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**2019**

**Mário Jorge Faria dos Santos Araújo**

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Marta Sofia Soares Craveiro Alves Monteiro dos Santos, Investigadora do CESAM e Departamento de Biologia da Universidade de Aveiro.

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Aos meus pais

**o júri**

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**palavras-chave**

4-metilbenzilideno cânfora, comportamento, desenvolvimento larvar, ecotoxicologia aquática, efeitos combinados, genes do eixo da tiroide, linguado branco, marcadores bioquímicos, metamorfose, peixe marinho, radiação ultravioleta, triclosan.

**resumo**

O aumento da radiação ultravioleta (UV) e a utilização crescente de produtos de cuidado pessoal (PCP) têm sido apontados como duas das ameaças derivadas da ação humana que podem afetar espécies aquáticas. Os estadios larvares de linguado branco, *Solea senegalensis,* Kaup 1858 estão entre os vertebrados marinhos potencialmente afetados por estes stressores. Assim, o objetivo deste trabalho é estudar, ao longo do desenvolvimento larvar do linguado, os efeitos simples e combinados de radiação UV e de dois ingredientes de PCP, o biocida de largo espectro triclosan (TCS) e o filtro UV 4-metilbenzilideno-cânfora (4-MBC).

Foram realizados testes de exposição a TCS, 4-MBC e UV em duas fases do desenvolvimento larvar do linguado: desde ovo até aos 3 dias após eclosão (aproximadamente 96 horas pós-fertilização) e ao longo das 48 h iniciais da metamorfose (tipicamente ao décimo terceiro dia após a eclosão) com avaliação do desenvolvimento até ao final da metamorfose (cerca de dez dias mais tarde). Estudaram-se efeitos ao nível do indivíduo (sobrevivência, malformações, crescimento, comportamento e progressão da metamorfose) que se tentaram ligar com efeitos a níveis organizacionais inferiores (marcadores bioquímicos e moleculares).

De um modo geral, a resposta à exposição a níveis sub-letais dos diferentes stressores indica que o estadio larvar inicial é mais sensível que a fase da metamorfose. No final da primeira fase de desenvolvimento estudada, a exposição a cada um dos stressores causou diminuição do crescimento e induziu malformações. A nível bioquímico, a exposição a 4-MBC e UV não afetou a atividade colinérgica de forma evidente, no entanto observaram-se alterações a nível comportamental, nomeadamente uma diminuição da natação. Adicionalmente, nesta fase, observou-se a indução da GST pelo TCS (LOEC=30 µg $L^{-1}$ TCS), sugerindo ativação da via biotransformação II para destoxificação. Da análise dos efeitos dos stressores químicos no final da metamorfose, verifica-se que estes causam efeitos mesmo após manutenção durante nove dias em meio limpo, nomeadamente a nível de stress oxidativo e diminuição do crescimento. Adicionalmente, ambos os stressores químicos causaram uma aceleração da progressão da metamorfose durante a exposição e também nos momentos imediatamente subsequentes. Esta resposta sugere uma possível ação direta ou indireta destes stressores no eixo da tiroide. No caso do TCS, esta ação foi verificada através da observação da sub-expressão de genes do eixo da tiroide (NIS e TSHβ), que se prolongou até ao final da metamorfose num dos genes analisados (NIS). Verificou-se ainda uma sub-expressão de genes tanto imediatamente após a exposição aos UV (THRβ, Tpo e NIS) como no final da metamorfose (NIS). Estas alterações podem estar relacionadas com o dano oxidativo nos tecidos da tiroide causado diretamente pelos UV, o que pode levar à disfunção desta glândula. No entanto, esta sub-expressão dos genes da tiroide dos organismos expostos aos UV não foi acompanhada por alteração da progressão da metamorfose ao

nível do indivíduo. Globalmente, os resultados sugerem que a resposta do linguado ao TCS não foi alterada pela exposição combinada com UV.
De um modo geral, os resultados obtidos neste trabalho sugerem que os stressores estudados poderão ter impacto no desenvolvimento e na performance ecológica da espécie. Adicionalmente, tendo em conta os modos de ação dos stressores e os efeitos relevantes observados, deverão ser realizados estudos em cenários mais realistas, nomeadamente, por períodos de tempo mais prolongados e utilizando concentrações/doses ambientalmente relevantes. A utilidade de estadios larvares do linguado como modelos na avaliação de efeitos de stressores em ambiente marinho foi confirmada, sendo que o período inicial até aos três dias pós-fertilização se enquadra como modelo alternativo em experimentação animal.

**keywords**

4-methylbenzylidene-camphor, aquatic ecotoxicology, behavior, biochemical markers, combined effects, genes of thyroid axis, larval development, marine fish, metamorphosis, Senegalese sole, triclosan, ultratiovet radiation.

**abstract**

The increase of ultraviolet radiation (UV) and the increasing use of personal care products (PCP) are referred as two of the main anthropogenic-driven threats that can affect aquatic species. The early larval stages of Senegalese sole, *Solea senegalensis*, Kaup 1858 are among the marine vertebrates potentially affected by these stressors. Therefore, the objective of this work is to study the single and combined effects of UV and two PCP ingredients along early development of the sole, the wide-spectrum biocidal triclosan (TCS) and the UV filter 4-methylbenzylidene camphor (4-MBC).

The TCS, 4-MBC and UV exposure tests were performed on two stages of larval development: from egg to 3 days after hatching (approximately 96 hours post-fertilization) and throughout the initial 48 h of metamorphosis (typically near the thirteenth day after hatching) with evaluation of the development until the end of the metamorphosis (about ten days later). Effects at the individual level (survival, malformations, growth, behavior, and progression of metamorphosis) were linked with effects at lower organizational levels (biochemical and molecular markers).

In general, the response to the exposure to sub-lethal levels of different stressors indicates that the initial larval stage is more sensitive than the metamorphosis stage. At the end of the first stage of development, exposure to the stressors caused a decrease in growth and induced malformations. At biochemical level, the exposure to 4-MBC and UV did not affect the cholinergic activity in an evident manner, however changes were observed at behavioral level, namely a decrease of swimming activity. Additionally, at this stage, the GST was induced by TCS (LOEC = 30 μg $L^{-1}$ TCS), suggesting activation of the phase II of biotransformation for detoxification. At the end of the metamorphosis, effects of chemicals exposure were observed even after maintenance in clean medium for nine days, namely alterations on antioxidant system and decrease of growth. Additionally, both chemical stressors caused an acceleration of the metamorphosis progression during exposure and also in the immediately subsequent moments. This response suggests a possible direct or indirect action of these stressors on thyroid axis. In the case of TCS, this action was verified through the observation of the down-regulation of thyroid axis genes (NIS and TSHβ), which lasted until the end of metamorphosis in one of the analyzed genes (NIS). There was also a down-regulation of genes both immediately after exposure to UV (THRβ, Tpo and NIS) and at the end of metamorphosis (NIS). The changes may be related to oxidative damage in thyroid tissues caused directly by UV, which can lead to dysfunction of this gland. However, this under-expression of the thyroid genes of UV-exposed organisms was not followed by alterations in the progression of metamorphosis at individual level. Overall, the results obtained suggest that the sole response to TCS was not altered when in combination with UV.

In general, the results obtained in the present study suggest that exposure to

stressors may have an impact on the development and ecological performance of the species. Additionally, taking into account the modes of action of the stressors and the relevant effects observed, studies in more realistic scenarios should be performed, namely, considering longer periods of exposure using environmentally relevant concentrations/doses. The usefulness of larval stages of sole as a model species to evaluate stressors effects in the marine environment was confirmed, with the initial early period up to the third day after fertilization being a suitable alternative model for animal testing.

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| | |
|---|---|
| 4-MBC | 4-methylbenzylidene-camphor |
| AChE | acetylcholinesterase |
| AcSCh | acetylthiocholine iodide |
| BHT | butylated hydroxytoluene |
| BuSCh | s-butyrylthiocholine iodide |
| BW284C51 | 1,5-bis(4-allyldimethylammonimphenyl) pentan-3-one dibromide |
| CAT | catalase |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| ChE | cholinesterase |
| Dah | days after hatching |
| DNA | deoxyribonucleic acid |
| DTNB | 5-5'-dithiobis (2-nitrobenzoic acid) |
| EC | effect concentration |
| EDC | endocrine disruptor compound |
| Eserine | eserine hemisulfate |
| FET | fish embryo test |
| GSH | glutathione |
| GST | glutathione s-transferase |
| hpf | hours post fertilization |
| HPLC | high-performance liquid chromatography |
| IPCC | Intergovernmental Panel on Climate Change |
| iso-OMPA | tetraisopropyl pyrophosphoramide |
| LC | lethal concentraion |
| LDH | lactate dehydrogenase |
| LOEC | lowest observed effect concentration |
| LPO | lipid peroxidation |
| MCT8 | monocarboxylate transporter 8 |
| mRNA | messenger RNA |
| NAD | nicotinamide adenine dinucleotide |
| NIS | sodium-iodide symporter (SLC5A5, Na+I− symporter) |
| NOEC | no observed effect concentration |
| OECD | Organization for Economic Cooperation and Development |
| PCP | personal care products |

| | |
|---|---|
| PrSCh | propionylthiocholine |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| SPE | solid-phase extraction |
| T3 | triiodothyronine |
| T4 | thyroxine |
| TBARs | thiobarbituric acid-reactive substances |
| TCS | triclosan |
| Tg | thyroglobulin |
| TH | thyroid hormone |
| THRαA | thyroid hormone receptor αA |
| THRβ | thyroid hormone receptor β |
| TPO | thyroid peroxidase |
| TSHβ | thyroid stimulating hormone β |
| Ubq | ubiquitin |
| UV | ultraviolet radiation |
| WWTP | wastewater treatment plant |

# Chapter 1. General introduction – use of early life stages of marine fish for the study of anthropogenic-driven threats

# General Introduction

## Use of early life stages of marine fish for the study of anthropogenic-driven threats

### 1. Natural ecosystems on a changing world

Anthropogenic-driven activities have been reported to cause disturbances on natural ecosystems, including the decrease of habitat quality, species abundance and their distribution ranges. According with the Organization for Economic Cooperation and Development (OECD), the production of chemicals, including the ones classified as hazardous, is foreseen to increase, leading to their intentional or accidental, direct or indirect release to natural habitats at global scale with impacts on all living organisms (OECD, 2012). In addition, abiotic stressors, such as temperature, acidification or UV have also been altered as a reflex of the increase of human population and industrial activities. Since the earliest versions, the Assessment Reports of Intergovernmental Panel on Climate Change (IPCC) refer increasing “discernable human influences” on climate affecting both land and aquatic ecosystems (IPCC, 1990, 1996, 2007). Climate change scenarios, which are far from being fully understood, might be intensified if associated with the increase of industrial activities all over the world (Solomon *et al*., 2008; Hay *et al*., 2016). World-wide scale climatic shifts have already been pointed as responsible for several impacts on biodiversity across all ranges of habitats (Walther *et al*., 2002).

Aquatic habitats are the amongst the most threatened ecosystems. Effects of stressors on aquatic species have been widely reported, which include changes in bioenergetics and adaptive capacities of organisms (Holt and Jørgensen, 2015), physical transport of species, mainly small-size species and larval stages of larger species (Wespestad *et al*., 2000), trophic structures and dynamics and corresponding levels of primary and secondary production and changes of distribution ranges, habitat quality and biodiversity in sensitive habitats (Walther *et al*., 2000). Up-to-date information on concentrations, effects and adequate monitoring of chemical compounds on natural ecosystems at global scale has been a challenge (Walther, 2000; Douda *et al*., 2013; Garcia *et al*., 2014; Tornero and Hanke, 2016).

Coastal habitats have high productivity, providing resources for human exploration; however they are particularly vulnerable areas on a changing world perspective (Teal *et al*., 2008; Constanza *et al*., 2014; Manciocco *et al*., 2014). As higher density populated areas

are typically located near riverine systems and seashore, there has been increasing release of Personal Care Products (PCP) and other household products into marine ecosystems as direct or indirect discharges (including the ones from wastewater treatment plants without adequate treatments) lead to the exposure of organisms to these chemicals on the coastal environments (Kumar *et al*., 2010; Dhillon *et al*., 2015; Díaz-Garduño *et al*., 2018).

### 1.1. Chemical stressors

Aquatic species from marine, coastal and transition habitats can be directly exposed to chemicals from several sources. In addition, the exposure and consequences of some chemicals may not be immediate as they can be adsorbed in sediments and/or re-suspended depending on climate conditions and/or seasonal hydrodynamic patterns. Such chemicals can bioaccumulate, act as neurotoxicants and induce endocrine disruption even when present at low concentrations (Díaz-Cruz and Barceló, 2009; Gago-Ferrero *et al*., 2012; Krause *et al*., 2012; Ruszkiewicz *et al*., 2017a,b).

Some of these chemicals are considered endocrine disruptor compounds (EDCs). This means that they can interfere with production, release, transport, metabolism, binding, action, or elimination of natural hormones, which are responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock *et al*., 1996). Present knowledge is mainly focused on vertebrates, particularly mammals. Effects of EDCs on sexual development and reproduction have received particular attention (Handy, 2003; Coster and Larebeke, 2012). Estrogen receptor agonists cause alterations in reproduction or development through effects on the hypothalamic-pituitary-thyroidal or hypothalamic-pituitary-gonadal (HPG) axes (Kavlock *et al*., 1996; Ankley *et al*., 2009). EDCs may affect growth and development of many other organisms. Effects on thyroid and metamorphosis of amphibians have been reported (Veldhoen *et al*., 2006; Sowers and Klaine, 2008). Thyroid functioning has also a key-role during early life stages on flatfish, namely on the onset and development of metamorphosis (Klaren *et al*., 2008; Fernández-Díaz *et al*., 2001). In addition, the exposure to EDC might not cause immediate visible damaging effects on the organisms and long term sub-lethal exposure to EDCs might also have cascading effects. In fact, previous works have also reported ecologically relevant effects of EDCs from a sub-organism scale to population level (Scholz and Mayer 2008; Scholz *et al*., 2013; Windsor *et al*., 2018).

### 1.1.1. Triclosan

The modern lifestyle encourages frequent use of soaps and detergents for hygiene and asepsis purposes, defending users against bacteria and transmission of other pathogenic microbes. Therefore, the broad class of products known as Personal Care Products (PCPs) frequently includes chemicals such as antibacterial substances which can also have toxic effects for organisms in their natural ecosystems.

Triclosan (5-cloro-2-(2,4-diclorofenoxi)-fenol, TCS) is one of the most commonly used antibacterial compounds and has been reported to occur frequently in aquatic habitats with concentrations up to 0.3 µg $L^{-1}$ in transition waters and 0.1 µg $L^{-1}$ in sea water (Kolpin *et al*., 2002; Rodricks *et al*., 2010; Olaniyan *et al*., 2016). It belongs to the chemical family of chlorophenols, a group of compounds with varied applications, from phytosanitary/pesticide use, pharmacological, household products, and industry, and are relatively present in aquatic habitats (Czaplicka *et al*., 2004).

Despite there is no widely accepted information on the mechanisms of action of chlorophenols, phase II biotransformation have been described for TCS (Peng *et al*., 2013; Ding *et al*., 2018). However, an alternative phase I biotransformation have also recently been proposed (Ashrap *et al*., 2017). Nevertheless, TCS and its metabolites affect aquatic organisms at individual and also at subcellular level. Oxidative stress induction has been reported after TCS exposure in amphibians (Liang *et al*., 2013; Martins *et al*., 2017) and freshwater fish (Oliveira *et al*., 2009; Falisse *et al*., 2017). Besides, TCS is also pointed to interfere with endocrine functioning in several life stages of aquatic vertebrates which can result from its similar molecular structure with thyroid hormones (Ishibashi *et al*., 2004; Veldhoen *et al*., 2006; Luthe *et al*., 2008; Oliveira *et al*., 2009; Pinto *et al*., 2012; Marlatt *et al*., 2013; Sahu *et al*., 2018). Neurotoxicity of TCS have been previously reported through activation of apoptosis of neuronal cells and the induction of protein kinases with associated adverse effects on cell survival (Ruszkiewicz *et al*., 2017a).

### 1.1.2. 4-methylbenzylidene camphor

The inclusion of UV filters in PCPs for dermal protection has been increasing as response to the need of increasing protection against harmful effects of UV. In addition, UV filters are also used for increased shelf-life of plastic and household products (Chisvert and Salvador, 2018). This type of chemical products are usually very stable, lipophilic and suffer low degradation and such characteristics are also typical of organic priority pollutants (e.g. USEPA, 2012).

The 4-methylbenzylidene camphor (4-MBC) is reported to be one of the most common UV filters used in Europe and Australia (Krause *et al*., 2012; Chisvert and Salvador, 2018). This compound is widely detected on coastal environments (Balmer *et al*., 2005; Langford and Thomas, 2008; Tovar-Sánchez *et al*., 2013; Chisvert and Salvador, 2015) and can occur in higher levels during warmer seasons reaching concentrations up to 1.04 µg $L^{-1}$ in Gran Canaria coast (Sánchez-Rodríguez *et al*., 2015). Furthermore, 4-MBC can be detected in drinking water and aquatic ecosystems, since both potable water treatment systems and wastewater treatment plants are not able to completely eliminate 4-MBC by typical treatment methods (Balmer *et al*., 2005; Li *et al*., 2007; Fent *et al*., 2010; Braush and Rand 2011; Badia-Fabregat *et al*., 2012; Díaz-Cruz *et al*., 2012; Liu *et al*., 2012).

Bioaccumulation of 4-MBC in fish tissues have already been detected (Buser *et al*., 2006; Ruszkiewicz *et al*., 2017b) and toxicity to aquatic organisms can occur at concentrations below 10 ng $L^{-1}$ to small organisms, including protozoa (Gao *et al*., 2013), microalgae (*Isochrysis galbana*) and crustaceans (*Siriella armata*) (Paredes *et al*., 2014) and freshwater aquatic vertebrates, affecting its development and reproduction (Schlumpf *et al*., 2008; Kunz and Fent, 2006; Martins *et al*., 2017). In addition, 4-MBC has been previously reported to impair endocrine system (e.g. Inui *et al*., 2003; Kunz and Fent, 2009; Schmitt *et al*., 2008; Schlumpf *et al*., 2004).

### 1.2. Ultraviolet radiation

Long term data show large increases of ultraviolet radiation (UV) in UV-B range (between 280 and 315 nm) in both hemispheres mostly caused by changes in stratospheric ozone amounts as consequence of chlorofluorinated compounds and increasing carbon dioxide concentrations (Austin *et al*., 1992; Herman, 2010; Stolarski, 2011). UV penetration in water significantly decreases in the first few meters of depth. However, it can be increased when in combination with several abiotic (e.g. acidification, precipitation and temperature) and biotic conditions (dissolved organic carbon, colored non-living organic matter). The interaction of UV with other environmental variables is in fact one of the major threats to biodiversity on a global changing perspective (Häkkinen *et al*., 2002; Häder *et al*., 2015).

Response of organisms to UV exposure have long been studied, and some aquatic species seem to avoid excessive UV seeking protection under rocks, caves and other structures or diving to greater depths. Other species can also synthesize or sequester UV-absorbing substances through their diet and increasing concentrations of melanin for pigmentation darkening (Applegate and Ley, 1988; Ahmed and Setlow, 1993; Williamson *et al*., 1997; Adachi *et al*., 2005; Fukunishi *et al*., 2013; Häder *et al*., 2015). However, several

sub-lethal effects have been reported in fish, including sunburn (Berghahn *et al*., 1993; Blazer *et al*., 1997), lesions in the brain and retina and reduced growth rate (Hunter *et al*., 1979). Besides, several effects at lower levels of organization have been reported, including thickening and stratification of the epidermis and reduction in the diameter of the mucus-secreting goblet cells in the epidermis of sole larvae (Fadzen *et al*., 2000). DNA damage in the eggs and larvae of the Atlantic cod *Gadus morhua* (Browman *et al*., 2003) and massive apoptosis in larval embryos of Japanese flounders through the generation of reactive oxygen species (Yabu *et al*., 2003) has also been observed

The UV, in certain conditions, has been reported to be lethal to embryos and larvae of different fish species, namely to *Engraulis mordax* and *Scomber japonicus* (Hunter *et al*., 1979), *G*. *morhua* (Beland *et al*., 1999), eggs of yellow perch *Perca flavescens*, bluegill sunfish *Lepomis macrochirus*, and *Galaxias maculatus* (Gutiérrez-Rodríguez and Williamson, 1999; Battini *et al*., 2000) and flatfish (Steeger *et al*., 2001; Ylönem and Karjalain, 2004).

Combined effects of UV and abiotic factors or environmental contaminants depend on the stressor, its doses and species tolerance (Gevertz and Oris, 2014; Almeida *et al*., 2015; Häder *et al*., 2015; Wolinski *et al*., 2016). In certain conditions, UV can decrease toxicity of chemicals such as the insecticide carbaryl (Bridges and Boone, 2003). However, it is frequent to report increased toxicity of chemicals when in combination with UV. For instance, Hatch and Blaustein (2003) reported increased combined effects of UV and nitrate fertilizer to larval amphibians' survival and weight. In addition, combined exposure to UV and phototoxic PAHs was also reported to increase the formation of reactive oxygen species and oxidative stress in fish (Gevertz and Oris, 2014). Combined exposure to UV and TCS was reported to lead to synergistic or antagonistic effects on zebrafish embryos depending on the parameter analysed (Almeida *et al*., 2015).

## 2. Integrative approaches on ecotoxicology: from molecular to individual level

Accidental or chronic exposure to waterborne chemicals can affect pelagic species and also accumulate on the sediments affecting benthic species. In general, small-size species and early larval stages are pointed as two of the most affected groups by anthropogenic activities and climate variability (Wespestad *et al*., 2000; Harley *et al*., 2006; Hooper *et al*., 2013; Häder *et al*., 2015; Schramski *et al*., 2015; Tornero and Hanke, 2016). Information of single and combined effects of stressors, such as Personal Care Products (PCP) and UV on these organisms are still needed. Mortality and growth are two of the most

used endpoints transversely in live organisms on assessment of stressors effects. However, knowledge on the effects at lower levels of organization allows to understand the modes of action of the stressors.

Biomarkers are a vast set of tools used to demonstrate exposure to stressors and/or to study the effects of stressors on the organisms (Oost *et al*., 2003). Biomarkers of effect are frequently used to understand the response of organisms to a stressor and can include behavioral, biochemical or molecular and gene expression alterations (Oost *et al*., 2003; Scholz *et al*., 2008).

### 2.1. Behavior responses

Behavior responses are considered an high sensitivity tool and a non-invasive and non-lethal endpoint with growing interest to evaluate ecotoxicological responses to stressors at individual level (Richards *et al*., 2007; Archard *et al*., 2012; Andrade *et al*., 2016). The development of standardized methods and computer and video automation allowed the increase of the time of effective analysis of behavior, as well as its reproducibility and reliability (Scott and Sloman, 2004; Kane *et al*., 2005; Melvin and Wilson, 2013). In addition, behavior can be associated with responses measured at lower levels of organization, including at molecular or biochemical level (Richards *et al*., 2007; Vieira *et al*., 2009; Almeida *et al*., 2015). Behavior has been increasingly used to understand harmful effects of environmental and chemical stressors, including in fish. Behavior has high ecological relevance, as altered responses observed in laboratory can indicate alterations on organism predatory, feeding and reproduction success on natural habitats, which is further related with alterations at even higher levels of organization (e.g. population).

### 2.2. Biochemical and molecular responses

Biochemical markers are sensitive and useful tools on the study of effects of chemical exposure at organisms' subcellular level, since specific biochemical responses can be associated with particular modes of action of chemicals. Inhibition or induction of enzymes can occur as a response of the organism to stressors exposure. Effects on gene expression and alterations at physiological level can be linked to effects at morphological level.

The inhibition of ChE was primarily used as biomarker of effect and/or exposure to neurotoxic agents such as organophosphates and carbamate pesticides; however, they can also respond to different classes of stressors (Guilhermino *et al*., 1996; Monteiro *et al*., 2005; Nunes, 2011). There are two main forms of cholinesterases (ChEs) in fish species

are acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and the levels and relative proportions of the different types of Cholinesterase (ChE) depend on the species, organ/tissue, physiology and stages of life (Monteiro *et al*., 2005; Wilson, 2010; Nunes, 2011; Fisher and Wonnacott, 2012; Solé *et al*., 2012).

Exposure to chemicals and other type of stressors can enhance the production of reactive oxygen species (ROS). Organisms have developed defense mechanisms to deal with increased ROS levels (e.g. antioxidant defense system); and the induction or inhibition of antioxidant enzymes activity, such as catalase (CAT) or glutathione S-transferase (GST) can indicate increased oxidative stress by the exposure to stressors (Quintaneiro *et al*., 2008; Antunes *et al*., 2010). The GST family of enzymes also act as catalyst for the conjugation of various electrophilic compounds with the tripeptide glutathione on phase II of biotransformation. GST increase the availability of lipophilic toxicants to phase I enzymes by serving as carrier proteins. Also, by covalently binding to electrophilic compounds themselves, GST reduce the likelihood of these compounds to bind to other cellular macromolecules such as DNA. In some conditions, excessive oxidative stress may cause peroxidation of membrane lipids which can be directly measured through lipid peroxidation (LPO) chain reaction (Storey, 1996; Oost *et al*., 2003).

The lactate dehydrogenase (LDH) is an enzyme involved in the anaerobic metabolism that catalyzes the reversible reduction of pyruvate to lactate. An increase in LDH may be related with low availability of oxygen and exposure to xenobiotics might induce higher energy consumption (Guilhermino *et al*., 1994; Cohen *et al*., 2001; Quintaneiro *et al*., 2006).

The expression of target genes can be used as a molecular tool to understand modes of action of chemicals, relating the effects of exposure to contaminants observed at molecular level with those observed at higher levels of biological organization (Scholz and Mayer, 2008; Scholz *et al*., 2008). Alterations on gene expression might allow to integrate effects from molecular to phenotypic and functional level. For instance, thyroid hormones regulate metamorphosis, besides several associated genes of thyroid axis fluctuate during early development of *S*. *senegalensis* (Klaren *et al*., 2008; Power *et al*., 2008; Fernandez *et al*., 2018); therefore, thyroid disrupting compounds might induce changes on such levels altering metamorphosis progression.

### 3. Relevance of fish early development stages on ecotoxicity testing

The use of fish for laboratory toxicity studies has been increasingly used to obtain information on effects and modes of action of stressors. Several fish species have been

recommended for toxicity testing (OECD, 1992). More recently, Fish Embryo Acute Toxicity Test (Test 236) became more used since the zebrafish (*Danio rerio*) early life stages fit into the definition of alternative models to animal testing (OECD, 2013). However, even more restricted regulations exist regarding ethics on animal use in chemical testing and therefore, alternative approaches and methods for risk assessment of stressors are still being asked due to the increasing awareness and recent regulations on animal testing and welfare (Lilienblum *et al*., 2008; European Union, 2010). The scientific community needs to do more with fewer animal resources with emphasis on reduction of animal testing (Ankley *et al*., 2009) and in this context, the 3Rs' principles which were initially suggested in 1959 (Russel and Burch, 1959), have been gaining increasing attention. Replacement refers to the use of methods which avoid or replace the use of animals, reduction refers to the use of methods which minimize the number of animals used per experiment and refinement refers to the use of methods which minimize animal pain, suffering, distress, lasting harm and improve welfare. Early development stages of vertebrates are among the most sensitive organisms for eco/toxicology testing. In addition, the importance of new alternative methods and model species that are robust/reliable, repeatable and appropriate for regulatory use are also needed.

### 3.1. Flatfish as model species

The freshwater zebrafish is one of the most used aquatic vertebrate species since its biology is well known and complete life-cycle in laboratory can be performed easily (Scholz *et al*., 2008). Other freshwater fish species such as Medaka *Oryzias latipes* are also becoming widely used during early development; however, new species to study targeted questions are needed (Schartl, 2014). A limited number of brackish and saltwater species have been suggested as models for acute toxicity testing (OECD, 2018); however, with the exception of *Dicentrarchus labrax*, the European sea bass, none of them is suitable for use in embryo testing. Other saltwater species recommended include silverside *Menidia* sp. and sheepshaead minnow *Cyprinodon variegatus*, which have wide distribution range, their ecology is well known and they can be easily maintained and spawn in aquaria. However, their early life is a relatively slow growing period with hatching occurring only after the fifth day after the fertilization and yolk sac is depleted before hatching occurs (Kunz, 1916; Bengston *et al*., 1987; Haney *et al*., 2007).

The recent expansion of flatfish aquaculture has contributed for the development of their physiological knowledge, even at genomic level (Imsland *et al*., 2003; Martinez and Bolker, 2003; Cerdà and Manchado, 2013; Morais *et al*., 2016). There are studies using

flatfish as model species for assessment of stressor effects; however those mostly include studies with juveniles or adults, namely biomonitoring studies (e.g. Vinagre *et al*., 2007; Oliva *et al*., 2012; Siscar *et al*., 2013). Metamorphosis is a remarkable transition period of all flatfish species. This taxa appeared about 50 million years ago and eye migration was one of the earliest events in the gradual development into a fully asymmetric form (Friedman, 2008; Campinho *et al*., 2018). Some flatfish species have fast growth and the early metamorphosis is an important stage of life where profound morphological, molecular and endocrine changes occur (Yúfera *et al*., 1999; Power *et al*., 2001, 2008; Klaren *et al*., 2008). Therefore, alterations caused by stressors during early development, particularly stressors with endocrine disruption action, may critically affect these organisms.

### 3.2. Senegalese sole, *Solea senegalensis*

The order Pleuronectiformes is a broad taxa of the class Actinopterygii with 786 species. One of the four families of these order is Soleidae which comprehends a total of 179 species, including Senegalese sole (*Solea senegalensis* Kaup, 1858) along with eight other species of *Solea* genus (Froeser and Pauly, 2018). *Solea senegalensis* is commonly found in coastal waters from Bay of Biscay southward and Western Mediterranean and southward to Angola and South Africa. Its presence in Eastern Mediterranean also occur but is uncommon (Quéro *et al*., 1986; Golani *et al*., 2013) and the sympatry with other *Solea* genus species across distribution range has led to hybridization of species (Ouanes *et al*., 2011).

Spawning of *S. senegalensis* takes place between May and August with a peak in June in Iberian Peninsula and Bay of Biscay. After fertilization, eggs float on coastal currents and are influenced by both active and passive transport processes, while nearly metamorphosed larvae are transported to shallow waters and inland nurseries in early summer (Cabral, 2003).

Early development and growth of *S. senegalensis* can be strongly influenced by water temperature, lighting conditions and feeding regimes among other factors (Yúfera *et al*., 1999; Fernández-Díaz *et al*., 2001; Blanco-Vives *et al*., 2012). Early development stages have been described in detail by several authors; however, soles present relatively wide ranges of size and age at the onset and end of metamorphosis. Therefore, only external morphology and ecology can be safely used when trying to describe metamorphosis progression (table 1.1). During *S. senegalensis* metamorphosis, this species changes from a symmetrical pelagic stage, moving the left eye to the right side and start a bilateral asymmetric benthic morphology with other drastic changes in physiology

(Yúfera *et al*., 1999; Manchado *et al*., 2009). Skin pigmentation also becomes distinct between dorsal and ventral sides, and the anterior head bone and brain are remodeled (Campinho *et al*., 2018). This event starts around nearly at 15 days after hatching (dah); however, in exceptional conditions it can start at 11 dah and it is complete before the end of the first month of life (Yúfera *et al*., 1999; Fernández-Díaz *et al*., 2001).

Table 1.1. Morphology and ecology of *Solea senegalensis* along metamorphosis progression. Adapted from Dinis (1986), Yúfera *et al*. (1999); Fernández-Díaz *et al*. (2001), Klaren *et al*. (2008), Blanco-Vives *et al*. (2012).

| Development stages | External morphology | Ecology |
|---|---|---|
| A | • Slightly asymmetric larvae<br>• Enlargement of dorsal and ventral fins.<br>• Pigmentation of the posterior part of dorsal fin. | • Pelagic |
| B | • Further enlargement of dorsal and ventral fins<br>• Pigmentation of the anterior part of dorsal fin and ventral fin<br>• Beginning of left eye on migration | • Bentho-pelagic |
| C | • Continuation of left eye migration<br>• Further enlargement of dorsal and ventral fins | |
| D | • Left eye on the anterior head<br>• Fully enlargement of dorsal and ventral fins<br>• Mouth shape changes to an inverted “U” shape | |
| E | • Both eyes in the same side<br>• Pigmentation intensification | • Benthic |
| F | • Further migration of both eyes<br>• Anterior part of the head becomes round | |
| G | • Growth of anal fin<br>• Shrink of pectoral fin<br>• Orbital arch is evident | |

After the metamorphosis, juveniles remain feeding and growing in the bottom coastal and estuarine habitats until reaching maturity when they migrate downstream to spawn at sea. Sexual dimorphism occurs naturally around 45 dah and female to male ratio is usually 1:1, however it can be changed in accordance to environmental conditions (light, temperature). Adult soles occur in shallow sand and muddy bottoms mainly up to 100 m thriving best when water temperatures range between 16º and 22ºC and being more active in the first part of the dark period (Quéro *et al*., 1986; Blanco-Vives *et al*., 2012).

### 3.2.1. The thyroid-regulated metamorphosis in *Solea senegalensis*

Thyroid hormones (TH) play a key role in growth, development and also during metamorphosis of flatfish (Power *et al*., 2001, 2008; Klaren *et al*., 2008; Manchado *et al*., 2008; Isorna *et al*., 2009; Fernandez *et al*., 2018).

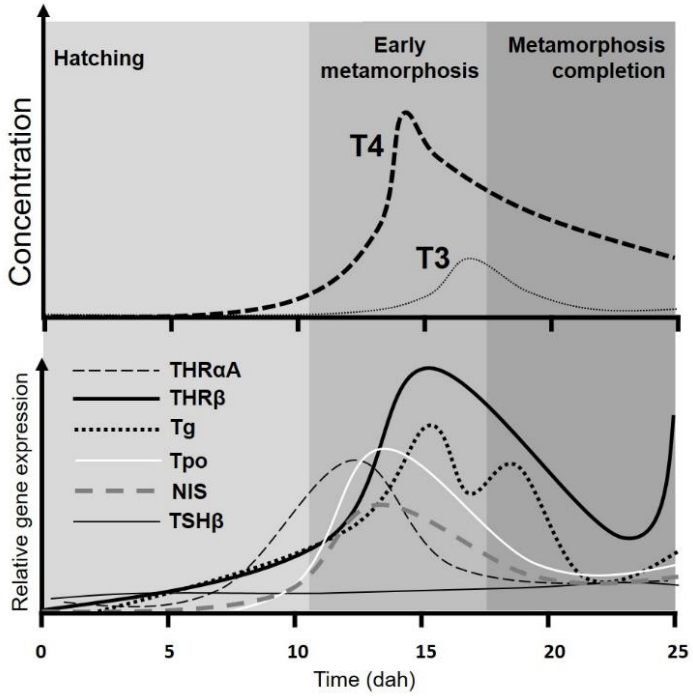


Figure 1.1. Representation of hormone levels and expression of thyroid-related genes in *Solea senegalensis* during metamorphosis. dah - days after hatching. Adapted from Klaren *et al*. (2008), Manchado *et al*. (2008), Campinho *et al*. (2015) and Fernandez *et al*. (2018).

Thyroxine (T4) and triiodothyronine (T3) are the two main thyroid hormones that are present in fish; which have active roles on metamorphosis (Miwa *et al*., 1988; Mayer *et al*., 1992; Klaren *et al*., 2008; fig. 1.1). The T4 is the primary hormone synthesized in thyroid follicle, which appears in *S. senegalensis* sub-pharyngeal region at early pre-metamorphosis stages (Klaren *et al*., 2008). This hormone is the precursor of the T3, the biologically active form that is produced through deiodination in the peripheral tissue (Power *et al*., 2001; Klaren *et al*., 2008). The release of these hormones is regulated by the pituitary (via thyroid stimulating hormone, TSH) which in turn is mediated presumably through inhibitory control (negative feedback) by the hypothalamus (via thyrotropin releasing hormone, TRH). TH mediate metamorphosis, down-regulating TSH β and also thyroglobulin (Tg) at transcriptional level in *S. senegalensis* (Manchado *et al*., 2008). Furthermore, TH signaling have been associated with skeletal development that occurs during metamorphosis of this species (Fernandez *et al*., 2018). TH mediate their effects through interacting with thyroid hormone receptors (THR), namely THRα and β, which present a

peak of gene expression during metamorphosis. The proliferation of skin pigmentation and the eye migration, which is reported to be forced by ossification on the blind-side sub-optical neurocranium is driven by TH which together give rise to the observed asymmetric development of the head (Bao *et al*., 2011; Campinho *et al*., 2018).

### 3.2.2. *Solea senegalensis* aquaculture

Fish consumption in southern Europe is amongst the highest in the world and flatfish are a major fisheries resource; however, conservation concerns on the fluctuations of catching have been mentioned (Claireaux *et al*., 2004; Teixeira and Cabral, 2009). Therefore, *Solea senegalensis* has been gaining interest in an increasingly diversified aquaculture industry and currently it is also produced in Southern Europe where the production is performed both in extensive and also intensive conditions (Recirculating Aquaculture Systems, RAS) (Imsland *et al*., 2003; Morais *et al*., 2016). The culture of early developmental stages is performed similarly on both production methods. However, despite the efforts for establishing protocols for reproduction of reared *S*. *senegalensis*, produced eggs from hatchery reared stocks are still not viable and stocks of spawners rely exclusively on wild individuals (Anguis and Cañavate, 2005). Wild spawners are kept in tanks with abiotic conditions similar to the ones found in the wild and seasonal variations of photoperiod and water temperature are artificially induced for production of eggs. Since the eggs are obtained from wild individuals, there is high genetic diversity within production batches. The production of eggs is also typically stimulated in excess for ensuring the necessary material for the following farming stages. Therefore, viable eggs end-up being discarded while they still have potential for use on laboratory testing.

### 3.2.3. Early life stages of *Solea senegalensis* as model species

Research is still needed regarding laboratory trials for understanding marine or coastal vertebrate responses to stressors at several levels. There is scarce knowledge on effects of chemicals on saltwater species and, in addition, behavior of chemicals can be distinct when comparing freshwater and saltwater environments so it is important to test effects on saltwater species. In this sense, several advantages exist on using early development stages of *Solea* sp. and particularly *S*. *senegalensis* on the study of effects of stressors, which are detailed below.

*Solea senegalensis* is native and common in the eastern Atlantic and Mediterranean Sea where several chemical stressors are reported as result of high density of human population. In addition, the commercial interest of this species has promoted the

raise of its biology and physiology knowledge in recent years, as well as an increase in the available genomic tools (Morais *et al*., 2016; Campinho *et al*., 2018). In addition, currently, several species of *Solea* genus are reared in private and rearing facilities in several European countries, including The Netherlands, France, Spain, Italy and Portugal (Imsland *et al*., 2003), making the species early life stages commercially available.

After screening for viable eggs, mortality of *S. senegalensis* is expected to be low and hatching will occur at nearly 36 hpf. Effects of stressors on survival should be easily identified. In addition, modifications induced by stressors on embryo development while inside the egg or as larvae can also be performed easily as the eggs and larvae are transparent. In addition to typical endpoints (such as mortality and growth), several endpoints can be studied during early life stages of *S. senegalensis*, including behavior, biochemical markers or gene expression.

Until 80-100 hpf, *S. senegalensis* larvae have the mouth closed and rely on endogenous energy reserves (Yúfera *et al*., 1999). Therefore, this species can be used as alternative method to animal testing by performing 96h embryo acute testing following the Fish Embryo Testing Guideline 236 (OECD, 2013) with adaptations.

The metamorphosis of *S. senegalensis* is a fast period that occurs nearly between 15 and 25 days after hatching, dah. Effects of stressors, particularly endocrine disruptors, on metamorphosis of aquatic organisms have mostly been studied on amphibians, namely frog species which undergo metamorphosis (e.g. Veldhoen *et al*., 2006; Sowers and Klaine, 2008). In this context, The Amphibian Metamorphosis Assay Test Guideline OECD 231 (2009) focus particularly on the screening of substances which may interfere with the normal functioning of the hypothalamo-pituitary-thyroid axis. Studies of factors affecting metamorphosis of flatfish address mostly issues that affect improvement of aquaculture production. During this stage, defense mechanisms against stressors may not be fully developed and specific stressors (such as those with endocrine disruption action) may affect their correct development with strong impacts on later life stages.

## 4. Objectives and outline of the thesis

The main objective of the thesis is to understand if widely used chemicals with potential endocrine disruption action which are included in personal care products formulations (TCS and 4-MBC) and also ultraviolet radiation (UV-B range) affect early development of *Solea senegalensis*. In addition, other objective is to verify if the UV affect the response of *S. senegalensis* to the PCPs exposure, through studying single and combined effects of the stressors.

This will be achieved by evaluating the effects of stressors at individual (growth, metamorphosis progression or behavior) and sub-individual level (genomic or biochemical markers) in two early development stages of *S*. *senegalensis*: between egg stage and three days after hatching and during the metamorphosis. The effects on lower and higher organizational levels will be linked as a way to understand the modes of action of the stressors.

This work will contribute to understand if *S. senegalensis* can be successfully used as a model species on early life toxicity testing for the marine environment. This work will also provide background information for regulatory purposes and for risk assessment of the stressors tested.

### 4.1. Chapters outline

This thesis is organized in seven chapters which are detailed below.

*Chapter 1*. **General Introduction - use of early life stages of marine fish for the study of anthropogenic-driven threats**

In this chapter, an overall introduction to the subject the main topics and fundamental concepts that are used along the thesis are presented.

*Chapter 2*. **Effects of triclosan on early development of *Solea senegalensis*: from biochemical to individual level**

In this chapter, the characterization of cholinesterases and biochemical markers baseline levels were studied in three moments of *Solea senegalensis* early development: early larval stage, early metamorphosis and after metamorphosis. Then, the hypothesis that the endocrine disruptor triclosan (TCS) affect early development of *S. senegalensis* is studied in two moments, at the early larval stage and during metamorphosis. Triclosan effects were studied on mortality, malformations, growth, metamorphosis progression and biochemical markers.

The following two chapters refer to the study of exposure effects of UV filter 4-methylbenzylidene camphor (4-MBC) on two development stages: early larvae and along metamorphosis.

*Chapter 3*. **Effects of the UV filter 4-methylbenzylidene camphor during early development of *Solea senegalensis* Kaup, 1858**

In this chapter, the hypothesis that the UV filter 4-MBC affects early larvae of *Solea senegalensis* is studied through analysis of effects on mortality, malformations, growth, behavior and biochemical markers.

*Chapter 4*. **Exposure effects of the UV-filter 4-MBC on *Solea senegalensis* metamorphosis**

In this chapter, it is studied the hypothesis that the UV filter 4-MBC affects *Solea senegalensis* during metamorphosis. Effects of 4-MBC exposure were studied on mortality, malformations, metamorphosis progression, growth, behavior and biochemical markers.

*Chapter 5*. **Effects of extreme ultraviolet radiation on *Solea senegalensis* during early development**

In this chapter, it is studied if UV exposure during early development of *Solea senegalensis* affects mortality, malformations, metamorphosis progression, growth, behavior and biochemical markers.

*Chapter 6*. **Effects of single and combined exposure to ultraviolet radiation and triclosan in metamorphosing *Solea senegalensis***

In this chapter, sub-lethal effects of single and combined exposure to UV and TCS were studied on malformations, metamorphosis progression, growth and behavior of metamorphosing *Solea senegalensis*. In addition, since alterations on metamorphosis progression caused by TCS were observed in the previous chapter, study of TCS effects on thyroid axis (through the study of expression of thyroid related genes) was also performed.

*Chapter 7*. **Effects of PCP ingredients and UV to the early life stages of *Solea senegalensis***

In this chapter, discussion of the main results and final conclusions of the work are integrated and future perspectives are presented.

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# Chapter 2. Effects of triclosan on early development of *Solea senegalensis*: from biochemical to individual level

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# Effects of triclosan on early development of *Solea senegalensis*: from biochemical to individual level

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**Abstract**

Harmful effects of triclosan (TCS) have been reported on several organisms; however, effects on early life stages of marine vertebrates are limited. Therefore, the objective of this work was to assess the effects of TCS during early development of the flatfish *Solea senegalensis* after initial characterization of cholinesterases (ChEs) and determination of selected biochemical markers baseline levels.

Characterization of ChEs and determination of biochemical markers baseline levels of cholinergic activity, energy metabolism and oxidative stress were analysed in sole at 3 days after hatching (dah) and at the onset and end of metamorphosis. To assess TCS effects, fish were exposed during 96h to 30-500 µg $L^{-1}$ TCS until 3 dah. Fish at 13 dah were exposed during 48h to 200-1,500 µg $L^{-1}$ TCS and maintained until complete metamorphosis. Effects on survival, malformations, length, metamorphosis progression and biochemical markers were evaluated.

The main ChE active form present in sole early life stages is acetylcholinesterase and baseline levels of oxidative stress and energy metabolism biomarkers changed according to fish developmental stage. Triclosan induced malformations ($EC_{50}$=180 µg $L^{-1}$ at 3 dah), decreased growth (95 µg $L^{-1}$ at 3 dah; 548 µg $L^{-1}$ at 24 dah) and affected metamorphosis progression (391 µg $L^{-1}$ at 17 dah). Impairment of antioxidant system was observed, with TCS affecting catalase at the end of metamorphosis test, however, no oxidative damage on lipids was detected. Glutathione S-transferase was the most sensitive endpoint during the early larval test (LOEC=30 µg $L^{-1}$).

Exposure to TCS affected *S. senegalensis* at individual and sub-individual levels, both at early larval stage and during the critical period of metamorphosis.

**Keywords:** cholinesterases; flatfish; growth; metamorphosis; oxidative stress; personal care products.

## 1. Introduction

The use of personal care products (PCP) has been rising and some of their compounds are not effectively eliminated through conventional water treatment processes, reaching aquatic ecosystems. Triclosan (5-chloro-2(2,4-dichlorophenoxy)phenol, TCS) is one of the most commonly used ingredients in soaps, toothpastes and deodorants and it is also found in clothing, kitchenware, furniture, and toys (Orvos *et al*., 2002; Fang *et al*., 2010).

The increase of TCS in environment due to the widely use of PCPs is of growing concern, in fact TCS is one of the most frequently detected organic micropollutants in the aquatic environment (Luo *et al*., 2014; Dhillon *et al*., 2015). Triclosan is widely present in wastewater influents, although the most efficient wastewater treatment plants achieve removal rates of 92-99 % (Kumar *et al*., 2010; Buth *et al*., 2011; Dhillon *et al*., 2015), in conventional water treatment processes the TCS clearance rate is 24-95% (Dhillon *et al*., 2015). Therefore, TCS end up in effluents reaching concentrations of about 0.08-5.37 μg $L^{-1}$ (SCCS, 2010; Dann and Hontela, 2011; Kookana *et al*., 2011; Díaz-Garduño *et al*., 2018). This highly lipophilic compound (log octanol–water partition coefficient, $K_{ow}$ of 4.8) has an estimated half-life of 60 days and environmental concentrations reach up to 40 μg $L^{-1}$ in freshwater ecosystems, 0.3 μg $L^{-1}$ in estuaries and 0.1 μg $L^{-1}$ in saltwater environments (SCCS, 2010; Dann and Hontela, 2011; Pintado-Herrera *et al*., 2014; Gasperi *et al*., 2014; Lehutso *et al*., 2017; Nag *et al*., 2018).

As an anti-bacterial, TCS inhibits the enzyme Fab1 (enoyl-acyl-carrier-protein reductase) which is responsible for catalyzing the terminal reaction in the fatty acid elongation of cell wall in bacteria (Lund *et al*., 2005; Massengo-Tiassé and Cronan, 2009; Fang *et al*., 2010). Triclosan can be bioaccumulated in non-target species, including in marine organisms, potentially causing adverse effects (Álvarez-Muñoz *et al*., 2015). It is known that TCS and other phenolic xenobiotics are metabolized through biotransformation phase I (pathway mediated by cytochrome P450 enzymes) and phase II generating more water-soluble glucuronide and sulfate conjugates (Liang *et al*., 2013; Ashrap *et al*., 2017; Wu *et al*., 2017). Oxidative stress induction has been reported after TCS exposure in amphibians (Martins *et al*., 2017) and freshwater fish (Oliveira *et al*., 2009; Liang *et al*., 2013; Falisse *et al*., 2017). For instance, TCS is reported to induce catalase (CAT) in different freshwater fish species (Li *et al*., 2018; Ku *et al*., 2014; Banerjee *et al*., 2016), to enhance glutathione levels and decrease the total antioxidant capacity in the fish *Carassius auratus* leading to oxidative damage of lipids (Li *et al*., 2018; Wang *et al*., 2018). In addition induction of the neurotransmission enzyme acetylcholinesterase (AChE) and lactate

dehydrogenase (LDH), an enzyme of the anaerobic metabolism was reported to occur in early life stages of *Danio rerio* exposed to TCS (Oliveira *et al*., 2009). In addition, endocrine disruption has also been described in different life stages of aquatic vertebrates after TCS exposure (Ishibashi *et al*., 2004; Pinto *et al*., 2012; Marlatt *et al*., 2013). However, knowledge on its effects and modes of action on early life stages of marine vertebrates are still scarce.

The determination of *a priori* biochemical markers baseline levels is an important initial step to understand normal physiological conditions in model species used in ecotoxicology (Quintaneiro *et al*., 2006; 2008; Antunes *et al*., 2010; Ferreira *et al*., 2010). Key life events and physiological status are known to influence responses at biochemical level (Monteiro *et al*., 2005; Nunes, 2011; Nunes *et al*., 2015). In addition, biochemical responses of organisms may be altered by the exposure to stressors and can provide relevant information on their mode of action and toxicity (Oost *et al*., 2003; Fernández-Díaz *et al*., 2006; Pimentel *et al*., 2015). For instance, stressors can elicit increased production of reactive oxygen species (ROS) and/or impairment of antioxidant system through enzymatic inactivation, which might lead to oxidative damage in DNA, proteins and lipids, and increase cellular degenerative processes which might lead to death (Storey, 1996; Oost *et al*., 2003; Park *et al*., 2017; Wang *et al*., 2018). Assessment of effects on neurotransmission can be performed through the determination of cholinesterases (ChEs) activity. There are two main forms of ChEs that break down esters of choline in fish species: AChE and butyrylcholinesterase (BChE), with an high affinity for the substrates acetylcholine and butyrylcholine, respectively (Rodríguez-Fuentes and Gold-Bouchot, 2004; Monteiro *et al*., 2005; Lionetto *et al*., 2013; Hampel *et al*., 2016). The levels and proportions of these key enzymes of the nervous system depend on the species, organ/tissue, physiology and stages of life, with AChE generally being the most common type from fish brain (Monteiro *et al*., 2005; Wilson, 2010; Nunes, 2011; Fisher and Wonnacott, 2012; Solé *et al*., 2012). Therefore, characterization of ChEs should be performed before using these enzymes for neurotoxicity assessment. The inhibition of AChE was primarily used as biochemical marker of effect and/or exposure to neurotoxic agents such as organophosphates and carbamate pesticides (e.g. Bocquené and Galgani, 1998); however, they can also respond to different classes of stressors (Guilhermino *et al*., 1996; Nunes, 2011; Quintaneiro *et al*., 2014).

Early life stages of fish, namely before beginning of independent feeding, stand as a good alternative to animal testing (EU, 2010; Scholz, 2013; Lillicrap *et al*., 2016); however, the use of early life stages of estuarine and marine fish species as alternative models has

been very limited. In this context, early life stages of Senegalese sole (*Solea senegalensis* Kaup, 1858) arise as a potential model organism. This species occurs naturally on Southwestern Europe and Northern African Atlantic waters and has an high ecological relevance, belonging to medium-top trophic level. In response to economical interest and aquaculture potential of *S. senegalensis*, commercial exploitation has been increasing since the early 90's (Imsland *et al*., 2003; Morais *et al*., 2016), which had supported further advances on the scientific knowledge of the species. While adult *S. senegalensis* have been widely used as sentinel species for environmental pollution monitoring and assessment (e.g. Riba *et al*., 2004; Costa *et al*., 2008; Oliva *et al*., 2012; Solé *et al*., 2012; and others), there is also an high potential to use early life stages of this species for laboratory toxicity testing (Pimentel *et al*., 2015; Pavlaki *et al*., 2016; Araújo *et al*., 2018). Contributing to this potential is the fact of Senegalese sole early development stages have been already described by several authors (e.g. Dinis, 1986; Fernández-Díaz *et al*., 2001; Klaren *et al*., 2008). Besides, the fast growth of larvae with an early thyroid regulated metamorphosis ending in the first month of life (Yúfera *et al*., 1999) are interesting features for studying compounds acting as endocrine disruptors.

The main aim of this study was to assess the effects of TCS on the early development of *S. senegalensis* at individual and biochemical level. In order to achieve this, firstly, characterization of the main ChE form(s) present in *S. senegalensis* was performed through the use of different substrates and specific inhibitors. This was evaluated in different early development stages (immediately after yolk sac depletion, at the beginning and at the end of metamorphosis). Secondly, baseline levels of selected biochemical markers were determined at the same stages. Thirdly, survival, growth, malformations, metamorphosis progression and biochemical markers on *S. senegalensis* were evaluated after 96h and 48h exposure to TCS at the early larval phase and at the onset of sole metamorphosis, respectively.

## 2. Material and Methods

### 2.1. Chemicals

Triclosan (Irgasan 97%) and all chemicals used for characterization of cholinesterases and biochemical analysis were purchased from Sigma-Aldrich Co. LLC (St Louis, USA), except Bradford reagent, which was purchased from Bio-Rad (Germany). Acetone, acetonitrile, methanol and dichloromethane were supplied by Merck. All chemicals used on chemical analysis of TCS were of analytical or HPLC grade quality.

### 2.2. Biological material

Eggs of *S. senegalensis* were obtained from a commercial hatchery (Sea8, Portugal) and were transported to the lab (2h maximum) within 12h post fertilization. Transportation was performed in a thermal box, within plastic bags (5 L of recirculatory system saltwater). Floating lipid-rich eggs in gastrula stage were washed and kept in glass jars in previously matured synthetic saltwater (Coral Pro Salt, Red Sea, Saudi Arabia, salinity 35, 19 °C, pH = 8.15) until further use. Observation of egg stage and viability was performed using a stereosmicroscope (Nikon SMZ 1270, Nikon, Japan).

For the present work, three development stages were considered (fig. 2.S1). The first stage referred to 3 days after hatching (dah). At this stage, full depletion of yolk sac has occurred (Dinis, 1986; Yúfera *et al*., 1999; Klaren *et al*., 2008). The second stage studied refers to the onset of metamorphosis, which highly depends on maintenance and feeding conditions. According to Fernández-Díaz *et al*. (2001), 50% of fish starts the metamorphosis between 9 and 16 days after hatching depending on the typical feeding regimes. At this stage, the larvae remain pelagic and with bilateral symmetry. The third stage refers to post-metamorphic organisms. Complete metamorphosed fish are benthic, laterally flattened and asymmetric, the left eye has reached its final position in the right (and dorsally) side of the body and orbital arches are clearly visible. This stage is achieved between 16 and 24 dah (Fernández-Díaz *et al*., 2001). The different sole stages used in the present study were within these time frames. Pre-metamorphic fish with 13 dah and post-metamorphic fish with 22 dah were used in ChE characterization and biochemical basal levels determinations. In the TCS test during metamorphosis the 48h of exposure began at 13 dah and fish were maintained until completion of metamorphosis, at 24 dah. All experimental procedures were carried out following the European and Portuguese legislation concerning animal experimentation (authorized by the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária, Ref. 009804).

### 2.3. Maintenance conditions of fish used for ChE characterization and biochemical markers baseline levels

*Solea senegalensis* eggs were placed in conical culture tanks (approx. 30 L, 50 eggs $L^{-1}$) with aeration, external biological filter, protein skimmer and refrigeration (19ºC, HC series chiller, Hailea, China), photoperiod 16:8 h (light:dark) and salinity 35 (Coral Pro), which was adjusted daily. The larvae feeding regime included increasing densities of rotifers (*Brachionus plicatilis*) from 1 to 6 dah (between 5 and 10 rotifers $mL^{-1}$), *artemia* nauplii from 5 to 10 dah (between 2 and 9 nauplii $mL^{-1}$) and from 10 dah with *artemia* metanauplii

(between 9 up to 35 metanauplii $mL^{-1}$) until the end of metamorphosis (Fernández-Díaz *et al*., 2001). Green algae (*Nannochloropsis* sp.) was also added since 1 dah. Randomly chosen fish from the three development stages previously described (larvae, 3 dah, 3.3±0.04 mm length; pre-metamorphosis, 13 dah, 4.5±0.04 mm length; and post-metamorphosis, 22 dah, 8.2±0.09 mm; 30 fish were measured for length in each life stage) were snap frozen with liquid nitrogen and kept at -80°C until further procedures for ChE characterization and determination of biochemical markers baseline levels.

### 2.4. Characterization of ChEs

Previously frozen samples of fish in the following life stages were used: 3 dah (n=3, 25 organisms per replicate), 13 dah (n=3, 9 organisms per replicate) and 22 dah (n=3, 9 organisms per replicate). After homogenization (Sonifier S-250A, Branson Ultrasonics, USA) in potassium buffer solution (pH= 7.2, 0.1 M), samples were centrifuged (6,000 rpm; 5 min; 4ºC) and supernatants were used for ChE characterization as described below.

#### 2.4.1. Substrates

To determine the substrate preference of the enzyme present along early development of *S. senegalensis*, three different substrates (acetylthiocholine iodide, AcSCh; S-butyrylthiocholine iodide, BuSCh and propionylthiocholine, PrSCh) were used in increasing concentrations in the enzymatic reactions, from 0.08 to 20.48 mM in the early larval stage and between 0.005 and 20.480 mM in the two metamorphosing stages.

#### 2.4.2. Inhibitors

To understand which esterase enzymes are present in the three development stages considered, the action of selective ChE inhibitors was studied in *in vitro* enzymatic assays using the two substrates AcSCh and BuSCh. To this end, initial incubation of samples was performed with eserine hemisulfate (selective inhibitor of ChEs), 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51, selective inhibitor of AChE) or tetraisopropyl pyrophosphoramide (iso-OMPA, selective inhibitor of BChE). Samples (495 µL of supernatant) were incubated with each inhibitor (5 µL) for 30 min at 25±1ºC. Eserine and BW284C51 were used with concentrations ranging from 6.25 to 200 µM and iso-OMPA from 250 to 8000 µM. In the different *in vitro* experiments, ultrapure water was used as negative control with the three inhibitors and ethanol was also used as solvent control for iso-OMPA, as it is not soluble in water.

### 2.5. Determination of biochemical markers baseline levels

Previously frozen samples of fish in the following life stages were used: 3 dah (n=9, 25 organisms per replicate), 13 dah (n=9, 9 organisms per replicate) and 22 dah (n=9, 9 organisms per replicate). After homogenization in potassium buffer solution (pH= 7.4, 0.1 M) by sonication, the homogenate was centrifuged for 20 min at 10,000 g (4°C) and the supernatant was used for the enzymatic analysis. AChE activity was measured by Ellman's method (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al*., 1996) using acetylthiocholine as substrate and following the increase of absorbance at 412 nm. Glutathione S-transferase (GST) activity was measured following the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene at 340 nm as described by Habig and Jakoby (1981) adapted to microplate reader (Frasco and Guilhermino, 2002). CAT activity was determined by measuring decomposition of the substrate hydrogen peroxide ($H_2O_2$) at 240 nm (Clairborne, 1985). The LDH activity was determined by measuring the conversion of pyruvate to L-lactate with the concomitant conversion of NADH to NAD+ during glycolysis which is measured at 340 nm as described by Vassault (1983) with the modifications introduced by Diamantino *et al*. (2001). The protein concentration was determined in triplicate according to the Bradford method (Bradford, 1976) adapted to microplate using bovine $\gamma$-globuline as a standard and measurements were performed at 595 nm.

The enzymatic activity is expressed in Units (U) per mg of protein. One U is a nmol of substrate hydrolyzed per minute using a molar extinction coefficient of $13.6 \times 10^3$ $M^{-1}cm^{-1}$ for AChE and $9.6 \times 10^3$ $M^{-1}cm^{-1}$ for GST, one µmol of substrate hydrolyzed per minute per mg protein, using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$ for CAT and $6.3 \times 10^3$ $M^{-1}cm^{-1}$ for LDH. All spectrophotometric measurements were performed in 96 well microplates (3-4 technical replicates per sample) using a Labsystem Multiskan EX microplate reader.

### 2.6. Fish exposure to TCS

Saltwater (salinity 35) and TCS solutions were prepared with synthetic salt (Coral Pro). Stock solutions of 9 or 18 mg $L^{-1}$ TCS (for the early larval or metamorphosis assays, respectively) were previously prepared in acetone and diluted in saltwater to achieve the selected concentrations. Exposure to TCS was performed in three independent trials according to fish life stage.

In the first trial, the early larval test, eggs were exposed during 96h until 3 dah (n=4, 10 organisms per replicate in 10 mL of test solution) to six concentrations of TCS (30, 53, 95, 169, 300 and 500 µg $L^{-1}$) and to the respective negative (saltwater) and solvent control solutions (33.3 µL $L^{-1}$ of acetone in saltwater). Exposure to TCS was performed on 6-well

plastic plates (10 mL in each well) previously incubated during 24h with TCS solutions at the respective concentrations to avoid TCS depletion through adsortion of the chemical to the plastic. The exposure was performed in semi-static conditions (solution renewal at 48h) without feeding, under the same temperature and photoperiod regimes as described above for fish maintenance. At 3 dah, *S*. *senegalensis* larvae (whole-body) previously exposed during 96h to TCS near or below the medium lethal concentration of 20% of organisms ($LC_{20}$: 164±10 µg $L^{-1}$ TCS) and controls were snap frozen in liquid nitrogen and kept at -80ºC for biochemical markers quantification.

Senegalese sole were maintained in culture conditions as described previously until the onset of metamorphosis (13 dah), then fish were divided in two trials, one for biochemical markers determination after 48h of TCS exposure and another to evaluate metamorphosis progression and biomarkers at 24 dah. One group of randomly selected fish (n=6, 10 fish per replicate in 10 mL test solution) was exposed to seven concentrations of TCS (200, 280, 391, 548, 766, 1072 and 1500 TCS µg $L^{-1}$) and to negative (saltwater) and solvent control solutions (83.3 µL $L^{-1}$ of acetone in saltwater). Exposure to TCS was performed in 6-well plastic plates (10 mL in each well) previously incubated during 24h with TCS solutions. After 48h of fish exposure, the organisms were snap frozen in liquid nitrogen and kept at -80°C for biochemical markers quantification. Another group of fish (n=6, 6 fish per replicate in 10 mL test solution) was also exposed to the same concentrations of the previous trial plus negative and solvent controls and after 48h of exposure, fish were transferred to new 6-well plastic plates with clean media (saltwater) and daily fed with live food (artemia). Maintenance was performed until more than 80% of fish from negative control completed metamorphosis (24 dah). At the end of metamorphosis, fish from treatments presenting mortality below 10% (five lowest concentrations of TCS) were snap frozen with liquid nitrogen and kept at -80°C for biochemical markers quantification. The physico-chemical parameters were controlled in both tests (pH=8±0.5, oxygen saturation over 80%, salinity 35±0.5 and temperature 19±1ºC).

In all experiments, survival and malformations were recorded on a daily basis with a stereomicroscope. Hatching was checked at 24h and 48h in larval test. Length of fish (from snout to tip of caudal fin) was determined at the end of early larval test (3 dah, n=12-16 for each treatment or control group) and at the end of metamorphosis test (24 dah, n=6-9 for each treatment or control group). All measurements were performed using a Nikon stereomicroscope coupled with a Nikon camera and with a millimetric ocular. While randomly selected fish were measured at 3 dah, at the end of metamorphosis only fish with complete metamorphosis were considered for length determination. Teratogenic index of

TCS was estimated using the ratio between $LC_{50}$ and $EC_{50}$ at 3 dah; a xenobiotic is considered teratogenic when index is above 1 (Selderslaghs *et al*., 2012).

Evaluation of metamorphosis progression was performed according to literature (Dinis, 1986; Fernández-Díaz *et al*., 2001; Klaren *et al*., 2008) and seven stages of development based on external morphology were considered: A - beginning of enlargement of dorsal and ventral fins, occasional sinking; B - beginning of migration of left eye to the right side, initial sinking; C - further migration of left eye and pigmentation, alteration of mouth shape; D - further migration of left eye and pigmentation (eye on the anterior edge), fully enlargement of dorsal and ventral fins, further alteration of mouth shape; E - fully flattened body, left eye on the dorsal side; F – further migration of left eye on the dorsal side, further pigmentation, anterior profile becomes more curved; G - orbital eye membrane becomes thicker, growth of anal fin, shrink of pectoral fin (complete metamorphosis).

In addition to the determination of AChE, CAT, GST and LDH activity levels as previously described, lipid peroxidation (LPO) was also determined in samples of TCS tests, by quantifying the thiobarbituric acid-reactive substances at a wavelength of 535 nm (Bird and Draper, 1984). An aliquot (150 µL) of the initial sample homogenates were placed in a microtube with 4 µL of 4% butylated hydroxytoluene (BHT) in methanol to avoid posterior oxidation of lipids. These samples were maintained at -80ºC until being further procedure according to Bird and Draper (1984). The LPO is expressed in nmol of TBARs hydrolyzed per mg protein using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$.

### 2.7. Chemical analysis of TCS

Chemical analysis of TCS was performed in initial test solutions and also in solutions after 48h of exposure (metamorphosis tests).

Solid-phase extraction (SPE) of TCS from testing solutions was adapted from Kookana *et al*. (2013). The SPE cartridges (C18/17%, 100 mg 1 $mL^{-1}$, Finisterre, Teknokroma, Spain) were initially conditioned with 4 mL of methanol followed by 4 mL of ultra-pure water. A volume of 20 mL from each sample were loaded at an approximate velocity of 1 mL $min^{-1}$ followed by the same volume of ultra-pure water for desalting. After that, the columns were vacuum dried during 5 min. TCS was then eluted with 4 mL of methanol and 4 mL of dichloromethane into a glass jar and samples were then dried with a gentle stream of nitrogen. Triclosan was reconstituted in 2 mL of methanol and sodium sulphate was added to remove any water content. Reconstituted samples were filtered with a mixed cellulose ester membrane filter (0.22 µm, 25 mm) and diluted to 0.5 mg $L^{-1}$ of TCS in methanol. The analyses were performed in triplicate using HPLC with PDA detector

(SPD-M20A, Shimadzu Co.) and a 15x0.46 cm column with a particle size of 5 µm (Brisa "LC$^2$", Teknokroma). The volume of injection was 10 µL, the flow rate was set at 1 mL min$^{-1}$, the mobile phase was 70% acetonitrile and 30% ultra-pure water and oven temperature was 25ºC. The TCS peak was detected at 7.0 min at a wavelength of 280 nm. Area calculation was performed using Labsolutions Series Workstation software (Shimadzu Co). For TCS quantification, three standards of TCS in artificial saltwater (10 mg L$^{-1}$) were prepared after dilution of an initial stock solution in acetone (5 mg mL$^{-1}$). Standard solutions followed the same SPE procedure as samples and were diluted for concentrations between 0.025 and 1.200 mg L$^{-1}$ of TCS in methanol. Standards were measured in triplicate and used to calculate a calibration line. The determination coefficient ($R^2$) of the calibration line and the limit of detection (LOD) were 0.9959 and 7.8 µg L$^{-1}$, respectively. The LOD was calculated as ($3S_{y/x}$)/m, where m is the slope of the regression line and $S_{y/x}$ is the sum of residuals that estimates the random errors in the yy axis (Leal *et al*., 2017).

### 2.8. Statistical analysis

In the *in vitro* assays with ChE inhibitors for ChEs characterization, statistical differences of ChE activity between control and ChE inhibitor treated samples were tested by One-way ANOVA followed by pairwise Dunnett's test after testing normality (Kolmogorov-Smirnov) and homoscedasticity (Levene's mean test). Non-parametric Kruskal-Wallis was followed by pairwise multiple comparison procedures (Student-Newman-Keuls) when normality and/or homoscedasticity was not observed. Solvent control was used to compare inhibition of iso-OMPA concentrations on both substrates, after an initial t-test comparison between solvent and negative control.

In order to determine differences on baseline levels of biochemical markers (AChE, CAT, GST or LDH) between each of the three life stages studied, One-way ANOVA was performed after verifying normality and homoscedasticity of data. Pairwise multiple comparison procedures (Tukey Test) were performed as post-hoc test.

In the bioassays with TCS, logistic three parameter regression model was used to determine lethal and effect concentrations (LC and EC, respectively) of TCS. Student t-tests were performed to test for differences between negative and solvent controls for all appropriate endpoints. Being significantly different or not from negative control, solvent control was always used in further data analyses to determine significant differences with TCS treatment groups. One-way ANOVA followed by Dunnett's test was used for comparison between treatments and solvent control for hatching, length and biochemical markers levels. Non-parametric Kruskal-Wallis test followed by pairwise Dunn's test was

performed when normality and/or homoscedasticity were not obtained (malformations at the metamorphosis test). Metamorphosis progression was studied using Chi-Square test. For significant ages, pairwise post-hoc Chi-Square with Bonferroni adjustment were used to test differences between solvent control and TCS groups individually (Arnholt, 2016).

All the statistical procedures were performed using SigmaPlot version 12.0 (Systat Software, Inc.). Results are expressed as mean ± standard error (SE).

## 3. Results

### 3.1. Characterization of ChEs

#### 3.1.1. Substrates

The preference of the ChE(s) present in the three stages of *S. senegalensis* for each of the substrates used in the enzymatic reactions is depicted in figure 2.1.

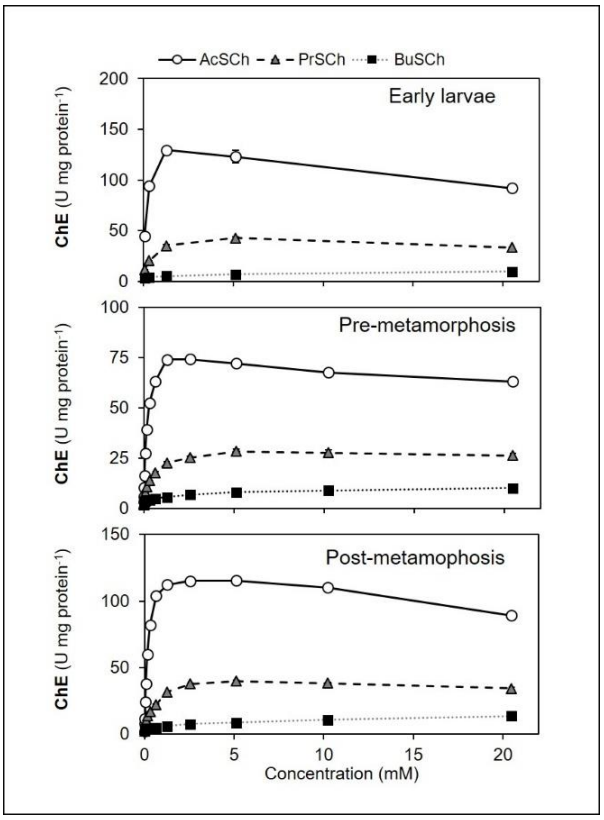


Fig. 2.1. Cholinesterase (ChE) activity with increasing concentrations of substrate at three stages of development of *Solea senegalensis*: early larvae (3 days after hatching, dah, n=3, 25 organisms per replicate); pre-metamorphic larvae (13 dah,.n=3, 9 organisms per replicate) and post-metamorphic larvae (22 dah, n=3, 9 organisms per replicate). Acetylthiocoline iodide (AcSCh), propionylthiocholine iodide (PrSCh) and S-butyrylthiocholine iodide (BuSCh) were used as substrates.

The AcSCh was the substrate cleaved at highest rate in all development stages of *S. senegalensis* followed by the PrSCh and BuSCh substrates. The maximum ChE activity was obtained using AcSCh at 1.28 mM for 3 dah fish (with 129.6±3.12 U mg protein$^{-1}$), 2.56 mM for fish at the beginning of metamorphosis (with 74.2±1.30 U mg protein$^{-1}$) and at 5.12 mM for fish at the end of metamorphosis (with 115.4±1.53 U mg protein$^{-1}$). Furthermore, a reduction of ChE activity was observed with the highest AcSCh concentrations tested in all development stages studied. Using PrSCh as substrate, maximum ChE activity was measured at 5.12 mM in the three development stages tested, with values ranging between 28.3±1.21 and 42.6±1.08 U mg protein$^{-1}$ (in pre-metamorphosing fish and 3 dah larvae, respectively). The BuSCh was the substrate cleaved at a lower rate in the three development stages, with maximum ChE activity measured at the highest substrate concentration tested (20.48 mM). In addition, the highest ChE activity observed using this substrate in sole was at the end of metamorphosis (13.5±0.23 U mg protein$^{-1}$).

### 3.1.2. Inhibitors

The selective inhibitor of ChEs, eserine sulfate, almost completely inhibited ChE activity in all *S. senegalensis* early life stages even at the lowest concentration tested (6.25 µM, fig. 2.2A, $p<0.05$), with percentages of inhibition over 85.5%.

In the BW284C51 assay using AcSCh as substrate, there was a significant decrease (above 80%) of enzyme activity with all concentrations of the inhibitor ($p<0.05$, fig. 2.2B) for all development stages.

Using AcSCh as substrate, no significant decrease of ChE activity was observed in 3 dah larvae with iso-OMPA, the selective inhibitor of BChE (fig. 2.2C, $p>0.05$). However, in the other two later life stages, significant inhibition in ChE activity was observed for all tested concentrations of iso-OMPA ($p<0.05$) with maximum percentages of inhibition of about 35.8% in post-metamorphosing *S. senegalensis* at 8000 µM.

Using the preferred substrate of BChE, BuSCh, there was no inhibition with BW284C51, except for the highest concentration used in fish homogenates of pre- and post-metamorphosing *S. senegalensis* with inhibition percentages up to 19.1% at the end of metamorphosis ($p<0.05$, fig. 2.2D). On the contrary, using the same substrate, there was significant inhibition of ChE activity with all concentrations of iso-OMPA tested in fish of the three life stages studied ($p<0.05$, fig. 2.2E) with a minimum of 70.9% inhibition at the earliest life stage.

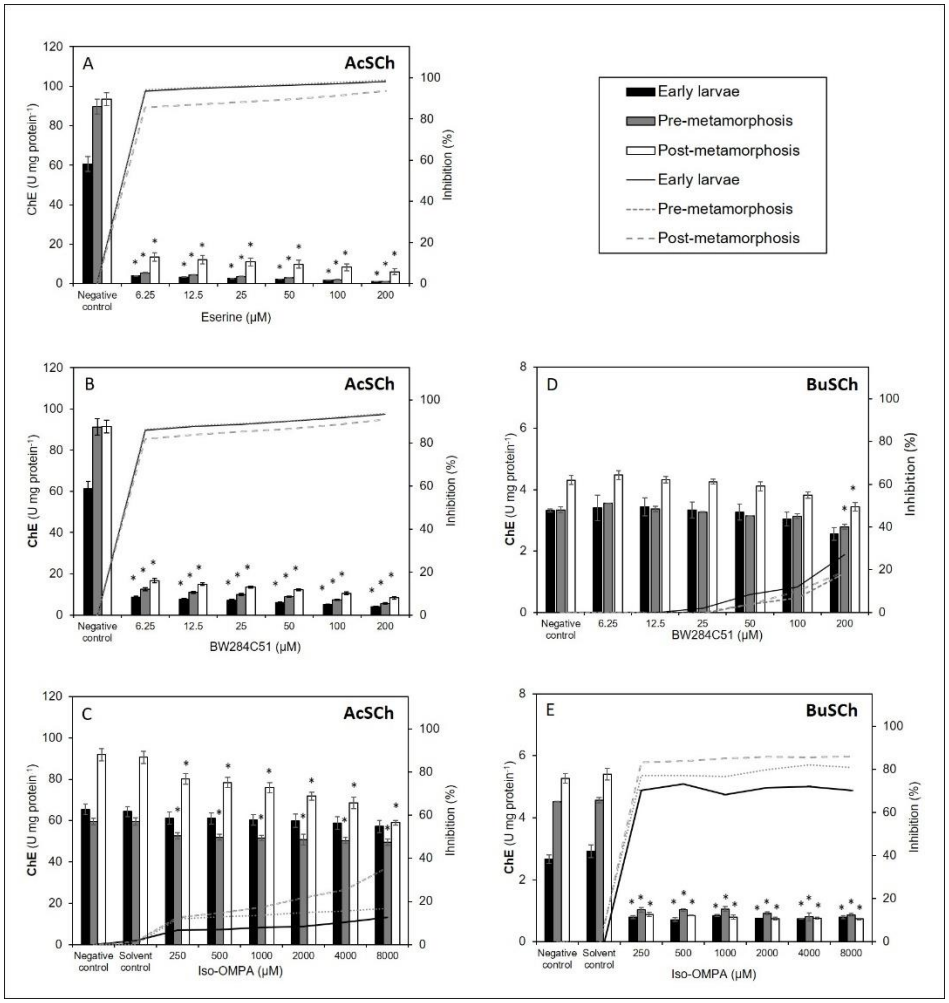


Fig. 2.2. Cholinesterases (ChE) activity (bars) and percentage of enzymatic inhibition (lines) of ChE activity in whole-body samples of *Solea senegalensis* in three stages of development: early larvae (3 days after hatching, dah, n=3, 20 organisms per replicate); pre-metamorphic larvae (13 dah, n=3, 9 organisms per replicate) and post-metamorphic larvae (22 dah, n=3; 9 organisms per replicate). Three selective inhibitors (eserine, BW284C51 and Iso-OMPA) were used and enzymatic assays were performed with two different substrates (acetylthiocholine, AcSCh and butyrylthiocholine, BuSCh). * represent the existence of significant differences of ChE activity between inhibitor-treated fish samples and solvent control or negative control fish samples if solvent control was not used ($p<0.05$). Results are expressed as mean±standard error.

### 3.2. Biochemical markers baseline levels

Biochemical markers baseline levels of *S. senegalensis* on three development stages are presented in table 2.1. No significant difference on AChE activity were found between fish development stages ($p>0.05$) with values ranging between 85.1±4.69 U mg protein$^{-1}$ at the beginning of metamorphosis and 96.3±2.67 U mg protein$^{-1}$ at the end of metamorphosis.

The activity of CAT was significantly higher in the beginning of metamorphosis (11.6±0.72 U mg protein$^{-1}$) when comparing to other development stages ($p<0.05$). The

CAT activity levels in earliest life stage and after the metamorphosis were 7.7±0.29 and 6.9±0.47 U mg protein$^{-1}$, respectively.

The levels of GST activity decreased along fish development, with significantly lower activity at the end of metamorphosis (10.8±0.30 U mg protein$^{-1}$, $p<0.05$) when compared with the two earlier stages (16.5±0.94 U mg protein$^{-1}$ at the beginning of metamorphosis and 18.4±0.60 U mg protein$^{-1}$ at 3 dah).

Activity of LDH increased near three-fold between 3 dah and the beginning of metamorphosis (0.046±0.0017 and 0.172±0.0036 U mg protein$^{-1}$, respectively, $p<0.05$) and significantly decreased at the end of metamorphosis (0.067±0.0096 U mg protein$^{-1}$, $p<0.05$).

Table 2.1. Baseline levels of biochemical markers in *Solea senegalensis* along early development: larvae (3 days after hatching, dah), at the onset of metamorphosis (13 dah) and at the end of metamorphosis (22 dah).

| **Biomarker** | **3 dah** (U mg protein$^{-1}$) | **13 dah** (U mg protein$^{-1}$) | **22 dah** (U mg protein$^{-1}$) |
|---|---|---|---|
| AChE | 87.5±2.21 | 85.1±4.69 | 96.3±2.67 |
| CAT | 7.7±0.29$^{a}$ | 11.6±0.72$^{b}$ | 6.9±0.47$^{a}$ |
| GST | 18.4±0.60$^{a}$ | 16.5±0.94$^{a}$ | 10.8±0.30$^{b}$ |
| LDH | 0.046±0.0017$^{a}$ | 0.172±0.0036$^{b}$ | 0.067±0.0096$^{a}$ |

Acetylcholinesterase, AChE; Catalase, CAT; Glutathione S-transferase, GST; Lactate dehydrogenase, LDH. Different superscript letters represent the existence of significant differences ($p<0.05$) within each biochemical marker between life stages. Results expressed as mean±standard error.

### 3.3. Effects of TCS

#### 3.3.1. Chemical analysis of TCS

Values of nominal and measured concentrations of TCS are presented in table 2.S1. The difference between measured and nominal concentrations was below 20% and therefore, nominal concentrations were used for all data analyses. The depletion of TCS after 48h ranged between 67.5% and 84.7% (for nominal concentrations of 200 and 1500 µg L$^{-1}$ TCS, respectively) in the fish metamorphosis assay.

#### 3.3.2. Effects of TCS on sole early larvae

Hatching after 24h of exposure to TCS ranged between 87.5±2.50% and 96.7±3.33% (for fish exposed to 95 µg L$^{-1}$ TCS and solvent control, respectively) without the existence of significant differences ($p>0.05$). In all testing groups, hatching was 100% after 48h of exposure to TCS.

Survival of *S. senegalensis* in negative and solvent control was above 90% at the end of the early larval test. Exposure to the highest concentration (500 µg L$^{-1}$) induced 100%

of mortality in *S. senegalensis* eggs and larvae at 1 dah. The 96h $LC_{50}$ for TCS exposure was 218±10.8 µg $L^{-1}$ (fig. 2.S2).

There were no differences on total length of fish between negative control and solvent control (3.6±0.05 mm for both groups, $p>0.05$). After 96h of exposure, the length fish exposed to concentrations higher than 53 µg $L^{-1}$ TCS were significantly lower than the length of fish from solvent control group (between 11% and 20% lower) ($p<0.05$, fig. 2.S3).

Oedema was observed in 19±7% of *S. senegalensis* larvae exposed to the second highest concentration of TCS tested (300 µg $L^{-1}$) at 1 dah and was not present on following days. Abnormal spinal malformation was detected in organisms exposed to the two highest treatment groups (300 and 500 µg $L^{-1}$) at 24hpf (5.0±5.0% and 90.5±9.52%, respectively) and also at 95 and 169 µg $L^{-1}$ treatment groups at 1 dah (8.4±2.85% and 26.8±15.5%, respectively). At 2 dah and at the end of the test (3 dah) all treatment groups presented fish with this malformation (fig. 2.3). An overall $EC_{50}$=180±18.0 µg $L^{-1}$ TCS was obtained when considering malformations present in the organisms at the 3 dah (fig. 2.S4). The teratogenic index was estimated as 1.1.

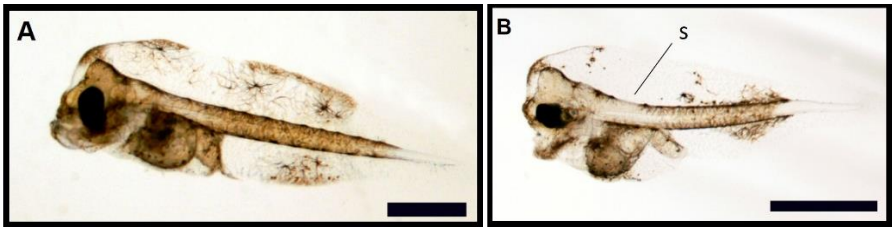


Fig. 2.3. *Solea senegalensis* early larvae (3 days after hatching) after 96h exposure to triclosan (TCS). A) negative control; B) larvae after exposure to 169 µg $L^{-1}$ TCS presenting spinal curvature (S). Black bars represent 1 mm.

Effects of TCS at biochemical level on *S. senegalensis* exposed during 96h from egg stage until 3 dah are presented in figure 2.4. Significant differences were not observed between solvent control and negative control on AChE, CAT, GST activities and LPO levels ($p>0.05$). No significant differences were observed on AChE, CAT and LPO when comparing TCS exposed larvae with solvent control at the early larval test ($p>0.05$). On the contrary, a significantly higher GST activity was observed in larvae after exposure to all tested concentrations of TCS ($p<0.05$).

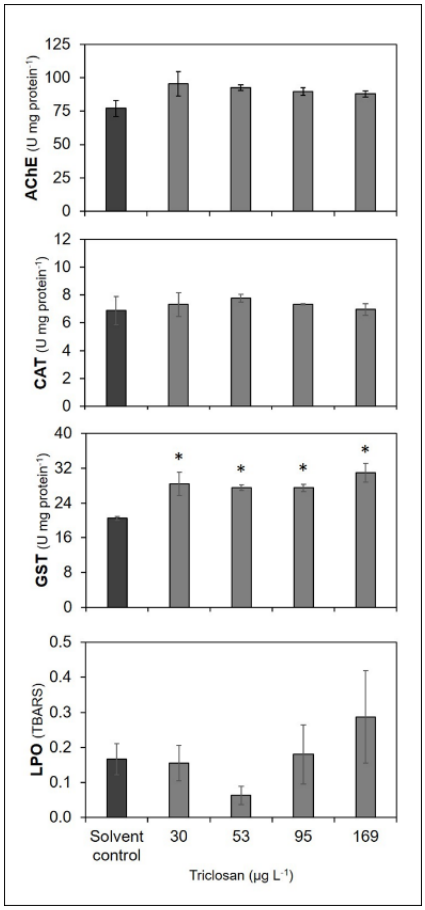


Fig. 2.4. Biochemical markers of *Solea senegalensis* early larvae (3 days after hatching) after 96h exposure to triclosan. AChE – acetylcholinesterase; CAT – catalase; GST – glutathione S-transferase; LPO – lipid peroxidation. * represent the existence of significant differences with solvent control ($p<0.05$). n=4, 10 fish per replicate, results are expressed as mean±standard error.

### 3.3.3. Effects of TCS on sole metamorphosis

Survival of metamorphosing *S. senegalensis* after 48h of exposure to TCS was above 90% for groups exposed below or equal to 1072 µg $L^{-1}$ TCS. Fish exposed to 1500 µg $L^{-1}$ TCS presented 25±9.4% of survival at 48h of exposure and a $LC_{50}$ of 1357±31.5 µg $L^{-1}$ TCS was obtained. Fish exposed to the highest concentration tested (1500 µg $L^{-1}$ TCS) presented 100% of mortality at 18 dah. The test ended at 24 dah, when 80% of organisms from control groups completed the metamorphosis. At 24 dah, survival was 91.7±3.73% for negative control and 79.2±5.16% for solvent control, with no significant differences observed between these two controls ($p>0.05$). The lowest survival percentage (77.8±8.24%) was observed for fish exposed to 548 µg $L^{-1}$, however no significant differences were observed between exposed groups and solvent control ($p>0.05$, fig. 2.S5).

At the end of metamorphosis no differences were observed on total length between fish from negative and solvent control groups (8.9±0.22 and 9.1±0.21 mm, respectively,

p>0.05). Fish exposed to TCS concentrations higher or equal to 548 µg $L^{-1}$ presented significantly lower length than fish from solvent control (8.1±0.24 mm, p<0.05, fig. 2.S6).

The percentage of malformations along fish metamorphosis (fig. 2.5) was lower than 10% in fish from control groups and was not significantly different when comparing solvent and negative control groups (p>0.05). The malformations observed in fish exposed to TCS included altered pigmentation, abnormal migration of the eye and underdeveloped structure of head bones. At 17 dah (after 48h in clean media post exposure to TCS), the maximum percentage of development abnormalities was observed, with significant differences between solvent control fish and fish exposed to concentrations higher than 391 µg $L^{-1}$ (p<0.05) reaching up to 42.0±8.27% for fish exposed to 766 µg $L^{-1}$. However, the frequency of such malformations tended to decrease over time in the clean media and, at the end of metamorphosis, no significant differences were observed between TCS exposed fish and fish in solvent control (p>0.05).

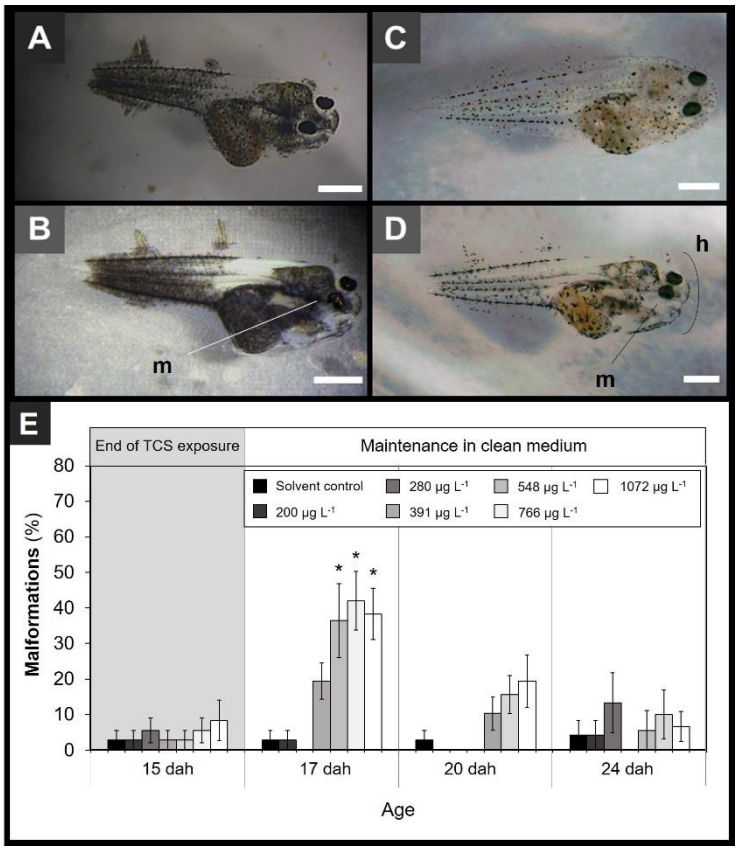


Fig. 2.5. Metamorphosing *Solea senegalensis* after 48h exposure to triclosan (TCS) at 13 days after hatching (dah). A) solvent control (18 dah); B) fish exposed to 766 µg $L^{-1}$ TCS (18 dah); C) solvent control (24 dah); D) fish exposed to 766 µg $L^{-1}$ TCS (24 dah). (m) abnormal migration of the eye and (h) underdeveloped head structure. White bar represents 1 mm. E) Total malformations (%) at 15, 17, 20 and 24 dah. * represent the existence of significant differences with solvent control within each age (p<0.05). Results are expressed as mean±standard error.

The frequency of fish in each metamorphic stage at 14, 17, 20 and 24 dah in the different treatment groups are presented in figure 2.6. Significant differences on sole metamorphosis progression stages between control groups were only found at 20 dah, with fish from solvent control showing a delay in development when comparing to negative control (48% of negative control fish were in stage E while 39% of solvent control fish were in stage D, $p<0.05$). At 14 dah no differences were observed on metamorphosis stages between solvent control and TCS exposed fish ($p>0.05$). Metamorphosis of fish exposed to 391, 548, 766, and 1072 µg $L^{-1}$ TCS presented a significantly faster progression than fish from solvent control at 17 dah ($p<0.05$). At 20 dah fish exposed to 1072 µg $L^{-1}$ TCS was still more developed than solvent control ($p<0.05$). However, at the end of the maintenance in clean media (24 dah) no significant differences were observed on sole development stages when comparing solvent control and TCS treatment groups ($p>0.05$).

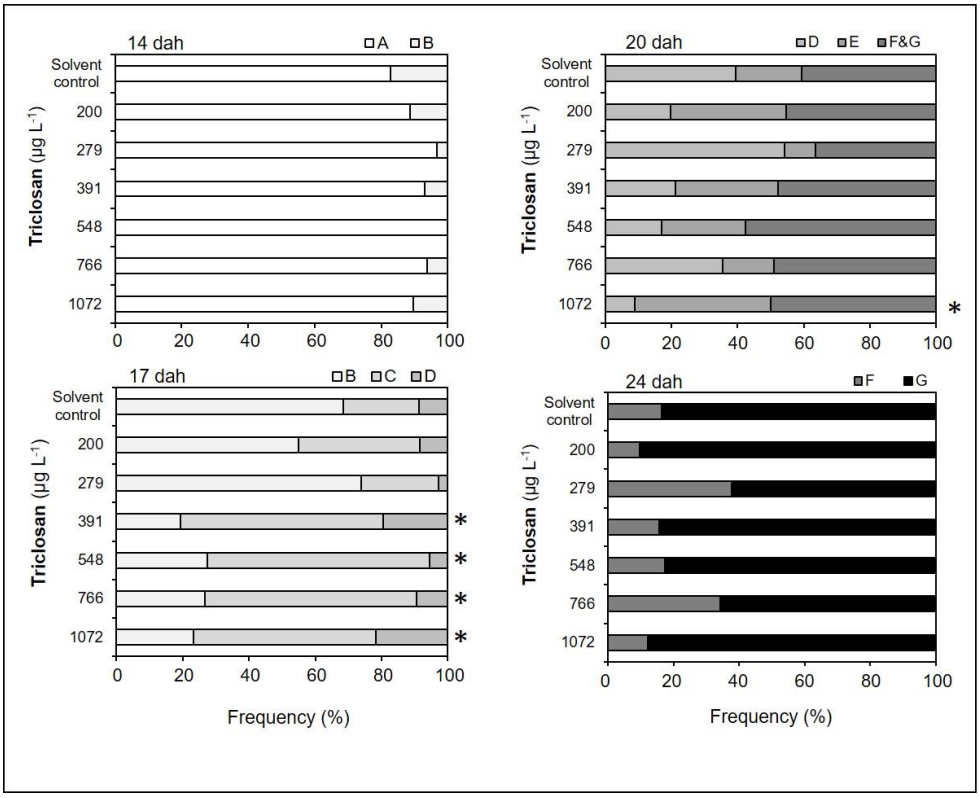


Fig. 2.6. Metamorphosis stages of *Solea senegalesins* after 48h of exposure to triclosan at the beginning of metamorphosis (13 days after hatching, dah). n=6, 10 fish per replicate. A, B – early metamorphosis (enlargement of fins, beginning of migration of left eye); C, D - alteration of mouth shape and pigmentation darkening; E - fully flattened body; F - anterior profile becomes more curved; G – complete metamorphosis: orbital eye membrane becomes thicker. * represent the existence of significant differences with solvent control for each age ($p<0.05$).

Effects of TCS at biochemical level during metamorphosis of *S. senegalensis* are presented in figure 2.7. No significant differences were observed when comparing the AChE, CAT, GST and LDH activities between solvent and negative control fish groups

immediately after the 48h exposure test (15 dah, $p>0.05$). LPO was significantly lower in negative control (0.60±0.035 nmol TBARS mg protein$^{-1}$) when comparing to solvent control fish (0.72±0.034 nmol TBARS mg protein$^{-1}$) immediately after the 48h test ($p<0.05$). At the end of the test (24 dah), differences between solvent and negative control were not observed for any of the biochemical markers studied ($p>0.05$).

Immediately after 48h of exposure to TCS (15 dah), a significantly higher AChE activity was observed in fish exposed to 548 and 766 µg L$^{-1}$ TCS ($p<0.05$). At the end of metamorphosis (24 dah), no TCS effects were observed on AChE activity ($p<0.05$).

There was no significant differences on CAT activity between solvent control and fish exposed to TCS immediately after the 48h of exposure ($p>0.05$). However, at the end of metamorphosis (24 dah), significantly lower CAT activity was observed in fish exposed to 280 and 766 µg L$^{-1}$ TCS, and significantly higher CAT activity was observed in fish exposed to 391 µg L$^{-1}$ when comparing to solvent control ($p<0.05$), while for the other two TCS exposed fish groups no differences were observed on CAT activity when comparing to solvent control ($p<0.05$).

For the groups of fish exposed to the three highest concentrations of TCS (391, 548 and 766 µg L$^{-1}$), GST was significantly lower immediately after 48h exposure, when comparing to solvent control ($p<0.05$). However, no significant differences were observed on GST activity at the end of metamorphosis when comparing TCS exposed fish to solvent control ($p>0.05$).

No significant effects were observed on LPO levels both immediately after 48h TCS exposure and at the end of metamorphosis when comparing TCS exposed fish and solvent control ($p>0.05$).

Lactate dehydrogenase was significantly lower for fish with ongoing metamorphosis exposed to TCS concentrations above 280 µg L$^{-1}$ when comparing to solvent control ($p<0.05$). However, at the end of metamorphosis LDH activity levels were similar in fish allocated to the different treatments ($p>0.05$).

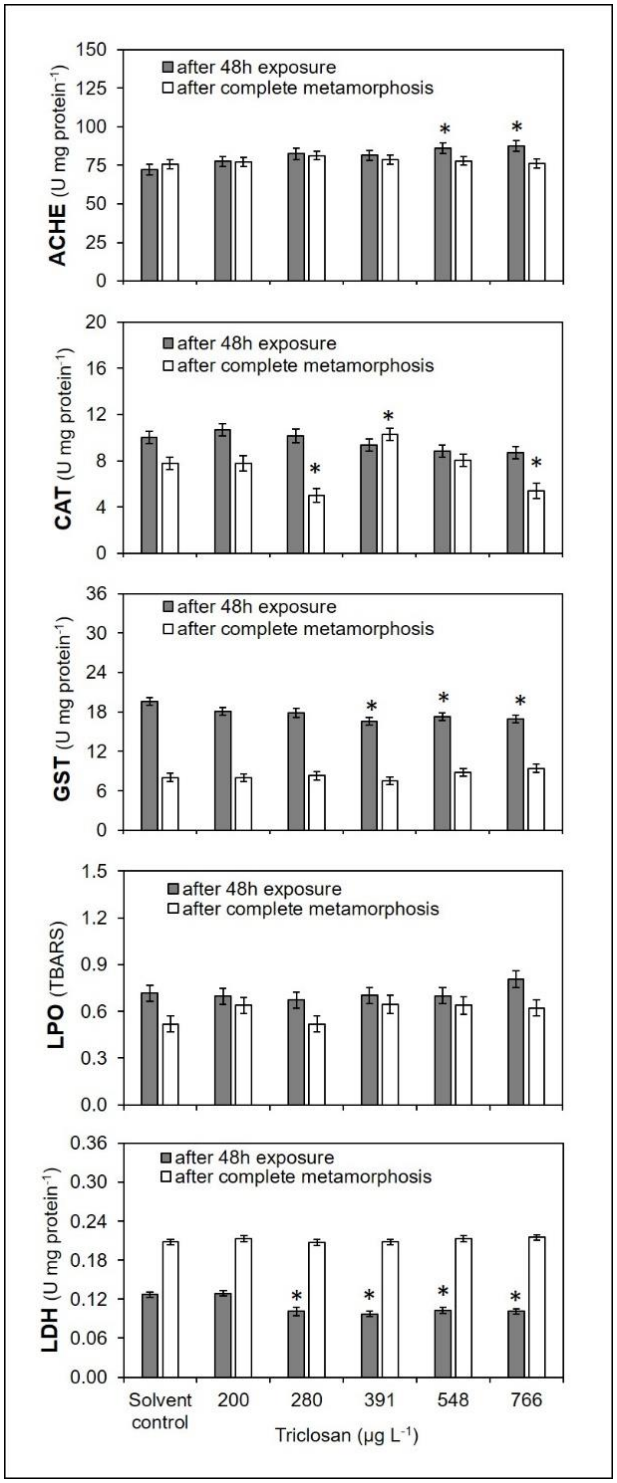


Fig. 2.7. Biochemical markers in *Solea senegalensis* after 48h of exposure to triclosan (15 days after hatching, dah) and at the end of metamorphosis (24 dah). AChE – acetylcholinesterase; CAT – catalase; GST – glutathione S-transferase; LPO – lipid peroxidation; LDH – lactate dehydrogenase. * represent the existence of significant differences with solvent control for the same age ($p<0.05$). n=6, 10 fish per replicate for 15 dah fish and n=6, 6 fish per replicate for 24 dah fish. Results are expressed as mean±standard error.

## 4. Discussion

In this work, effects of exposure to TCS along early development of *S. senegalensis* were evaluated after characterization of ChEs and the study of biochemical markers baseline levels in whole body homogenates.

### 4.1. Characterization of ChEs

While measuring ChE activity on *S. senegalensis* during early development, the high inhibition by eserine along all stages studied indicates that only ChEs are present. There was an higher preference for AcSCh over the other substrates and ChE activity was strongly inhibited by the AChE selective inhibitor BW284C51 when using the AcSCh. Therefore, AChE is the most abundant active ChE form on whole body samples of *S. senegalensis* larvae. Furthermore, the enzymatic activity decreased with increasing concentration of the substrate AcSCh, which is a typical characteristic of vertebrate AChE as described by other authors (Eto, 1974; Sturm *et al*., 1999; Lionetto *et al*., 2013). There was a low but continuously increasing ChE activity with the preferred substrate of BChE (BuSCh) along all *S. senegalensis* development stages studied. Such increase might be related with organ and tissue differentiation along development, since levels and proportions of ChE forms might change between different organs and tissues. For instance, AChE is the main predominant form in the brain of vertebrates, in blood is BChE (e.g. Monteiro *et al*., 2005; Santos *et al*., 2012). Nevertheless, a negligible influence of BChE on total ChE activity measurement is expected.

The ChE activity is resistant to the specific BChE inhibitor, iso-OMPA, at the earliest life stage tested, but a low inhibition percentage was observed at later development stages. Therefore, together with the very low BChE activity detected in later stages, our results suggest that despite the main ChE form present is AChE, since it preferably cleaves ASCh and is almost completely inhibited by BW284C51 (selective inhibitor of AChE), this enzyme form also presents an atypical characteristic which is the sensitivity to iso-OMPA. This atypical characteristic has also been described for AChE present in other marine fish species (e.g. Monteiro *et al*., 2005).

### 4.2. Biochemical markers baseline levels

Several biochemical changes occur during development processes of a wide range of organisms, namely during early larval stages and metamorphosis (Kashiwagi, 1995; Dandapat *et al*., 2003; Jovanović-Galović *et al*., 2004). The present work shows several

biochemical changes along *S. senegalensis* development. The activity of AChE was not significantly altered along the development of *S. senegalensis* which is in accordance with the fact that vertebrate cholinesterases appear early during ontogenesis (Layer, 1990; Pezzementi *et al*., 2010). On the other hand, CAT, GST and LDH changed significantly as discussed below. In our study, CAT baseline activity levels significantly increased in the beginning of *S. senegalensis* metamorphosis which is in accordance with the work of Fernández-Díaz *et al*. (2006). These authors also observed an increase of this enzyme at beginning of sole metamorphosis (10 dah) followed by a decrease at 15 and 20 dah. This increase of CAT can be associated with the increase of ROS production (such as $H_2O_2$), which is usually associated to several processes of metamorphosis progression. On the one hand, an increase of $H_2O_2$ can be expected with increase in metabolism that might occur during metamorphosis (Yúfera *et al*., 1999; Fernández-Díaz *et al*., 2001; Geffen *et al*., 2007). On the other hand, cell death naturally occurs during flatfish metamorphosis (Sun *et al*., 2015) and CAT alterations and production of $H_2O_2$ have been associated with normal mechanisms of cell death, namely during amphibian metamorphosis (Kashiwagi, 1995; Kashiwagi *et al*., 1997). In addition to CAT, GST also works on organism defense against ROS. The activity of GST has already been shown to change along sole metamorphosis. For instance, Pimentel *et al*. (2015) reported increasing GST activity in *S. senegalensis* from 10 to 30 dah grown in similar conditions as in the present study. Such trend was not observed in our study since at 22 dah the activity of GST was lower than at early ages (3 dah and 13 dah). These different patterns of GST activity might be due to the different ages considered between both studies. Furthermore, the decrease in GST activity observed at the end of sole metamorphosis might be related with lower levels of the substrate glutathione, which was found to be decreased during metamorphosis of anurans (Menon and Rozman, 2007).

The LDH enzyme has been directly associated with several energetic-related processes of organisms such as metamorphosis or periods of increased growth rate (Pelletier *et al*., 1995; Geffen *et al*., 2007; Wen *et al*., 2017). In our work, there was a significant increase of LDH at the beginning of metamorphosis followed by a decrease at the end. Since the onset of metamorphosis is an high demanding energetic process on flatfish development (Yúfera *et al*., 1999), our results suggests the use of anaerobic metabolism by the fish during this development period.

### 4.3. Effects of TCS

Regarding the chemical analysis of TCS in testing solutions, the difference obtained between nominal and measured concentrations were within acceptable range (below 20%). However, high depletion of TCS along the experiments occurred, which can be associated with the photochemical degradation of the compound. During the experiments, adsorption by lipid droplets during egg stage or absorption by the organism tissues after hatching stages may also have occurred along with eventual metabolization of TCS by the fish (Dhillon *et al*., 2015).

Triclosan has been reported to affect several aquatic organisms at concentrations from 3.4 to 300 µg $L^{-1}$ TCS (Tatarazako *et al*., 2004). In this study, effects on *S*. *senegalensis* below this value were also observed for several endpoints, namely on mortality, growth, prevalence of malformations in larvae and in biomarkers at both life stages studied (table 2.2). Exposure to TCS is reported to decrease and delay hatching of medaka *Oryzias latipes* after exposure to 313 µg $L^{-1}$ TCS or above (Ishibashi *et al*., 2004) and to significantly decrease zebrafish *Danio rerio* hatching until 72 h after exposure to 500 µg $L^{-1}$ TCS (Oliveira *et al*., 2009). However, TCS (up to 500 µg $L^{-1}$) did not affect *S*. *senegalensis* hatching in the present study. In the case of *S*. *senegalensis* eggs, their highly lipidic outer layer may adsorb organic compounds during first hours of development not affecting the species hatching. In addition, when comparing to these species (medaka and zebrafish) the hatching of *S*. *senegalensis* occurs earlier during development.

The $LC_{50}$ of *S*. *senegalensis* exposed to TCS at the early larval test (218 µg $L^{-1}$) is lower than the observed for other fish species, namely for the freshwater fish species zebrafish (96h $LC_{50}$=420 µg $L^{-1}$, Oliveira *et al*., 2009) and *Lepomis macrochirus* (96h $LC_{50}$=370 µg $L^{-1}$, Orvos *et al*., 2002), revealing an higher sensitivity of the species used in the present study during larval stages. Indeed, saltwater fish tend to be more sensitive to chemicals than freshwater fish. For instance, in a study including substances from several classes, in 50% of the cases saltwater fish were more sensitive than freshwater species, while in 25% of the studies they were less sensitive (Hutchinson *et al*., 1998). Besides this, the six-fold increase of $LC_{50}$ between 3 dah larvae and after 48h exposure to TCS at the beginning of metamorphosis suggests a decrease of sensitivity with the development progression of the species. This can be related with development of defense and detoxification mechanisms in older life stages, that enable fish to better cope with TCS exposure.

Growth of *S*. *senegalensis* was affected by the exposure to TCS, both in the early larval and metamorphosis tests (LOEC=95 and 548 µg $L^{-1}$, respectively), which is in

accordance with previous observations in fish species exposed to TCS (Orvos *et al.,* 2002; Ishibashi *et al*., 2004; Oliveira *et al*., 2009). In addition, in the marine fish white seabream (*Diplodus sargus*) significant negative correlations were obtained between morphometric data, including total length, and TCS accumulation in fish liver (Maulvault *et al*., 2019). The effects observed on growth of fish species might have severe ecological implications, including delayed or unsuccessful metamorphosis, effects on reproduction and/or ultimately decreased survival.

Abnormal spinal curvature was previously reported on *S. senegalensis* larvae in response to xenobiotics exposure (Pavlaki *et al*., 2016; Araújo *et al*., 2018) and was also observed in present work in response to TCS exposure at the larval stage test. This malformation has also been reported in zebrafish exposed to TCS (Orvos *et al*., 2002). Triclosan is pointed to be a low teratogenic compound to zebrafish (Ducharme *et al*., 2013) and according to our study, TCS can also be considered relatively low teratogenic to *S. senegalensis*, as the teratogenic index is relatively close to the threshold (1).

In our work, the malformations observed in TCS exposed metamorphosing sole at 17 dah, after 2 days in clean medium, suggest delayed morphological effects of TCS. Although, at the end of metamorphosis (24 dah) no significant percentage of malformations was observed, which might indicate a possible recovery from the exposure to TCS when the period of non-exposure is prolonged.

Sole metamorphosis was also affected by TCS that induced a faster progression at intermediate development stages. During metamorphosis of anurans the exposure of the organisms to TCS have also been associated with acceleration rate and abnormal timing of metamorphic events, in addition, TCS was suggested to interact with receptors of the thyroid hormones (TH) T3 and T4 (Veldhoen *et al*., 2006; Sowers and Klaine, 2008). Progression of metamorphosis is directly dependent on the fluctuation of TH levels (Yamano *et al*., 1991; Okada *et al*., 2003; Klaren *et al*., 2008). Furthermore, TCS is pointed to interfere with thyroid axis, acting as thyroid disrupting chemical following its structural similarity with the TH (Crofton, 2008; Veldhoen *et al*., 2006; Luthe *et al*., 2008). However, the specific molecular mechanisms through which TCS interfere with metamorphosis progression of *S. senegalensis* are still unknown; the transient acceleration of metamorphosis observed suggests a possible pro thyroid activity of TCS which needs to be further studied and confirmed. As well, other possible mechanisms not directly related with thyroid axis, which might also be responsible for the appearance of malformations phenotype, should also be considered.

In our study, assessment of biochemical effects of TCS on *S. senegalensis* showed alterations on AChE, CAT, GST and LDH biochemical markers in both sole life stages studied, revealing different effects on neurotransmission, antioxidant defense system and anaerobic metabolism. Oliveira *et al*. (2009) also reported different biochemical effects of TCS on zebrafish depending on life stage. While AChE, GST and LDH were affected in larvae of zebrafish exposed to 250 µg $L^{-1}$; adults were not significantly affected at concentrations up to 350 µg $L^{-1}$ (Oliveira *et al*., 2009).

Triclosan exposure has been shown to cause AChE inhibition in some species, namely in juveniles of the marine fish white seabream *Diplodus sargus* (Maulvault *et al*., 2019), amphibian larvae (Martins *et al*., 2017) and in the brain tissue of *Pangasianodon hypophthalmus* fingerlings, a freshwater fish (Sahu *et al*., 2018). However, the contrary has also been reported. For instance, AChE activity in zebrafish larvae was induced with exposure to 250 µg $L^{-1}$ TCS (Oliveira *et al*., 2009) and also in another study, when exposed to 50 and 100 µg $L^{-1}$ TCS (Falisse *et al*., 2017). In the present work, induction of AChE by TCS on *S. senegalensis* seems also to occur. While the AChE induction trend was not significant at the early larval stage, a significant induction was observed immediately after 48h exposure in metamorphosing fish for the two highest TCS concentrations tested. At the end of metamorphosis, AChE activity returned to control levels in TCS exposed fish indicating possible a recovery. Previous works with compounds suspected of thyroid disruption have been reported to induce AChE, namely, in *S. senegalensis* larvae exposed to the organic UV filter 4-MBC (Araújo *et al*., 2018) and in zebrafish larvae exposed to carbendazim (Andrade *et al*., 2016). The involvement of TH in the regulation of AChE activity has also been previously suggested (Puymirat *et al*., 1995; Andrade *et al*., 2016), therefore, effects of TCS on thyroid function may explain the differences in AChE activity and should be further studied. In addition, TCS have been previously reported to induce neurotoxicity through the activation of apoptosis of neuronal cells (Ruszkiewicz *et al*., 2017) and apoptosis has been associated with increased AChE activity (Zhang and Greenberg *et al*., 2012). Therefore the AChE induction observed in our work on sole larvae might be related with TCS induction of neuronal apoptosis.

In our study, the TCS exposure triggered different responses on antioxidant enzymes depending on the fish life stage assessed. While effects on CAT activity were not observed at the early larval test and immediately after the 48h exposure at the beginning of metamorphosis, a bell-shaped response of CAT activity was observed at the end of metamorphosis. Previous works showed an induction of CAT in muscle of the marine fish *D. sargus* exposed to TCS through diet (Maulvault *et al*., 2019). Furthermore, induction of

CAT were also reported in other studies with different freshwater fish species, namely in the yellow catfish, *Pelteobagrus fulvidraco* (0.5 µg $L^{-1}$ TCS; Ku *et al*., 2014), in *P. hypophthalmus* (above 97 µg $L^{-1}$; Sahu *et al*., 2018) and in goldfish *Carassius auratus* (above 280 µg $L^{-1}$; Wang *et al*., 2018). As previously discussed, CAT activity levels are already relatively higher during early metamorphosis and TCS seem not to have the ability to induce CAT activity above the naturally expected levels during normal progression of metamorphosis. However, effects of TCS on CAT activity were observed at the end of fish metamorphosis, after the period of maintenance in clean media, suggesting later effects on the mechanism of this specific antioxidant enzyme.

In our work, GST activity was affected immediately after exposure to TCS. While a clear induction of GST activity occurred at the end of the early larval test (Lowest Observed Effect Concentration, LOEC: 30 µg $L^{-1}$), a GST inhibition after 48h TCS exposure at the metamorphosis test was observed (LOEC: 391 µg $L^{-1}$). Triclosan also caused alterations in GST activity in several fish species, including inhibition in concentrations equal and over 50 µg $L^{-1}$ TCS on adult yellow catfish (*Pelteobagrus fulvidraco*, Ku *et al*., 2014) and induction in the liver of swordtail fish (*Xiphophorus helleri*) above 20 µg $L^{-1}$, Liang *et al*., 2013), in *P. hypophthalmus* fingerlings (above 97 µg $L^{-1}$, Sahu *et al*., 2018) and in zebrafish larvae (above 250 µg $L^{-1}$, Oliveira *et al*., 2009). The GST plays an important role in phase II of biotransformation, catalysing the conjugation of the reduced form of glutathione with xenobiotics to increase their hydro-solubility (Oost *et al*., 2003; Rudneva *et al*., 2010; Haluzová *et al*., 2011). The GST induction in TCS exposed fish at the end of the early larval test are in agreement with the fact that besides effects on antioxidant system, TCS might also be detoxified through phase II of biotransformation as proven to occur in several species (Ku *et al*., 2014; Ashrap *et al*., 2017; Wu *et al*., 2017; Ding *et al*., 2018; Peng *et al*., 2018). At the metamorphosis test, GST inhibition might have compromised phase II of biotransformation and the maintenance in clean medium after TCS exposure seems to have allowed the recovery of fish GST activity. Therefore, the mechanisms of recovery used by *S. senegalensis* during metamorphosis still need to be further studied.

Exposure of goldfish to concentrations between 280 and 560 µg $L^{-1}$ TCS during 14 days increased malondialdehyde levels, indicating oxidative damage of TCS in this species (Wang *et al*., 2018). Mauvault *et al*. (2019) reported an increase of LPO levels in liver of the marine fish *D. sargus* fish exposed to the TCS diet, however in brain and muscle this increase was not observed. In our study, the exposure to TCS did not cause lipid peroxidation during and/or after exposure to TCS at the early larvae and at the metamorphosis tests. Despite the alterations observed in the antioxidant enzymes, no

oxidative damage of lipids occurred in the tested conditions. Nevertheless, attention should be given to the fact that effects on antioxidant system of *S. senegalensis* were observed and decreased defense capacity might increase vulnerability to other stressors on the environment.

The LDH activity in zebrafish have been shown to increase in the presence of 250 µg $L^{-1}$ TCS (Oliveira *et al*., 2009). However, in our study, inhibition of LDH activity just after the exposure in metamorphosing sole to almost all concentrations of TCS tested suggests that the anaerobic energy metabolism is a less used pathway. A direct inhibition of LDH might be occurring leading to an impairment of anaerobic metabolism or the use of aerobic metabolism might be preferred in detriment of the former (Teodorescu *et al*., 2012). This fact, together with a possible increased energy demand for oxidative stress response, might justify the found effects on growth of fish observed at the end of metamorphosis.

Table 2.2. Lowest and non-observed effect concentration (LOEC, NOEC) and 10 and 50% effect concentration ($EC_{10}$ and $EC_{50}$, respectively) in *Solea senegalensis* larvae after 96h of exposure to triclosan (endpoints measured at 3 days after hatching, dah), just after exposure to 48h at the beginning of metamorphosis (15 dah), and after maintenance in clean medium until completed metamorphosis (24 dah).

| **Endpoint** | **Life stage** | **NOEC** (µg $L^{-1}$) | **LOEC** (µg $L^{-1}$) | **$EC_{10}$** (µg $L^{-1}$) | **$EC_{50}$** (µg $L^{-1}$) |
|---|---|---|---|---|---|
| **Mortality** | Early larvae (3 dah) | - | - | 133 (c.i.: 94 - 162) | 218 (c.i.: 196 - 236) |
| | Metamorphosis (15 dah) | - | - | 1083 (c.i.: 1001 - 1207) | 1357 (c.i.: 1284 - 1404) |
| **Length** | Early larvae (3 dah) | 53 | 95 | - | - |
| | Metamorphosis (24 dah) | 391 | 548 | - | - |
| **Malformations** | Early larvae (3 dah) | - | - | 80 (c.i.: 56 – 105) | 180 (c.i.: 156 – 210) |
| | Metamorphosis (17 dah) | 280 | 391 | - | - |
| **Biomarkers** | Early larvae (3 dah) | <30 | 30 (GST) | - | - |
| | Metamorphosis (15 dah) | 200 | 280 (LDH) | - | - |
| | Metamorphosis (24 dah) | 200 | 280 (CAT) | | |

c.i. - 95% confidence interval. GST – glutathione S-transferase, LDH – lactate dehydrogenase, CAT – catalase.

### 4.4. Conclusions

Biochemical enzymatic markers were the most sensitive endpoint to TCS exposure at both development stages tested. Exposure to TCS in levels as low as 30 µg $L^{-1}$ induced effects at biochemical level on the larvae test, which was the lowest LOEC obtained. In addition, growth impairment and malformations were also detected at low concentrations (LOEC: 95 µg $L^{-1}$ and EC10: 80 µg $L^{-1}$ TCS, respectively). Despite the effects observed occurred in fish exposed to concentrations above the reported environmental TCS levels for marine and transitional waters, harmful effects of longer exposure periods to environmental relevant levels of TCS can occur and should be further investigated. Furthermore, higher LOECs (equal or above 280 µg $L^{-1}$ TCS) were obtained during metamorphosis when compared to early larval stage, revealing an higher degree of tolerance to TCS. Despite this, relevant responses to TCS were also detected. Exposure to TCS during the critical period of metamorphosis onset led to alterations at biochemical (impairment of antioxidant system) and individual level (malformations and alterations on metamorphosis progression). These alterations might be linked to the observed effects with higher ecological relevance, namely on fish growth. Furthermore, as sole metamorphosis is a thyroid regulated process, a possible interference of TCS on thyroid-axis functioning should be further explored and understood.

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# Effects of triclosan on early development of *Solea senegalensis*: from biochemical to individual level

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**Supplementary Data**

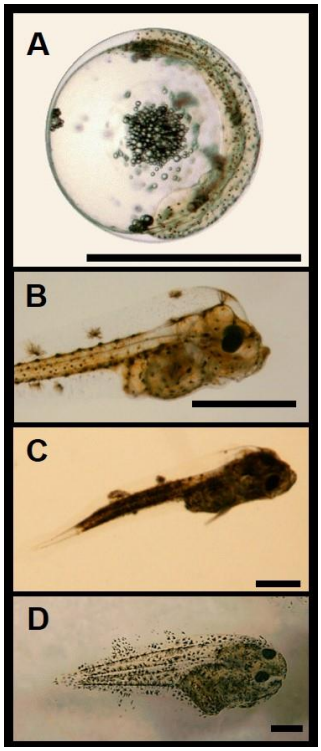


Fig. 2.S1. Development stages of *Solea senegalensis*. A) egg in gastrula stage, B) early larvae (3 days after hatching); C) pre-metamorphic larvae; D) post-metamorphic larvae. Each black bar represents 1 mm.

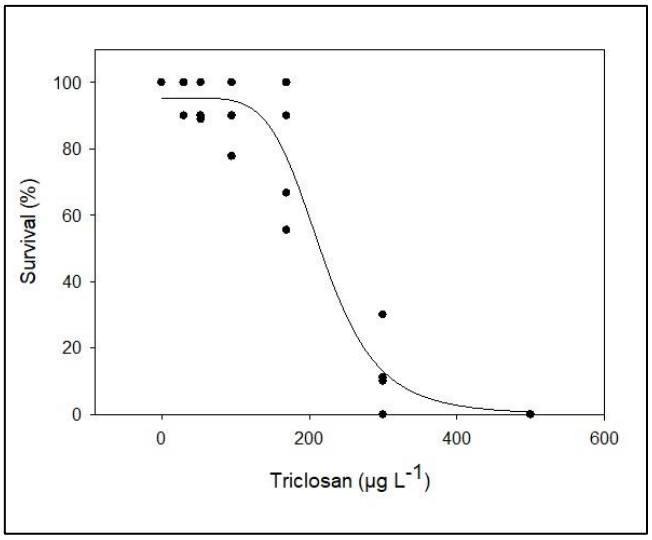


Fig. 2.S2. Survival of *Solea senegalensis* after 96h of exposure to triclosan from egg stage until 3 days after hatching. Logistic three parameter regression model, N=4, 10 fish per replicate.

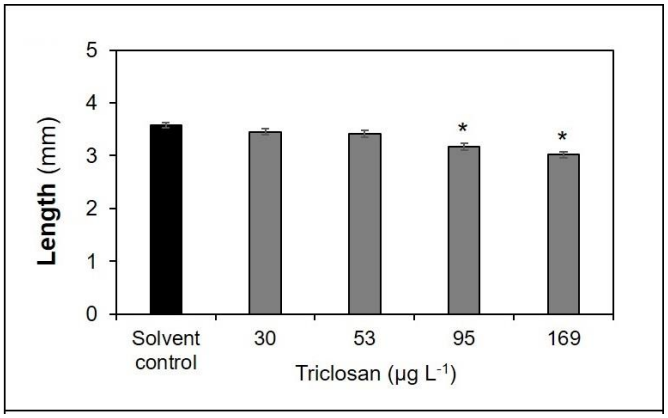


Fig. 2.S3. Triclosan effects on total length of *Solea senegalensis* early larvae. Exposure was performed between egg stage and 3 days after hatching (dah), length measured at 3 dah (n=12-16). * represent the existence of significant differences with solvent control ($p<0.05$). Results are expressed as mean±standard error.

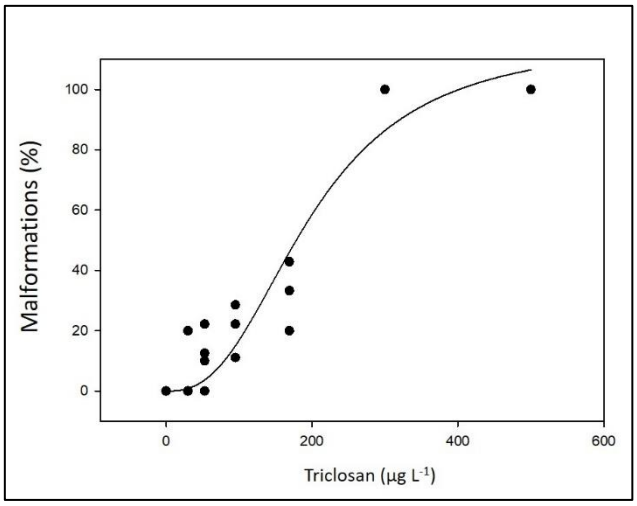


Fig. 2.S4. Malformations present in *Solea senegalensis* after 96h exposure to triclosan from egg stage until 3 days after hatching. Logistic three parameter regression model, N=4, 10 fish per replicate.

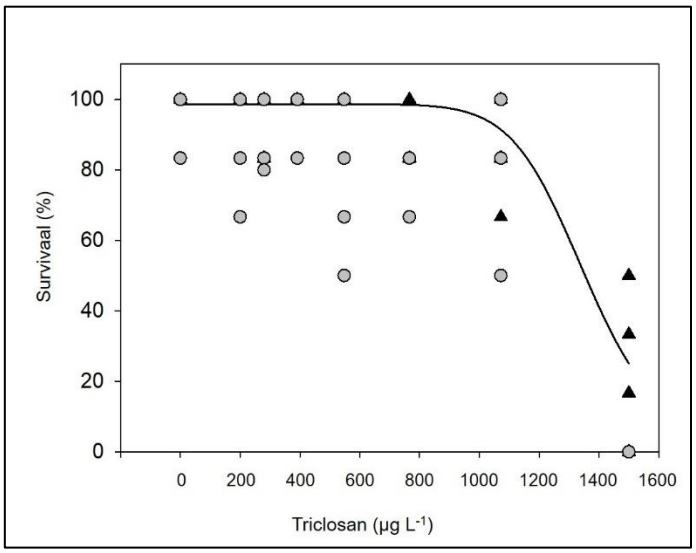


Fig. 2.S5. Survival of *Solea senegalensis* after 48h exposure to triclosan at the beginning of metamorphosis (13 days after hatching, dah). Dark line and triangles represent survival at 15 dah (n=6, 10 fish per replicate) and grey circles represent survival after maintenance in clean medium until complete metamorphosis (24 dah, n=6, 6 fish per replicate). Line based on logistic three-parameter regression model.

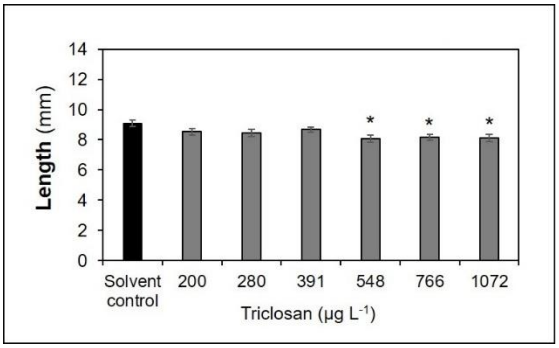


Fig. 2.S6. Triclosan effects on total length of *Solea senegalensis* at the end of metamorphosis. Exposure was performed at the beginning of metamorphosis (13 dah) during 48h and length measured at 24 dah (n=6-9) after maintenance in clean medium. * represent the existence of significant differences with solvent control ($p<0.05$). Results are expressed as mean±standard error.

Table 2.S1. Nominal and measured concentrations of triclosan (TCS) solutions at 0h and after 48h of fish exposure.

| **Experiment** | **Nominal concentrations of TCS** (µg $L^{-1}$) | **Measured concentrations of TCS** (µg $L^{-1}$) | **Difference** (%) | **TCS after 48h exposure** (µg $L^{-1}$) | **Loss of TCS after 48h exposure** (%) |
|---|---|---|---|---|---|
| Early larval test | 30 | 34±1.2 | 113.3 | - | - |
| | 300 | 259±8.1 | 86.3 | - | - |
| | 3000[a] | 2603±42.5 | 86.8 | - | - |
| Metamorphosis test | 200 | 209±5.0 | 104.5 | 37±3.6* | 82.3 |
| | | | | 68±2.6** | 67.5 |
| | 766 | - | - | 176±10.1* | - |
| | 1500 | 1722±31.6 | 114.8 | 263±28.7** | 84.7 |

Measured concentrations are mean ± standard error of 3 technical replicates. Limit of Detection was 7.8 µg $L^{-1}$ TCS. [a] stock solution. * Group with higher fish density used for study of TCS biochemical effects at 48h. ** Group with lower fish density used for study of TCS individual effects and biochemical level determination at the end of metamorphosis. - No data available. Difference was calculated as *M*/*N*x100, with *M* and *N* as measured and nominal concentrations, respectively.

# Chapter 3. Effects of UV filter 4-methylbenzylidene camphor during early development of *Solea senegalensis* Kaup, 1858

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# Effects of UV filter 4-methylbenzylidene camphor during early development of *Solea senegalensis* Kaup, 1858

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**Abstract**

The inclusion of organic UV filters in personal care products (PCPs) has increased in recent years. 4-Methylbenzylidene camphor (4-MBC) is one of the most used UV filters, and thus it is commonly found in aquatic ecosystems, with proved negative effects on aquatic organisms. Effects on early life stages of marine vertebrates are largely unknown. Therefore, the main goal of this work was to evaluate 4-MBC effects on Senegalese sole (*Solea senegalensis* Kaup, 1858) larvae at different levels of biological organization. *S. senegalensis* were exposed to increasing concentrations of 4-MBC from egg stage until 96h. Mortality, growth, malformations, behavior and biochemical responses, including enzymatic biomarkers were studied. The exposure to 4-MBC until 96 hours post-fertilization (hpf) induced mortality and malformations in a dose-response manner. Besides, reduced growth with increasing concentrations was observed. The exposure to 4-MBC also caused alterations on behavior, including overall lower swimming time during light and dark periods. Biomarkers alterations caused by 4-MBC included imbalance of neurotransmission related endpoints (increased acetylcholinesterase activity) and decreased activity of enzymes related to anaerobic metabolism (lower cellular lactate dehydrogenase activity) at the lower concentrations tested. Furthermore, our results suggest that 4-MBC do not induce oxidative stress in *S. senegalensis* larvae, since catalase and lipid peroxidation levels were not significantly altered by 4-MBC.

*Solea senegalensis* revealed to be a good model species for vertebrate animal testing in the marine environment. Sub-lethal concentrations of 4-MBC induced toxic effects at all organizational levels. Swimming behavior was a sensitive endpoint and showed that exposure to 4-MBC causes impairment on response to light stimulus which is possibly linked with the observed imbalances on cholinesterase activity in larvae. Conservation concerns along distribution range of *S. senegalensis* should consider that increasing levels of UV filters in marine environment might have impact on the ecology of the species.

**Keywords:** behavior, biochemical markers, flatfish, Personal Care Products, fish Early Life Stage test

## 1. Introduction

In last decades, the knowledge on harmful effects of ultraviolet radiation (UV) led to the increasing use of cosmetic products containing UV filters (Chisvert and Salvador, 2007). These personal care products (PCPs) include such compounds for dermal protection against deleterious UV, but UV filters are also used as additives in plastics and household products for increasing shelf-life. However, UV filters exhibit some characteristics typical of organic priority pollutants, such as high stability and lipophilicity and low biotic degradation (USEPA, 2012). Besides, they tend to bioaccumulate and are reported to act as neurotoxicants and endocrine disruptors (Díaz-Cruz and Barceló, 2009; Gago-Ferrero *et al*., 2012; Krause *et al*., 2012; Ruszkiewicz *et al*., 2017).

Usually, more than one UV filter are combined on sunscreen formulations. 4-methylbenzylidene camphor (4-MBC) is one of the most common UV filters in Europe and Australia, with a maximum allowed concentration of 4% (Chisvert and Salvador, 2007; Krause *et al*., 2012). Bathing and swimming activities are one of the most common direct sources of this compound in aquatic ecosystems, particularly during warmer seasons when levels in water are prone to be high. Maximum reported concentrations are found at recreational coastal areas with concentrations up to 1.043 µg $L^{-1}$ (Atlantic Gran Canaria) with peaks during daylight and warmer months (Balmer *et al*., 2005; Langford and Thomas, 2008; Sánchez-Rodríguez *et al*., 2015; Tovar-Sánchez *et al*., 2013). In addition, aquatic ecosystems can also receive 4-MBC from wastewater treatment plants (WWTP), since this compound is not totally eliminated by typical treatment methods, reaching concentrations up to 2.7 µg $L^{-1}$ (Badia-Fabregat *et al*., 2012; Balmer *et al*., 2005; Braush and Rand 2011; Fent *et al*., 2010b; Li *et al*., 2007; Liu *et al*., 2012). Besides, 4-MBC has also been found in tap water (maximum concentration of 35 ng $L^{-1}$) (Díaz-Cruz *et al*., 2012).

The ubiquity of 4-MBC on coastal environments (Chisvert and Salvador, 2015) and high toxicity to marine organisms raises environmental concerns. Toxicity has already been described at concentrations below 10 ng $L^{-1}$ to small organisms, such as protozoa (Gao *et al*., 2013), microalgae (*Isochrysis galbana*) or small crustaceans (*Siriella armata*) (Paredes *et al*., 2014). Besides, toxic effects have been detected in freshwater aquatic vertebrates, affecting development and reproduction (Inui *et al*., 2003; Kunz and Fent, 2006, 2009; Martins *et al*., 2017; Schmitt *et al*., 2008; Schlumpf *et al*., 2004, 2008). UV filters accumulate in fish tissues, and 4-MBC have been detected in concentrations as high as 1.8 µg $g^{-1}$ lipid

in muscle tissue of *Salmo trutta fario,* brown trout (Balmer *et al*., 2005; Buser *et al*., 2006; Fent *et al*., 2010b; Gago-Ferrero *et al*., 2015; Zenker *et al*., 2008). However, effects of 4-MBC at larval stages of marine fish are still very scarce and should be further assessed.

New alternative testing methods to animal experimentation are necessary for testing chemicals with toxicological risk (e.g. REACH regulations). Early life stages of aquatic vertebrates have been used as models for evaluating the effects of contaminants (Lammer *et al*., 2009; Scholz *et al*., 2008; 2013). While several vertebrate freshwater species have been widely used and accepted by scientific community (e.g. *Danio rerio*, zebrafish; *Pimephales promelas*, fathead minnow; *Xenopus laevis*, African clawed frog; among others), the use of marine vertebrates as testing model species are still very scarce. Eggs of flatfish marine species from aquaculture facilities are becoming more frequently used for ecotoxicology studies (e.g. Foekema *et al*., 2008; Pavlaki *et al*., 2016).

Senegalese sole (*Solea senegalensis* Kaup, 1858) is a coastal flatfish species native to the Eastern Atlantic waters. This high economic important species is commercially exploited in Southwestern European aquaculture and its increasing scientific knowledge in different fields of biology has gained momentum in recent years (Imsland *et al*., 2003; Morais *et al*., 2016). Early development, metamorphosis and physiology of this species is well understood (Cañavate and Fernández-Diaz, 1999; Cañavate *et al*., 2006; Fernández-Diaz *et al*., 2001). Eggs can be obtained from several companies and national agencies throughout southern Europe. Besides, eggs and larvae are transparent and malformation screening is easily performed and endogenous energetic reserves last until nearly 80-100 hours after fertilization, time when external feeding is in fact needed (Cañavate *et al*., 2006; Parra *et al*., 1999). Therefore, larval stages of this species arise as alternative animal models for toxicological evaluation of marine environmental contaminants, such as 4-MBC.

Biochemical markers are sensitive and useful tools on the study of effects of chemical exposure at organisms' subcellular level. Specific biochemical responses can be associated with particular modes of action of chemicals. Neurotoxicity can be assessed through estimation of cholinergic activity (e.g. acetylcholinesterase, AChE). Exposure to specific chemicals can induce production of reactive oxygen species (ROS) exceeding basal cell levels. In such conditions, defense capacity of organism is surpassed and damage on DNA, peroxidation of membrane lipids and proteins, and cellular degenerative processes can occur (Oost *et al*., 2003; Storey, 1996). Therefore, oxidative stress can be detected through increased antioxidant enzymes activity (such as catalase, CAT) or measured through lipid peroxidation (LPO) levels (Antunes *et al*., 2010; Quintaneiro *et al*., 2008). Another relevant biomarker is lactate dehydrogenase (LDH), which catalyses the

reversible reduction of pyruvate to lactate and may be related with low availability of oxygen and exposure to xenobiotics (Cohen *et al*., 2001; Guilhermino *et al*., 1994; Quintaneiro *et al*., 2006).

Non-invasive and non-lethal tools, are also becoming increasingly used as indicators of toxic effects in the assessment of chemicals. Behavior is a sensitive and non-invasive tool for the evaluation of contaminants and the recent development of standardized methods and computer and video automation has been improving the analysis of behavior in terms of reproducibility, reliability and turning it less time-consuming (Kane *et al*., 2005; Little *et al*., 1993; Melvin and Wilson, 2013; Scott and Sloman, 2004).

In this context, the main objective of this study was to understand the toxic effects of 4-MBC to larval stages of *S. senegalensis* at different levels of biological organization using apical, biochemical and behavioral endpoints.

## 2. Material and Methods

### 2.1. Chemicals

The 3-(4-Methylbenzylidene) camphor (4-MBC) (CAS Number 36861-47-9) used in bioassays 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 (Sigma-Aldrich Co.).

In the chemical analysis, 4-MBC and deuterated benzophenone (benzophenone-d10, Sigma–Aldrich) were used as analytical standard and surrogate, respectively. Liquid chromatography-grade absolute ethanol from Scharlau (Barcelona, Spain) was used as solvent to prepare the 4-MBC standard stock solution. Working standard solutions were freshly prepared daily by dilution with deionized water obtained by a Connect water purification system from Adrona (Riga, Latvia). Extra pure chloroform and extra pure acetone from Scharlau (Barcelona, Spain) were used as extraction solvent and disperser solvent, respectively, in the chemical analysis procedure. Analytical reagent grade sodium chloride 99.5%, and glacial acetic acid and analytical reagent grade sodium hydroxide all from Scharlau (Barcelona, Spain) were used to adjust the ionic strength and the pH, respectively, in the analytical procedure. High purity helium (99.999%) from Carburos Metálicos S.A. (Paterna, Spain) was used as carrier gas in the gas chromatography-mass spectrometry (GC–MS) system used in the chemical analysis.

### 2.2. Experimental design

Fertilized eggs of *S. senegalensis* were obtained from a commercial hatchery (Sea8, Portugal). Spawning of wild adults is artificially induced through the regulation of temperature and photoperiod. Eggs were taken to the lab less than 12 hours post-fertilization (hpf). Gastrula eggs were chosen and those presenting abnormalities were discarded. Eggs were then exposed to different concentration levels of 4-MBC. Sterilized (T= 121 ºC, 10 min) natural seawater collected during high tide in Barra channel (Aveiro, Portugal, salinity 35 usp) was used in lab procedures.

Three experiments were performed in order to assess: 1) mortality and malformations; 2) length and behavior; and 3) biochemical markers. The first two experiments were performed in 24 well plates (N=4, 5 eggs/group, individual exposure) at 0.235, 0.331, 0.447, 0.599 and 0.935 mg $L^{-1}$ of 4-MBC and then using the sub-lethal concentrations 0.068, 0.132, 0.229 and 0.360 mg $L^{-1}$ of 4-MBC, respectively, plus negative and solvent controls. The third experiment was performed in 6 well plates (N=6, 10 egg/well) at 0.025, 0.051, 0.085, 0.140 and 0.216 mg $L^{-1}$ of 4-MBC, plus negative and solvent controls. Lethal concentrations (LC) and relevant Effect concentrations (EC), Lowest observed effect concentrations (LOEC) and Non-observed effect concentrations (NOEC) were estimated for each test. In order to verify the stability of 4-MBC during the experimental conditions, 4-MBC concentrations were quantified by chemical analysis. Since the differences between measured/real concentrations and nominal concentrations were higher than 20% (supplementary table 3.S1) the concentrations presented throughout this work report to real concentrations. Solvent controls were prepared with ethanol for each test. Solvent control solutions were prepared with the same concentration of ethanol of highest 4-MBC treatment group in each of the three experiments, namely, 0.100, 0.028 and 0.015 mL $L^{-1}$, respectively. To avoid 4-MBC depletion by adsorption to plastic, plates were previously incubated for 24h with test media which was completely renewed at beginning of the test and at 48 hpf. All experiments were conducted in controlled conditions of temperature (19±1 ºC) and photoperiod (18:6h light:dark) (supplementary table 3.S2).

#### 2.2.1. Mortality and malformations

In the first experiment, hatching success was checked at 24 and 48 hpf, and mortality/survival and malformations were checked daily until 96 hpf, using a stereomicroscope (Nikon SMZ 1270, Nikon, Japan). Development of larvae (opening of mouth and development of fins) was also checked at 96 hpf. Teratogenic index was

calculated as the ratio between $LC_{50}$ and $EC_{50}$ values (Selderslaghs *et al*., 2012). TI values of tested compounds higher than 1 indicate teratogenicity.

### 2.2.2. Length and behavior

In the second experiment, 4-MBC effect on growth (as total length, in mm) and behavioral responses were evaluated after 96 hpf. Length was recorded in at least 15 larvae of each treatment. For the behavior analysis, exposed larvae were placed in new 24 well plates filled with 2 mL of sterilized natural saltwater. Randomly chosen larvae (one per well, completing a total of 9 larvae for each control and 6 for each treatment) were analysed in Zebrabox® (Viewpoint, France). Two alternating periods with light and dark were set with 15 minute each, after an initial 5 minute acclimation light period, completing a total of 60 minutes of behavior video recording. Light intensity used was set at 0.26 mW $cm^{-2}$ and background threshold was set at 20 pixels. In addition to the white light irradiated by Zebrabox, also an infra-red light (not seen by the fish, constant at 2.3±0.11 mW $cm^{-2}$) was needed for video recording. The software provided (Zebralab, Viewpoint) automatically records distance (mm), duration (seconds) and number of swimming events (n) during specific integration periods (set at 1 minute). Based on software output, swimming distance, number of movements and duration of swimming time were calculated for each treatment considering the one minute integration time (mm $min^{-1}$, n $min^{-1}$ and sec $min^{-1}$), 15 minutes light/dark periods and along all test (60 minutes).

### 2.2.3. Biochemical markers

In the third experiment, six replicates with 10 organisms per treatment were kept until 96 hpf. At that time, larvae were snap-frozen in liquid nitrogen and kept at -80°C until further procedure. Samples were defrosted in ice and homogenized with potassium phosphate buffer (pH 7.4, 0.1 M) by sonication. A volume of 150 µL of whole-body homogenates was separated into a microtube with 4 µL of 4% butylated hydroxytoluene (BHT) in methanol for endogenous LPO determination by measuring thiobarbituric acid-reactive substances (nmol TBARS $mg^{-1}$ of protein) at 535 nm (Bird and Draper, 1984). Remaining homogenate was centrifuged for 20 min at 10,000 g (4°C). Supernatant was used in following enzymatic analyses. AChE activity was measured following the Ellman's method (Ellman *et al*., 1961) adapted to microplate (Guilhermino *et al*., 1996) using acetylthiocholine as substrate and 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) as chromogen. AChE activity was read at 412 nm of absorbance. CAT activity was determined by measuring decomposition of the substrate $H_2O_2$ at 240 nm (Clairborne, 1985). For LDH

determinations, the methodology described by Vassault (1983) with the modifications introduced by Diamantino *et al*. (2001) was used. Pyruvate is converted in lactate by LDH oxidation of reduced nicotinamide adenine dinucleotide (NADH) into its oxidizing form (NAD+). The decreasing of NADH can be measured at 340 nm for 5 minutes. Protein concentration was determined according to the Bradford method (Bradford, 1976), adapted to microplate using bovine γ-globuline as a standard and a wavelength of 595 nm. Enzymatic activities are expressed in Units (U) per mg of protein (1 U is a nmol of substrate hydrolyzed per minute for AChE and LDH and a µmol of substrate hydrolyzed per minute for CAT). Readings were performed in 96 well plates using a Labsystem Multiskan EX microplate reader for all enzymatic and protein determinations.

#### 2.2.4. Chemical analysis of 4-MBC in test media

Chemical analysis based on dispersive liquid-liquid microextraction (DLLME) followed by gas chromatography-mass spectrometry (GC-MS) (Benedé *et al*., 2014) was performed at initial stocks and test solutions at 0 and 48h after exposure.

In order to construct the calibration curve, a stock solution containing 500 µg $mL^{-1}$ of 4-MBC was prepared in ethanol. Then, an aliquot of this solution was properly diluted with deionized water to prepare an aqueous stock solution (10 µg $mL^{-1}$), from which different aqueous working solutions (20–250 ng $mL^{-1}$) were prepared and then adjusted to pH 2.5 with glacial acetic acid. Aliquots of 5 mL of each one of these aqueous working solutions were transferred to 7.5 mL glass centrifuge tubes to which 50 µL of the surrogate aqueous solution at 500 ng $mL^{-1}$ were added. Then, they were subjected to DLLME by rapid injection of pre-mixed 250 µL of acetone (as disperser solvent) and 50 µL of chloroform (as extraction solvent). Once the cloudy solutions were formed, they were centrifuged at 3500 rpm for 5 min. After centrifugation, the organic sedimented phases were collected and transferred into 100 µL inserts placed inside 1.5 mL injection vials for GC–MS analysis.

Regarding water samples, they were previously pH-adjusted to 2.5 with glacial acetic acid. Then, by triplicate, aliquots were properly diluted with deionized water, and then 5 mL of each one of these solutions were subjected to the DLLME procedure as previously described for standards.

The chromatographic separations were carried out in a HP-5MS Ultra Inert (95% dimethyl-5% diphenylpolysiloxane, 30 m length, 0.25 mm i.d., 0.25 µm film thickness) column from Agilent Technologies (Palo Alto, CA, USA). Two microliters of each sedimented phase were injected into the GC–MS system. The inlet temperature was 280 °C and the injection was accomplished in splitless mode (splitless time 1.5 min). The

separation was run at a 1 mL $min^{-1}$ helium constant flow rate. The oven temperature program was: from 70 ºC (1 min) to 170 ºC at 10 ºC $min^{-1}$, then to 200 ºC at 2 ºC $min^{-1}$ and finally to 280 ºC (6 min) at 10 ºC $min^{-1}$. The transfer line and ion source temperatures were set at 280 and 250 ºC respectively. The chromatograms were recorded in selected ion monitoring (SIM) mode at mass/charge (m/z) ratios of 254 (quantifier) plus 128 and 211 (qualifiers). Calibration graph was performed by plotting $A_{4\text{-MBC}}/A_{SUR}$ (where $A_{4\text{-MBC}}$ is the peak area of the target analyte and $A_{SUR}$ that of the surrogate (i.e., benzophenone-d10) each one obtained by its quantifier ion) versus 4-MBC concentration.

### 2.3. Statistical analysis

Four-parameter-logistic-curves were used for determination of $LC_{10}$ and $LC_{50}$ and $EC_{50}$ of hatching and malformations. Statistical differences between negative and solvent control were tested initially by performing a t-test. Whenever such differences exist they are stated in the results section. One-way ANOVA followed by post-hoc Dunnett's tests were performed to test statistical differences between treatments and solvent control for length, behavior (total swimming distance, total number of movements and total duration of swimming time), and biochemical markers endpoints. Non-parametric ANOVA followed by Dunns' test was performed when normality or homoscedasticity were not observed. Since total duration of swimming time presented significantly differences with solvent control this parameter was also tested with Two-way ANOVA (2 factors: concentration with 5 levels and 4 periods of light and dark) followed by Tukey's post-hoc test. All statistical analyses were performed in Sigmaplot v. 12.5, Systat Software Inc. and results are expressed as mean ± standard error (SE).

## 3. Results

### 3.1. Mortality, hatching and malformations

In the first experiment, there was no significant effects of 4-MBC on hatching of *S. senegalensis* ($p>0.05$). However, relatively lower hatching rates were observed at 24 hpf in sole early larvae exposed to 0.447 and 0.599 mg $L^{-1}$ 4-MBC (60%) when compared to hatching rates observed in solvent control and 0.935 mg $L^{-1}$ 4-MBC treatment (100%). At 48 hpf all the fish in the different treatments have hatched.

There was no mortality of hatched larvae nor egg abortion in the treatments exposed to 4-MBC at 24 hpf. At 48 and 72 hpf mortality was 40±18% and 85±5% (mean±SE) in sole larvae exposed to the highest concentration tested (0.935 mg $L^{-1}$),

respectively. At the second highest concentration (0.599 mg $L^{-1}$), mortality was below 10% at 48 hpf and was 25±10% at 72 hpf. At 96 hpf a $LC_{50}$ of 0.439±0.016 mg $L^{-1}$ was obtained (supplementary fig. 3.S1). Sole larvae mortality in negative control reached 4% at 96 hpf while in solvent control mortality was 0%. Similarly to mortality, malformations and effects on development occurred mostly in higher concentrations groups (between 0.447 mg $L^{-1}$ and 0.935 mg $L^{-1}$), besides they have preceded the death of affected larvae. Longitudinal curved notochord was observed at 48 hpf for 0.447 mg $L^{-1}$ and 0.599 mg $L^{-1}$ with prevalence of 5.0% and 5.3% respectively (fig. 3.1). This malformation was not observed at 48 hpf in larvae exposed to the highest concentration group, however at 72 hpf it affected all larvae in this treatment. At 72 hpf the prevalence of this malformation was 0% for 0.447 mg $L^{-1}$ and 17% for 0.599 mg $L^{-1}$. At 96 hpf this malformation was detected at 0.331 mg $L^{-1}$ (15%) and responded in a dose-response manner with a $EC_{50}$ of 0.372±0.014 mg $L^{-1}$ (table 3.1, supplementary fig. 3.S1). Malformations were not observed in solvent and negative controls. Muscular spasms were observed in larvae at 72 hpf at 0.599 mg $L^{-1}$ (65%) and at the end of the test (96 hpf) at 0.331 and 0.447 mg $L^{-1}$ (5 and 63% respectively). In addition, all larvae exposed to 0.447 and 0.599 mg $L^{-1}$ of 4-MBC were affected by delayed development. The estimated TI value for 4-MBC was 1.18, indicating low teratogenicity level.

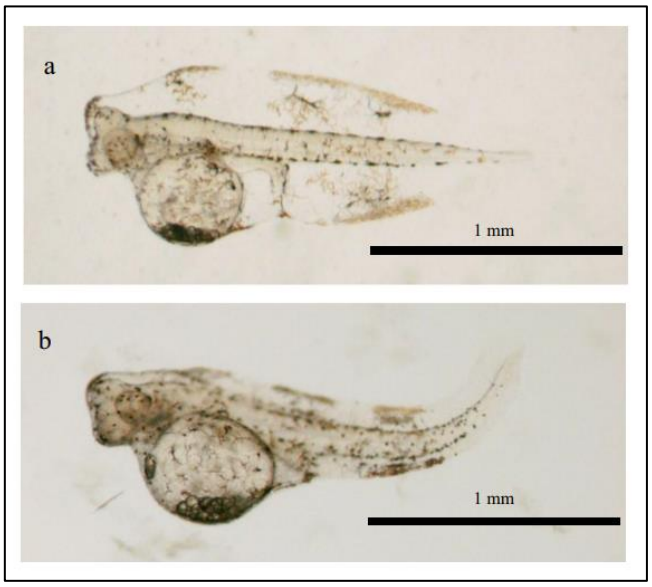


Fig. 3.1. *Solea senegalensis* at 48 hpf. Control larvae (a) and larvae affected by notochord curvature after exposure to 4-MBC (b).

Table 3.1. Endpoints and correspondent low and non-observed effect concentration (LOEC, NOEC) and 10% and 50% effect concentration ($EC_{10}$ and $EC_{50}$) in *Solea senegalensis* after 96 h of exposure to 4-MBC. CI - 95% confidence interval.

| **Endpoint** | **NOEC at 96 hpf** (mg $L^{-1}$) | **LOEC at 96 hpf** (mg $L^{-1}$) | **$EC_{10}$** (mg $L^{-1}$) | **$EC_{50}$** (mg $L^{-1}$) |
|---|---|---|---|---|
| Mortality | - | - | 0.336 (CI: 0.290-0.387) | 0.439 (CI: 0.419-0.479) |
| Notochord curvature | 0.235 | 0.331 | 0.324 (CI: 0.299-0.344) | 0.372 (CI: 0.355-0.400) |
| Length | 0.229 | 0.360 | - | - |
| Behavior | <0.068 | 0.068 | - | - |

### 3.2. Length and behavior

The second experiment, performed with concentrations of 4-MBC below the $EC_{50}$ for malformation determined in the first experiment, revealed a decrease on length of *S. senegalensis* at 96 hpf (F= 2.850, p=0.029, fig. 3.2). The LOEC for length was registered at 0.360 mg $L^{-1}$ of 4-MBC.

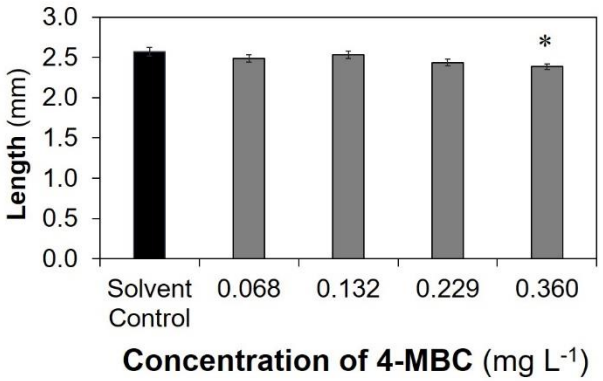


Fig. 3.2. Total length (mm) of *Solea senegalensis* after 96 h of exposure to 4-MBC (N = 14, mean ± SE). * represents significant differences with solvent control (p<0.05).

At behavioral level, no significant differences were detected on total swimming distance between larvae of different treatments and solvent control during the 60 min of analysis (F=1.873, p=0.144, data not shown), with values ranging between 3.7±1.1 m (0.229 mg $L^{-1}$ 4-MBC) and 8.0±1.3 m (solvent control). Also, total number of movements during the 60 min period did not statistically differ between groups (F=1.897, p=0.144, data not shown) with highest values obtained for 0.360 mg $L^{-1}$ 4-MBC ($6.4x10^3$±$0.9x10^3$ movements) and lowest for 0.068 mg $L^{-1}$ 4-MBC ($4.0x10^3$±$0.6x10^3$ movements). However, 4-MBC affected significantly the larvae swimming time (F=4.430, p=0.013) with less activity

time for larvae exposed to the highest concentration tested (0.360 mg $L^{-1}$ treatment group, 37±1.5 sec $min^{-1}$) when comparing to larvae of solvent control (47.0±1.2 sec $min^{-1}$, $p<0.05$, supplementary fig. 3.S2). Significant differences on swimming time between larvae of negative and solvent controls within periods were inexistent (t-test, $p>0.05$). Two-way ANOVA analysis of larvae swimming time revealed no interaction between the factors concentration and periods of light/dark ($p>0.05$). However, concentration and period of light were both significant ($p<0.05$, fig. 3.3).Considering the factor period of light/dark, a higher swimming time during light periods was observed ($p<0.05$). which can be particularly observed in solvent control with larvae revealing higher swimming time in first and second light periods when compared to the first and second dark periods, respectively ($p<0.05$). In addition, larvae exposed to the lowest concentration (0.068 mg $L^{-1}$) also presented higher swimming time in second light period when compared with the second dark period ($p<0.05$). When considering the factor concentration, larvae exposed to highest 4-MBC concentration (0.360 mg $L^{-1}$) presented a significantly lower swimming time than solvent control ($p<0.05$). Significant differences between light and dark periods were not observed for larvae exposed to any concentrations above 0.068 mg $L^{-1}$ ($p>0.05$). Furthermore, significantly lower swimming time was obtained between larvae of highest concentration group (0.360 mg $L^{-1}$) and solvent control larvae at both light periods and at the first dark period ($p<0.05$).

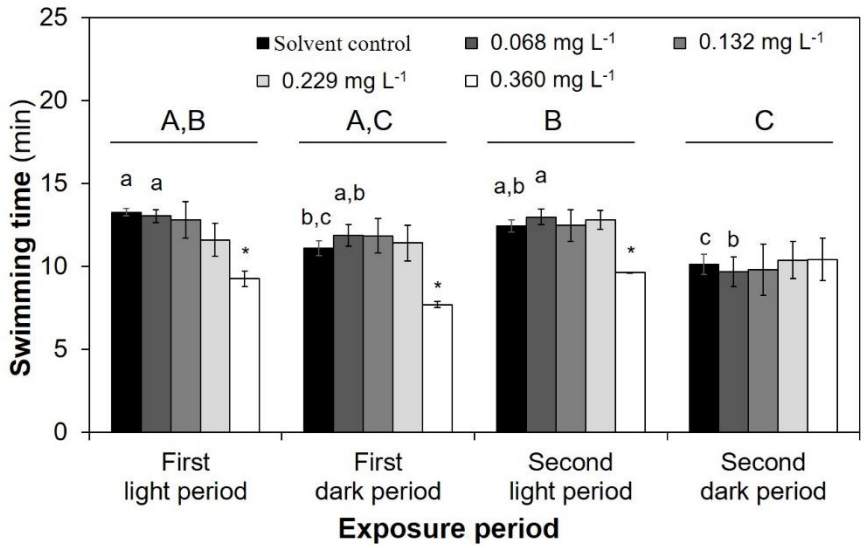


Fig. 3.3. Swimming time of *Solea senegalensis* within each 15 minute light and dark cycles during the 60 minute behavioral analysis after 96h exposure to 4-MBC. Uppercase letters represent significant differences between periods of light/dark, lowercase letters represent significant differences within treatments and * represents significant differences with solvent control within each light/dark periods (Two-way ANOVA, $p<0.05$).

### 3.3. Biochemical markers

In the third experiment, a range of sub-lethal concentrations of 4-MBC below $LC_{10}$ was tested to quantify biochemical biomarkers. Mortality registered was below 10% in all treatment groups, including control groups. Significant differences between negative and solvent controls were inexistent for AChE and CAT, while significant differences between controls occurred in LDH and LPO, with higher values for solvent control group in LDH and higher values on negative control group for LPO (t-test, $p<0.05$). Exposure to 4-MBC caused significant increased activity of AChE on larvae exposed to 0.085 mg $L^{-1}$ 4-MBC when comparing to solvent control (fig. 3.4, $p<0.05$). Significantly lower LDH activity ($p<0.05$) was also observed between solvent control larvae and larvae exposed to the two lower concentrations tested (0.025 and 0.051 mg $L^{-1}$). CAT activity and LPO levels were not significantly different between solvent control and 4-MBC treatment groups ($p>0.05$).

A loss of 4-MBC between 71 and 74% after 48h of fish larvae exposure, at the time of test medium renewal was detected by chemical analysis of the test mediums used in all experiments. Supplementary table 3.S1 shows this observation for three randomly selected solutions (one from each experiment).

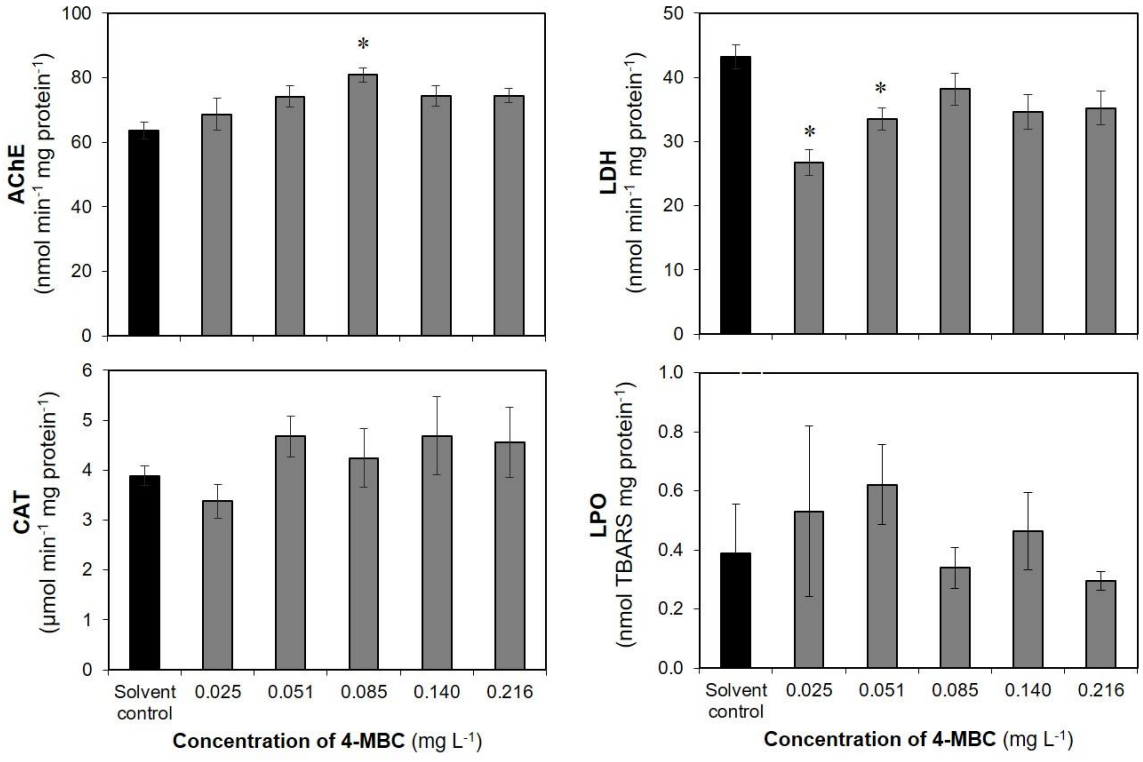


Fig. 3.4. Biochemical response of *Solea senegalensis* after 96 h of exposure to 4-MBC. AChE, acetylcholinesterase; LDH, lactate dehydrogenase; CAT, catalase; LPO, lipid peroxidation. * represents significant differences with solvent control ($p<0.05$).

## 4. Discussion and conclusions

In this study, toxicological effects of the organic UV-filter 4-MBC on *S. senegalensis* larvae at different levels of biological organization were evaluated using apical behavioral and biochemical endpoints.

Despite the relatively low solubility of 4-MBC, this compound can be frequently found in coastal ecosystems (Sánchez-Rodríguez *et al*., 2015; Tovar-Sánchez *et al*., 2013) and has high potential for adsorption to bed sediments and aquatic organism tissues, including fish (Balmer *et al*., 2005; Buser *et al*., 2006; Gago-Ferrero *et al*., 2012). Therefore, 4-MBC loss verified by chemical analysis after 48h can be related with assimilation by the fish, but also adsorption to plastic plate walls and photo-degradation might have contributed to 4-MBC degradation.

Globally, *S. senegalensis* larvae responded in a dose-response manner to 4-MBC exposure regarding mortality, malformations, delayed development and growth. Effects at sub-lethal level, namely on behavior and biochemical biomarkers were also obtained.

4-MBC has been pointed previously to decrease hatching rate of zebrafish (Torres, 2016), on the contrary, it did not alter *Pelophylax perezi* hatching (Martins *et al*., 2017). Although hatching of *S. senegalensis* can be affected by exposure to xenobiotics such as metals (Pavlaki *et al*., 2016), in our study most *S. senegalensis* hatched at 38 hpf and all hatched at 48 hpf as expected (Yúfera *et al*., 1999). Hatching effects caused by exposure to 4-MBC were not observed, suggesting that the chorionic membrane and the hatching process of *S. senegalensis* were not affected by this UV-filter.

The 96 hpf $LC_{50}$ obtained for *S. senegalensis* larvae was 0.439 mg $L^{-1}$ 4-MBC. This value is in the same range of the value obtained for *Daphnia magna* (0.56 mg $L^{-1}$ at 48h) (Fent *et al*., 2010a) and for frog embryos (0.79 mg $L^{-1}$) (Martins *et al*., 2017). However, it was lower than 72 hpf or 96 hpf $LC_{50}$ obtained for zebrafish larvae by Li *et al*. (2016) or Quintaneiro *et al*. (2019) (5.04 and 2.4 mg $L^{-1}$, respectively), revealing an higher sensibility to 4-MBC of the fish species used in our work. This can be related with typical richer lipid content of eggs, namely on yolk sac, when comparing marine species to freshwater species, which can be associated with highest assimilation of lipophilic organic contaminants and consequently higher toxic effects might be expected.

Zebrafish has exhibited negative developmental effects, including tail deformities after exposure to 4-MBC, leading to locomotion deficiency by impairment of muscles and neuronal development (Li *et al*., 2016; Ruszkiewicz *et al*., 2017; Torres, 2016). Skeleton deformities are common in reared *S. senegalensis* during larval development (Gavaia *et al*., 2009) and therefore this endpoint needs to be carefully checked when using this

species. In the present study, tail deformities were not detected neither in controls or treatment groups. However, abnormal notochord curvature in *S. senegalensis* exposed to 4-MBC occurred in a similar way to that previously described for 4-MBC exposed zebrafish (Li *et al*., 2016). Such effects might have ecologically relevant implications in swimming behavior of fish and in ability of feeding of later life stages, impairing their growth.

4-MBC has been previously pointed to affect growth of several aquatic organisms, such as larvae of the echinoderm *Paracentrotus lividus* (LOEC=2 µg $L^{-1}$ during 48h of exposure to 4-MBC, Torres, 2016), diptera larvae *Chironomus riparius* (LOEC=2.05 mg $kg^{-1}$, 10 days adult emergence, Campos *et al*., 2017b), green alga *Desmodesmus subspicatus* (growth inhibition $IC_{10}$=0.81 mg $L^{-1}$ during 72 h of exposure) and adult *D. magna* (LOEC=0.2 mg $L^{-1}$ during 21 days exposure, Sieratowicz *et al*., 2011). Growth of *S. senegalensis* larvae was similarly affected in our study (LOEC=0.360 mg $L^{-1}$).

Growth restriction is one of the parameters commonly used to verify development toxicity and teratogenicity of xenobiotics (e.g. Van den Bulck *et al*., 2011). However, little information is known about effects of 4-MBC on early vertebrate development. Previous report of European Commission Scientific Committee on Consumer Products (SCCP, 2008) states assuming there is no evidence of teratogenicity by this compound in mammals. Concerning fish embryo development, Li *et al*. (2016) reported evidences of 4-MBC teratogenic potential in zebrafish. For comparison purposes, the TI of 4-MBC for 72 hpf zebrafish is estimated as 1.37 (using data from Li *et al*. (2016)) which is similar to the TI of 4-MBC estimated in our work (1.18). Both values near the threshold limit (1), suggesting that 4-MBC can be slightly teratogen to these fish species.

Exposure to 4-MBC affected behavior *S. senegalensis* larvae, as previously described on zebrafish (Li *et al*., 2016). While total swimming distance and number of movements were not affected by exposure to 4-MBC, *S. senegalensis* exposed to 4-MBC took more time to swim similar distances. At concentrations lower than the LOEC of abnormal notochord curvature (which is similar to LOEC of growth) behavior was significantly affected. This might be related with a decrease of resistance and/or ability of swimming.

Larvae of *S. senegalensis* are typically more active in light periods until 15 days post fertilization (Blanco-Vives *et al*., 2011; 2012). When analysing behavior and light and dark phases are manipulated, the pattern of circadian rhythms of fish are usually maintained, for instance the diurnal zebrafish still presents more activity during light periods (Kopp *et al*., 2016). Our study is in accordance with these observations, as non-exposed sole larvae (negative and solvent control) were always more active during light periods.

However, the exposure to the highest concentration tested of 4-MBC reduced clear responses to alternating light and dark phases. Since this might have consequences in circadian rhythms of *S. senegalensis* with relevant ecological consequences to natural fish populations, this effect of 4-MBC should be further confirmed in 24h behavioral studies, under normal photoperiod regimes. Other EDCs have already been shown to disturb circadian rhythms in zebrafish, including another UV-filter, the benzophenone 3 (Kopp *et al*., 2017). Furthermore, it should be highlighted that thyroid system of *S. senegalensis* has a key role during larval neurodevelopment (Klaren *et al*., 2008). In addition, 4-MBC has been previously reported to impair endocrine system (e.g. Inui *et al*., 2003; Kunz and Fent, 2009; Schmitt *et al*., 2008; Schlumpf *et al*., 2004). Therefore, the enrolment of thyroid system on the observed behavioral effects by 4-MBC in *S. senegalensis* should be further investigated.

Along test progression, significant differences on swimming time between controls and 4-MBC exposure groups present tended to decrease which could be related with the fact that behavioral recording was performed in clean media and post-exposure recovery of exposed fish may have occurred. While, some neurotransmission and endocrine effects of 4-MBC can be permanent, the cause for behavioral effects in *S. senegalensis* should be confirmed and prolonged behavioral analysis should be performed., because the natural circadian cycles of this species suffers a deep change after metamorphosis, with highest activity during night periods (Blanco-Vives *et al*., 2012) and effects on natural patterns at individual level can have severe ecological implications at population or community level. Behavioral effects observed, might also be a consequence of effects registered at biochemical level.

Biomarkers have long been considered useful tools for understanding effects of chemicals on low organizational levels, namely for the elucidation of their mode of action. However, it is of upmost relevance use biomarkers in combination with endpoints of higher levels of organization (e.g. malformations, behavior, growth) in order to fully understand 4-MBC adverse effects.

Neurotoxic damage can result from the accumulation of the neurotransmitter acetylcholine with effects on normal development of larvae (Ruszkiewicz *et al*., 2017; Torres, 2016). Inhibition of AChE by 4-MBC is expected to occur because it is a camphor belonging to terpene family, which are known to have this effect (Miyazawa *et al*., 1997). Indeed, inhibition of AChE by 4-MBC has been observed previously in zebrafish embryos (NOEC= 0.254 mg $L^{-1}$; LOEC=2.54 mg $L^{-1}$) and confirmed in vitro using neuro-2a cell-line exposed indicating a possible mechanism for inducing muscular and neuronal defects (Li

*et al*., 2016). However, no significant alterations were obtained for diptera and tricoptera species (Campos *et al*., 2017a, b) or amphibian embryos (Martins *et al*., 2017). In the present work, an overall trend of AChE induction was observed in 96 hpf *S*. *senegalensis*, with significant induction registered at 0.085 mg $L^{-1}$ of 4-MBC. Similarly, AChE induction was also observed in 96 hpf zebrafish larvae, with a LOEC of 0.33 mg $L^{-1}$ 4-MBC (Quintaneiro *et al*., 2019). In several studies, apoptosis has been pointed out to up-regulate AChE genes (e.g. Zhang *et al*., 2002) and as an explanation for AChE enzyme activity induction (e.g. Andrade *et al*., 2016). Indeed, 4-MBC is pointed to be one of the UV filters with highest capacity of increasing Caspase-3 activity, even in very lower concentrations 2.54 µg $L^{-1}$ (Broniowska *et al*., 2016), which is a protein with an important role on cell apoptosis. Besides, expression of thyroid related genes (among others), including thyroid hormone receptors (TRα and TRβ), and iodothyronine deiodinases (Dio1 and Dio2) have been suggested previously to be involved in regulation of AChE activity (Jiang *et al*., 2014; Puymirat *et al*., 1995). Therefore, the association of AChE induction with an eventual thyroid disruptor action of 4-MBC on *S*. *senegalensis* should be investigated.

AChE alterations have been related to neurological impairment and stress-related responses which are pointed to occur after exposure to contaminants with possible implication on behavior of fish (Kannan *et al*., 2012; Lionetto *et al*., 2003, 2013; Rao *et al*., 2005; Weis *et al*., 2001). Such fact was observed in our study, with AChE activity increased and swimming behavior affected after exposure to 4-MBC at similar values (0.085 mg $L^{-1}$ and 0.068 mg $L^{-1}$, respectively). However, clear response of AChE activity was not obtained with increasing concentrations of 4-MBC and still need further understanding.

Despite variation of LDH can occur in response to changes on environmental conditions, certain compounds also lead to LDH changes pointing toxicity on animal tissues and cells. 4-MBC did not affect LDH in zebrafish embryos (Martins *et al*., 2017), while in the present work the lower 4-MBC concentrations tested decreased LDH enzyme activity of *S*. *senegalensis* larvae, suggesting an impairment of anaerobic metabolism in sole larvae exposed up to 0.051 mg $L^{-1}$. However, this result should be interpreted with caution, since differences between negative and solvent control occurred in our study (higher LDH levels in solvent control; data not shown) and effects on LDH of the use of different solvents in fish bioassays should be tested and further studied.

Catalase occur in natural conditions to cope with regular cellular metabolism by hydrolyzing the ROS hydrogen peroxide and can be induced in response to oxidative stress conditions. Hydrogen peroxide is formed internally on biochemical processes of organisms and plays an important role as a signalling molecule. On the other hand, it is one of the

most abundant harmful ROS to cellular components. 4-MBC has been pointed to inhibit CAT in *T*. *thermophile* at 1.0 µg $L^{-1}$ (Gao *et al*., 2013) and at concentrations of 14.13 mg $kg^{-1}$ in *C*. *riparius* (Campos *et al*., 2017b). Catalase was however, not significantly altered on frog embryos after 144h of exposure to 4-MBC up to 1.3 mg $L^{-1}$ (Martins *et al*., 2017) nor in zebrafish embryos after 96h exposure up to 1.09 mg $L^{-1}$. Similarly, in our study, CAT was not significantly altered in *S*. *senegalensis* larvae. In fact, camphors have generally been shown dimorphic behavior as pro-oxidant or antioxidant, such as in the work of Sedaghat *et al*. (2016) in bird testis. In our study, a high variability of LPO in response to 4-MBC exposure was obtained. This has also occurred with *C*. *riparius* at concentrations ranging from 0.09 and 14.13 mg $kg^{-1}$ (Campos *et al*., 2017b), suggesting that no oxidative lipid damage was induced by 4-MBC in sole larvae, which is in good agreement with the no alterations on CAT activity, suggesting overall no induction of oxidative stress by 4-MBC in our experimental conditions.

In this study, swimming behavior is the most sensitive endpoint (LOEC at 0.068 mg $L^{-1}$ 4-MBC), followed by malformations, and finally, growth was the least sensitive. Biochemical biomarkers were not significantly altered, despite from the induction of AChE at 0.085 mg $L^{-1}$ and the inhibition of LDH at the lowest concentration tested (0.025 mg $L^{-1}$ 4-MBC). Integration of behavior is then recommended in ecotoxicology studies with sole, because effects of exposure to 4-MBC on swimming behavior were observed at concentrations lower than the malformation NOECs. Such effects can be associated with unsuccessful growth and development of species in the wild. Among all endpoints, 0.025 mg $L^{-1}$ of 4-MBC induced significant responses on LDH activity. This value is in the same range of environmental levels (up to 0.01 mg $L^{-1}$) in coastal waters (Tovar-Sánchez *et al*., 2013) and potential environmental effects of this compound might be affecting aquatic vertebrates.

As the use of sunscreens by humans has been recommended by national and international health institutions in recent years, the spread of UV filters in natural ecosystems is expected to increase. Therefore, the environmental risk of 4-MBC to the marine environment should be considered. Furthermore, effects of mixtures of UV filters and other organic compounds and effects of each specific conformation are still difficult to predict and mostly unknown (Fent *et al.,* 2008; Gago-Ferrero *et al*., 2012; Ruszkiewicz *et al*., 2017; Torres, 2016).

We conclude that *S*. *senegalensis* larvae has potential as alternative model species for vertebrate animal testing of chemicals in the marine environment. The levels of UV filters in environment are expected to continue to increase and might have severe

impacts on the ecology of this marine species. Sub-lethal concentrations of 4-MBC induced toxic effects at all organizational levels on this marine vertebrate as previously reported for other aquatic organisms. Swimming behavior revealed to be a very sensitive endpoint and due to its ecological relevance is an highly valuable endpoint recommended for ecotoxicology studies with sole. Conservation concerns along distribution range of *S. senegalensis* should consider the threat of 4-MBC. Furthermore, *S. senegalensis* has economic and ecological interest in Atlantic and Mediterranean temperate regions and aquaculture reproduction still rely on wild spawners. The continuous understanding of the impacts of widespread emergent contaminants over this species is needed and might contribute for defining management actions.

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# Effects of UV filter 4-methylbenzylidene camphor during early development of *Solea senegalensis* Kaup, 1858

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## Supplementary Data

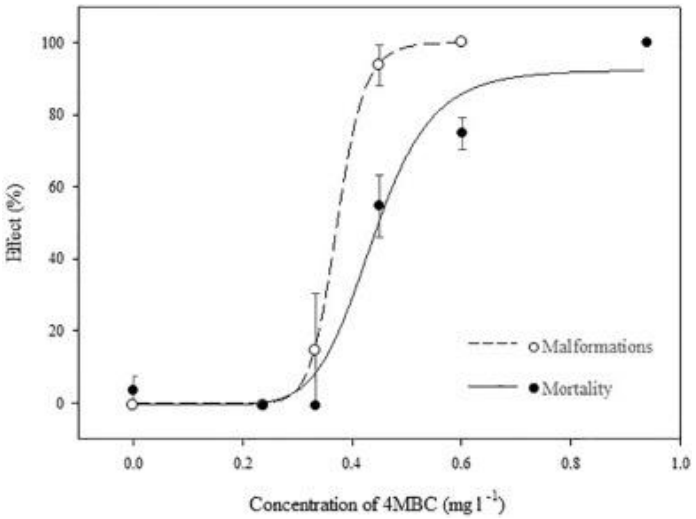


Fig. 3.S1. Concentration-response curve for notochord curvature and mortality of *Solea senegalensis* after 96 h of exposure to 4-MBC (N = 4, 5 larvae per replicate). Effects were fitted to four parameter logistic curves ($EC_{50}$ = 0.372 ± 0.014 mg $l^{-1}$, adj. $R^2$ = 0.958, p ≤ 0.001; $LC_{50}$ = 0.439 ± 0.016 mg $l^{-1}$, adj. $R^2$ = 0.92, p < 0.001).

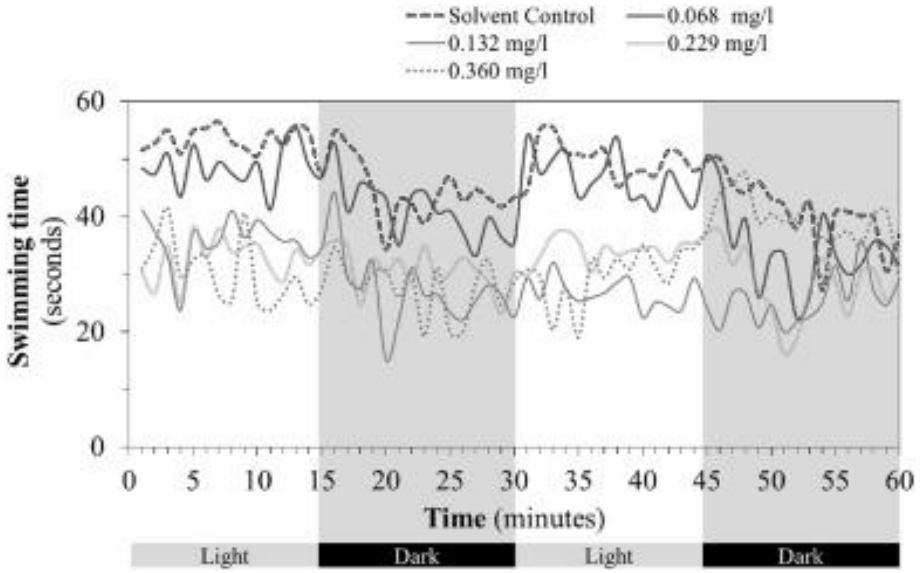


Fig. 3.S2. Swimming time of *Solea senegalensis* per minute during the 60 min behavioral analysis with 15 minute light and dark cycles after 96 h of exposure to 4-MBC.

Table 3.S1. Nominal and measured concentrations of 4-MBC at 0h and after 48h of exposure.

| **Nominal concentrations of 4-MBC** (mg $L^{-1}$) | **Measured concentrations of 4-MBC** (mg $L^{-1}$) | **Concentrations of 4-MBC after 48h** (mg $L^{-1}$) | **Loss of 4-MBC at 48h** |
|---|---|---|---|
| 0.05 | 0.025 | 0.006 | 73 % |
| 0.56 | 0.360 | 0.103 | 71 % |
| 1.75 | 0.935 | 0.245 | 74 % |
| 2.00[a] | 1.190 | - | . |

[a] stock solution

Table 3.S2. Physical-chemical parameters of 4-MBC solutions at 0h of exposure. *na*: not available.

| | **4-MBC** (mg $L^{-1}$) | **Physical-chemical parameters** | | | |
|---|---|---|---|---|---|
| | | **Temperature** (ºC) | **Salinity** | **$O_2$** (%) | **pH** |
| First Experiment | Solvent control | 20.3 | 34.7 | 66.3 | 8.1 |
| | Negative control | 19.3 | 34.5 | 62.2 | 8.1 |
| | 0.235 | 20.2 | 34.4 | 64.2 | 8.2 |
| | 0.331 | 19.9 | 34.4 | 63.5 | 8.3 |
| | 0.447 | 20.2 | 34.5 | 65.0 | 8.2 |
| | 0.599 | 20.2 | 34.4 | 63.5 | 8.2 |
| | 0.935 | 20.2 | 34.4 | 60.0 | 8.2 |
| Second experiment | Solvent control | 21.0 | 35.9 | *na* | 8.1 |
| | Negative control | 20.5 | 35.0 | *na* | 7.9 |
| | 0.068 | 20.9 | 35.6 | *na* | 8.1 |
| | 0.132 | 20.7 | 35.4 | *na* | 8.1 |
| | 0.229 | 21.8 | 35.1 | *na* | 8.1 |
| | 0.360 | 20.9 | 35.3 | *na* | 8.0 |
| Third experiment | Solvent control | 20.4 | 35.2 | *na* | 8.0 |
| | Negative control | 20.3 | 35.0 | *na* | 7.9 |
| | 0.025 | 20.8 | 35.1 | *na* | 8.0 |
| | 0.051 | 21.0 | 35.1 | *na* | 8.0 |
| | 0.085 | 21.1 | 34.9 | *na* | 8.0 |
| | 0.140 | 21.1 | 34.8 | *na* | 8.0 |
| | 0.216 | 21.1 | 34.8 | *na* | 8.0 |

# Chapter 4. Exposure effects of the UV-filter 4-MBC on *Solea senegalensis* metamorphosis

# Exposure effects of the UV-filter 4-MBC on *Solea senegalensis* metamorphosis

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**Abstract**

Harmful effects of ultraviolet radiation (UV) is leading to growing use of UV-filters, such as 4-methylbenzylidene camphor (4-MBC). This organic compound is included in sunscreens formulations and other cosmetics and it has been detected in aquatic habitats; however, the effects of 4-MBC on early life stages of marine vertebrates, namely during fish metamorphosis, have been scarcely studied. Therefore, the main objective of this work is to understand the effects of exposure to 4-MBC during *S. senegalensis* metamorphosis.

To achieve this, at the beginning of metamorphosis (13 days after hatching, dah) fish were exposed to 4-MBC (0.2 - 2.0 mg $L^{-1}$) during 48h. 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 energy metabolism (lactate dehydrogenase, LDH) were determined at the end of the experiment.

Increased mortality and malformations of *S. senegalensis* and alterations on metamorphosis progression were observed during and after the 4-MBC exposure. In addition, decreased length and induced oxidative damage (LOEC=0.928 mg $L^{-1}$ 4-MBC) and altered behavior responses at one of the lowest concentrations tested (0.294 mg $L^{-1}$ 4-MBC) were observed at the end of sole metamorphosis.

A short-term exposure to 4-MBC in 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 effects on growth and metamorphosis progression, suggesting possible long-term adverse effects to this species.

**Keywords:** behavior; biochemical markers; early development; flatfish; marine vertebrates; oxidative stress; personal care products

## 1. Introduction

The release of several chemicals to the environment has been occurring as a consequence of human population increasing growth. Early life stages of coastal and marine species are particularly vulnerable to anthropogenic driven stressors with action on aquatic environment because most part of world population inhabits near such areas (Shoji *et al*., 2011; Tovar-Sánchez *et al*., 2013; Pimentel *et al*., 2015; Wen *et al*., 2017b; Chisvert and Salvador, 2018). However, studies on the effects of widely used organic chemicals and on how marine fish early life stages cope with a global changing environment linking biochemical, physiological and morphological effects from sub-cellular to organism level are still scarce.

The harmful effects of UV that reach earth surface is leading to growing consumption of sunscreens (Lowe, 2006; Young, 2006; Young *et al*., 2017). Organic UV filters are key compounds of these personal care products, therefore, increasing levels of this class of chemicals are expected in coastal areas (Chisvert and Salvador, 2018). The 4-methylbenzylidene camphor (4-MBC) is one of the most common UV filters used in sunscreens and other cosmetics (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 $l^{-1}$ (Atlantic Gran Canaria) (Tovar-Sánchez *et al*., 2013; Sánchez-Rodríguez *et al*., 2015). Besides, 4-MBC is highly stable, lipophilic, suffers low biotic degradation and 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). It has also high potential for adsorption to bed sediments and aquatic organism tissues, including fish (Balmer *et al*., 2005; Buser *et al*., 2006; Gago-Ferrero *et al*., 2012).

Previous works have shown that 4-MBC affects aquatic vertebrates on development, behavior and at biochemical level 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 disruption action of 4-MBC have also been previously reported, which included androgen and oestrogen activity and effects on reproductive organs (Krause *et al*., 2012; Wang *et al*., 2016). In addition, effects of 4-MBC 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). Previous studies also reported that exposure to endocrine active chemicals, namely polychlorinated biphenyls during the thyroid regulated metamorphosis

can retard the metamorphic processes of flatfish (Dong *et al*., 2017); however, ecotoxicological studies during this sensitive window of flatfish development are still very scarce.

Flatfish have been increasingly used in European marine aquaculture industry (Imsland *et al*., 2003; Morais *et al*., 2016). Their commercial availability together with their life cycle features and characteristics make them a relevant and high potential model species for use in ecotoxicological studies (Pimentel *et al*., 2015; Pavlaki *et al*., 2016; Araújo *et al*., 2018). Senegalese sole (*Solea senegalensis* Kaup, 1858) inhabits coastal areas during larval stages and eggs are 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). 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. Besides, 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 involved with the fluctuation of thyroid hormones levels (Yamano *et al*., 1991; Okada *et al*., 2003; Klaren *et al*., 2008). Therefore, chemicals with structural similarity with these hormones are pointed to interfere with thyroid axis (Crofton, 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 increasing thyroid hormone levels and inducing faster metamorphosis progression (Jarque and Piña, 2014; Yue *et al*., 2017; Dong *et al*., 2017; Araújo *et al*., 2019).

Exposure to stressors at very low levels may induce clear behavioral responses and therefore, behavior is becoming more used as an endpoint on ecotoxicology studies (Sloman and McNeil, 2012; Sharma, 2019). Different techniques and methods have been developed to assess behavior as it can be integrated with responses at lower (e.g. physiological) or higher levels of organization (e.g. populations) (Scott and Sloman, 2004). In fact, behavior is among the most sensitive non-invasive tools when comparing to traditional developmental endpoints or sensitive molecular techniques such biochemical

enzymatic markers (Vieira *et al*., 2009; Almeida *et al*., 2015; Andrade *et al*., 2016; Henriques *et al*., 2016; Araújo *et al*., 2018). Behavioral response to light stimulus can reveal effects of exposure to chemicals. Besides, 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, namely on ecological relevant endpoints, such as behavior and fish growth and can provide information to understand the mechanisms and modes of action of stressors (Oost *et al*., 2003). Inhibition of acetylcholinesterase (AChE) activity can indicate neurotoxicity that result from the exposure to chemicals, typically organophosphates (Guilhermino *et al*., 1996) and carbamates (Andrade *et al*., 2016), but also organic compounds such as the UV filter 4-MBC have 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 study of molecular and enzymatic responses to 4-MBC exposure showed that this compound affect antioxidant system leading to oxidative damage (Gao *et al*., 2013, Liu *et al*., 2015; Campos *et al*., 2017a; Martins *et al*., 2017). The enzyme glutathione S-transferase (GST) and catalase (CAT) have central role on oxidative stress response and also these enzyme levels fluctuate in response to organisms physiological or developmental stages including metamorphosis (Oost *et al*., 2003; Menon and Rozman, 2007; Rudneva *et al*., 2010; Araújo *et al*., 2019). Lipid peroxidation (LPO) can occur when antioxidant capacity of organisms is surpassed and this can occur as a consequence to the presence of different classes of stressors (Oost *et al*., 2003).

Lactate dehydrogenase (LDH) activity can provide information on the increased demand of energy from anaerobic metabolism. This can occur when higher metabolic activity is needed, namely for detoxification mechanisms to cope with exposure to chemical stressors (Diamantino *et al*., 2001; Güngördü *et al*., 2016).

Effects of environmental contaminants, such as UV-filters on key stages of life cycle of marine vertebrates, in particular the flatfish thyroid-regulated metamorphosis, are scarcely studied in ecotoxicology. In this context, the objective of this work is to study the effects of 4-MBC exposure during *S*. *senegalensis* metamorphosis, a sensitive window of

development of flatfish, 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. Biological samples and experimental design

Eggs from a commercial aquaculture in north of Portugal (Safiestela/Sea8, Póvoa de Varzim) were brought to laboratory within 12 hours after fertilization and placed in recirculating system with artificial saltwater (35 of salinity, Coral Pro salt, Saudi Arabia) equipped with biological filtering medium, UVR sterilizer and protein skimmer. Refrigeration was set at 19ºC and photoperiod 16h:8h (l:d). Feeding included rotifers (*Brachionus plicatilis*) from 2 dah until 6 dah and *Artemia salina* nauplii and metanauplii (from 5 until 10 and from 9 until 13 dah, respectively). The green algae *Nannochloropsis gaditana* was also provided daily for improvement of maintenance conditions by increasing live feed visual contrast and their nutrients, increase of oxygen and reduction of metabolic by-products.

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; *in preparation*), which occurred at 13 dah. At this age, fish had a total length of 4.7±0.23 mm (n=24). Randomly selected organisms were then exposed to different 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 6 well plates (n=6, 5 fish in 10 mL of testing solution per replicate). After 48h of exposure unfed fish were transferred to new plates with clean medium and fed daily with artemia *metanauplii* until 24 dah. At that age, 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 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 0.10%) were also used. The pH of 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 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 (authorized by the

Portuguese competent authority, Direcção Geral de Alimentação e Veterinária, Ref. 009804 ).

### 2.2. 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. Metamorphosis development stages of fish were determined in accordance with literature (table 4.1). Length of organisms in each experimental group was determined at the end of metamorphosis (n=6, 3 fish per replicate).

Table 4.1. Metamorphosis stages of *Solea senegalensis* in accordance with Dinis (1986), Fernández-Díaz *et al*. (2001) and Klaren *et al*. (2008).

| Stage | Morphology |
|---|---|
| A | Beginning of enlargement and pigmentation of dorsal and ventral fins |
| B | Beginning of migration of left eye to the right side<br>Further pigmentation of fins |
| C | Further migration of left eye and pigmentation<br>Alteration of mouth shape |
| D | Further migration of left eye and pigmentation<br>Fully enlargement of dorsal and ventral fins<br>Further alteration of mouth shape |
| E | Fully flattened body<br>Left eye on the dorsal side |
| F | Further migration of left eye on the dorsal side |
| G | Growth of anal fin<br>Shrink of pectoral fin<br>Orbital arch becomes clearly visible |

### 2.3. 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 organism per replicate) in 24 well plates with 2 mL of clean medium in each 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 duration of swimming and total distance at 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 infra-red light (2.30±0.11 mW $cm^{-2}$).

### 2.4. Biochemical analysis

Previously frozen samples (n=6, 3-5 fish per replicate) were used to measure the activity of the enzymes AChE, CAT, GST 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 412 nm (Ellman *et al*., 1961; Guilhermino *et al*., 1996). 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). CAT activity was determined by measuring the rate of hydrogen peroxide ($H_2O_2$) consumption at 240 nm (Clairborne, 1985). 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 $40M^{-1}cm^{-1}$ for CAT and $6.3x10^{3}M^{-1}cm^{-1}$ for LDH. For LPO, U represented one nmol of TBARs hydrolyzed per minute 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.

### 2.5. 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 (Sigma-Aldrich Co.).

### 2.6. Statistical analysis

After initial t-test for testing existence of differences between solvent and negative control, One-way ANOVA were used to study differences between solvent control and 4-MBC treatment groups on mortality, development effects, length and biochemical markers. For the statistical analysis of behavior, One-way ANOVA was used to test for differences on total duration and distance of swimming during the 60 min of testing. When normality or equality of variance were not achieved, non-parametric ANOVA on Ranks were used. For significant endpoints, post-hoc Dunnett's pairwise test with solvent control were performed. Two-way Repeated Measures (RM) ANOVA was used to study the effect of 4-MBC concentration and consecutive light and dark periods on swimming duration and distance. Since in both behavioral parameters, the interaction between factors (4-MBC concentration and light/dark periods) was not significant, Tukey post-hoc test was performed to study differences between different 4-MBC concentrations or between different light/dark periods.

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).

Sigmaplot 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

Fish mortality was 0% in solvent and negative control treatments along the test. A dose-response curve could not be determined at any age. Significantly higher mortality was observed only in fish groups exposed to the highest concentration (2.000 mg $L^{-1}$) at 14, 15 and 24 dah when comparing to solvent control ($p<0.05$, fig. 4.1), with the highest mortality occurring at 15 dah (70.0±8.56%) which did not increase during maintenance in clean medium, until the end of metamorphosis. Mortality of the second highest 4-MBC

concentration (1.363 mg $L^{-1}$) at 15 dah and at the end of metamorphosis were 10.0±6.83% and 13.3±6.67%, respectively. At the end of metamorphosis, the mortality of fish exposed to 4-MBC concentrations up to 1.363 mg $L^{-1}$ was below 15%. Therefore, only fish groups exposed up to 1.363 mg $L^{-1}$ were used in the following endpoints.

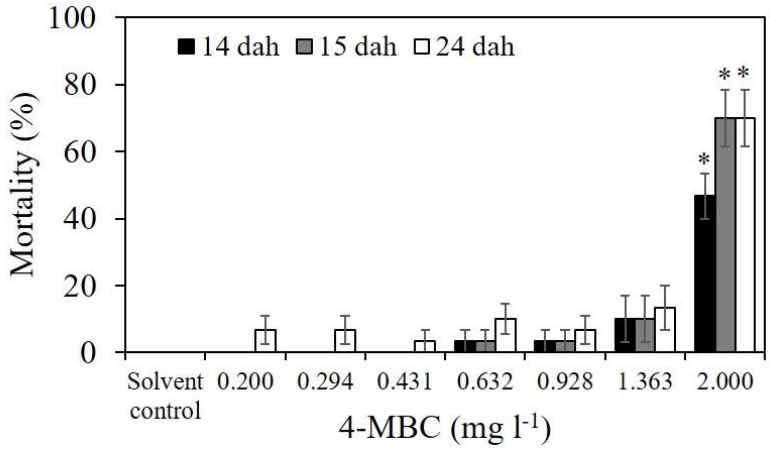


Fig. 4.1. Mortality of *Solea senegalensis* along metamorphosis after exposure to 4-MBC. Exposure was performed at 13 days after hatching (dah) during 48h. Metamorphosis ended at 24 dah. * represent significant differences from solvent control at same day ($p<0.05$, ANOVA on ranks followed by pairwise Dunn's test).

### 3.2. Malformations

No malformations in fish were observed on both controls during all the metamorphosis progression. Alterations to normal fish development were observed in fish exposed up to 1.363 mg $L^{-1}$ of 4-MBC which referred to the simultaneous alterations on tissues, pigmentation, fin and abdominal cavity (fig. 4.2). The percentage of these alterations were significantly increased in fish exposed to 0.928 mg $L^{-1}$ at 14 and 15 dah (24.2±8.00% at both ages, $p<0.05$).

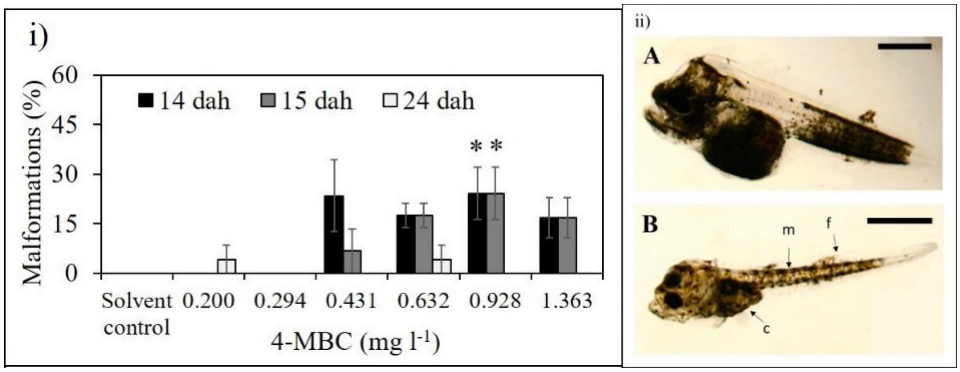


Fig. 4.2. Total malformations of *Solea senegalensis* along metamorphosis after exposure to 4-MBC. Exposure was performed at 13 days after hatching (dah) during 48h. Metamorphosis ended at 24 dah. i) Percentage of fish with alteration on tissues, abdominal cavity, fins and pigmentation. * represent significant differences between each 4-MBC exposure group with solvent control at same day ($p<0.05$, ANOVA on ranks followed by pairwise Dunn's test). ii) A – control fish; B – fish exposed to 1.363 mg $L^{-1}$ of 4-MBC with simultaneous alteration of tissues (m), abdominal cavity (c), fins (f) and altered pigmentation. Black bar represents 1 mm.

At 24 dah, after the maintenance in clean medium, the percentage of affected fish ranged between 0 and 8.3±5.27% in fish groups exposed to 4-MBC without significant differences between 4-MBC exposed fish and solvent control ($p>0.05$).

### 3.3. 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) at 14, 17, 20, 22 and 24 dah ($p<0.05$, Chi-square test) with solvent control fish constantly presenting retarded metamorphic progress comparing to negative control.

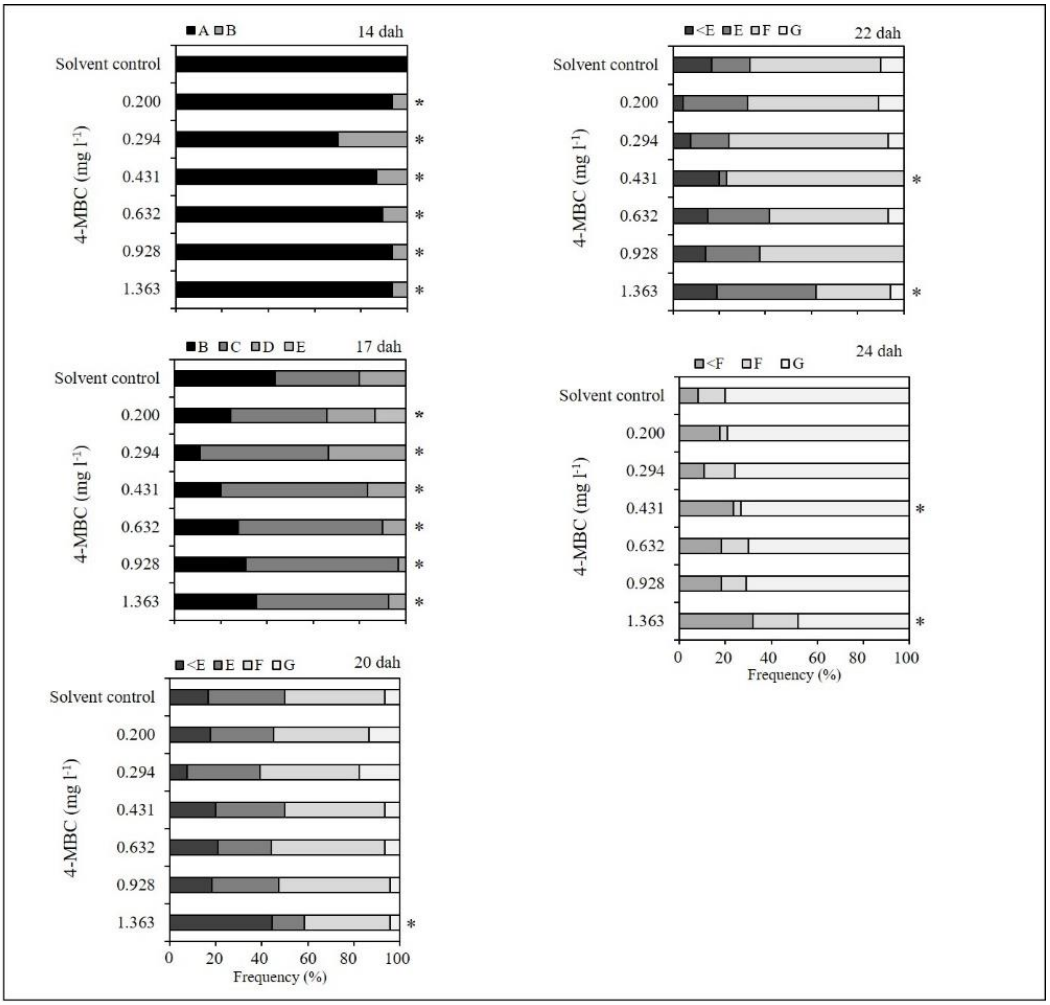


Fig. 4.3. Metamorphosis progression of *Solea senegalensis* after 48h exposure to 4-MBC. Exposure was performed at 13 days after hatching (dah) during 48h. Metamorphosis ended at 24 dah. Data represent the percentage of fish in each development stage (A, B, C, D, E, F, and G stages) according to literature (Dinis, 1986; Fernández-Díaz *et al*., 2001; Klaren *et al*., 2008) and as described in table 4.1 in each treatment group. * represent significant differences between fish in 4-MBC exposure group with solvent control group at same day ($p<0.05$, Chi-square test).

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 and 17 dah, with 4-MBC exposed fish groups presenting faster metamorphosis progression than solvent control group ($p<0.05$, fig. 4.3). 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 ($p<0.05$).

### 3.4. Length

There were no 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. 4.4).

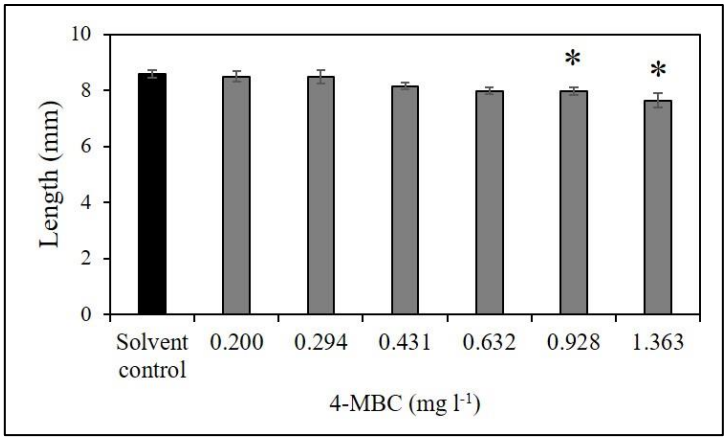


Fig. 4.4. Length of *Solea senegalensis* at the end of metamorphosis after exposure to 4-MBC. Exposure was performed at 13 days after hatching (dah) during 48h. Metamorphosis ended at 24 dah. * represent significant differences from solvent control ($p<0.05$, one-way ANOVA followed by pairwise Dunnett's test).

### 3.5. Behavior

During the 60 minute behavior test, 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. 4.5).

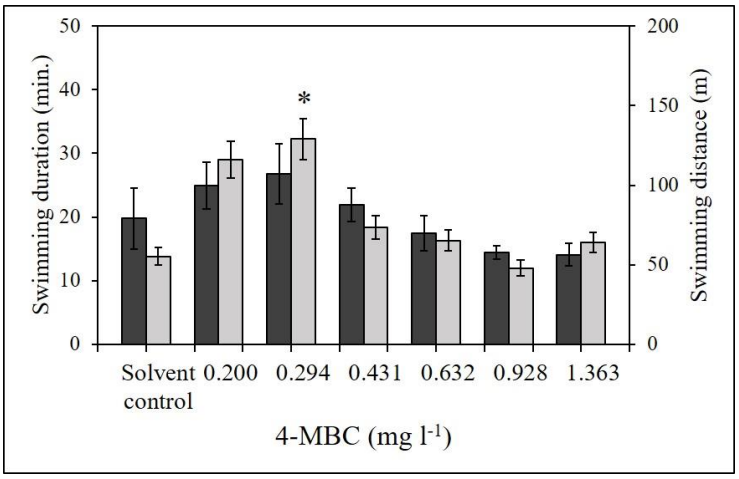


Fig. 4.5. Swimming duration (black bar) and swimming distance (grey bar) of *Solea senegalensis* at the 60 min behavior test at the end of metamorphosis after exposure to 4-MBC. Exposure was performed at 13 days after hatching (dah) during 48h. Metamorphosis ended at 24 dah. * represent significant differences between fish in 4-MBC exposure group with solvent control group ($p<0.05$, one-way ANOVA followed by pairwise Dunnett's test).

When analysing the 15 min light and dark periods, to assess solvent effect and light/dark periods effect, no significant interaction was found between both factors (treatment and light/dark periods) and none of the factors were significant in both behavioral parameters analysed, swimming duration and distance (data not shown, $p>0.05$).

When analysing the 15 min light and dark periods, to assess the effect of 4-MBC treatments and the effect of light/dark periods during the behavior test on swimming duration and distance, the interaction of both factors (4-MBC concentration and period) was not significant for the two parameters analysed ($p>0.05$, fig. 4.6). The factor 4-MBC concentration was not significant ($p>0.05$). Significant differences were observed between periods on both fish swimming duration and distance ($p<0.05$), namely fish swimming duration was lower in the second dark period ($p<0.05$) and swimming distance was higher in the first dark period than in the other light/dark periods ($p<0.05$).

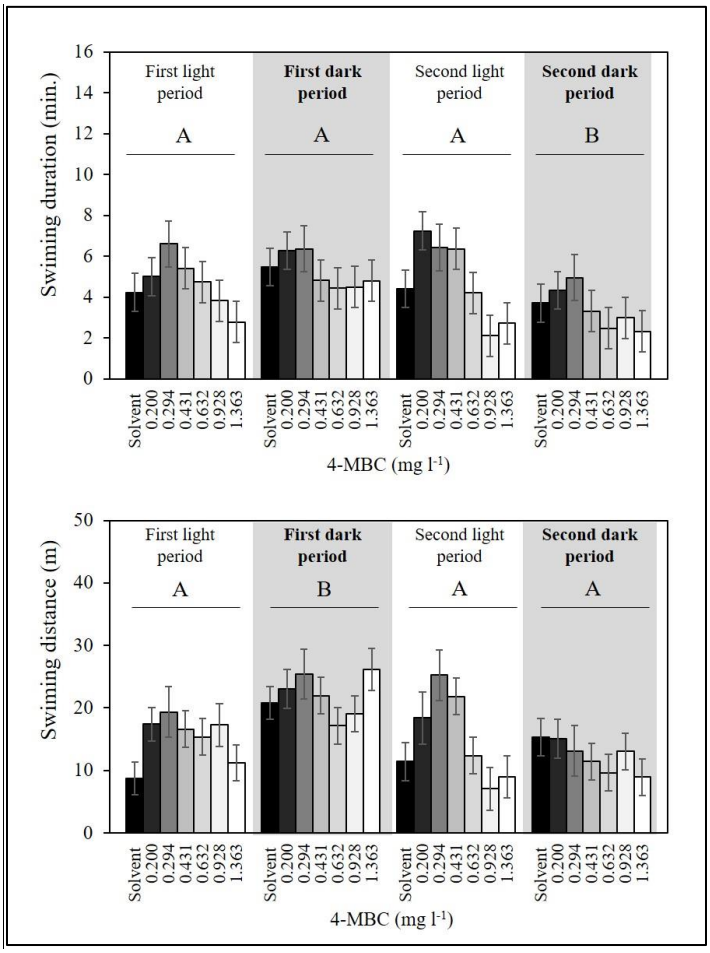


Fig. 4.6. Swimming duration and swimming distance of *Solea senegalensis* within each 15 minute light and dark period of the 60 min behavioral test at the end of metamorphosis after exposure to 4-MBC. Exposure to 4-MBC was performed at 13 days after hatching (dah) during 48h. Different letters represent significant differences between periods ($p<0.05$, Two-way Repeated Measures ANOVA followed by Tukey multiple comparison test).

### 3.6. Biochemical markers

The effects of 4-MBC exposure on *S. senegalensis* biochemical markers analysed at the end of metamorphosis are presented in figure 4.7. No significant differences were obtained between fish from negative and solvent control groups for AChE, CAT, GST and LPO ($p>0.05$). On the contrary, LDH activity of negative control (0.190±0.0042 U mg of protein$^{-1}$) was significantly higher than solvent control (0.172±0.0023 U mg of protein$^{-1}$, $p<0.05$).

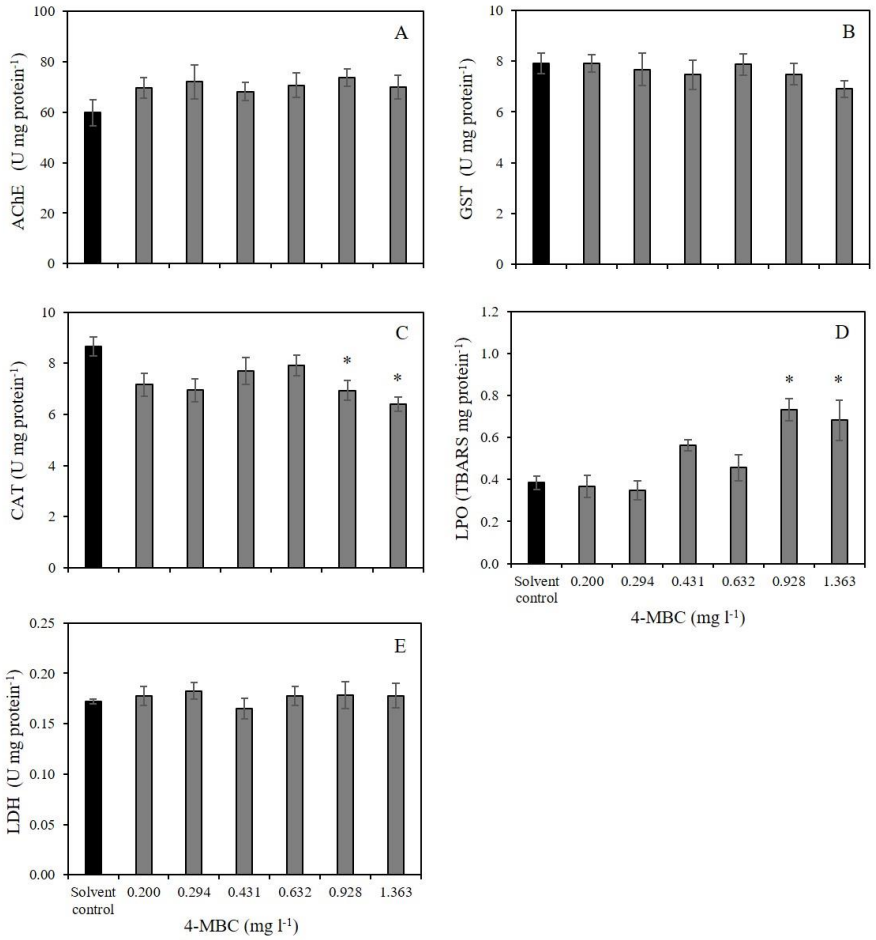


Fig. 4.7. Biochemical responses of *Solea senegalensis* at the end of metamorphosis (24 days after hatching (dah) after 48h exposure to 4-MBC at 13 dah. A - Acetilcholinesterase (AChE) activity, B - Glutathione S-Transferase (GST) activity, C - Catalase (CAT) activity, D - Lipid peroxidation (LPO) levels and E – Lactate dehydrogenase (LDH) activity. * represent the existence of significant differences with respective solvent control ($p<0.05$, one-way ANOVA followed by pairwise Dunnett's test).

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

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, effects of exposure to 4-MBC during *S. senegalensis* metamorphosis were studied at different levels of organization. Table 4.2 summarizes the

results obtained, showing that deleterious effects were observed during exposure to 4-MBC, 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.

Table 4.2. No observed (NOEC) and lowest observed (LOEC) effect concentration of 4-MBC during metamorphosis. 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 after maintenance in clean medium until complete metamorphosis (17-24 dah). CAT – catalase, LPO – lipid peroxidation.

| **Endpoint** | **NOEC** (mg $L^{-1}$) | **LOEC** (mg $L^{-1}$) |
|---|---|---|
| Mortality (14, 15 and 24 dah) | 1.363 | 2.000 |
| Metamorphosis progression (14, 17 dah) | - | 0.200 |
| Growth (24 dah) | 0.632 | 0.928 |
| Biochemical markers – CAT, LPO (24 dah) | 0.632 | 0.928 |

### 4.1. Apical and developmental endpoints

The mortality data obtained with *S*. *senegalensis* exposed to 4-MBC in the onset of metamorphosis indicate that *S*. *senegalensis* in this life stage is less sensitive to 4-MBC than earlier life stages. While an $LC_{50}$ of 0.439 mg $L^{-1}$ 4-MBC was obtained for eggs exposed during 96 hours (Araújo *et al*., 2018), in the present work the mortality was lower than 15 % for all concentrations below or equal to 1.363 mg $L^{-1}$. 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 several 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 preparation*). In the present work, 4-MBC induced several malformations to *S*. *senegalensis,* which have occurred simultaneously during the 48h exposure period. Nevertheless, *S*. *senegalensis* was able to recover from the induced effects on development after the interruption of 4-MBC exposure, since no differences were observed at 24 dah between exposed and control fish. However, the recovery from these effects might have led to lasting implications in 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 have been reported in amphibian larvae (Martins *et al*., 2017). Such

effects of 4-MBC on normal mechanisms of development of metamorphosing species still needs further study.

Despite the solvent used delayed metamorphosis progression stages of *S. senegalensis* in relation to negative control, the exposure to some concentrations of 4-MBC also induced delayed metamorphosis at the end of metamorphosis comparing with solvent control. However, a faster development rate was observed at the earlier moments of metamorphosis. Normal progression of flatfish and anuran metamorphosis can be affected by the exposure to chemicals, including the ones with endocrine disruptor potential. Such effect can be related with the similar structure of some of these chemicals with thyroid hormones (Veldhoen *et al*., 2006; Crofton, 2008; Dong *et al*., 2017; Yue *et al*., 2017). Previous works with *S. senegalensis* showed that after an acceleration of metamorphosis caused by TCS exposure, at the end of metamorphosis no lasting effects from this exposure were observed (Araújo *et al*., 2019). However, in the present work, some fish groups exposed to 4-MBC presented a lower rate of fish with complete metamorphosis when comparing to control groups, indicating lasting effects of this chemical. The mechanism by which this occurs needs to be studied in order to verify if there is any relation with a possible interference in the thyroid axis, that 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 which is in accordance with similar work carried out in an earlier life stage with this species (Araújo *et al*., 2018). Furthermore, the lowest observed effect concentration (LOEC) on length was lower in the earlier life stage (96 h - $LOEC_{length}$ =0.360 mg $L^{-1}$ 4-MBC; sole exposed during 4 days between egg stage and 3 dah) when comparing to the present test conditions (48h - LOEC=0.928 mg $L^{-1}$). This is in accordance with the generally observed decrease of sensitivity to chemicals with fish increasing age, and was also observed in *S. senegalensis* with other chemical stressors (Araújo *et al*., 2019). However, it should be noted that a shorter exposure period 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 the 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. However, concerning growth, besides

this maintenance period after exposure, metamorphosing *S. senegalensis* did not recover from the 4-MBC exposure, further demonstrating lasting effects of this chemical, which can be also associated with 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. While for higher 4-MBC concentrations no significant differences were observed when comparing exposed fish with solvent control, for lower concentrations (0.294 mg $L^{-1}$ 4-MBC) an induction of fish swimming behavior was observed, even after nine days in clean medium after the exposure to 4-MBC. However, 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; however, increased energy expenditure through an increase of LDH activity (see below) were not perceived at the end of metamorphosis.

### 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 camphors 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 stages of these organisms life (e.g. later juveniles or adult stages), which might impair their fitness (e.g. decreased resistance to disease, or feeding and preying success) and this should be further studied. 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 through the increase of total GSH observed in this species (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 time in clean medium after 4-MBC exposure before the biochemical analysis. In addition, strong biochemical changes occur along *S. senegalensis* metamorphosis, as denoted by the fluctuation observed in biochemical markers basal levels, which include a significant decrease of GST activity (Araújo *et al*., 2019). Therefore, a possible GST increase immediately after the 48h 4-MBC exposure might have occurred, followed by a return to control levels at the end of metamorphosis.

Under stress conditions, LDH activity can be affected by increased consumption of energy by anaerobic metabolism due to increased energy demand (Wen *et al*., 2017a). Variation of LDH can occur in response to changes on environmental conditions and chemical stressors can also lead to LDH changes which are related with toxicity effect on animal tissues and cells. First of all, inhibition of LDH activity in fish from solvent control when comparing to negative control was observed in our study, which might be related with the fact that ethanol is a metabolic product that prevents lactate accumulation (Torres *et al*., 2012). Furthermore, considering 4-MBC effects, and similarly to the toxicity test at earlier stages of *S. senegalensis* exposed to 4-MBC (Araújo *et al*., 2018) and a test using *P. perezi* embryos (Martins *et al*., 2017), significant effect of this chemical on LDH levels were not observed. These results indicate that 4-MBC exposure during the *S. senegalensis* early metamorphosis apparently do not induce lasting effects on energy metabolism.

### 4.4. Conclusions

The present work showed relevant 4-MBC exposure responses of *S. senegalensis* during metamorphosis. Despite malformations were not observed at the end of metamorphosis, exposure of *S. senegalensis* to 4-MBC induced oxidative damage and affected metamorphosis progression, inhibited growth and induced alterations on swimming patterns even after nine days in clean medium. 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.

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# Chapter 5. Effects of extreme ultraviolet radiation on *Solea senegalensis* during early development

# Effects of extreme ultraviolet radiation on *Solea senegalensis* during early development

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**Abstract**

Ultraviolet radiation (UV) reaching the Earth surface is increasing and scarce information is available regarding its effects on early life stages of marine vertebrates. Therefore, this work aims at studying effects of exposure to extreme UV on *Solea senegalensis* during early development.

Firstly, *S. senegalensis* were exposed to six distinct doses of UV (UV index 13 or 17 during 180, 240 or 330 min) at the following days: gastrula stage (24 hours post fertilization, hpf), 1 day after hatching (dah, approx. 48 hpf) and 2 dah (approx. 72 hpf). In a second test, fish at the beginning of metamorphosis (13 dah) were exposed to different doses of UV (UV index 12 or 21 during one or two daily 360 min exposure) and then were maintained until the end of metamorphosis. Mortality, malformations, behavior, growth and development effects of UV exposure were evaluated at the end of the early larvae (3 dah, approx. 96 hpf) and metamorphosis (18 dah) tests. Biochemical markers of neurotransmission (acetylcholinesterase, AChE) and oxidative stress (catalase, CAT and glutathione S-transferase, GST) were also determined at the end of the early larval test, and metamorphosis progression was evaluated on the second stage test.

The UV exposure caused distinct damaging effects depending on the life stage. Altered fish pigmentation and decreased growth, impaired fish behavior and inhibition of AChE and GST were observed at the earlier larval phase. Whereas, the main effects observed at the metamorphosis stage were increased mortality and higher malformations percentage and decreased growth, which lasted until the end of metamorphosis. In sum, the exposure of *S. senegalensis* early stages to environmental relevant doses of UV led to adverse responses at different levels of biological organization, which might led to adverse effects at population level.

**Keywords:** behavior; biochemical markers; climate change; marine vertebrates; growth; metamorphosis; oxidative stress.

## 1. Introduction

Long term data show large increases of ultraviolet (UV) radiation in both Earth hemispheres, mostly caused by changes in ozone at stratospheric level (Stolarski *et al*., 1992; Herman, 2010; Čížková *et al*., 2018). The UV is exclusively emitted by sun and can be divided in several wavelength regions of the electromagnetic spectrum: ultraviolet C (UVC) region is comprehended between 100 and 280 nm and is the most energetic region; however, it does not reach Earth surface as it is filtered by atmosphere; ultraviolet A (UVA) is the lowest energetic region (between 315 nm and 400 nm); ultraviolet B (UVB) is located between the two previous spectral regions and is the most energetic wavelength reaching Earth surface.

The correct way to measure UV has been widely debated (Zaratti *et al*., 2014; Moshammer *et al*., 2016). Energy irradiance (measured as Watts per unit of area) and dose (time-dependent amount of effective energy that reach surfaces, in Joules per unit of area) are two of the main factors. Erythemal UV index is a non-dimensional value proportional to effective energy irradiance (Fioletov *et al*., 2010). This index was adopted worldwide in early 1990s to easily estimate effects on immediate short-term impact on human cells (skin reddening) and increase public awareness on damaging effects of UV (McKinlay and Diffey, 1987; WHO, 2002; ICNIRP, 2004; Gies *et al*., 2018). A value of 10 can be reached under clear-sky conditions at noon for mid-latitude locations during summer (Kerr and Fioletov, 2008). UV index as high or higher than 11 are reported as extreme values (WHO, 2002); however, altitude or latitude can affect the values and index above 20 have been estimated in extreme conditions (near equator, higher altitude or tropical regions) (Kerr and Fioletov, 2008; Cordero *et al*., 2014; McKenzie *et al*., 2015).

Despite the global ban on ozone depleting substances progressively since early 1980's, the increase of UV can still occur along next decades with the increase of anthropogenic activities and abnormal climate conditions leading to frequent extreme UV indices (IPCC, 2014; Häder *et al*., 2015; McKenzie *et al*., 2015).

The UVB radiation penetrates water up to the first meters of depth and is attenuated by some environmental factors, such as turbidity and aquatic vegetation (Häder *et al*., 2015). The 10% irradiation depth of UVB radiation in Eastern Atlantic and Mediterranean Sea ranges between 2.5 and 16 m (Tedetti and Sempere, 2006). However, such depth can increase, as UV can interact with other environmental factors which altogether will decrease the concentrations of dissolved organic carbon (DOC) and coloured non-living organic matter, in a way that will further increase the penetration of solar radiation (Zagarese and Williamson, 2001; Häkkinnen *et al*., 2002; Wang *et al*., 2014).

The sensitivity to UV varies widely between species and life history stages and ecological performance can also be affected (Häkkinen *et al*., 2002; Blaustein *et al*., 2003; Solomon, 2008; Fukunishi *et al*., 2012; Gao, *et al*., 2012; Häder *et al*., 2015). Planktonic animals drifting into top water column can be particularly exposed to UV. Among them, ichthyoplankton, namely fish embryos and larvae, are known to be highly sensitive to UV as they still lack photoprotective pigments (Béland *et al*., 1999; Battini *et al*., 2000; Zagarese and Williamson, 2001). Long-term excessive UV has been pointed to affect early development stages of anadromous fish causing mortality in later stages (Walters and Ward, 1998). UV can also cause brain, dermal, and ocular damage (Ahmed and Setlow,1993; Häder *et al*., 2015), loss of osmoregulatory capability in fish larvae (Dethlefsen *et al*., 2001; Sucré *et al*., 2012), which can lead to effects at individual level such as reduced growth rate in several aquatic species (Häder *et al*., 2015). Behavioral responses, such as physical avoidance and shifts in selective transport of species (mainly small-size species and larval stages of larger species) in response to UV exposure have also been reported (Speekmann *et al*., 2000; Ylönen *et al*., 2005; Fukunishi *et al*., 2012; Häder *et al*., 2015). At sub-individual level, UV exposure can initiate a series of redox reactions generating reactive oxygen species (ROS) and leading to oxidative stress in cells and tissues (Zagarese and Williamson 2001; Häder *et al*., 2015). UV can also cause DNA and protein damage leading to cell apoptosis (Applegate and Ley, 1991; Lesser *et al*., 2001; Lesser, 2006; Charron *et al*., 2000; Zagarese and Williamson 2001). Such effects have high potential of leading to mortality of fish early life stages. However, the underlying mechanism and adverse effects of UV exposure on fish larvae still needs further research (Häkkinen *et al*., 2002; Ylönen and Karlajainen, 2004; Häder *et al*., 2015).

Behavior has becoming widely used to study chemical-induced stress responses in fish (Sloman and McNeil, 2012; Sharma, 2019). It allows to understand harmful effects of environmental and chemical stressors (Andrade *et al*., 2017; Araújo *et al*., 2018) and to link behavior, with ecologically relevant endpoints measured at individual level (e.g. growth), but also with responses measured at molecular or biochemical level, such as effects on enzymes involved in neurotransmission (Richards *et al*., 2007; Vieira *et al*., 2009; Archard *et al*., 2012; Almeida *et al*., 2015). In addition, type of light and photoperiod regimes are known to affect behavior and development of fish (Volpato and Barreto, 2001; Blanco-Vives *et al*., 2012). In this sense, tools for behavioral assessment have been improved, revealing high sensitivity and the technology is becoming more robust, reliable, and arise as a complement to other relevant endpoints.

The flatfish *Solea senegalensis* is a rapid development flatfish species with great potential for use in assessment of environmental stressors (Pavlaki *et al*., 2016; Araújo *et al*., 2018; 2019; *in preparation*). It occurs in Atlantic coastal areas of Southern Europe and Northern Africa and the exposure of this species to harmful UV can mainly occur before the pelagic to benthic metamorphosis. Typical spawning, fertilization and early growing season of *S*. *senegalensis* corresponds to the spring and summer warmer months in Northern hemisphere (Imsland *et al*., 2003; Vinagre *et al*., 2013) during which highest UV reach northern hemisphere (McKenzie *et al*., 2003). During initial life stage, eggs may fluctuate close to aquatic surface due to high lipid content (Yúfera *et al*., 1999). After hatching the increase of swimming ability may help the organism to avoid excessive radiation. The onset of metamorphosis starts nearly at 10 days after hatching (dah) and ends before the third week of development under laboratory conditions and is deeply influenced by light rhythms or feeding regimes (Yúfera *et al*., 1999; Blanco-Vives *et al*., 2012; Fernández-Díaz *et al*., 2001). Flatfish have particular capacity to change skin color to mimic texture and color of the background at adult stage; however, pigmentation can be affected by environmental factors since very early days of development (Burton, 2002; Darias *et al*., 2013).

Therefore, in this work, we aim at understanding the effects of exposure to extreme UV from subcellular to individual level during early development of *S*. *senegalensis*, namely from early larval stage until 3 dah and also during the progression of metamorphosis. To achieve this UV effects will be studied on mortality, malformations, growth, behavior, metamorphosis progression and biochemical markers.

## 2. Material and methods

### 2.1. Biological material and husbandry conditions

Eggs of *S*. *senegalensis* were provided by a commercial fish farm (Sea8, Póvoa de Varzim, Portugal) and were maintained in a recirculating system with artificial saltwater (salinity 35, pH 8.2-8.4; temperature 19ºC; red Sea, Coral Pro, Saudi Arabia). The recirculating saltwater system included biological filtering medium, UV sterilizer, refrigeration and protein skimmer and was placed in a room with controlled photoperiod (16h:8h, light:dark). At the metamorphosis test (Fernández-Díaz *et al*., 2001; Klaren *et al*., 2008), fish were fed with increasing concentrations of rotifers (*Brachionus plicatilis*) from 2 to 6 dah (between 5 and 10 rotifers $mL^{-1}$), *Artemia salina* nauplii from 5 to 10 dah (between 2 and 9 nauplii $mL^{-1}$) and from 10 dah with *A*. *salina* metanauplii (between 9 up to 35 metanauplii $mL^{-1}$, Fernández-Díaz *et al*., 2001). All experimental procedures were carried

out following the European and Portuguese legislation concerning animal experimentation (authorized by the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária, Ref. 009804).

### 2.2. Experimental design

To understand the effects of different UV doses on *S. senegalensis* early development, two tests were performed which are described below.

In a first test, eggs of *S. senegalensis* were placed individually in 24 well plates (n=3, 8 fish per replicate, 2 mL of artificial saltwater per fish). Fish were exposed to UV in three consecutive days: gastrula stage, at 24 hours post fertilization (hpf, 100 % unhatched eggs), 1 and 2 dah, at two different distances from the UV lamp (50 or 60 cm, table 5.1), which correspond to two different levels of total irradiance (1.15±0.029 and 1.60±0.017 W $m^{-2}$, respectively) and two levels of the extreme UV index range (13 and 17 respectively). Within each level, fish were exposed to these UV conditions during 180, 240 or 330 min while in light phase of photoperiod (16h:8h, light:dark). One control group was kept in same conditions (without UV exposure) along the testing period. At 3 dah, fish were frozen in liquid nitrogen and kept at -80ºC until further procedures.

In the second test, *S. senegalensis* at the beginning of metamorphosis (13 dah) were randomly selected and placed individually in 24 well plates (n=4, 6 fish per replicate, 2 mL artificial saltwater per well; salinity 35; T=19ºC; no feeding). Fish were then exposed to UV at two different distances from the lamp (45 or 60 cm, table 5.2), which correspond to two levels of total energy irradiance (1.17±0.032 and 1.97±0.052 W $m^{-2}$, respectively) and two levels of the extreme UV index range (12 and 21, respectively). Fish exposure under each of these conditions was performed once (360 min at 13 dah) or twice (360 min at 13 and 14 dah) within light photoperiod. One control group, without UV exposure, was kept in similar conditions along the testing period. After 15 dah onwards fish were maintained under the same conditions (except for no UV exposure) and daily fed with *A salina*; daily water renewal) until at least 80% of the control group ended the metamorphosis.

Table 5.1. Irradiance, ultraviolet (UV) index and total energy dose of UV exposure test using *Solea senegalensis* early larvae. Fish were exposed to UV three times, namely at gastrula stage (24 hours post fertilization) and at 1 and 2 days after hatching (dah). Each fish group were exposed during 180, 240 or 330 min. Corrected irradiance intensity was calculated in accordance to McKinlay and Diffey (1987).

| **Distance to lamp** (cm) | **Peak intensity** ($mW\ m^{-2}\ nm^{-1}$) | | **Irradiance intensity** ($W\ m^{-2}$) | **Corrected intensity** ($W\ m^{-2}$) | **Erythema UV index** | **Exposure duration** (min) | **Daily dose** ($kJ\ m^{-2}$) | **Σ Dose** ($kJ\ m^{-2}$) |
|---|---|---|---|---|---|---|---|---|
| | **UVB** (306 nm) | **UVA** (358 nm) | | | | | | |
| 60 | 33.1±0.9 | 19.2±0.9 | 1.15±0.029 | 0.31±0.007 | 13 | 180 | 3.4±0.08 | 10.2 |
| | | | | | | 240 | 4.5±0.10 | 13.6 |
| | | | | | | 330 | 6.2±0.14 | 18.7 |
| 50 | 48.7±0.8 | 28.3±0.2 | 1.62±0.017 | 0.44±0.007 | 17 | 180 | 4.7±0.08 | 14.1 |
| | | | | | | 240 | 6.3±0.10 | 18.8 |
| | | | | | | 330 | 8.6±0.14 | 25.9 |

Table 5.2. Irradiance, ultraviolet (UV) index and total energy dose of UV exposure test using metamorphosing *Solea senegalensis*. Each fish group was exposed once, at 13 days after hatching (dah), or twice, at 13 and 14 dah, during 360 minutes each day. Corrected irradiance intensity was calculated in accordance to McKinlay and Diffey (1987).

| **Distance to lamp** (cm) | **Peak intensity** (mW $m^{-2}$ $nm^{-1}$) | | **Irradiance intensity** (W $m^{-2}$) | **Corrected intensity** (W $m^{-2}$) | **Erythema UV index** | **Exposure duration** (min) | **Daily dose** (kJ $m^{-2}$) | **Σ Dose** (kJ $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|
| | **UVB** (306 nm) | **UVA** (358 nm) | | | | | | |
| 60 | 35.6±0.6 | 21.4±0.4 | 1.17±0.032 | 0.31±0.012 | 12 | 360 | 7.2±0.39 | 7.2 |
| | | | | | | 2 x 360 | 7.2±0.39 | 14.4 |
| 45 | 55.7±0.1 | 33.8±1.4 | 1.97±0.052 | 0.53±0.023 | 21 | 360 | 11.1±0.49 | 11.1 |
| | | | | | | 2 x 360 | 11.1±0.49 | 22.2 |

Physico-chemical parameters were measured in artificial saltwater of all treatments during both experimental procedures and are presented in supplementary table 5.S1. Mortality and malformations were checked daily with a stereoscope in both experiments. Length was also checked with stereoscope at 3 dah for the first experiment and at 14 dah, 15 and 18 dah (end of metamorphosis) for the second experiment. Additionally, for the second experiment, metamorphosis progression was observed on a daily basis at stereoscope in accordance with previous studies (Dinis, 1986; Fernandez-Díaz *et al*., 2001). Behavior was assessed at the end of each test (3 and 18 dah, respectively). The biochemical markers AChE, CAT and GST were analysed at the end of the first experiment (3 dah).

### 2.3. Behavior analysis

Randomly selected *S*. *senegalensis* were used for the behavior analysis at the end of each experiment. Behavior of fish after exposure to UV was assessed using Zebrabox® (Viewpoint, FR) at 3 dah (n=6) and at the end of metamorphosis (n=8). Zebrabox white light was set at an intensity of 10% (0.26 mW $cm^{-2}$) during four alternating periods of 10 min (starting with a dark period for first stage and with a light period for second stage test) after an initial 5 min of acclimation period (dark at first stage and light at the second stage test). An infra-red light (not perceived by the fish and constant at 2.3 ± 0.11 mW $cm^{-2}$) is used for video recording purposes. Background threshold was set at 2 pixels for fish at the end of early larval test and 40 pixels for fish at the end of metamorphosis. Duration of swimming (seconds), swimming distance (mm) along the 40 min test were automatically recorded by ZebraLab (Viewpoint, FR) during integration periods of 1 min, which allow estimation of average speed during light or dark periods (mm/sec). Specific movement thresholds for each development stage were used for each stage test (above 0.2 or 6 mm $sec^{-1}$ for small and large movements of 3 dah fish, respectively and above 2 or 8 mm $sec^{-1}$ for fish at the end of metamorphosis for the same movements, respectively).

### 2.4. Biochemical markers

Three replicates per treatment (n=3, 5-8 organisms per sample) were initially homogenized with potassium buffer solution (pH= 7.4, 0.1 M) by sonication and centrifuged for 20 min at 10,000 g (4°C). The supernatant was used for AChE, CAT and GST activity determination. AChE activity was measured by Ellman's method adapted to microplate (Ellman *et al*., 1961, Guilhermino *et al*., 1996). To this end, acetylthiocholine is used as substrate, 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) as chromogen and the reaction was

followed by measuring the increase of absorbance at 414 nm. CAT activity was determined by measuring the anabolic decomposition of oxygen peroxide substrate at 240 nm (Clairborne, 1985). GST activity was measured following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm adapted to microplate reader (Habig and Jakoby, 1981; Frasco and Guilhermino, 2002). Enzymatic activity were expressed in Units (U) per mg of protein; U is a nmol of substrate hydrolyzed per min, 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 one µmol of substrate hydrolyzed per min, using a molar extinction coefficient of 40 $M^{-1}$ $cm^{-1}$ for CAT. Readings were performed in 96 well plates (3-4 replicates per each sample) using a Labsystem Multiskan EX microplate reader for all enzymatic and protein determinations.

Protein was determined in all samples according to the Bradford method (Bradford, 1976) and adapted from BioRad's Bradford micro-assay setup adapted to microplate using bovine γ-globuline as a standard and absorbance at a wavelength of 595 nm.

Chemicals for biochemical procedures were purchased from Sigma-Aldrich Co. LLC (St Louis, USA). Bradford reagent was purchased from Bio-Rad (Germany). All chemicals used were of analytical or HPLC grade quality.

### 2.5. UV lamp and energy measurement

One UV lamp (Spectroline XX15A series 2x15-Watt lamps, Spectronics Corporation, NY, USA with peak emission at 313 and 365 nm corresponding to UVB and UVA, respectively) was used in all experiments performed, with clear cellulose acetate sheets (0.003 mm, Grafix plastics, USA) for filtering UVC radiation. These sheets were previously exposed to the UV lamp during 12 h for radiation stabilization. Values of energy were measured (each 330 and 360 min for the first and second state test, respectively) with a double monochromator (Bentham DMC150-USB, Bentham Instruments Ltd, UK) with a high voltage supply (Bentham 215) and using the software Benwin+ (Bentham Instruments Ltd). The irradiance was corrected and final UV dose is expressed using the Commission Internationale de l'Éclairage (CIE) reference action spectrum for the erythema in human skin (McKinlay and Diffey, 1987). The UV index was estimated as the product of erythemally weighted total irradiance in W $m^{-2}$ multiplied by 40 (Fioletov *et al*., 2010).

### 2.6. Statistical analysis

One-way ANOVA was used to compare differences between different fish groups (control and fish exposed to UV) on mortality and length for both stages and biochemical markers for the earlier larval stage test. Non-parametric ANOVA on Ranks was performed

on malformations analysis since normality or equal variance were not achieved. Dunnett's or Dunn's post-hoc tests were performed in each case, respectively, to check which sub-group of UV were significantly different from control group.

For the second stage test, effects of UV exposure on metamorphosis progression were studied using Chi-Square test.

At the end of both experiments, significant differences between control and UV exposed fish on the 40 min behavior test (total swimming distance and time) were studied using One-Way ANOVA or non-parametric ANOVA on Ranks followed by Dunnett's or Dunn's tests. Two-way Repeated Measures ANOVA for detection of significant factors or significant interactions between factors (UV dose and light/dark periods) on swimming speed. Six UV doses plus control were used in the first stage test and four UV doses plus control were used in the second stage test and four consecutive periods of light and dark were tested in the behavior analysis (four periods: two alternate light and dark periods). The interaction between factors (UV dose and light/dark period) was not significant for the both life stages and therefore, multiple comparison Tukey test was used to analyse each significant factor individually.

Sigmaplot v.12.5 ® (Systat Software Inc.) was used for all statistical procedures. All results are expressed as mean±standard error.

## 3. Results

### 3.1. UV effects on early larvae

#### 3.1.1. Mortality

The mortality of *S. senegalensis* at the end of the early larval test (3 dah) was 4.2±4.17% in the control group. In fish exposed to UV, the mortality ranged between 12.5±7.22% (for 18.7 kJ $m^{-2}$, UV index 13 during 330 min) and 21.8±15.0% (14.1 kJ $m^{-2}$, UV index 17 during 180 min); however, no significant differences were obtained when comparing to control group ($p>0.05$).

#### 3.1.2. Malformations and pigmentation alterations

Spine curvature was the most common malformation observed in flatfish larvae exposed to UV. Namely, spine curvature was observed at 3 dah on all groups of fish, with a maximum percentage of 24.6±9.15% in groups exposed to the highest UV dose (25.9 $Jm^{-}$

$^{2}$, UV index 17 during 330 min), but no significant differences were registered when comparing to control group (4.2±4.17%; p>0.05, fig. 5.1).

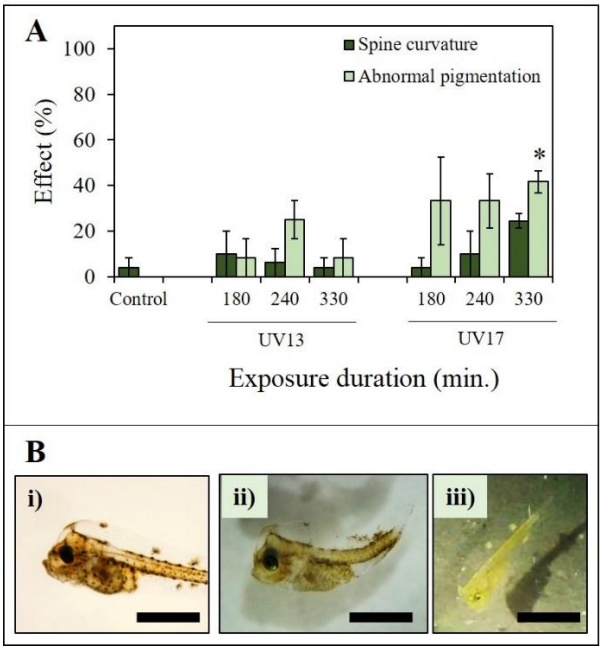


Figure 5.1. Spine curvature and lack of pigmentation of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UV). Fish were exposed at three periods of time (24 hours post fertilization, 1 and 2 dah). The six doses were obtained as combination of two UV index (13 and 17, which correspond to two irradiance levels: 1.15 and 1.62 W m$^{-2}$, respectively) and three durations of exposure (180, 240 or 330 minutes), which correspond to the following UV doses: 10.2, 13.6 and 18.7 kJ m$^{-2}$ for the lower UV index, respectively and 14.1, 18.8 and 25.9 kJ m$^{-2}$ for the higher UV index, respectively. i) control fish; ii) spine curvature (14.1 kJ m$^{-2}$); iii) lack of pigmentation (25.9 kJ m$^{-2}$). Black bar represents 1 mm.

In addition, fish exposed to UV showed abnormal pigmentation characterized by a clearer overall pigmentation, with fish presenting less dark pigments (fig. 5.1B iii). In the highest UV dose (41.7±4.81%, 25.9 kJ m$^{-2}$, UV index 17 during 330 min), a significantly higher percentage of fish presented abnormal pigmentation when comparing to control group (0%, p>0.05, fig. 5.1Bi).

### 3.1.3. Length

The total length of fish exposed to UV during 180 min (10.2 and 14.1 kJ m$^{-2}$) was not significantly different from control group (2.91±0.05 cm, p>0.05). However, fish exposed to UV during longer periods (240 and 330 min) were significantly smaller (p<0.05, fig. 5.2), compared to control fish group, with fish exposed to UV index 17 during 330 min or 25.5 kJ m$^{-2}$ presenting the minimum value registered for length (2.6±0.08 mm).

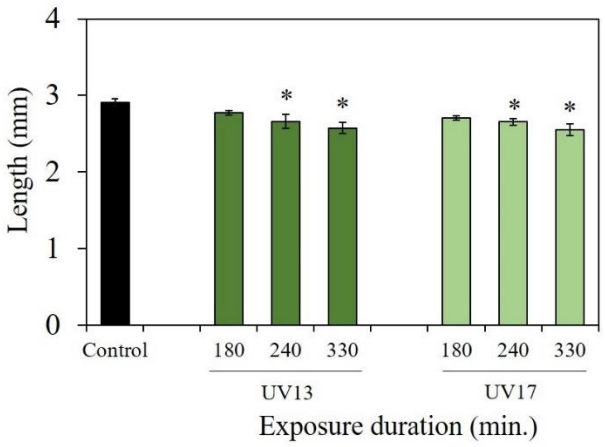


Figure 5.2. Length of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UV). Fish were exposed at three periods of time (24 hours post fertilization, 1 and 2 dah). The six doses were obtained as combination of two UV index (13 and 17, which correspond to two irradiance levels: 1.15 and 1.62 W $m^{-2}$, respectively) and three durations of exposure (180, 240 or 330 minutes), which correspond to the following UV doses: 10.2, 13.6 and 18.7 kJ $m^{-2}$ for the lower UV index, respectively and 14.1, 18.8 and 25.9 kJ $m^{-2}$ for the higher UV index, respectively. * represent the existence of significant differences between control and organisms exposed to UV ($p<0.05$).

### 3.1.4. Behavior

When analysing the entire period of behavior testing (40 min), the percentage of time spent swimming was not significantly different between UV exposed fish and control group (93.7±1.53%), with values in UV exposed fish ranging between 89.2±3.00% (18.8 kJ $m^{-2}$, UV index 17 for 240 min) and 94.7±1.14% (14.1 kJ $m^{-2}$, UV index 17 for 180 min, $p>0.05$, data not shown). However, the total swimming distance was significantly lower in fish exposed to 13.6 and 18.8 kJ $m^{-2}$ (17.2±4.71 and 16.6±8.47 m, exposure during 240 min at UV index 13 and 17, respectively; $p<0.05$, data not shown) than in fish from control group (36.9±1.36 m).

When analysing the effect of exposure to alternate periods of light or dark during the behavior test and the effect of previous UV exposure (Two-Way Repeated Measures ANOVA, fig. 5.3), no interaction between the two factors was obtained on swimming speed ($p>0.05$). Both factors, UV exposure and alternate light/dark periods, affected swimming speed ($p<0.05$). Lower swimming speeds were obtained in fish exposed to 13.6 and 18.8 kJ $m^{-2}$ doses when comparing to control fish (exposure during 240 min to UV index 13 and 17, respectively, $p<0.05$). Furthermore, fish swimming speed was higher in light periods of behavior testing in relation to dark periods ($p<0.05$).

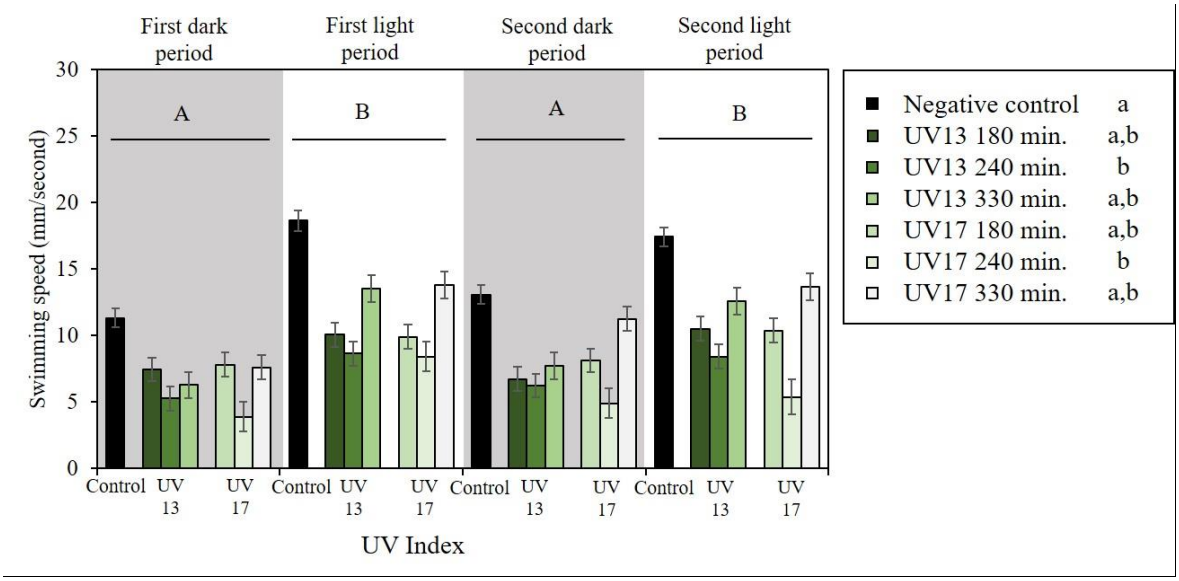


Figure 5.3. Swimming speed of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UV). Fish were exposed at three periods of time (24 hours post fertilization, 1 and 2 dah). The six doses were obtained as combination of two UV index (13 and 17, which correspond to two irradiance levels: 1.15 and 1.62 W $m^{-2}$, respectively) and three durations of exposure (180, 240 or 330 minutes), which correspond to the following UV doses: 10.2, 13.6 and 18.7 kJ $m^{-2}$ for the lower UV index, respectively and 14.1, 18.8 and 25.9 kJ $m^{-2}$ for the higher UV index, respectively. Swimming speed was estimated based on distance recorded in two alternate dark and light periods of 10 minutes each. Two-way Repeated Measures ANOVA was performed considering six UV doses and one control (seven levels) and four light and dark periods (four levels). Upper case letter represent differences between dark/light periods and lower case letters in the legend represent the differences between UV treatments ($p<0.05$).

### 3.1.5. Biochemical markers

Response of biochemical markers of *Solea senegalensis* early larvae to UV exposure is presented in figure 5.4. In general, AChE activity of fish exposed to UV were similar to those of control group, except in fish exposed to 14.1 kJ $m^{-2}$ (lowest duration of exposure, 180 min, to UV index 17) that presented a significantly decrease of about 19,3% on AChE activity ($p<0.05$). Considering CAT activity, this enzyme was not affected by UV exposure in none of tested conditions ($p>0.05$).

When comparing GST activity between fish in different treatment groups to control group, this enzyme was significantly reduced on fish groups subjected to the shortest UV exposure time to both UV indexes (180 min, $p<0.05$); the highest reduction on GST activity, of about 31.7%, was observed on fish exposed to UV index 17 ($p<0.05$).

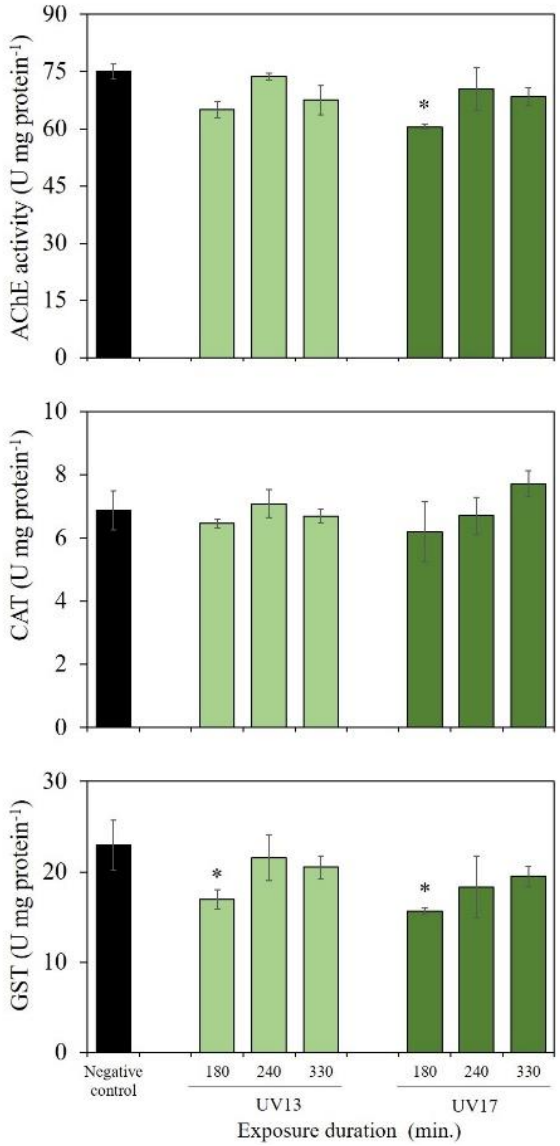


Fig. 5.4. Biochemical markers of *Solea senegalensis* early larvae at 96 hours post fertilization (hpf) after exposure to ultraviolet radiation (UV). Fish were exposed at three periods of time (24, 48 and 72 hpf). The six doses were obtained as combination of two UV index (13 and 17, which correspond to two irradiance levels: 1.15 and 1.62 W $m^{-2}$, respectively) and three different durations of exposure (180, 240 or 330 minutes), which correspond to the following UV doses: 10.2, 13.6 and 18.7 kJ $m^{-2}$ for the lower UV index, respectively and 14.1, 18.8 and 25.9 kJ $m^{-2}$ for the higher UV index, respectively. A: Acetylcholinesterase (AChE), B: Catalase (CAT) and C: Glutathione S-transferase (GST). * represent the existence of significant differences with control ($p<0.05$).

### 3.2. UV effects on metamorphosing sole

Fish used at the second stage test started the metamorphosis at 13 dah with a length of 5.4±0.03 mm (n=80) and the tested ended when control group completed the metamorphosis in 90% of organisms at 18 dah.

### 3.2.1. Mortality

No mortality of *S. senegalensis* was registered for control group until the end of the second stage test. While no significant differences on fish mortality rate was observed just after UV exposure, at 14 and 15 dah ($p>0,05$), fish exposed to UV index 21 (during 360 and 2x360 min, corresponding to 11.1 and 22.2 kJ $m^{-2}$ doses, respectively) had significantly higher mortality when compared to control at the end of metamorphosis (18 dah, $p<0.05$, fig. 5.5), registering 19.6±8.83% and 22.7±7.99% mortality, respectively.

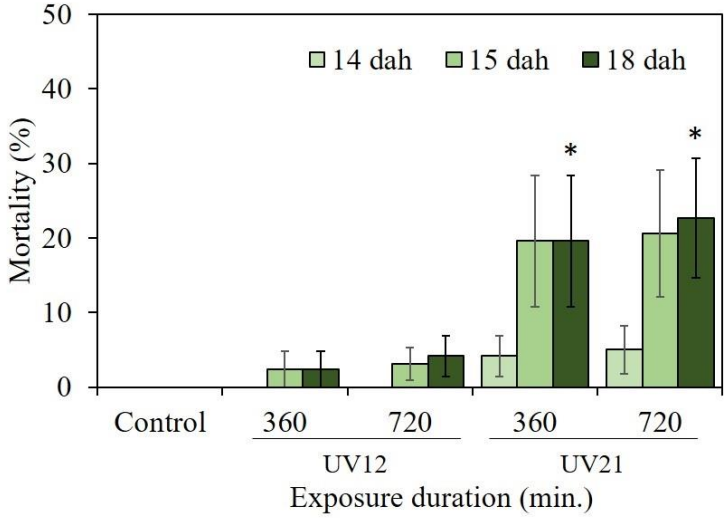


Fig. 5.5. Mortality of *Solea senegalensis* during metamorphosis after exposure to ultraviolet radiation (UV). Total doses were obtained as combination of two UV index (12 and 21, or two irradiance levels: 1.17 and 1.97 W $m^{-2}$, respectively) and two periods of exposure (360 minutes at 13 days after hatching, dah or 2x360 minutes at 13 and 14 dah), which correspond to the following UV doses: 7.2 and 14.4 kJ $m^{-2}$ for the lowest UV index and 11.1 and 22.2 kJ $m^{-2}$ for the highest UV index. Metamorphosis was complete at 18 dah. * represent the existence of significant differences between control and organisms exposed to UV within each age ($p<0.05$).

### 3.2.2. Malformations

The total percentage of organisms with malformations occurring during flatfish metamorphosis (namely, damaged fin, abnormal migration of the eye and malformations in cephalic structure) was significantly higher in all UV exposed groups than in control group after the first UV exposure (14 dah, fig. 5.6A). At 15 dah, all UV exposed groups except those exposed to UV index 12 during 360 min (7.2 kJ $m^{-2}$) had significantly more organisms with malformations than control ($p<0.05$). However, after UV exposure, at the end of metamorphosis, only groups previously exposed to UV index 21 had significantly more fish with malformations than control ($p<0.05$), with 64.7±0.11% and 45±10.7% of organisms with

malformations in fish groups exposed once (11.1 kJ $m^{-2}$) or twice (22.2 kJ $m^{-2}$) to UV, respectively.

The most prevalent malformations was fin damage, which was significantly higher in all UV exposed fish groups than in control groups at 14 and 15 dah ($p<0.05$, fig. 5.6B), affecting more than 90% of fish after the second exposure to both UV index intensities. However, after the end of UV exposure, this percentage was below 5% for all UV exposed groups at 18 dah, with no significant differences observed between UV exposed groups and control fish (0%, $p>0.05$).

Malformations related with metamorphosis progression, namely incorrect migration of the eye, incorrect cephalic development, were also detected along the test and increased with age of fish (figures 5.6C and 5.6D). At 18 dah, only fish exposed earlier to both doses with highest UV index (21, 11.1 and 22.2 kJ $m^{-2}$) showed significantly higher malformations related with metamorphosis progression when compared to control (reaching 64.8±7.94% and 42.5±7.94% for fish exposed once or twice during 360 min, respectively, $p<0.05$).

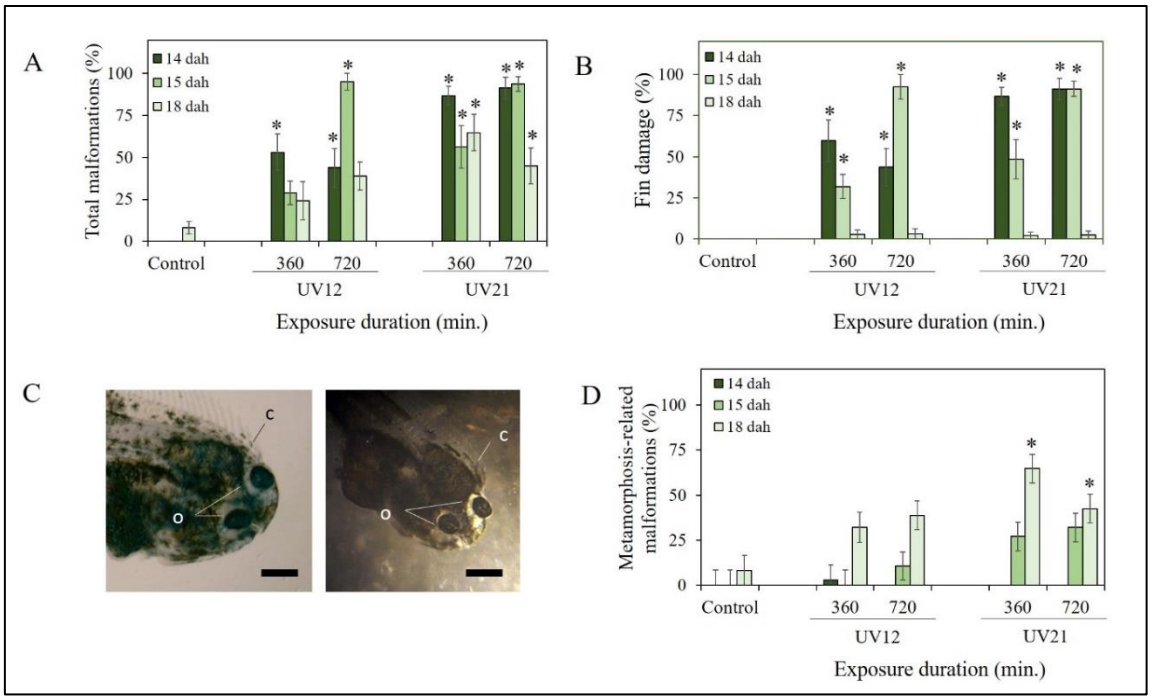


Fig. 5.6. Malformations (A), fin damage (B) and metamorphosis-related malformations (C, D) in *Solea senegalensis* after exposure to ultraviolet radiation (UV). Total doses were obtained by combination of two UV index (12 and 21, or two irradiance levels: 1.17 and 1.97 W $m^{-2}$, respectively) and two periods of exposure (360 minutes at 13 days after hatching, dah or 2x360 minutes at 13 and 14 dah), which correspond to the following UV doses: 7.2 and 14.4 kJ $m^{-2}$ for the lowest UV index and 11.1 and 22.2 kJ $m^{-2}$ for the highest UV index. Metamorphosis was complete at 18 dah. * represent the existence of significant differences between control and organisms exposed to UV within each age ($p<0.05$). C – The orbital arch (o) is well developed on the i) control and ii) UV exposed organism (22.2 kJ $m^{-2}$) at 18 dah; however, the anterior cranial shape (c) is not round on the UV exposed organism. Black bar represents 1 mm.

### 3.2.3. Length

After UV exposure, at 14 and 15 dah, no differences were observed on fish length when comparing UV exposed fish with control group ($p>0.05$; fig. 5.7). However, at the end of the metamorphosis, a decrease in length of 8.3% and 6.9% on the groups of fish exposed to UV index 21, once during 360 min or twice, respectively (doses of 11.1 and 22.2 kJ $m^{-2}$ $p<0.05$), was recorded when comparing to the length of fish in control group.

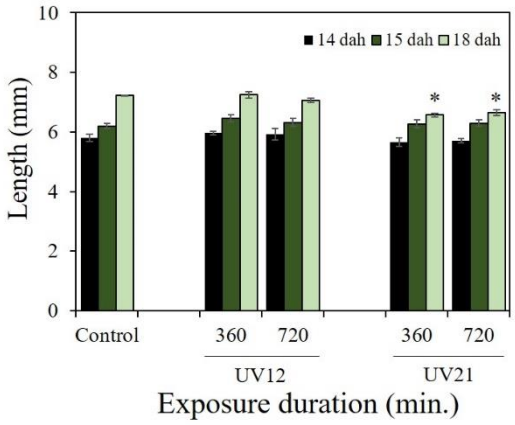


Fig. 5.7. Length of *Solea senegalensis* at the end of metamorphosis (18 days after hatching, dah) after exposure to ultraviolet radiation (UV). Total doses were obtained as combination of two UV index (12 and 21, or two irradiance levels: 1.17 and 1.97 W $m^{-2}$, respectively) and two periods of exposure (360 minutes at 13 days after hatching, dah or 2x360 minutes at 13 and 14 dah), which correspond to the following UV doses: 7.2 and 14.4 kJ $m^{-2}$ for the lowest UV index and 11.1 and 22.2 kJ $m^{-2}$ for the highest UV index. Metamorphosis was complete at 18 dah. * represent the existence of significant differences between control and organisms exposed to UV ($p<0.05$).

### 3.2.4. Metamorphosis progression

Significant differences between control and UV exposed fish on metamorphosis progression did not occur neither at 14, 15 nor at 18 dah (Chi-square test $p>0.05$; data not shown). In control group most fish were at stages B (58.6%), D (65.5%) and G (89.7%) at 14, 15 and 18 dah, respectively. Within UV exposed groups, at 18 dah, the frequency of organisms with complete metamorphosis ranged between 77.9±7.10% for UV index 21 during 720 min (22.2 kJ $m^{-2}$) and 89.5±5.22% for UV index 12 during 360 min (7.2 kJ $m^{-2}$).

### 3.2.5. Behavior

There was no effect of UV exposure on the percentage of time spent swimming and total swimming distance during the 40 min behavior test performed at the end of metamorphosis ($p>0.05$; data not shown). The percentage of time spent swimming was 57.7±4.74% for control group and ranged between 49.4±5.30% for fish exposed to UV index 12 during. 360 min (7.2 kJ $m^{-2}$) and 58.5±5.69% for UV index 21 during 720 min (22.2 kJ $m^{-}$

$^{2}$). The total swimming distance of control group was 3.1±0.16 m for control group and ranged between 3.2±0.75 for UV index 12 during 360 min (7.2 kJ $m^{-2}$) and 4.0±0.31 m for fish exposed to UV index 21 during 360 min (11.1 kJ $m^{-2}$).

The UV exposure and alternate light/dark periods, revealed no interaction on swimming speed of flatfish (Two-Way Repeated Measures ANOVA, $p>0.05$, supplementary fig. 5.S1). While UV exposure did not alter significantly the swimming speed ($p>0.05$), light/dark periods had a significant effect in this parameter, with fish swimming faster during dark periods ($p<0.05$).

## 4. Discussion

In this study, the effects of UV within extreme range were studied in two stages of early development of *S. senegalensis*: between egg stage and 3 dah and during the metamorphosis.

Effects were observed at subcellular level, namely on biochemical markers (first stage test) and individual level, namely on mortality (second stage test), malformations and growth (both tests) and behavior (first stage test) (supplementary tables 5.S2 and 5.S3).

### 4.1. Early larval test

In the first part of our study, the exposure to the extreme UV conditions tested have not affected sole survival until 3 dah, which was not observed in early larval stages of other fish species exposed to excessive UV (Béland *et al*., 1999; Battini *et al*., 2000; Zagarese and Williamson, 2001; Jokinen *et al*., 2008; Häder *et al*., 2015). The response of our study indicates possibly a lower sensibility to UV by *S. senegalensis*. Similarly, a previous study with eggs and early larvae of the flatfish plaice (Steeger *et al*., 2001) showed no clear dose-response of UV-B dose on mortality. A lower sensibility to high UV levels might be associated with a possible increased capacity of production of external mucous and goblet cell numbers by these organisms during early days which make is reported to fish more tolerant in contact to stressors (Blazer *et al*., 1997; Dahms and Lee, 2010; Elliott *et al*., 2011). Nevertheless, in our study, several sub-lethal effects occurred, namely on development (e.g. malformations), growth, behavior and biochemical markers, which are discussed below.

Spine curvature and pigmentation alterations were observed on UV exposed *S. senegalensis* early larvae. Despite malformations of *S. selengalensis* are relatively uncommon during early development days in typical rearing conditions, spine curvature has been reported previously after exposure to chemical stressors (Pavlaki *et al*., 2016; Araújo

*et al*., 2018). UVA also caused this type of malformation on medaka larval stages (Sayed and Mitani, 2016) and in another study, both UVA and UVB exposures led to spinal deformities in zebrafish embryos (Dong *et al*., 2007).

While the epidermis of some fish tolerate excessive UV exposure, pigmentation alterations and increased melanin levels in response to UV have also been previously reported (Blazer *et al*., 1997; Zagarese and Williamson, 2001; Häder *et al*., 2015). Melanin at external dermal layers and interior organs has also immunological and antioxidant functions. The melanin altered levels after UV exposure can be directly related with activation of natural repairing mechanisms through increased immune system and molecular responses (Jobling, 2010; Franco-Belussi *et al*., 2016). In the case of our study, the opposite seems to occur after excessive UV exposure of *S*. *senegalensis*, through an apparent decrease of pigmentation, possibly as consequence of DNA damage or inactivation of photo-repairing molecular mechanisms (Blazer *et al*., 1997) and therefore, further histological and molecular studies should focus in such effect on *S*. *senegalensis* early life stages.

In our study, UV exposure inhibited growth of *S*. *senegalensis* at 3 dah, which is in accordance with previous studies in other aquatic species, including fish (e.g. Häder *et al*., 2015) and amphibians (Misra *et al*., 2002). Previous studies have associated DNA damage induced by UV exposure and protein decrease with effects on growth (Zagarese and Williamson, 2001). In addition, immune system response in UV exposed fish larvae has also been linked with poor growth (Sharma *et al*., 2010). At higher levels of biological organization, reduced growth of UV exposed fish have also been linked to decreased ecological performance (Fukunishi *et al*., 2012).

It is expected that the behavior activity pattern of *S*. *senegalensis* might change between light and dark periods according to its natural diurnal or nocturnal activity (Blanco-Vives *et al*., 2012; Araújo *et al*., 2018). In the present study, such pattern was observed in the alternating light/dark periods for both sole life stages, with earlier larvae (3 dah) presenting faster swimming speed in light periods, while metamorphosed sole larvae presented higher swimming speed in dark periods. In addition, 3 dah larvae exposed to UV were less active when comparing to control, namely by presenting an overall decrease on swimming distance and swimming speed. This impairment on *S*. *senegalensis* behavior might negatively affect growth, as fish with less ability to swim might have lower feeding success. These combined effects can also be associated with individual decrease in ecological performance and fitness (e.g. reproduction, escaping from predators), which may have further implications at population level (Häder *et al*., 2015). Furthermore, our results

suggest that behavior is a very sensitive endpoint for studying effects of UV on early larvae of *S. senegalensis* as effects on behavior were observed at the two lowest doses tested, which can be assumed as environmental relevant.

Considering UV effects on biochemical markers of sole at 3 dah, while effects on CAT activity were not detected, AChE and GST were inhibited in fish groups treated with lowest UV exposure duration. Exposure to UV has been shown to inhibit AChE in several species of copepods (Souza *et al*., 2010). Dysfunctions in AChE are pointed to be ecologically relevant as continuous and excessive stimulation of the nerve/muscle fibers, can affect organism behavior and ultimately lead to death (Scott and Sloman, 2004; Souza *et al*., 2010). The reduction on AChE that occurred in *S. senegalensis* larvae after the lowest UV exposure duration treatment suggest an impairment on cholinergic activity that might be associated with the observed lower swimming activity in fish of the same UV treatments. In addition, cholinergic mechanisms of eye might also have been impaired by UV exposure, which in turn can also be associated with the behavior changes observed in *S. senegalensis*, as effects on fish optic nerve have previously been associated with behavioral changes (Matsukawa *et al*., 2004).

*Solea senegalensis* larvae may have an undeveloped antioxidant defense system until pre-metamorphic stage (Pimentel *et al*., 2015). This might explain the non-activation of antioxidant enzymes studied, since no effect was observed on CAT activity with UV exposure and GST was inhibited/non-altered. Different studies report distinct effects of GST in organisms following UV exposure. For instance, while GST was increased in *Daphnia commutata* (Wolinski *et al*., 2016), GST was inhibited in the anellidae tubifex (Misra *et al*., 2002). Several biochemical processes might be involved in such different responses, namely UV induction of oxidative stress or inactivation of antioxidant defense system. In the present study, the lack of response of the antioxidant system, verified by the non-induction of both CAT and GST, might have led to accumulation of ROS in fish larvae. The increase of ROS occurs as consequence of UV irradiation exposure on mammals skin (Afaq and Mukhtar, 2001) and previous studies have associated oxidative damage with epidermis effects of UV on fish (Gevertz and Oris, 2014). The effects reported as lack and/or reduced pigmentation in *S. senegalensis* in response to UV, might be a consequence of the lack of response of the enzymes related with antioxidant system. Further evaluation of oxidative damage status (e.g. lipid or DNA damage) after UV exposure in sole larvae would clarify if this lack of response leads to ROS accumulation. In addition to the occurrence of oxidative damage, effects of UV on epidermis should be further studied at molecular and histological level to better understand the extent of UV damage to *S. senegalensis* early larvae as

already previously referred. Furthermore, the ability of UV to inhibit GST during early stages can be critical to the normal development of *S. senegalensis* while dealing with other stressors present in environment.

Comparable UV doses can be achieved by different combinations of irradiance energy and exposure duration. In the case of the early larval test, several parameters did not respond constantly with increasing UV doses, including on growth, behavior and biochemical markers (supplementary tables 5.S2 and S5.S3).

It should be also mentioned that the aimed UV irradiation intensities (measured as energy per unit of space) or doses (measured as energy per unit of time) to be used on experiments can fluctuate due to a number of reasons (e.g. variability of lamp energy along the test). Nevertheless, despite our results indicate that UV exposure lead to effects on *S. senegalensis* early larvae, they revealed not to be intensity or dose dependent. However, to clarify the occurrence of eventual mechanisms of protection and/or damage, further works are needed to better understand the impacts of UV exposure, namely at cellular level.

### 4.2. Metamorphosis test

While solar radiation has an important role on normal fish early development, excessive UV might disrupt natural development and induced a cascade of events with lasting effects. For instance, a previous study with salmonids exposed to UV during critical life events exhibited increased mortality at later life stages (Walters and Ward, 1998). Similar effects were also observed in our study. Namely, the 48h-exposure to extreme UV levels during the *S. senegalensis* metamorphosis caused mortality, leading up to 23 % of mortality at the end of the metamorphosis (4 days after the exposure) at the highest intensities and doses tested. The increased mortality seem to be the consequence of several effects that were also detected on metamorphosing sole in response to UV exposure (increased malformations and decreased growth), while some of the endpoints studied apparently were not affected (behavior and metamorphosis progression), which are discussed below.

The occurrence of abnormal metamorphosis in flatfish has been previously described and is usually characterized by the occurrence of pigment abnormalities, bone deformities and lack or abnormal migration of the eye (Power *et al*., 2008). Previous studies with amphibians have shown that UV can induce abnormalities during metamorphosis and also later life-history effects even if no immediate damage is observed (Blaustein *et al*., 1997; Ceccato *et al*., 2016). In our study, damage of fin was increased during exposure to UV. However, fish recovered from this induced malformation by the end of metamorphosis,

suggesting the existence of specific recovery mechanisms for dealing with occasional excessive UV. In the other hand, UV exposure elicited metamorphosis related malformations and also growth inhibition of fish that were notorious by the end of metamorphosis (18 dah), even with no UV exposure in the preceding 4 days. The occurrence of lasting malformations is of concern as they can be associated with impacts at later development stages. Similarly to the effects observed during early larval stage, lasting effects on growth and malformations can affect fish individual fitness with consequences on fish populations.

The induction of malformations and altered metamorphosis progression of *S. senegalensis* can be affected by the exposure to stressors (Araújo *et al*., 2019*, in preparation*). In the present study, excessive UV did induce malformations but have not affected the metamorphosis progression of this species even at higher UV doses. A similar experimental design with *S. senegalensis* exposed to a chemical stressor, triclosan, revealed altered metamorphosis progression. Thus, such distinct results suggest that mechanisms ruling metamorphosis progression and the alterations at molecular level that lead to the observation of malformations might be distinct and stressor-dependent.

The UV exposure affected behavioral pattern at earlier sole life stage, however such effects were not observed at the second stage test. Metamorphosing fish exhibited higher swimming speeds at dark periods as expected, since with metamorphosis, larvae switch from diurnal to nocturnal behavior (Blanco-Vives *et al*., 2012). Furthermore, our study suggests that UV effects at behavioral level depend on development stage and a remark should also be given to the fact that early pelagic *S. senegalensis* larvae swim much longer distance than benthic post-metamorphosed fish during behavior tests. During light period at nearly 3 dah, fish are starting to actively search for food; while the behavior tests with benthic post-metamorphosed *S. senegalensis* were performed during their typical rest periods which are the day light hours (Blanco-Vives *et al*., 2012). Therefore, periods longer than 40 min of behavior analysis and/or a longer acclimation period to dark conditions at the beginning of the behavior test might worth consideration with post-metamorphosic *S. senegalensis*.

As occurred for the early stage test, at the metamorphosis test similar UV doses lead to different responses, with effects apparently being more dependent on the UV index. For instance, fish exposed once to the highest UV index presented effects on mortality, malformations and growth which were not observed on fish exposed twice to a lower UV index, but corresponding to a similar UV dose (see supplementary table 5.S3).

### 4.3. Conclusions

Our results indicate that UV induced adverse effects on both early life stages of *S. senegalensis*. However, the responses obtained were dependent on sole life stage. Impairment on behavior and decreased growth by UV exposure were the main observed effects in the first early life stage (until 3 dah), while survival was not affected. In the other hand, the UV exposure during metamorphosis have not affected fish behavior nor metamorphosis progression but led to adverse effects that lasted until complete metamorphosis, namely increased mortality, malformations percentage and decreased growth. This suggests irreversible effects of UV exposure during this critical sole life stage period. Moreover, the exposure of *S. senegalensis* early stages to environmental relevant doses of UV led to adverse responses at different levels of biological organization, which might led to adverse effects at population level. The ability of this abiotic stressor to affect early stages of *S. senegalensis* can be critical while dealing with environmental contamination and other types of stressors.

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**Effects of extreme ultraviolet radiation on *Solea senegalensis* during early development**

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**Supplementary Data**

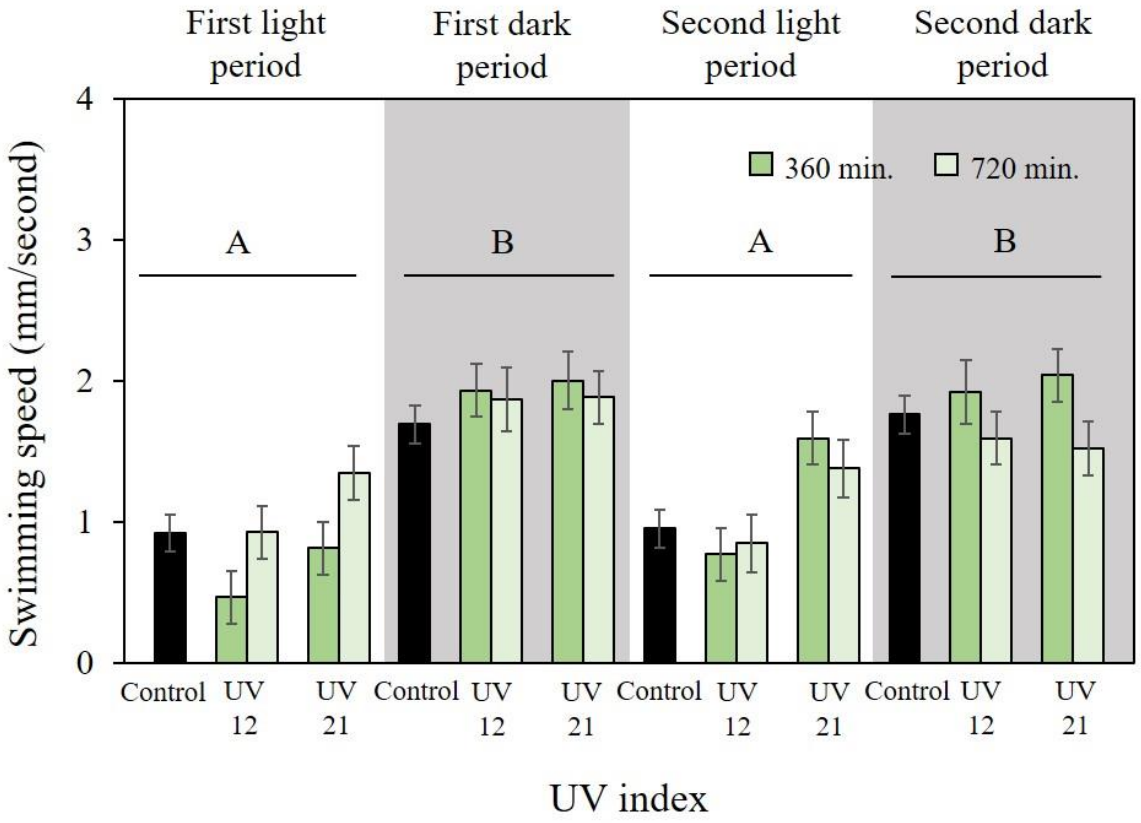


Fig. 5.S1. Swimming speed of *Solea senegalensis* at the end of metamorphosis (18 days after hatching, dah) after exposure to ultraviolet radiation (UV). Total doses were obtained as combination of two UV index (12 and 21, or two irradiance levels: 1.17 and 1.97 W $m^{-2}$, respectively) and two periods of exposure (360 minutes at 13 days after hatching, dah or 2x360 minutes at 13 and 14 dah), which correspond to the following UV doses: 7.2 and 14.4 kJ $m^{-2}$ for the lowest UV index and 11.1 and 22.2 kJ $m^{-2}$ for the highest UV index. Metamorphosis was complete at 18 dah. Swimming speed was estimated based on distance recorded in two alternate dark and light periods of 10 minutes each. Two-way Repeated Measures ANOVA was performed considering two UV doses and one control (five levels) and four light and dark periods (four levels). Upper case letter represent differences between dark/light periods ($p<0.05$).

Table 5.S1. Physico-chemical parameters of saline saltwater solutions of the ultraviolet radiation (UV) tests.

| Life Stage Test | Samples | pH | Salinity | Temperature (ºC) | $O_2$ (mg $L^{-1}$) |
|---|---|---|---|---|---|
| Early larvae | 0 hours | 8.15 | 34.7 | 18.8 | NA |
| | CTR (3 dah) | 7.72 | 34.8 | 19.3 | NA |
| | UV (3 dah) | 7.75 | 35.1 | 18.9 | NA |
| Metamorphosis | 0 hours | 7.15 | 35.3 | 19 | 6.9 |
| | CTR (15 dah) | 7.01 | 35.5 | 19.3 | 6.8 |
| | UV (15 dah) | 7.20 | 35.4 | 19.4 | 6.0 |

CTR – control. dah – days after hatching. NA – not available.

Table 5.S2. Summary of ultraviolet radiation (UV) effects on early larvae of *Solea senegalensis* in comparison to control group.

| UV dose (kJ $m^{-2}$) | UV index | Exposure duration (min) | Mortality | Malformations | Growth | Behavior | Biochemical markers |
|---|---|---|---|---|---|---|---|
| 10.2 | 13 | 180 | n.s. | n.s. | n.s | ↓ SS | ↓ GST |
| 13.6 | 13 | 240 | n.s. | n.s. | ↓ | ↓ SD; ↓ SS | n.s |
| 14.1 | 17 | 180 | n.s. | n.s. | n.s | ↓ SS | ↓ AChE; ↓ GST |
| 18.7 | 13 | 330 | n.s. | n.s. | ↓ | n.s | n.s |
| 18.8 | 17 | 240 | n.s. | n.s. | ↓ | ↓ SD; ↓ SS | n.s |
| 25.9 | 17 | 330 | n.s. | ↑ | ↓ | n.s | n.s |

n.s. - not significant; ↓ - significantly lower; ↑ - significantly higher. Level of significance: 0.05. SD - swimming distance; SS - Swimming speed; GST - glutathione S-transferase, AChE - acetylcholinesterase.

Table 5.S3. Summary of ultraviolet radiation (UV) effects on metamorphosing *Solea senegalensis* in comparison to control group.

| **UV dose** (kJ $m^{-2}$) | **UV index** | **Exposure duration** (min) | **Mortality** | **Malformations** | **Growth** | **Metamorphosis progression** | **Behavior** |
|---|---|---|---|---|---|---|---|
| 7.2 | 12 | 360 | n.s. | ↑ 14 dah | n.s. | n.s. | n.s. |
| 11.1 | 21 | 360 | ↑ 18 dah | ↑ 14,15,18 dah | ↓ | n.s. | n.s. |
| 14.4 | 12 | 720 | n.s. | ↑ 14, 15 dah | n.s. | n.s. | n.s. |
| 22.2 | 21 | 720 | ↑ 18 dah | ↑ 14,15,18 dah | ↓ | n.s. | n.s. |

dah - days after hatching; n.s. - not significant; ↓ - significantly lower; ↑ - significantly higher. Level of significance: 0.05.

# Chapter 6. Effects of single and combined exposure to ultraviolet radiation and triclosan in metamorphosing *Solea senegalensis*

# Effects of single and combined exposure to ultraviolet radiation and triclosan in metamorphosing *Solea senegalensis*

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**Abstract**

The ultraviolet radiation (UV) and the bactericide triclosan (TCS) are known to affect early development of the flatfish *Solea senegalensis*. However, the mechanisms of such effects are still not understood, particularly during the thyroid-regulated metamorphosis, a critical life stage of flatfish. In addition, no information exists on the effects of combined exposure to these stressors. Therefore, in this work, we aim to study the single and combined effects of the exposure to UV and TCS at the onset of metamorphosis on growth, behavior, malformations, metamorphosis and on the expression of thyroid-related genes of *S. senegalensis*.

Exposure of *S. senegalensis* to sub-lethal UV dose (5.89 kJ $m^{-2}$) and/or sub-lethal TCS concentrations (0.546 and 1.090 mg $L^{-1}$) was performed at the beginning of metamorphosis (13 and 14 days after hatching, dah) and fish were maintained in clean medium until complete metamorphosis (24 dah). Malformations and metamorphosis progression were evaluated along the test. Total length and behavior were studied at 24 dah and expression of thyroid-axis related genes was evaluated at 15 dah and 24 dah.

Triclosan exposure induced malformations, decreased swimming activity and altered metamorphosis progression, inducing an acceleration of metamorphosis at 15 dah, followed by a delay at 24 dah. The UV exposure only induced a transient delay in metamorphosis (16-18 dah). The combined exposures to TCS and UV induced an acceleration of metamorphosis on transient stages (15-18 dah) in relation to single exposures.

The stressors caused an overall down-regulation trend of thyroid-axis genes in sole larvae. At 15 dah, TCS down-regulated NIS and TSHβ and UV down-regulated THRβ, Tpo and NIS. At 24 dah, only NIS gene was down-regulated in single exposures to stressors.

Overall, our results suggest that the response of *S. senegalensis* to TCS was not altered by UV exposure, except for the metamorphosis progression at transient stages.

Furthermore, a clear action of TCS on thyroid-axis was associated with alterations on metamorphosis. In addition, sole larvae were still affected at the end of metamorphosis even after the period of maintenance in clean medium, which might have further implications on the ecological performance of the species.

**Keywords:** abiotic stressor; flatfish; gene expression; marine vertebrates; metamorphosis; personal care products; thyroid axis.

## 1. Introduction

The flatfish Senegalese sole (*Solea senegalensis* Kaup, 1858) occurs naturally in coastal areas of Southern Europe and Northern Africa and has a great economic and aquaculture interest (Imsland *et al*., 2003; Power *et al*., 2008; Morais *et al*., 2016). Metamorphosis of this species is a fast life event that takes place during the first month of life, which involves a shift from pelagic larvae to benthic organisms with deep alterations on morphology, feeding and activity patterns (Yúfera *et al*., 1999; Blanco-Vives *et al*., 2012; Gomes *et al*., 2015; Campinho *et al*., 2018). The profound changes in external morphology throughout metamorphosis has the advantage to easily allow to track its progression until climax (Gomes *et al*., 2015). It starts with the appearance of skin pigmentation alterations that become different between dorsal and ventral sides at the end of metamorphosis. The asymmetric development of the head forces eye migration and the anterior head bone and brain are remodeled, while the remainder of the head and organs therein stay symmetric (Bao *et al*., 2011; Campinho *et al*., 2018). Such morphological changes are deeply associated with alterations at molecular and biochemical level and are mediated by the thyroid axis (Fernández-Díaz *et al*., 2001; Klaren *et al*., 2008; Manchado *et al*., 2009; Gomes *et al*., 2015). The thyroxine (T4) and triiodothyronine (T3) are the two main thyroid hormones (TH), which have active roles on this process (Miwa *et al*., 1988; Yamano *et al*., 1991; Inui *et al*., 1994; Okada *et al*., 2003; Klaren *et al*., 2008; Gomes *et al*., 2015). At the beginning of metamorphosis, T4 is the primary hormone synthesized in thyroid follicle, which appears in *S. senegalensis* sub-pharyngeal region at early pre-metamorphosis stages (Klaren *et al*., 2008). This hormone is the precursor of T3, the biologically active form that is produced through deiodination in the peripheral tissue (Power *et al*., 2001; Klaren *et al*., 2008). The deiodination is regulated by deiodinases 1 and 2 (Dio), which are also able to activate/inactivate the TH. Besides Dio, several transporters or receptors genes are also involved in these alterations (Bao *et al*., 2011; Campinho *et al*., 2018). The release of these

hormones is regulated by the pituitary (via thyroid stimulating hormone, TSH), which in turn is mediated through inhibitory control (negative feedback) by the hypothalamus (via thyrotropin releasing hormone, TRH). The TH mediate metamorphosis down-regulating TSHβ and thyroglobulin (Tg) at transcriptional level in *S. senegalensis* and by interacting with thyroid hormone receptors (THR), such as THR α and β (Manchado *et al*., 2008; Gomes *et al*., 2015). The peak of both TH, T3 and T4, coincides with the metamorphic climax in *S. senegalensis* (Gomes *et al*., 2015). In an ecological context, metamorphosis represents a critical period of development and increased vulnerability in flatfish lifecycle.

Homeostasis of hypothalamus-pituitary-thyroid (HPT) axis might be disrupted by environmental contaminants. Disruption of thyroid cascade can occur by interfering in the synthesis, regulation, metabolism, and/or action of TH (Brown *et al*., 2004), namely by inhibiting thyroid iodide uptake (Darrouzet *et al*., 2014) or by affecting thyroid stimulating hormone (TSH), the protein thyroglobulin (Tg) or the thyroid peroxidase (Tpo) enzyme (Campinho *et al*., 2015; Mackenzie, 2018). Therefore, the normal progression of flatfish metamorphosis might be affected by the exposure to chemical contaminants present in coastal ecosystems. In addition, environmental and abiotic factors, namely the type or regime of light (Blanco-Vives *et al*., 2012) or the food regime (Fernández-Díaz *et al*., 2006) can also affect the normal rhythmic behavior of fish larvae during early ontogenesis and metamorphosis of this species. Hereupon, the study of specific stressors effects, from chemical stressors to abiotic factors changing in a global climate change context (e.g. ultraviolet radiation (UV) increase), on thyroid axis during flatfish metamorphosis is still needed.

Triclosan (5-chloro-2(2,4-dichlorophenoxy)phenol, TCS) is one of the most used biocides, which are included in personal care products (PCP) such as soaps, toothpaste and deodorants and it is also found in clothing, kitchenware, furniture, and toys (Orvos *et al*., 2002; Fang *et al*., 2010). This organic compound inhibits the type II fatty acid synthesis of bacteria through inhibition of the Enoyl-acyl carrier protein reductase (Lund *et al*., 2005; Fang *et al*., 2010). TCS can be almost completely degraded in wastewater treatment plants using specific treatments; however, it has been frequently detected in freshwater (concentrations up to 40 μg $L^{-1}$), estuarine (up to 0.31 μg $L^{-1}$) and saltwater habitats (up to 0.1 μg $L^{-1}$) (Kolpin *et al*., 2002; Reiss *et al*., 2002; Capdevielle *et al*., 2008; Kumar *et al*., 2010; Buth *et al*., 2011; Dhillon *et al*., 2015). Bioaccumulation of TCS and toxic effects of this chemical have been reported from highest to lowest organizational levels on a wide range of aquatic organisms (Orvos *et al*., 2002; Tatarazako *et al*., 2004; Liang *et al*., 2013; Martins *et al*., 2017; Park *et al*., 2017), including freshwater (Oliveira *et al*., 2009; Falisse *et*

*al*., 2017; Sahu *et al*., 2018) and marine fish (Araújo *et al*., 2019; Maulvault *et al*., 2019). The interference of TCS with thyroid-axis has been increasingly reported, and it might occur due to its structural similarity with the TH (Crofton, 2008; Luthe *et al*., 2008). Effects of TCS exposure on thyroid-axis of adult zebrafish includes morphological alterations of thyroid tissue (through reduction of thyrocyte height and increased follicle area) suggesting a possible reduction of circulating TH (Pinto *et al*., 2012). Alterations on rate of metamorphosis of aquatic species have also been reported in response to TCS exposure (Marlatt *et al*., 2013). Indeed, our previous study with *S*. *senegalensis* also reported that TCS induces an acceleration of metamorphosis progression (Araújo *et al*., 2019); however, the understanding of TCS mode of action at thyroid level is still needed, namely on expression of HPT-axis related genes.

Abiotic stressors (such as temperature, acidification or UV) in certain conditions can change the toxicity of chemicals (e.g.: Bridges and Boone, 2003). However, they can also alter the resilience of aquatic species to cope with environment chemical contaminants, affecting their survival or growth and physiological status (Hatch and Blaustein, 2003; Gevertz and Oris, 2014; Maulvault *et al*., 2019; Pirone *et al*., 2019). Since increasing UV is still being reported as the result to changes in ozone at stratospheric level (Stolarski *et al*., 1992; Herman, 2010; Čížková *et al*., 2018), assessing UV effects in combination with other stressors is relevant. The UV is expected to significantly decrease in the first few meters of water; however, global changes are promoting the increase of water penetration of UV through decreased dissolved organic carbon (DOC) and colored non-living organic matter (Zagarese and Williamson, 2001; Häkkinen *et al*., 2002; Tedetti and Sempere, 2006; Wang *et al*., 2014). The sensitivity of organisms to UV varies widely between species and life stages (Häkkinen *et al*., 2002; Blaustein *et al*., 2003; Solomon, 2008; Fukunishi *et al*., 2012; 2013; Häder *et al*., 2015). In our previous work (Araújo *et al*., *in prep*. *b*), exposure to extreme levels of UV increased mortality and malformations and decrease growth in post-metamorphosing *S*. *senegalensis*.

Previous studies on combined effects of TCS or UV with other stressors indicate that response patterns depend on the stressor, its doses and species tolerance (Gevertz and Oris, 2014; Häder *et al*., 2015; Wolinski *et al*., 2016; Freitas *et al*., 2019). Combined exposure to UV and TCS on zebrafish also leads to synergistic or antagonistic effects depending on the analysed parameter (Almeida *et al*., 2015). Evaluation of the joint effects of these stressors in other species are still needed.

Therefore, the aim of this work was to study if exposure to UV and TCS at the beginning of metamorphosis of *Solea senegalensis* induced alterations on growth, behavior,

malformations, metamorphosis progression and on the expression of genes related with thyroid axis. In addition, it was studied if (and how) the response of *S. senegalensis* to TCS is altered by UV exposure.

## 2. Material and methods

### 2.1. Biological material

Eggs of *S. senegalensis* were provided by Estação Piloto de Piscicultura de Olhão (EPPO/IPMA, Portugal). After arrival to the laboratory, eggs (total weight approx. 20 g) were placed in a nearly 200 L recirculating water system with artificial saltwater (35 of salinity, Coral Pro, Coral Reef, Saudi Arabia). The system also includes a biological medium filter, UV sterilizer and protein skimmer. Refrigeration was set at 20ºC (Hailea HC Chiller Series, China) and the photoperiod was 16h:8h (Light:Dark). Fish were fed with rotifers (*Brachionus plicatilis*, between 5 and 10 rotifers $mL^{-1}$) from hatching until 6 days after hatching (dah), with *Artemia salina* nauplii (Inve, Belgium) from 5 to 10 dah (between 2 and 9 nauplii $mL^{-1}$) and from 10 dah with *A. salina* metanauplii (between 9 and 35 metanauplii $mL^{-1}$ (Fernández-Díaz *et al*., 2001). Frozen green algae (*Nannochloropsis* sp.) was also provided daily since egg stage. All experimental procedures were carried out following the European and Portuguese legislation concerning animal experimentation (authorized by the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária, Ref. 009804).

### 2.2. Experimental design

The beginning of metamorphosis was evaluated as described by Klaren *et al*. (2008) and Fernández-Díaz *et al*. (2001), which was achieved at 13 dah (total length of approximately 5.2±0.11 mm, measured in 10 randomly selected fish) and were then selected (checked for abnormalities). Randomly placed in 20 mL glass petri dishes (N=10) with 10 mL of the respective testing solution. Ten replicates per treatment (10 fish per replicate/petri dish) were used. Treatments included a negative control (artificial saltwater, 35 of salinity), a solvent control of acetone at 0.01% (molecular grade, Merck) and six treatment groups with the following UV doses and/or TCS (Irgasan, 97 %, Sigma-Aldrich Co. LLC) concentrations:

- Exposure to UV: artificial saltwater (*UV* treatment) or 0.01% acetone artificial saltwater solution (*Solvent UV* treatment; used for comparison purposes with combined exposures) were used as test solution. Fish were exposed to UV during two sessions of 3h each in two consecutive days (13 and 14 dah). The intended

effective UV dose (7.2 kJ $m^{-2}$) was half of the lowest observed effect concentration (energy dose) for malformations (LOEC=14.4 kJ $m^{-2}$, a total of 6h of exposure in two consecutive days) obtained in previous studies (Araújo *et al*., *in prep*. b). The effective dose obtained was 5.9 kJ $m^{-2}$, corresponded to total corrected daily energy of 1.1 W $m^{-2}$ (UV index 11) (supplementary table 6.S1).

- Exposure to TCS during 48h: the nominal concentrations of 1.091 mg $L^{-1}$ (*High TCS* treatment, $LC_{10}$ according to Araújo *et al*., *2019*) and 0.546 mg $L^{-1}$ (*Low TCS* treatment, $LC_{10}/2$) of TCS were used. A stock solution of 10 g $L^{-1}$ TCS in acetone was used to prepare *Low* and *High* TCS solutions. A final concentration of 0.01% of acetone was used as solvent in treatments with *High TCS* and 0.005% acetone in *Low TCS* solutions.
- Combined exposure to TCS and UV: exposure to 1.091 or 0.546 mg $L^{-1}$ TCS and same UV dose as described before for UV treatments was performed simultaneously (*High TCS*+*UV* and *Low TCS*+*UV*, respectively).

All test solutions were renewed at 13 and 14 dah immediately after UV exposure in order to avoid TCS degradation and depletion. The physico-chemical parameters of the testing solutions are presented in supplementary table 6.S2. After 48h (15 dah), fish of half of replicates (n=5, 9-10 fish per replicate) from each treatment group were snap frozen in liquid nitrogen and kept at -80ºC until further procedures. Since during metamorphosis *S. senegalensis* size increases significantly, fish density was lowered at 15 dah by transferring eight fish (out of the ten) from the remaining five replicates of each treatment to new petri dishes with artificial saltwater. The remaining two fish from each petri dish were discarded. Fish were then fed daily with *A. salina* (>50 metanauplii $mL^{-1}$) until more than 80% of fish from both controls completed the metamorphosis, which occurred at 24 dah. At the end of the metamorphosis, fish were also snap frozen in liquid nitrogen and kept at -80ºC for gene expression analysis. Mortality, malformations and metamorphosis progression were recorded along the test. Total fish length (n=5, 4 organisms per replicate) and behavior (n=3, 3 fish per replicate) were analysed at the end of the test. Samples of fish from all groups with exception of *High TCS* and *High TCS*+*UV* were used for gene expression analysis at 15 dah (n=3, 9-10 organisms per replicate) and 24 dah (n=3, 7-8 organisms per replicate).

### 2.3. UV lamp and energy measurement

The UV was obtained with an UV lamp (Spectroline XX15F series 2x15 Watt, Spectronics Corporation, NY, USA, peak emission at 313 and 365 nm corresponding to UV-

B and UV-A wavelength range, respectively) with clear cellulose acetate sheets (0.003 mm, Grafix plastics, USA) for UV-C radiation filtering. These sheets were previously used in the UV lamp during 12 h for radiation stabilization. Energy was measured with a double monochromator (Bentham DMC150-USB, Bentham Instruments Ltd, UK) with an high voltage supply (Bentham 215) and using the software Benwin+ (Bentham Instruments Ltd). The irradiance was corrected and final UV dose is expressed using the Commission Internationale de l'Éclairage (CIE) reference action spectrum for the erythema in human skin according to McKinlay and Diffey (1987) and WHO (2002). The UV Index was calculated multiplying the erythema weighted total irradiance (in W $m^{-2}$) by 40 (Fioletov *et al*., 2010).

### 2.4. Behavior analysis

Randomly chosen *S*. *senegalensis* of all treatment groups were individually placed in 24 well plates filled with 2 mL of saltwater and analysed in Zebrabox® (Viewpoint, France). Fish behavior was analysed during a total of 60 minutes by video recording and automatic data acquisition (Zebralab software, Viewpoint). Two alternating periods of light and dark were set (15 minute each), after an initial acclimation light period (5 min). The movement threshold was set at 15 and 8.0 mm/sec (for small/fast and “inact”/small movements, respectively). Light intensity used was set at 0.26 mW $cm^{-2}$ and background threshold was set at 70 pixels (“transparent” color). In addition to the white light irradiated by Zebrabox, an infra-red light (not seen by the fish, constant at 2.3±0.11 mW $cm^{-2}$) was used for video recording purposes in dark periods. Based on software results, the mean of total swimming distance (m), total duration of swimming time (min) and number of fast movements (counts) were calculated for each treatment for the complete testing period (60 min) and for each 15 min light/dark period.

### 2.5. Gene expression analysis

Three biological replicates of each of the two periods (15 dah and 24 dah) from negative control, solvent control, UV, solvent treated UV, *Low TCS* and *Low TCS+UV* treatments were used for gene expression analysis. Extraction of RNA was carried out using TRIzol® method (Invitrogen, Belgium). Samples were further treated with DNAse (Invitrogen) to purify the RNA from DNA contamination. For cDNA synthesis purposes, the quantity of RNA was measured using a NanoDrop® ND 1000 spectrophotometer (Thermo Fisher Scientific, USA) and purity was verified by checking the absorbance ratios 260/280 nm and 260/230 nm (between 1.8 and 2.2 for both ratios). The integrity of RNA

was verified through formaldehyde agarose gel electrophoresis by checking the existence of large and small subunit ribosomal RNAs (rRNA) correspondent bands (Taylor *et al*., 2010) before proceeding with cDNA synthesis. Total RNA (1 µg per each sample) was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), according to manufacturer instructions.

The qPCR expression analysis of selected genes (table 6.1) was performed in triplicate for each of the three biological samples from each treatment in 96-well plates (BIO-RAD) using Platinum SYBR® Green PCR Master Mix (Invitrogen) in a CFX Connect TM Real-Time PCR Detection System (BIO-RAD) with CFX Manager 3.1 Software (BIO-RAD). A total volume of 20 µL was used in each reaction with different primer concentrations: 200 µM for ubiquitin (Ubq), thyroid hormone receptor αA (THRαA) and β (THRβ), Tpo, Tg, sodium-iodide symporter SLC5A5 (NIS) and 300 µM for thyroid stimulating hormone β (TSHβ). To ensure amplification of a single product, primer specificity was determined by analysis of melting curves. Primer efficiencies of all qPCR reactions were near 100%. For each gene, gene-specific primers were selected according to sequences published previously (table 6.1). The Ubq gene was used as housekeeping gene (i.e. gene uniformly expressed with low variance under both control and experimental conditions).

The amplification qPCR conditions consisted of an initial step at 50 ºC for 2 minutes, then 95 °C for 7 minute and then 40 amplification cycles at 95 °C for 15 seconds and 70 °C for 30 seconds (Boglino *et al*., 2017). A final dissociation reaction (melting curve) was performed between 65 ºC and 95 °C with 0.5 seconds and 0.5 ºC increments. Relative Expression Software Tool (REST©-MSC) was used to determine relative transcriptional abundance of each gene with corresponding control (Pfaffl *et al*., 2002). Data was normalized with $\log_2$ transformed factor relative to all expression data.

Table 6.1. Selected genes of *Solea senegalensis* used for gene expression analysis. dah – days after hatching.

| Gene identification | Abbreviation | Sequence | Efficiency (15 dah/24 dah) | Reference |
|---|---|---|---|---|
| Ubiquitin (housekeeping) | *Ubq* | Fw – AGCTGGCCCAGAAATATAACTGCGACA<br>Rv – ACTTCTTCTTGCGGCAGTTGACAGCAC | 1.90/1.96 | Infante *et al.*, 2008 |
| Thyroid hormone receptor αA | *THRαA* | Fw – CGCAAGTTCATGCCGGATGATATCG<br>Rv – TGTGATGGCGGGTGTCATTATCTTGGT | 1.94/1.89 | Manchado *et al.*, 2009 |
| Thyroid hormone receptor β | *THRβ* | Fw – AAACAGAAGCGGAAGTTCCTGAGTGCAG<br>Rv – CTTTGTTTCCTTCAGGTGTGTTTGCCATC | 2.01/2.03 | Manchado *et al.*, 2009 |
| Thyroglobulin / thyroid gland | *Tg* | Fw – CACCTCCTCTGACCGCCGCCTCT<br>Rv – CGGGGCATAACTGACTCCTCCCACA | 1.94/2.19 | Manchado *et al.*, 2008 |
| Thyroid peroxidase | *TPO* | Fw – CGTTCCAAAGATCATAGGCCCCGAGT<br>Rv – CAGTGGCAAACACATTGGAGGCTGA | 1.90/2.19 | Campinho *et al.*, 2015 |
| Sodium-iodide symporter (SLC5A5, Na+I− symporter) | *NIS* | Fw – TTCTTCACTGGTGGCCGGAGCTTG<br>Rv – GACGGCTGACATGAAACTGGCACA | 2.03/2.19 | Campinho *et al.*, 2015 |
| Thyroid stimulating hormone β | *TSHβ* | Fw – CACGAGGGCTACTCAAGGGACAGCAA<br>Rv – CCTGGCAGTATGGCGCTGTGG | 2.06/1.85 | Manchado *et al.*, 2008 |

### 2.6. Statistical analysis

In order to test the effect of the solvent and UV in fish mortality, malformations, length and behavior (total swimming duration, distance and number of fast movements), student t-tests or Mann-Whitney rank sum tests were performed between negative control and solvent control or UV exposed fish.

One Way Analysis of Variance (ANOVA) were used to compare mortality, malformations, length and behavior (total swimming duration, distance and number of fast movements) of solvent control, TCS and/or UV treated fish groups when the criteria of normality and homocedasticity were satisfied. Non-parametrical Kruskal-Wallis test were used on the other cases. Significant differences between treatments were checked using post-hoc Tukey tests.

For swimming behavior, Two Way Repeated Measures ANOVAs were performed for studying the effect of treatments and of the 15 min alternating light and dark periods. The first one was with negative control and solvent control treatments; the second one with negative control and UV exposed fish, and the last one with the main data, namely solvent control, TCS and/or UV exposed fish. The existence of significant interaction between factors were checked. For significant factors, Tukey test was performed for multiple comparison procedures.

Regarding metamorphosis progression, a Chi-square tests were used initially to test differences between fish from negative and solvent control group and also between negative and non-solvent treated UV exposed group. Furthermore, a Chi-square tests were then used to compare differences between solvent control and TCS and/or UV exposed fish. When significantly differences on metamorphosis progression were found, pairwise Chi-square tests were performed with Bonferroni adjustment (Arnholt, 2016).

Statistical analysis were performed using SigmaPlot v.12.5 (Systat Software Inc., California, USA). A significance level of 5% was considered in all statistical procedures. Results are expressed as mean±standard error.

## 3. Results

### 3.1. Mortality

The mortality recorded at 48h after the beginning of the test was below 10% for all experiment groups; and no significant differences were found between controls groups ($p>0.05$) or between controls and treatments ($p>0.05$). At the end of metamorphosis, mortality was below 10% for all groups except for *High TCS* (10.4±4.74%) and also no

significant differences between control and the different treatments groups were found ($p>0.05$).

### 3.2. Length

At the end on metamorphosis, no significant differences were observed in length of fish from negative and solvent control groups (8.2±0.09 mm and 8.3±0.09 mm, respectively, $p>0.05$, data not shown). Also, the length of fish exposed to UV (8.4±0.12 mm) was not significantly different from length of negative control fish ($p>0.05$).

Fish length of UV group (8.5±0.12 mm for solvent treated UV group) or TCS group (8.2±0.12 mm and 8.1±0.14 mm for *Low TCS* and *High TCS*, respectively) was similar to the length of fish from solvent control ($p>0.05$). Furthermore, no significant differences were observed on the length of fish simultaneously exposed to TCS and UV (8.2±0.11 mm and 8.0±0.11 mm for *Low TCS + UV* and *High TCS + UV*, respectively) when comparing to solvent control, UV or TCS exposed fish ($p>0.05$).

### 3.3. Behavior

In the 60 min behavior test performed at the end of metamorphosis (24 dah) behavior was evaluated through total swimming duration, distance and total number of fast movements. A significant increase ($p<0.05$) was observed on solvent control when comparing to negative control on the total swimming duration (30.6±2.82 min), total swimming distance (53.8±13.45 m) and number of fast movements (2295.6±330.83 counts).

A significant increase ($p<0.05$) was also observed on fish exposed to UV when comparing to negative control fish on total swimming duration (18.5±1.79 min and 14.3±1.69 min, respectively), total swimming distance (28.1±2.99 m and 12.4±4.67 m, respectively) and number of fast movements (1206.3±100.97 and 488.5±210.04 counts, respectively).

When comparing solvent control fish to fish exposed to TCS or UV, single or in combination (fig. 6.1), a significant lower swimming duration ($p<0.05$) was observed in fish exposed to *Low TCS*, *Low TCS+UV* and *High TCS* (with 16.3±3.37, 16.1±3.89 and 14.9±3.64 min, respectively). Similarly, the number of fast movements (fig. 6.1) of the fish exposed to *Low TCS*, *Low TCS+UV* and *High TCS* (with 917.7±154.09, 1004.3±284.50 and 1137.89±244.46 counts, respectively) was also significantly lower than in fish from solvent control ($p<0.05$). Furthermore, no significant differences ($p>0.05$) were found between single treatments (TCS or UV) and combined treatments (TCS+UV) for these behavior parameters.

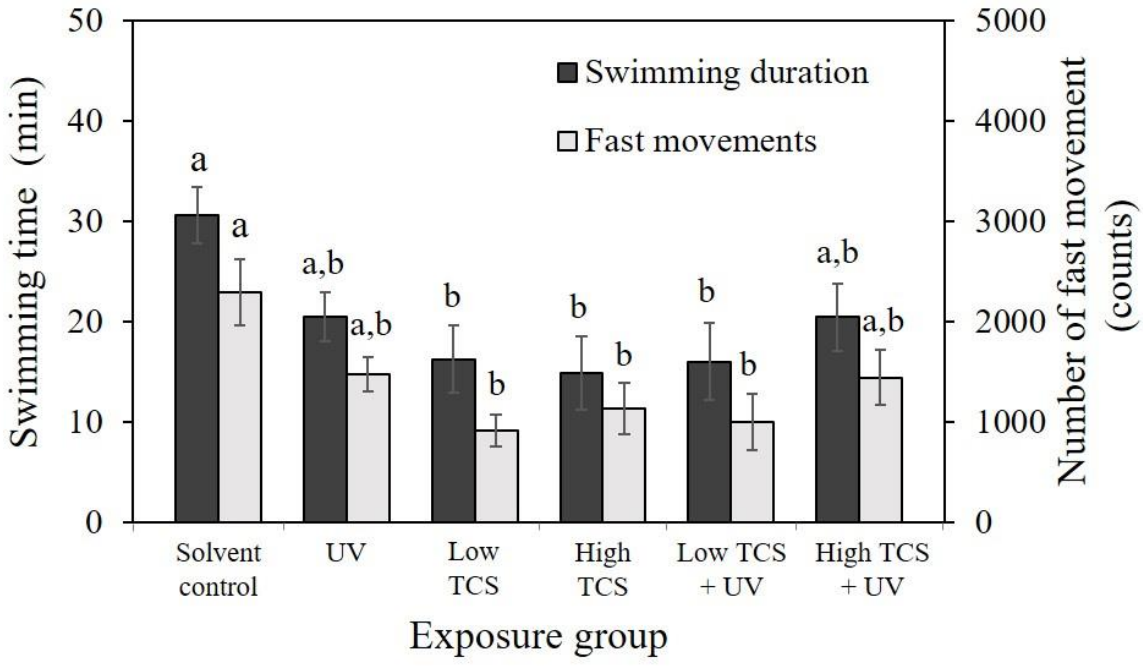


Fig. 6.1. Total swimming duration and total number of fast movements during the 60 min behavior test of *Solea senegalensis*. The behavior test was performed at the end of metamorphosis (24 days after hatching, dah), after exposure to triclosan (TCS, 0.546 and 1.091 mg $L^{-1}$, *Low TCS* and *High TCS*, respectively) and/or UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$) at 13 and 14 dah. Acetone (up to 0.01%) was used as solvent in all treatments. Results are expressed as mean±SE. Different letters represent significant differences between treatments (Tukey test; $p<0.05$).

No significant differences were observed on total swimming distance of fish (data not shown) between solvent control, TCS and/or UV exposure treatments ($p>0.05$), with values ranging between 22.8±5.57 m for *High TCS* and 53.8±13.45 m for solvent control.

When considering the alternate 15 min periods of light/dark in the analysis of behavior, no significant interactions were found between the periods light/dark and treatments (TCS and/or UV, controls) in all behavioral endpoints analysed (Two-way ANOVA; $p>0.05$). The swimming distance, swimming duration and the number of fast movements of solvent control were significantly higher than negative control ($p<0.05$, supplementary fig. 6.S1). A significant decrease along periods was observed in all of these behavioral endpoints between negative control and solvent control or UV exposed fish ($p<0.05$, supplementary fig. 6.S1 and 6.S2), except for the number of fast movements when comparing UV against negative control ($p>0.05$). Furthermore, no significant differences were observed on these three parameters between negative control and UV ($p>0.05$).

When comparing different treatments (solvent control, TCS and/or UV), significant differences were not observed on fish swimming duration ($p>0.05$), while decreasing

swimming duration along the periods were observed for all fish groups ($p<0.05$, data not shown). Considering the effects of treatments on swimming distance, fish exposed to *High TCS* and *Low TCS+UV* presented a significant decrease when compared to solvent control group ($p<0.05$; fig. 6.2A). Regarding the number of fast movements, significant decreases were observed between solvent control and fish exposed to *Low* and *High TCS* and *Low TCS+UV* ($p<0.05$, fig. 6.2B). Furthermore, swimming duration, swimming distance and number of fast movements are similar in fish exposed to combined treatments in relation to the respective single exposure treatments ($p>0.05$).

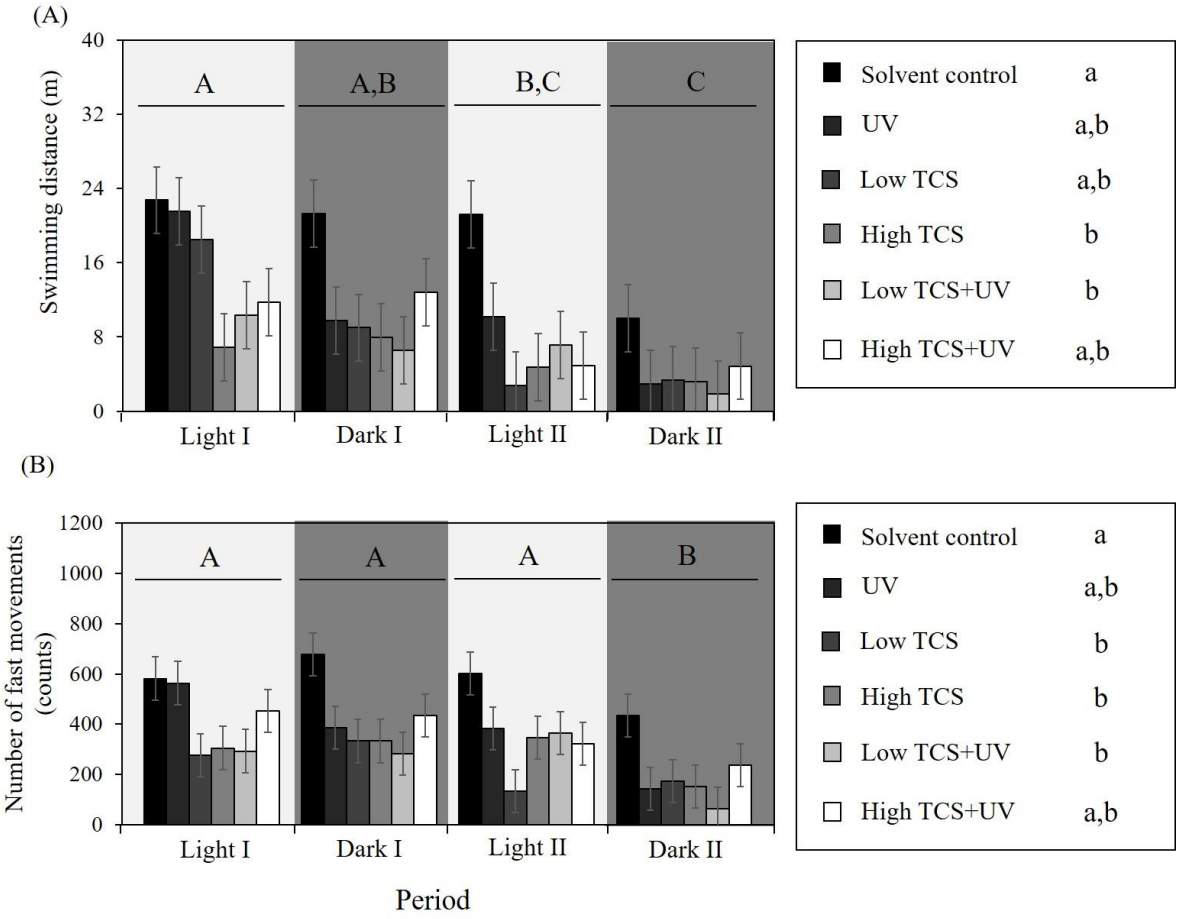


Fig. 6.2. Behavior in each 15 min light or dark periods of *Solea senegalensis* at the end of metamorphosis (24 days after hatching, dah) after 48h exposure to triclosan (0.546 and 1.091 mg $L^{-1}$, *Low TCS* and *High TCS*, respectively) and/or UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 dah). Acetone (up to 0.01%) was used as solvent in all treatments. (A) Swimming distance (m) and (B) number of fast movements (above 15 mm $sec^{-1}$). Results are expressed as mean±SE. Different upper letters represent significant differences between different light/dark periods and different lowercase letters (in legend) represent significant differences between TCS and/or UV treatments ($p<0.05$).

### 3.4. Malformations

Along the metamorphosis, several malformations were observed at 14, 15, 16, 21 and 24 dah on organisms, namely on fins, pigmentation, cephalic structure and eyes.

No significant differences were observed on total malformations when comparing fish from negative and solvent control groups ($p>0.05$; up to 12.5±3.9% for negative control and up to 10.0±4.68% for solvent control, both at 16 dah). In addition, significant differences on total malformations between negative control and UV exposed fish were not observed at 14, 15, 16, 21 and 24 dah ($p>0.05$), ranging between 3.1±1.59% and 27.5±4.68% at 14 and 16 dah respectively for fish exposed to UV.

Significantly higher percentage of total malformations were observed at 16 dah in fish of *High TCS+UV* group (42.3±10.21%) and at 24 dah in fish of *High TCS* group (82.4±22.75%, $p<0.05$) when compared with solvent control fish. No significant differences were observed on total malformations when comparing TCS or UV exposed fish with fish simultaneously exposed to TCS and UV ($p>0.05$).

Simultaneous underdevelopment of fish on size, fins and altered pigmentation was observed in fish groups exposed to TCS and/or UV (less than 10%), which was significantly increased at 14 dah for fish exposed to *High TCS+UV* (6.9±1.92%) when comparing to solvent control (0%, $p<0.05$, data not shown; fig. 6.3A).

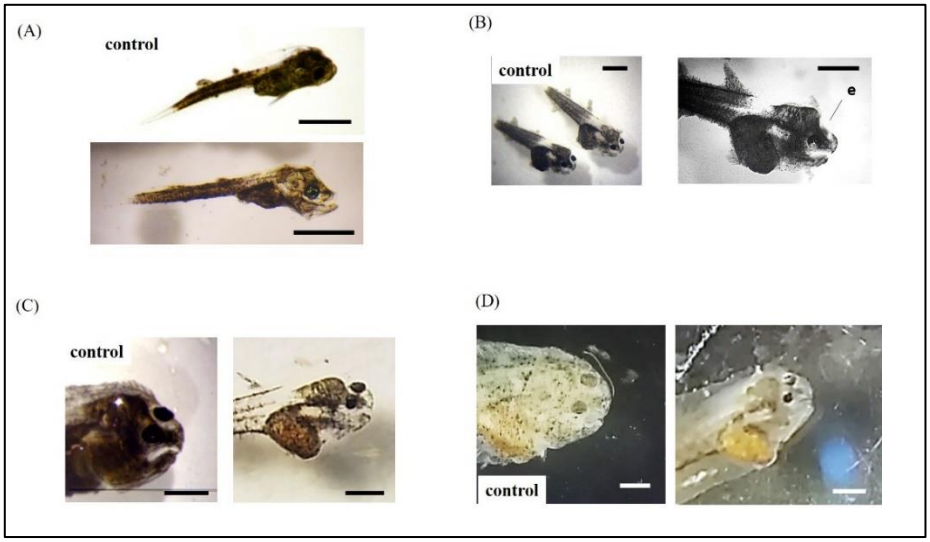


Fig. 6.3. Malformations of *Solea senegalensis* after 48h exposure to triclosan (0.546 and 1.091 mg $L^{-1}$, *Low TCS* and *High TCS*, respectively) and/or UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 days after hatching, dah). Acetone (up to 0.01%) was used as solvent in all treatments. A – Negative control fish (up) and underdeveloped fish (lack of dorsal fin, pigmentation and skeletal development, down) after exposure to *High TCS+UV* at 14 dah; B – Negative control fish (left) and fish exposed to *High TCS+UV* with abnormal migration of the eye (e, right) at 16 dah; C – Solvent control fish (left) and fish exposed to *High TCS+UV* with abnormal migration of the eye and cephalic development and underdeveloped pigmentation (right) at 21 dah; D - Control fish (left) and fish exposed to *High TCS+UV* (right) at 24 dah. Horizontal bars in pictures represent 1 mm.

Abnormal migration of eye was observed throughout the test with significantly higher values at 16 dah for fish exposed to *High TCS+UV* (42.3±10.21%) when comparing to solvent control fish (10.0±4.68 for 16 dah, $p<0.05$, figures 6.3B and 6.4A). At 16 dah, abnormal migration of eye of *High TCS+UV* fish group was also significantly higher than in UV (solvent treated) fish group (12.5±5.59%, $p<0.05$). Despite no significant higher number of fish with abnormal migration of the eye than in solvent control was observed on *High TCS* fish group at 16 dah (23.3±4.29%, $p>0.05$), a significantly higher number was observed at 24 dah (41.2±11.38%) when comparing to solvent control ($p<0.05$).

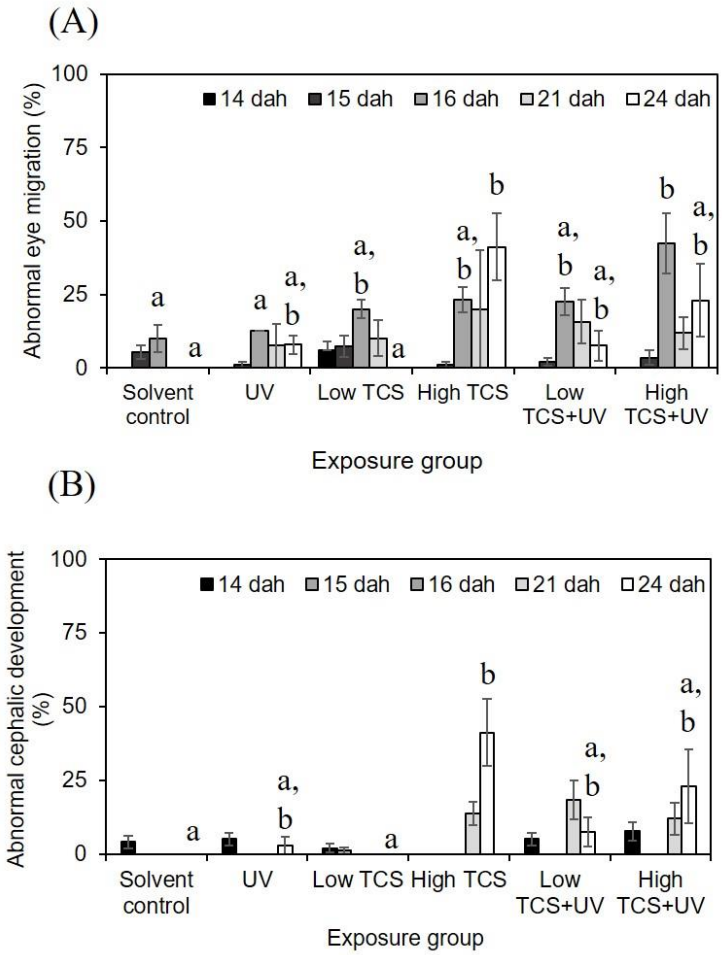


Fig. 6.4. Percentage of abnormal migration of the eye (A) and cephalic development (B) of *Solea senegalensis* larvae after 48h exposure at to triclosan (0.546 and 1.091 mg $L^{-1}$, *Low TCS* and *High TCS*, respectively) and/or UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 days after hatching, dah). Acetone (up to 0.01%) was used as solvent in all treatments. Results are expressed as mean±SE. Significant differences between treatments at 16 and 24 dah are denoted through different letters (Tukey test; $p<0.05$).

Abnormal cephalic development was also observed in fish along the test, which was significantly increased at 24 dah on fish exposed to *High TCS* (41.2±7.70%) when comparing to solvent control (0%, $p<0.05$, figures. 6.3C, 6.3D and 6.4B).

### 3.5. Metamorphosis progression

When comparing metamorphosis stages, significant differences between negative and solvent controls at 15, 18 and 21 dah ($p<0.05$); fish of solvent control group presented a faster progression at 16, 18 and 21 dah, when compared with fish from negative control group.

Metamorphosis progression in UV exposed fish was significantly delayed when compared to negative control at 16 dah while at 18 dah the opposite was observed ($p<0.05$; supplementary fig. 6.S3).

In figure 6.5 is presented the progression of sole metamorphosis after exposure to TCS and UV (single or in combination). At 14 dah no significant differences were observed on metamorphosis progression between all treatments ($p>0.05$). At 15 dah, just after exposures, all fish groups exposed to TCS except *Low TCS+UV* were significantly more developed than solvent control fish ($p<0.05$). However, after the beginning of maintenance in clean medium, at 16 dah, no effect or a significant delay (*Low TCS+UV*; $p<0.05$) in metamorphosis progression were denoted in exposed fish. At 18 dah, fish exposed simultaneously to TCS and UV were more developed than the ones from solvent control ($p<0.05$). At 21 dah, all groups of fish exposed to TCS were significantly less developed than fish from solvent control ($p<0.05$). At 24 dah, fish exposed to *Low TCS* and *Low TCS+UV* remained less developed than the solvent control group ($p<0.05$).

Furthermore, metamorphosis progression was significantly faster ($p<0.05$) in fish exposed to combined treatments (*Low* or *High* TCS + UV) than to the respective single exposures (*Low* or *High* TCS or UV) in the following cases: *Low TCS+UV* vs. *Low TCS* at 18 dah; *High TCS+UV* vs. *High TCS* (15 dah and 18 dah); *Low TCS+UV* vs. UV (from 14 dah until 18 dah); *High TCS+UV* vs. *UV* (from 14 dah until 24 dah).

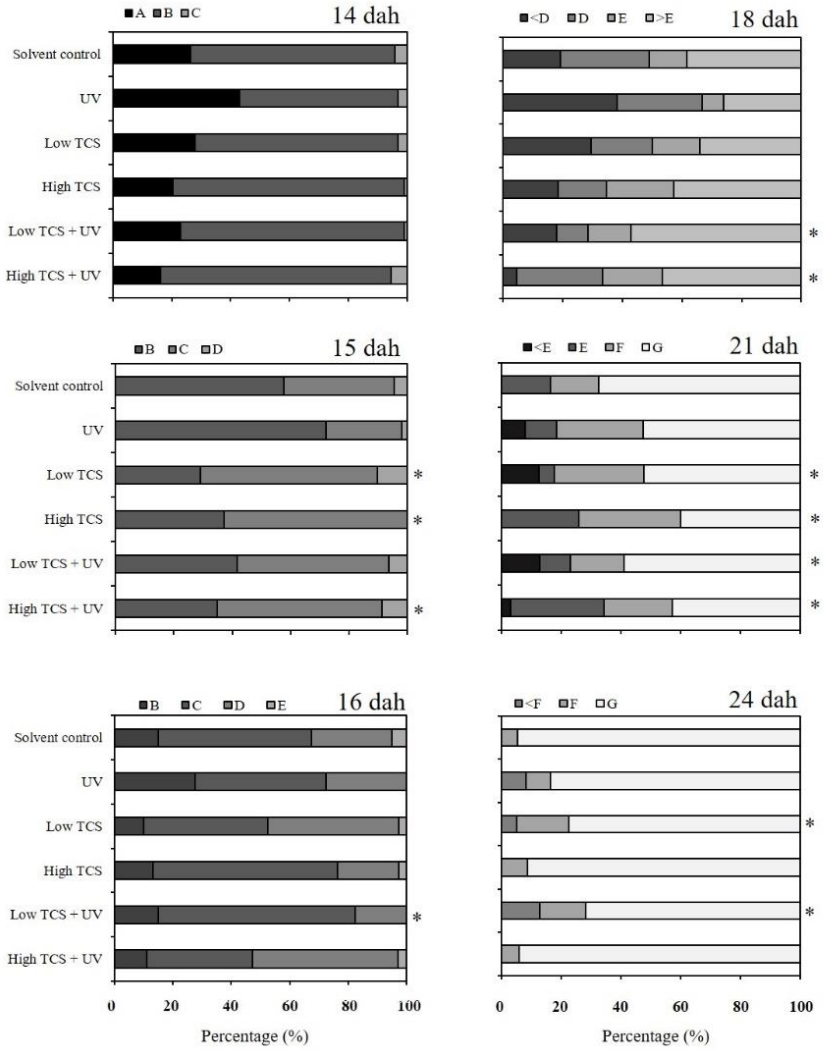


Fig. 6.5. Metamorphosis stages of *Solea senegalensis* after exposure to triclosan (0.546 and 1.091 mg $L^{-1}$, *Low TCS* and *High TCS*, respectively) and/or UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 days after hatching, dah). Acetone (up to 0.01%) was used as solvent in all treatments. A&B – early metamorphosis (enlargement of fins, beginning of migration of left eye); C&D - alteration of mouth shape and pigmentation darkening; E - fully flattened body; F - anterior profile becomes more curved; G – complete metamorphosis: orbital eye membrane becomes thicker. * represent the existence of significant differences between each treatment group with solvent control for each age ($p<0.05$).

### 3.6. Gene expression

Relative gene expression in solvent control and UV exposed fish is presented in supplementary figure 6.S4. Expression of thyroid-axis genes from solvent control fish were not significantly up or down expressed in relation to negative control at 15 dah ($p>0.05$). However, at the end of the metamorphosis (24 dah), there was a significant down-regulation of THRβ, Tg, Tpo and TSHβ genes of solvent control fish in relation to negative control ($p<0.05$).

At 15 dah, just after exposure to UV, only the NIS gene of *S. senegalensis* was significantly down-regulated when compared with fish from negative control group ($p<0.05$; supplementary fig. 6.S4A). On the other hand, after maintenance in clean medium until complete metamorphosis (24 dah), there was a significant down-regulation of THRβ, Tg, Tpo and TSHβ genes of UV exposed fish in relation to negative control ($p<0.05$, supplementary fig. 6.S4B).

When analysing gene expression of TCS and/or UV exposed fish in relation to solvent control, at 15 dah the THRβ, Tpo and NIS genes of fish from UV group were significantly down-regulated ($p<0.05$, fig. 6.6A). In addition, NIS and TSHβ genes of *Low TCS* exposed group were also down-regulated ($p<0.05$). Furthermore, combined exposure to TCS and UV resulted in a significant down-regulation of THRβ and NIS genes ($p<0.05$).

At the end of metamorphosis (24 dah), NIS was the only gene affected by exposure to stressors when comparing to solvent control, being down-regulated by single exposure to UV and *Low TCS* ($p<0.05$, fig. 6.6B).

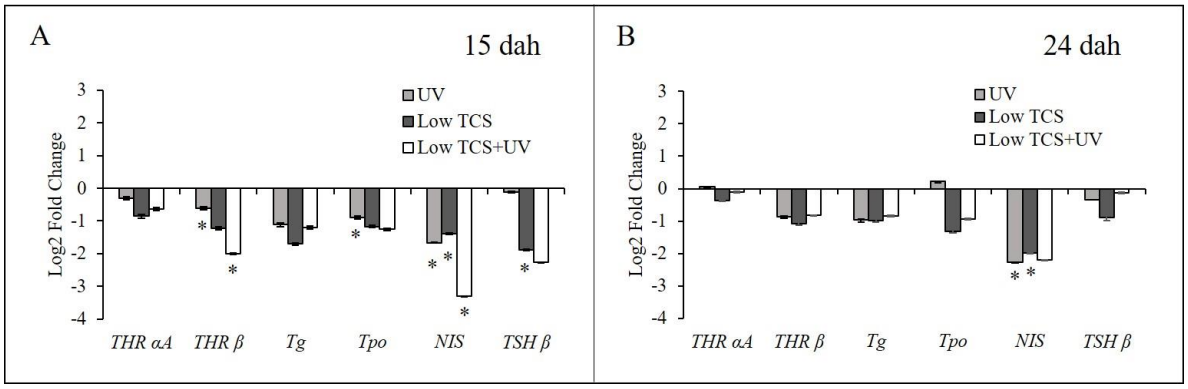


Fig. 6.6. Relative gene expression in *Solea senegalensis* at 15 (A) and 24 days after hatching (dah, B) after 48h exposure to triclosan (0.546 mg $L^{-1}$, Low TCS) and/or UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 dah). Acetone (up to 0.01%) was used as solvent in all treatments. Results are expressed relatively to solvent control as log 2 Fold Change±SE. * indicate significant differences from solvent control group ($p<0.05$).

## 4. Discussion

In this study, the single and combined effects of exposure to UV and TCS at the onset of metamorphosis were studied on *S. senegalensis* at different levels of biological organization, namely from individual (e.g. behavior, malformations) to genomic (e.g. HPT-axis genes) level. The link between the induced alterations by stressors in the different endpoints will be discussed, particularly the link between the induced changes on expression of thyroid-axis related genes and alterations on the metamorphosis of *S. senegalensis* larvae.

### 4.1. Behavior

In our work, different behavioral responses to the stressors were observed, depending on the parameters measured, namely on swimming distance, duration and number of fast movements. Overall, the behavioral parameters assessed on *S. senegalensis* were increased by the exposure to UV when compared to negative control during the 60 minutes of testing. Moreover, it should be highlighted that these effects in *S. senegalensis* lasted beyond the UV exposure period, since the behavioral responses were studied nine days after exposure to the stressors. These later effects observed on behavior might have further consequences and negative effects on the ecological performance of this species. Other authors have also reported negative ecologically relevant effects of UV on fish behavior, namely on escape from predators after short-term exposure to UV (Fukunishi *et al*., 2012). In addition, an indirect effect of UV exposure on behavior was described, namely the produced alarm cues by UV exposed fish were shown to induce increased shoaling behavior (Manek *et al*., 2012).

Exposure of *S. senegalensis* to TCS decreased larvae swimming duration and decreased the number of fast movements of larvae as well. This is in accordance with previous studies that also showed TCS affects behavior of the adult shark catfish *Pangasianodon hypophthalmus*, namely by inducing hyperactivity and erratic movements in tanks (Sahu *et al*., 2018). These alterations on behavior due to TCS exposure can be related with alterations on AChE, an enzyme involved in the neurotransmission process. Alterations in AChE activity were in fact observed in our previous work on metamorphosing sole, just after TCS exposure (Araújo *et al*., 2019) and also in the brain of shark catfish (Sahu *et al*., 2018).

When combining both stressors, TCS and UV, the total swimming time, duration and number of fast movements was similar to the observed in the fish exposed to each isolated stressor, suggesting that UV did not change the response in fish behavior induced by TCS.

Considering the behavior responses in each 15 min alternating light/dark periods of sole exposed to the stressors UV and/or TCS, the light/dark period did not influence the response of sole to UV and/or TCS, as no interactions were observed in each of the behavioral endpoints evaluated. Considering the effects of the stressors UV and/or TCS in the light/dark periods, fish behavior responses followed a similar trend to those observed for the total of the 60 min of the test. Furthermore, in general, fish swimming time, duration and number of fast movements in alternating light/dark periods decreased along the behavior test, which was not the expected activity pattern for this life stage of sole. Higher

activity during dark periods was expected since after metamorphosis fish change to a nocturnal behavior (Blanco-Vives *et al*., 2012).

Acetone has been widely used as solvent in aquatic toxicology, inclusively in bioassays regarding the study of TCS effects (Oliveira *et al*., 2009; Fang *et al*., 2010; Almeida *et al*., 2015; Martins *et al*., 2017). However, our results indicate that low concentrations of acetone (0.01%) affect *S*. *senegalensis* larvae swimming behavior, namely increasing the swimming time and the travelled distance as well increasing the number of fast movements. Apart from the effects on behavior, acetone also affected metamorphosis progression and down-regulated thyroid axis genes at 24 dah in the present work with *S*. *senegalensis* larvae. Therefore, the sensitivity to acetone demonstrated by *S*. *senegalensis* larvae suggest that other solvents should be considered when assessing effects of stressors in fish larvae.

### 4.2. Malformations

Several malformations have been reported during flatfish metamorphosis, including abnormal migration of the eye, arrest of metamorphosis and pigmentation defects (Bisbal and Bengston, 1995; Ellis *et al*., 1997; Pittman *et al*., 1998; Power *et al*., 2008).

In the present work, an induction of malformations was observed during metamorphosis in *S*. *senegalensis* exposed to the highest tested concentration of TCS, namely underdevelopment of fish fins, altered pigmentation, abnormal migration of the eye and/or abnormal development of cephalic bones; however, single UV exposure did not induce an increase in malformations. The combined exposure to UV and TCS also induced malformations during metamorphosis progression in relation to solvent control, but in a similar percentage than the respective single exposures, suggesting that UV did not alter the toxicity induced by TCS for this endpoint. Furthermore, at the end of metamorphosis total malformations or abnormalities were only observed on fish exposed to *High TCS*. While fish were partially able to recover from such malformations or abnormalities, the type of malformations observed at 24 dah seem directly related with metamorphosis progression. At the end of metamorphosis, abnormal migration of the eye was significantly higher for fish exposed to *High TCS* when comparing to solvent control, even after 9 days maintenance in clean medium. These effects on eye migration might negatively influence fish vision and subsequently their fitness. Such results might be associated with an effect of TCS on thyroid axis, as the eye migration is forced by ossification on the blind-side sub-optical neurocranium which is driven by TH which together give rise to the observed asymmetric development of the head (Bao *et al*., 2011; Campinho *et al*., 2018; Fernández *et al*., 2018).

### 4.3. Metamorphosis progression

After the UV exposure, fish presented alterations in metamorphosis progression at transient stages of metamorphosis, firstly inducing a delay, and then an acceleration. Despite this, by the end of metamorphosis, all fish were in similar stages when compared to negative control. When using solvent, no effects of UV exposure were detected.

In the present work, single exposure to TCS induced a faster metamorphosis rate of *S. senegalensis* immediately after exposure. These results are in accordance with those observed in our previous work with TCS for this life stage, since a faster metamorphosis rate was induced in transient stages after exposure to similar TCS concentrations (Araújo *et al*., 2019). While increased growth during metamorphosis of anurans in the presence of TCS was attributed to the decrease of bacteria in cultures (Fort *et al*., 2011), an increase in the rate of metamorphosis of anurans was also reported and associated with alterations on TH metabolism (Sowers and Klaine, 2008). Alterations at endocrine level as advanced or precocious metamorphosis were also previously reported to occur after administration of TH in flatfish (Power *et al*., 2001, 2008; Klaren *et al*., 2008). Triclosan has already been suggested to act as thyroid hormone receptor agonist, mimicking TH action during metamorphosis (Sowers and Klaine, 2008). Therefore, the effects observed in our work suggest that TCS might affect thyroid axis of *S. senegalensis*. In addition, at 21 dah, metamorphosis of all fish groups exposed to TCS were significantly delayed when comparing to solvent control while such delay at 24 dah was only observed in fish exposed to *Low TCS*.

In general, the combination of the stressors TCS and UV, induced a faster metamorphosis in the transient stages in relation to the effect of the isolated stressors. Particularly, at 15 dah, the highest concentration of TCS tested in combination with UV induced a faster metamorphosis rate in relation to the single treatments (UV or High TCS). Similarly, at 18 dah both concentrations of TCS in combination with UV accelerated metamorphosis in relation to the respective isolated treatments. These results suggest an exacerbation of the effect of TCS alone by the UV exposure. The mechanisms through which this occurs should be further studied.

### 4.4. Gene expression

In general, a down-regulation trend of thyroid axis related genes in UV and/or TCS exposed fish when comparing to solvent control was observed. Such trend was more clear immediately after the exposure to stressors (15 dah) than after 9 days in clean medium (24 dah) when comparing TCS and/or UV (solvent treated) groups with solvent control. When

comparing UV group with negative control in both periods, down-regulation was more accentuated at 24 dah.

The TH have a key role on normal development of many species including fish. These hormones are poorly soluble in water and while circulating in blood they are bound to carrier proteins which are mediated by THR (Zhang and Lazar, 2000). In our work, while THRαA gene expression was not affected by TCS and/or UV exposure, on the contrary THRβ was down-regulated on UV exposure groups, both immediately after exposure (15 dah) relatively to solvent control and at the end of metamorphosis relatively to negative control. In addition, the combined exposure to TCS and UV resulted in a significant down-regulation of THRβ in relation to solvent control. These interferences in THRβ gene expression and their subsequent activity might lead to misregulation of gene expression under THRβ control.

THRα and β occur in flatfish with several isoforms, with distinct temporal and spatial patterns of expression with a peak at flatfish metamorphosis and subsequent decline towards post-climax (Yamano and Miwa, 1998; Power *et al*., 2001; Klaren *et al*., 2008; Manchado *et al*., 2009; Campinho *et al*., 2018). Several patterns of expression of THR have been reported for different flatfish species (Yamano and Miwa, 1998; Power *et al*., 2001, 2008; Galay-Burgos *et al*., 2008; Manchado *et al*., 2009). During metamorphosis in *S. senegalensis* only THRβ presented altered mRNA levels in a similar way to the T4 levels (Manchado *et al*., 2009). The (in)activation of THRβ can be associated with several development problems including abnormal development of fish organs and tissues (Zoeller, 2007; Zoeller *et al*., 2005). Despite no marked changes on sole metamorphosis were observed in the present work, the down-regulation of THRβ gene on UV exposed fish might be associated with the misregulation of the other HPT-genes studied, which can have further implications on fish normal development.

The release of TH is regulated by the pituitary via TSH. The TSH and Tg are strictly connected as the TSH stimulation of thyroid follicular cells leads to increased production of Tg, the major protein in thyroid gland, that acts as a substrate for TH synthesis (Sturgeon, 2014). It should be noted that, both TSH and Tg are up-regulated during *S. senegalensis* metamorphosis (Manchado *et al*., 2008; Campinho *et al*., 2015). Previous studies reported that *S. senegalensis* metamorphosis can be blocked by the exposure to the anti-thyroid drug methimazole, which results on increased Tg and TSH (Campinho *et al*., 2015). In our work, TCS exposure seemed to work on the opposite direction causing a down-regulation of TSHβ and a non-significant reduction Tg expression, which might have contributed to the faster progression of metamorphosis that occurred after TCS exposure. While at 15 dah

TCS induced significant TSHβ down-regulation and the advance on metamorphosis progression was clear, at the end of metamorphosis the down-regulation of this gene was not significant and a delay in metamorphosis was observed. On the contrary, the effect of UV observed on TSHβ down-regulation in fish at 24 dah could not be associated with any alteration on metamorphosis progression at that age.

The enzyme Tpo catalyzes iodide oxidation, thyroglobulin iodination and iodothyronine coupling. Its reduced activity or expression impairs thyroid follicles by reducing iodide trapping and impairing thyroid hormone synthesis (Stathathos and Ringel, 2006). Tpo is highly up-regulated during *S. senegalensis* metamorphosis; however, further increased Tpo occur when *S. senegalensis* metamorphosis is blocked indicating pituitary–thyroid feedback (Campinho *et al*., 2015). Inhibitory control (negative feedback) between the thyroid and the pituitary gland is functional well before metamorphosis while the contribution of the hypothalamus (via thyrotropin releasing hormone, TRH) in flatfish metamorphosis still needs further understanding (Campinho *et al*., 2015). While *S. senegalensis* metamorphosis interruption results on increased Tpo (Campinho *et al*., 2015), in our study, the opposite effect on Tpo expression was observed at 15 dah, with a significant down-regulation in UV exposed fish which might be related with the faster metamorphosis observed 3 days after, at 18 dah. Furthermore, no effect of combined exposure on Tpo expression was observed.

The iodide uptake ability of thyroid follicular cells is highly dependent on the transport protein Sodium-iodide symporter (NIS) (Darrouzet *et al*., 2014; Campinho *et al*., 2015). Several chemicals have been reported to affect the active transport of iodide on the thyroid cascade (Gerard *et al*., 1994; Farwell and Braverman, 2001; Brown *et al*., 2004; Darrouzet *et al*., 2014). Stressors with effects on iodide uptake are particularly harmful for aquatic organisms as while terrestrial organisms obtain iodide mainly from the diet, fish also obtain this ion from the water via the gills (Brown *et al*., 2004). Effects on iodide uptake can therefore lead to long term effects on development of the organism. A previous study with zebrafish (Pinto *et al*., 2012) found a significant increase in NIS expression after exposure to TCS. In our study, the opposite effect was obtained with both UV and/or TCS immediately after exposure (15 dah) and this effect on NIS remained after further maintenance in clean medium (until 24 dah) on fish exposed to single treatments. Such fact is in agreement with a previous UVC study performed with a mammal cell line, where a down-regulation of genes involved in thyroid hormone production was observed, namely on NIS, Tpo and Tg genes (Baldini *et al*., 2013). Our results suggest that TCS and UV might be able to induce

alterations on iodide uptake during *S. senegalensis* metamorphosis through NIS down regulation.

## 5. Conclusions

The exposure to UV and TCS lead to different responses of metamorphosing *S. senegalensis*. In general, TCS induced malformations, decreased swimming behavior parameters and altered metamorphosis progression, while no effects on these endpoints were observed on UV exposed fish. An acceleration of metamorphosis just after TCS exposure was observed, followed by a delay at the end of metamorphosis. Combined exposure to TCS and UV only enhanced effects of single exposures by inducing a faster metamorphosis progression at transient stages.

The effects observed on metamorphosis were associated with down-regulation of specific genes of thyroid axis. Immediately after the exposure, at 15 dah, more genes were affected with TCS (NIS and TSHβ) and UV (TRHβ, Tpo and NIS), than after maintenance on clean medium, at 24 dah, when only NIS gene, responsible for iodide uptake was down-regulated with both stressors. This effect on NIS might have further implications on normal development of sole by impairing iodide uptake.

Overall, our results suggest that even though the period of maintenance in clean medium after exposure, sole larvae were still affected at the end of metamorphosis, which might have implications on the ecological performance of the species that can be compromised at medium or long-term.

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**Effects of single and combined exposure to ultraviolet radiation and triclosan in metamorphosing *Solea senegalensis***

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**Supplementary data**

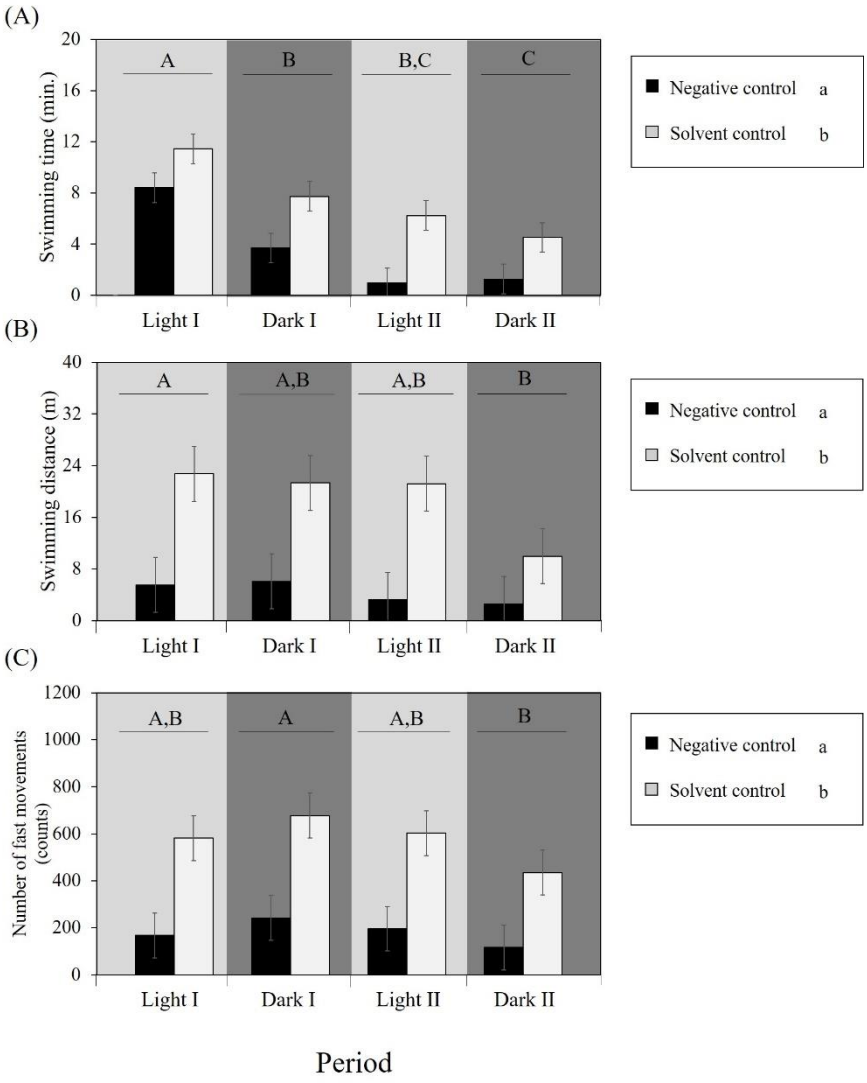


Fig. 6.S1. Behavior of *Solea senegalensis* (negative and solvent control (0.01% acetone) in each 15 min light or dark periods at the end of metamorphosis (24 days after hatching, dah). (A) Swimming duration (min), (B) swimming distance (m) and (counts) number of fast movements (above 15 mm $sec^{-1}$). Results are expressed as mean±SE. Different upper letters represent significant differences between light/dark periods and different lowercase letters in the legend represent significant differences between negative and solvent control ($p<0.05$).

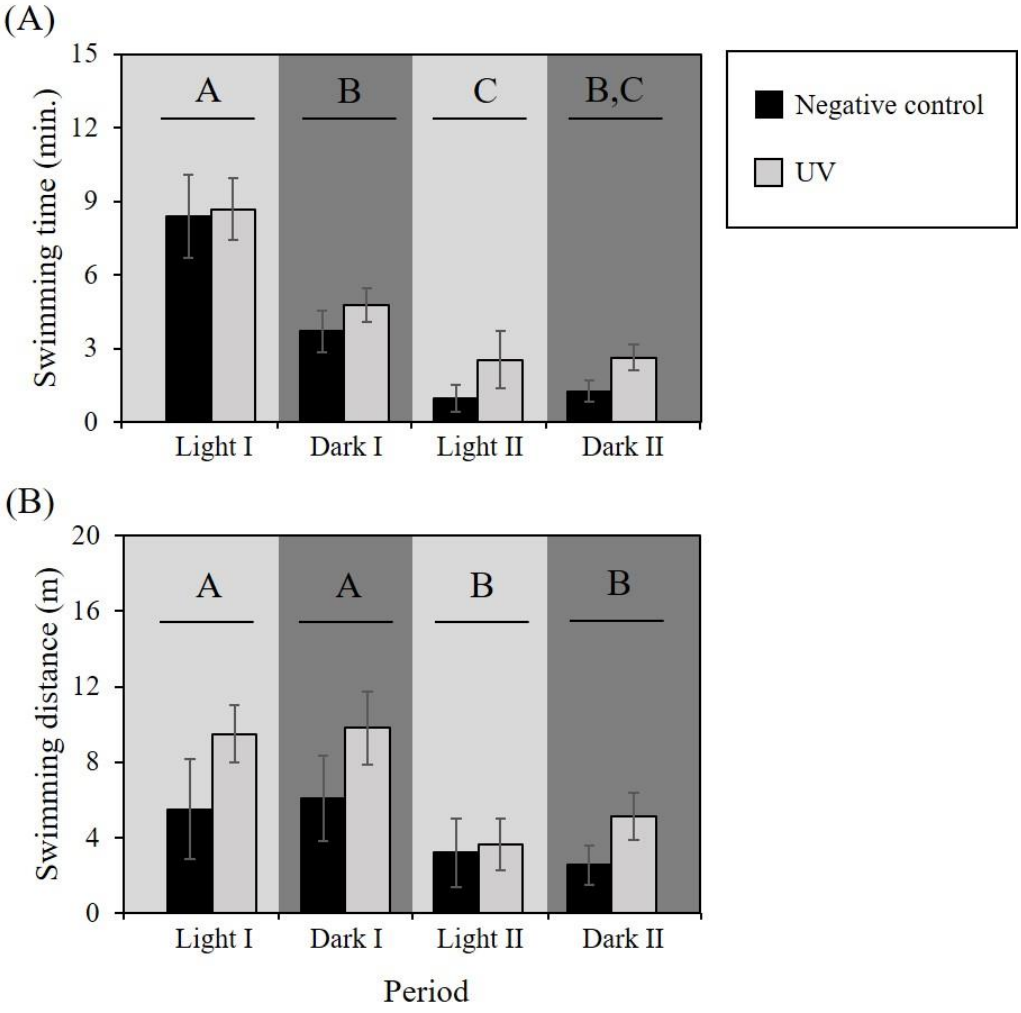


Fig. 6.S2. *Solea senegalensis* behavior in each 15 min light or dark periods at the end of metamorphosis (24 days after hatching, dah) after previous 48h exposure to ultraviolet radiation (UV, daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 dah). (A) Swimming duration (min) and (B) Swimming distance (m). Results are expressed as mean±SE. Different upper letters represent significant differences between light/dark periods ($p<0.05$).

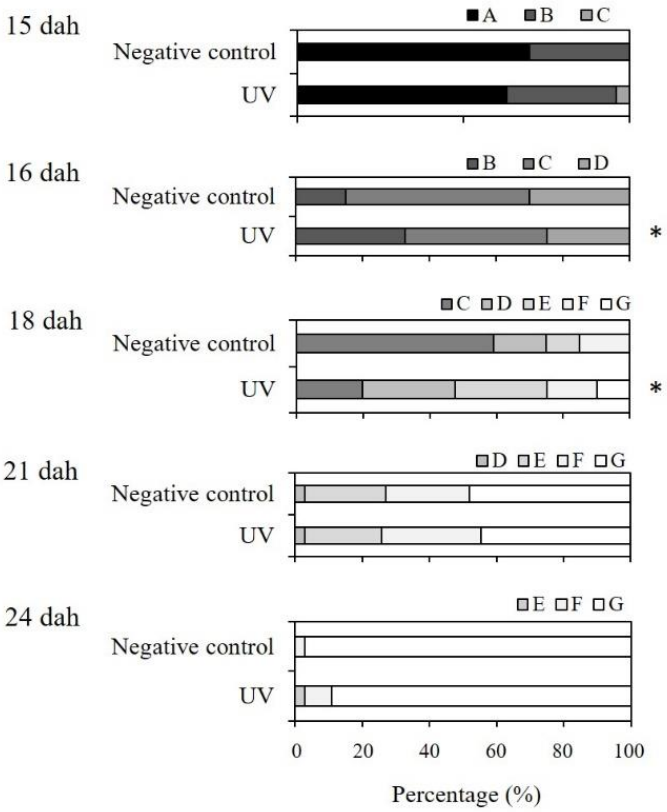


Fig. 6.S3. Metamorphosis stages of *Solea senegalensis* after exposure to ultraviolet radiation (UV, daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 days after hatching, dah). A&B – early metamorphosis (enlargement of fins, beginning of migration of left eye); C&D - alteration of mouth shape and pigmentation darkening; E - fully flattened body; F - anterior profile becomes more curved; G – complete metamorphosis: orbital eye membrane becomes thicker. *represent the existence of significant differences between UV exposed fish group and negative control at each age ($p<0.05$).

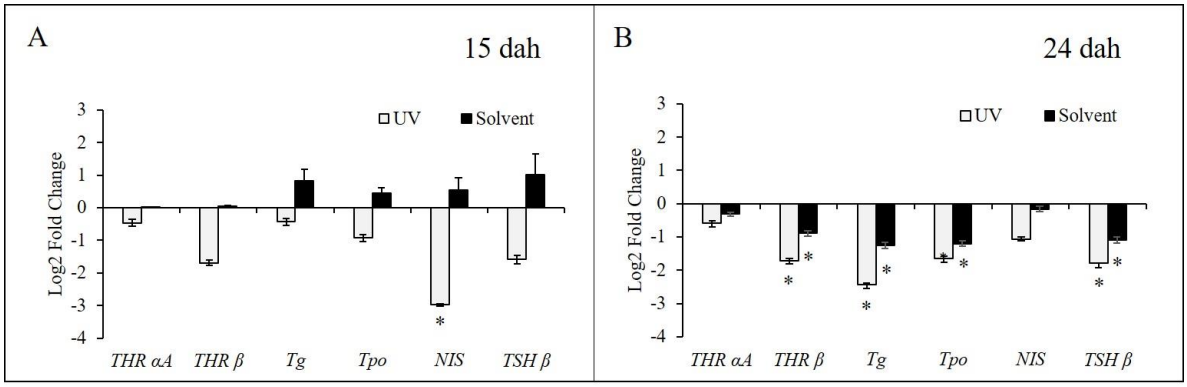


Fig. 6.S4. Relative gene expression in *Solea senegalensis* at (A) 15 days after hatching (dah) and (B) at 24 dah after exposure to UV (daily 3h sessions, total effective dose: 5.9 kJ $m^{-2}$ at 13 and 14 dah) and acetone (0.005%). Results are expressed relatively to negative control as log 2 Fold Change±SE. * indicate significant differences relatively to negative control ($p<0.05$).

Table 6.S1. Intensity and dose of ultraviolet radiation (UV). dah – days after hatching.

| Parameter | 13 dah | 14 dah |
|---|---|---|
| Total intensity ($W\ m^{-2}$) | 1.083 | 1.061 |
| Erythemal intensity[a] ($W\ m^{-2}$) | 0.280 | 0.265 |
| UV Index [b] | 11 | 11 |
| Dose (3h, $J\ m^{-2}$) | 3021.5 | 2867 |

[a] correction according to McKinlay and Diffey (1987).
[b] UV index estimated in accordance with Fioletov *et al*. (2010).

Table 6.S2. Physico-chemical parameters of the testing solutions.

| Exposure group | pH | Salinity | $O_2$ (%) | $O_2$ ($mg\ L^{-1}$) | Temperature (ºC) |
|---|---|---|---|---|---|
| Negative control | 7.91 | 36.8 | 98.7 | 8.83 | 19.3 |
| Solvent control | 7.90 | 36.7 | 96 | 8.61 | 19.3 |
| UV | 7.81 | 36.9 | 89.9 | 7.54 | 19.7 |
| UV (solvent treated) | 7.90 | 37.4 | 99.4 | 8.83 | 19.1 |
| TCS low | 7.79 | 36.5 | 98.6 | 8.74 | 18.9 |
| TCS low + UV | - | 36.5 | 96 | 8.51 | 18.7 |
| TCS high | - | 37 | 95 | 8.4 | 19.5 |
| TCS high + UV | - | 36.5 | 95.9 | 8.51 | 19.5 |

# Chapter 7. General discussion – Effects of PCP ingredients and UV to the early life stages of *Solea senegalensis*

# General Discussion
# Effects of PCP ingredients and UV to the early life stages of *Solea senegalensis*

## 1. Introduction

The increasing production and use of chemicals is leading to their unintentional release and widespread presence on the aquatic habitats. Personal care products (PCP) ingredients are amongst the chemicals with increasing presence on ecosystems and organisms tissues (Balmer *et al*., 2005; Dhillon *et al*., 2015; Buser *et al*., 2016; Chisvert and Salvador, 2018). In addition, increasing ultraviolet radiation (UV) reaching Earth surface has also been reported as a consequence of stratospheric ozone depletion (Stolarski, 2011). Early life stages of aquatic organisms are amongst the most vulnerable organisms to these stressors (Manciocco *et al*., 2014). Limited information exists on the effects of these stressors on early life stages of marine vertebrates, such as the flatfish *Solea senegalensis*. This species has economic and ecological interest in Atlantic and Mediterranean temperate regions and aquaculture reproduction still rely on wild spawners (Morais *et al*., 2016). *Solea senegalensis* larvae has a great potential for use as alternative model species for vertebrate animal testing of chemicals in the marine environment. The hatching of *S. senegalensis* occurs relatively soon (36 hours post fertilization, hpf) and endogenous energy reserves last until 80-100 hpf (Yúfera *et al*., 1999). In addition, this species metamorphosis occurs during the first month of life and is regulated by thyroid hormones (Klaren *et al*., 2008; Power *et al*., 2008). Thus, theoretically this species might be useful to study the effects of stressors acting on the thyroid axis. In this context, this work aimed at studying the effects of two chemicals included in PCP formulations with proved or potential endocrine disruption action, single and in combination with UV on several levels of organization in distinct stages of early life of the marine flatfish *S*. *senegalensis*.

## 2. Cholinesterase characterization and biochemical markers baseline levels along *Solea senegalensis* early development

Through a characterization study of the main cholinesterase forms present along early development of *S*. *senegalensis*, it was concluded that AChE is the most abundant ChE form in whole body samples. In addition, the baseline activity of AChE on *S*. *senegalensis* was similar along the three early development stages studied.

Baseline levels of other biochemical markers in different early life stages of *S. senegalensis* were also determined. These biochemical markers were related to oxidative stress (GST, CAT) and energy production (LDH). Metamorphosis of *S. senegalensis* is a fast developmental stage that ends during the first month of life (with fish total length near 10 mm) and in the present work, the results confirm that marked biochemical fluctuations occur during this period (Yúfera *et al*., 1999; Fernández-Díaz *et al*., 2001; 2006; Power *et al*., 2008; Pimentel *et al*., 2015). Namely, alterations on enzymes related with oxidative stress following the natural progression of metamorphosis are reported to occur (Yúfera *et al*., 1999; Fernández-Díaz *et al*., 2001; Pimentel *et al*., 2015). The variation of basal activity of CAT and GST observed in the different sole early life stages are in agreement with these expected fluctuations. An increasing activity of the enzymes CAT at the onset of metamorphosis was observed, which might be associated with its role on programmed processes of cell death involved in distortion of frontal bone structure (Sun *et al*., 2015) and also in the definition of fins that occurs during this period (Power *et al*., 2008). These alterations are comparable to the processes that occur during tail regression in amphibians metamorphosis (Kashiwagi, 1995; Kashiwagi *et al*., 1997). The GST activity in the present work was also higher at the onset than at the end of sole metamorphosis, possibly in order to cope with the increase of oxidative stress generated during this process. During the metamorphosis of other organisms, the reduction of glutathione levels occurs simultaneously with the depletion of CAT (Menon and Rozman, 2007). In addition, an induction on LDH activity was observed in the present work at the onset of metamorphosis indicating that this stage requires anaerobic energy metabolism pathways, which might be due to the previously reported fact that *S. senegalensis* metamorphosis is an energy demanding period (Yúfera *et al*., 1999).

As early life stages of sole are critical and sensitive periods in the development of this species with profound changes at biochemical, physiological and morphological level, any disturbance induced by environmental stressors might have severe adverse effects on the normal development of these organisms.

### 3. Effects of personal care products ingredients and UV to early life stages of *Solea senegalensis*

There was a wide range of malformations observed on *S. senegalensis*, depending on stressor and development stage, including abnormal spinal curvature at early larval stage, while abnormal migration of the eye and bone structure development were observed during metamorphosis. Moreover, metamorphosing fish seemed to recover from the

malformations along time after the interruption of the exposure to the stressors. *Solea senegalensis* also responded to each stressor at different levels of biological organization which is useful to understand the defense mechanism of the organism in each development stage and also allows to study the specific modes of action of each stressor. Overall, growth of *S*. *senegalensis* was negatively affected by exposure to all stressors tested and at both stages of development. Distinct behavior responses of the larvae to each stressor were also observed, which included decrease of swimming distance and/or swimming duration along the behavior tests. In addition, behavioral responses to the stressors depended on *S*. *senegalensis* early life stage, with the earlier larval stage, in general, being more sensitive than the metamorphosing stage. Effects of stressors on metamorphosis progression rate were also observed and, in the case of triclosan (TCS) they were linked with altered expression of thyroid-axis related genes.

### 3.1. Effects of triclosan to early life stages of sole

The exposure to TCS induced several effects on *S*. *senegalensis* at both development stages studied, including increased mortality and malformations, decreased growth and acceleration of metamorphosis progression at transient stages of metamorphosis. Biochemical markers were the most sensitive endpoint affected at both development stages; however, the pattern of biochemical responses to TCS exposure depended on development stage. Biochemical responses to TCS exposure indicated effects on neurotransmission, oxidative stress and detoxification processes in *S*. *senegalensis* early development stages which are in agreement with previous studies reporting alterations on neurotransmitter and antioxidant system enzymes of vertebrate species (Liang *et al*., 2013; Ku *et al*., 2014; Falisse *et al*., 2017; Ruszkiewicz *et al*., 2017a).

While no alterations on AChE were observed at the sole earlier larval stage, a significant induction was observed immediately after 48h exposure in metamorphosing fish exposed to the two highest TCS concentrations tested. The involvement of thyroid hormones in the regulation of AChE gene transcription has been previously suggested (Puymirat *et al*., 1995; Andrade *et al*., 2016), and therefore, a possible dysregulation of thyroid function by TCS may explain the induction observed in AChE activity. In addition, TCS have been previously reported to activate apoptosis of neuronal cells (Ruszkiewicz *et al*., 2017a). Since apoptosis has been associated with increased AChE activity (Zhang *et al*., 2002; Zhang and Greenberg, 2012), the AChE induction observed in the present work might be related with TCS induction of neuronal apoptosis on *S*. *senegalensis* larvae.

Effects of TCS on CAT activity were not observed at the early larval test nor after the 48h exposure test performed at the onset of metamorphosis. Nevertheless, CAT activity of fully metamorphosed fish were altered by TCS after the period of maintenance in clean media, suggesting later effects on antioxidant mechanisms of this organism.

The increase of GST levels at the earlier larval test, suggests activation of phase II of biotransformation which has been previously reported to occur as a detoxification mechanism of TCS in other species (Ku *et al*., 2014; Wu *et al*., 2017; Peng *et al*., 2018). Among all endpoints studied at this earlier life stage, GST presented the lowest observed effect concentration (LOEC=30 µg $L^{-1}$). On the other hand, during the metamorphosis test, TCS exposure caused GST inhibition, which might be related with compromised phase II of biotransformation during the 48h exposure period. Since different GST responses seem to occur, the specific role of GST in the antioxidant and detoxification mechanisms used by *S*. *senegalensis* in each early life stage still need to be further studied.

### 3.2. Effects of 4-MBC to early life stages of sole

The exposure to 4-MBC increased malformations rate and reduced growth in 3 dah sole larvae. Decreased swimming time was also observed. The exposure to 4-MBC induced alterations on AChE and LDH enzymes; however, relationship between effects at sub-individual level and effects at individual level could not be directly associated since they were observed at different concentrations. Nevertheless, the results suggest exposure to 4-MBC (especially behavior and growth) can have long-term implications on performance of the species, namely by reducing feeding and reproduction success or the ability to escape from predators.

The exposure to 4-MBC also induced effects on metamorphosing *S*. *senegalensis*, including increased malformations rate, delayed metamorphosis progression, decreased growth and increased swimming behavior. Effects on oxidative stress biomarkers (namely through inhibition of enzyme catalase) suggests that 4-MBC affects the antioxidant defense mechanism of metamorphosing sole and causes oxidative damage, since increased lipid peroxidation was observed in the present study. A similar response to 4-MBC on antioxidant system enzymes was also observed in frog larvae (Martins *et al*., 2017). The fact that growth, behavior and biochemical markers were affected at the end of metamorphosis (after nine days of cessation of 4-MBC exposure) should also be highlighted and the alterations on these endpoints can also be related with long-term implications on this species ecological performance. The fact that AChE activity was not affected by the exposure to 4-MBC suggests that this chemical is not neurotoxic; while previous *in vitro* and *in vivo* studies

have suggested the opposite (Ruszkiewicz *et al*., 2017b). Despite not causing alterations on AChE activity, 4-MBC affected metamorphosing sole behavior which was even the most sensitive endpoint. After an initial faster metamorphosis progression of 4-MBC exposed organisms, a delayed metamorphosis at the end of the test was observed. Therefore, effects of this chemical at molecular level should be further studied in order to understand if the observed effects on development induced by 4-MBC are associated with an eventual ability of this substance to interfere with the thyroid axis or if other mechanisms are involved.

### 3.3. Effects of ultraviolet radiation to early life stages of sole

The exposure of *S*. *senegalensis* to UV induced distinct harmful effects depending on development stage of this species. While extreme UV ranges did not cause increased mortality during the early larval test, extreme UV induced mortality to *S*. *senegalensis* at the end of the metamorphosis four days after the end of the exposure, which suggests increased sensitivity to this stressor with organisms' development. In addition, decreased growth of UV exposed fish was observed in both development stages studied, while the progression of metamorphosis was not affected. The UV exposure induced malformations at the beginning of metamorphosis; however, fish exposed to the lower UV index tested were able to recover four days after the end of UV exposure. On the contrary, malformations persisted on fish exposed to higher UV index at the end of metamorphosis, along with a decrease on fish growth. Further study of UV damage at histological level on sole fish fins and epidermis is recommended to deepen the understanding of the UV effects observed on *S*. *senegalensis* fins and pigmentation.

At the earlier stage, the UV decreased swimming distance and speed of fish. On the contrary, UV exposure did not affect behavior at the end of the sole metamorphosis. *Solea senegalensis* change to a nocturnal activity behavior during the metamorphosis and since the behavior test was performed during the diurnal period, this might have hindered the response of the organisms to UV exposure during this life stage. The evaluation of the swimming behavior of metamorphosed sole should considered both nocturnal and diurnal periods to assess if different responses to stressors might occur.

### 3.4. Single and combined effects of triclosan and UV during sole metamorphosis

Scarce information exists regarding the effects of PCP with endocrine disruption action in combination with abiotic stressors on marine fish species in the context of global changes (e.g. Maulvault *et al*., 2019). In certain circumstances, the exposure to endocrine

disruptors may not cause immediate visible damaging effects on the organisms. In addition, sub-lethal exposure to endocrine disruptors might have cascading effects from sub-cellular to higher levels of organization. In this context, effects of UV and TCS were studied at the onset and end of metamorphosis (13 dah and 24 dah, respectively) on *S. senegalensis*. Alteration on the expression of thyroid related genes was linked to effects on metamorphosis progression, and fish development and behavior were also evaluated.

In general, the single exposure to TCS decreased swimming activity and increased malformations rate. In addition, TCS accelerated metamorphosis development at transient stages while a lower metamorphosis progression rate was observed at the end of metamorphosis on TCS exposed fish. On the contrary, effects of single UV exposure on these endpoints were not observed. Overall, responses to TCS exposure were not altered by UV co-exposure with the exception of a faster metamorphosis progression of sole at transient stages. Besides, in general, UV effects were more accentuated when TCS was also used, namely on the acceleration of the metamorphosis and in the percentage of abnormal migration of the eye. Overall, these results suggest that when in combination, the effects observed on these endpoints in metamorphosing *S. senegalensis* are mainly caused by TCS. Therefore, these results also suggest that toxicity effects of TCS in sole are not altered by UV exposure.

Effects of the exposure to UV and TCS were observed in several genes involved in normal thyroid functioning of metamorphosing *S. senegalensis*. In the case of the single exposure to UV or TCS, *S. senegalensis* revealed both immediate and lasting responses, indicating possible thyroid axis impairment. Regarding the TCS exposed fish, altered expression of specific thyroid axis genes was related with the acceleration of metamorphosis progression at transient stages. Therefore, a link between effects on thyroid axis by TCS during sole metamorphosis and effects observed at individual level is suggested, which can be linked to effects on normal development of the species. In the case of UV effects, the down-regulation of genes observed both immediately after exposure to UV and also at the end of metamorphosis, might be related to oxidative damage in thyroid tissues caused directly by UV, which can lead to the dysfunction of this gland. While damaging effects of UV on thyroid gland through the action of reactive oxygen species was previously reported (Rai *et al*., 2018) which still needs further understanding; effects of UV on sole observed at genomic level were not followed by alterations in the progression of metamorphosis.

## 4. Conclusions

The establishment of links between effects of TCS and 4-MBC on early life stages of *S. senegalensis* at sub-individual and individual levels were performed. For instance, the alteration of expression of thyroid axis genes by TCS might have led to altered metamorphosis rate in *S. senegalensis*, indicating a possible thyroid disruption action by this chemical in this organism during this particularly life stage when thyroid hormones have an important role by regulating metamorphosis. Furthermore, since these effects of TCS were observed immediately after the exposure and also at metamorphosis completion, the normal development and fitness of this species might be jeopardized beyond this period as well.

The overall higher resistance or tolerance to the stressors at the metamorphosing stage of *S. senegalensis* when comparing to the earlier stage might be a consequence of the lower exposure period to the stressors performed during this stage. Nevertheless, mechanisms of defense are also more developed at the later development stage enabling the organisms to better cope with the induced stress.

In general, all the stressors studied induced effects just after the exposure period, but effects were also observed after a period maintained in clean water until the end of metamorphosis suggesting lagged and/or lasting effects. Such effects can have further implications on ecological performance of the organisms.

*Solea senegalensis* arises as an useful species for studying different toxicity mechanisms as well as the consequences at individual level of the exposure to stressors that might lead to adverse effects on the ecological performance of this marine species, providing useful information for regulatory and risk assessment purposes. Short periods of exposure to increasing concentrations/doses of the stressors induced relevant effects at several levels of biological organization on both stages of sole early life development studied.

Globally, the results of this work suggest that earlier larval stages of sole were more sensitive than the later metamorphosis life stage. Besides, as the use of this species during the earliest stage (until 3 dah) is not considered animal testing by EU (2010), this *S. senegalensis* life stage can be considered as a good alternative model for ecotoxicological testing for the marine environment. Nevertheless, as the type of response to some endpoints heavily depended on fish development stage, early larvae should not be the only life stage considered in ecotoxicity studies with *S. senegalensis*. The thyroid mediated metamorphosis is also a critical stage of *S. senegalensis* life with deep physiological

alterations; and therefore the use of that stage can provide relevant information to evaluate the effects of stressors suspected of thyroid disrupting action in marine vertebrates.

The present work contributed to increase the knowledge of chemical and abiotic stressor effects on marine species which are still scarce. Since extrapolation of effects from freshwater to saltwater fish species cannot be directly performed and in general saltwater species are more sensitive to chemical stressors (Hutchinson *et al*., 1998) there is a need to assess toxicity effects in marine model species. Indeed, the early larval stages of *S. senegalensis* revealed in general an higher sensitivity when comparing to freshwater fish species.

## 5. Future perspectives

In the present work, relevant adverse effects and mechanisms of action of TCS, 4-MBC and UV were disclosed for a marine fish species; however, the ranges tested were in general above their environmental concentrations/doses. Therefore, the study of these stressors in sole during longer periods and at environmental relevant concentrations/doses should be performed in order to verify effects of stressors in more realistic scenarios. This will increase the complexity of the experimental design in order to fulfill the sole larval stage requirements, which include live food and recirculatory or flow through systems. This can be accomplished performing for instance micro or mesocosm studies and using marine species from different trophic levels (e.g. algae, rotifers, crustaceans) needed for *S. senegalensis* culture. This will require considering several factors, such as the accumulation of the chemicals on food and the degradation and/or metabolization products by the filtering medium or excreted by fish. Besides the exposure to chemical stressors through water, the exposure can also be performed through diet, allowing to assess bioaccumulation through a marine trophic chain.

In addition, performing longer periods of monitoring (i.e. until adult stage) after exposure to the stressors during early life stages should also be performed. This will allow to understand if the exposure during critical periods of early development induce later effects on the ecological performance of the species (e.g. successful preying/feeding, reproduction, escape from predators).

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