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Effects of ultraviolet radiation to *Solea senegalensis* during early development

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Abstract

Ultraviolet radiation (UVR) reaching the Earth surface is increasing and scarce information is available regarding effects of this stressor to early life stages of marine vertebrates. Therefore, this work aims to study the effects of UVR exposure during early development stages of the flatfish *Solea senegalensis*.

Firstly, fish were exposed to UVR (six daily doses between 3.4±0.08 and 8.6±0.14 kJ m^{-2}) at the following moments: gastrula stage (24 hours post fertilization, hpf), 1 and 2 days after hatching (dah, 48 and 72 hpf, respectively). In a second bioassay, fish at the beginning of metamorphosis were exposed to UVR (one or two daily doses of 7.2±0.39 or 11.1±0.49 kJ m^{-2}) and then maintained until the end of metamorphosis. Mortality and effects on development, growth and behaviour were evaluated at the end of both bioassays (3 dah and 18 dah, respectively). Biomarkers of neurotransmission (acetylcholinesterase, AChE) and oxidative stress (catalase, CAT and glutathione S-

transferase, GST) were also determined at the end of the early larvae bioassay, and metamorphosis progression was evaluated during the second bioassay.

UVR exposure caused distinct effects depending on life stage. Altered pigmentation and decreased growth, impaired fish behaviour and AChE and GST inhibition were observed at the earlier larval phase. Whereas, decrease in growth was the main effect observed at the metamorphosis stage. In summary, the exposure of *S. senegalensis* early stages to environmentally relevant UVR doses led to adverse responses at different levels of biological organization, which might lead to implications in later life stages.

Keywords: behaviour; biochemical markers; climate change; flatfish; growth; metamorphosis.

1. Introduction

The ultraviolet radiation (UVR) emitted by the sun can be divided in several wavelength regions of the electromagnetic spectrum. The ultraviolet C (UVC) region is between 100 and 280 nm and is the most energetic region, however, it does not reach the Earth's surface as it is filtered by the atmosphere. The ultraviolet A (UVA) is the lowest energetic region (between 315 nm and 400 nm) and ultraviolet B (UVB) is located between the two previous spectral regions and is the most energetic wavelength reaching the Earth's surface.

The UVR can be quantified by measuring the irradiance (in Watts per unit of area) or dose (time-dependent amount of effective energy that reach surfaces, in Joules per unit of area). Erythemal UV index is a non-dimensional value proportional to the corrected

irradiance. This index was adopted worldwide in the early 1990s to easily estimate the effects on immediate short-term impact on human cells (skin reddening) and increase public awareness on the damaging effects of UVR (McKinlay and Diffey, 1987; WHO, 2002; ICNIRP, 2004; Fioletov *et al*., 2010; 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). An UV index equal or higher than 11 is reported as an extreme value (WHO, 2002). Moreover, UV index changes with altitude or latitude and values above 20 have been recorded in extreme conditions, such as near the equator, higher altitude or tropical regions (Kerr and Fioletov, 2008; Cordero *et al*., 2014; McKenzie *et al*., 2015).

Long-term data show large increases of UVR in both Earth hemispheres, mostly caused by changes in ozone in the stratosphe (Stolarski *et al*., 1992; Herman, 2010; Čížková *et al*., 2018). Despite the progressive global ban on ozone-depleting substances since the early 1980's, an increase of UVR can still occur during the following 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).

UVB radiation penetrates the ocean's upper layers and is attenuated by some environmental factors such as turbidity and aquatic vegetation (Häder *et al*., 2015). For instance, the depth where 10% of surface UVR remains (Z10%) in Mediterranean Sea can range between 2.5m and 16m (Tedetti and Sempere, 2006). However, this depth of UVR penetration into the water column can be affected by other environmental factors, which together will decrease the concentrations of dissolved organic carbon and coloured non-living organic matter, in a way that will further increase the penetration of solar radiation in the water column (Zagarese and Williamson, 2001; Häkkinnen *et al*., 2002; Wang *et al*., 2014). Furthermore, irradiation depth might change due to global warming and ozone depletion interactions (Tedetti and Sempere, 2006).

Zooplankton drifting into the upper water column can be particularly exposed to UVR. Among them, ichthyoplankton, namely fish embryos and larvae, are known to be highly sensitive to UVR as they still lack photoprotective pigments (Béland *et al*., 1999; Battini *et al*., 2000; Zagarese and Williamson, 2001).

Natural repairing mechanisms in response to UVR exposure have been described, which include cellular responses that precede responses at physiological level in fish (Blazer *et al*., 1997; Zagarese and Williamson, 2001; Dahms and Lee, 2010). However, excessive UVR is thought to severely affect early development stages of fish causing effects in tissues and organs such as brain, skin and eye, increasing mortality, while also increasing the susceptibility of fish to environmental contaminants (Ahmed and Setlow, 1993; Walters and Ward, 1998; Häder *et al*., 2015; Alloy *et al*., 2016; Sweet *et al*., 2017). Physiological effects of UVB exposure in fish include loss of osmoregulatory capability in larvae (Dethlefsen *et al*., 2001; Sucré *et al*., 2012), decreased oxygen allocation for digestion (Ylönen *et al*., 2004), decreased haematocrit value and plasma protein concentration (Jokinen *et al*., 2008). These physiological alterations can be linked to effects at the individual level such as reduced growth rate and immune depression (Hader et al., 2015). Fish behavioural responses, such as physical avoidance and changes of larvae vertical distribution in response to UVR exposure have also been reported (Speekmann *et al*., 2000; Ylönen *et al*., 2005; Fukunishi *et al*., 2012; Häder *et al*., 2015). The exposure to UVB initiates a series of redox reactions that can lead to oxidative damage in cells and tissues (Zagarese and Williamson 2001; Häder et al., 2015). For instance, effects of UVB on mosquitofish swimming performance have been suggested to be linked with the increased formation of reactive oxygen species (ROS), since ROS were found to impair muscle function through the damage of muscle proteins (Kazerouni *et al*., 2015). The UVB induced damage on DNA and proteins might lead to cell apoptosis (Applegate and Ley,

1991; Lesser *et al*., 2001; Lesser, 2006; Charron *et al*., 2000; Zagarese and Williamson 2001). In addition, effects of UVR on neurotransmission enzymes of aquatic organisms have also been reported (Souza *et al*., 2010). Overall, the UVB-induced effects have high potential to compromise the survival of fish during early life stages. However, the underlying mechanisms resulting from the adverse effects of UV exposure on fish larvae still needs further research.

The *Solea senegalensis* is reported to grow faster and more efficiently during larval development than other flatfish (Sarasquete *et al*., 2019), allowing great potential for its use in assessment of environmental stressors during early life stages (Pavlaki *et al*., 2016; Araújo *et al*., 2018; 2019). The distribution of this species includes the Atlantic coastal areas of Southern Europe and Northern Africa, including the Mediterranean Sea. Their typical spawning, fertilization, and early growing seasons occur during the spring and summer (Imsland *et al*., 2003; Vinagre *et al*., 2013). During this period of the year UV index up to 10 are measured on the Mediterranean coast (McKenzie *et al*., 2003; Marín *et al*., 2005). The *S. senegalensis* are more susceptible to UVR during their pelagic life stages. As eggs, they emerge along water column due to their high lipid content (Yúfera *et al*., 1999). After hatching, despite the ability to avoid excessive radiation; they do not reach great depths until they become benthic at the end of metamorphosis.

Therefore, in this work, we aim at determining the effects of exposure to UVR from subcellular to individual level during early development of *S*. *senegalensis*, namely from egg stage until 3 dah (days after hatching) and also during the progression of metamorphosis. To achieve this, mortality, malformations, growth, behaviour and metamorphosis progression will be studied at individual level and linked with biochemical markers measured at the subcellular level.

2. Material and methods

2.1. Biological material and husbandry conditions

Eggs (less than 12h after fertilization) of *S. senegalensis* were provided by a commercial fish farm (Sea8, Póvoa de Varzim, Portugal) and were maintained with artificial saltwater (red Sea, Coral Pro, Saudi Arabia) with same conditions as in the fish farm (salinity 35; pH 8.2-8.4; temperature 19ºC). A recirculating saltwater system was used to maintain the fish until the beginning of the metamorphosis bioassay. This system included a biological filtering medium, UVR sterilizer, refrigeration (also set at 19 ºC) and a protein skimmer was placed in a room with controlled photoperiod (16h:8h, light:dark). In addition, those fish were fed according to Fernández-Díaz *et al*. (2001) and the diet consisted in rotifers (*Brachionus plicatilis*) from 2 to 6 dah (in increasing concentrations, between 5 and 10 rotifers mL^{-1}), and/or *Artemia salina* nauplii from 5 to 10 dah (between 2 and 9 nauplii mL^{-1}) and *A. salina* metanauplii from 10 dah until the end of metamorphosis (between 9 up to 35 metanauplii mL^{-1}). The green algae *Nannochloropsis gaditana* was added to the system and also to *Brachionus plicatilis* for enriched feeding, All experimental procedures were carried out following the European and Portuguese legislation concerning animal experimentation and authorized by the Portuguese competent authority (Direcção Geral de Alimentação e Veterinária, Ref. 009804).

2.2. Sole early life stages assays

To understand the effects of different UVR doses on *S. senegalensis* early development, two distinct bioassays were performed considering two different exposure periods, namely until 3 dah and at the onset of metamorphosis.

In the first bioassay, eggs of *S. senegalensis* earlier arrived from the commercial fish farm were rinsed with clean artificial medium and individually and randomly placed in 24

polystyrene well plates (n=24, 2 mL of artificial saltwater per well; salinity 35; T=19ºC; no feeding). Fish were exposed to UVR in three consecutive days in the uncovered plates, namely at 24 hours post fertilization (hpf, gastrula stage), and at 1 and 2 dah, at two vertical distances from the UVR lamp (50 or 60 cm, supplementary table S1). The distances between the horizontally placed lamp and the plates correspond to two different levels of total irradiance (1.15±0.029 and 1.62±0.017 W m^{-2}, respectively), and two UVR indices (13 and 17, respectively), which are considered within extreme UVR range. Fish were exposed for 180, 240 or 330 min per day in the light phase of the photoperiod, which corresponded to a total of six daily doses: 3.4±0.08, 4.5±0.10, 6.2±0.14, 4.7±0.08, 6.3±0.10 and 8.6±0.14 kJ m^{-2}. One control group was kept in same conditions (without UVR exposure) along the testing period.

In the second bioassay, *S. senegalensis* just starting metamorphosis (13 dah) were randomly selected and placed in 24 polystyrene well plates (n=24, 1 fish per well, 2 mL artificial saltwater per well; salinity 35; T=19ºC; no feeding). At the beginning of the bioassay, total length of fish was 5.4±0.03 mm (n=80; randomly selected). Fish were then exposed to UVR at two vertical distances from the horizontal placed lamp (45 or 60 cm, supplementary table S2), which correspond to two levels of total irradiance (1.17±0.032 and 1.97±0.052 W m^{-2}, respectively) and two UVR indices (12 and 21, respectively), which are also considered within extreme UVR range. Fish were exposed to UVR once (360 min at 13 dah, or twice (360 min at 13 and 14 dah). The UVR daily doses were 7.2±0.39 or 11.1±0.49 kJ m^{-2}, for fish exposed twice the total dose was 14.4 or 22.2 kJ m^{-2}. Exposure was performed during the light phase of the photoperiod. One control group, without UVR exposure, was kept in similar conditions along the testing period. After 15 dah onwards, all fish groups were maintained under the same conditions (without UVR exposure, daily

water renewal and feeding with *A. salina*) until at least 90% of the fish from control group completed the metamorphosis.

Physico-chemical parameters were measured on both experimental bioassays and are presented in supplementary table S3. Mortality and malformations were checked daily with a stereomicroscope in both bioassays. Length (n=12) was checked with stereomicroscope at 3 dah for the first experiment and at 14, 15 and 18 dah (end of metamorphosis) for the second experiment. Behaviour was assessed at the end of each fish bioassay (3 and 18 dah, respectively). Additionally, at the end of the first bioassay (3 dah), fish were frozen in liquid nitrogen and kept at -80ºC until biochemical markers determination, namely acetylcholinesterase (AChE), catalase (CAT) and glutathione S-transferase (GST). In the second experiment, metamorphosis progression was observed on a daily basis with a stereomicroscope and the stages of metamorphosis (A-G) were registered in accordance with previous studies (Dinis, 1986; Fernández-Díaz *et al*., 2001).

2.3. Behaviour analysis

Randomly selected *S. senegalensis* were used for the behaviour analysis at the end of each experiment. The behaviour of fish after exposure to UVR was assessed using Zebrabox® (Viewpoint, FR) at 3 dah (n=6) and at the end of metamorphosis (n=8). Selected fish were placed individually in new 24-well plates for behaviour analysis. Zebrabox white light was set at an intensity of 10% (0.26 mW cm^{-2}) during four alternating dark/light periods of 10 min after initial 5 min acclimation. In the first stage bioassay, the organisms were acclimated for 5 min in dark and the test began with dark period. In the second bioassay, the acclimation was in light and the test started with light period. An infra-red light (not perceived by the fish and constant at 2.3 mW cm^{-2}) was 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^{-1}). Specific movement thresholds for each development stage were used for each stage test, namely 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

The biochemical markers AChE, CAT and GST were analysed in the 3 dah sole larvae from the first bioassay. Three replicates per treatment (n=3, 5-8 fish per sample) were 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 then used for enzymatic activity determination. The AChE activity was measured by Ellman's method adapted to microplate (Ellman *et al*., 1961, Guilhermino *et al*., 1996) using acetylthiocholine as a substrate and 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) as chromogen. The reaction was followed by measuring the increase of absorbance at 414 nm. The CAT activity was determined by measuring the catabolic 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 (Habig and Jakoby, 1981; Frasco and Guilhermino, 2002). Enzymatic activities are expressed in Units (U) per mg of protein; U represents one nmol of substrate hydrolyzed per min for AChE and GST, using a molar extinction coefficient of $13.6x10^3$ M^{-1} cm^{-1} and $9.6x10^3$ M^{-1} cm^{-1}, respectively. For CAT, U represents one µmol of substrate hydrolyzed per min, using a molar extinction coefficient of 40 M^{-1} cm^{-1}. Protein was determined at 595 nm according to the Bradford method (Bradford, 1976) adapted to microplate using bovine γ-globuline as a standard. All spectrophotometric measurements were performed in 96 well plates using a Labsystem

Multiskan EX microplate reader. All chemicals used for biochemical procedures were purchased from Sigma-Aldrich Co. LLC (St Louis, USA), except the Bradford assay kit, which was purchased from Bio-Rad (Germany).

2.5. UVR lamp and energy measurement

A UVR 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, Fig. S1) 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 UVR lamp during 12 h for radiation stabilization. Values of energy were measured (each 330 and 360 min for the first and second sole bioassay, respectively) with a double monochromator (Bentham DM150-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 UVR dose was 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-weighed total irradiance in W m^{-2} multiplied by 40 (Fioletov *et al*., 2010).

2.6. Statistical analysis

One-way ANOVA, followed by Dunnett's post-hoc tests when applicable, were used to compare differences between control and UVR exposed fish groups on length for both stages and biochemical markers for the earlier larval stage bioassay. For the second stage test, effects of UVR exposure on metamorphosis progression were studied using Chi-Square test.

At the end of both experiments, significant differences between control and UVR exposed fish on the 40 min behaviour test (total swimming distance and time) were studied using One-Way ANOVA or Kruskal-Wallis test followed by Dunnett's or Dunn's tests. A two-way Repeated Measures ANOVA was used to test for the existence of significant interactions between the factors UVR dose and alternating light/dark periods on fish swimming speed. The interaction between factors (UVR dose and light/dark period) was not significant for both life stages and therefore, multiple comparison Tukey test was used to analyse each significant factor individually.

For easiness of reading, the irradiance levels are presented as "lower irradiance level" (corresponding to 1.15±0.029 W m^{-2} and 1.17±0.032 W m^{-2} of un-weighted irradiance for first and second bioassay, respectively) or "higher irradiance level" (1.62±0.017 W m^{-2} and 1.97±0.052 W m^{-2} for the first bioassay and second bioassay, respectively).

Sigmaplot v.12.5 ® (Systat Software Inc.) was used for all statistical procedures.

3. Results

3.1. UVR 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% in control fish (supplementary table S4). In fish exposed to UVR, the mortality ranged between 12.5% (lower irradiance level for 330 min and higher irradiance level for 240 min) and 20.8% (higher irradiance level for 180 min).

3.1.2. Malformations

Overall, the exposure to the highest UVR dose (higher irradiance level for 330 min) caused a higher percentage of organisms with malformations at 3 dah (50.1%, fig. 1, supplementary table S4). Fish exposed to UVR showed abnormal pigmentation characterized by less dark pigments. The fish exposed to higher irradiance level for 180 min presented the highest percentage of abnormal pigmentation (42.1%), while 0% of control fish presented abnormal pigmentation. Spine curvature was also observed in fish exposed to UVR. This malformation was observed at 3 dah in all fish groups, with a maximum percentage of 15.0% in the fish exposed to the highest UVR dose (higher irradiance level for 330 min), whereas control group presented 4.4 % of fish displaying spine curvature.

3.1.3. Length

The total length of fish exposed to UVR for 180 min (for both irradiance levels) was not significantly different from the total length of fish in the control group (2.91±0.05 mm, $p>0.05$). However, fish exposed to UVR for longer periods (240 and 330 min) were significantly smaller ($p<0.05$, fig. 2) with fish exposed to highest UVR dose (higher irradiance level for 330 min) presenting the maximum decrease in length (10.7%).

3.1.4. Behaviour

When analysing the entire period of behaviour testing (40 min), the percentage of time spent swimming was not significantly different between UVR exposed fish and control group (93.7±1.53%), with values in UVR exposed fish ranging between 89.2±3.00% (higher irradiance level for 240 min) and 94.7±1.14% (higher irradiance level for 180 min, $p>0.05$, data not shown). However, considering the total swimming distance, a significant reduction of more than 40% was observed in fish exposed for 240 min to both irradiances

levels tested ($p<0.05$, fig. 3A; 17.2±4.71 and 16.6±8.47 m, respectively) when compared to the swimming distance of fish from the control group (36.9±1.36 m).

Regarding the effect on swimming speed of the alternating periods of light or dark during the behaviour test and the effect of previous exposure of fish to UVR (Two-Way Repeated Measures ANOVA, fig. 3B), no interaction between the two factors was obtained ($p>0.05$). Both factors, UVR exposure and alternate light/dark periods, affected fish swimming speed ($p<0.05$). Lower swimming speeds were observed in fish exposed to both irradiance levels for 240 min ($p<0.05$) when compared to control fish. Furthermore, fish swimming speed was higher in light periods of behaviour testing in relation to dark periods ($p<0.05$).

3.1.5. Biochemical markers

The response of biochemical markers of *S. senegalensis* early larvae to UVR exposure is presented in figure 4. In general, AChE activity of fish exposed to UVR were similar to those of the control group, except in fish exposed for 180 min, to lower irradiance level, which presented a significant decrease of about 19,3% on AChE activity ($p<0.05$). Considering CAT activity, this enzyme was not affected by UVR exposure in any of the tested conditions ($p>0.05$). The activity of GST was significantly lower in fish exposed to both irradiance levels during the shortest exposure time (180 min; $p<0.05$) when comparing to fish from the control group, and the greatest decrease (31.7%) was observed in fish exposed to lower irradiance level.

3.2. UVR effects on metamorphosing sole

3.2.1. Mortality

The cumulative mortality of *S. senegalensis* during the metamorphosis bioassay with UVR exposure is presented in supplementary table S5. No mortality was registered in control fish until the end of the metamorphosis bioassay. Mortality percentage in fish exposed to lower irradiance level was below 10% at the end of the bioassay (18 dah). However, the mortality of fish exposed to higher irradiance level ranged between 19% and 23% at 15 and 18 dah, respectively.

3.2.2. Malformations

The highest percentage of organisms with malformations during flatfish metamorphosis (i.e. damaged fin, abnormal migration of the eye and malformations in cephalic structure) was observed in fish exposed twice to higher irradiance level (14 dah and 15 dah, supplementary table S5). At 15 dah, about 100% of fish exposed twice to lower irradiance level presented malformations. After the UVR exposure, at the end of metamorphosis, all exposed fish groups presented malformations (between 30 and 60%). The most prevalent malformation was fin damage, reaching over 80% of fish exposed to higher irradiance level at 14 dah and at 15 dah of fish exposed twice to both irradiance levels. However, after the UVR exposure, at the end of metamorphosis, the percentage of fish with this malformation decreased to less than 5% in all UVR exposed fish groups. Malformations related with metamorphosis progression, namely incorrect migration of the eye and incorrect cephalic development, were also detected in fish exposed to UVR (fig. 5) presenting a percentage below 10% in fish exposed to UVR at 14 dah. However, the malformations related with metamorphosis increased to near 30% at 15 dah for fish exposed to higher irradiance level

and at the end of metamorphosis they increased in all UVR exposed groups to percentages between 30% and 60%.

3.2.3. Length

After UVR exposure, at 14 and 15 dah, no differences were observed in fish length when comparing UVR exposed fish with the control group ($p>0.05$; fig. 6). However, at the end of the metamorphosis (18 dah), a significant decrease in length of about 8.3% and 6.9% was observed in fish exposed for 360 and 720 min, respectively, to the higher irradiance level ($p<0.05$).

3.2.4. Metamorphosis progression

No significant differences were observed between control and UVR exposed fish on metamorphosis progression between 14 and 18 dah (Chi-square test $p>0.05$; data not shown). In the 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 UVR exposed groups, at 18 dah, the frequency of organisms with complete metamorphosis (stage G) ranged between 77.9±7.10% for fish exposed to higher irradiance level for 720 min and 89.5±5.22% for fish exposed to lower irradiance level for 360 min.

3.2.5. Behaviour

There was no effect of UVR on the percentage of time spent swimming and total swimming distance during the 40 min of the behaviour test observed at the end of metamorphosis ($p>0.05$; data not shown). The percentage of time spent swimming was 57.7±4.74% for fish in the control group and ranged between 49.4±5.30% for fish exposed to lower irradiance level for 360 min and 58.5±5.69% for fish exposed to higher irradiance level for 720 min. The total swimming distance of fish from control group was 3.1±0.16 m

and ranged between 3.2±0.75 m and 4.0±0.31 m for fish exposed to lower irradiance level for 360 min and to fish exposed to higher irradiance level for 360 min, respectively.

The factors UVR exposure and alternate light/dark periods, revealed no interaction on swimming speed of flatfish (Two-Way Repeated Measures ANOVA, $p>0.05$, supplementary fig. S2). While UVR exposure did not significantly alter the fish swimming speed ($p>0.05$), light/dark periods had a significant effect in this parameter, with fish globally swimming faster during dark periods ($p<0.05$).

4. Discussion

Solar radiation has an important role on normal development of fish such as skin pigmentation, circadian rhythms, hormonal cycles, enabling primary production and preying or contributing for heat generation (Boeuf & Bail, 1999). However, excessive UVR exposure might disrupt normal development and induce a cascade of events with lasting adverse effects. In this study, the effects of UVR within an extreme range were studied in two early life stages of development of *S. senegalensis*, namely between egg stage and 3 dah and during the phase of metamorphosis. The exposure to UVR induced effects both at individual and subcellular level, which are summarized in the supplementary table S6.

4.1. Early larvae bioassay

The survival of sole larvae exposed to extreme UVR conditions was higher than 80% until 3 dah in the present work. Lack of standardized UVR assay conditions (e.g. wavelength predominance, energy intensity or duration) among other variables (e.g. organism life stage) impair proper comparisons between different studies. Nevertheless, in a study with eggs and early larvae of another flatfish species, plaice *Pleuronectes platessa*, no clear dose-response of UVB on mortality was observed (Steeger *et al*., 2001). In other studies,

lower survival was observed in early larval stages of other fish species when exposed to high doses of UVR radiation (Béland *et al*., 1999; Battini *et al*., 2000; Sucré *et al*., 2012).

Different malformations were observed in *S. senegalensis* early larvae exposed to UVR, including spine curvature and pigmentation alterations. The exposure to UVR have also led to significant increase of spinal deformities at UVR doses as low as 1.188 kJ m^{-2} in zebrafish embryos (Dong *et al*., 2007; Nuñez *et al*., 2012; Aksakal *et al*., 2018), 1.238 kJ m^{-2} in rainbow trout larvae (Dargaei *et al*., 2014). The exposure to UVA also caused similar malformations in medaka larval stages (Sayed and Mitani, 2016). Furthermore, Nuñez et al. (2012) have linked increased expression of osteonectin in zebrafish larvae with the presence of UVR induced spinal curvature. Therefore, one of the possible molecular mechanisms of these UVR induced phenotypic anomalies might be related with an interference in the expression of osteonectin, a protein described as a major noncollagenous constituent of vertebrate bones whose expression appears early in development and has an essential role in skeletal development (Nuñez *et al*., 2012).

Several authors have suggested a relation between increased pigmentation in fish skin, namely increased melanin levels with activation of natural repairing mechanisms in response to UVR exposure (Blazer *et al*., 1997; Zagarese and Williamson, 2001; Häder *et al*., 2015). In the present study, instead of increased pigmentation, an apparent decrease in pigmentation and/or whitening of *S. senegalensis* skin was observed after excessive UVR exposure. This might be related with effects already described by different authors for sunburn, namely dermal lesions characterized by white necrotic areas (Bullock, 1982) and/or epidermis thickening as shown in another sole species exposed to UVR (MacFadzen *et al*., 2000). Goblet cells develop early in fish epidermis and are responsible for the production of mucous, which have been described to confer protection against injuries and infections (Dash *et al*., 2018; Reverter *et al*., 2018) that can be developed after

UVR exposure. However, in *S. senegalensis*, the goblet cells only became evident in the epidermis from 15 dah (Sarasquete *et al*., 1998), therefore, until 3 dah sole larvae do not have this mechanism of protection. Further histological studies focused on fish epidermis should be performed in order to understand the UVR effects on skin tissues of *S. senegalensis* early life stages.

Growth of *S. senegalensis* was inhibited at 3 dah by UVR exposure, which is in accordance with previous works in other aquatic species, including fish (e.g. Häder *et al*., 2015) and amphibians (Misra *et al*., 2002). Growth inhibition in fish early stages due to UVR exposure has been related with DNA damage, decrease in protein levels (Zagarese and Williamson, 2001) and impairment on immune system (Sharma *et al*., 2010). Furthermore, reduced growth in UVR exposed fish have also been linked to decreased ecological performance, which might have consequences at higher levels of biological organization (Fukunishi *et al*., 2012).

Distinct effects of UVR on behaviour of several fish species have been reported, including impaired escape behaviour in Atlantic cod larvae (Fukunishi *et al*., 2012) or decrease of swimming activity of *Danio rerio* larvae (Hurem *et al*., 2018). However, in the *D. rerio* study, only very high UVA doses affected behaviour. In our work, an overall decrease of the swimming activity of 3 dah larvae exposed to UVR (which included mostly UVB radiation and low UVA energy levels) was also observed. This impairment in behaviour might negatively affect fish growth, as fish with less ability to swim might have lower feeding success. As a consequence of these effects individual decrease in ecological performance and fitness of the organisms can occur (e.g. reproduction, escaping from predators), which may have further implications at population level (Häder *et al*., 2015).

Considering UVR effects on the biochemical markers of sole at 3 dah, the activity of the enzyme AChE was inhibited by UVR exposure to the higher irradiance level for 180 min,

suggesting an impairment in neurotransmission in sole larvae. Exposure to UVR has been shown to inhibit AChE in two species of copepods (Souza *et al*., 2010) and in a terrestrial isopod (Morgado *et al.*, 2013; Ferreira *et al.*, 2016). The inhibition of AChE might occur by direct action of UVR denaturing the enzyme as described in vitro by Bishop *et al.* (1980) or indirectly through the increase production of reactive oxygen species (ROS), namely hydrogen peroxide (H_2O_2). ROS is known to have the ability to up-regulate AChE expression, but when present in high levels H_2O_2 has the ability to inactivate the enzyme (Kurzen and Schallreuter, 2004). However, in the present study, AChE was only inhibited in sole larvae with an intermediate dose of UVR, and no effect was observed with the other doses of UVR tested. Ferreira *et al*. (2016) have obtained similar results when exposing a terrestrial isopod to increasing doses of UVR, registering inhibition of AChE only with intermediate doses. The observed pattern in AChE and the associated mechanism(s) require further investigation.

UVR radiation is a known pro-oxidant agent and is able to induce the production of ROS in organism tissues and organs (Zagatese *et al*., 2001; Seebacher *et al*., 2016). Organisms have complex antioxidant systems that can act against ROS and is constituted by enzymatic (e.g. CAT, GST) and non-enzymatic antioxidants acting synergistically. In the present study, while CAT activity was not affected by UVR exposure, GST activity was inhibited at the lower time of exposure (180 min) in both irradiance levels studied. Different studies report distinct effects on GST activity in organisms following UVR exposure. For instance, while GST was increased in daphnia (Wolinski *et al*., 2016), in copepods (Souza *et al*., 2012) and in butterflies (Meng *et al*. 2009), GST was inhibited in tubifex worms (Misra *et al*., 2002), in an insect species (Karthi *et al.*, 2014), in the epidermis of mice and in cultured human keratinocytes (Seo *et al*., 1996). Several biochemical processes might be involved in such different responses, namely UVR induction of GST to cope with

oxidative stress, or as described by Seo *et al*. (1996), through direct inactivation or malfunction of GST due to UVR exposure. In the present study, the non-activation of the antioxidant system, verified by the non-induction of both CAT and GST, might lead to increased levels of ROS in fish larvae. Furthermore, since the spectrum shape of the lamp used in UVR exposure testing (e.g. peak > 300 nm) might also favour ROS formation (Myakishev-Rempel *et al*., 2011), the possible existence and extent of oxidative damage should be evaluated.

Overall, in the early larval bioassay, despite some of the endpoints assessed (i.e. behaviour and biochemical markers) not responding constantly to increasing UVR doses, sole larvae growth was affected in longer exposures to higher UVR intensities. Therefore, further studies, including other sub-individual endpoints that can lead to growth impairment, need to be addressed.

4.2. Sole metamorphosis bioassay

The adverse effects of UVR radiation during critical windows of development in the life history of aquatic organisms, namely fish metamorphosis is poorly studied. In the present study, the 48h-exposure to extreme UVR levels at the onset of *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 mortality registered, as well as the decrease in growth, might be a consequence of alterations at lower levels of biological organization on metamorphosing sole in response to UVR exposure. The observed metamorphosis-related malformations (incorrect eye migration and cephalic development) may have also contributed to these observations. Impairments in behaviour and metamorphosis progression are also known to have further implications on survival; however in this study these parameters were not affected in UVR-exposed metamorphosing sole.

The occurrence of abnormal metamorphosis in flatfish has been previously described and is usually characterized by the occurrence of pigment alterations, bone deformities and abnormal or non-migration of the eye (Power *et al*., 2008). In the present study, sole exposed to UVR presented fin damage, from which fish recovered by the end of metamorphosis. This suggests the existence of specific recovery mechanisms to deal with occasional excessive UVR. On the other hand, UVR exposure affected sole growth and elicited metamorphosis related malformations that were notorious by the end of metamorphosis (18 dah), even after 4 days without exposure to UVR. The later occurrence of malformations is of particular concern as they can be associated with impacts at later development stages. Similar to the effects of UVR exposure observed during early larval stage, the effects observed on metamorphosing sole might affect individual fitness with consequences on fish populations. Previous studies with amphibians have also shown that UVR can induce abnormalities, delayed development and growth inhibition during metamorphosis (Ankley *et al.*, 1998, 2002) and can led to carry-over effects in later life-history traits even if no immediate damage is observed (Ceccato *et al*., 2016; Pahkala *et al.*, 2001).

The production of thyroid hormones (TH) and expression of TH-responsive genes play an important role in the development of flatfish and amphibians towards metamorphic climax (Galton 1992; Klaren et al., 2008; Power et al., 2008; Buchholz, 2017). Different studies have shown that UVR exposure can interfere with the thyroid system of vertebrates, namely in mammals and amphibians (Croteau *et al.*, 2008, 2009). For instance, Croteau *et al*. (2009) reported a potential thyroid-based mechanism of action for the developmental delay observed in metamorphosing amphibians exposed to UVR. Given this finding, we hypothesized that exposure to UVR in the onset of *S. senegalensis* metamorphosis would lead to alterations in metamorphosis progression. However, despite the metamorphosis-

related malformations observed, no effect on metamorphosis progression was observed in any UVR conditions tested. Therefore, to better understand the potential interference of UVR on thyroid axis during flatfish metamorphosis, a mechanistic based approach at genomic level is recommended.

It is expected that the behaviour activity pattern of *S. senegalensis* might change between light and dark periods according to its natural diurnal or nocturnal activity, as observed in pre-metamorphic and metamorphosed larvae, respectively (Blanco-Vives *et al*., 2012; Araújo *et al*., 2018). In the present study, such a 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. The UVR exposure affected behavioural patterns at an earlier sole life stage, however such effects were not observed during the metamorphosis lifestage bioassay. As expected, metamorphosed fish exhibited higher swimming speeds at dark periods, as metamorphosis larvae switch from diurnal to nocturnal behaviour (Blanco-Vives *et al*., 2012). Furthermore, our study suggests that UVR effects at the behavioural level depend on the development stage and a remark should also be given to the fact that early pelagic *S. senegalensis* larvae seem to swim much longer distances than benthic post-metamorphosed fish during behaviour tests. During light period at nearly 3 dah, fish are starting to actively search for food; while the behaviour 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, performing the behaviour test during the dark phase of a photoperiod might be worth consideration with post-metamorphic *S. senegalensis*.

4.3. Conclusions

Our results indicate that UVR induced adverse effects on both early life stages of *S. senegalensis*. However, the responses obtained were dependent on the sole's life stage. Impairments on behaviour and decreased growth induced by UVR exposure were the main observed effects in the first early life stage (until 3 dah). However, survival was not affected. On the other hand, the UVR exposure during metamorphosis did not affect fish behaviour, nor metamorphosis progression, but led to adverse effects at the end of metamorphosis, namely a decrease in fish growth. This suggests that exposure to UVR during this critical sole life stage period might compromise normal fish growth. Moreover, the exposure of *S. senegalensis* early stages to UVR led to adverse responses at sub-individual and individual levels, which might lead to adverse effects at higher levels of biological organization. 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. Further studies considering other endpoints and other life stages should be performed in order to understand effects of UVR at higher levels of biological organization.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1. Malformations of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UVR). Fish were exposed three times to 1.15±0.029 W m^{-2} or 1.62±0.017 W m^{-2} of irradiance at 24 hours post fertilization, 1 and 2 dah, during 180, 240 or 330 min per day. i) control fish; ii) fish with lack of pigmentation (330 min of exposure to 1.62±0.017 W m^{-2}); iii) fish with spine curvature (180 min of exposure to 1.62±0.017 W m^{-2}). Black bar represents 1 mm.

Figure 2. Length of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UVR). Fish were exposed three times to 1.15±0.029 W m^{-2} ("Low irradiance") or 1.62±0.017 W m^{-2} ("High irradiance") at 24 hours post fertilization, 1 and 2 dah, during 180, 240 or 330 min per day. * represent the existence of significant differences between control and organisms exposed to UVR ($p<0.05$).

Figure 3. Swimming distance (A) and speed (B) of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UVR). Fish were exposed three times to 1.15±0.029 W m^{-2} ("Low") or 1.62±0.017 W m^{-2} ("High") of irradiance at 24 hours post fertilization, 1 and 2 dah, during 180, 240 or 330 min per day. A - * represent the existence of significant differences between control and organisms exposed to UVR ($p<0.05$). B - Swimming speed was estimated based on distance recorded in four alternate dark and light periods of 10 min each. Two-way Repeated Measures ANOVA was performed considering as factors the UVR exposure and alternating dark/light periods. CTR – control. Upper case letters represent differences between dark/light periods and lower case letters in the legend box represent the differences between UVR treatments ($p<0.05$).

Figure 4. Biochemical markers of *Solea senegalensis* early larvae at 3 days after hatching (dah) after exposure to ultraviolet radiation (UVR). Fish were exposed three times to 1.15±0.029 W m^{-2} ("Low irradiance") or 1.62±0.017 W m^{-2} ("High irradiance") at 24 hours post fertilization, 1 and 2 dah, during 180, 240 or 330 min per day. AChE - Acetylcholinesterase, CAT – Catalase, GST - Glutathione S-transferase. * represent the existence of significant differences with control ($p<0.05$).

Figure 5. Malformations related with metamorphosis in 18 days after hatching (dah) *Solea senegalensis* after exposure to ultraviolet radiation (UVR). Fish were exposed to 1.17±0.032 W m^{-2} or 1.97±0.052 W m^{-2} during 360 min at 13 dah or 2x360 min per day at 13 and 14 dah. Metamorphosis was complete at 18 dah. At this moment, the organisms are fully flatened and pigmented and eyes are at the final position. The orbital arch (o) is also well developed on the control (left) and UVR exposed organism (right, 2x360 minutes to 1.97±0.052 W m^{-2} of irradiance); however, the anterior cranial region (c) is not correctly round and developed on the UVR exposed organism affecting the eye position. Black bar represents 1 mm.

Figure 6. Length of *Solea senegalensis* during metamorphosis bioassay (14, 15 and 18 days after hatching, dah) after exposure to ultraviolet radiation (UVR) at 13 and/or 14 dah. Fish were exposed to 1.17±0.032 W m^{-2} ("Low irradiance") or 1.97±0.052 W m^{-2} ("High irradiance") during 360 min at 13 dah or 2x360 min per day at 13 and 14 dah. * represent the existence of significant differences between control and organisms exposed to UVR ($p<0.05$).

Graphical abstract

Highlights

- Effects of UVR during early life of the flatfish *Solea senegalensis* were studied
- Effects of UVR were distinct depending on larvae development stage
- Pigmentation, behavior and enzymatic activity were affected at earlier stage
- Decreased growth were observed at both development stages
- Increasing UVR affected this species at different levels of biological organization

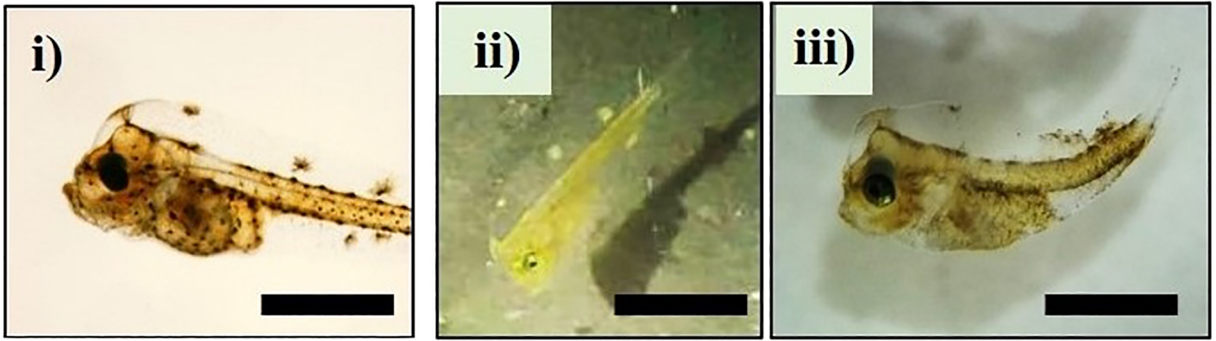

Figure 1

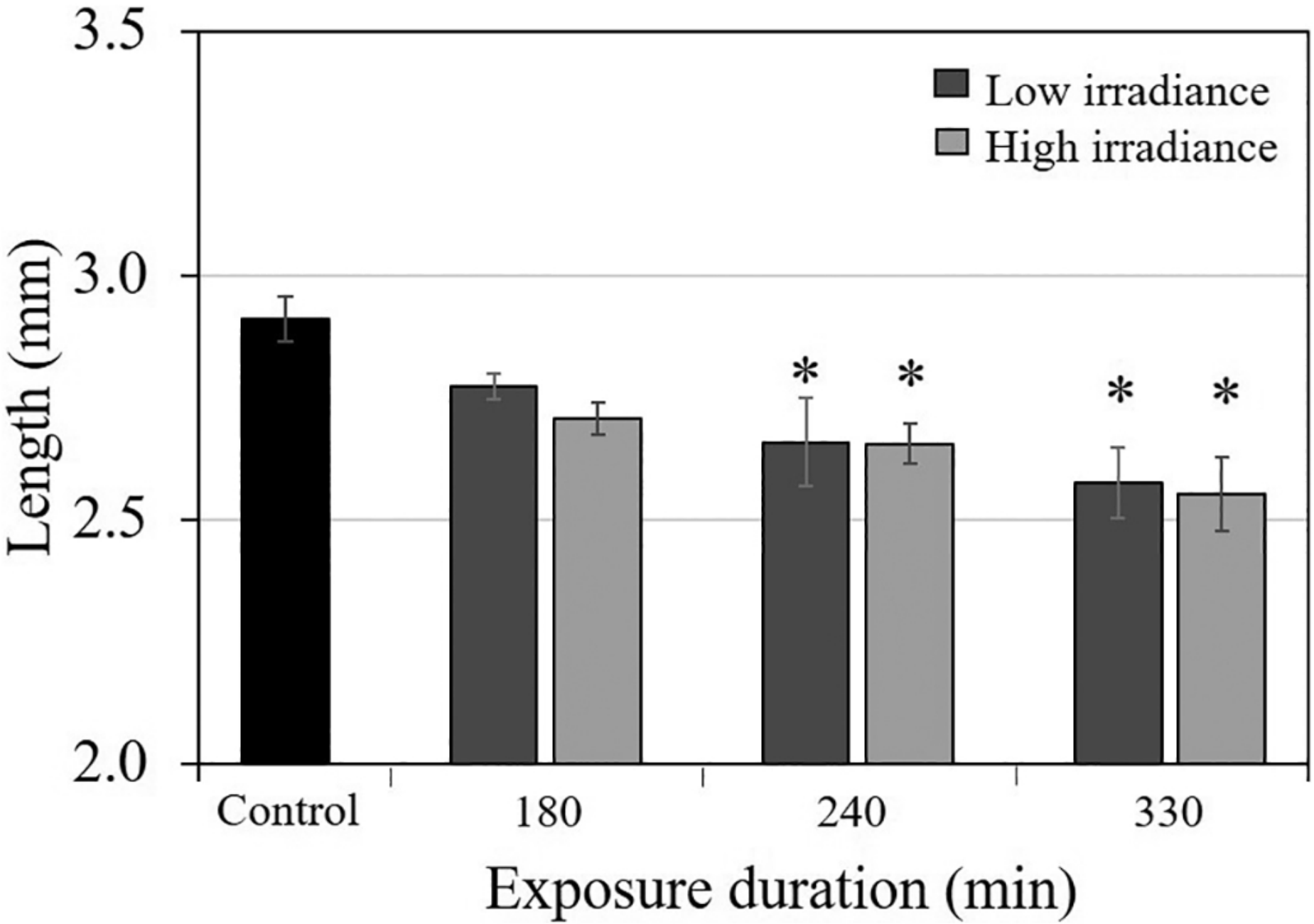
3.5
3.0
2.5
2.0
Length (mm)
Low irradiance
High irradiance
*
*
*
*
Control
180
240
330
Exposure duration (min)

Figure 2

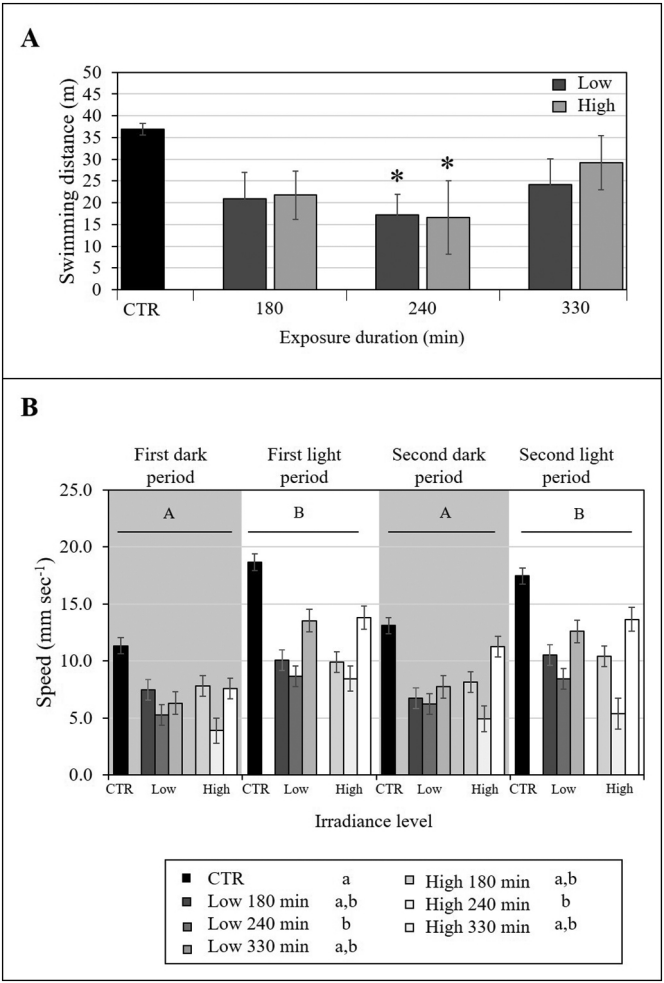
A
Swimming distance (m)
50
45
40
35
30
25
20
15
10
5
0
Low
High
*
*
CTR
180
240
330
Exposure duration (min)
B
First dark period
First light period
Second dark period
Second light period
A
B
A
B
Speed (mm sec-1)
25.0
20.0
15.0
10.0
5.0
0.0
CTR Low High CTR Low High CTR Low High CTR Low High
Irradiance level
CTR a
Low 180 min a,b
Low 240 min b
Low 330 min a,b
High 180 min a,b
High 240 min b
High 330 min a,b

Figure 3

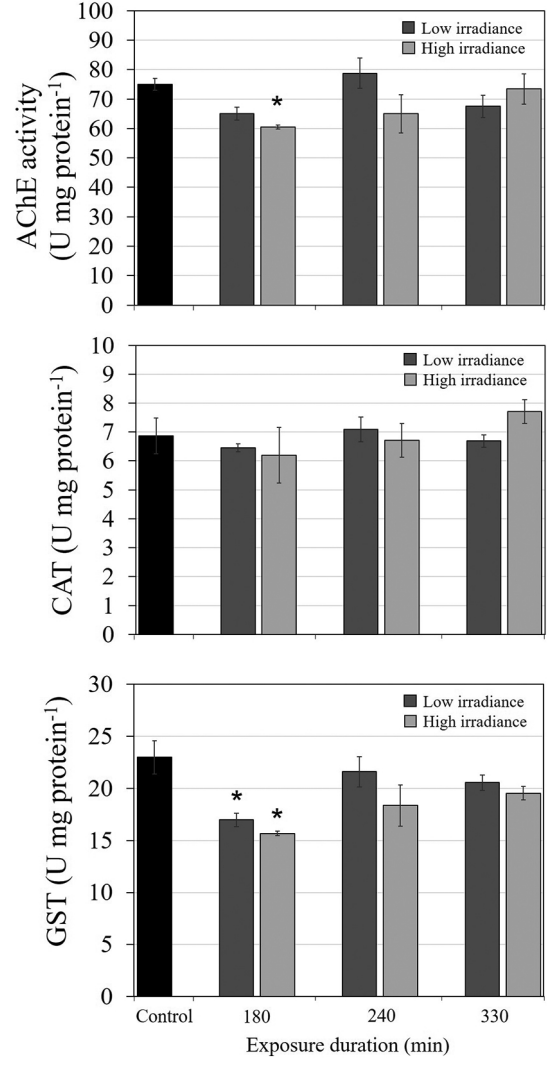
AChE activity (U mg protein-1)
Low irradiance
High irradiance
*
CAT (U mg protein-1)
Low irradiance
High irradiance
GST (U mg protein-1)
Low irradiance
High irradiance
*
*
Control
180
240
330
Exposure duration (min)

Figure 4

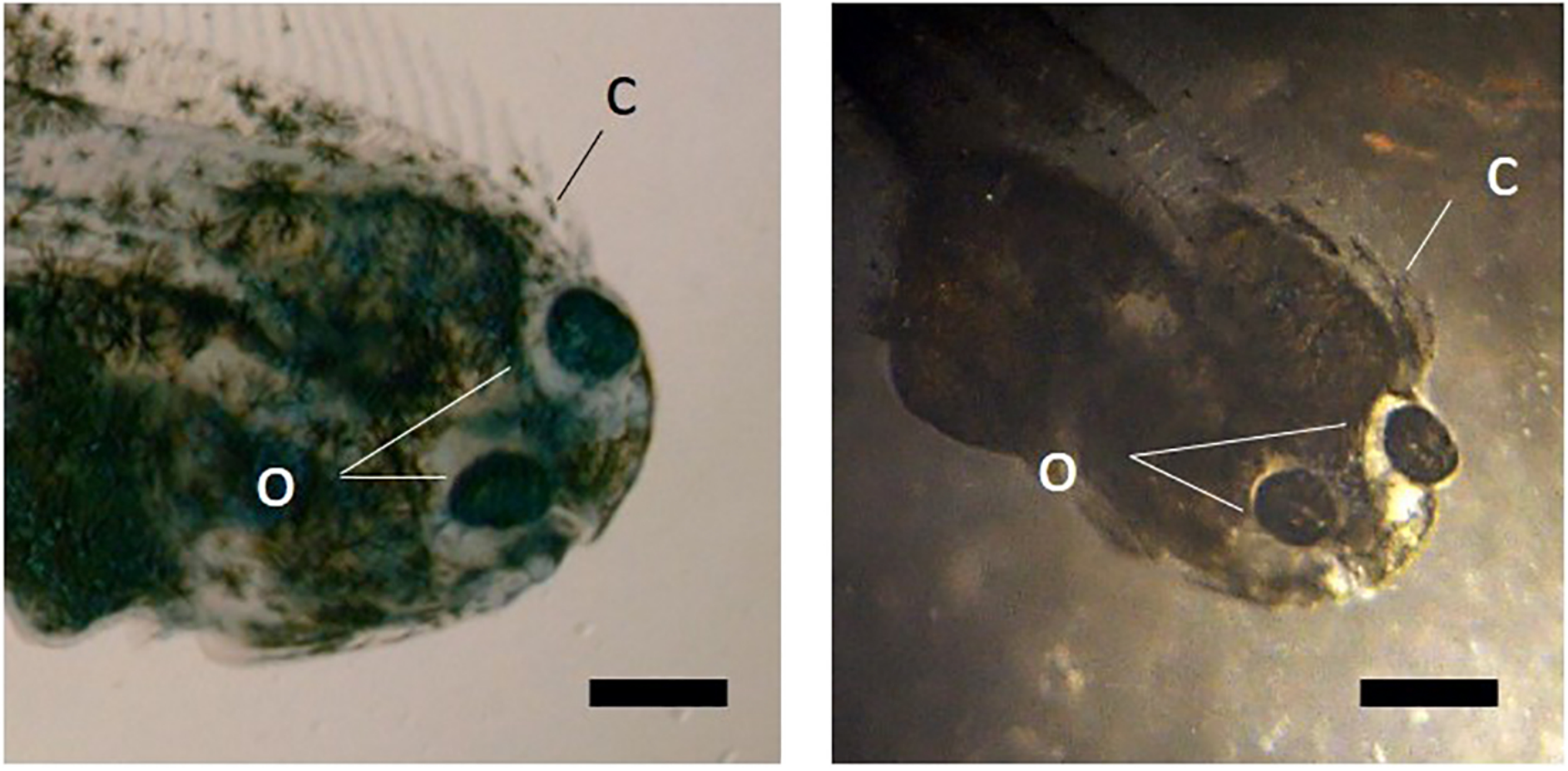

Figure 5

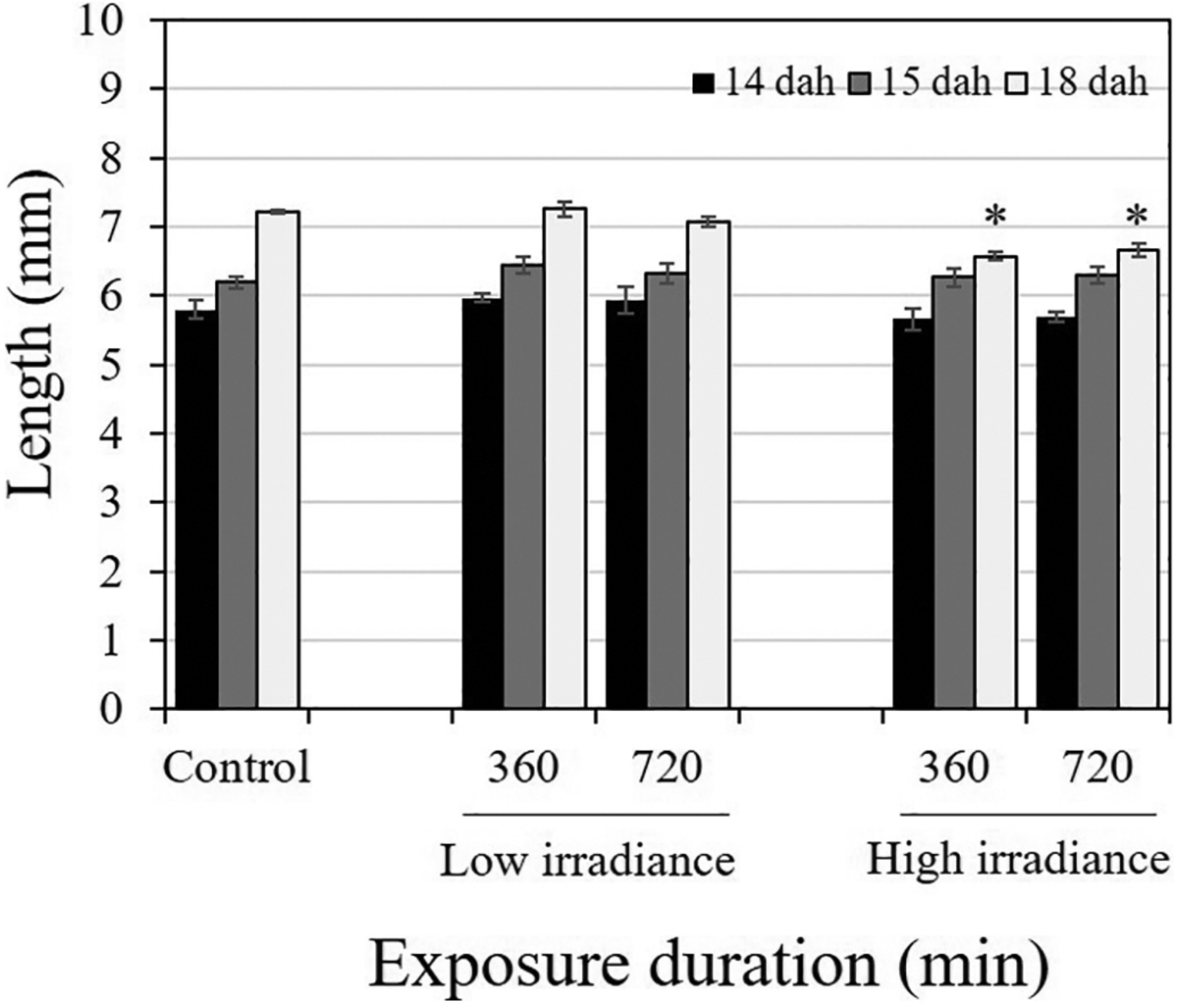
10
9
8
7
6
5
4
3
2
1
0
Length (mm)
14 dah
15 dah
18 dah
*
*
Control
360
720
360
720
Low irradiance
High irradiance
Exposure duration (min)

Figure 6