In the case of fish, the abundance of pCREB in all known neurogenic regions seems to be responsible for the triggering of cell proliferation and modulation of embryonic brain development as reported for zebrafish (Dworkin et al., 2007). It is thus tempting to speculate that a conspicuous presence of pCREB in mz-exposed wrasses, by stimulating neurogenesis, assures tolerance against toxic conditions along with neuroprotective ability toward the repairing of brain damages.

In a same manner, it is plausible that the up-regulation of HSP90 mRNA, detected in many encephalic nuclei after mz-exposure, may constitute a part of the pro-survival program activated by pCREB. Such a feature is strongly supported on the one hand by HSP90/Akt/CREB pathways upregulating the glial cell line-derived neurotrophic factor, a protein used for the treatment of neurodegenerative disorders such as Parkinson's disease (Cen et al., 2006) and on the other hand by CREB-dependent transactivation of HSP70.3 above all during heat-shock/ischemia-like conditions (Sasi et al., 2014). This should not be so surprising since studies confirm that high levels of HSPs are involved with protective mechanisms against different stressful conditions (Mahanty et al., 2017), including hypoxia (Giusi et al., 2012). In addition, our recent findings demonstrated that high HSP90 mRNAs are precocious elements activating protective events in the brain of both marine and freshwater fish especially after a recovery period from exposure to some heavy metals like copper (Zizza et al., 2014, 2016). At the same time, it has been reported that pesticides are also responsible for the activation of HSP90 in other teleosts (Peng et al., 2015; Xing et al., 2015) thus proposing this chaperone as a crucial biomarker of environmental contamination.

Taken together, these first results on mz-dependent motor deficits and anxiety-like states of a marine fish point to interesting molecular and behavioral responses adopted by wrasses during toxic conditions. In particular, the contemporary activation of pCREB and HSP90 during behavioral alterations strengthen them as early indicators of aquatic contamination that may assure cellular tolerance with eventual repairing of brain damages following exposure to mz. Hence, the present study would surely benefit from evidences on cross-talking ability of the above molecular factors with some neuropeptidergic systems, which will be considered in a future work (MS in preparation). This type of condition may be achieved by their interactions with key neuropeptides like orexin (ORX) as previously demonstrated (Sokołowska et al., 2014), very likely in concert with other neuroreceptor circuits (Facciolo et al., 2011; Crudo et al., 2013). The fact that the ORXergic system may exert a key role on encephalic neurogenic events in fish during pesticide toxicity is turning out to be of major concern given that ORXs play a determinant role during stressful responses against metal contamination (Zizza et al., 2011, 2014, 2017). We are still at the beginning but our results plus recent findings on a genetic hazard to fish contaminated by mz may encourage biomonitoring programs of aquatic ecosystems and regulatory policies regarding the utilization of this agrochemical (Marques et al., 2016).

## Transparency document

The Transparency document associated to this article can be found, in the online version.

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