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Influence of the dark/light rhythm on the effects of UV radiation in the eyestalk of the crab *Neohelice granulata*

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ABSTRACT

Crustaceans are interesting models to study the effects of ultraviolet (UV) radiation, and many species may be used as biomarkers for aquatic contamination of UV radiation reaching the surface of the Earth. Here, we investigated cell damage in the visual system of crabs *Neohelice granulata* that were acclimated to either 12L:12D, constant light, or constant dark, and were exposed to UVA or UVB at 12:00 h (noon). The production of reactive oxygen species (ROS), antioxidant capacity against peroxyl radicals (ACAP), lipid peroxidation (LPO) damage, catalase activity, and pigment dispersion in the eye were evaluated. No significant differences from the three groups of controls (animals acclimated to 12L:12D, or in constant light, or not exposed to UV radiation) were observed in animals acclimated to 12L:12D, however, crabs acclimated to constant light and exposed to UV radiation for 30 min showed a significant increase in ROS concentration, catalase activity, and LPO damage, but a decrease in ACAP compared with the controls. Crabs acclimated to constant darkness and exposed to UV for 30 min showed a significantly increased ROS concentration and LPO damage, but the ACAP and catalase activity did not differ from the controls (animals kept in the dark while the experimental group was being exposed to UV radiation). Pigment dispersion in the pigment cells of eyes of animals acclimated to constant light was also observed. The results indicate that UVA and UVB alter specific oxidative parameters; however, the cell damage is more evident in animals deviated from the normal dark/light rhythm.

1. Introduction

Ultraviolet (UV) radiation comprises electromagnetic wavelengths between 200 and 400 nm. In order to analyze its capacity to cause damage in cell targets, UV is divided into UVA (320–400 nm), UVB (290–320 nm), and UV-C (200–290 nm). The wavelengths of UV that include UVA and UVB are also called Solar UV (Diffey, 2002). The increased incidence of UV radiation on the Earth's surface is receiving more attention because it can produce biological changes and some impact on biodiversity (McKenzie et al., 2007). However, additional studies are necessary in order to understand the responses of biological systems to UV radiation damage.

The first and main target-structure for UV radiation in animals is the body surface, including the skin and eyes. The influence of UV radiation on the human skin has been investigated: in general, the effects of UV radiation on the epidermis are harmful (Tran et al., 2008), except for stimulation of vitamin D synthesis (Holick, 2008). The adverse effects of UV radiation include immunosuppression (Timares et al., 2008), production of reactive oxygen species (ROS) (Heck et al., 2003), photoaging, DNA mutation, and cancer (Albert and Ostheimer, 2003). In the eye, damage has been reported in the cornea, characterized by inflammation; in the lens, with cataracts the most common pathology (Sliney, 2001; Meyer-Rochow, 2000); and in the retina, where apoptosis, especially in the photoreceptors, was observed (Miguel et al., 2003). The mechanism underlying this damage is oxidative stress, which results from an imbalance between reactive oxygen species (ROS) and antioxidants, i.e., oxidative stress occurs when ROS production exceeds the capacity of the antioxidant system to repair the deleterious effect of ROS. The most commonly encountered ROS in biological systems include the hydroxyl radical, the superoxide anion, hydrogen peroxide (H_2O_2), and singlet oxygen (Sies, 1991). ROS results from the incomplete reduction of oxygen species and can be produced by both exogenous, such as solar radiation, and endogenous sources, such as the mitochondrial

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electron transport chain and the endoplasmic reticulum (Dean et al., 1997), the activity of enzymes, *viz.* cytochrome P450, xanthine oxidase, urate oxidase, and D-amino acid oxidase (Stadtman and Levine, 2000).

Animals may respond to environmental conditions, the most important being the photoperiod. Circadian and exogenous daily variations, including those related to locomotor and brain activity, as well as environmental temperature and light fluctuations, result in corresponding daily patterns of reactive oxygen species (ROS) (Hardeland et al., 2003). Some investigators have proposed that circadian clocks organize metabolic functions into a coherent daily schedule, assuring their synchrony with environmental changes (Wijnen and Young, 2006).

If in vertebrates, mainly mammals, the adverse effects of UV radiation are well known, this is not true for invertebrates. To our knowledge, in crustaceans only Gouveia et al. (2005) have reported oxidative and DNA damage in the cephalothorax and pereiopod epithelia in the crab *Neohelice granulata*, and Miguel et al. (2002) reported morphological damage in the retina and *lamina ganglionaris* cells in the mangrove crab *Ucides cordatus*.

The crab *N. granulata* (=*Chasmagnathus granulatus*) is a semi-terrestrial animal of the southern coast of Brazil, Uruguay, and Argentina, and it has been used by our group as a model for studies on the effects of UV. This species shows pigment dispersion in the melanophores during UV exposure, which disappears when the stimulus ceases (Gouveia et al., 2004; Vargas et al., 2008). Although the effects of UV radiation on the epithelium of this crab have been studied, the effects of UV on the visual system have not so far been investigated. Thus, the aim of this study was to investigate the effects of UV radiation on the visual system of *N. granulata* in response to oxygen species production, using a biochemical approach and the evaluation of pigment dispersion. This may help to understand how crustaceans, which are an integral part of estuarine environments and contribute substantially to their dynamics, are affected by UV rays. In addition, because UVB radiation reaching the surface of the Earth has been increasing in recent decades (Casiccia et al., 2003; Bertagnolli et al., 2007; Kirchhoff et al., 2000), these animals may eventually be used as biomarkers for aquatic contamination.

2. Materials and methods

2.1. Animals

Adult male crabs *N. granulata* weighing 7.0 ± 0.5 g (mean ± S.E.M) were collected in salt marshes near the city of Rio Grande, Brazil. They were transferred to the laboratory for an acclimation period of at least 10 days in tanks under constant conditions of temperature (20 °C) and salinity (20 ppt), and in three photoperiods, consisting of 12L:12D, constant light, and constant darkness. In the 12L:12D regime, the lights went on at 6:00 h and went off at 18:00 h. All procedures adopted in this study were performed after approval by the National Environmental Committee (IBAMA document number 1.637.714), and every effort was made to minimize animal suffering.

2.2. Assays

Within each set of crabs acclimated to one of the three different photoperiods, a group was exposed to UVA radiation, another group was exposed to UVB radiation, and still another group was the control. This control group was constituted by the animals that were not exposed to UV radiation (UVA or UVB), but to visible light (the animals acclimated to 12L:12D and constant light) or maintained in the dark (animals acclimated to constant darkness) during the same period of time that the experimental groups were exposed to UV radiation.

Therefore, after the acclimation period, two groups of crabs were irradiated at 12:00 h (noon), since this is the time when UV radiation is maximal, with different doses of UVA (1.575 J/cm^2) and UVB (1.294 J/cm^2), respectively, for 30 min. The UVA (VL: 115 L, 30 W) or UVB (VL: 115 C, 30 W; Vilber Lourmat, Marne Lavalee, France) lamps were monitored using a radiometer/photometer (model IL 1400A, International Light, Newburyport, MA, USA). The UVA lamp irradiation was 1.39 mW/cm^2 UVA, with contamination of 0.006 mW/cm^2 UVB and 0.000928.0 mW/cm^2 of visible light. The UVB lamp irradiation was 1.195 mW/cm^2, produced with contamination of 0.493 mW/cm^2 of UVA and 0.000113 mW/cm^2 of visible light. Both lamps showed no contamination with UV-C. The control group, formed with crabs that were not irradiated with UV (animals acclimated to 12L:12D and constant light), was maintained under fluorescent lamps (Philips TLT 40 W/75, São Paulo, Brazil) irradiating 96.0 mW/cm^2 visible light. After the exposure, the animals were killed by severing the supra-esophageal ganglion, and the eyes were removed for further analyses.

2.2.1. ROS production

Two hundred and seventy eyestalks (90 for each group: control, exposed to UVA, and exposed to UVB) were weighed and homogenized (1:20 w/v) in a cold (4 °C) buffer solution containing sucrose (250 mM), PMSF (1 mM), and EDTA (5 mM), with pH adjusted to 7.6. The samples were centrifuged twice (2000*g*, 4 °C for 20 min) and the supernatant was collected and centrifuged again (10,000 *g*, 4 °C for 45 min). The supernatant resulting from this last centrifugation was used for the determination of the ROS (Viarengo et al., 1999). For ROS detection, we used 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA, Molecular Probes). This molecule in the presence of ROS generates a fluorochrome, detected at 488 and 525 nm wavelengths for excitement and emission, respectively. The analyses were carried out in a fluorescence microplate reader (Victor 2, Perkin Elmer) with readings every 5 min for 60 min. The total fluorescence production was calculated by integrating the fluorescence units (FU) during the period of the measurement, after adjusting the FU data to a second-order polynomial function. The ROS concentration was referred to the total protein content present in the biological sample and expressed in FU (mg of protein)$^{-1}$.

2.2.2. Antioxidant capacity against peroxyl radical analysis

Another 270 eyestalks (separated into three groups as explained in Section 2.2.1) were weighed and homogenized (1:20 w/v) in a cold (4 °C) buffer solution containing sucrose (250 mM), PMSF (1 mM), and EDTA (5 mM), with pH adjusted to 7.6. The samples were centrifuged twice at 2000*g*, 4 °C, for 20 min, and the supernatant was collected and centrifuged again (10,000*g*, 4 °C, for 45 min). The supernatant of this last centrifugation was then employed for the analyses. The antioxidant capacity against peroxyl radicals (ACAP) was measured according to the method of Amado et al. (2009). Briefly, 10 µL of the supernatant prepared for the enzyme analysis was pipetted into a white 96-well microplate, six wells per sample. The reaction buffer (127.5 µL) containing 30 mM HEPES (pH 7.2), 200 mM KCl, and 1 mM $MgCl_2$ was added to the wells with the samples. In three of the six wells of each sample, 7.5 µL of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) was added, and the same volume of ultrapure water was pipetted into the other three wells. The microplate was inserted into a fluorescence microplate reader (Victor 2, Perkin Elmer), at a programmed temperature of 35 °C, and the peroxyl radicals were produced by thermal decomposition of ABAP. Immediately before reading, 10 µL of the fluorescent probe 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA) was added to the wells at a final concentration of 40 µM (Ferreira-Cravo et al., 2007). H_2DCF-DA is cleaved by esterases present in samples, and the non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound DCF, which is detected at