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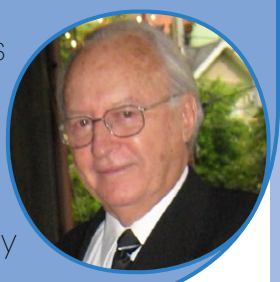

ORIGINAL ARTICLE

Biometrical and morphological analyses of *Macrobrachium olfersii* (Wiegmann, 1836) (Crustacea, Decapoda, Palaemonidae) embryos exposed to UVA and UVB radiation

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ABSTRACT

Macrobrachium olfersii (Wiegmann, 1836) is a prawn that lives in shallow and transparent freshwater in Southern Brazil. Aquatic organisms that inhabit these systems can be exposed to incident UV radiation. Many studies have demonstrated the effects of ultraviolet (UV) radiation on the development, survival rate, morphological abnormalities, and impairments in swimming behavior in crustaceans. However, fewer studies have elucidated how the embryos of crustaceans respond to the exposure of UV radiation. Thus, the aim of this study was to evaluate whether UVA and UVB affect biometrical and morphological parameters of eggs and embryos of *M. olfersii*. Embryos were divided into two groups: Group I, composed of embryonic day 7 (E7) to E10 and Group II, composed of E11 to E14. Both groups were irradiated with 60 min UVA and 30 min UVB. Non-irradiated embryos at the same

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stages were used as controls. UVA and UVB radiation induces variations in morphometric parameters, such as egg volume, egg water content, and eye index. UVB radiation also induced an increase of embryonic cell death. We conclude that the embryonic cells of *M. olfersii* respond differentially to UVA and UVB radiation in accordance with the evaluated parameters.

Key words

Prawn, embryonic development, egg volume, cell proliferation and death.

Introduction

The freshwater prawn *Macrobrachium olfersii* (Wiegmann, 1836) is widely distributed in tropical and subtropical regions, ranging from the southwestern United States to the south of Brazil (Holthuis and Provenzano, 1970). On the Island of Santa Catarina (Brazil), this species was collected in rivers and streams, particularly in the Ratones River and Peri Lagoon (Müller *et al.*, 1999; Ammar *et al.*, 2001).

Males and females of *M. olfersii* present similar body size until sexual maturity, similar to other palemonids (Bond-Buckup and Buckup, 1989). From sexual maturity, males invest energy in somatic growth, becoming the bigger individuals of the population, while females invest energy in yolky egg production and parental care, such as egg incubation in a brood pouch (Adiyodi and Subramonian, 1983). Decapods with yolky eggs exhibit different incubation times, ranging from 10 days for *Palaemon argentinus* (Nobili, 1901) (Nazari *et al.*, 2000), 20 days for *Macrobrachium rosenbergii* (de Man, 1879) (Habashy *et al.*, 2012), and 180 days for *Homarus americanus* H. Milne Edwards, 1837 (Helluy and Beltz, 1991). *Macrobrachium olfersii* ovigerous females carry out approximately 2,000 eggs per spawning in an external brood pouch for 14 days (24 ± 1°C) (Simões-Costa *et al.*, 2005).

Different abiotic factors can affect the size and number of eggs and, therefore, may compromise reproduction of crustacean species (Charniaux-Cotton *et al.*, 1992). The breeding season of *M. olfersii* occurs in late spring and summer in Southeast and South Brazil (Ammar *et al.*, 2001; Mossolin and Bueno, 2002; Pescinelli *et al.*, 2016), where high incidence of UV radiation is registered. The reduction of the ozone layer is related to the increase in the incidence of UVB radiation on the Earth's surface and depending on the dose received, this radiation can promote adverse biological effects on organisms (Hollmann *et al.*, 2015; Almeda *et al.*, 2016; Bonaventura and Matranga, 2017). The UVB radiation has been reported to be an environmental stressor for *M. olfersii* embryos. In general, DNA is the cellular chromophore for UVB radiation, because its maximum absorbance coincides with the UVB wavelength (Hadshiew *et al.*, 2000; Petit-Frère *et al.*, 2000). Moreover, UVB radiation also interferes with the cell cycle, mitochondrial dynamics, cell viability, and induces DNA damage (Nazari *et al.*, 2010, 2013; Zeni *et al.*, 2015; Quadros *et al.*, 2016).

Taking into account that (i) UV radiation is able to penetrate into aquatic environments, depending on the depth of the water column and the amount of suspended organic matter (Häder *et al.*, 2007); (ii) *M. olfersii* inhabit shallow and clear waters (Ammar *et al.*, 2001), and (iii) the ovigerous females carry embryos in an external brood pouch, we suggest that the eggs of this species are subject to the direct incidence of environmental UV radiation. Thus, UV radiation can compromise the viability of the embryos and cause greater consequences to the population. The aim of this study was to evaluate whether UVA and UVB radiation interfere with biometrical and morphological parameters of eggs and embryos of *M. olfersii.*

Material and Methods

Animals

We collected females (25.40–58.20 mm and 0.18–4.30 g) and males (42.70–93.00 mm and 2.25–16.57 g) of *M. olfersii* during the spring and summer seasons from the Peri Lagoon/Santa Catarina Island (27°45'S 48°32'W). The prawns were maintained in aquaria with dechlorinated, aerated tap water at 24°C (± 1°) and a 12 h light: 12 h dark regime. The light cycle was set with a fluorescent lamp (Philips TLT 40 W/75 RS) irradiating visible light at 96 $mW.cm^{-2}$. The prawns were fed once a day with commercial pellet food Alcon Bottom Fish. To obtain embryos under laboratory conditions, males

and non-ovigerous females (1:3) were placed in small aquaria, where courtship and fertilization occurred. The collection procedures adopted in this study were approved by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Certificate number 15294-1/IBAMA/2008).

Irradiation procedures

To evaluate the effects of UVA and UVB radiation, ovigerous females were divided into two experimental groups. In group I, ovigerous females with eggs from embryonic day 3 (E3) to E6 were exposed once to a UVA lamp (Vilber Lourmat, 6W - 365 nm) for 60 min or once to a UVB lamp (Vilber Lourmat, 6W - 312 nm) for 30 min. Following this, ovigerous females were kept in the dark for 4 days. Thus, in group I, embryos were analyzed from E7 to E10, which corresponds to initial eye pigmentation to the formation of oval shaped eyes. In group II, ovigerous females with eggs from E7 to E10 were exposed to a UVA lamp (Vilber Lourmat) for 60 min or UVB lamp (Vilber Lourmat) for 30 min. Following this, ovigerous females were kept in the dark for 4 days. Group II embryos were analyzed from E11 to E14, which corresponds to initial rounded eyes until hatching. Non-irradiated embryos from E7 to E14 were used as controls. The irradiance of UVA was 0.94 $mW.cm^{-2}$ with contamination of 0.003 $mW.cm^{-2}$ UVB and the irradiance of UVB was 310 $mW.cm^{-2}$ with contamination of 0.055 $mW.cm^{-2}$ UVA. Measurements of UVA and UVB irradiances were made using a radiometer (International Light, model IL 1400A) and the doses were calculated according to Diffey (2002).

Egg volume and water content

Samples of irradiated eggs from E7–E10 (group I) and E11–E14 (group II) were measured using a morphometric eyepiece (Olympus; 40× magnification) to obtain the long and short axis of the eggs (n = 20 eggs per group/5 ovigerous females/irradiation procedure). The egg volume was calculated according to the formula: $(\pi lh^2/6)$, where l is the long axis and h is the short axis, according to Odinetz-Collart and Rabelo (1996). To determine the water content, samples of approximately 100 mg of wet eggs (n = 2 ovigerous females for each embryonic day/irradiation procedure) were dehydrated at 50°C for 48 h. The dried eggs were then weighed, and the water content was calculated as the difference between the wet and dry weight of the eggs, and expressed as a percentage.

Eye index

The eye index was calculated according to Perkins (1972) by the average of the length and width of the pigmented area of the eyes. Eye measurements were performed using a morphometric eyepiece (Olympus; 400× magnification) (n = 10 embryos for each embryonic day/5 ovigerous females/irradiation procedure).

Developmental rhythms

Developmental rhythms were characterized by daily analysis (Olympus; 70× magnification) of UVA and UVB-irradiated embryos from E7 to E14 (n = approximately 100 embryos per group/5 ovigerous females/irradiation procedure) and compared with non-irradiated embryos to verify the external morphological features predictable for each embryonic day, as described by Simões-Costa *et al.* (2005). Morphological features considered were (i) shape of the embryos and egg size, (ii) eye shape and pigmentation, (iii) growth of caudal papilla, naupliar, and post-naupliar appendages, and (iv) presence of chromatophores.

Immunohistochemical analysis

To evaluate whether UVA and UVB radiation interferes with cell proliferation and cell death, embryos were fixed in 10% formaldehyde and embedded in paraffin and serial sections (6 μm) were obtained using a rotary microtome. Sections were dewaxed, washed in 0.1 M phosphate-buffered saline (PBS; pH 7.4), and maintained for 10 min in 3% hydrogen peroxide in methanol to inactivate endogenous peroxidases. The sections were washed in 0.1 M PBS + 0.3% Triton X-100 and maintained for 45 min in 5% fetal bovine serum to block non-specific binding of immunoglobulins. The sections were incubated overnight at 4°C with the mitosis marker, rabbit anti-phospho-histone H3 (PHH3) IgG antibody (1:200; Upstate/Millipore) and with rabbit anti-caspase-3 IgG antibody (1:100, Santa Cruz Biotechnology) to recognize apoptotic cells. Afterwards, the sections were washed with 0.1 M PBS + 0.1% Triton X-100 and incubated with peroxidase-conjugated secondary anti-rabbit IgG antibody (1:200;

Sigma) for 3 h at room temperature. Antibody binding sites were revealed with 3,3'-diaminobenzidine (DAB; Sigma). Negative controls for the immunohistochemical reactions were treated in the same way as above, except that the primary antibodies were replaced with 0.1 M PBS buffer (pH 7.4). The numerical density per area (NA) of immunolabeled cells was determined in a 14.544 μm^2 frame (Mandarim-de-Lacerda, 2003). Five random fields of embryos were counted for each section (n = 45 embryos per group/5 ovigerous females/irradiation procedure).

Statistical analysis

Quantitative data were analyzed using Statistica 13.0 for Windows. The differences among the groups after UVA and UVB radiation were evaluated by one-way analysis of variance, followed by Tukey's post hoc test. $p < 0.05$ was considered statistically significant.

Results

Effects of UVA and UVB radiation on biometrical parameters

During embryonic development, non-irradiated eggs of *M. olfersii* showed a significant increase in volume between groups I (0.042 ± 0.0005 mm^3) and II (0.060 ± 0.001 mm^3; $p < 0.01$) (Tab. 1). However, for UVA and UVB-irradiated eggs, no significant difference was found between the volumes of groups I (0.053 ± 0.001 and 0.051 ± 0.001 mm^3, respectively) and II (0.05 ± 0.002 and 0.06 ± 0.0007 mm^3, respectively). When egg volume was compared among the irradiation procedures in the same group, no significant difference was found in group I. For group II, only UVA-irradiated eggs showed a lower egg volume (0.050 ± 0.002 mm^3; $p < 0.0001$) compared with non-irradiated and UVB-irradiated eggs.

The water content of the eggs showed a significant increase between groups I (67.8 ± 0.8%) and II (76.9 ± 0.9%; $p < 0.0001$) in the non-irradiated controls. The same was observed between groups I and II of UVA- (68.3 ± 1.0 and 76.9 ± 0.4%; $p < 0.0001$) and UVB-irradiated eggs (70.9 ± 0.3 and 77.2 ± 0.3%; $p < 0.0001$). UVB irradiation induced a significant increase in water content only in group I (Fig. 1).

Table 1. Egg volume of *Macrobrachium olfersii* from groups I and II in each UVA and UVB irradiation procedure.

Eggs Volume (mm^3)	Group I	Group II
Non-irradiated	$0.042^{a,A}$ (± 0.0005)	$0.060^{b,A}$ (± 0.001)
UVA	$0.053^{a,A}$ (± 0.001)	$0.050^{a,B}$ (± 0.002)
UVB	$0.051^{a,A}$ (± 0.001)	$0.061^{a,A}$ (± 0.0007)

Data are presented as mean ± SEM. Lowercase letter indicates significant difference of the egg volume in each irradiation procedures between groups I and II. Uppercase letter indicates significant difference of the egg volume between irradiation procedures in the same group.

Effects of UVA and UVB on the eye index

The eye index was obtained from E7, when the eye pigmentation was first recognized. During development, the eye index progressively grows. The eye index showed slightly variations from E7 to E14 in embryos exposed to UVA and UVB radiation and also non-irradiated embryos. Therefore, UVA and UVB radiation did not induce significant variations in the eye index in both groups in comparison to non-irradiated embryos (Fig. 2).

Effects of UVA and UVB on developmental rhythms

Accompaniment of the embryonic development rhythm of *M. olfersii* observed that 10% of UVA and UVB-irradiated embryos in group I did not show a developmental delay (Fig. 3). We also observed that 15% of UVA- and 10% of UVB-irradiated embryos showed a developmental delay of 12 h, 70% of UVA- and 60% of UVB-irradiated embryos showed a developmental delay of 24 h, and 5% of UVA- and 15% of UVB-irradiated embryos showed a developmental delay of 48 h. The development delay in group II gradually decreased, evidenced by the absence of embryos with a developmental delay greater than 24 h.

Effects of UVA and UVB irradiation on cell proliferation and cell death

Immunohistochemical reaction using antibodies against PHH3 and caspase-3 was used to evaluate if UV radiation affects cell proliferation and death, which are essential mechanisms of embryonic development. Labeling was observed in various embryonic structures, including the caudal papilla, appendages, and optic lobes.

The density per area (NA) of PHH3-positive cells, which indicated cell proliferation, showed that

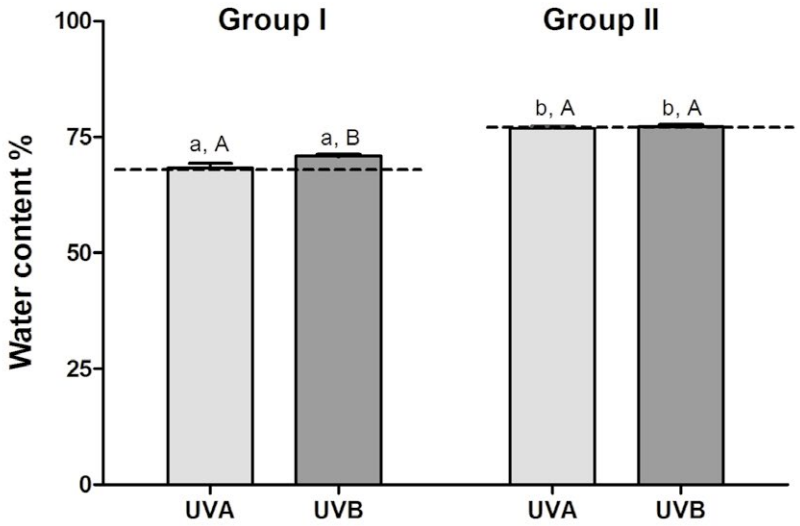

Figure 1. Percentages of water content of eggs of *Macrobrachium olfersii* from groups I and II following UVA and UVB exposure. Data are presented as the mean ± standard error of the mean (SEM). Dashed lines correspond to the percentage of water content of non-irradiated eggs from groups I and II. Lowercase letter indicates significant difference in each irradiation procedure between groups I and II. Uppercase letter indicates significant differences of the water content among irradiation procedures in the same group.

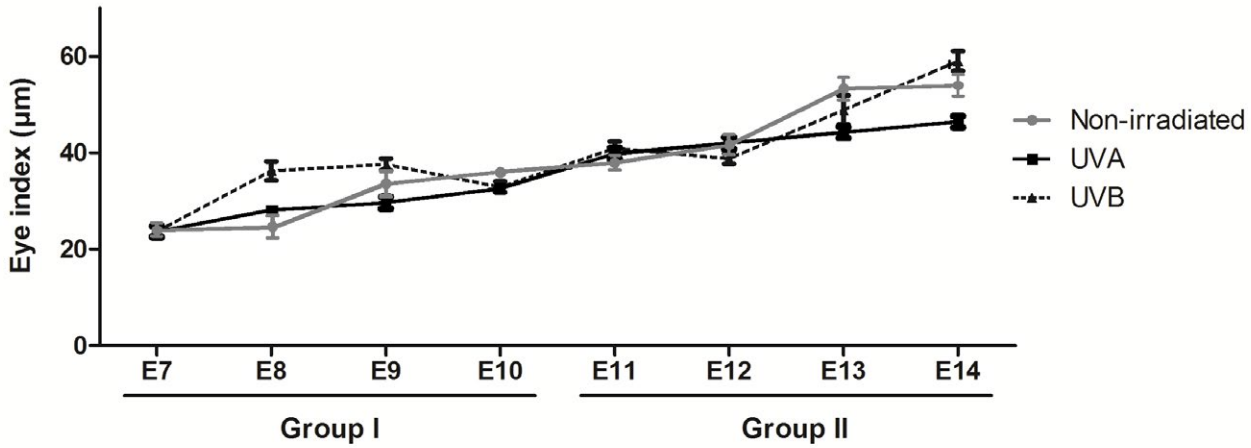

Figure 2. Eye index of embryos of *Macrobrachium olfersii* from groups I and II following UVA and UVB exposure. Data are presented as the mean ± SEM. Gray, black, and dashed lines correspond to non-irradiated, UVA-irradiated, and UVB-irradiated embryos, respectively.

embryos exposed to UVA in both group I (95.42 ± 6.01 mm^2) and group II (114.59 ± 12.9 mm^2) did not differ compared with non-irradiated embryos (94.01 ± 5.2 mm^2). Following UVB exposure, the density of PHH3-positive cells decreased in both group I (91.67 ± 10.0 mm^2; $p < 0.03$) and II (92.61 ± 4.0 mm^2; $p < 0.04$) respectively, compared with non-irradiated embryos (Fig. 4A–G).

The density per area (NA) of caspase-3-positive cells, which is indicative of cell death, showed a significant increase in the embryos of group I exposed to UVA (143.5 ± 9.7 mm^2; $p < 0.02$) and UVB (140.32 ± 8.0 mm^2; $p < 0.03$), compared with non-irradiated embryos (113.6 ± 6.3 mm^2). In group II, only embryos exposed to UVB showed a significant increase (236.5 mm^2 ± 12.3; $p < 0.01$) compared with non-irradiated embryos (127.7 ± 17.6 mm^2). No significant difference was found in embryos exposed to UVA (171.89 ± 23.6 mm^2) (Fig. 4H–N).

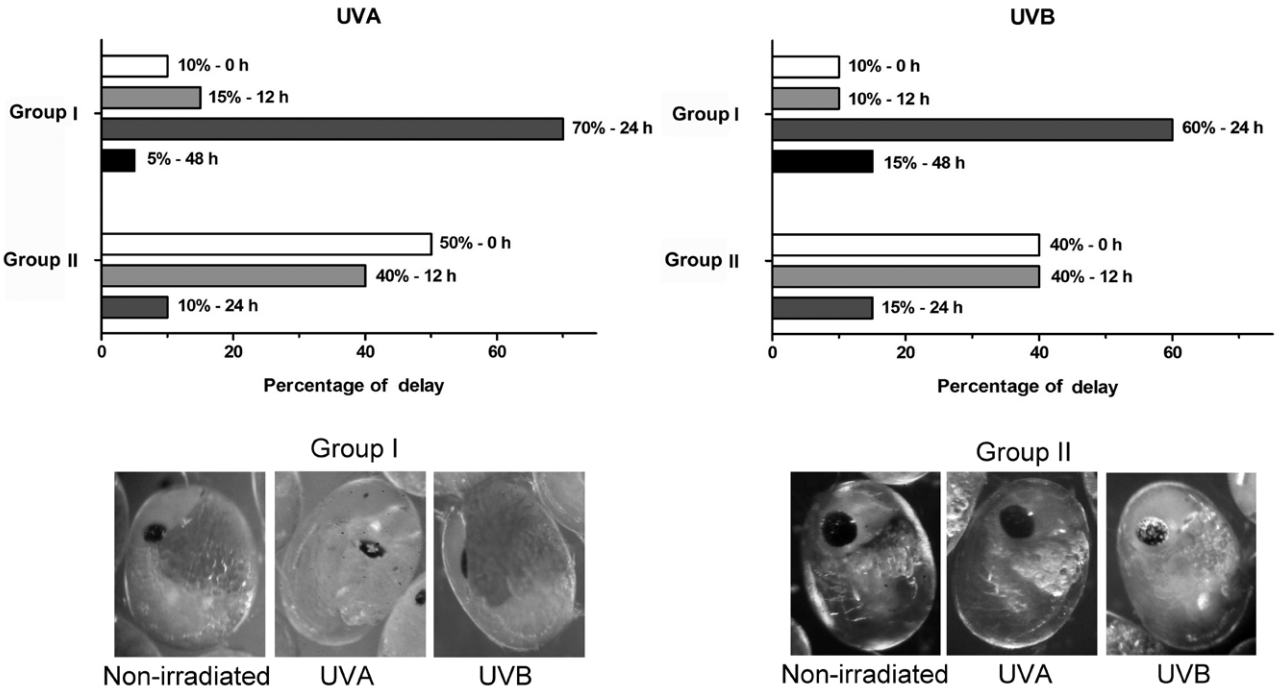

Figure 3. Developmental timelines of *Macrobrachium olfersii* embryos from groups I and II following UVA and UVB exposure. Data are presented as a percentage.

Discussion

Aquatic animals are subject to UV radiation, which depends on water clarity as well as other factors. Several studies have demonstrated that different species have different sensitivities to UV exposure, and there are few studies reporting the effects on aquatic invertebrate embryos. Moreover, it is important to consider that embryos are more sensitive to environmental stressors than organisms at later life stages. Together, these considerations highlight the importance of studies that evaluate the responses of aquatic embryos to UV exposure.

It has been well established that the eye index is an important morphological landmark in the development of crustaceans (Helluy and Beltz, 1990) and in *M. olfersii*, the presence of the lateral eyes can be observed at approximately 50% of the embryonic development time (Müller *et al.*, 2003). In this study, the irradiance and the exposure time of UVA and UVB radiation did not disturb the eye index. The same way, the eye index was not disturbed in a study performed by Nazari *et al.* (2010), which evaluated the effect of 310 $mW.cm^{-2}$ UVB in *M. olfersii* embryos. These authors also demonstrated that this index was not disturbed in embryos exposed to solar UVB radiation.

Under controlled temperature conditions, the development of *M. olfersii* occurs in 14 days (Simões-Costa *et al.*, 2005). However, UVA and UVB irradiation results in a significant developmental delay. This delay may be related to the unbalance between proliferation and apoptosis observed in embryonic cells following UV irradiation. It is important to note that during embryonic development, cell division is fast (Ninov *et al.*, 2009), which may compromise the detection of damage and promote the propagation of damage to descendent cells.

Apoptosis also serves to maintain the integrity of tissues of organisms when they are exposed to extrinsic stimuli that promote cell damage (Zhou and Steller, 2003). Caspase-3 is a protein involved in the cascade that triggers apoptosis, and is one of the last signals to be activated, promoting the cleavage of DNA and the formation of apoptotic bodies (Cohen, 1997). Cell proliferation and death are essential for morphogenesis and organogenesis events. The interference of external agents, such as UV radiation, may compromise the adequate development of embryos (Nazari *et al.*, 2013). In the present work, it was verified that exposure to both UVA and UVB was able to induce alterations in

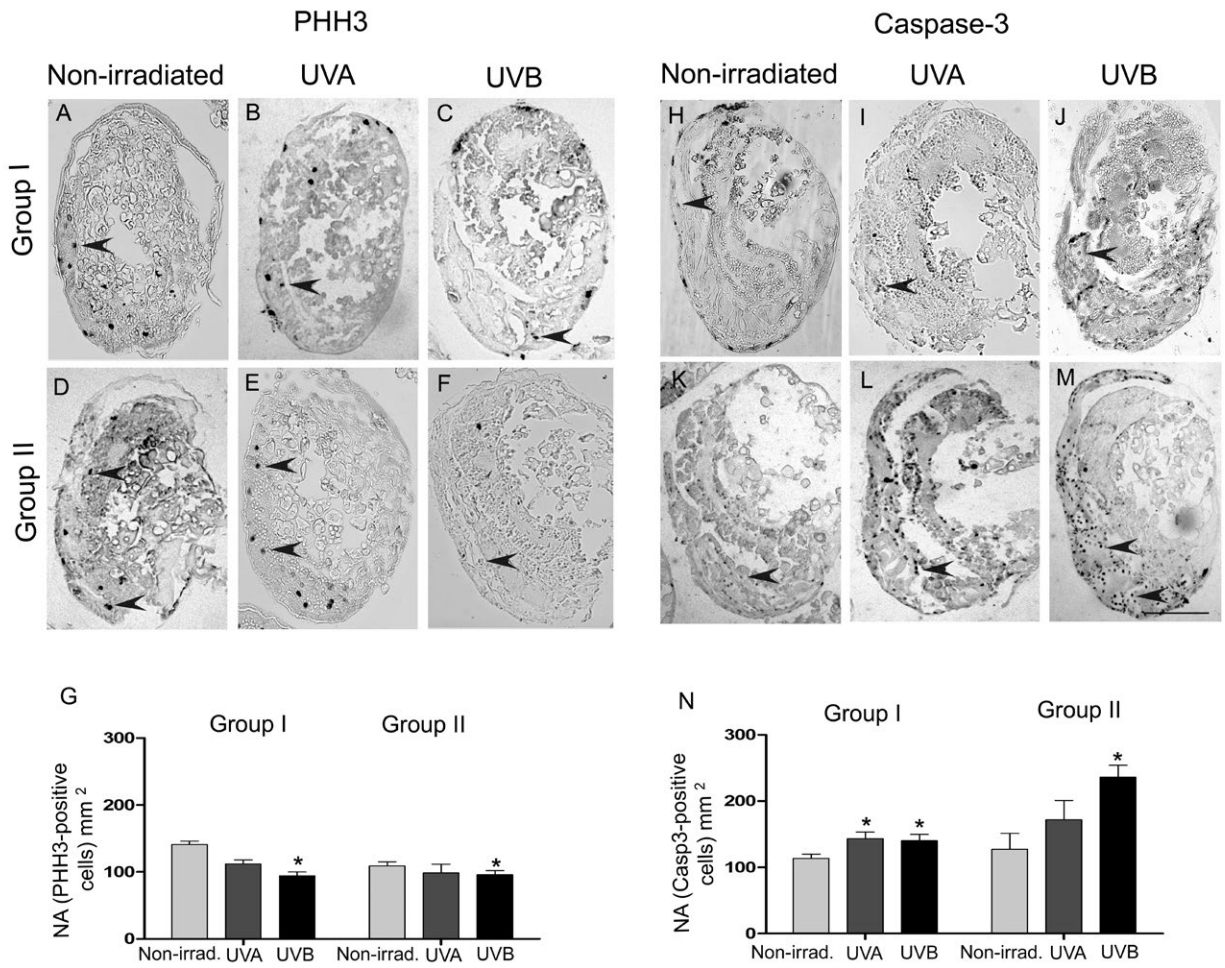

Figure 4. Cell proliferation and cell death labeled with anti-PHH3 (arrowhead) and anti-caspase-3 (arrowhead) antibodies in embryos exposed to UVA and UVB. Numerical density per area (NA) of proliferating cells of embryos from group I and II (A–G). NA of death cells from groups I and II (H–N). Data are presented as the mean ± SEM.

the embryos of *M. olfersii*, promoting an unbalance between proliferation and apoptosis in embryonic cells.

Studies performed with other crustaceans, such as the crab *Ucides cordatus* (Linnaeus, 1763) (Vargas *et al.*, 2010; Hollmann *et al.*, 2016), have shown that the cells of these organisms also trigger apoptosis in response to UVB exposure. In embryos of *M. olfersii*, UVB radiation induced cell cycle arrest, DNA damage, and apoptosis (Zeni *et al.*, 2015; Schramm *et al.*, 2017). Apoptosis occurs normally during embryogenesis (Agnello and Roccheri, 2010), however, the increase in the number of apoptotic cells in the UV-exposed group indicates the action of radiation on this cell mechanism, and suggests it may interfere with the processes of morphogenesis and organogenesis.

In summary, we conclude that the embryonic cells of *M. olfersii* respond differentially to UVA and UVB radiation in accordance with the evaluated parameters. Considering the alterations observed, new studies focusing on the photobiological effects of UV radiation on aquatic organisms will be necessary, since UV radiation has been considered an environmental stressor and these organisms are essential for the maintenance of aquatic biodiversity.

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