Exposure effects of the UV-filter 4-MBC to "Solea senegalensis" metamorphosis

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Abstract

Many personal care products integrate UV-filters, such as 4-methylbenzylidene camphor (4-MBC) which has been detected in aquatic habitats. Possible effects of 4-MBC to aquatic organisms have been poorly studied. Therefore, the main objective of this work is to study the effects of 4-MBC exposure to *Solea senegalensis* during metamorphosis, a sensitive life stage of this flatfish. To achieve this, at the beginning of metamorphosis (13 days after hatching, dah) fish were exposed to 4-MBC (0.2–2.0 mg L$^{-1}$) for 48 h. After this period, fish were transferred to clean medium and were fed and maintained until more than 80% of fish in control group completed the metamorphosis (24 dah). Mortality, malformations and metamorphosis progression were studied on a daily basis. In addition, growth, behavior and biochemical markers of neurotransmission (acetylcholinesterase, AChE), oxidative stress (catalase, CAT; glutathione S-transferase, GST, and lipid peroxidation, LPO) and anaerobic metabolism (lactate dehydrogenase, LDH) were determined at the end of the experiment. An acceleration of metamorphosis progression was observed during and 2 days after the 4-MBC exposure in all concentrations tested. In addition, decreased length, inhibition of CAT activity and induction of oxidative damage (LOEC = 0.928 mg L$^{-1}$ 4-MBC for length, CAT and LPO) were observed. A short-term exposure to 4-MBC at the onset of metamorphosis, a critical period of development, affected *S. senegalensis* at several levels of organization, even after nine days in clean medium, including growth and metamorphosis progression, suggesting possible long-term adverse effects to this species.

1. Introduction

Harmful effects of UV reaching the Earth surface is leading to growing consumption of sunscreens, which results in the increasing presence of their ingredients in the environment (Lowe, 2006; Young, 2006; Young *et al*., 2017). Early life stages of coastal and marine species can be highly exposed to these chemical compounds, since most part of world population lives and uses such chemical products near aquatic habitats (Shoji *et al*., 2011; Tovar-Sánchez *et al*., 2013; Pimentel *et al*., 2015; Wen *et al*., 2017a). Organic UV filters are amongst the key ingredients of personal care products (PCPs), including sunscreens. Therefore, contamination of coastal areas by PCPs is receiving increasing attention (e.g. Tovar-Sánchez *et al*., 2013; Chisvert and Salvador, 2018; Capela *et al*., 2019). The 4-methylbenzylidene camphor (4-MBC) is one of the most used UV filters (Krause *et al*., 2012; Wang *et al*., 2016; Chisvert and Salvador, 2018). As a consequence, this compound is present in bathing areas, mainly during daylight and warmer months, reaching concentrations up to 1.043 μg L$^{-1}$ in Atlantic Gran Canaria (Sánchez-Rodríguez *et al*., 2015). 4-MBC can also reach aquatic ecosystems through the release of untreated or ineffectively treated effluents from wastewater treatment plants (Balmer *et al*., 2005; Li *et al*., 2007; Langford and Thomas, 2008; USEPA, 2012; Sánchez-Rodríguez *et al*., 2015). This highly stable and lipophilic compound, suffers low biotic degradation and has high potential for adsorption to bed sediments, presenting levels up to 7.6 ng g$^{-1}$ dry weight of 4-MBC in beach sand of Porto coast (Portugal).
(Capela et al., 2019) and is also bioaccumulated in aquatic organisms, including fish (e.g. Balmer et al., 2005; Buser et al., 2006; Gago-Ferrero et al., 2012).

Previous works have shown that 4-MBC affects development, behavior and biochemical endpoints during larval stages of amphibians (Martins et al., 2017) and freshwater (Li et al., 2016; Torres et al., 2016; Quintaneiro et al., 2019) or marine fish (Araújo et al., 2018). Endocrine action of 4-MBC has been previously reported (Krause et al., 2012; Ozáez et al., 2013; Wang et al., 2016), which included androgen and oestrogen activity and effects on reproductive organs. In addition, effects on thyroid axis of vertebrates have also been reported, which included increased thyroid weight, altered thyroid hormones levels and decreased iodide uptake (Gotthardt et al., 2007; Krause et al., 2012).

Flatfish, such as Senegalese sole (Solea senegalensis Kaup, 1858), have been increasingly used in European marine aquaculture industry (Imsland et al., 2003; Morais et al., 2016). Besides, their complex and fast life cycle are interesting for ecotoxicological studies (Pimentel et al., 2015; Pavlaki et al., 2016; Araújo et al., 2018; 2019; 2020). The eggs can be obtained from wild spawners in rearing facilities in Southern Europe throughout the year (Imsland et al., 2003; Anguis and Cañavate, 2005; Morais et al., 2016). This species typically hatch at 38 hours post fertilization (hpf) and after the thyroid-mediated metamorphosis (10 to 15 days after hatching, dah) the organisms become laterally asymmetric and benthic (Yúfera et al., 1999; Klaren et al., 2008; Sarasquete et al., 2019). The metamorphosis of S. senegalensis ends at nearly three weeks of life and is a fast and deep morphologically and biochemically changing stage in flatfish life history. Additionally, the circadian rhythm of this species also changes during metamorphosis: their activity is higher during day light before metamorphosis; while after this event the organism becomes nocturnal (Blanco-Vives et al., 2012). Previous studies have shown that metamorphosis of S. senegalensis can be affected by environmental conditions such as temperature (Campos et al., 2013) and feeding conditions (Fernández-Diáz et al., 2001). The progression of metamorphosis is directly regulated by the fluctuation of thyroid hormones levels (Yamano et al., 1991; Okada et al., 2003; Klaren et al., 2008). Therefore, chemicals might interfere with thyroid axis, namely those having structural similarity with thyroid hormones, or by interfering in their synthesis, transport and/or metabolism (Crofton, 2007; 2008; Veldhoen et al., 2006; Luthe et al., 2008; Sowers and Klaine, 2008). Flatfish metamorphosis has been reported to be affected by the exposure to chemical stressors with endocrine disrupting properties by retarding the metamorphic process (Dong et al., 2017) or increasing thyroid hormone levels and/or inducing faster metamorphosis progression (Yue et al., 2017; Araújo et al., 2019).

Behavior can be integrated with responses at lower (e.g. physiological) or higher levels of organization (e.g. populations) and is among the most sensitive endpoints with increasing use in ecotoxicology (Scott and Sloman, 2004; Vieira et al., 2009; Sloman and McNeil, 2012; Almeida et al., 2015; Henriques et al., 2016; Araújo et al., 2018; 2020; Sharma, 2019). Previous studies have shown that S. senegalensis behavior during early pelagic stage was affected by 4-MBC exposure (Araújo et al., 2018); however, the effects of the exposure to this chemical during metamorphosis, a sensitive window of flatfish development is still unknown.
Biochemical markers are commonly used on evaluation of stressors effects at molecular level. Their changes might be associated with effects at higher level of biological organization, such as behavior and fish growth and can provide information to understand the mechanisms and modes of action of stressors (Oost et al., 2003). Acetylcholinesterase (AChE) is one main neurotransmission enzymes that can work as biomarker. Organic compounds such as the UV filter 4-MBC have also been shown to inhibit AChE (Li et al., 2016). Previous results with *S. senegalensis* during early pelagic stage were unclear to associate exposure of 4-MBC with behavioral and anticholinergic activity (Araújo et al., 2018) and information about the effects of this chemical during metamorphosis progression are still lacking. The oxidative stress caused by reactive oxygen species can be detected through determination of increased antioxidant enzymes activity (such as catalase, CAT or glutathione S-transferase, GST). The study of molecular and enzymatic responses to 4-MBC exposure already showed that this compound affects the antioxidant system of different aquatic species (Gao et al., 2013; Campos et al., 2017a; Martins et al., 2017). The GST and CAT have a central role on oxidative stress response and also these enzyme levels fluctuate in response to organisms physiological or developmental stages including during metamorphosis (Oost et al., 2003; Menon and Rozman, 2007; Rudneva et al., 2010; Araújo et al., 2019). Lipid peroxidation (LPO) can be observed when antioxidant capacity of organisms is surpassed. Peroxidation can occur in lipids of cell membranes as result of exposure to of different classes of stressors (Oost et al., 2003). Under stress conditions, lactate dehydrogenase (LDH) enzyme activity can be used as biomarker since anaerobic metabolism might be switched on due to increased energy demand such as detoxification mechanisms to cope with exposure to chemical stressors (Diamantino et al., 2001; Güngördü et al., 2016; Wen et al., 2017a).

Studies on the effects of environmental contaminants such as UV-filters on key stages of life cycle of marine vertebrates, in particular during the thyroid-regulated metamorphosis of *S. senegalensis*, linking biochemical, physiological and morphological effects from sub-cellular to organism level are still lacking. In this context, the objective of this work is to study the effects of 4-MBC exposure during metamorphosis, a sensitive period of this flatfish early development, at different levels of biological organization. Effects on mortality, development, metamorphosis progression, growth, behavior and biochemical markers were evaluated.

2. Material And Methods

2.1. Chemicals

The 4-MBC was purchased from Sigma-Aldrich Co. LLC (St Louis, USA) and the ethanol used to prepare 4-MBC stock solution and solvent control was supplied by Merck. All other chemicals used in biochemical marker analysis were of analytical grade quality and were also purchased from Sigma-Aldrich Co., except the protein assay kit, which was purchased from Bio-Rad (Germany).

2.2. Biological material and experimental design
Eggs from a commercial aquaculture in north of Portugal (Safiestela/Sea8, Póvoa de Varzim) were brought to laboratory within 12 hours of fertilization and placed in a recirculating system equipped with one 25 L fiber tank, biological filtering medium, UVR sterilizer, protein skimmer. The flow rate in the main tank increased with fish age refrigeration unit set at 19ºC and room light photoperiod at 16h:8h (l:d). Medium was composed by artificial saltwater (35 of salinity, Coral Pro salt, Saudi Arabia), daily feeding included rotifers (*Brachionus plicatilis*, from 2 dah until 6 dah in increasing concentrations between 5 and 10 rotifers mL\(^{-1}\)) and *Artemia salina* nauplii and metanauplii (from 5 until 10 between 2 and 9 nauplii mL\(^{-1}\) and from 9 until 13 dah between 9 up to 35 metanauplii mL\(^{-1}\), respectively). The microalgae *Nannochloropsis gaditana* was also provided daily for improvement of maintenance conditions (Brown & Blackburn, 2013).

The beginning of the metamorphosis was evaluated in accordance with morphological features of fish (Yúfera *et al.*, 1999; Fernández-Díaz *et al.*, 2001; Araújo *et al.*, 2019), which occurred at 13 dah. At this age, fish had a total length of 4.7±0.23 mm (n=24; random measurement). Randomly selected organisms were then exposed to concentrations of 4-MBC (0.200, 0.294, 0.431, 0.632, 0.928, 1.363 and 2.000 mg L\(^{-1}\)) and respective controls in glass petri dishes (n=6, 5 fish per biological replicate/petri dish, 10 mL of testing solution per replicate). The choice of the concentration range was based on the dose-response effects of 4-MBC in earlier life stages of *S. senegalensis* from previous work (Araújo *et al.*, 2018). These concentrations are higher than expected in saltwater natural ecosystems but were applied during a short duration of exposure, chosen to fit the onset of metamorphosis and to mimic an high contaminant input during summer season.

The testing solutions were prepared by dilution of a stock solution of 4-MBC in ethanol (20 mg mL\(^{-1}\)) in artificial saltwater (Coral Pro salt). A negative control (only saltwater) and a solvent control (ethanol in saltwater at 0.01% v/v, as used in the highest tested concentration of 4-MBC) were also used. The pH of 4-MBC solutions ranged between 8.10 and 8.25 (8.06 for solvent control and 8.17 for negative control) while the salinity ranged between 33.8 and 35.5 for 4-MBC testing solutions (34.2 for solvent control and 34.6 for negative control). The bioassay was performed with an oxygen saturation level over 80%.

During a period of 48h, exposure to testing solutions was performed with no feeding of fish. After that period, fish were transferred to six well plastic plates with clean artificial saltwater medium (10 ml) and fed daily with *artemia* metanauplii until 24 dah. Artificial saltwater was also renewed daily. At 24 dah, the experiment was stopped as more than 80% of fish from negative and solvent control groups completed the metamorphosis (93±6.7% and 80±5.8% respectively). Fish were then snap frozen in liquid nitrogen and kept at -80ºC until biochemical analysis. The test was performed in a room with controlled temperature (T=19ºC). All experimental procedures were carried out following the European and Portuguese legislation concerning animal experimentation and authorized by the Portuguese competent authority (Direcção Geral de Alimentação e Veterinária, Ref. 009804).

### 2.3. Apical and developmental endpoints
Mortality, malformations, development effects and metamorphosis development stages were recorded daily during exposure to 4-MBC and until the end of the experiment with a stereoscope in all sampling groups and replicates. Metamorphosis development stages of fish were determined in accordance with literature (Dinis, 1986; Fernández-Díaz et al., 2001; Klaren et al., 2008; supplementary table S1). Length of organisms in each experimental group was determined at the end of metamorphosis (n=6, 3 fish per replicate).

2.4. Behavior analysis

Effects of 4-MBC exposure on behavior of S. senegalensis was studied at the end of the metamorphosis (at 24 dah). The Zebrabox® (Viewpoint, France) was used to record behavioral response (n=6, 1 randomly selected organism from each biological replicate) in 24 well plates with 2 mL of clean medium in each well (1 organism per well). Behavior was analysed after an initial 5 min acclimation period (light), during four alternate light (L)-dark (D) periods (LDLD) with 15 min each. Zebralab software (Viewpoint) recorded movement as total swimming duration and distance (integration periods of 1 minute). Background threshold was set at 60 pixels, and light intensity at 10% (0.26 mW cm\(^{-2}\)). For recording purposes, this device also irradiates a constant infrared light (2.30±0.11 mW cm\(^{-2}\)).

2.5. Biochemical analysis

Previously frozen samples (n=6, pools of 3-5 fish from each biological replicate) were used to measure the activity of the enzymes AChE, CAT, GST, LDH and LPO levels in fish at the end of metamorphosis. Samples were initially homogenized with potassium buffer solution (pH=7.4, 0.1M, 150 µL per organism) by sonication. An aliquot of tissue homogenate was separated into a microtube with 4 µL of 4% butylated hydroxytoluene (BHT) in methanol and was used for LPO determination by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird and Draper, 1984). The remaining homogenate was centrifuged during 20 min at 10,000 g (4ºC) and the supernatant was used for the following enzymatic analyses and protein quantification. AChE activity was quantified by using acetylthiocholine as substrate and 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) as chromogen and measuring the increase of absorbance at 414 nm (Ellman et al., 1961; Guilhermino et al., 1996). CAT activity was determined by measuring the rate of hydrogen peroxide (H\(_2\)O\(_2\)) consumption at 240 nm (Clairborne, 1985). LDH activity was determined following the methodology of Vassault (1983) with the modifications introduced by Diamantino et al. (2001) by measuring the conversion of pyruvate to L-lactate with the concomitant conversion of NADH to NAD\(^+\) during glycolysis which is monitored at 340 nm. GST activity was measured following the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig and Jakoby, 1981; Frasco and Guilhermino, 2002). The protein concentration was determined in triplicate according to the Bradford method (Bradford, 1976), adapted to microplate from BioRad’s Bradford protein micro-assay kit, using bovine γ-globuline as a standard and a wavelength of 595 nm. The enzymatic activity is expressed in Units (U) per mg of protein where one U is a nmol of substrate.
hydrolyzed per minute, using a molar extinction coefficient of 13.6x10^{3} M^{-1} cm^{-1} for AChE and 9.6x10^{3} M^{-1} cm^{-1} for GST and U is one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1} for CAT and 6.3x10^{3} M^{-1} cm^{-1} for LDH. The LPO is expressed in nmol of TBARs hydrolyzed per mg protein using a molar extinction coefficient of 1.56x10^{5} M^{-1} cm^{-1}. Spectrophotometric determinations were performed in 96 well microplates (3-4 technical replicates per sample) using a Labsystem Multiskan EX.

2.6. Statistical analysis

Three-parameter logistic regression were performed for mortality and malformations at 14, 15 and 24 dah. After initial t-test for testing existence of differences between solvent and negative control, One-way ANOVA were used to verify differences between solvent control and 4-MBC treatment groups for length, biochemical effects and behaviour (total duration and distance of swimming). When normality or equality of variance of were not achieved, Kruskal-Wallis test (one-way non-parametric ANOVA) was used. When significant differences were found, post-hoc Dunnett’s pairwise tests against solvent control were performed. Further statistical analysis of behaviour was performed, testing the effect of 4-MBC concentrations and the response to light and dark cycles with two-way Repeated Measures ANOVA.

Effects of 4-MBC on metamorphosis progression were studied using Chi-Square test, followed by pairwise Chi-square test for detection of significant differences between 4-MBC groups and solvent control, with Bonferroni adjustment (Arnholt, 2016).

Sigmplot v.12.5 (Systat Software, Inc.) was used for all statistical procedures and all results are expressed as mean±standard error.

3. Results

3.1. Mortality and malformations

Fish mortality was 0% in solvent and negative controls along the test. Increasing mortality was observed in fish groups during exposure to 4-MBC (fig. 1A). The highest mortality occurred in fish exposed to 2.000 mg L^{-1} 4-MBC at 15 dah (70.0±8.56%), which after changing to clean medium, did not increase until the end of metamorphosis at 24 dah. At the end of metamorphosis, the LC_{20} was 1.442 mg L^{-1} (95% confidence interval, c.i.: 1.234-1.816, Supplementary table S2).

No malformations were observed in fish from both controls groups during exposure to 4-MBC and until complete metamorphosis. Alterations to normal fish development were observed in fish exposed up to 2.000 mg L^{-1} of 4-MBC, mainly during the 48h exposure period with a LC_{20} of 0.883 (c.i.: 0.617-1.219) mg L^{-1} and 1.044 (c.i. 0.750-1.399) mg L^{-1} at 14 dah and 15 dah, respectively (fig. 1B). The malformations decreased after nine days in clean media (LC_{20} at that time was over 1.363 mg L^{-1}) while mortality did not
increase during the same period. Malformations observed refer to the simultaneous alterations on skin pigmentation, fin and abdominal cavity (fig. 2). Only fish groups exposed up to and including 1.363 mg L$^{-1}$ were used to assess the remaining endpoints.

### 3.2. Metamorphosis

Significant differences between negative and solvent control fish were observed when analysing the distribution of relative percentages of fish in each metamorphic stage (A to G) with solvent control fish constantly presenting retarded metamorphic progress comparing to negative control at 14, 17, 20, 22 and 24 dah (p<0.05, Chi-square test, fig. S1).

Significant differences on relative percentages of fish in each metamorphic stage were observed between solvent control and all groups of fish exposed to 4-MBC at 14 dah (24h of 4-MBC exposure) and 17 dah (2 days after the end of 4-MBC exposure), with 4-MBC exposed fish groups presenting faster metamorphosis progression than fish in solvent control group (p<0.05, fig. 3). However, at 20 dah and afterwards, such faster development is absent and fish exposed to the highest 4-MBC concentration tested (1.363 mg L$^{-1}$) presented even a retarded metamorphic progress compared to solvent control fish (p<0.05). In addition, at 22 and 24 dah, fish exposed to 0.431 mg L$^{-1}$ 4-MBC presented also retarded metamorphic progress compared to fish from solvent control (p<0.05).

### 3.3. Length

There were no significant differences on total length of fish between solvent control (8.6±0.14 mm) and negative control (8.6±0.21 mm) at the end of metamorphosis, at 24 dah (p>0.05). Exposure to 4-MBC induced a significant decrease in length of fish exposed to the two highest concentrations tested, 0.928 mg L$^{-1}$ and 1.363 mg L$^{-1}$, with fish presenting 6.9% and 11.6% of reduction in length in relation to fish in solvent control, respectively (p<0.05, fig. 4).

### 3.4. Behavior

When analysing the effect of 4-MBC on behaviour at 24 dah during the 60 min. in each 15 min. light or dark periods, significant differences on swimming duration and distance between controls and between solvent control and 4-MBC treatment groups were not observed (p>0.05, fig. S2). Nonetheless, light absence seems to induce a general significant increasing trend for swimming distance (p<0.05). The interaction of both factors (4-MBC and light/dark) for both parameters (swimming distance and duration) was not significant (p>0.05).

No significant differences were observed between negative and solvent controls on total duration of swimming (19.8±4.75 and 18.2±3.08 min, respectively) and total swimming distance (70.2±11.79 and 55.4±12.66 m, respectively, p>0.05). An increasing trend in the total swimming duration and distance was
observed on fish exposed to the lowest 4-MBC concentrations tested, with significantly higher swimming distance registered in fish exposed to 0.294 mg L\(^{-1}\) of 4-MBC when comparing to solvent control (p<0.05, fig. 5).

3.5. **Biochemical markers**

The effects of 4-MBC exposure on *S. senegalensis* biochemical markers analysed at the end of metamorphosis are presented in fig. 6. No significant differences were obtained for AChE, CAT, GST enzymatic activities and LPO levels between fish from negative and solvent control groups (p>0.05). On the contrary, LDH activity of fish from solvent control group (0.172±0.0023 U mg of protein\(^{-1}\)) was significantly lower than in fish from negative control (0.190±0.0042 U mg of protein\(^{-1}\), p<0.05).

Exposure to 4-MBC did not induce alterations in AChE, GST nor in LDH activity of fish exposed to 4-MBC when comparing with fish in solvent control group (p>0.05).

Considering the oxidative stress biomarkers analysed, exposure to 4-MBC inhibited the activity of CAT, with significant lower activity recorded in the two highest concentrations tested, namely in 0.928 mg L\(^{-1}\) and 1.363 mg L\(^{-1}\) of 4-MBC, corresponding to a decrease in activity of 19.8% and 26.1%, respectively (p<0.05). Fish exposed to these concentrations also presented significantly higher LPO levels, corresponding to an increase of 89.7% and 77.1%, respectively, in relation to solvent control (p<0.05).

4. **Discussion**

In the present work, we aimed at studying effects of exposure to 4-MBC during *S. senegalensis* metamorphosis at different levels of organization. Metamorphosis is a high demanding energetic and sensible period in the development of flatfish species. Therefore, exposure to 4-MBC during a relatively short period of time, concomitantly to the onset of metamorphosis represents an additional challenge to the normal development of sole. Although, the exposure to lower concentrations for longer periods would be more realistic, the studied 4-MBC concentrations and conditions provided relevant insights on mechanisms of toxicity and recovery of sole early life stages. Indeed, deleterious effects were observed during exposure to 4-MBC (table 1), but also at the end of metamorphosis, nine days after the exposure ended, suggesting lasting and/or lagged effects of 4-MBC in this species.

4.1. **Apical and developmental endpoints**

The mortality observed with *S. senegalensis* exposed to 4-MBC at the onset of metamorphosis indicate that *S. senegalensis* in this life stage is less sensitive to 4-MBC than earlier life stages. In a previous work with *S. senegalensis* eggs, after 48h exposure to 0.935 mg L\(^{-1}\) of 4-MBC (highest concentration tested) mortality was 40.0±16.96% and a 96h-LC\(_{50}\) of 0.439 mg L\(^{-1}\) 4-MBC was obtained (Araújo *et al*., 2018). Whereas, in the present work, the mortality observed in metamorphosing sole was lower than 15% for
concentrations up to 1.363 mg L\(^{-1}\) (48h exposure). Nevertheless, metamorphosing \(S.\ senegalensis\) is more sensitive to 4-MBC than other aquatic vertebrate species, including zebrafish embryos, as an 72h-LC\(_{50}\) of 5.042 mg L\(^{-1}\) 4-MBC was reported by Li et al. (2016) and concentrations up to 1.3 mg L\(^{-1}\) 4-MBC did not affect survival of the frog \(Pelophylax\ perezi\) larvae during 144h exposure (Martins et al., 2017).

The exposure to stressors can induce alterations on metamorphosis progression and lead to permanent or short-temporal abnormalities such as lack and/or erratic eye migration or bone deformities in metamorphosing \(S.\ senegalensis\) (Araújo et al., 2019). In the present work, 4-MBC induced malformations to \(S.\ senegalensis\), which have occurred during the initial 48h exposure period of the bioassay. Such malformations might be related with the effects observed on metamorphosis progression and fish growth, as the total number of organisms that completed metamorphosis and their total length were still affected by 4-MBC exposure by the end of metamorphosis, at 24 dah. Delayed development caused by 4-MBC exposure has been reported in amphibian larvae (Martins et al., 2017). Such effects of 4-MBC on normal mechanisms of development of aquatic vertebrate species still needs further study.

A faster progression of metamorphosis was observed in fish exposed to 4-MBC at the beginning of the metamorphic phase, during the exposure and immediately after the two day exposure. Despite the solvent used (ethanol at 0.01% v/v) delayed metamorphosis progression of \(S.\ senegalensis\) in relation to negative control, the exposure to some concentrations of 4-MBC induced a further progression delay at the end of metamorphosis when comparing with solvent control. Normal progression of flatfish and anuran metamorphosis are known to be affected by the exposure to other chemicals, some of them with endocrine disruptor potential (e.g. Dong et al., 2017; Yue et al., 2017; Araújo et al., 2019). For instance, our previous work with \(S.\ senegalensis\) using a similar experimental design, showed that the exposure to the wide spectrum biocide triclosan (TCS) induced an immediate acceleration of the metamorphosis while no effects were observed at the end of metamorphosis (Araújo et al., 2019). While TCS is reported to have a similar structure to the thyroid hormone thyroxine, since both are halogenated biphenyl ethers (Crofton et al., 2007), such similarity is not so clear with 4-MBC. Nevertheless, specific effects by 4-MBC on organs or tissues associated with thyroid function have also been previously reported and associated with pro or anti-thyroid action (Gotthardt et al., 2007; Schmutzler et al., 2007; Wang et al., 2016). In addition, it should also be mentioned that in the present work, some fish groups exposed to 4-MBC presented a lower rate of fish with complete metamorphosis at 24 dah when comparing to control groups, indicating lasting effects of this chemical since exposure to this chemical was interrupted nine days earlier. Therefore, the mechanism by which this occurs needs to be studied in order to verify if there is any relation with a medium or long-term possible interference in the thyroid axis, which regulates metamorphosis in sole. Furthermore, the morphological effects of 4-MBC on metamorphosis indicate possible occurrence of lasting ecological implications that can be anticipated as severe and should be further studied. Longer exposure periods to lower concentrations of 4-MBC (environmental relevant) may affect differently development and metamorphosis progression and should be considered in further studies.

Growth of \(S.\ senegalensis\) was affected in the present study in response to exposure to 4-MBC. Growth of \(S.\ senegalensis\) was also affected by 4-MBC after 96h exposure period carried out in an earlier life stage
with the same species (Araújo et al., 2018). The lowest observed effect concentration (LOEC) on length in the earlier life stage was 0.360 mg L\(^{-1}\) 4-MBC and – 0.928 mg L\(^{-1}\) 4-MBC in present test conditions (48h exposure followed by 9 days in clean medium). This results seem to follow the generally observed decrease of sensitivity to chemicals with fish increasing age which was also observed in *S. senegalensis* with another chemical stressor, namely TCS (Araújo et al., 2019). A short exposure period of exposure to 4-MBC was performed (48h) in the present study than in earlier stage test (96 h) (Araújo et al., 2018) and longer periods of exposure to lower 4-MBC levels could induce further decrease of the LOEC. In addition, attention should also be given to the fact that length measurement was only performed nine days after maintenance in clean medium and feeding, demonstrating lasting effects of this chemical on the normal development of this species, which can have further implications at higher levels of organization.

### 4.2. Behavior

In the present work, effects of 4-MBC on *S. senegalensis* behavior were observed at the end of the metamorphosis. Namely, for the lower concentrations tested (0.294 mg L\(^{-1}\) 4-MBC) an increase of fish swimming behavior was observed, even after nine days in clean medium after the exposure to 4-MBC. However, in previous studies a distinct effect of 4-MBC on behavior was observed in earlier life stages of sole (Araújo et al., 2018); a significant decrease on total sole swimming time was observed in fish exposed to the highest concentration tested, 0.360 mg L\(^{-1}\) 4-MBC (Araújo et al., 2018). In addition, Li et al. (2016) have reported an impairment of swimming of zebrafish embryos with exposure to 3.81 mg L\(^{-1}\) 4-MBC.

Different distinct activity patterns are expected to be observed on *S. senegalensis* depending on life stage, with metamorphosis inducing a change to higher nocturnal activity while early larvae are diurnal (Bayarri et al., 2004; Blanco-Vives et al., 2011, 2012). The different swimming activity pattern of metamorphosing sole in response to 4-MBC in relation to earlier life stages, might be due to the distinct behavioral patterns of each life stage of *S. senegalensis*.

Swimming is a major component of energy expenditure for many fishes (McKenzie, 2011). The increased energy used by *S. senegalensis* exposed to lower 4-MBC through increased swimming distance can be associated with effects on energetic budget. In fact, effects of chemical stressors on energetic balances have already been reported (Agbohessi et al., 2014; Rabasa and Dickson, 2016; Anacleto et al., 2018). Such excitatory swimming response after 4-MBC exposure may also have later implications on growth or successful feeding and reproduction.

### 4.3. Biochemical markers

Biochemical endpoints were studied with post-metamorphic *S. senegalensis* nine days after exposure to 4-MBC. In the present study, effects on AChE activity levels were not detected at the end of the experiment after exposure to 4-MBC. Similarly, previous studies also reported the inexistence of neurotoxic effects of
this chemical in other species, namely on freshwater caddisfly Sericostoma vittatum (Campos et al., 2017a), aquatic midge Chironomus riparius (Campos et al., 2017b) and embryos of the amphibian Pelophylax perezi after exposure to 4-MBC (Martins et al., 2017). On the contrary, induction of AChE by 4-MBC was observed in Solea senegalensis early life stages (Araújo et al., 2018) and in zebrafish embryos (Quintaneiro et al., 2019). Such diverse responses should be further studied as 4-MBC is a camphor derivative and camphor’s belong to the terpenes family, which are considered AChE inhibitors (Ahmed et al., 2013; Li et al., 2016). Additionally, 4-MBC exposure seem to induce different AChE activity patterns depending on the life stages of S. senegalensis (Araújo et al., 2018), and this was also observed with TCS exposure, while no effects were observed on sole 3 dah-larvae, an induction of AChE was observed in metamorphosing larvae (Araújo et al., 2019), reflecting different biochemical responses.

Alterations of AChE (whether as inhibition or induction) under stress conditions can be associated with effects at behavioral level (Mach et al., 2004; Rao et al., 2005; García-de-la-Parra et al., 2006; Li et al., 2016; Araújo et al., 2018). However, in the present work, the increase in swimming activity observed with 4-MBC cannot be related with an alteration in AChE activity. The 4-MBC increased fish swimming distance at 0.294 mg L$^{-1}$, suggesting an excitatory effect at the lower concentrations tested, but no significant alteration on AChE was detected, despite the slightly higher activity registered in all 4-MBC concentrations.

In the present study, CAT activity was inhibited in S. senegalensis exposed to the highest concentrations of 4-MBC tested and increased LPO levels occurred at the same concentrations, indicating the occurrence of oxidative damage. During metamorphosis of amphibians (Tata, 1994; Kashiwagi et al., 1999) and flatfish (Fernández-Díaz et al., 2006; Yu et al., 2006; Klaren et al., 2008; Power et al., 2008; Sun et al., 2015; Araújo et al., 2019), CAT levels are expected to fluctuate in response to programmed cell death. The exposure to 4-MBC induced effects on S. senegalensis, inhibiting normal CAT activity levels by the end of fish metamorphosis, reflecting an impairment of the role of this enzyme in the antioxidant system, which might have contributed to the oxidative damage observed in lipids. Since our results indicate lasting oxidative damage by 4-MBC, even after 9 days in clean medium, awareness should be given to the fact that effects at higher levels of biological organization might appear or become observed in later life stages of these organisms (e.g. later juveniles or adult stages), which might impair their fitness (e.g. decreased resistance to disease, or feeding and preying success). Similarly to our results, exposure to 4-MBC enhanced LPO levels in embryos of the frog P. perezi (Martins et al., 2017). However, no alterations in lipid peroxidation levels were observed after 4-MBC exposure in caddisfly Sericostoma vittatum since ROS detoxification might have occurred in this species as suggested in Campos et al. (2017a).

Previous studies with amphibian and zebrafish early life stages showed that GST activity was increased just after 4-MBC exposure (Martins et al., 2017; Quintaneiro et al., 2019). In the present work, GST levels were not affected. This might be associated with the period of fish maintenance in clean medium after 4-MBC exposure before the biochemical analysis. Therefore, possible GST alterations in response to 4-MBC immediately after the 48h exposure might have occurred, followed by a return to control levels at the end of metamorphosis.
Variation of LDH can occur in response to changes on environmental conditions. Chemical stressors can also lead to LDH changes, which are related with toxicity effect on animal tissues and cells. Considering the effect of the solvent treatment on sole LDH, namely the observed inhibition of LDH activity in solvent control fish when comparing to negative control fish, this might be related with the fact that ethanol is a metabolic product that prevents lactate accumulation (Torres et al., 2012). Furthermore, when considering 4-MBC effects, and similarly to the results obtained in a toxicity test performed at earlier stages of *S. senegalensis* (Araújo et al., 2018) and a test using *P. perezi* embryos (Martins et al., 2017), no significant effect of 4-MBC on LDH activity was observed. These results suggest that 4-MBC exposure during the *S. senegalensis* early metamorphosis apparently do not induce alterations on anaerobic metabolism.

4.4. Conclusions

The present work showed that a 48h exposure to 4-MBC during the onset of metamorphosis affected *S. senegalensis* at different levels of biological organization, and part of these effects were observed even after nine days in clean medium. An acceleration of metamorphosis in all concentrations tested was observed during and just after the end of the exposure. Whereas, by the end of metamorphosis, 4-MBC showed in general to be able to retard metamorphosis progression, induce oxidative damage and inhibit growth in the highest concentrations tested in *S. senegalensis*. This indicates long-term effects of short exposure to this UV-filter during a critical period in the development of this marine species, which might have further implications at higher levels of organization. Moreover, the interference of 4-MBC in the progression of metamorphosis should be further studied in order to understant if its mode of action is through the interference in the thyroid axis of this flatfish model.

Declarations

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Ethics approval and consent to participate

- Not applicable
Consent for publication

- Not applicable

Availability of data and materials

- All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

- The authors declare that they have no competing interests

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Authors' contributions

- Mário J. Araújo: Conceptualization, Methodology, Investigation, Writing- Original draft preparation.
- Amadeu M.V.M. Soares: Supervision, Funding acquisition, Project administration.
- Marta S. Monteiro: Conceptualization, Investigation, Writing- Reviewing and Editing.

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**Tables**

Table 1. Effect concentration (EC), no observed (NOEC) and lowest observed (LOEC) effect concentration of 4-MBC during metamorphosis of *Solea senegalensis*. Exposure to 4-MBC was performed at 13 days after hatching (dah) during 48h. Endpoints were analysed during and/or at the end of exposure (14 and 15 dah) and during maintenance in clean medium until complete metamorphosis (24 dah). Concentrations under square brackets express the 95% confidence interval of the three parameter logistic regression. CAT – catalase, LPO – lipid peroxidation.
| Endpoint                                      | EC_{20}         | NOEC          | LOEC          |
|----------------------------------------------|-----------------|---------------|---------------|
| Mortality                                    |                 | (mg L^{-1})   | (mg L^{-1})   |
| 14 dah                                       | 1.606 [1.389-1.886] | -             | -             |
| 15 dah                                       | 1.547 [1.373-1.923] | -             | -             |
| 24 dah                                       | 1.442 [1.234-1.816] | -             | -             |
| Malformations                                |                 |               |               |
| 14 dah                                       | 0.883 [0.617-1.219] | -             | -             |
| 15 dah                                       | 1.044 [0.750-1.399] | -             | -             |
| Metamorphosis progression (14, 17 dah)       | -               | -             | 0.200         |
| Growth (24 dah)                              | -               | 0.632         | 0.928         |
| Biochemical markers – CAT, LPO (24 dah)      | -               | 0.632         | 0.928         |