



Brain morphometric profiles and their seasonal modulation in fish (*Liza aurata*) inhabiting a mercury contaminated estuary[☆]

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ARTICLE INFO

Article history:

Received 30 November 2017

Received in revised form

9 February 2018

Accepted 16 February 2018

Available online 15 March 2018

Keywords:

Mercury

Neurotoxicity

Brain morphology

Seasonal variation

Environmental exposure

ABSTRACT

Mercury (Hg) is a potent neurotoxicant known to induce important adverse effects on fish, but a deeper understanding is lacking regarding how environmental exposure affects the brain morphology and neural plasticity of specific brain regions in wild specimens. In this work, it was evaluated the relative volume and cell density of the lateral pallium, hypothalamus, optic tectum and molecular layer of the cerebellum on wild *Liza aurata* captured in Hg-contaminated (LAR) and non-contaminated (SJ) sites of a coastal system (Ria de Aveiro, Portugal). Given the season-related variations in the environment that fish are naturally exposed, this assessment was performed in the winter and summer. Hg triggered a deficit in cell density of hypothalamus during the winter that could lead to hormonal dysfunctions, while in the summer Hg promoted larger volumes of the optic tectum and cerebellum, indicating the warm period as the most critical for the manifestation of putative changes in visual acuity and motor-dependent tasks. Moreover, in fish from the SJ site, the lateral pallium relative volume and the cell density of the hypothalamus and optic tectum were higher in the winter than in summer. Thus, season-related stimuli strongly influence the size and/or cell density of specific brain regions in the non-contaminated area, pointing out the ability of fish to adapt to environmental and physiological demands. Conversely, fish from the Hg-contaminated site showed a distinct seasonal profile of brain morphology, presenting a larger optic tectum in the summer, as well as a larger molecular layer of the cerebellum with higher cell density. Moreover, Hg exposure impaired the winter-summer variation of the lateral pallium relative size (as observed at SJ). Altogether, seasonal variations in fish neural morphology and physiology should be considered when performing ecotoxicological studies in order to better discriminate the Hg neurotoxicity.

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1. Introduction

The central nervous system (CNS) is highly vulnerable to

[☆] This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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mercury (Hg) (Clarkson, 2002; Farina et al., 2011), a contaminant released to the environment from a multitude of natural and anthropogenic sources (Pirrone et al., 2010; UNEP, 2013; AMAP/UNEP, 2015). In the last decades, several studies documented the accumulation of Hg compounds, as methylmercury (MeHg) and inorganic forms (iHg), in fish brain under environmental and experimental conditions (Rouleau et al., 1999; Berntssen et al., 2003; Mieiro et al., 2010; Korbas et al., 2011; Pereira et al., 2014). However, the majority of the studies is focused on the neurotoxicology of Hg compounds in humans where the poisoning due to acute or chronic exposure causes fine motor alterations, cognitive, social and emotional dysfunctions, as well as sensory loss, hearing and visual deficiencies (Harada, 1995; Farina et al., 2013). Within the human CNS, two of the most often affected areas by Hg are the

cerebellum and cerebral cortex, usually presenting neuronal loss and glial cell proliferation (Ceccatelli et al., 2010; Syversen and Kaur, 2012). Rodent models have been used to further explore the deleterious effects of Hg in brain morphology, showing adverse effects in cell populations within the hippocampus and cerebellum (Sager et al., 1984; Møller-Madsen and Danscher, 1991; Roegge et al., 2006; Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). By opposition, in fish, which are a major source of nutrients to people worldwide (FAO, 2014) and a vital component of marine ecosystems, the impact of Hg on brain morphology has received less attention. Berntssen et al. (2003) performed a histopathological analysis of Atlantic salmon brain after long-term dietary exposure to MeHg, which revealed vacuolation and necrosis in the medulla oblongata, cerebellum, ventral regions of the optic tectum and cerebrum, while dietary iHg elicited astrocyte proliferation especially in the dorsal regions of the optic tectum and throughout the medulla oblongata. Additionally, the impairment of optic tectum integrity was described in zebrafish, with a decrease of cell density after long-term dietary exposure to MeHg (Cambier et al., 2012). Also in zebrafish, a decrease in the telencephalon cell body density was observed after developmental exposure to MeHg, causing life-long morphological changes in this brain region (Smith et al., 2010). Recently, our research group performed an in-depth and extensive stereological analysis of fish brain morphology, showing that laboratory exposure of the white seabream to environmentally realistic levels of waterborne iHg elicited a deficit on the number of brain cells (neurons plus glial cells) in the optic tectum, hypothalamus, and molecular layer of the cerebellum (Pereira et al., 2016). On the other hand, dietary MeHg affected medial pallium, optic tectum and hypothalamus of the same fish species (Puga et al., 2016). Altogether, these controlled laboratory exposures indicated significant Hg-induced adverse alterations on brain morphology involving discrete brain regions. Thus, transferring the same approach to wild fish populations under real Hg contamination scenarios appears as a critical subsequent step.

It is known that Hg availability in the sediments and water can vary between seasons, with a tendency towards the occurrence of higher concentrations in winter comparing to summer (Pereira et al., 2014). In parallel, it was also documented seasonal variations of Hg concentrations in the fish tissues, being fish length and age, changes in trophic position, and summer growth dilution effect the putative responsible factors for the Hg fluctuations (Zhang et al., 2012; Braaten et al., 2014; Moreno et al., 2014). Also seasonal surveys have been used to study the oxidative stress and biotransformation responses in the fish liver and muscle under environmental Hg exposure (Guilherme et al., 2008). Nevertheless, in the brain, the influence of seasonal variables has been assessed only regarding the Hg bioaccumulation and oxidative stress profiles of environmentally exposed fish (Mieiro et al., 2011). Interestingly, fish brain not only faithfully reflected environmental spatial differences in levels of different Hg forms, but also responded to seasonal changes found in water and sediments (Pereira et al., 2014). Thus, the evaluation of Hg effects in wild fish should be performed in contrasting seasons, as winter and summer. Importantly, it is recognized that fish brain remains plastic throughout a fish's life, which makes them able to adapt their physiology and behaviour to the challenges posed by the surrounding environment, e.g. avoiding predation, dealing with spatial complexity or finding a mate (Ebbesson and Braithwaite, 2012; McCallum et al., 2014; Eifert et al., 2015; White and Brown, 2015b). These adaptations are assumed to be caused or supported by neural plasticity comprising structural reorganisation, as well as biochemical switching in the brain (Ebbesson and Braithwaite, 2012; Sørensen et al., 2013). As wild fish naturally experience seasonal

environmental variations, also periodic changes in the brain morphology are expected, since the tissue investment and brain plasticity are related to their needs (Ebbesson and Braithwaite, 2012; Kotrschal et al., 2013; McCallum et al., 2014; Näslund and Johnsson, 2014). Nevertheless, the brain morphology regarding cell density and size of individual brain regions of wild fish environmentally exposed to Hg has not yet been assessed. Most of the studies addressed neurodegeneration (Berntssen et al., 2003; Keyvanshokooh et al., 2009; Wang et al., 2015), disturbances on sensory processing (Baattrup et al., 1990), and behavioural changes (Berntssen et al., 2003; Grippo and Heath, 2003; Webber and Haines, 2003; Pereira et al., 2016; Puga et al., 2016) in fish under laboratory conditions with uptake routes alternatively restricted to waterborne or dietary exposure, using different chemical forms of Hg, and neglecting the impact of seasonal variation of Hg levels, as well as oscillations in all other environmental factors. Moreover, considering a scenario of climate change and anthropogenic pollution that organisms inhabiting the coastal environments will be subjected, there is an increasing interest in determining how these wild animals adapt and respond to environmental stressors. Under this new light, it is important to establish now the baseline knowledge on the fish brain morphology and plasticity under a Hg contaminated scenario in two contrasting seasons.

In the present study, we used a stereological approach successfully established in laboratory-controlled studies (Pereira et al., 2016; Puga et al., 2016), in order to evaluate the long-term impact of Hg on the brain morphology of wild golden grey mullet (*Liza aurata*) inhabiting an Hg contaminated system, analysing the extension of morphological changes in the brain under the influence of winter-summer associated conditions.

2. Materials and methods

2.1. Study area

This study was carried out in a coastal lagoon (Ria de Aveiro, Portugal) subjected during decades, in the past, to effluent discharges from a chlor-alkali industry. Nowadays, high Hg levels are still found in sediments and fish body inhabiting this area (Guilherme et al., 2008; Pereira et al., 2014). A commonly occurring fish in this lagoon is the golden grey mullet (*L. aurata*), which has been often used as a bioindicator of water quality since it is a pelagic species frequently in contact with sediments, feeding of small benthic organisms and detritus (Ferrari and Chieregato, 1981; Pacheco et al., 2005). Hence, *L. aurata* juveniles were sampled in the winter (February 2013) and summer (June 2013) at two different locations (Fig. 1), a Hg contaminated site (Laranjo - LAR) and a reference site (São Jacinto – SJ). These locations were chosen for being representative of highly Hg contaminated and non-contaminated sites (Mieiro et al., 2010, 2011; Pereira et al., 2014). Hg has been established as the main contaminant in LAR, as the levels for other compounds were within the permitted thresholds (García-Seoane et al., 2016). In the winter and summer of 2013, surface sediments, sub-surface water and *L. aurata* were collected in the same locations for Hg determination (Pereira et al., 2014), and several water physical-chemical parameters were also measured (Table S1). The brain of *L. aurata* from LAR showed a higher accumulation of MeHg and iHg than the brain of SJ fish, in both seasons. Moreover, a seasonal variation was found for LAR site, with the winter levels of tHg and MeHg in the brain being higher than the levels accumulated during the summer, while no seasonal differences were found for iHg levels. The levels of tHg and MeHg in sediments and water followed a similar pattern in LAR site, and no seasonal variation was found for the SJ site regarding Hg levels in sediments, water and fish brain (Pereira et al., 2014).

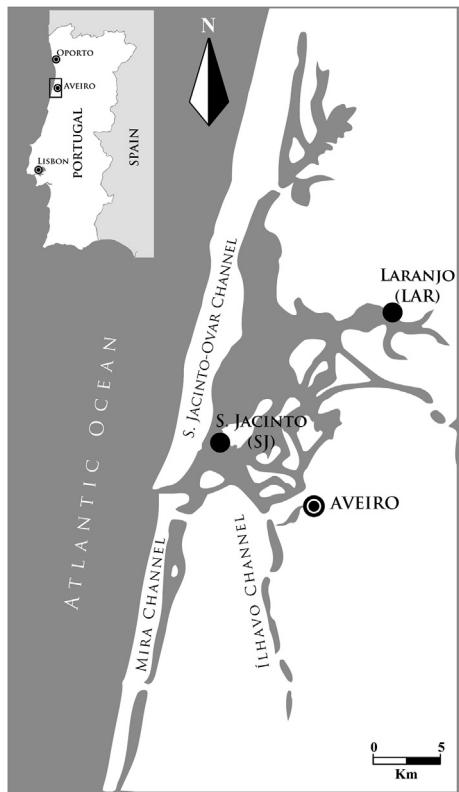


Fig. 1. Location of the sampling sites (●) at Ria de Aveiro, Portugal: São Jacinto (SJ; 40°41'00" N, 8°42'44"W) non-contaminated site; Laranjo (LAR; 40°43'28.98" N, 8°37'35.80" W) Hg-contaminated site.

2.2. Fish collection and histological procedures

Fish were caught using a traditional beach-seine net and immediately anaesthetized, being then sacrificed by cervical transection and properly bled. The brain was excised and preserved in 4% paraformaldehyde (PFA) in phosphate-buffered saline (pH 7.4) solution until further processing ($n = 6$ per group). In the winter at SJ and LAR, fish total length was 13.0 ± 2.1 and 15.1 ± 3.4 cm, respectively, while in summer it was 18.3 ± 0.6 and 13.9 ± 0.7 cm, respectively.

After 72 h in 4% PFA, brains were embedded in glycolmethacrylate (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany), 30 µm coronal sections were obtained and stained a solution of 20% Giemsa's azur eosin methylene blue (Merck, Darmstadt, Germany). All animals were blind coded to eliminate any bias by the experimenter.

2.3. Morphological evaluation of the *L. aurata*'s brain

To the best of our knowledge, no brain atlas has been published concerning the brain anatomy of *L. aurata*. Therefore, the overall approach for identifying the different brain regions was based on comparisons between the morphology of *L. aurata* brain and several neuroanatomical studies of other fish species, such as grey mullet, Japanese eel, zebrafish, gold fish, mormyrid fish, sea bass, cichlid fish (Rupp et al., 1996; Precht et al., 1998; Cerdá-Reverter et al., 2001; Ahrens and Wullmann, 2002; Mukuda and Ando, 2003; Lamas et al., 2007; Durán et al., 2010; Simões et al., 2012). Thus, noticeable cytoarchitectonic features were used to establish the limits of the lateral pallium (LP; the dorsolateral region of the telencephalon), hypothalamus (Hyp), optic tectum (OT) and

molecular layer of both the valvula and corpus cerebelli (Ce ML; Fig. 2A–C).

2.3.1. Determination of the relative volume

To obtain estimates of the brain overall size, we calculated its volume (Table S2) according to the ellipsoid volume formula: Volume = (length × width × height) × $\pi/6$ (Pollen et al., 2007; Kotrschal et al., 2012a; Eifert et al., 2015; Herczeg et al., 2015; White and Brown, 2015b). PFA-fixed whole brains were used to perform the linear measurements of the brain length (from the rostral edge of the telencephalic lobes to the caudal edge of the cerebellum excluding the medulla oblongata as it was cut imprecisely), width (measured across the two optic tecta) and height (dorsal edge of the midbrain to the ventral edge of the hypothalamus). In order to control for differences in brain size between the experimental groups, it was calculated the relative volume of the different brain regions for each fish specimen. Therefore, it was divided the volume of the individual brain regions (obtained by the Cavalieri method described in Section 2.3.2 and supplementary material) by the total brain volume to obtain the relative value (e.g. lateral pallium volume/total brain volume = relative lateral pallium volume; Fig. 3), as described by White and Brown (2015b).

2.3.2. Determination of the cell density

Examining the cell density together with the relative volume of the brain regions help to determine if the responses are mediated by changes in cell size or cell number. The cell densities (Fig. 4) were calculated as the ratio of the total cell number (obtained by the optical fractionator method) and the Cavalieri-estimated volume of the individual brain regions, as described by Höistad et al. (2013) and Richards et al. (2013). As a result, we estimated both the volume and total cell number (Table S2; for detailed information on the methodology see supplementary material) using an unbiased stereological approach as previously described (Pereira et al., 2016; Puga et al., 2016) with a minor modification. During the cell number estimation, the x and y step size used was $200 \times 200 \mu\text{m}$. Examples of neuronal and glial cell profiles are shown in Fig. 2 D–G. During the counting procedure no distinction was made between neurons and glial cells due to difficulties discriminating between the cell types, as previously explained (Pereira et al., 2016; Puga et al., 2016), and endothelial cells and occasional erythrocytes were identified and not counted. The data for the lateral pallium, hypothalamus and optic tectum correspond to the average of the right and left hemispheres, while in the molecular layer of the cerebellum the total values were estimated due to its central location in the encephalon.

2.4. Photomicrographs

An Olympus BX61 microscope (Olympus Europe, Hamburg, Germany) coupled with a digital camera (Olympus DP70) was used to shot representative photomicrographs of *L. aurata*'s coronal sections (Fig. 2A–C) using a $4\times$ (UPlan SAPO, N.A. 0.16) objective, and the Cell-P software (Olympus, Germany). A $100\times$ (UPlan SAPO, N.A. 1.4) oil objective was used to obtain the photomicrographs of stained sections in Fig. 2 D–G.

2.5. Statistical analysis

The distribution of the variables was considered to be normal if the absolute skew value was less than 2 and the absolute kurtosis less than 7 (West et al., 1995). As all dependent variables fell within these cut-off values, differences in relative volumes of brain regions and cell density of SJ and LAR groups in winter and summer were assessed by a two-way ANOVA (2W ANOVA) coupled with

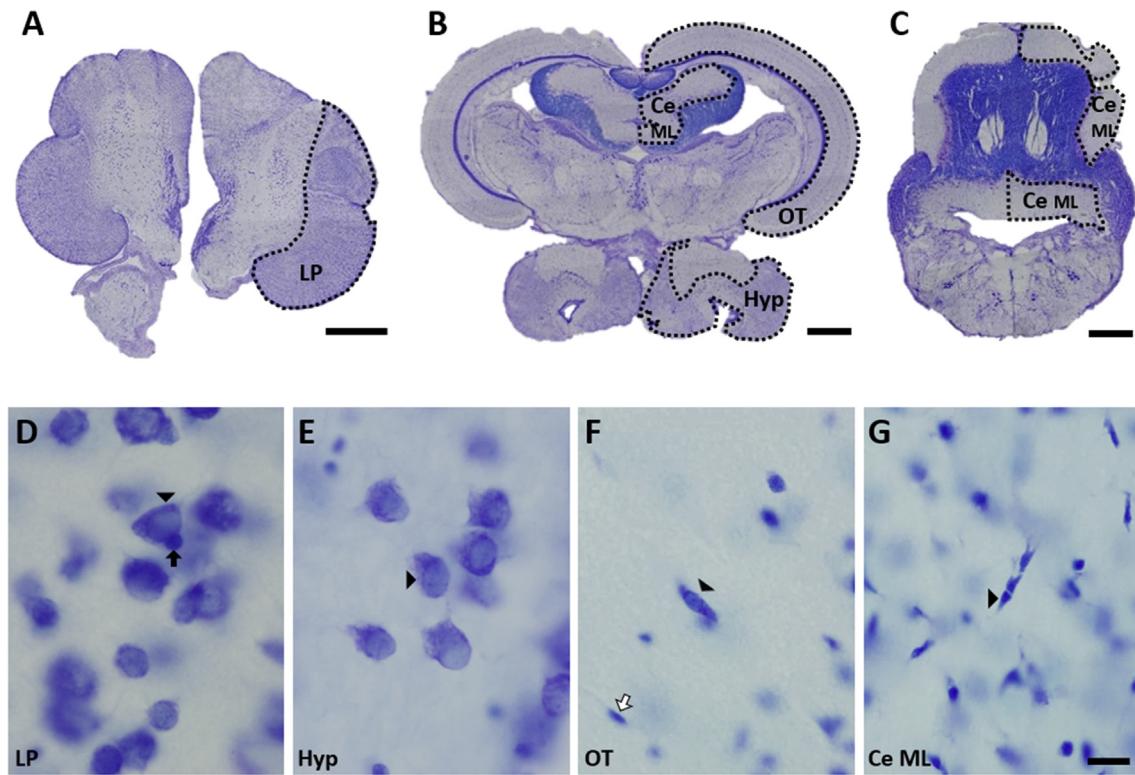


Fig. 2. Representative coronal sections through the *L. aurata*'s brain, illustrating the locations of the brain regions analysed (A–C). High magnification photomicrographs showing different cell types within each brain region analysed (D–G). There was no discrimination between neurons (black arrowhead) and some of the identifiable glial cells (black arrow) during the counting procedure. Occasional erythrocytes and endothelial cells (white arrow), which are easily distinguished, were excluded from the analysis. LP – lateral pallium; Hyp – hypothalamus; OT – optic tectum; Ce ML – molecular layer of cerebellum. Scale bar: A–C = 500 μm ; D–G = 10 μm .

Bonferroni correction for multiple comparisons, using the GraphPad software (GraphPad Prism version 6.01 for Windows, San Diego, USA). The results were considered statistically different when $p < 0.05$.

3. Results

We found the relative volume of the lateral pallium to be significantly affected by season (2W ANOVA, $p = 0.034$), but only in fish collected in the non-contaminated site. Thus, SJ fish in the summer had a 76% smaller lateral pallium than fish collected in the winter (*post-hoc*, $p = 0.047$; Fig. 3A). However, no significant effect of season, site or interaction between season and site was found in cell density of this brain region, indicating that cell density across seasons and sites was similar (Fig. 4A).

A significant effect of season was also found in the relative volume of the optic tectum (2W ANOVA, $p = 0.045$), besides the marginal interaction (2W ANOVA, $p = 0.057$). Nevertheless, the seasonal differences were not found in SJ fish, but rather in LAR fish that in the summer had a 48% larger optic tectum than winter-collected fish (*post-hoc*, $p = 0.019$; Fig. 3C). Moreover, *post-hoc* analysis revealed the relative volume of the optic tectum of this summer-collected LAR fish to be also larger (around 36%) than SJ fish collected in the same season (*post-hoc*, $p = 0.045$), highlighting differences between sites in the summer. In addition, it was found a season main effect in cell density of the optic tectum (2W ANOVA, $p = 0.004$). Interestingly, the cell density is approximately 19% lower in SJ fish collected in the summer than in the winter (*post-hoc*, $p = 0.045$; Fig. 4C), while in the LAR fish this reduction was around 18%, corresponding only to a tendency to inter-seasonal change (*post-hoc*, $p = 0.092$; Fig. 4C).

The summer-collected LAR fish had a 52% larger molecular layer

of the cerebellum than winter-collected LAR fish (*post-hoc*, $p = 0.0097$; Fig. 3D), and its size had doubled compared to SJ fish collected in the same season (*post-hoc*, $p < 0.001$). These results evidenced significant main effects of season (2W ANOVA, $p = 0.026$) and site (2W ANOVA, $p < 0.001$), as well as significant interaction between season and site (2W ANOVA, $p = 0.040$) in the cerebellum relative volume. In addition, it was found a borderline main effect of season in cell density in the cerebellum (2W ANOVA, $p = 0.088$). The cerebellum of LAR fish had a higher cell density (26%) in the summer compared to winter (*post-hoc*, $p < 0.001$; Fig. 4D). No seasonal variations of volume and cell density were observed in this brain region in fish from the SJ site.

A significant main effect of site was observed in the relative volume of the hypothalamus (2W ANOVA, $p = 0.021$), yet no significant differences were found in the *post-hoc* analysis (Fig. 3B). Moreover, this parameter did not seem to be dependent of season (2W ANOVA, $p > 0.050$). Nonetheless, a main effect of season (2W ANOVA, $p < 0.001$) and interaction between season and site (2W ANOVA, $p = 0.017$) were found for the hypothalamus cell density. SJ and LAR fish had around 38% (*post-hoc*, $p < 0.001$) and 21% (*post-hoc*, $p = 0.006$), respectively, lower cell density in the summer compared with winter (Fig. 4B). Additionally, during winter LAR fish had a 13% reduction of cell density compared with SJ fish (*post-hoc*, $p = 0.042$), pointing out differences between sites in the winter.

4. Discussion

This study addressed, for the first time, the brain morphology of wild *L. aurata* inhabiting a Hg-contaminated (LAR) and a non-contaminated site (SJ), in order to unveil if Hg occurrence could alter the fish brain morphology and modulate its winter-summer

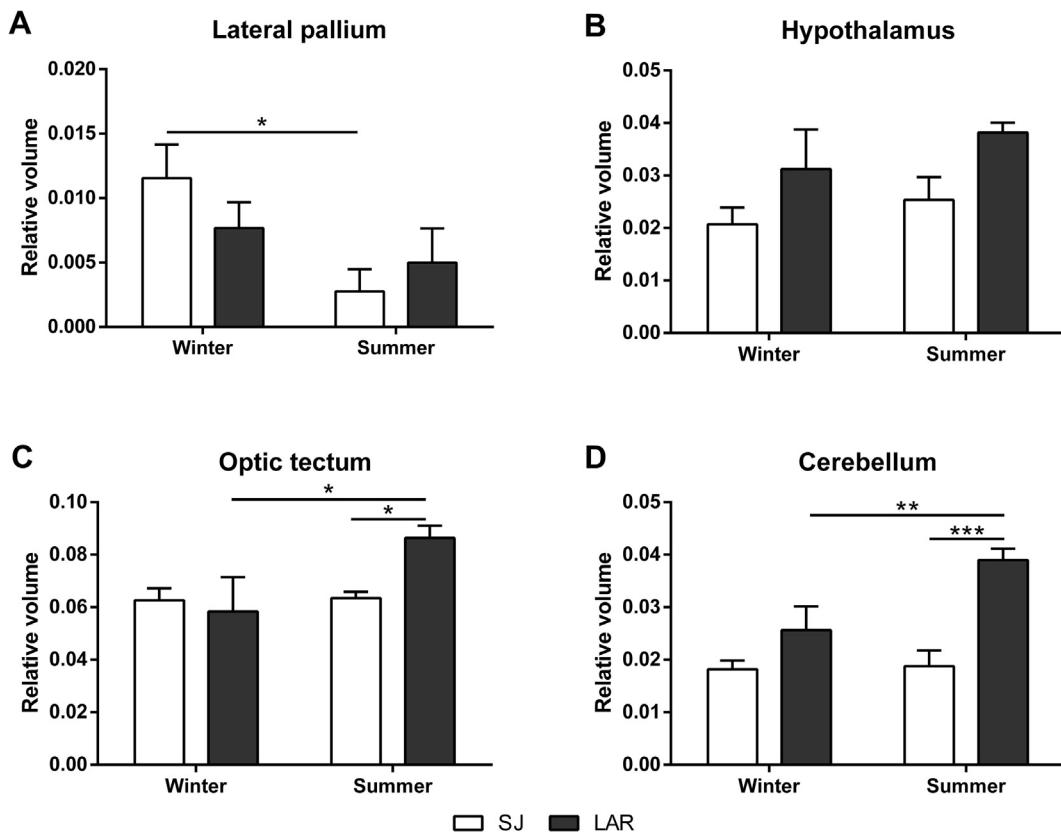


Fig. 3. Relative volumes of individual brain regions of wild *L. aurata* captured in winter and summer in Hg-contaminated (LAR) and non-contaminated (SJ) sites. Data presented as mean \pm S.E.M; * p < 0.05, ** p < 0.01, *** p < 0.001.

profiles.

4.1. Spatial-related variations of brain morphology in association with Hg occurrence

In the winter, when the tHg and MeHg levels in fish brain from LAR are, respectively, around 6 and 3 times higher than in SJ (Pereira et al., 2014), the hypothalamus of LAR fish has a lower cell density. In line with these results, exposure to iHg elicited a deficit on the cell number of white seabream hypothalamus (Pereira et al., 2016), and triggered hypothalamic neuron degeneration in *Channa punctatus* (Ram and Joy, 1988; Crump and Trudeau, 2009). Although proliferative and regenerative potential were described for the hypothalamus (Maruska et al., 2012; Ganz and Brand, 2016; McPherson et al., 2016), Hg is likely inhibiting cell proliferation, as observed in the hippocampus and cerebellum of rat exposed to MeHg (Burke et al., 2006; Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). Yet, up-to-now no studies have addressed this issue in fish. Alternatively, the lower cell density could be caused by Hg-induced cell death (Nagashima et al., 1995; Nagashima, 1997; Toimela and Tähti, 2004; Fujimura et al., 2009; Chang et al., 2013), which was not compensated by a putative cell proliferation response, since the basal winter levels of SJ fish were not reached at LAR. Future studies should evaluate this question. The alteration currently reported point out a risk of LAR fish to develop endocrine dysfunction, which is corroborated by a previous study in the same area where *L. aurata* (capture in autumn) displayed a disruption on hypothalamus–pituitary–thyroid axis (Oliveira et al., 2011).

In the summer, the recorded levels of tHg and MeHg in fish brain from LAR are around three times higher than the levels found in SJ

fish (Pereira et al., 2014), but it was observed a larger optic tectum and cerebellum in LAR fish. The cerebellum is particularly susceptible to Hg in humans, monkeys and rodents (Sager et al., 1984; Berntssen et al., 2003; Syversen and Kaur, 2012). Even though scientific evidences of Hg 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). Nonetheless, mechanical lesions in the fish cerebellum can induce high proliferative activity together with the occurrence of apoptosis to remove damaged cells in order to repair the injured tissue (Zupanc and Ott, 1999; Clint and Zupanc, 2001; Zupanc, 2009). While it is not known if a chemical-induced injury has a similar effect, this region displayed a decrease in cell size and cell number in white seabreams after a 7-day exposure to iHg, but in a post-exposure period it was able to recover to the level of non-exposed fish (Pereira et al., 2016). On the contrary, the underlying cause of the LAR specific increase in cerebellum relative volume in the summer might result from cell proliferation induced by Hg toxicity and not by a regenerative process. In this line, glial cell proliferation in the cerebral cortex of mammalian brain has been documented after Hg poisoning, being glial reactivity regarded as the central phenomenon of brain inflammation (Monnet-Tschudi et al., 2007; Ceccatelli et al., 2010; Syversen and Kaur, 2012). As a matter of fact, proliferation of microglia, upregulation of GFAP (astrocyte reactivity marker) and interleukin-6 (released during the inflammatory processes), and cell hypertrophy (typical of reactive astrocytes) were found after Hg exposure (Charleston et al., 1995; Monnet-Tschudi et al., 1996, 2007; Eskes et al., 2002). Berntssen et al. (2003) showed that pyramidal cells in the medulla and apex of the brain appeared rounded in salmon exposed to MeHg, which is an indication of cell swelling, and several studies

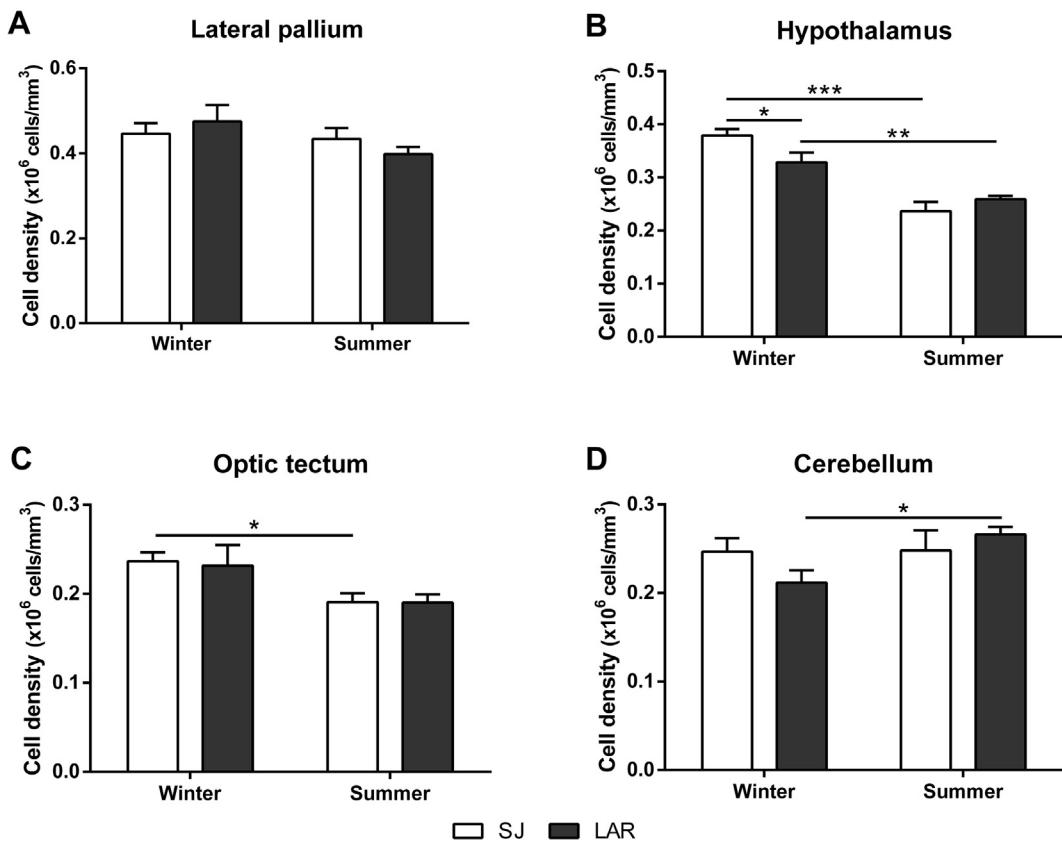


Fig. 4. Stereology-based estimates for cell density of individual brain regions of wild *L. aurata* captured in winter and summer in Hg-contaminated (LAR) and non-contaminated (SJ) sites. Data presented as mean \pm S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

in vitro also demonstrated astrocytic swelling upon MeHg exposure (Brookes and Kristt, 1989; Aschner et al., 1990, 1998a, 1998b; Vitarella et al., 1996).

Cell hypertrophy due to Hg neurotoxicity can be contributing to the increased volume of the optic tectum as no changes in cell density were found between sites. Despite the fact that a potential for regeneration after damage was also described for the optic tectum (Maruska et al., 2012; Zupanc and Sîrbulescu, 2013; Bhumika et al., 2015; Ganz and Brand, 2016), in the context of Hg neurotoxicity this region is highly susceptible. A decrease in cell density was described in zebrafish after long-term dietary exposure to MeHg (Cambier et al., 2012), and a cell loss was found in white seabream during either exposure and post-exposure to waterborne iHg (Pereira et al., 2016). In addition, a long-term dietary exposure to iHg resulted in increased cellularity of astrocytes, especially in the dorsal regions of the optic tectum and throughout the medulla, of Atlantic salmon (Berntssen et al., 2003). As such, glial cells appear to have a prominent role towards Hg neurotoxicity, mainly in the molecular layer of the cerebellum and optic tectum. Since astrogliosis is detected often before the appearance of overt neuronal damage, it was proposed to use this criterion as an early marker of neurotoxicity (Monnet-Tschudi et al., 2007). Another possibility to explain a larger optic tectum and cerebellum in LAR fish during the summer is that high Hg levels invoke compensatory processes more efficiently compared to exposure to lower levels, an effect that has been attributed to non-monotonic dose-response relationships (Weiss et al., 2002; Syversen and Kaur, 2012). This type of effect was previously observed in *Diplodus sargus* brain, where higher accumulation of iHg was not followed by increased cells loss (Pereira et al., 2016).

Importantly, the accumulation of iHg in fish brain from LAR was higher than SJ in both seasons and no seasonal variation was found (Pereira et al., 2014). The iHg form displayed a higher neurotoxic potential in *D. sargus*, as unveiled by the poor activation of brain antioxidant defences and recurrent oxidative damage, while the opposite was recorded upon MeHg exposure (Cardoso et al., 2017). As a consequence, the exposure to waterborne iHg resulted in a reduction in the number of cells in several brain areas, as well as impaired swimming behaviour (Pereira et al., 2016), while milder deleterious effects were found for dietary exposure to MeHg (Puga et al., 2016). In the present study, *L. aurata* from LAR was exposed to both inorganic and organic Hg as well as diet and water as Hg sources, being thus, difficult to associate the observed effects in brain morphology with a particular Hg form or exposure route.

Altogether, our results point out a region-specific difference in susceptibility to Hg-induced neurotoxicity that might be explained by regional differences in brain Hg levels (Rouleau et al., 1999; Korbas et al., 2012). Nevertheless, Hg accumulation in fish brain *per se* might not imply toxicity; instead, the cytotoxic effects can relate to the intracellular chemical form of Hg, as well as its sub-cellular distribution (Baatrup and Danscher, 1987; Le Faucheur et al., 2014). In addition, Hg compounds have cell selectivity, with *in vitro* studies using human cell cultures showing that neurons are more susceptible to Hg-induced cytotoxicity compared to astrocytes (Lohren et al., 2015). Following a previous study from our group, in the present work, both neurons and glia were counted together, and a future follow up analysis of the ratio of neurons and glia within different brain regions may help to identify the cell (or cells) type affected.

4.2. Seasonal modulation of brain morphology and plasticity

4.2.1. Interference of non-contamination related variables

Alterations in fish brain structures are partly shaped by the needs imposed by environmental and ecological demands (Ebbesson and Braithwaite, 2012; McCallum et al., 2014; Näslund and Johnsson, 2014). However, most of the research has been devoted to address the effect of habitat-specific differences (wild vs. laboratory, enriched vs. non-enriched environment, or differences among ecologically divergent populations) in the variation of brain structure and size (Kotschal et al., 2012b, 2013; Eifert et al., 2015; White and Brown, 2015a; b). Nonetheless, changes in brain morphology due to year periods and their season-related stimuli remain unexplored, despite fluctuations in brain oxidative profiles according to season had been reported in the wild European sea bass (*Dicentrarchus labrax*) captured in Aveiro lagoon (Mieiro et al., 2011). The current work showed that *L. aurata* specimens inhabiting the non-contaminated area (SJ), where the potential interference of seasonality by contaminant bioavailability is negligible, present clear inter-season variations in brain morphology. In the winter, these fish developed larger lateral pallia as well as higher cell density in both hypothalamus and optic tectum, which may reflect increased physiological demands in response to biotic and abiotic challenges. For instance, the dorsolateral region of the telencephalon, the lateral pallium, is mainly associated to spatial learning in fish (Portavella and Vargas, 2005; Vargas et al., 2009); therefore, variation in telencephalon size appears to relate closely to challenges in spatial environmental complexity (McCallum et al., 2014; Näslund and Johnsson, 2014; White and Brown, 2015a). The increased investment in this structure may be related to greater spatial demands experienced by *L. aurata* in the winter (e.g. predator avoidance, food foraging, water turbidity). Furthermore, even fish living in structurally simple enclosures but with complex water flow regimes have greater rates of cell proliferation in specific regions of the telencephalon (Lema et al., 2005). Thus, due to more extreme weather conditions during the winter, a higher hydrodynamic complexity probably occurs in the lagoon resulting in an overriding stimulatory effect in the lateral pallium of winter collected SJ fish. In agreement with this hypothesis, in rodents, voluntary physical activity associated with enrichment of the housing environment is widely described to promote neurogenesis in the hippocampus (Van Praag et al., 1999; Brown et al., 2003; Mustroph et al., 2012), the region that is proposed as the homologue of the lateral pallium (Portavella and Vargas, 2005; Vargas et al., 2009). Interestingly, inter-season volume changes of lateral pallium were not followed by alterations in cell density in SJ fish; therefore, it is apparent that all volume divergences were mediated by changes in cell number. In mammals, hippocampus is believed to be involved in memory and learning, as well as in the regulation of mood with several studies linking depression and stress with reduced hippocampal volume (Lee et al., 2002). This is caused in part by cellular atrophy, reduced arborisation of the neurons, and reduced neurogenesis, while environmental enrichment has an opposite effect (Brown et al., 1999, 2003; Klintsova and Greenough, 1999; Lee et al., 2002). Hence, a larger lateral pallium in winter-collected fish at SJ can be explained by increased neurogenesis in response to winter-specific environmental challenges.

The higher cell density in hypothalamus and optic tectum during the winter in SJ fish is not straightforward to interpret: despite that we hypothesize that it is also related to this species' repertoire of winter survival strategies. For instance, the hypothalamus has been recently shown to mediate swimming behaviour in fish (Godoy et al., 2015; Huang et al., 2016; McPherson et al., 2016), but it plays a major role in energy balance and reproductive behaviour (Leal et al., 2013; Tinoco et al., 2014). As juvenile specimens were

surveyed, it is not expected a contribution of gender and sexual maturation for the changes in cell density. However, temperature and photoperiod are two of the most important seasonal factors regulating feeding, and the hypothalamus integrates input from factors that stimulate or inhibit food intake (Volkoff et al., 2005). Moreover, in ectothermic species such as fish, the role of the hypothalamus in thermosensitivity and behavioural thermoregulation has been established, and seems to be an important way to protect against large variations in the brain temperature of these animals that are directly influenced by daily and seasonal fluctuations (Tsai and Wang, 1997; Bicego et al., 2007). It has been suggested that *L. aurata* is able to modulate its feeding behaviour in order to save energy for other activities such as locomotion (Como et al., 2014). This is particularly important because grey mullets are generally active swimming foragers, allowing them to avoid predators and keep hunting or grazing while still digesting (Fu et al., 2009). Despite the increased number of feeding events and the swimming velocity with temperature, the cumulative distance covered during feeding was three times higher in 10 °C than in 20 °C-acclimated *L. aurata* (Como et al., 2014). Due to the complexity of the fish hypothalamus and its functional diversity, it is difficult to directly infer why the cell density of this brain region is higher during winter, but a tight orchestration of all these processes is crucial for the homeostasis maintenance.

The optic tectum integrates retinal, auditory, lateral line and somatosensory inputs (Nguyen et al., 1999; Ben-Tov et al., 2013), being important for phototaxis, prey capture, and predator avoidance (Nevin et al., 2010). It is widely accepted that visual input, i.e. the amount of light, positively affects tectum growth and/or maintenance, and thus, increased turbidity during winter in SJ (Table S1 and García-Seoane et al., 2016) may limit visual orientation and communication, making expectable a reduced volume/cell density. Another factor potentially influencing optic tectum size is the social environment (isolated vs. group-reared fish) (Gonda et al., 2009; Kotschal et al., 2012b). However, it seems incongruent a higher cell density during the winter and its decrease in the summer when visual input and fish density are probably much higher. In the present case, it is difficult to ascertain why there is a greater development of this structure during the winter, but it might be part of this species coping strategy where higher turbidity in winter creates a greater requirement for visual function. However, optic tectum is also important for integration of sensory information from other modalities, as a result, it would make sense that *L. aurata*, similarly to other pelagic species, would invest more brain tissue in visual/sensory processing structures that will ultimately help them to forage effectively and avoid predation (White and Brown, 2015a).

The major contributor to these morphological changes, translated into alterations in cell population size, is the widespread proliferation and neurogenesis that occur during the entire life span of several teleost species (Zupanc, 2008; Ganz and Brand, 2016). These processes are suggested to be influenced by different environmental parameters, such as social interactions, stress, growing conditions, and environmental complexity (Zupanc, 2009; Maruska et al., 2012; Kaslin and Brand, 2013; Ganz and Brand, 2016). Future studies should address this hypothesis by evaluating regional rates of proliferation/neurogenesis as these are most likely responses to specific environmental changes and thereby adaptive; on the contrary, global proliferative changes across the brain are more likely a consequence of nonspecific factors as temperature, growth, or sex change (Dunlap, 2016).

The molecular layer of the cerebellum showed no seasonal alterations in brain morphology at the non-contaminated site. Since the cerebellum is important for the execution of swimming gait, control of the vestibulo-ocular reflex and emotional learning in

teleosts (Johansson et al., 2007; Broglio et al., 2011; Ikenaga, 2013), this may indicate an unaltered recruitment of this region between seasons. Although alterations in other cerebellar layers cannot be excluded, in accordance with our findings, in the intertidal gobies the relative size of the telencephalon, optic tectum, and hypothalamus are correlated with variation in the environment, while the cerebellum was largely independent of environmental variation (White and Brown, 2015a).

In general, repertoires of physiological and behavioural strategies that characterize many fish species are shaped by the adverse environmental conditions. Indeed, fish in temperate regions anticipate winter as part of their annual phenology and attempt to prepare accordingly (e.g., energy allocation, seasonal habitat shifts) (Shuter et al., 2012). In this context, it seems reasonable that the brain morphology of *L. aurata* changes in order to promote survival strategies that reflect adaptation to local conditions.

4.2.2. Interference of mercury contamination

Fish from the Hg-contaminated site (LAR) displayed a pattern of seasonal changes in the brain morphology different from that recorded at the non-contaminated site (SJ). For instance, in the lateral pallium of LAR fish, the seasonal variation in the relative volume appears disrupted. Hg seems to impair its growth promoted by winter-induced environmental challenges (as observed in SJ fish), possibly by interfering with cell proliferation/neurogenesis, as no alterations in cell density were detected. In rats, perinatal exposure to MeHg impaired neurogenesis in the hippocampus and led to profound deficits in juvenile hippocampal-dependent learning (Falluel-Morel et al., 2007; Sokolowski et al., 2013; Obiorah et al., 2015). Also, in zebrafish, a decrease in cell density in the telencephalon was present in the adult animals when exposure to MeHg occurs during developmental stages (Smith et al., 2010). Interestingly, several fish species have the ability to repair physical and chemical damage to the telencephalon through stimulation of neurogenesis (Baumgart et al., 2012; Kizil et al., 2012; Kyritsis et al., 2012; Skaggs et al., 2014). Although it has not yet been described if a Hg-induced insult has similar effect, no significant changes in volume and cell number of this region occurred in white seabream juveniles exposed to waterborne iHg or dietary MeHg (Pereira et al., 2016; Puga et al., 2016). The different outcomes observed in early developmental stages and in juveniles/adults might indicate that the negative impact of Hg upon lateral pallium could be a consequence of *L. aurata* exposure somewhere during early developmental stages resulting in long-lasting effects. In further support of this hypothesis, it has also been proposed that vulnerability to MeHg declines with age in rats and zebrafish (Dreiem et al., 2005, 2009; Xu et al., 2012b; Obiorah et al., 2015).

The larger volume of optic tectum accompanied by a marginally decrease in cell density and larger cerebellum with higher cell density in the summer might result from hypertrophy and proliferation of glial cells, as previously discussed in Section 4.1. Not surprisingly, one of the mechanisms proposed for Hg neurotoxicity suggests that Hg accumulates mainly in astrocytes and microglial cells, which would correspond to a latent phase without manifestation of symptoms (Syversen and Kaur, 2012). Then, the following transfer of Hg to neurons results in development of neurotoxicity. Therefore, this could explain the delay in the onset of symptoms from winter to summer, when Hg levels in LAR fish brain decreased comparatively to winter (Pereira et al., 2014). Moreover, the presence of compensatory mechanisms occurring in certain brain regions, along with the redundancy and plasticity of neural functions in teleosts, can often mask Hg-induced damages for an extended period of time (Falluel-Morel et al., 2007; Syversen and Kaur, 2012; Weis, 2014). Noteworthy, these phenomena were described mainly

in fish that experience a depuration/post-exposure period, or after acute poisoning in humans (Syversen and Kaur, 2012; Huang et al., 2016; Puga et al., 2016). In opposition, *L. aurata* seems to be subjected to a long-term Hg exposure with seasonal fluctuations of the contaminant level.

The hypothalamus is the only brain region that presented the same seasonal variation for LAR and SJ fish, i.e. the cell density decreases from winter to summer, albeit without changes in volume, thereby an effect mediated by cell loss. This can be explained by the pressure of season-related factors stimulating the fish to adapt to the environmental challenges, as discussed for SJ fish, or by the deleterious effects of Hg, such as glutamate and calcium dys-homeostasis, and oxidative stress that ultimately can lead to cell death (Berntsen et al., 2003; Toimela and Tähti, 2004; Farina et al., 2011; Xu et al., 2012a; Chang et al., 2013).

The herein documented changes in brain morphology most likely represent Hg induced toxicity, as several studies indicate relatively low levels of other contaminants in the lagoon (Monterroso et al., 2007; García-Seoane et al., 2016). However, we cannot exclude additional non-contamination related differences between SJ and LAR that could potentially affect the interpretation of the results. Environmental factors, such as water salinity, pH, and temperature are known to modulate Hg speciation, mobility and bioavailability in the aquatic systems (Ullrich et al., 2001), which further affect the bioaccumulation process in the aquatic organisms. The most striking variation in LAR site between winter and summer is the water salinity (Table S1), since the values were 4-fold lower in winter than in summer, while almost no variation in salinity between seasons occur in SJ (Pereira et al., 2014). There are also considerable differences in salinity between sites, particularly in winter. It should be highlighted that salinity was measured under low-tide conditions, which maximizes differences between sites. Low salinity favours Hg accumulation (Ullrich et al., 2001; Wang and Wang, 2010), which is in accordance with the higher Hg levels in the brain during winter (Pereira et al., 2014). Besides its role favouring Hg accumulation, salinity also leads to changes in brain energy metabolism associated to the osmotic adaptation (Sangiao-Alvarellos et al., 2003). Importantly, changes in brain size or structure require high energetic demands (Niven and Laughlin, 2008; Kotrschal et al., 2013), and it has been suggested that fish exposure to Hg (or other metals) might lead to a trade-off where the metabolic costs of detoxification are met by a reduction of locomotor activity (Handy et al., 1999; Berntsen et al., 2003; Vieira et al., 2009). Future studies should explore how adaption to salinity, changes in brain morphology, and Hg detoxification impact brain metabolism of *L. aurata*. Finally, and interestingly, mullets are often present in highly impacted areas (Mieiro et al., 2012; García-Seoane et al., 2016), suggesting some degree of tolerance to Hg contamination.

5. Conclusions

Hg plays a clear role in the brain morphology differences observed between sites, which might be further enhanced by the seasonal fluctuations of the contaminant level. This is the first study showing that fish brain morphology fluctuates with year periods (winter and summer) most likely due to changes in ecological factors. The present study also provides, for the first time in a wild fish population, evidence that chronic exposure to Hg in the environment can disturb the normal process of seasonal variations in brain morphology. Future follow up studies, such as year-to-year assessments and the investigation of Hg effects on fish brain morphometry from other contaminated systems, are needed to unravel the occurrence of cyclic annual fluctuations in brain morphology and its effects on fish's fitness and survival.

Acknowledgments

This work has been supported by the research project funded by Fundação para a Ciência e a Tecnologia (FCT-PTDC/AAG-REC/2488/2012), as well as by CESAM research funds (UID/AMB/50017). Sónia Puga and Vera Cardoso benefited from a research grant from NEUTOXMER project (FCT-PTDC/AAGREC/2488/2012). Patrícia Pereira benefited from SFRH/BPD/69563/2010 and SFRH/BPD/107718/2015 post-doctoral grants supported by FCT.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.enpol.2018.02.047>.

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