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Unveiling the neurotoxicity of methylmercury in fish (*Diplodus sargus*) through a regional morphometric analysis of brain and swimming behavior assessment

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ABSTRACT

The current study aims to shed light on the neurotoxicity of MeHg in fish (white seabream – *Diplodus sargus*) by the combined assessment of: (i) MeHg toxicokinetics in the brain, (ii) brain morphometry (volume and number of neurons plus glial cells in specific brain regions) and (iii) fish swimming behavior (endpoints associated with the motor performance and the fear/anxiety-like status). Fish were surveyed for all the components after 7 (E7) and 14 (E14) days of dietary exposure to MeHg (8.7 $\mu g\,g^{-1}$), as well as after a post-exposure period of 28 days (PE28). MeHg was accumulated in the brain of *D. sargus* after a short time (E7) and reached a maximum at the end of the exposure period (E14), suggesting an efficient transport of this toxicant into fish brain. Divalent inorganic Hg was also detected in fish brain along the experiment (indicating demethylation reactions), although levels were 100–200 times lower than MeHg, which pinpoints the organic counterpart as the great liable for the recorded effects. In this regard, a decreased number of cells in medial pallium and optic tectum, as well as an increased hypothalamic volume, occurred at E7. Such morphometric alterations were followed by an impairment of fish motor condition as evidenced by a decrease in the total swimming time, while the fear/anxiety-like status was not altered. Moreover, at E14 fish swam a greater distance, although no morphometric alterations were found in any of the brain areas, probably due to compensatory mechanisms. Additionally, although MeHg decreased almost two-fold in the brain during post-exposure, the levels were still high and led to a loss of cells in the optic tectum at PE28. This is an interesting result that highlights the optic tectum as particularly vulnerable to MeHg exposure in fish. Despite the morphometric alterations reported in the optic tectum at PE28, no significant changes were found in fish behavior. Globally, the effects of MeHg followed a multiphasic profile, where homeostatic mechanisms prevented circumstantial morphometric alterations in the brain and behavioral shifts. Although it has become clear the complexity of matching brain morphometric changes and behavioral shifts, motor-related alterations induced by MeHg seem to depend on a combination of disruptions in different brain regions.

1. Introduction

Methylmercury (MeHg) is known to have devastating effects on the mammalian nervous system (Harada, 1995; Ceccatelli et al., 2010; Farina et al., 2011; Syversen and Kaur, 2012). In contrast, there are only a few studies investigating MeHg neurotoxicity in

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fish (Berntssen et al., 2003; Branco et al., 2011; Mieiro et al., 2011; Korbas et al., 2012; Pereira et al., 2013), being most of them a simple report of the toxicant presence in the brain. Korbas et al. (2012) went further into the neurotoxicity of MeHg by mapping the toxicant distribution in zebrafish brain. They found that MeHg accumulated in the diencephalon of zebrafish with progressively higher levels with increasing distance from the ventricle (Korbas et al., 2012). While many publications have focused on MeHg neurotoxicity research in zebrafish (Gonzalez et al., 2005; Smith et al., 2010; Cambier et al., 2012; Gentès et al., 2015), non-model species have received much less attention and this is a major gap in the published research since MeHg is widely distributed in aquatic ecosystems, it reaches high levels in contaminated areas (Canário et al., 2005) and is biomagnified through aquatic food webs (Ceccatelli et al., 2010). Therefore, fish are highly vulnerable to MeHg, since it can accumulate in their brain, leading to probable neurological alterations and motor dysfunctions. In order to provide new insights for the protection of ichthyo-fauna and for the preservation of ecosystem health, studies are needed to fill the existing knowledge gap on the neurotoxicity of MeHg in non-model fish species.

Interestingly, divalent inorganic Hg (iHg) may occur in fish brain upon dietary MeHg exposure, as observed in the human brain (Korbas et al., 2010). As such, both Hg forms could be potential neurotoxicants in fish exposed to a MeHg contaminated diet, but this hypothesis still needs confirmation. It has been shown that MeHg is demethylated to iHg in the intestines of mice (Rowland et al., 1984) and this form can thereafter be transported into the brain. Additionally, there is some evidence of MeHg demethylation in the brain of mammals (Vahter et al., 1995; Korbas et al., 2010). Although identical processes may be inferred based on iHg occurrence in fish brain upon exposure to dietary MeHg, this aspect remains poorly understood.

The neurotoxicity of MeHg in fish has been disclosed by a very limited number of biological effects in the brain. For instance, Mieiro et al. (2011) reported changes in the antioxidant system of wild fish from a Hg contaminated area, while Berntssen et al. (2003) found that the salmon brain is particularly susceptible to dietary MeHg (in comparison with iHg), which induced lipid peroxidation at relatively low exposure levels. Indeed, after 4 months of exposure to MeHg, the salmon brain showed a severe vacuolation that differed among brain areas (*i.e.* it was worst in the medulla, followed by cerebellum, then by ventral regions of the tectum and by cerebrum and other regions) and was occasionally accompanied by necrotic cell bodies (Berntssen et al., 2003). Such evidence is in line with several studies in rodents (Nagashima, 1997) and humans (Sanfeliu et al., 2001; Ceccatelli et al., 2010) that have described the occurrence of neuronal damage upon MeHg exposure. In order to unveil the neurotoxic effects of MeHg, rodent brain has been scrutinized by stereological methods comprising an evaluation of cell numbers and volumes (Sager et al., 1984; Larsen and Brændgaard, 1995; Roegge et al., 2006; Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). This methodology has provided clarification about the localized effects of MeHg in the nervous system of rodents. Indeed, a pronounced axonal degeneration occurred in the peripheral nervous system of rats exposed to MeHg (Larsen and Brændgaard, 1995; Schiønning et al., 1998). Moreover, adverse effects in cell populations within the hippocampus and cerebellum have also been found in rodents exposed to MeHg (Sager et al., 1984; Roegge et al., 2006; Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). Unfortunately, this is a time consuming method, which has mostly been used for toxicology purposes to assess the neurotoxicity of contaminants in humans. To the best of our knowledge, there is only one study that employed stereological methods to evaluate the effects of Hg in fish, and this was performed by our research group (Pereira et al., 2016). This study pinpointed that waterborne iHg elicits a significant reduction in the number of cells in the hypothalamus, optic tectum and cerebellum of *Diplodus sargus* after as few as 7 days of exposure. In this direction, the assessment of brain morphometric alterations (by stereological methods) in specific brain regions of fish exposed to MeHg provides, for the first time, an indication of potential brain dysfunctions. Additionally, a follow-up evaluation upon the cessation of exposure can determine the occurrence of regenerative processes, as previously described for *D. sargus* (Pereira et al., 2016).

Alterations in the number of cells and volumes in brain of rats exposed to MeHg were associated with behavioral changes. The chronic intrauterine exposure to a low dose of MeHg induced a decrease in neuronal population of the limbic system (amygdala and hippocampus) of the rat's offspring, as well as a significant learning disability (Kakita et al., 2000). Similarly, in zebrafish, the developmental exposure to MeHg triggered a decrease in the telencephalon cell body density and learning deficits in adulthood (Smith et al., 2010). Salmon exposed to dietary MeHg displayed lower swimming activity together with cellular damages in the brain (Berntssen et al., 2003). As far as we know, only the study by Berntssen et al. (2003) has combined the evaluation of fish brain lesions and behavior alterations after exposure to dietary MeHg. Despite the study having the merit of an integrative approach, Berntssen et al. (2003) only considered overall swimming activity of the fish. A more complex evaluation of swimming behavior was employed, for the first time, by Pereira et al. (2016) to investigate the effects of iHg in *D. sargus*, with the demonstration of alterations related both to motor function and mood/anxiety-like status, together with the loss of brain cells in specific regions.

The current study aimed to shed light on the neurotoxicity of MeHg in white seabream (*D. sargus*) after a dietary exposure to MeHg, through the assessment of MeHg toxicokinetics in the brain, regional brain morphometry and swimming behavior. In addition, the recovery of alterations in brain morphometry and swimming performance were assessed along with MeHg depuration. For that purpose, a combined approach was designed, comprising: (i) determination of the accumulation of MeHg and iHg in the brain; (ii) stereological evaluation of the total number of cells (neurons plus glia) and volume of medial and lateral pallia, hypothalamus, optic tectum and cerebellum; (iii) assessment of fish swimming behavior through the evaluation of motor performance and potential fear/anxiety-like status. Fish were surveyed after 7 and 14 days of dietary MeHg exposure, as well as after a post-exposure period that lasted 28 days. Realistic levels of MeHg in natural contaminated food of *D. sargus* were considered ($8.7\,\mu g\,g^{-1}$) in order to produce reliable data for environmental health assessment and fish populations' preservation.

2. Material and methods

2.1. Experimental set-up and fish exposure to dietary MeHg

The white seabream *Diplodus sargus* was selected as a test organism since it is abundant in estuarine systems where Hg contamination is frequent (Pereira et al., 2009). In this context, *D. sargus* was previously employed to investigate the iHg toxicokinetics (Pereira et al., 2015) and neurotoxicity (Pereira et al., 2016). Moreover, *D. sargus* is easily maintained in the laboratory and is easy to handle, which are important traits when performing behavioral studies.

Juvenile specimens (sexually immature and with undifferentiated gender at this stage of development) were provided by the Aquaculture Research Station (IPMA – Olhão, Portugal), from the same cohort (weight: 124 ± 11 g; total length: 18 ± 0.6 cm). Fish were kept in 300 L fiberglass tapered-cylindrical tanks with an average initial density of $0.0050\,kg\,L^{-1}$, under a 14:10 light:dark

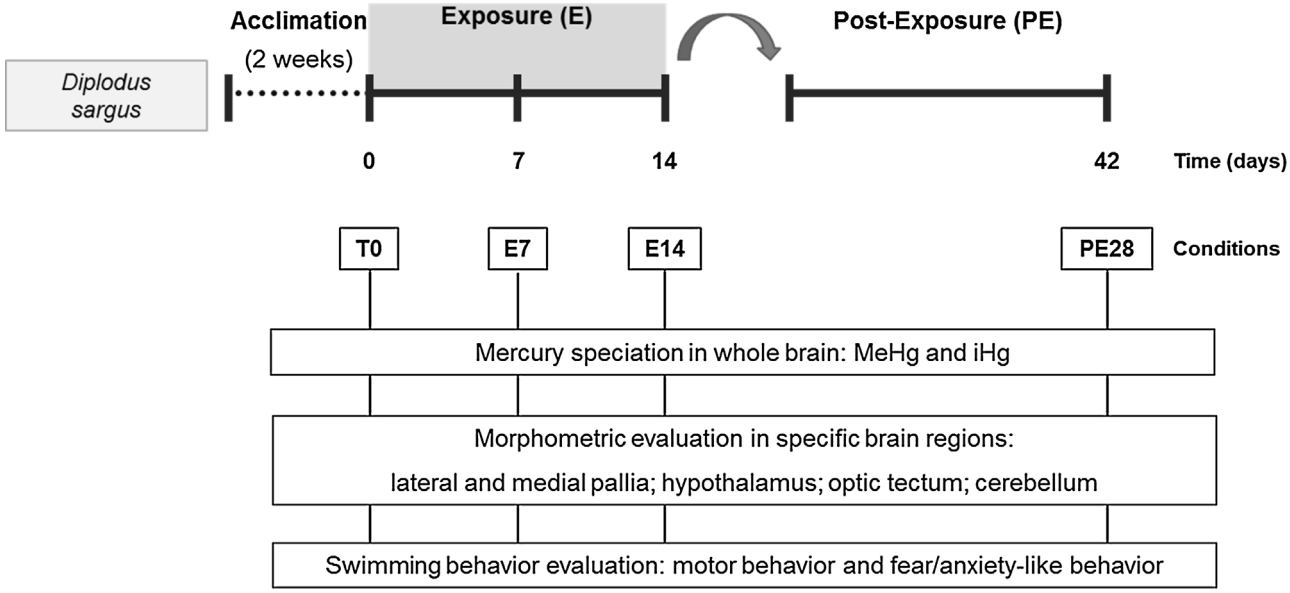

Fig. 1. Schematic overview of the experimental study design. After a two-week period of acclimation (T0), the juvenile white seabreams (*Diplodus sargus*) were fed with either MeHg spiked feed (8.7 $\mu g\,g^{-1}$) or regular feed, over a 7 (E7) and 14 (E14) day period. Exposed fish were then shifted to the regular diet and allowed to recover for 28 days (PE28).

photoperiod. A total of 12 tanks with the same characteristics were placed in the same room for the experiment (6 for control conditions, and 6 for MeHg exposure).

Contaminated feed (3 mm pellets), produced by SPAROS Company (Portugal), was used to expose fish to MeHg. A solution of MeHg chloride (Sigma-Aldrich; prepared in ethanol) was added during the process of pellet production, with homogenous distribution of toxicant throughout the batch. Uncontaminated pellets, with the same size and nutritional formulation, were also produced following an identical methodology but without addition of MeHg solution. The levels of MeHg were determined in both the uncontaminated and contaminated pellets. The MeHg levels in the uncontaminated pellets were lower than 0.01 $\mu g\,g^{-1}$ (0.05 $nmol\,g^{-1}$), while the contaminated pellets presented a mean value (±standard deviation of three replicates) of $8.7 \pm 0.5\ \mu g\,g^{-1}$ ($40 \pm 2.3\ nmol\,g^{-1}$). This MeHg concentration corresponds to that found in prey species of white seabream in a Portuguese Hg-contaminated lagoon (Pereira et al., unpublished data), which is also in agreement with levels found in benthic species from Hg contaminated areas (Locarnini and Presley, 1996). Fish were fed at a daily rate of 3% (as percentage of biomass), corresponding to 3.72 g food/day/fish and a daily intake of 32.4 μg MeHg/day/fish. In all tanks, fish were fed once a day. In addition, water temperature, salinity, dissolved oxygen and pH were monitored daily throughout the experiment, and were found to average: $17.0 \pm 2.0\ ^{\circ}C$, 35 ± 1 psu and $6.3 \pm 0.6\ mg\,L^{-1}$ (mean values in all 12 tanks ± standard deviation) for temperature, salinity, and dissolved oxygen, respectively, while pH ranged between 7.6 and 7.9.

Prior to MeHg exposure, all fish were allowed to acclimatize to experimental conditions and routines for two weeks. Twenty fish were sacrificed at the beginning of the experiment and used as the initial reference group (time zero; T0) (n = 5 for brain Hg quantifications, n = 5 for brain morphometric analyses and n = 10 for evaluation of swimming behavior) (Fig. 1). Fish were exposed to dietary MeHg for 7 (E7) and 14 (E14) days. Thereafter, fish were fed uncontaminated pellets (post-exposure) for 28 (PE28) days (Fig. 1). At each sampling time point, a total of 20 fish were sampled per condition, and these were divided into the different parameters of the study as follows: n = 5 for brain Hg quantifications; n = 5 for brain morphometric analyses; n = 10 for behavioral evaluation. The experiment ran for a total of 42 days. On the sampling days, fish were not fed in the 12 h preceding handling.

Fish well-being and all the procedures were carried out in accordance with national and international guidelines (Directive 2010/63/EU) to ensure minimal animal use and discomfort. Moreover, three of the co-authors are authorized by the competent Portuguese authorities to perform animal experiments.

2.2. Brain collection

Immediately after collection, fish were anesthetized with tricaine methanesulfonate (MS-222) for approximately 15 min (Gilderhus and Marking, 1987). After being weighed and measured, fish were sacrificed by cervical transection and properly bled, and the whole brain (rostral and caudal portions) was removed. One set of brain samples was frozen at −20 °C for MeHg and iHg determinations. Another set of brain samples was preserved for morphometric evaluation by immersion in a solution of 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for a minimum of 72 h, with no rinses.

2.3. Mercury speciation analysis in the brain

All samples were processed and analysed in the Center for Analytical Research on the Environment (CARE) at Acadia University (Nova Scotia, Canada). Freeze dried and homogenized brain samples were weighed using a Sartorius ultra balance and approximately 10 mg of sample were transferred to a 2 mL polypropylene vial for Hg speciation analysis.

MeHg and divalent mercury (Hg(II)) were quantified in brain samples using alkaline digestion, ethylation, and purge and trap gas chromatography – atomic fluorescence spectrometry (Liang et al., 1994; Edmonds et al., 2012). Quality control measures included the use of alkaline digest method blanks, sample replicates, analytical replicates, and analysis of two certified standard reference materials – Dogfish Liver Tissue (DOLT-4) and Dorsal Fish Muscle (DORM-3) – from the Canadian National Research Council. Detection limits were calculated as 3 times the standard deviation of method blanks. Mean method blanks for MeHg (n = 9; MeHg = 0.09 ± 0.069 pg) were below the calculated detec-

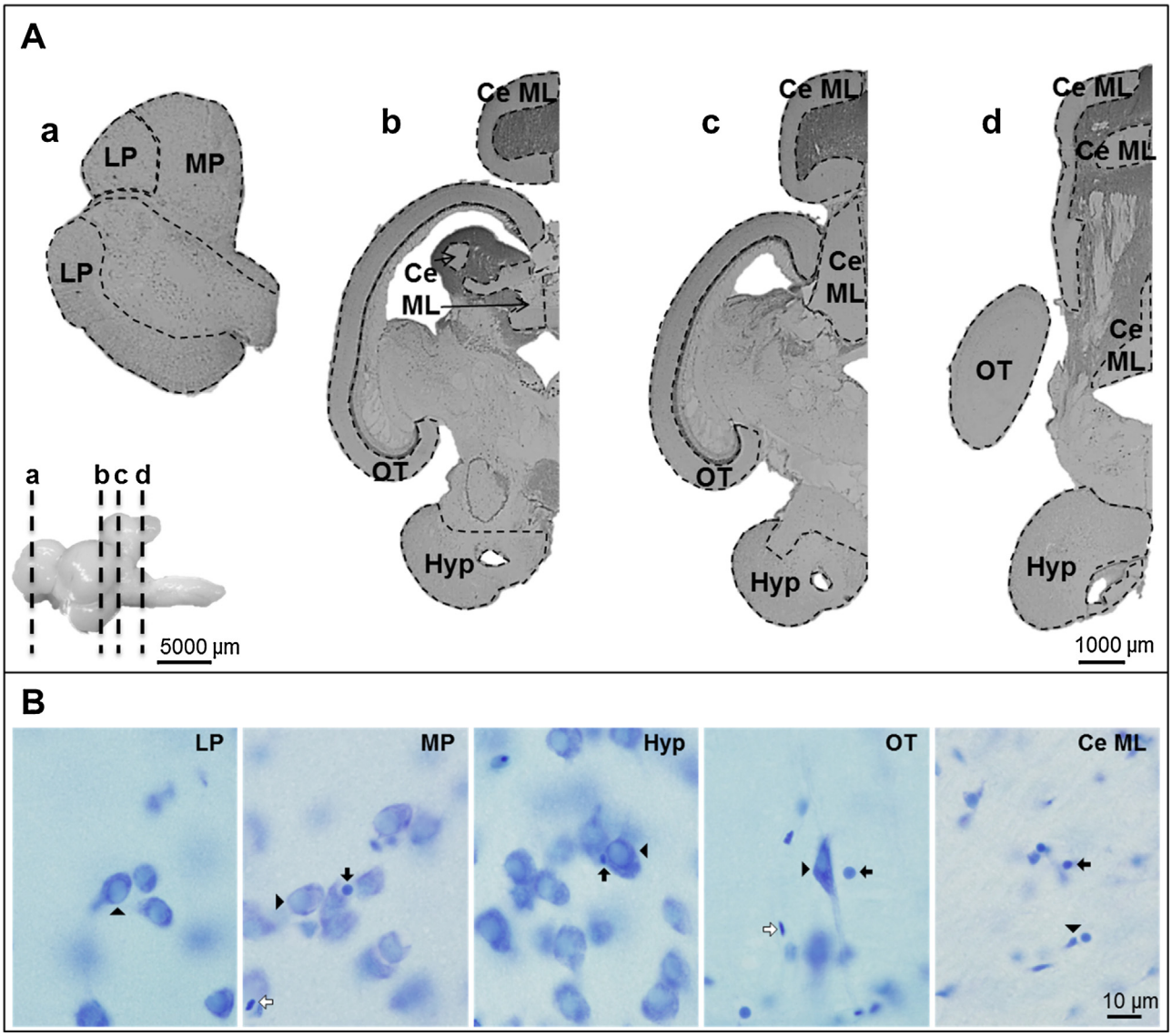

Fig. 2. Examples of the brain tissue of *D. sargus*. A macroscopic lateral view of the whole brain with approximate locations of coronal sections (a–d) are shown to illustrate the regions analysed (A). Representative high magnification photomicrographs of typical neuronal and glial cells within each brain region analysed (B). Neurons (black arrowheads) and glia (black arrows) were not distinguished during the counting procedure. Erythrocytes (white arrows) and some occasional endothelial cells, which are easily located, were excluded from the analysis. LP – lateral pallium; MP – medial pallium; Hyp – hypothalamus; OT – optic tectum; Ce ML – molecular layer of the cerebellum.

tion limit for MeHg (0.21 pg). Mean method blanks for divalent Hg (n = 9; Hg(II) = 1.26 ± 1.27 pg) were lower than the calculated detection limit for divalent Hg (3.81 pg) for many of the samples. All samples were blank corrected, by subtracting the method blank MeHg/Hg(II) concentration from each sample. Samples replicates were within accepted norms for MeHg (mean% RSD = 5.8%, n = 18) but were more variable for Hg(II) (mean% RSD = 21%, n = 18). The certified reference materials DOLT-4 and DORM-3 showed excellent recoveries for MeHg (n = 6; MeHg = 98.8 ± 4.7% and 97.4 ± 5.7% respectively) and Hg(II) in DOLT-4 (n = 6; 109.8% ± 16.8%). Hg(II) in DOLT-4 was calculated by assuming the difference between total Hg and MeHg was Hg(II). This technique has been used in previously published works (Liang et al., 1994; Edmonds et al., 2012).

2.4. Morphometric analyses of brain regions

All the histological procedures and morphological measurements were performed as described previously (Pereira et al., 2016). Briefly, the brains were dehydrated through graded concentrations of ethanol and embedded in glycolmethacrylate resin (Technovit 7100 embedding kit; Heraeus Kulzer, Wehrheim, Germany). The brain tissue was then serially cut in 30 µm coronal sections (microtome; Leica Microsystems, Nussloch, Germany), and stained with 20% Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany).

To the best of our knowledge, no brain atlas is available for *D. sargus*. Thus, for the identification of the lateral and medial pallia, hypothalamus, optic tectum and molecular cell layer of both the valvula and corpus cerebelli (Fig. 2A), which are key regions in controlling neurobehavioral functions, we used the brain atlas of the Sparidae gilthead seabream *Sparus aurata* (Muñoz-Cueto, 2001). The volume of each brain region was estimated according to the Cavalieri's principle (Gundersen and Jensen, 1987; Gundersen et al., 1988) as previously described (Pereira et al., 2016). In brief, each brain region was outlined (final magnification of 25×) and its cross-sectional area was measured in every 8th section throughout the brain, with the first section chosen randomly. The unbiased stereological estimation of total number of cells (neurons plus glia)

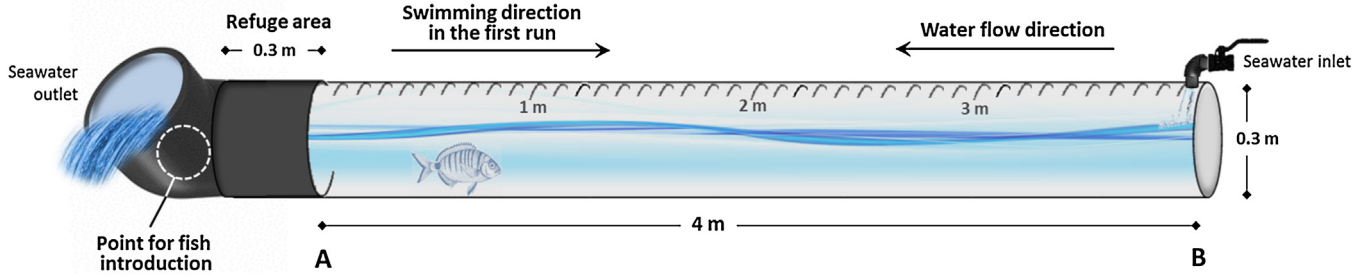

Fig. 3. Illustration of the behavior evaluation device (BED). The water flow direction, the swimming direction of fish in the first run, point for fish introduction, refuge area, as well as the tube extremes, corresponding to the seawater outlet (A) and inlet (B), are represented.

was performed in the same sections used for volume estimation, and applying the optical fractionator method (West et al., 1991; Keuker et al., 2001; West, 2013a,b; Pereira et al., 2016). The following sampling scheme was used: the dimensions of the optical disector were 30 × 30 × 20 μm, with a guard zone of 5 μm above the disector box. The used x and y step size, which define the location of the disector box, was 500 × 500 μm in lateral and medial pallia and hypothalamus, 550 × 550 μm in optic tectum and 600 × 600 μm in the molecular layer of the cerebellum. The cells were counted (at a magnification of 1000×) when the cell nuclei came into focus within the optical disector or intersected the inclusion planes without touching the exclusion planes. Typical neuronal and glial cell morphology is shown in Fig. 2B. However, during the counting procedure no distinction was made between neurons and glial cells, as previously reported (Pereira et al., 2016). The volume and cell number data for the lateral and medial pallia, hypothalamus and optic tectum correspond to the average of the right and left hemispheres, while in the molecular layer of the cerebellum the estimated values correspond to the whole region. All fish were blind coded to eliminate any bias by the experimenter.

2.5. Photomicrographs

The lateral view of a whole brain of *D. sargus* (Fig. 2A) was shot with a digital camera (Sony Handycam DCR-SR52). The representative photomicrographs of the coronal sections (Fig. 2A, a–d) were captured with a digital camera (Olympus Sc30) mounted on Olympus SZX7 zoom stereo microscope, and using the Cell-P software (Olympus, Germany). An Olympus BX61 microscope (Olympus, Germany), coupled with a digital camera (Olympus DP) and running the Cell-P software (Olympus, Germany) was used to obtain the photomicrographs of typical cell morphology (Fig. 2B) at a final magnification of 1000×.

2.6. Evaluation of fish swimming behavior

The construction of the behavior evaluation device (BED; Fig. 3) and the selection of the behavioral endpoints were based on previous works (Vieira et al., 2009; Pereira et al., 2016). The BED consists in a 4 m long polypropylene tube (transparent) with marked graduations every 10 cm, 30 cm in diameter, and a refuge area (black polypropylene tube) in the extreme A (Fig. 3). At each sampling time point, fish were caught individually and randomly, and rapidly transported into the behavioral test room in a 10 L bucket. Fish were introduced to the BED, close to the water outlet in a vertical position, with their head against the seawater flow (150 L min^{-1}), allowing them to swim against the current. For 3 min, the swimming behavior of each fish was videotaped (Sony Handycam DCR-SR52) by an experimenter placed 4 m away from the BED, for posterior visualization and analyses. Unnecessary movements or noise were avoided during the tests. Several endpoints were registered during the behavioral testing and further confirmed upon examination of the video following completion of the test. These endpoints were divided in two major sets: (i) those concerned mainly with motor skills, and (ii) those evaluating fine aspects related with potential fear/anxiety-like behavior of fish, as defined in Pereira et al. (2016).

i) Motor skills:

- First run distance (m) – distance that each fish swims (from point A to B; see Fig. 3) against the water flow on their first run (until being immobilized or dragged for the first time by the water flow);
- First run time (s) – time spent on the first run;
- First run velocity (m/s) – calculated by dividing the first run distance (m) by the respective run time (s);
- Swimming frequency – number of times that fish swam actively (at least 10 cm) during the 3 min of the test, independent of the swimming direction;
- Total swimming distance (m) – total distance swam during the test (3 min), obtained by summing all runs, independent of the swimming direction;
- Total swimming time (s) – time spent swimming actively (displacement ≠ 0) during the test.

• Fear/anxiety-like behavior:

- Refuge latency (s) – time elapsed until fish hide in the refuge area;
- Immobility latency (s) – time spent swimming until immobilization for at least 3 s;
- Dragging latency (s) – period of time swimming until being dragged towards the dragging zone, which was considered to be the half of the tube farthest from the water inflow (in the latter 2 m towards the extreme A of the tube; see Fig. 3).

The white seabream has a vigorous swimming activity that allows it to swim against a strong water flow; this makes the chosen fish species suitable for evaluation of swimming activity with the designed BED (Pereira et al., 2016). As a result, it is likely that some endpoints are more appropriate as measures of the strength capacity and indicators of gross motor alterations. Additionally, novelty, in which an animal is confronted with an unfamiliar object or environment – as putting the fish in the BED device without a habituation period – has been proposed as one of the categories of fear-inducing stimuli for animals, including fish (Maximino et al., 2012). Numerous novelty-based paradigms have been used as experimental approaches to assess fish anxiety-like behaviors, and have been adapted from rodents and applied to teleost fish showing striking similarities (Egan et al., 2009; Cachat et al., 2010; Maximino et al., 2012; Stewart et al., 2012; Ibrahim et al., 2014). Furthermore, increasing evidence indicates that the

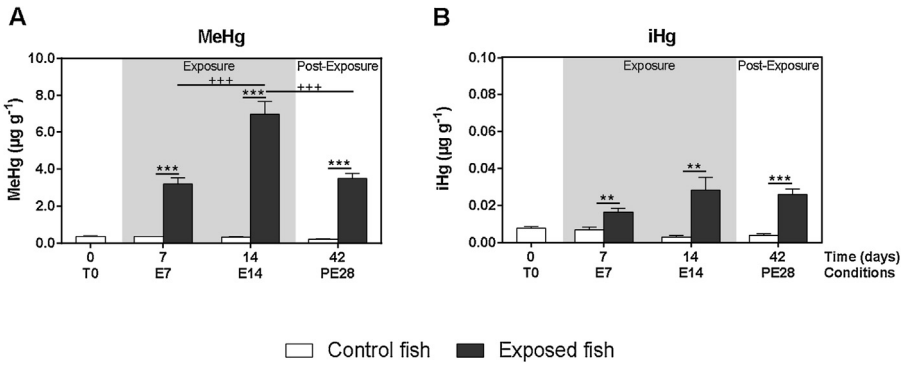

Fig. 4. Hg speciation in the brain tissue of *D. sargus*. The levels of MeHg (A) and iHg (B) in the whole brain were measured before exposure (T0), as well as in white seabreams fed with MeHg spiked feed and in control fish, during the exposure period (E7 and E14–shaded area), and in the post-exposure period (PE28). For each experimental time, the significant differences between the MeHg-exposed and the control groups are indicated by ** ($p < 0.01$) and *** ($p < 0.001$). Differences along time are indicated by +++ ($p < 0.001$). Data are expressed as mean ± SEM.

anxiety-like behavior of the teleost zebrafish, and most likely also in *D. sargus*, is driven by similar environmental factors as that of rodents, and involves evolutionarily conserved circuits that regulate aversive learning (Stewart et al., 2012). However, novelty is not the sole dimension controlling behavior in the BED, as we included in the apparatus design a more aversive region (lighted portion of the BED) and a less aversive region (dark extremity that can be used as a refuge/shelter area). Several fish species were shown to demonstrate a marked preference for the dark compartment in the dark/light preference (scototaxic testing) protocol (Maximino et al., 2010). The white seabream has an increased propensity for sheltering in novel environments (D'Anna et al., 2012; Näslund and Johnsson, 2014), thus a reduced refuge latency can be an expression of defensive behavior, a feature of anxiety (Maximino et al., 2010). Moreover, as several of the behavioral paradigms used in fish are conceptually similar to the rodent tests, and while the behavioral response of rodents to fear/anxiety has been extensively documented, the research with fish is mostly limited to the zebrafish. Taking into consideration the studies performed in both rodents and zebrafish, and as part of an exploratory and pioneer analysis of fish behavior made by our group in the context of Hg neurotoxicity in fish (Pereira et al., 2016), the division of the behavioral endpoints in our work was made broadly into behaviors that can be allocated to motor capacity and those that may represent fear/anxiety-like aspects.

It should be noted that fish sometimes do not attain all endpoints. For example, if the fish swims continuously until refuge in the dark area of the BED, the dragging, immobility and refuge latency are the same for statistical purposes. On the contrary, if the fish does not take refuge or does not stop swimming, the immobility, dragging and refuge latency are considered to be the time limit of the test (3 min).

All the tests were performed between 12:00 h and 16:00 h, since the white seabream has been described as a diurnally active fish, influenced by light intensity (Figueiredo et al., 2005; Abecasis et al., 2013). After testing, fish were removed carefully from the BED and transferred to a discard tank. Discarded animals were not used in any other evaluation (accumulation of Hg species, stereological or behavioral).

2.7. Data analysis

All the statistical analyses were performed using the GraphPad software (GraphPad Prism version 6.01 for Windows, GraphPad Software). The normal distribution of all variables was assessed by observation of the absolute values of skewness and kurtosis, as West and collaborators proposed a reference of substantial departure from normality as an absolute skew value > 2 and an absolute kurtosis > 7 (West et al., 1995). If the data fell within these cut-off values, an unpaired Student's *t*-test with a significance level of 0.05 (two-tailed) was employed to assess the differences between control and exposed fish at each sampling time. For data that did not fall within the cut-off range, a nonparametric Mann-Whitney *U*-test (two-sided, unpaired) was used. Additionally, the effect of time on the accumulation of Hg species within the experimental groups (exposed or control) was tested using a one-way ANOVA. When the level of significance was less than 0.05, *post hoc* pairwise comparisons using Bonferroni's test were performed (*p* value presented in results section). Data are presented as mean ± standard error of the mean (SEM).

3. Results

No fish mortality was observed during the experiment. Although feeding was not strictly monitored, no perceptible alterations in fish feeding behavior were observed during and after treatment. Fish condition was assessed during the experiment through the use of Fulton's condition factor (K), according to the expression $K = (Wx100)/L^3$, where W = weight (g) and L = total length (cm). At E7, E14 and PE28 the condition factor of control (2.1 ± 0.15; 2.3 ± 0.07; 2.3 ± 0.15, respectively) and exposed fish (2.2 ± 0.15; 2.3 ± 0.07; 2.2 ± 0.14, respectively) showed no significant differences within each sampling time, with K values also found to be statistically similar to T0 fish (2.1 ± 0.16). When performing the behavioral evaluation, fish that sporadically escaped through the seawater outlet immediately after being placed in the BED were discarded and not considered for further analyses. Moreover, brain regions of interest that were severely damaged from the histological and/or sectioning procedures were not included in the stereological study.

3.1. MeHg and iHg accumulation in the brain

The levels of MeHg and iHg in the brain of white seabream exposed to dietary MeHg are presented in Fig. 4A and B, respectively, together with levels in control fish. MeHg concentrations were 9 times higher in the brain of exposed fish after 7 days of exposure (*t*-test, $p < 0.001$), and reached a maximum 21 times higher than controls at 14 days of exposure (*t*-test, $p < 0.001$). In the post-exposure period (PE28) the accumulated levels of MeHg have become nearly 16 times higher in exposed than in control fish (*t*-test, $p < 0.001$). Moreover, concentrations of MeHg doubled between E7 and E14 (ANOVA, Bonferroni *post hoc* test, $p < 0.001$), with maximum values recorded at E14. However, MeHg levels at

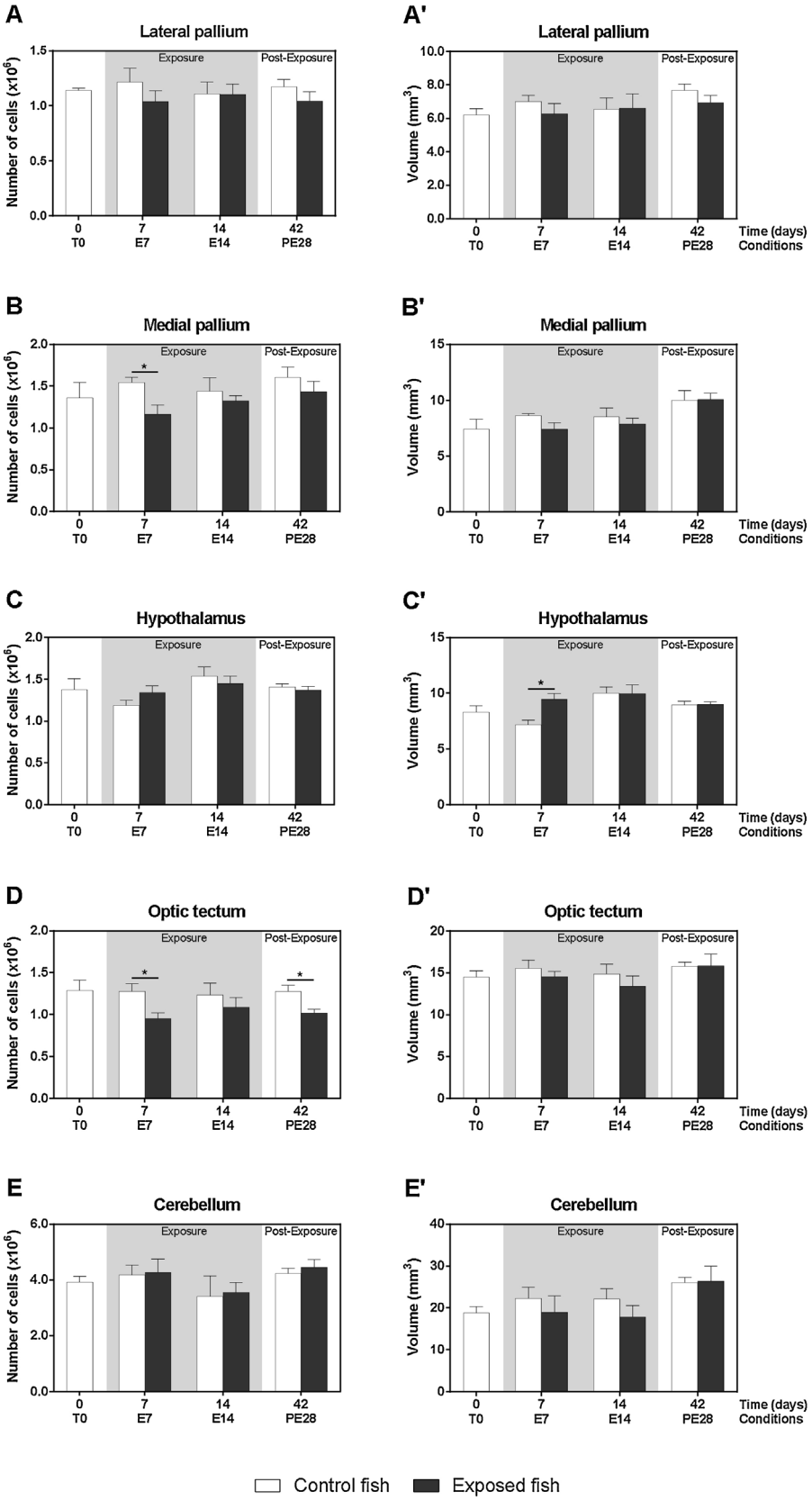

Fig. 5. Effects of dietary MeHg in the brain morphology of *D. sargus*. The cell number (A–E) and volume (A'–E') of lateral pallium, medial pallium, hypothalamus, optic tectum and molecular layer of the cerebellum were assessed in controls and in white seabreams fed with MeHg spiked feed. The morphometric evaluation of cell number (neurons and glial cells) and brain region volumes was performed before exposure (T0), during the exposure period (E7 and E14–shaded area), and in the post-exposure period (PE28). For each experimental time, the significant differences between the MeHg-exposed and the control groups are indicated by * ($p < 0.05$). Data are expressed as mean ± SEM.

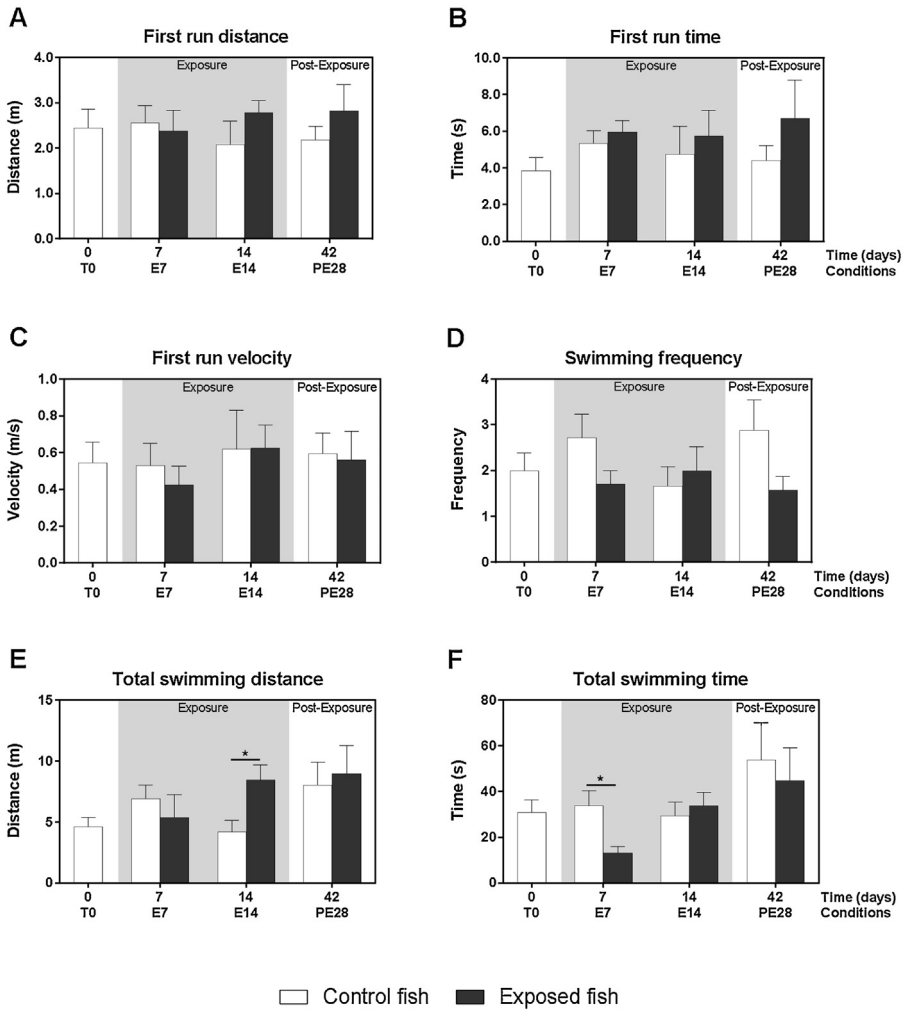

Fig. 6. Effects of dietary MeHg in the motor-related behavior of *D. sargus*. First run distance (A), first run time (B), first run velocity (C), swimming frequency (D), total swimming distance (E), and total swimming time (F) were measured before exposure (T0), during the exposure period (E7 and E14–shaded area), and in the post-exposure period (PE28). Data correspond to mean ± SEM. For each experimental time, the significant differences between the MeHg-exposed and the control groups are indicated by * ($p < 0.05$).

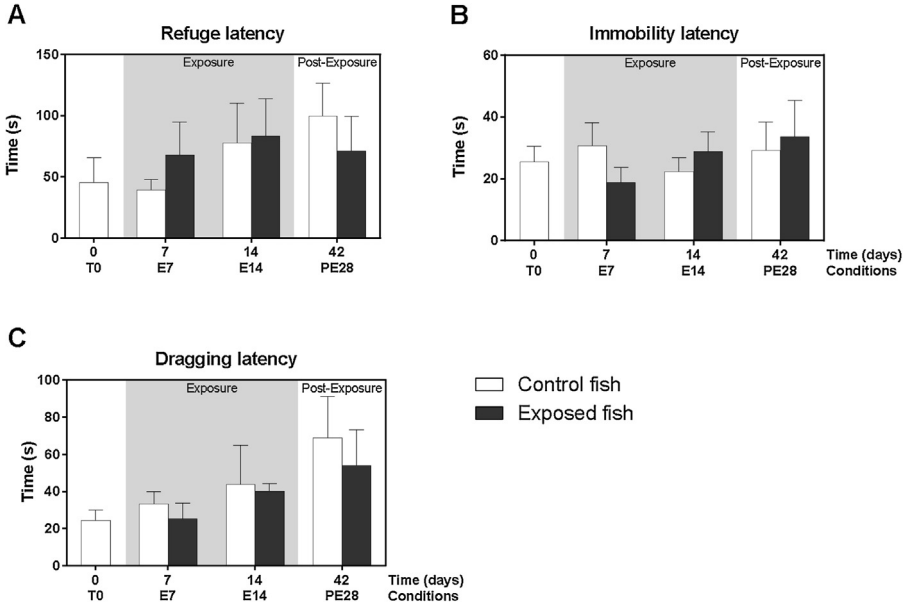

Fig. 7. Effects of dietary MeHg in the fear/anxiety-like behavior of *D. sargus*. Refuge latency (A), immobility latency (B), and dragging latency (C) were measured before exposure (T0), during the exposure period (E7 and E14–shaded area), and in the post-exposure period (PE28). Data correspond to mean ± SEM. No significant differences were observed between exposed and control fish.

PE28 were similar to the levels accumulated after 7 days of exposure, thus half than those found at E14 (ANOVA, Bonferroni *post hoc* test, $p < 0.001$). Finally, MeHg concentrations in the post-exposure period never reached those found in control fish (*t*-test, $p < 0.001$) and, in general, no temporal variations were recorded for MeHg in brain of control fish (ANOVA, $p > 0.05$).

Levels of iHg (Fig. 4B) in the exposed fish were 100- to 200-fold lower than those of MeHg. Despite that, similar temporal patterns were found between the two Hg counterparts, namely iHg levels in the brain of exposed fish were 2 times higher than in control fish at E7 (*t*-test, $p = 0.006$), at E14 it was reached the maximum accumulation of nearly 10 times higher (*t*-test, $p = 0.006$), and at PE28 the levels were still almost 7 times higher (*t*-test, $p < 0.001$). In contrast to MeHg, levels of iHg were not significantly different at E7, E14 and PE28 in exposed fish (ANOVA, $p > 0.05$), despite the fact that iHg appeared to almost double between E7 and E14. Interestingly, almost no change in the accumulation of iHg was observed in exposed fish between E14 and PE28, and the levels of iHg in control brains did not significantly change throughout the experiment (ANOVA, $p > 0.05$).

3.2. Number of cells and volume of brain regions

After 7 days of exposure to dietary MeHg, a 25% reduction of the total number of neurons plus glial cells was found in medial pallium (*t*-test, $p = 0.016$; Fig. 5B), as well as in the optic tectum that has almost 26% less cells in exposed fish (*t*-test, $p = 0.021$; Fig. 5D). The number of brain cells was also approximately 20% lower in the optic tectum of exposed fish at PE28 (*t*-test, $p = 0.015$). The number of cells did not change significantly in the lateral pallium, hypothalamus and cerebellum of fish exposed to MeHg (for all time points: *t*-test, $p > 0.05$; Fig. 5A, C and E).

A 32% increase of the hypothalamus volume was found at E7 in exposed fish (*t*-test, $p = 0.01$; Fig. 5C'). No other volume changes were recorded in the surveyed brain regions during the experiment (Fig. 5A', B', D' and E').

3.3. Swimming behavior

Fish exposed to dietary MeHg swam about twice the distance of the controls during the 3 min of observation at E14 (*t*-test, $p = 0.02$; Fig. 6E). Additionally, the total swimming time was around 61% lower in exposed fish at E7 (*t*-test, $p = 0.018$; Fig. 6F). Otherwise, no significant changes were found between exposed and control fish, during the entire experiment, regarding the first run distance, the first run time and the first run velocity, or the swimming frequency (*t*-test and *U*-test, $p > 0.05$; Fig. 6A–D). Moreover, no significant differences were recorded between exposed and control fish with regard to fear/anxiety-like status endpoints, namely the refuge latency, immobility latency and dragging latency (*t*-test and *U*-test, $p > 0.05$; Fig. 7A–C).

4. Discussion

4.1. Toxicokinetics of MeHg in the brain

The elevated neurotoxic potential of MeHg in mammals is, in general, attributed to its efficient transport into the brain (Ceccatelli et al., 2010; Farina et al., 2013). Results from this study point in the same direction, since high levels of MeHg were found in the brain of exposed white seabream just after 7 days of exposure. This result is consistent with studies on zebrafish that also recorded a significant accumulation of MeHg in the brain after 7 days of exposure to a contaminated diet (Gonzalez et al., 2005; Feng et al., 2015; Gentès et al., 2015). While little is known about the toxicokinetics of dietary MeHg in fish, it is widely accepted that MeHg is nearly completely absorbed in the gastrointestinal tract of humans and other mammals (Clarkson, 1972). From the gastrointestinal tract, MeHg quickly enters the bloodstream and is distributed to various organs including all areas of the brain where approximately 10% of the ingested metal is deposited (Clarkson, 2002). MeHg crosses biological membranes easily due to its single valence, relatively small size, and high solubility in lipids (100 times greater than in water). In particular, MeHg transport across the blood-brain barrier (BBB), as well as its uptake by neural cells, occurs *via* a MeHg-L-cysteine complex that has a similar structure to methionine, which is transported by the L-type neutral amino acid transporter (Aschner and Clarkson, 1988). MeHg is proposed to have followed a pathway in *D. sargus* similar to that proposed by Clarkson (1972) for mammals, before being accumulated in the brain.

The significant increase of MeHg levels in the brain of *D. sargus* between the 7th and the 14th day of exposure confirmed the high mobility of this toxicant in fish, and identified the brain as a target organ for MeHg. In fact, MeHg levels doubled within that time frame, suggesting an absorption rate of MeHg in the brain of 0.54 $\mu g\, g^{-1}\, day^{-1}$. MeHg levels in the brain of *D. sargus* at E14 were higher than those recorded in zebra-seabream exposed to waterborne MeHg (Branco et al., 2011) probably due to differences on the exposure routes. Although no data exist concerning the absorption of MeHg in fish brain after dietary exposure, a high exchange rate was reported between liver and blood ($1.27\, d^{-1}$) in a marine fish (Wang and Wang, 2015), confirming its rapid distribution through fish body. Results in white seabream also suggest an easy mobility of MeHg through the BBB, thus explaining its high accumulation in the brain.

Levels of MeHg in the brain of *D. sargus* reached a maximum at E14 while at 28 days post-exposure (PE28) MeHg levels had decreased to almost half. This considerable decrease of MeHg may be due to either the demethylation of MeHg to iHg and/or due to MeHg elimination. Indeed, results from a number of studies on humans exposed to MeHg for many years have shown high concentrations of iHg in the brain in relation to total Hg, revealing the potential for demethylation of MeHg in the human brain (Friberg and Mottet, 1989). Similar evidence of demethylation is available from long-term studies on monkeys exposed to MeHg (Vahter et al., 1995). Overall, previous works indicate that a significant accumulation of iHg takes place in the brain with time, despite the fact that the demethylation rate is very slow (Friberg and Mottet, 1989). Our data from *D. sargus* showed that iHg is accumulated in fish brain along with MeHg exposure. The presence of iHg in the brain of *D. sargus* is probably the result of MeHg demethylation in digestive organs (mainly intestine and liver) followed by its transport to the brain, as previously hypothesized by Branco et al. (2011) for fish. The demethylation of MeHg in fish brain has never been reported before, but it can also be hypothesized in light of observations in mammals (Vahter et al., 1995).

When considering the previous discussion on MeHg demethylation, the most probable explanation for the decrease of MeHg levels in the brain of *D. sargus* between E14 and PE28 (two-fold) is its elimination. In fact, iHg levels in the brain did not significantly change in that period and concentrations were very low, which strongly supports the MeHg elimination hypothesis. *In vitro* studies performed by Kerper et al. (1996) with bovine brain capillary endothelial cells revealed that the complexation of MeHg with glutathione and subsequent transport of the complex by an ATP-independent mechanism might be involved in the transport of MeHg out of the cells. The decrease of MeHg levels between E14 and PE28 is in line with the estimation of MeHg half-life in captive mink (15.4 days) (Evans et al., 2016). After 28 days of MeHg elimination in fish, a significant fraction of this form was still found in the head probably associated with the brain nervous system (Wang and Wong, 2003).

A very interesting result is that iHg was not eliminated in the post-exposure period as occurred for MeHg. Although MeHg is well recognized as a neurotoxicant that acts at specific biomolecular sites (for a review, see Farina et al., 2011), the demethylation of MeHg into iHg likely accounts for Hg's persistence in the brain, and potentially its long-lasting neurological outcomes (Grandjean et al., 1997; Ninomiya et al., 2005). iHg has a great affinity for selenium in the brain, being mainly accumulated as mercuric selenide (HgSe) and this is a very stable chemical form in the brain (Korbas et al., 2010).

4.2. MeHg-induced brain morphometric alterations

Taking into account the substantially lower levels of iHg compared with MeHg, as discussed above, the morphometric alterations currently detected in *D. sargus* should be mostly attributed to the MeHg form, confirming its neurotoxic potential in fish. Hence, a decrease in the number of cells in the medial pallium and optic tectum of *D. sargus* was observed after 7 days of exposure (E7), concomitantly with the significant accumulation of MeHg in the brain. In the current study, MeHg levels were determined in the whole brain of fish mainly due to analytical restrictions, although a heterogeneous distribution of MeHg is expected based on evidences presented for other species. For instance, MeHg was highly accumulated in the calcarine region of the occipital cortex, the corpus striatum and the lateral geniculate body of squirrel monkeys when compared to the medulla or cerebral cortex (Evans et al., 1977). Moreover, Korbas et al. (2012) performed a screening of MeHg distribution in zebrafish larvae and found a preferential accumulation in the pineal gland. Such a heterogeneous distribution of MeHg in seabream's brain would explain the preferential deleterious effects of MeHg in medial pallium and optic tectum, while no changes in the number of cells were recorded for lateral pallium, hypothalamus and cerebellum. Accordingly, MeHg induced neuropathological changes in the cerebral cortex of mouse but not in the hippocampus (Fujimura et al., 2009). Additionally, a severe vacuolation was recorded in brain of fish exposed to dietary MeHg with an extension varying largely with the brain area (Berntssen et al., 2003). Current data is also in line with the effects of iHg in the brain of white seabream reported in our previous study (Pereira et al., 2016), mainly characterized by the loss of cells in hypothalamus, optic tectum and cerebellum after 7 days of exposure *via* water.

Although the mechanisms of MeHg neurotoxicity still remain elusive, apoptosis has been described as a key process in mammals (Toimela and Tähti, 2004), leading eventually to cells loss in the brain. Apoptosis is a highly conserved cell death process involved in tissue remodeling and degeneration in a variety of cell types (Ceccatelli et al., 2010). Indeed, the loss of cells in hypothalamus, optic tectum and cerebellum of *D. sargus* exposed to iHg was hypothetically related with apoptosis (Pereira et al., 2016), in line with a number of evidences in mammals (Charleston et al., 1995; Nagashima et al., 1995; Nagashima, 1997; Fujimura et al., 2009). Accordingly, the current loss of cells in medial pallium and optic tectum after 7 days of exposure to MeHg may be an evidence of apoptosis, but future studies should be undertaken to confirm this hypothesis. Aside from apoptosis, also autophagy has been recently shown to be induced in human neural stem cells (Chang et al., 2013), and in rat primary astrocytes (Yuntao et al., 2014) after MeHg exposure. In fish brain, diffuse necrosis was also observed after prolonged MeHg exposure (Berntssen et al., 2003). In addition, glutamate and calcium dyshomeostasis, as well as oxidative stress, are important and interrelated phenomena that mediate a toxic cycle culminating in cell death upon exposure to MeHg (Farina et al., 2011; Xu et al., 2012a).

Alternatively, the loss of cells in medial pallium and optic tectum may also be related to an inhibition of cell proliferation promoted by MeHg, as previously hypothesized in *D. sargus* exposed to iHg (Pereira et al., 2016) and as observed in the hippocampus and cerebellum of rats exposed to MeHg (Burke et al., 2006; Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). Studies with MeHg have indicated that the decreased number of brain cells in mammals is a result of the inhibition of proliferation rather than cell death (Lewandowski et al., 2003 and references herein). Interestingly, cell proliferation in the brain of teleost fish occurs along life (Maruska et al., 2012), reinforcing the hypothesis that a mitotic arrest associated to MeHg can occur in juveniles of white seabream, leading to a lower number of cells in medial pallium and optic tectum. Several mechanisms may contribute to the effects of MeHg on mitotic progression, namely the disruption of microtubule formation, cellular signaling, gene expression, protein phosphorylation and oxidative stress (Lewandowski et al., 2003). With this regard, a parallel study was conducted with *D. sargus* to clarify the effects of MeHg on the oxidative stress status of the brain. Our accompanying results pointed to the activation of several antioxidant enzymes in the brain after 7 days of exposure to MeHg, suggesting an overproduction of reactive oxygen species (Albuquerque et al., unpublished data).

An increase of hypothalamus volume was recorded in *D. sargus* after 7 days of exposure to MeHg. To the best of our knowledge, a volume increase of a brain region in reaction to MeHg neurotoxicity has not been recorded in fish. However, this may reflect a compensatory mechanism given the regenerative capacity of fish brain in response to physical and chemical insults (Skaggs et al., 2014; Ganz and Brand, 2016; McPherson et al., 2016). Since in the present case the enlargement of the hypothalamus was not accompanied by a rise in the cell number, cell hypertrophy seems likely. Indeed, MeHg exposure induced astrocytic swelling *in vitro* (Brookes and Kristt, 1989; Aschner et al., 1990, 1998a,b; Vitarella et al., 1996). Furthermore, the pyramidal cells in the medulla and apex of brain appeared rounded in the salmon exposed to MeHg, which is indicative of cell swelling (Berntssen et al., 2003). Future studies should clarify the mechanism involved in the increase of hypothalamic volume in *D. sargus* following MeHg exposure.

The brain morphometric evaluation at E14 pointed out a recovery of the medial pallium and optic tectum since an identical number of cells was found between exposed and control fish. The volume of hypothalamus did not differ between exposed and control fish, corroborating a recovery after 14 days of exposure to MeHg. Keeping in mind that the accumulation of MeHg in whole brain increased between E7 and E14, this is quite an interesting result. It is likely that the activation of some adaptive mechanisms occurred within this exposure period, allowing the brain to recover. An identical recovery phenomenon has been suggested for the effects of iHg in *D. sargus* (Pereira et al., 2016). There are several mechanisms that can be on the basis of brain cell adaptation to MeHg, being the activation of antioxidant systems one of the most relevant. Indeed, after 14 days of exposure to MeHg, an activation of antioxidant defenses was found in brain of *D. sargus*, as indicated by increased activity of superoxide dismutase, catalase, glutathione peroxidase, glutathione and glutathione S-transferase (Albuquerque et al., unpublished data). The recovery of the number of cells in medial pallium and optic tectum implies the formation of new cells at E14. The teleost fish has a great potential to regenerate brain cells and this can be achieved by multiple mechanisms (Zupanc, 2009).

The adaptive mechanisms that were on the basis of the medial pallium recovery were likely maintained at PE28. MeHg levels in the whole brain of *D. sargus* decreased two-fold between E14 and PE28, facilitating the probable recovery of the medial pallium. In contrast, a deficit in the number of cells in the optic tectum reemerged at

PE28, highlighting the greater vulnerability of this brain area when compared with all the other areas. Identical results were found in optic tectum of white seabream exposed to iHg (Pereira et al., 2016). As previously argued in that work, the distinct profile found between medial pallium and optic tectum indicates that the former area was only transiently affected by MeHg exposure, while effects on the optic tectum are more persistent over time.

The response of the *D. sargus* brain to MeHg exposure followed a multiphasic profile, comprising an initial phase of cells loss in the medial pallium and optic tectum at the 7th day of exposure, as well as the increase in hypothalamus volume. Then, a phase of homeostatic adjustments occurred in medial pallium and optic tectum at the 14th day of exposure when the accumulation of MeHg reached a maximum in the whole brain. Finally, 28 days after exposure to MeHg had ceased, those adaptive mechanisms failed in the optic tectum, leading to a loss of cells. In general, effects of iHg in fish brain followed an identical multiphasic pattern, particularly for the optic tectum (Pereira et al., 2016). This finding is a quite relevant result for the study of Hg neurotoxicity in fish, both because it highlighted the optic tectum as the most vulnerable brain area, and also because it suggests some convergence of effects of the two Hg forms in the fish brain.

4.3. Swimming behavior and association with brain morphometric alterations

Changes on the motor status of *D. sargus* due to MeHg exposure were scarce. After 7 days of exposure, fish swam for a shorter time, suggesting an impairment of motor activity. Coincidently, this behavior shift occurred at the point in time where brain morphometric alterations were more pronounced, embracing three different regions (*i.e.* loss of cells in medial pallium and optic tectum, and increase of hypothalamus volume). In line with pathological conditions in brain stem and mid brain of Atlantic salmon, Berntssen et al. (2003) also observed an adverse behavior upon dietary exposure to MeHg. Interestingly, no changes were observed in the molecular layer of the cerebellum, being this region important for the modulation of motor coordination, as well as motor and associative learning in fish (Kaslin and Brand, 2013; Liu, 2013). Moreover, the cerebellum has been described as particularly susceptible to MeHg in rodents, monkeys and humans (Sager et al., 1984; Berntssen et al., 2003; Syversen and Kaur, 2012). Although scientific evidences of MeHg neurotoxicity in fish are by far scarcer than those in mammals, it was observed a severe vacuolation of cerebellum upon exposure to a MeHg contaminated diet over 4 months (Berntssen et al., 2003). Our study used a different fish species than Berntssen et al. (2003) and exposure was shorter (14 days), which can contribute to the dissimilar results in cerebellum. An aspect that may contribute chiefly to a lower susceptibility of teleost fish brain to MeHg is the fact that cell proliferation is orders of magnitude higher than that observed in mammals (Maruska et al., 2012). Remarkably, cell proliferation was reported in the cerebellum of adult teleost fish after lesions with a maximum of proliferative activity at 5 days after lesion (Zupanc and Ott, 1999).

Despite the described role of the optic tectum as a visual center, knowledge of its function in fish is still limited. Interestingly, the removal of the tectum in zebrafish larvae had no effect on the processing of second-order motion, suggesting that the tectum is not an integral part of the circuit that extracts higher-order cues in the motion pathway (Roeser and Baier, 2003). To the best of our knowledge, the medial pallium has not been associated with the regulation of fish motor behavior. On the other hand, the hypothalamus has been shown to mediate swimming behavior in fish (Godoy et al., 2015; Huang et al., 2016; McPherson et al., 2016), as well as energy balance (Leal et al., 2013; Tinoco et al., 2014). However, the current impairments of *D. sargus* motor function cannot be explained by a decrease in fish condition, since k values did not vary significantly between control and exposed fish at any experimental time. For the same reason, the hypothesis of swimming impairment due to energy allocation towards detoxification, as previously reported (Berntssen et al., 2003), is largely unlikely.

The dopaminergic neurons in the hypothalamus were described to be involved in the regulation of motor activity in fish (McPherson et al., 2016), and MeHg has been suggested as a dopaminergic toxicant (Götz et al., 2002; Huang et al., 2016). Recently, Huang et al. (2016) showed a significant reduction in locomotor ability accompanied by a reduced number of mitochondria in dopaminergic cells of specific brain regions (pretectum and caudal hypothalamus) in zebrafish larvae exposed to MeHg. The authors proposed that dopaminergic neuron function, rather than neuron numbers, was compromised (Huang et al., 2016). The same might have occurred in our study where the reduced total swimming time in fish exposed to MeHg *via* diet could be triggered by cellular dysfunction rather than cell loss.

Curiously, after 14 days of exposure to MeHg fish swam greater distances than controls, but no morphometric alterations were found in any of the brain areas. It is possible that alterations in neurotransmission activity could have occurred at E14 due to MeHg exposure, leading to more active fish. Indeed, adult zebrafish hatched from embryos exposed to MeHg were described as hyperactive for frequently swimming back and forth (Xu et al., 2012b). This enhancement of the total swimming distance at E14 was concomitant with the recovery of brain cells in medial pallium and optic tectum, and thus, probably related to neurophysiological and structural compensatory mechanisms.

MeHg did not change significantly the behavioral endpoints related with the fish fear/anxiety-like status in *D. sargus*. This is a very interesting result since MeHg has been identified to predispose humans (Siblerud et al., 1994) and rodents (Onishchenko et al., 2008; Maia et al., 2009) to depression and anxiety. Moreover, iHg induced a mood/anxious-like behavior in *D. sargus*, as evidenced by significant changes in related endpoints during exposure and post-exposure periods (Pereira et al., 2016). Considering the increase of hypothalamus volume after 7 days of exposure to MeHg, an alteration in the fear/anxiety-like status of *D. sargus* could be expected, since this area is essential for autonomic and neuroendocrine responses to stress in fish (Nardocci et al., 2014). Also a significant decrease of cells occurred in medial pallium after 7 days of exposure to MeHg without a translation in the fear/anxiety-like behavior. The medial telencephalic pallium of actinoptrygian fish has been hypothesized to be the homologous to the mammalian amygdala (Broglio et al., 2005). In this context, it was found that lesions on the medial pallium of goldfish were followed by deficits in avoidance learning, in line with the role of mammalian amygdala (Portavella and Vargas, 2005). Despite this close interplay between medial pallium and emotional learning in fish (Portavella and Vargas, 2005), *D. sargus* did not show alterations in fear/anxiety-like related endpoints, even with the loss of cells in that brain region. Also the loss of cells in optic tectum after 7 days of exposure to MeHg did not change the fear/anxiety-like status of *D. sargus*, although an opposite result was found in the same species exposed to iHg (Pereira et al., 2016).

Overall, morphometric changes in medial pallium, hypothalamus and optic tectum of *D. sargus* were not followed by alterations in fear/anxiety-like behavior, which suggests the occurrence of compensatory mechanisms preventing effects at high levels of complexity. It has been suggested that the redundancy and plasticity of neural functions in teleosts can often mask MeHg-induced damages for an extended period of time (Weis, 2014). It has also been reported that the deleterious effects of MeHg may only manifest later in the depuration period (Huang et al., 2016). In fact, as

consequence of exposure to MeHg, delayed or transient behavioral effects have been recurrently described in several teleost species (Weis and Weis, 1995; Fjeld et al., 1998; Samson et al., 2001; Smith et al., 2010; Xu et al., 2012b; Huang et al., 2016). Interestingly, a delay period before the onset of symptoms, lasting from weeks to several months after exposure to MeHg, has been also observed in humans, monkeys and rodents (Weiss et al., 2002; Syversen and Kaur, 2012). In order to capture any delayed effects of MeHg in *D. sargus*, an extended depuration period should be considered. The present results suggest, specifically, that significant shifts in motor-related behavior induced by MeHg may depend on a combination of changes in different brain regions of fish.

In contrast to MeHg, waterborne iHg triggered changes in hypothalamus, optic tectum and cerebellum related to numerous changes in both motor function and mood/anxiety-like status (Pereira et al., 2016). The waterborne iHg study by Pereira et al. (2016) and the present work showed an amplified differentiation of effects of the two Hg forms at higher levels of biological organization, such as the integration of cognitive and behavioral responses. Future studies would benefit from the implementation of behavior tests closely related with the integrity and function of specific brain areas in fish. For instance, the assessment of detailed optokinetic and optomotor responses in fish, as well as of visual acuity, would probably provide more clarifying information on the causal association with the morphometry and integrity of the optic tectum.

5. Conclusions

According to the present results, it can be concluded that:

1. MeHg was significantly accumulated in the brain of *D. sargus* after a short exposure time (7 days), suggesting an efficient transport of this toxicant to the fish brain. MeHg levels in the brain doubled at end of the exposure period (14 days) and halved after 28 days of depuration, confirming the high mobility of MeHg.
2. iHg also occurred in the brain of *D. sargus*, probably as a result of demethylation, although at levels 100–200 times lower than MeHg. Interestingly, iHg was not eliminated from the fish brain 28 days after exposure had ceased.
3. MeHg elicited the loss of cells (neurons plus glial cells) in the medial pallium and optic tectum after 7 days of exposure, as well as an increase in hypothalamus volume. These morphometric alterations were followed by an impairment of fish motor condition as evidenced by a decrease of the total swimming time; however, the fear/anxiety-like status of fish was not altered.
4. The optic tectum showed a higher vulnerability to MeHg than other brain regions, since a loss of cells occurred once more at the end of the post-exposure period (PE28). Despite that, no alterations in fish behavior were found at that time, indicating that the loss of brain cells in *D. sargus* due to MeHg does not always imply changes in behavior.
5. The effects of MeHg in *D. sargus* brain followed a multiphasic profile characterized by the occurrence of homeostatic mechanisms at the maximum of accumulation, preventing circumstantially morphometric alterations in the brain and behavioral shifts.

Ethical statement

This study was conducted in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, under the supervision of a team member (Mário Pacheco) authorized by the competent authorities.

Conflicts of interest

There are no conflicts of interest in this work.

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