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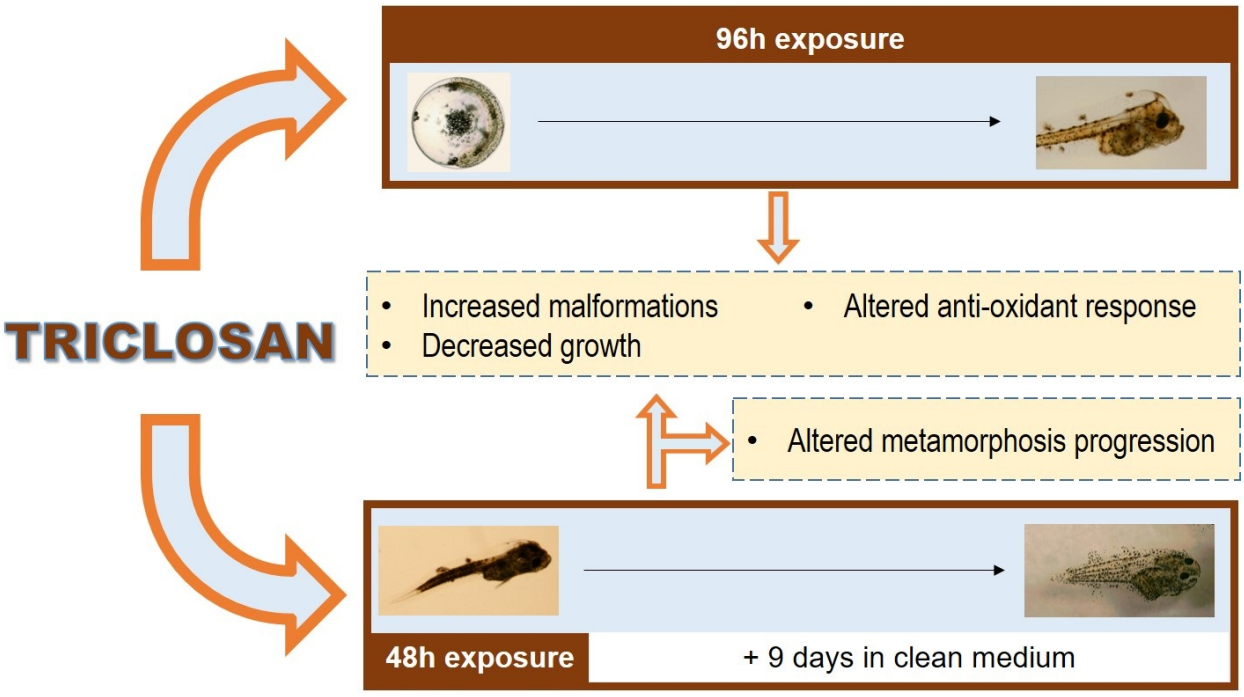
96h exposure
TRICLOSAN
• Increased malformations
• Decreased growth
• Altered anti-oxidant response
• Altered metamorphosis progression
48h exposure
+ 9 days in clean medium

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 along metamorphosis. 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 cholinesterase active form present in sole early life stages is AChE 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; LOEC=391 µg L^{-1} at 17 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 causing long-term effects on catalase at the metamorphosis test, however, no oxidative damage was detected. Glutathione S-transferase was the most sensitive endpoint during early larval test (LOEC=30 µg L^{-1}).

Exposure to TCS affected the development of *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 AChE and of 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*., 2013; 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*., 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: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), with a high affinity for the substrates acetylcholine or 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 in 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 ChEs 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 estuarine and marine fish species early life stages 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*., 2014), 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.

147 The main aim of this study was to assess the effects of TCS on early
148 development of *S. senegalensis* at individual and biochemical level. In order to achieve
149 this, firstly, characterization of the main ChE form(s) present in *S. senegalensis* was
150 performed through the use of different substrates and specific inhibitors. This was
151 evaluated in different early development stages (immediately after yolk sac depletion, at
152 the beginning and at the end of metamorphosis). Secondly, baseline levels of selected
153 biochemical markers were determined at the same stages. Thirdly, survival, growth,
154 malformations, metamorphosis progression and biochemical markers on *S. senegalensis*
155 were evaluated after 96h exposure to TCS at the early larval phase and after 48h at the
156 onset of sole metamorphosis.
157

158 ## 2. Material and Methods

159 ### 2.1. Chemicals

160 Triclosan (Irgasan 97 %) and all chemicals used for characterization of
161 cholinesterases and biochemical analysis were purchased from Sigma-Aldrich Co. LLC
162 (St Louis, USA), except Bradford reagent, which was purchased from Bio-Rad
163 (Germany). Acetone, acetonitrile, methanol and dichloromethane were supplied by
164 Merck. All chemicals used on chemical analysis of TCS were of analytical or HPLC
165 grade quality.
166

167 ### 2.2. Biological material

168 Eggs of *S. senegalensis* were obtained from a commercial hatchery (Sea8,
169 Portugal) and were transported to the lab (2 hours maximum) within 12 hours post
170 fertilization. Transportation was performed in a thermal box, within plastic bags (5L
171 recirculatory system saltwater). Floating lipid-rich eggs in gastrula stage were washed

172 and kept in glass jars in previously matured synthetic saltwater (19ºC, salinity 35, Coral
173 Pro Salt, Red Sea, Saudi Arabia, pH=8.15) until further use. Observation of egg stage
174 and viability was performed using a stereosmicroscope (Nikon SMZ 1270, Nikon,
175 Japan).

176 For the present work, three development stages were considered (fig. S1). The
177 first stage referred to 3 days after hatching (dah). At this stage, full depletion of yolk sac
178 has occurred (Dinis, 1986; Yúfera *et al*., 1999; Klaren *et al*, 2008). The second stage
179 studied refers to the onset of metamorphosis, which highly depends on maintenance and
180 feeding conditions. According to Fernández-Díaz *et al.* (2001), 50% of fish starts the
181 metamorphosis between 9 and 16 days after hatching depending on the typical feeding
182 regimes. The larvae remain pelagic and with bilateral symmetry. The third stage refers
183 to post-metamorphosis organisms. Complete metamorphosed fish are benthic, laterally
184 flattened and asymmetric, the left eye has reached its final position in the right (and
185 dorsally) positioned side of the body and orbital arches are clearly visible. This stage is
186 achieved between 16 and 24 days (Fernández-Díaz *et al*., 2001). The different sole
187 stages used in the present study were within these time frames. Pre-metamorphic fish
188 with 13 dah and post-metamorphic fish with 22 dah were used in ChE characterization
189 and biochemical basal levels determinations. In the TCS test during metamorphosis the
190 48h exposure began at 13 dah and fish were maintained until completion of
191 metamorphosis, at 24 dah. All experimental procedures were carried out following the
192 European and Portuguese legislation concerning animal experimentation (authorized by
193 the Portuguese competent authority, Direcção Geral de Alimentação e Veterinária).
194

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. 30L, 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 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.

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.

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-allyldimethylammonimphenyl) 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 was used with concentrations ranging from 6.25 to 200 µM, BW284C51 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 analyses. 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. GST activity was measured following the conjugation of GSH 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). 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 γ-globuline as a standard and measurements at 595 nm.

The enzymatic activity is expressed in Units (U) per mg of protein. One U is a nmol of substrate hydrolysed 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, one µmol of substrate hydrolysed per minute per mg protein, using a molar extinction coefficient of 40 $M^{-1}cm^{-1}$ for CAT and $6.3x10^3$ $M^{-1}cm^{-1}$ for LDH. All spectrophotometric measurements were performed in 96 well microplates (3-4 technical replicates per each 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 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 each well) previously incubated during 24h with TCS solutions at the same respective concentrations to avoid TCS depletion during the test. 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 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 negative control of fish 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 physical-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 early larval test. Length of fish (from snout to tip of caudal fin) was determined at the end of the 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 the 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, LPO was also measured in samples of TCS tests, by applying the method of 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 and further processed according to Bird and Draper (1984). LPO is expressed in U per mg of protein which represents one nmol of TBARs hydrolysed 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. Eluted 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 (Brisa "LC^2", Teknokroma), particle size 5 µm. 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 salt water (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, statistic differences of ChE activity between control and ChE inhibitor treated samples were tested by One-way Analysis Of Variance (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 for comparing 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 analysis 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 for testing 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 cholinesterases and baseline levels

3.1.1. Cholinesterases characterization

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 1. The AcSCh was the substrate cleaved at highest rate in all development stages of *S. senegalensis* followed by 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}).

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. 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. 2B) for all development stages.

Using AcSCh as substrate, no significant decrease in ChE activity was observed in 3 dah larvae with iso-OMPA, the selective inhibitor of BChE (fig. 2C, $p>0.05$). However, in the other two later life stages, significant inhibition in ChE activity was observed for all tested concentrations with 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 for the concentrations tested, 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. 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. 2E) with a minimum of 70.9% inhibition at the earliest life stage.

3.1.2. Biochemical markers baseline levels

Biochemical markers baseline levels of *Solea senegalensis* on three development stages are presented in table 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, $p<0.05$).

3.2. Effects of TCS

3.2.1. Chemical analysis of TCS

Values of nominal and measured concentrations of TCS are presented in table S1. The difference between measured and nominal concentrations was below 20% and therefore, nominal concentrations were used for all data analysis. 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.

3.2.2. Effects of TCS on sole larvae

Hatching after 24h 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 between exposure groups ($p>0.05$). Hatching was 100% after 48h of TCS exposure in all test groups.

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% mortality of *S. senegalensis* eggs and larvae at 1 dah. The 96h LC_{50} for TCS exposure was 218±10.8 µg L^{-1} (fig. 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, fish exposed to concentrations higher than 53 µg L^{-1} TCS were significantly smaller than fish from solvent control group (between 11% and 20% smaller) ($p<0.05$, fig. 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 24 hours post fertilization (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 this malformation (fig. 3) and an overall EC_{50}=180±18.0 µg L^{-1} TCS was obtained when considering malformations present in the organisms at the end of this test (fig. S4). The teratogenic index was estimated as 1.1.

Effects of TCS at biochemical level on *S. senegalensis* exposed during 96h from egg stage until 3 dah are presented in figure 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 ($p<0.05$).

3.2.3. Effects of TCS during 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% mortality at 18 dah. The test ended at 24 dah, when 80% of organisms from control groups completed the metamorphosis. At that time, 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. S5).

At the end of metamorphosis no differences were observed on total length between fish from solvent and negative control groups (8.9±0.22 and 9.1±0.21 mm for negative and solvent control, 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. S6).

The percentage of malformations along fish metamorphosis (fig. 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 reached, 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

517 exposed to 766 µg L^{-1}; however, the frequency of such malformations tended to
518 decrease over time in the clean media and, at the end of metamorphosis, significant
519 differences between TCS exposed fish and fish in solvent control were inexistent for all
520 concentrations of TCS ($p>0.05$).

521 The frequency of metamorphosis stages at 14, 17, 20 and 24 dah for TCS
522 exposed fish are presented in figure 6. Significant differences on sole metamorphosis
523 progression stages between control groups were only found at 20 dah, with fish from
524 solvent control showing a delay in development when comparing to negative control
525 (48% of negative control fish were in stage E while 39% of solvent control fish were in
526 stage D, $p<0.05$). At 14 dah no differences were observed on metamorphosis stages
527 between solvent control and TCS exposed fish ($p>0.05$). Metamorphosis of fish exposed
528 to 391, 548, 766, and 1072 µg L^{-1} TCS presented a significantly faster progression than
529 in fish from solvent control at 17 dah ($p<0.05$). At 20 dah fish exposed to 1072 µg L^{-1}
530 TCS was still more developed than solvent control ($p<0.05$). However, at the end of the
531 maintenance in clean media (24 dah) no significant differences were observed on sole
532 development stages when comparing solvent control and TCS treatment groups
533 ($p>0.05$).

534 Effects of TCS at biochemical level during metamorphosis of *S. senegalensis* are
535 presented in figure 2.7. No significant differences were observed when comparing the
536 AChE, CAT, GST and LDH activities between solvent and negative control fish groups
537 immediately after the 48h exposure test (15 dah, $p>0.05$). LPO was significantly lower
538 in negative control (0.60±0.035 nmol TBARS mg $protein^{-1}$) when comparing to solvent
539 control fish (0.72±0.034 nmol TBARS mg $protein^{-1}$) immediately after the 48h test
540 ($p<0.05$). At the end of the test (24 dah), differences between solvent and negative
541 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 ($p>0.05$).

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 a 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* lavae. 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, for instance in blood the BChE is the main predominant form (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 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 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. Thes 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. In 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, namelyduring amphibian metamorphosis (Kashiwagi 1995; Kashiwagi *et al.*, 1997). In addition to CAT, GST also works on organism defence 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. The reduction of GST activity is likely to occur considering the decreasing oxidative stress status that occurs along metamorphosis progression. For instance, in anuran metamorphosis, a reduction of glutathione levels occurs simultaneously with the depletion of catalase activity (Menon and Rozman, 2007).

The LDH enzyme has been directly associated with several energetic-related functions and it is expected to increase with growth rate of organisms (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. 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 the present study, effects on *S. senegalensis* below this value were also observed for several endpoints, namely on mortality, growth and prevalence of malformations in larvae and biomarkers at both life stages studied (table 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, the solutions of TCS tested (concentrations 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

664 defence and detoxification mechanisms in older life stages, that enable fish to better
665 cope with TCS exposure.

666 Growth of *S. senegalensis* was affected by the exposure to TCS, both in the
667 early larval and metamorphosis tests (LOEC=95 and 548 µg L^{-1}, respectively), which is
668 in accordance with previous observations in fish species exposed to TCS (Orvos *et al*.,
669 2002; Ishibashi *et al*., 2004; Oliveira *et al*., 2009). In addition, in the marine fish white
670 seabream (*Diplodus sargus*) significant negative correlations were obtained between
671 morphometric data, including total length, and TCS accumulation in fish liver
672 (Maulvault *et al*. 2019). The effects observed on growth of fish species might have
673 severe ecological implications, including delayed or unsuccessful metamorphosis,
674 effects on reproduction and/or ultimately decreased survival.

675 Abnormal spinal curvature was previously reported on *S. senegalensis* larvae in
676 response to xenobiotics exposure (Pavlaki *et al*., 2016; Araújo *et al*., 2018) and was also
677 observed in present work in response to TCS exposure at the early larval test. This
678 malformation has also been reported in zebrafish exposed to TCS (Orvos *et al*., 2002).
679 Triclosan is pointed to be a low teratogenic compound to zebrafish (Ducharme *et al*.,
680 2013) and according to our study, TCS can also be considered relatively low teratogenic
681 to *S. senegalensis*, as the teratogenic indexI is relatively close to the threshold (1).

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

687 Sole metamorphosis was also affected by TCS that induced a faster progression
688 at intermediate development stages. During metamorphosis of anurans the exposure of

689 the organisms to TCS have also been associated with acceleration rate and abnormal
690 timing of metamorphic events and proved to interact with receptors of TH, T3 and T4
691 (Veldhoen *et al*., 2006; Sowers and Klaine, 2008). Progression of metamorphosis is
692 directly dependent on the fluctuation of TH levels (Yamano *et al*., 1991; Okada *et al.*,
693 2003; Klaren *et al.*, 2008). Furthermore, TCS is pointed to interfere with thyroid axis,
694 acting as thyroid disrupting chemical following its structural similarity with the TH
695 (Crofton, 2007; 2008; Veldhoen *et al*., 2006; Luthe *et al*., 2008). Besides, the specific
696 molecular mechanisms through which TCS interfere with metamorphosis progression of
697 *S. senegalensis* are still unknown; the transient acceleration of metamorphosis observed
698 suggests a possible pro thyroid activity of TCS which needs to be further studied and
699 confirmed. As well, other possible mechanisms not directly related with thyroid axis,
700 which might also be responsible for the appearance of malformations phenotype, should
701 also be considered.

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

709 Triclosan exposure has been shown to cause AChE inhibition in some species,
710 namely in juveniles of the marine fish white seabream *Diplodus sargus* (Maulvault *et*
711 *al.*, 2019), amphibian larvae (Martins *et al*., 2017) and in the brain tissue of
712 *Pangasianodon hypophthalmus* fingerlings, a freshwater fish (Sahu *et al*., 2018).
713 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 recovery of exposed fish. Previous works with compounds suspected of thyroid disruption have been reported to induce AChE, namely, in *S. senegalensis* larvae exposed to organic UV filter 4MBC (Araújo *et al*., 2018) and in zebrafish larvae exposed to carbendazim (Andrade *et al*., 2016). The involvement of thyroid hormones 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

739 species, namely in the yellow catfish, *Pelteobagrus fulvidraco* (0.5 µg L^{-1} TCS; Ku *et*
740 *al*., 2014), in *P. hypophthalmus* (above 97 µg L^{-1}; Sahu *et al*., 2018) and in goldfish
741 *Carassius auratus* (above 280 µg L^{-1}; Wang *et al*., 2018). As previously discussed,
742 CAT activity levels are already relatively higher during early metamorphosis and TCS
743 seem not to have the ability to induce CAT activity above the naturally expected during
744 normal progression of metamorphosis. However, effects of TCS on CAT levels were
745 observed at the end of fish metamorphosis, after the period of maintenance in clean
746 media, suggesting long-term effects on antioxidant mechanisms of this organism.

747 In our work, GST activity was affected immediately after exposure to TCS:
748 while a clear induction of GST activity occurred at the end of the early larval test
749 (Lowest Observed Effect Concentration, LOEC: 30 µg L^{-1}), a GST inhibition after 48h
750 TCS exposure at the metamorphosis test was observed (LOEC: 391 µg L^{-1}). Triclosan
751 also caused alterations in GST activity in several fish species, including inhibition in
752 concentrations equal and over 50 µg L^{-1} TCS on adult yellow catfish (*Pelteobagrus*
753 *fulvidraco*, Ku *et al*., 2014) and induction in the liver of swordtail fish (*Xiphophorus*
754 *helleri*) above 20 µg L^{-1}, Liang *et al*., 2013), in *P. hypophthalmus* fingerlings (above 97
755 µg L^{-1}, Sahu *et al*., 2018) and in zebrafish larvae (above 250 µg L^{-1}, Oliveira *et al*.,
756 2009). The GST plays an important role in phase II biotransformation, catalysing the
757 conjugation of the reduced form of glutathione (GSH) with xenobiotics for their
758 increased hydro-solubility (Oost *et al*. 2003; Rudneva *et al*., 2010; Haluzová *et al*.,
759 2011). The GST induction in TCS exposed fish at the end of the early larval test are in
760 agreement with the fact that besides effects on antioxidant system, TCS might also be
761 detoxified through phase II biotransformation as has been proven to occur in several
762 species (Ku *et al*., 2014; Ashrap *et al*., 2017; Wu *et al*., 2017; Ding *et al*., 2018; Peng *et*
763 *al*., 2018). At the metamorphosis test, GST inhibition might have compromised phase II

764 biotransformation and the maintenance in clean medium after TCS exposure seems to
765 have allowed the recovery of GST activity. Therefore, the understanding of which
766 mechanisms of recovery are used by *S. senegalensis* during metamorphosis still need to
767 be further studied.

768 Exposure of goldfish to concentrations between 280 and 560 µg L-1 TCS during
769 14 days increased malondialdehyde levels, indicating oxidative damage of TCS in this
770 species (Wang *et al*., 2018). Mauvault *et al*. (2019) reported an increase of LPO levels
771 in liver of the marine fish *D. sargus* fish exposed to the TCS through diet, however in
772 brain and muscle this increase was not observed. In our study, the exposure to TCS did
773 not cause lipid peroxidation during and/or after exposure to TCS at the early larval and
774 at the metamorphosis tests, suggesting that despite the alterations observed in the
775 antioxidant enzymes, no oxidative damage occurred in the tested conditions.
776 Nevertheless, attention should be given to the fact that effects on antioxidant system of
777 *S. senegalensis* were observed and decreased defense capacity might increase
778 vulnerability to other stressors on the environment.

779 The LDH in zebrafish have been shown to increase in the presence of 250 µg L^{-1}
780 TCS (Oliveira *et al*., 2009). However, in our study, inhibition of LDH activity in sole
781 exposed to almost all concentrations of TCS tested indicates that anaerobic energy
782 metabolism is a less used pathway relatively to control organisms, just after the
783 exposure during early metamorphosis. A direct inhibition of LDH might be occurring
784 leading to an impairment of anaerobic metabolism or the use of aerobic metabolism
785 might be preferred in detriment of the former (Teodorescu *et al*., 2012). This fact,
786 together with a possible increased energy demand for oxidative stress response, might
787 justify effects on growth of the organisms, which were observed at the end of our test.
788

5. Conclusions

Biochemical enzymatic markers were the most sensitive endpoint to TCS exposure at both development stages tested in relation to the apical endpoints studied. Exposure to TCS in levels as low as 30 µg L^{-1} induced effects at biochemical level on the early larval test, which was the overall lowest LOEC obtained. In addition, growth impairment and malformations were also detected at low concentrations (LOEC: 95 µg L^{-1} and EC_{10}: 80 µg L^{-1} TCS, respectively). Despite the effects observed are above the reported environmental 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 life stage window 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 observed effects with higher ecological relevance, namely to on fish growth. Furthermore, as sole metamorphosis is a thyroid regulated process, a possible interference of TCS the existence of alterations on thyroid-axis functioning should be further explored and understood.

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Fig. 1. Cholinesterase (ChE) activity with increasing concentrations of substrate at three stages of development of *Solea senegalensis*: early larvae (3 days after hatching, n=3, 25 organisms per replicate); pre-metamorphic larvae (n=3, 9 organisms per replicate) and post-metamorphic larvae (n=3, 9 organisms per replicate). Acetylthiocoline iodide (AcSCh), propionylthiocholine iodide (PrSCh) and S-butyrylthiocholine iodide (BuSCh) were used as substrates.

Fig. 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, n=3, 20 organisms per replicate); pre-metamorphic larvae (n=3, 9 organisms per replicate) and post-metamorphic larvae (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.

Fig. 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.

Fig. 4. Biochemical markers of *Solea senegalensis* early larvae (3 days after hatching) after 96h exposure to Triclosan. AChE, acetilcolinesterase; CAT – catalase; GST – Gluthatione S-transferase. * 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.

Fig. 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) incorrect 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.

Fig. 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 ($p<0.05$).

Fig. 7. Biochemical markers of *Solea senegalensis* after 48h of exposure to Triclosan at the beginning of metamorphosis (13 days after hatching, dah). Grey columns: 15 dah; white columns: end of metamorphosis (24 dah). AChE, acetilcolinesterase; CAT – catalase; GST – Gluthatione S-transferas; 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.

Fig. 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. 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. 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. 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. 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. 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 1. Baseline levels of biochemical markers in *Solea senegalensis* along early development: early larvae (3 days after hatching, dah), at the onset of metamorphosis (13 dah) and at the end of metamorphosis (22 dah).

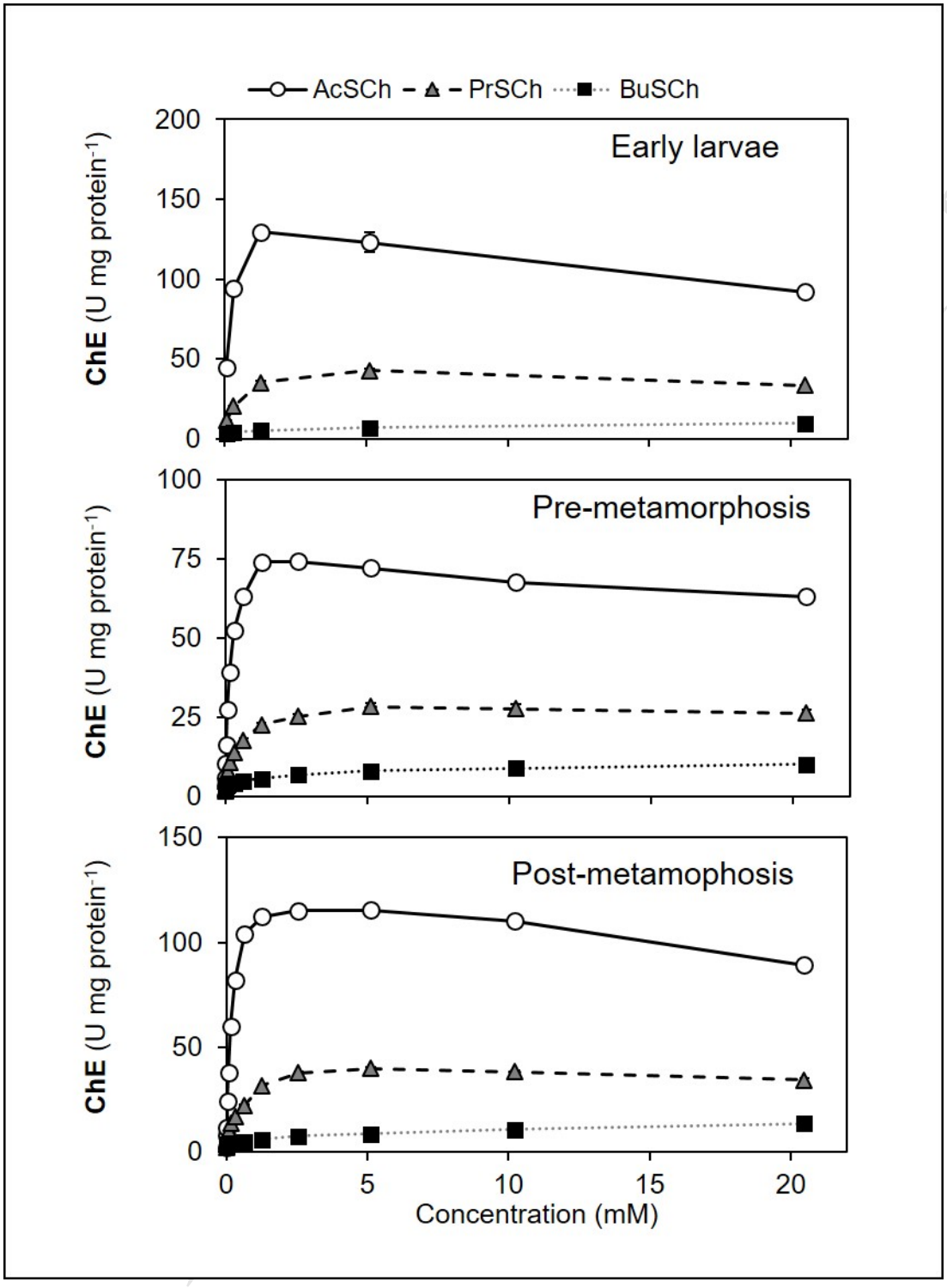
Biomarker	**3 dah** (U mg protein^{-1})	**13 dah** (U mg protein^{-1})	**22 dah** (U mg protein^{-1})
AChE	87.5±2.21	85.1±4.69	96.3±2.67
CAT	7.7±0.29^{a}	11.6±0.72^{b}	6.9±0.47^{a}
GST	18.4±0.60^{a}	16.5±0.94^{a}	10.8±0.30^{b}
LDH	0.046±0.0017^{a}	0.172±0.0036^{b}	0.067±0.0096^{a}

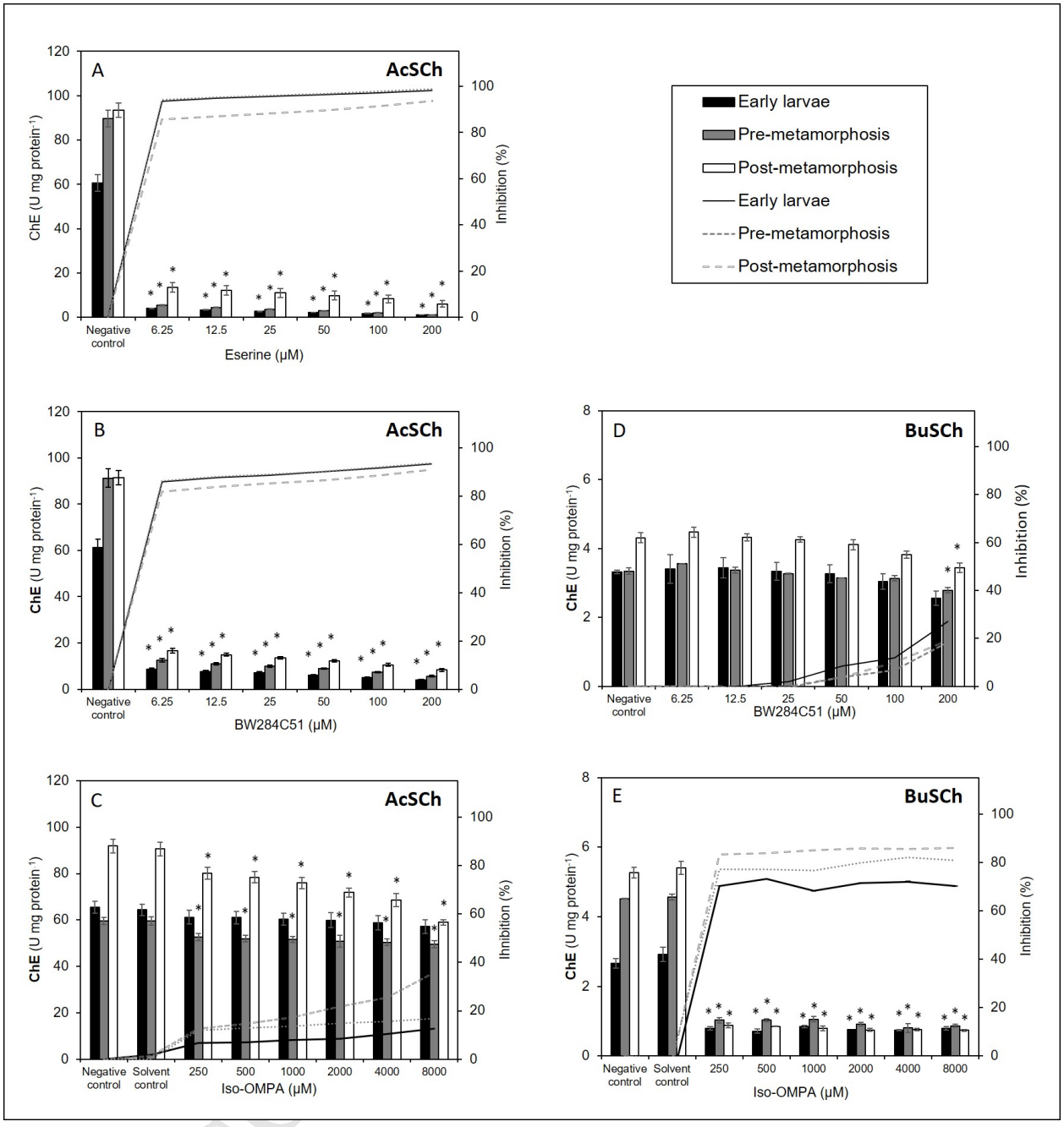
Acetylcholinesterase, AChE; Catalase, CAT; Glutathione S-transferase, GST; Lactate dehydrogenase, LDH. Different superscript letters represent the existence of significant differences within each biochemical marker between life stages. Results expressed as mean±standard error.

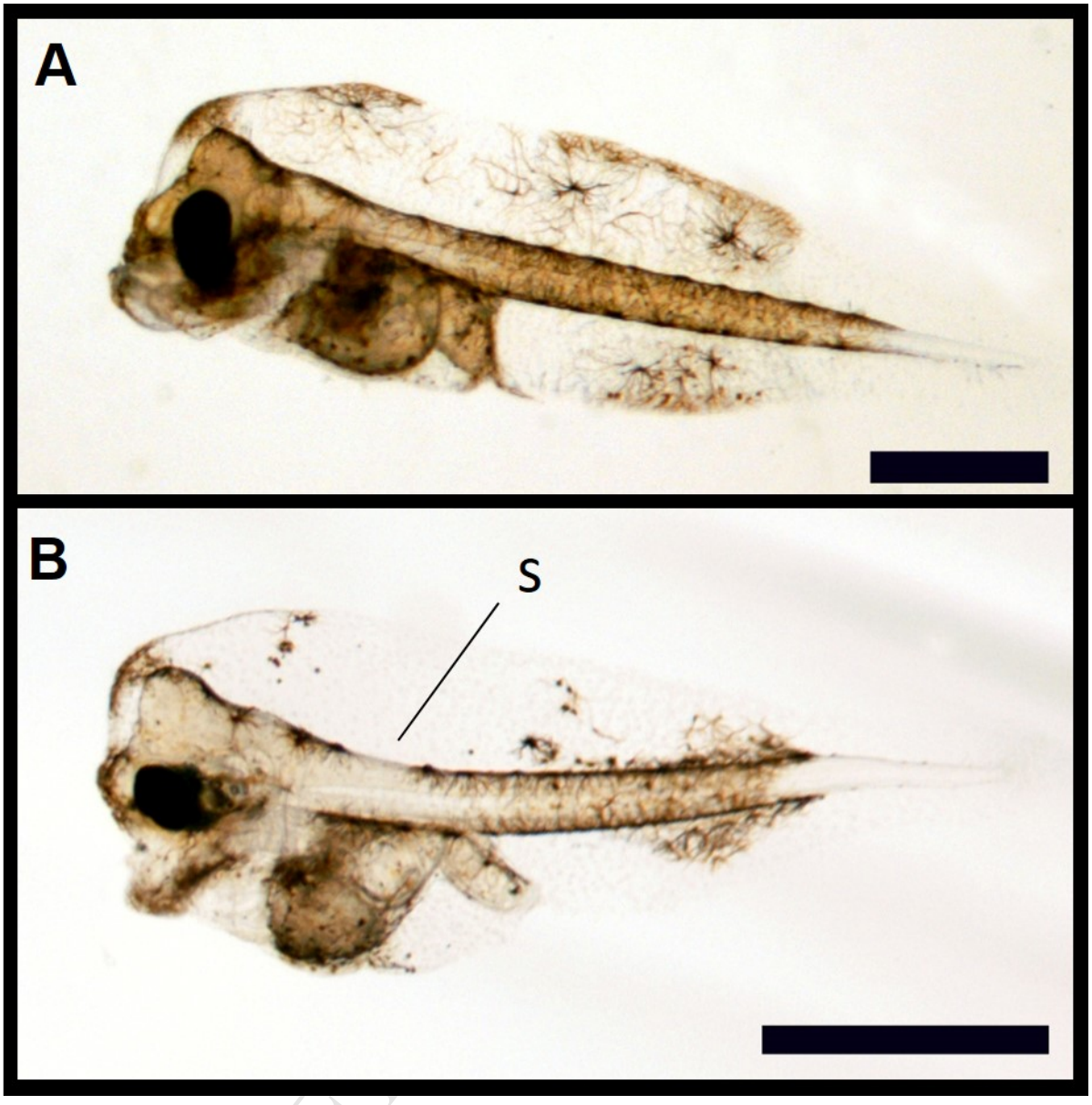
Table 2. Lowest and non-observed effect concentration (LOEC, NOEC) and 10 and 50% effect concentration (EC_{10} and EC_{50}, respectively) in *Solea senegalensis* larvae after 96h of exposure to Triclosan (endpoints measured at 3 days after hatching, dah), just after exposure to 48h at the beginning of metamorphosis (15 dah), and after maintenance in clean medium and completed metamorphosis (24 dah).

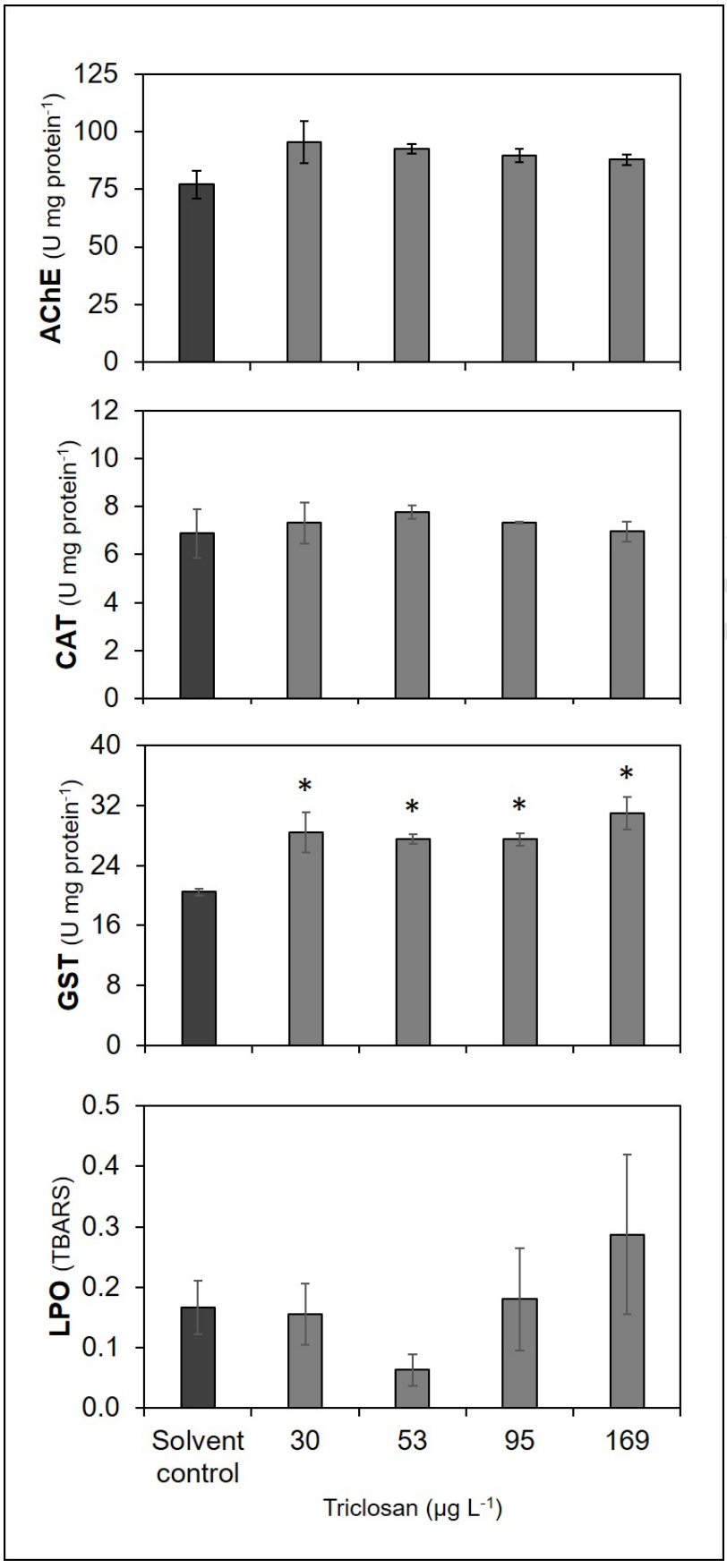
Endpoint	**Life stage**	**NOEC** ($\mu g\ l^{-1}$)	**LOEC** ($\mu g\ l^{-1}$)	**EC_{10}** ($\mu g\ l^{-1}$)	**EC_{50}** ($\mu g\ l^{-1}$)
Mortality	Early larvae (3 dah)	-	-	133 (c.i.: 94 - 162)	218 (c.i.: 196 - 236)
	Metamorphosis (15 dah)	-	-	1083 (c.i.: 1001 - 1207)	1357 (c.i.: 1284 - 1404)
Length	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)		

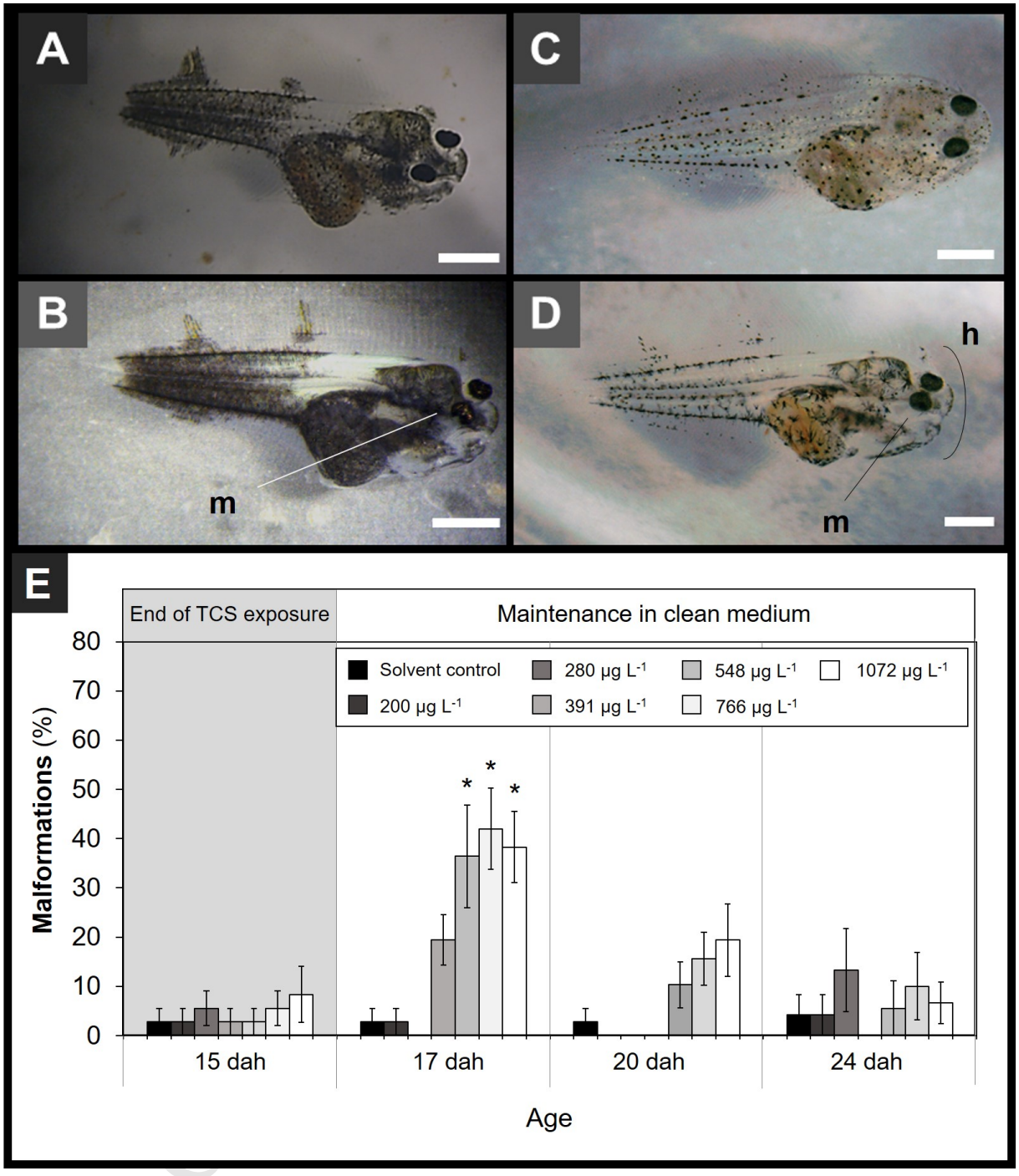
c.i. - 95% confidence interval. GST – Glutathione S-transferase, LDH – Lactate dehydrogenase, CAT – Catalase.

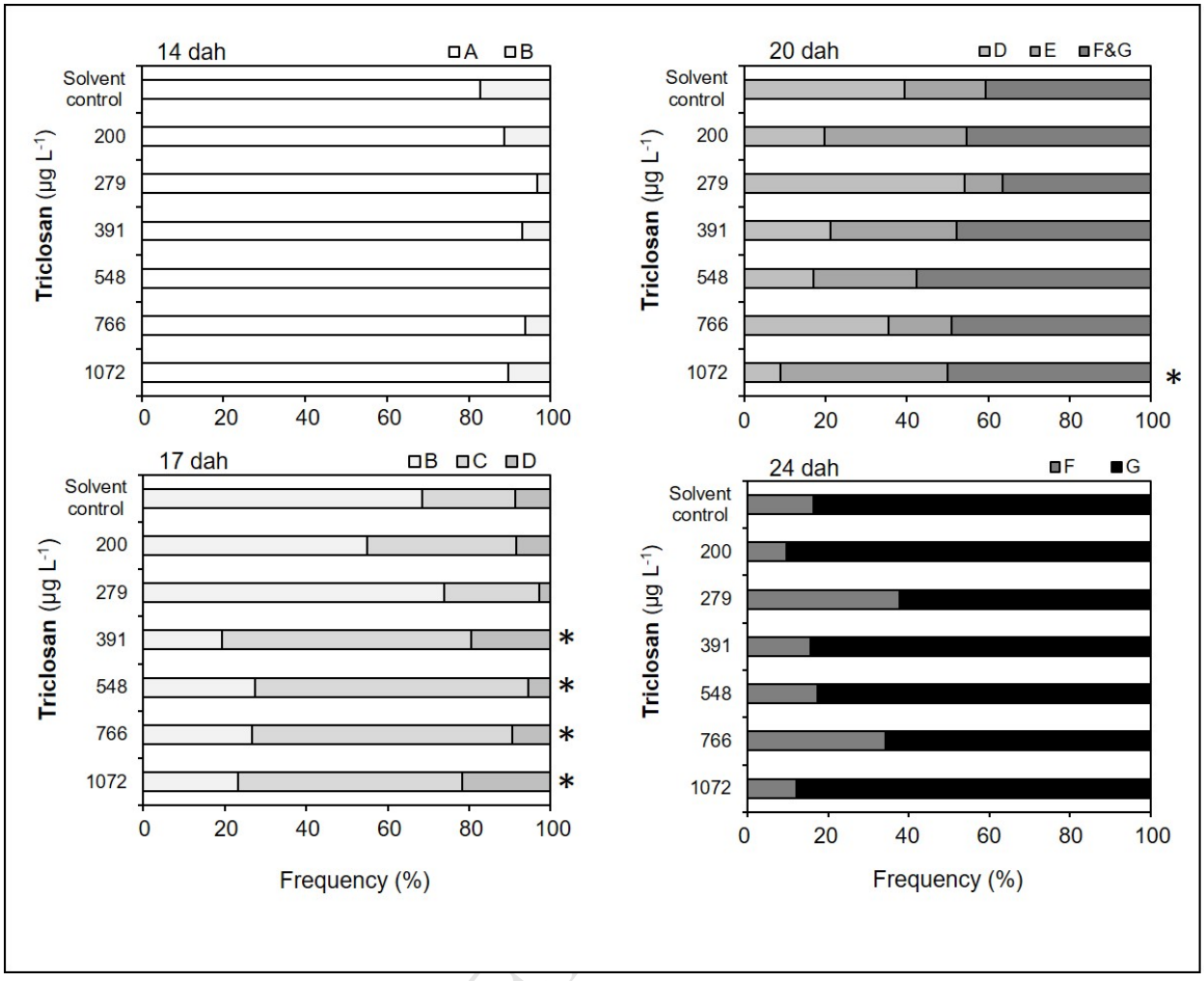

AcSCh
PrSCh
BuSCh
Early larvae
Pre-metamorphosis
Post-metamophosis
ChE (U mg protein-1)
Concentration (mM)
0
5
10
15
20
50
100
150
200
25
75

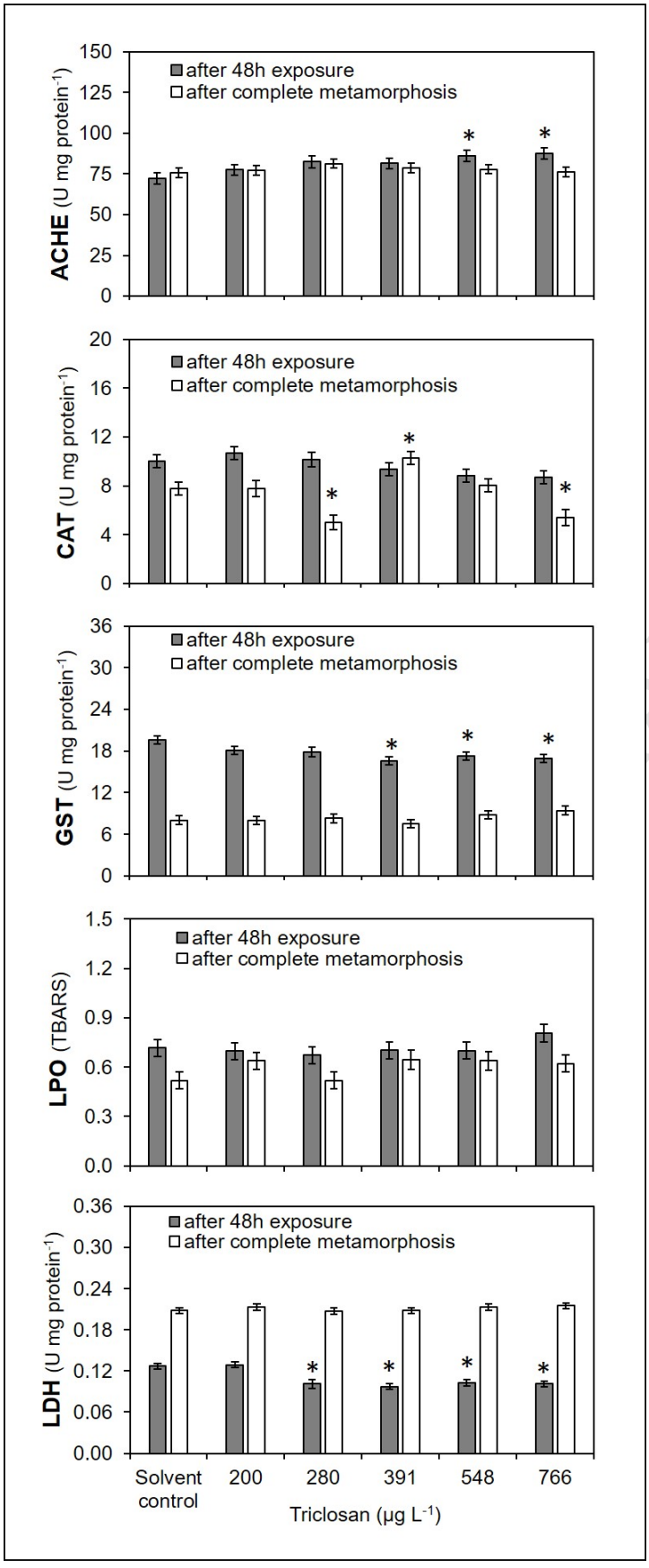

A
AcSCh
ChE (U mg protein⁻¹)
Inhibition (%)
Negative control
6.25
12.5
25
50
100
200
Eserine (μM)
Early larvae
Pre-metamorphosis
Post-metamorphosis
Early larvae
Pre-metamorphosis
Post-metamorphosis
B
AcSCh
ChE (U mg protein⁻¹)
Inhibition (%)
BW284C51 (μM)
D
BuSCh
ChE (U mg protein⁻¹)
Inhibition (%)
BW284C51 (μM)
C
AcSCh
ChE (U mg protein⁻¹)
Inhibition (%)
Solvent control
250
500
1000
2000
4000
8000
Iso-OMPA (μM)
E
BuSCh
ChE (U mg protein⁻¹)
Inhibition (%)
Iso-OMPA (μM)






A
C
B
m
D
h
m
E
End of TCS exposure
Maintenance in clean medium
Solvent control
200 µg L-1
280 µg L-1
391 µg L-1
548 µg L-1
766 µg L-1
1072 µg L-1
Malformations (%)
80
70
60
50
40
30
20
10
0
*
*
*
15 dah
17 dah
20 dah
24 dah
Age


14 dah
A
B
20 dah
D
E
F&G
17 dah
B
C
D
24 dah
F
G
Solvent control
200
279
391
548
766
1072
Triclosan (µg L-1)
0
20
40
60
80
100
Frequency (%)
*


ACHE (U mg protein-1)
CAT (U mg protein-1)
GST (U mg protein-1)
LPO (TBARS)
LDH (U mg protein-1)
after 48h exposure
after complete metamorphosis
Solvent control
200
280
391
548
766
Triclosan (µg L-1)

Highlights

Triclosan induced malformations and decreased growth on sole early life stages

Triclosan accelerated sole metamorphosis at transient stages

Biochemical enzymatic markers were the most sensitive endpoints to Triclosan exposure

Triclosan induced alterations on anti-oxidant system without causing oxidative damage