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Effects of melatonin in connection with the antioxidant defense system in the gills of the estuarine crab *Neohelice granulata*

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ABSTRACT

Numerous studies have shown that melatonin exerts some influence on the antioxidant defense system (ADS) in vertebrates, but for crustaceans no such effect has been demonstrated till now. However, earlier reports did show a similar profile of daily variations in the ADS of the gills and the melatonin content of the eyestalk in the crab *Neohelice granulata* and, thus, the aim of this study was to take a closer look at the effects of melatonin in the gill ADS of *N. granulata*. Gill ADS is to a minor extent modulated by reactive oxygen species (ROS), because only the nonproteic sulfhydryl (NP-SH) content increases ($p < 0.05$) in the presence of hydrogen peroxide (H_2O_2). No significant differences ($p > 0.05$) were observed in the melatonin content of the hemolymph between intact and eyestalkless crabs. Gills from intact and eyestalkless crabs injected with physiological saline showed a daily variation in the total peroxyl radical scavenging capacity (TPRSC) ($p < 0.05$) with two peaks, one at the photophase and another at the scotophase. However, in the gills of eyestalkless crabs injected with melatonin (2×10^{-12} mol crab^{-1}), the daily variation in TPRSC values was abolished ($p > 0.05$). This molecule did not change the NP-SH content ($p > 0.05$) in vitro, but decreased ($p < 0.05$) the oxygen consumption in gills when incubated for 120 min. In the in vivo experiments melatonin also decreased ($p < 0.05$) the oxygen consumption in eyestalkless crabs after 390 min. The results suggest that melatonin does not act directly on the ADS of the gills of *N. granulata*, but decreases the aerobic metabolism possibly involved in variations of tissue ADS.

1. Introduction

Melatonin, a ubiquitous molecule present from unicellular algae to vertebrates, undergoes circadian production in virtually all organisms. The most clearly established function of melatonin in vertebrates is to inform the organism of day/night conditions, which then allows for the synchronization of physiological parameters to environmental factors. This molecule forms part of the biological clock of organisms, signaling photoperiodic variations (Reiter, 1991). However, melatonin is considered a multifunctional molecule, also regulating physiological parameters such as body temperature, locomotor activity, reproduction, color changes amongst others (e.g., Mayer et al., 1997; Lutterschimidt et al., 2003; Underwood, 1981). Numerous studies have furthermore verified the influence of melatonin in the antioxidant defense system, regulating certain antioxidant enzymes like superoxide dismutase and glutathione peroxidase (Barlow-Walden et al., 1995; Liu and Ng, 2000) or acting as a scavenger of ROS, such as hydroxyl radical (Tan et al., 1993), peroxyl radical (Pieri et al., 1994), nitric oxide (Pozo et al., 1994), hydrogen peroxide (Barlow-Walden et al., 1995), and singlet oxygen (Cagnoli et al., 1995).

There are a few studies that have reported the presence of melatonin in the hemolymph, brain and eyestalk of crustaceans: e.g., *Carcinus maenas* (Vivien-Roels and Pévet, 1986), *Macrobrachium rosenbergii* (Withyachumnarnkul et al., 1992a,b), *Penaeus monodon* (Withyachumnarnkul et al., 1995), *Procambarus clarkii* (Agapito et al., 1995; Balzer et al., 1997), *Astacus fluviatilis* (Meyer-Rochow, 2001), *Saduria entomon* (Meyer-Rochow, 2001), *Uca pugilator* (Tilden et al., 1997, 2001a), *Euphausia superba* (Pape et al., 2008) and

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Neohelice granulata (Maciel et al., 2008). However, peak periods of melatonin production are not as consistent as they are in vertebrates. Regarding effects of this molecule in crustaceans, some reports have shown effects on eletroretinogram biorhythmicity (Balzer et al., 1997) and synaptic transmission (Tilden et al., 2003) in *Procambarus clarkii*, limb regeneration (Tilden et al., 1997) and rhythm of glucose and lactate levels in the hemolymph in *Uca pugilator* (Tilden et al., 2001b), and erythrophore responses to the red-pigment-concentrating-hormone in *Macrobrachium potiuna* (Nery et al., 1999). However, there are currently no reports on the influence of melatonin on the antioxidant defense system (ADS) in crustaceans.

Many abiotic parameters vary cyclically like temperature, tides, photoperiod, and others. In this way, the organisms might adapt their physiological processes anticipating or following these cyclic factors. One of these physiological processes is the aerobic metabolism, normally associated with locomotor activity and oxygen consumption. Taking into account that variations in aerobic metabolism should impose variations with regard to ROS generation, variations of the ADS should occur to minimize any deleterious effects due to the actions of the ROS. In fact, daily variations of enzymatic antioxidant activities or non-enzymatic antioxidants content were also reported in various tissues of vertebrates (Hardeland et al., 2003).

Daily variations in crustacean physiological parameters have been documented (Burggren et al., 1993; Crear and Forteath, 2000; Fanjul-Moles et al., 2003; Granato et al., 2004; Maciel et al., 2004; Pereyra et al., 1996; Rosas et al., 1992). Regarding the aerobic metabolism, such as oxygen consumption, higher values at night were measured in the crabs *Callinectes similis* and *Portunus spinicarpus* (Rosas et al., 1992), the shrimp *Farfantepenaeus aztecus* (Burggren et al., 1993) and the prawn *Jasus edwardsii* (Crear and Forteath, 2000). In the crab *Neohelice granulata* (previously named *Chasmagnathus granulata*), daily variations in locomotor activity were reported peaking during the scotophase (Pereyra et al., 1996).

Considering that variations in the aerobic metabolism do occur in crustaceans, daily variations in the antioxidant system should happen. In this sense, daily variations in glutathione peroxidase (GPx) and glutathione reductase (GR) activities, as well as reduced glutathione (GSH) levels in the hemolymph and hepatopancreas, of the crayfishes *Procambarus clarkii* and *P. digueti*, were reported (Fanjul-Moles et al., 2003). The crab *Neohelice granulata* displays a biphasic profile in the daily variations of oxygen consumption both in the gills and the hepatopancreas, with one peak at the photophase and another at the scotophase, as well as daily variations in some components of the antioxidant defense system and lipid peroxidation (Maciel et al., 2004).

In the crab *N. granulata* daily variations in oxygen consumption and in the ADS of gills have recently been reported (Maciel et al., 2004). Interestingly, Maciel et al. (2008) confirmed the biphasic profile of melatonin content in the eyestalk of *N. granulata*, with a peak at 15:00 h and another at 03:00 h in animals kept under a 12:12 dark/light regimen and constant dark photoperiods, similar (*i.e.*, one at photophase and one at scotophase peak) to those of oxygen consumption shown to operate in the gills (Maciel et al., 2004). This result adds support to the notion of this indoleamine's possible involvement in the ADS of *N. granulata*.

Thus the aim of this work was (i) to test whether the daily variation of some components of the ADS responded directly to hydrogen peroxide (H_2O_2), (ii) to establish whether the melatonin content in the hemolymph depended in some way on the eyestalks, and (iii) to demonstrate melatonin-induced fluctuations in the TPRSC, NP-SH content and aerobic metabolism of the gills in *N. granulata*.

2. Materials and methods

Adult male crabs of *Neohelice granulata* (formerly known as *Chasmagnathus granulata/granulatus* Dana 1851 – see Sakai et al., 2006) weighing 9.92 ± 0.25 g (means ± s.e.m.) were collected in the salt marshes around Rio Grande City – Brazil, and transported to the laboratory. The animals were acclimated in tanks for 15 days, temperature of 20 °C, salinity of 20 and fixed photoperiod of 12L:12D (lights on at 07:00 h). The crabs were fed *ad libitum* with ground beef 3 times a week.

All chemicals were purchased from Sigma (St. Louis, MO). Melatonin and vitamin E (-tocopherol acetate) stock solutions were made in ethanol. For each solution, the final dilution was made in physiological saline, and the final concentration of the nonaqueous solvents never exceeded 1%. The physiological saline contained: 1×10^{-2} M $MgCl_2$, 355×10^{-3} M NaCl, 16.6×10^{-3} M $CaCl_2$, 5×10^{-3} M H_3BO_3, 1×10^{-2} M $KHCO_3$, 8×10^{-3} M sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$); pH adjusted to 7.6.

2.1. In vitro experiments: incubation of gills with hydrogen peroxide (H_2O_2)

With the purpose of verifying whether the daily variation of the ADS of gills are a direct response to H_2O_2, crabs were sacrificed (n = 4–10) at 12:00 h or 24:00 h, and the gills were dissected and immediately incubated for 120 min with physiological saline only (control group) or physiological saline plus H_2O_2 (20×10^{-3} M). After the time of incubation, the tissues were immediately frozen at −80 °C prior to quantification of catalase (CAT) and glutathione-*S*-transferase (GST) enzymatic activities, nonprotein sulfhydryl (NP-SH) content, level of lipid peroxidation and protein analysis.

Moreover, to verify the effect of melatonin on the NP-SH content in gills in vitro, crabs were sacrificed (n = 3–5) at noon and the gills were obtained and immediately incubated for 120 min with physiological saline only (control group), physiological saline plus H_2O_2 (20×10^{-3} M), physiological saline plus melatonin (20×10^{-9} M), physiological saline plus vitamin E (10^{-7} M), physiological saline plus H_2O_2 and melatonin, or physiological saline plus H_2O_2 and vitamin E. After the time of incubation, the tissues were immediately frozen at −80 °C prior to quantification of NP-SH content.

For antioxidant enzyme analysis, gills were homogenized (1:10w/v) in a cold 4 °C buffer solution containing Tris base (20×10^{-3} M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with pH adjusted to 7.6. Homogenates were centrifuged at 9000g (4 °C) for 30 min and the supernatants were then employed as antioxidant-enzyme sources and for protein quantification. All enzymatic determinations were done at least in triplicate.

CAT and GST activities were determined using the spectrophotometric methods described by Beutler (1975) and Habig and Jakoby (1981), respectively. Specific enzyme activities were calculated considering the total protein content in the homogenates. All results are expressed in enzyme units. One unit of CAT represents the amount of enzyme needed to degrade 1 μmol of H_2O_2 $min^{-1} \cdot (mg\ of\ total\ protein)^{-1}$ present in the homogenates at 30 °C and pH 8.0. One unit of GST is the amount of enzyme necessary to conjugate 1 μmol of 1-chloro-2,4-dinitrobenzene $\cdot min^{-1} \cdot (mg\ of\ total\ protein)^{-1}$ present in the homogenates at 25 °C and pH 7.0. Absorbance readings were done at 240 and 340 nm for CAT and GST, respectively.

The NP-SH content assay was based on Sedlak and Lindsay (1968). Gills were homogenized (1:4w/v) in EDTA (0.02 M). The determination of sulfhydryl content in the samples was performed

after deproteinization with trichloroacetic acid (50%). Sulfhydryl groups were detected using 5,5-dithio-*bis*-2-nitrobenzoic acid (DTNB). Absorbance readings (405 nm) were done using a microplate reader (Elx 800, Bio-Tek Instruments, Inc., Winooski, Vermont). The NP-SH content was based on the protein concentration of each homogenate prior to deproteinization.

Protein content in homogenates was determined using a commercial reagent kit (Doles Reagents Ltda., Goiânia, Goiás, Brazil), based on the biuret reagent (550 nm).

In the analysis of lipid peroxidation (LPO) levels, the methodology was based on Hermes-Lima et al. (1995) and Monserrat et al. (2003). Gills were homogenized (1:9*w*/*v*) in cold methanol and centrifuged at 1000*g* for 10 min at 4 °C; the pellet was discarded. For LPO measurements, $FeSO_4$ (1×10^{-3} M), H_2SO_4 (0.25 M), xylenol orange (1×10^{-3} M), and MilliQ water were sequentially added. Samples (30 μL) or methanol (blanks) were added and incubated for 90 min. After that, absorbance (550 nm) was determined using a microplate reader. Cumene hydroperoxide (CHP) was employed as a standard. LPO values were expressed in CHP equivalents per gram of wet mass.

2.2. Quantification of melatonin in the hemolymph

With the purpose of revealing the concentration of melatonin in the hemolymph of the crab *N. granulata* and to know whether the eyestalk has some influence on it, two groups of crabs were kept in constant darkness for 3 days. After that, one group of animals had their eyestalks ablated (the eyestalks were cut off with scissors and then the crabs were cauterized) in the morning (9:00–11:00 h) 24 h prior to the experiment (eyestalkless group). The intact group was not manipulated. Then, on the first day (Day 1) of the experiment (96 h of constant darkness for both groups and 24 h after the ablation for the eyestalkless group) between 9:00 h – 11:00 h, 100 μl of hemolymph from intact and eyestalkless groups were collected with a syringe from the base of the 4th or 5th pairs of the walking legs and stored at –80 °C for further analysis. A pool of 5 animals was used to obtain a sample. Since 4 to 5 samples (n = 4–5) were used for each dosage, 20 to 25 animals had to be sacrificed. On the second day (Day 2), the same procedure, same time interval as on the previous day was carried out. All collecting and manipulation procedures took place under dim red light.

For melatonin quantification, 500 μl of methanol were added in each sample, centrifuged for 10 min at 10,000 rpm at 4 °C and the supernatants were collected and lyophilized. The melatonin concentration was measured according to Vakkuri et al. (1984a,b) and Maciel et al. (2008).

2.3. Daily variation of the total peroxyl radical scavenging capacity (TPRSC) in the gills and the effect of melatonin

To verify whether the total peroxyl radical scavenging capacity (TPRSC) in the gills of *N. granulata* exhibits a daily variation and to demonstrate a possible effect from melatonin, intact and eyestalkless (to eliminate a known site of melatonin production in crustaceans – Maciel et al., 2008) animals were utilized. After the acclimation period, 24 h prior to the experiment, the crabs were divided into three groups: one group with intact crabs, and the other two with ablated animals (the eyestalks were cut off with scissors and then the crabs were cauterized). Each group was distributed across 9 aquaria (giving a total of 27), corresponding to the 9 times that they had gills removed (every 3 h over a 24 h period = 9 times). Each time tissue was collected, an animal from a different aquarium was chosen, so that at the time of the sacrifice, animals left for the tissue collections at other times did not get stressed. Each aquarium contained 4 animals; thus, each group consisted of a total of 36 animals. The number of crabs used for the 3 groups together amounted to 108 crabs. Therefore, at each specific time, 4 animals from each of the three groups had their gills removed (a total of 12 animals at any one time). This procedure was repeated 9 times. Thirty minutes prior to the experiment, the eyestalkless crabs were injected with 100 μl of physiological saline (control group) or melatonin (20×10^{-9} M). The intact crabs received no injections. The tissues were immediately frozen at −80 °C prior to quantification of TPRSC.

TPRSC was determined according to Winston et al. (1998) and Regoli and Winston (1999). The gills were homogenized (1:4*w*/*v*) in phosphate buffer 50×10^{-3} M, pH 7.50, containing 2.5% NaCl. The homogenates were centrifuged at 13,000*g* at 4 °C for 25 min. The supernatants were collected and centrifuged at 33,000*g* at 4 °C for 1 h. These were later used for TPRSC quantification and protein content. Alpha-keto-γ-methiolbutyric acid (KMBA) (0.2×10^{-3} M) was employed as the substrate for ethylene production in the presence of peroxyl radicals, which were generated by thermal decomposition at 35 °C of 2,2′-azobis(2 methylpropionamidine) dihydrochloride (ABAP; 20×10^{-3} M) (Aldrich, Milwaukee, WI) dissolved in potassium phosphate buffer 0.1 M, pH 7.4. Ethylene production was measured by means of a Hewlett Packard (HP 4890 series) gas chromatograph equipped with a Supelco SPB-1 capillary column ($30 \times 0.32 \times 0.25$ μm) and a flame ionization detector (FID). The oven, injection and FID temperatures were 60, 280 and 190 °C, respectively. Helium was the carrier gas (flow rate: 30 ml/min) and a split ratio of 20:1 was used. TPRSC values were referred to the total protein content present in the biological sample. TPRSC values were calculated according to Winston et al. (1998).

2.4. The effect of melatonin in the aerobic metabolism of gills

To verify the effect of melatonin on the oxygen consumption of the gills in vitro and in vivo experiments were conducted. In the in vitro assay, crabs were sacrificed (n = 3–5) at noon and the gills were obtained and immediately incubated for 120 min with physiological saline only (control group), physiological saline plus melatonin (20×10^{-9} M), or physiological saline plus vitamin E (10^{-7} M). After the time of incubation, the oxygen consumption of the tissues was measured using a portable oxymeter. In the in vivo assay, intact and eyestalkless animals were used. After the 24-h acclimation period, crabs were divided into three groups: one group with intact crabs, and the other two groups with eyestalkless crabs. Each group consisted of 4 aquaria with 5 crabs each. Thirty minutes prior to the experiment, the eyestalkless crabs were injected with 100 μl of physiological saline (control group) or melatonin (20×10^{-9} M). The intact crabs received no injections. At 12:00, 13:00, 15:00, and 18:00 h, gills were collected and measured for oxygen consumption with a portable oxymeter. Oxygen consumption was expressed in milligrams of oxygen per gram-wet mass of tissue per hour.

2.5. Statistical analysis

Statistical analyses were made by analyses of variance (ANOVA) followed by Newman–Keuls test (α = 0.05). Normality and variance homogeneity were verified as ANOVA assumptions (Zar, 1984).

3. Results

CAT activity (Fig. 1A) showed a significant decrease ($p < 0.05$) in tissues exposed to H_2O_2 at 24:00 h (15.1 ± 0.97 U CAT (mg of protein)$^{-1}$) compared with the control groups (21.3 ± 2.3 U CAT (mg of protein)$^{-1}$). However, no significant differences ($p > 0.05$) were verified in GST activity (Fig. 1B) in gills exposed to H_2O_2 at

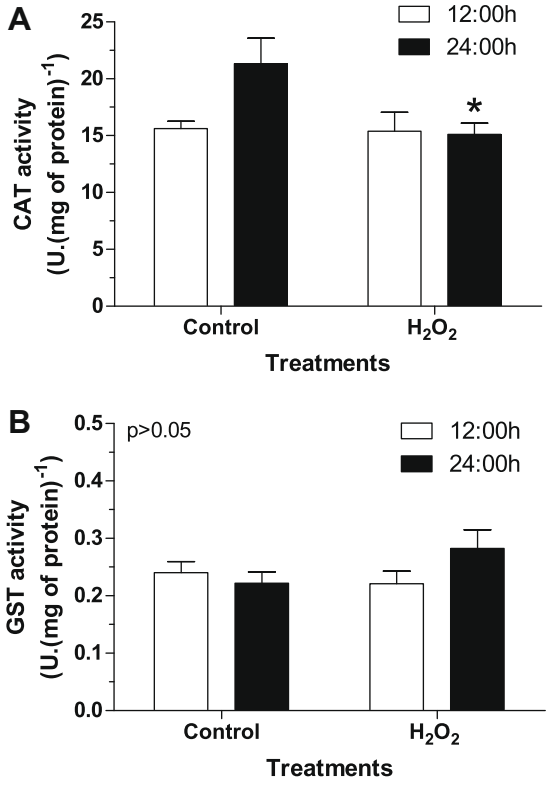

Fig. 1. Catalase (CAT) (A) and glutathione-*S*-transferase (GST) (B) activities in the gills of *Neohelice granulata* incubated for 120 min with H_2O_2 (20 mM) at 12:00 h (white bars) or at 24:00 h (black bars). Control groups were incubated with physiological saline only. One unit of CAT activity is defined as the amount of enzyme needed to degrade 1 μmol H_2O_2 min^{-1}·(mg total protein)$^{-1}$ present in the homogenates at 30 °C and pH 8.0 (n = 5–10). One unit of GST activity is defined as the amount of enzyme necessary to conjugate 1 μmol 1-chloro-2,4-dinitrobenzene·min^{-1}·(mg total protein)$^{-1}$ present in the homogenates at 25 °C and pH 7.0 (n = 5–10). Each point represents the mean ± 1 s.e.m. * Indicates significant differences (p < 0.05) from the control group.

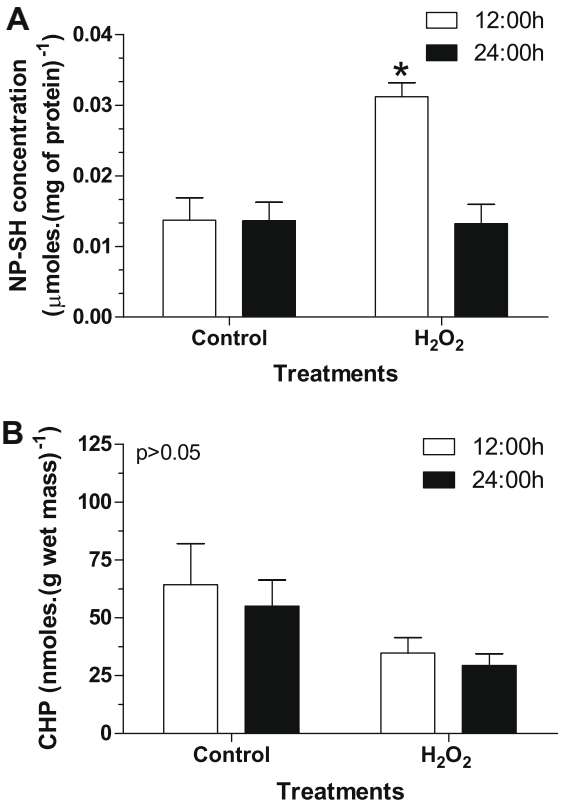

Fig. 2. Nonprotein sulfhydryl (NP-SH) (A) and lipid peroxidation (LPO) (B) contents in the gills of *N. granulata* incubated for 120 min with H_2O_2 (20 mM) at 12:00 h (white bars) or at 24:00 h (black bars). Control groups were incubated with physiological saline only. NP-SH content (n = 4–9) is express in μmoles·(mg total protein)$^{-1}$. LPO content (n = 5–6) is expressed as cumene hydroperoxide (CHP) equivalents per gram of wet mass. Each point represents the mean ± 1 s.e.m. * Indicates significant differences (p < 0.01) from the control group.

12:00 h or 24:00 h. Gills exposed to H_2O_2 during the day showed a significant increase (p = 0.005) in NP-SH contents (Fig. 2A) (0.03 ± 0.002 μmoles.(mg of protein)$^{-1}$) when compared with the control (0.01 ± 0.003 μmoles.(mg of protein)$^{-1}$). No significant differences (p > 0.05) were observed in LPO levels (Fig. 2B) in gills exposed to H_2O_2 at 12:00 or 24:00 h.

Interestingly, no significant differences between intact and eyestalkless crabs (p > 0.05) were observed with regard to the melatonin content of the hemolymph (Fig. 3) on Day 1 (81.75 ± 17 ρg of melatonin (ml of hemolymph)$^{-1}$ and 161.67 ± 49 ρg of melatonin (ml of hemolymph)$^{-1}$, respectively) and on Day 2 (123.75 ± 36 ρg of melatonin (ml of hemolymph)$^{-1}$ and 119.5 ± 23 ρg of melatonin (ml of hemolymph)$^{-1}$, respectively).

Daily variations (p < 0.05) in TPRSC were detected in the gills of intact crabs (Fig. 4). Two peaks were observed with a 12 h interval between them, a higher peak at the end of the photophase (2298 ± 225 U TPRSC (mg of protein)$^{-1}$) and another at the end of the scotophase (1063 ± 253 U TPRSC (mg of protein)$^{-1}$). Similar to the intact crabs, a daily variation (p < 0.05) in TPRSC was noticed in the gills of eyestalkless crabs injected with physiological saline (Fig. 4). Two peaks were also observed with a 12 h interval between them, but with a delay of 3 h for each peak (2386 ± 260 U TPRSC (mg of protein)$^{-1}$ for the higher and 1802 ± 339 U TPRSC (mg of protein)$^{-1}$ for the shorter one. However, in eyestalkless crabs injected with melatonin (2×10^{-12} mol $crab^{-1}$) there were no variations (p > 0.05) in TPRSC values during the 24 h period of analysis (Fig. 4).

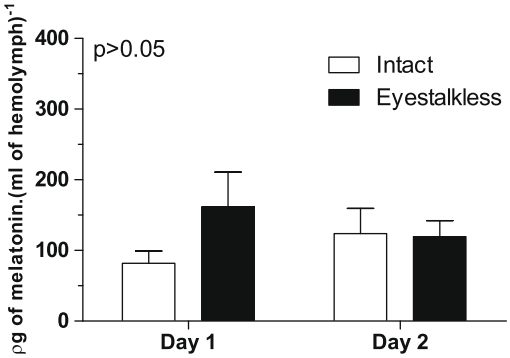

Fig. 3. Melatonin content (n = 4–5) in the hemolymph of intact (white bars) and eyestalkless (black bars) *N. granulata* on Day 1 and Day 2 (see Section 2 for additional information). No significant differences (p > 0.05) were observed between the groups.

Gills exposed only to H_2O_2 (20×10^{-3} M) at 12:00 h (Fig. 5) showed a significant increase (p < 0.05) in NP-SH content (0.023 ± 0.002 μmoles (mg of protein)$^{-1}$) compared with the control group (0.013 ± 0.001 μmoles (mg of protein)$^{-1}$). An increase in NP-SH content (p < 0.05) was also observed in tissues incubated with H_2O_2 (20×10^{-3} M) plus melatonin (20×10^{-9} M) or vitamin E (10^{-7} M) (0.03 ± 0.003 μmoles (mg of protein)$^{-1}$ and 0.025 ± 0.002 μmoles (mg of protein)$^{-1}$, respectively) compared to the control group. No significant differences (p > 0.05) were observed in the NP-SH contents of gills incubated only with melato-

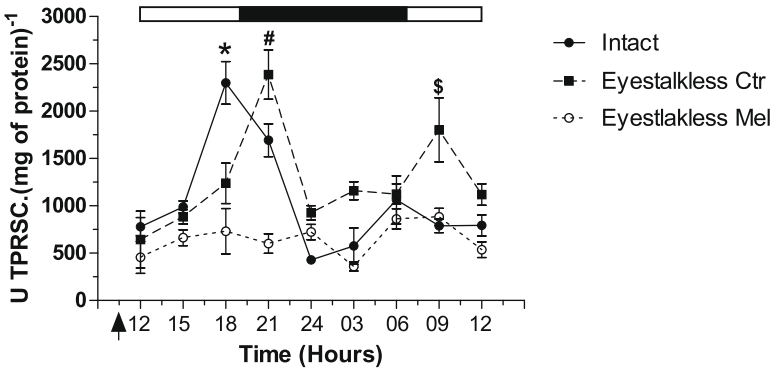

Fig. 4. Daily variations in total oxyradical scavenging capacity (TPRSC) in the gills of intact and eyestalkless *N. granulata* using a peroxyl radical generation system (ABAP, 20 mM), injected with 100 µl of physiological saline or melatonin (20×10^{-9} M) 30 min before the extraction of the tissues (indicated by the arrow). No injections were applied to intact crabs. Each point represents the mean ± 1 s.e.m. ($n = 4$). Hours when tissues were sampled are indicated on the *x* axis. Open and solid bars indicate light and dark phases, respectively. No significant differences ($p > 0.05$) were found during the 24 h period of analyses in the melatonin treated crabs. ∗, #, $ Indicates significant differences ($p = 0.0002$, $p = 0.001$ and $p = 0.012$, respectively) from the respectively smallest data point of each group.

nin (20×10^{-9} M) (0.012 ± 0.002 µmoles (mg of protein)$^{-1}$). However, in tissues incubated only with vitamin E (10^{-7} M) a rise ($p < 0.05$) in NP-SH content (0.021 ± 0.001 µmoles (mg of protein)$^{-1}$) in relation to the control group (0.013 ± 0.001 µmoles.(mg of protein)$^{-1}$) was noticed.

In the in vitro assay (Fig. 6), gills incubated with melatonin significantly decreased ($p < 0.05$) the oxygen consumption (0.087 ± 0.019 mg O_2 $g^{-1}h^{-1}$) compared with the control group (0.296 ± 0.026 mg O_2 $g^{-1}h^{-1}$). No significant differences ($p > 0.05$) were observed between gills incubated with vitamin E (0.214 ± 0.062 mg O_2 $g^{-1}h^{-1}$) and the control group (Fig. 6). In the in vivo assay (Fig. 7) the oxygen consumption of eyestalkless crabs injected with physiological saline was significantly higher ($p < 0.05$) (0.421 ± 0.046 mg O_2 $g^{-1}h^{-1}$) than that of intact crabs (0.262 ± 0.003 mg O_2 $g^{-1}h^{-1}$). When eyestalkless crabs were injected with melatonin, the oxygen consumption significantly decreased ($p < 0.05$) (0.225 ± 0.02 mg O_2 $g^{-1}h^{-1}$) when compared with eyestalkless crabs injected with only physiological saline at 18:00 h.

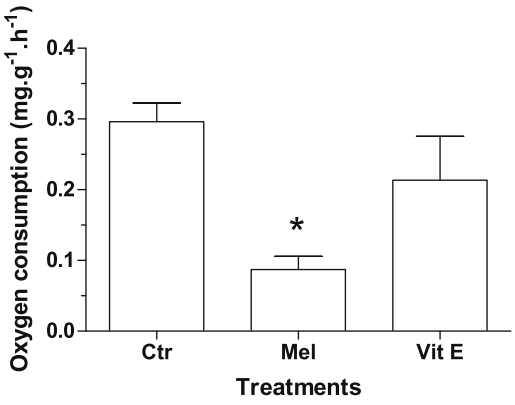

Fig. 6. Oxygen consumption in the gills of *N. granulata* incubated for 120 min with physiological saline (control group), physiological saline plus melatonin (20×10^{-9} M), and physiological saline plus vitamin E (10^{-7} M) at 12:00 h. Oxygen consumption ($n = 3$–5) is expressed in milligrams of oxygen per gram-wet mass of tissue per hour. Each point represents the mean ± 1 s.e.m. ∗ Indicates significant differences ($p < 0.05$) from the control group.

4. Discussion

In crustaceans, some studies have identified daily variations in oxygen consumption (Crear and Forteath, 2000; Maciel et al., 2004; Rosas et al., 1992), suggesting that there could also be fluctuations in the production of ROS. To counteract the rises in ROS concentrations and/or decrease the deleterious effects of ROS actions, it is necessary for organisms to adjust the ADS. Fanjul-Moles et al. (2003) reported a daily variation in some components of the ADS in the hemolymph and hepatopancreas of the prawns *Procambarus clarkii* and *P. digueti*. The activities of the enzymes GPx and GR are higher at night and a peak of the tripeptide GSH content was observed during the day period. It was suggested that different

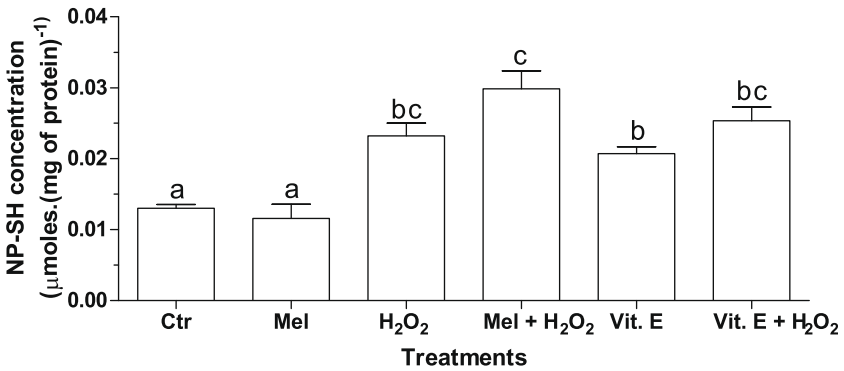

Fig. 5. Nonprotein sulfhydryl (NP-SH) content in the gills of *N. granulata* incubated for 120 min with physiological saline plus H_2O_2 (20 mM), physiological saline plus melatonin (20×10^{-9} M), physiological saline plus vitamin E (10^{-7} M), physiological saline plus H_2O_2 and melatonin, or physiological saline plus H_2O_2 and vitamin E at 12:00 h. Control group were incubated with physiological saline only. Nonprotein sulfhydryl (NP-SH) content ($n = 3$–5) are expressed in µmoles·(mg total protein)$^{-1}$. Each point represents the mean ± 1 s.e.m. Significant differences ($p < 0.05$) are indicated with different letters.

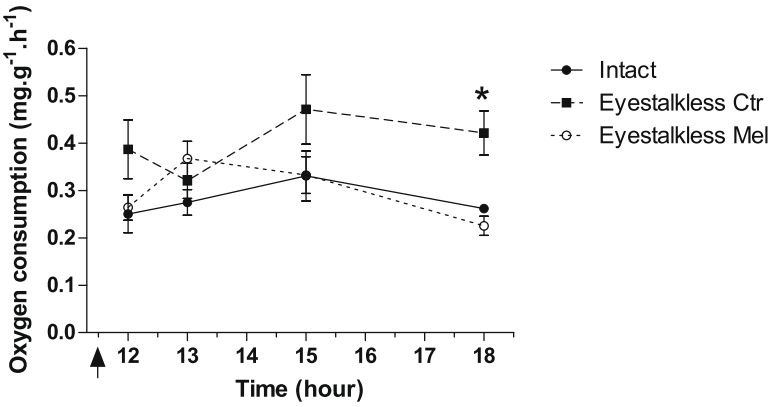

Fig. 7. Oxygen consumption in the gills of intact and eyestalkless *N. granulata* at 12:00, 13:00, 15:00 and 18:00 h, injected with 100 μl of physiological saline or melatonin (20×10^{-9} M) 30 min prior to the extraction of the tissues (indicated by the arrow). No injections were applied to intact crabs. Each point represents the mean ± 1 s.e.m. * Indicates significant differences ($p < 0.05$) of Eyestalkless CTR group from Intact and Eyestalk MEL groups only at 18 h.

strategies to counteract ROS generation are adopted depending on the time of day.

Maciel et al. (2004) found daily variation in oxygen consumption and some components of the ADS in the gills of the crab *N. granulata*. Two peaks of oxygen consumption with a 12 h interval between them were observed. Lipoperoxidation levels and NP-SH content were higher in the middle of the day and night periods, respectively. The enzyme GST showed a reduced activity at the end of the photophase and higher values during the night, but CAT activities did not show daily variations. The variations in the ADS component can be a direct response of ROS production or may be regulated by external signals. Therefore, the first step of this investigation was to know whether the ADS components and lipoperoxidation were a direct response to the ROS in the gills of this crab.

The in vitro assays showed that when gills were incubated with H_2O_2, depending on time of day, an inhibition of CAT was seen (Fig. 1A). CAT activity decreased during an exposure to H_2O_2 at night (24:00 h) by 29%. Although the oxidants employed diminish CAT activity, no significant differences were observed in the lipid peroxidation products. Escobar et al. (1996) reported an inactivation of SOD and CAT by singlet oxygen and peroxyl radicals and Gieseg et al. (2000) and Du and Gebicki (2004) verified that proteins are the major targets of peroxyl and hydroxyl radicals before lipids. It is possible that the duration of H_2O_2 incubation (120 min) was insufficient to induce an increase in lipoperoxidation (Fig. 2B) and/or some interference by other enzymatic or non-enzymatic antioxidants (SOD, GPx, β-carotenes) occurred. This may be one explanation for the differential inhibition of CAT activity during day and night phases, as some antioxidant levels (enzymatic or not) may peak at different times.

Interestingly, gills exposed to H_2O_2 during the day (12:00 h) increased the NP-SH content with respect to the control group (Fig. 2A). Seemingly, NP-SH content is regulated by a direct response to ROS; in this case by H_2O_2. An involvement of ROS in signaling cascades, promoting activation or inactivation of peptides and proteins has been reported (Dröge, 2002). In this study, H_2O_2 may induce a rise in the γ-glutamylcysteine ligase activity, increasing GSH synthesis, or in the GR activity, assisting restoration of GSH contents from oxidized glutathione (GSSG). Semchyshyn et al. (2005) showed an increase in GR activity of approximately 50% in bacterial cultures (*Escherichia coli*) exposed to 20×10^{-6} M H_2O_2 for 20 min, indicative of a rapid response of this enzyme to ROS.

Taking into account that some components of the ADS in the gills of *N. granulata* vary cyclically (Maciel et al., 2004) but that, a priori, only NP-SH content was modulated by ROS (H_2O_2), the next point to analyze was to verify whether the total antioxidant capacity against peroxyl radicals displayed a daily variation. In fact, a biphasic daily variation in TPRSC (with one peak in the photo- and one in the scotophase) was observed in the gills of intact *N. granulata* (Fig. 4), paralleling oxygen consumption in this crab (Maciel et al., 2004). If we consider that increased oxygen consumption may result in elevated ROS generation (Yan and Sohal, 2000), variations in ADS may be correlated to aerobic metabolism. Interestingly, although the highest NP-SH content and peak of oxygen consumption occurred during the scotophase (Maciel et al., 2004), the highest peak of TPRSC values were observed during the photophase. Regoli and Winston (1999) reported that differences in total oxidant scavenging capacity values could depend on the oxyradical generating system used. In fact, GSH is an important HO· scavenger (Storey, 1996; Gavin and Sies, 2001) and, therefore, its contribution to TPRSC values should be low. Other non-enzymatic antioxidants such as β-carotenes, vitamins (E and C) and enzymatic antioxidants (superoxide dismutase and GPx) could be most effective during the period (scotophase to photophase) of the highest peak of TPRSC, even if the biphasic profile were maintained. To identify the antioxidants responsible to generate this profile in TPRSC requires further study.

As mentioned before, melatonin had earlier been identified in some crustacean tissues, like brain and eyestalks (cf., Maciel et al., 2008), but with regard to hemolymph, only Agapito et al. (1995) in *Procambarus clarkii* and Pape et al. (2008) in *Euphausia superba* reported finding this indoleamine. The melatonin content of the hemolymph of *N. granulata* is more similar to that of the decapod *P. clarkii* (approximately 10^{-9} M) reported by Agapito et al. (1995) rather than that of the krill *E. superba*. However, this is the first report that measured the melatonin content in the hemolymph of eyestalkless crabs (Fig. 3). Interestingly, no significant differences were observed between the groups, including crabs ablated on Day 1 or Day 2. These results suggest that the eyestalk does not influence the melatonin content of the hemolymph and indicates that one or more sites besides the eyestalk are capable of producing melatonin, similar to mammals (Hardeland, 2008) and the honeybee (Meyer-Rochow and Vakkuri, 2002).

In the present work it was observed that a daily variation of TPRSC in the gills of eyestalkless *N. granulata* persists (Fig. 4), but with a phase-shift of three hours (the highest values of TPRSC in eyestalkless crabs were one data point behind those of the intact ones), suggesting that the antioxidant defense system in gills is controlled from outside the eyestalk. As previously stated, other sites may modulate (through melatonin or not) the antioxidant de-

fense system in this tissue. Moreover, the biphasic profile of the TPRSC values in eyestalkless crabs were similar to the biphasic profile of TPRSC in intact crabs and the oxygen consumption in the same tissue reported in this crab, one peak at photophase and another at scotophase (Maciel et al., 2004). It is possible that the daily variation of TPRSC in gills of *N. granulata* is related to the aerobic metabolism so that an increase in oxygen consumption would result in an increase in ROS generation and ADS.

However, we cannot rule out the possible involvement of the eyestalks in the control of the circadian rhythms. In fact, circadian rhythmicity of several physiological functions in crustaceans has been documented, e.g., locomotor activity, pigment migration, heart rate, glucose level, enzyme activities a few others (Aréchiga and Rodrigues-Sosa, 1997,2002). In mammals, the suprachiasmatic nucleus of the brain has been identified as the seat of the 'master clock', regulating cyclic physiological events chiefly through melatonin. In crustaceans the identity of the circadian clock, as well as its signaling molecules, is not well established. Some authors suggest that in this group of animals a multi-oscillatory clock could regulate biological rhythms (Aréchiga and Rodrigues-Sosa, 1997,2002) and some tissues or organs that synchronize some physiological parameters have, indeed, already been identified: for instance, the eyestalk with its X-organ/sinus gland complex as the main neurohaemal organ, the retina, supraoesophageal ganglion, and sixth abdominal ganglion (Aréchiga and Rodrigues-Sosa, 1997,2002; Fuentes-Pardo and Inclán-Rubio, 1987; Larimer and Smith, 1980; Saigusa, 2002). Therefore, the eyestalks of *N. granulata* may exert some influence on the daily variation of TPRSC in the gills of this crab.

Numerous studies have reported an involvement of melatonin in the regulation of the ADS, through the modulation of the antioxidant enzymes or by acting as an ROS scavenger. In this work melatonin significantly decreased the antioxidant capacity against peroxyl radicals in the gills of eyestalkless *N. granulata* (Fig. 4).

To substantiate the effect of melatonin in regulating the ADS components, NP-SH content, as an indication of the GSH level, was determined (Fig. 5). It could be shown that melatonin did not influence the NP-SH content in the gills exposed to H_2O_2, at least in the concentration used (20×10^{-9} M). Some studies showed an increase of GSH levels through the increase of γ-glutamylcysteine ligase activities (Abe et al., 1994; Urata et al., 1999), but according to other studies through glutathione reductase, responsible for GSH synthesis and/or recycling (Melchiorri et al., 1995; Sewerynek et al., 1995). However, Dadanpat et al. (2000) and Pinho et al. (2005) observed an increase of GSH and NP-SH levels in the gills of *Macrobrachium rosenbergii* and *N. granulata*, respectively, when these crustaceans were given supplementary vitamin E. Shang et al. (2003) reported that rabbit lens epithelial cell culture pre-treated with vitamin E ($5–40 \times 10^{-6}$ M) restored the resistance of GSH-depleted cells to H_2O_2 ($0–400 \times 10^{-6}$ M). Interestingly, the NP-SH content increased when exposed to H_2O_2 irrespective to whether the treatment was with or without melatonin or vitamin E. As remarked above, this result suggests that gill NP-SH content may be a direct response to ROS.

The direct reaction of melatonin or its metabolites with endogenous ROS (i.e., scavenging) (Hardeland, 2008) could lead to a decrease in the antioxidant status of the tissue, detectable in TPRSC measurements. Studies that demonstrated the scavenging capacity of melatonin involved higher concentrations (ranging from micromolar to millimolar) than the doses 2×10^{-12} mol crab^{-1} (equivalent to 10^{-9} M in the hemolymph, which in fact is the same amount of melatonin measured in intact and eyestalkless crabs – cf., Fig. 3) used in this work, suggesting that melatonin's direct scavenging capacity was not effective in this case (Barlow-Walden et al., 1995; Cagnoli et al., 1995; Hardeland et al., 2003; Pieri et al., 1994; Pozo et al., 1994; Tan et al., 1993).

In human medicine, melatonin is used as a chronobiotic agent in sleep-wake disorders (Arendt and Skene, 2005; Dijk and Cajochen, 1997). This molecule also influences body temperature, locomotor activity, and blood glucose levels in both vertebrates and invertebrates (Cagnacci et al., 1997; Lutterschimidt et al., 2003; Redman et al., 1983; Tilden et al., 2001a, 2001b; Underwood, 1981). Thus, it is clear that melatonin can affect the entire metabolism through physiological interactions. In fact, some reports have shown an effect of melatonin in mitochondrial electron flux, which, in turn, affects all parts of the metabolism (Hardeland, 2005; Hardeland and Pandi-Perumal, 2005; Hardeland et al., 2003). The significant decrease of TPRSC in gills of eyestalkless crabs after melatonin injection (Fig. 4) could be due to a reduction of the aerobic gill metabolism, reducing ROS production and allowing for a decrease in the antioxidant capacity in this tissue. The decrease of the aerobic metabolism of the gills was verified as a decrease of oxygen consumption in vitro, when the gills were incubated for 120 min with melatonin (Fig. 6). This effect was observed in vivo only 390 min after melatonin was injected into eyestalkless crabs (Fig. 7). The increase in oxygen consumption in eyestalkless crabs is a known effect in crustaceans (Rosas et al., 1991,1993). However, additional studies are necessary to confirm the role of melatonin in the reduction of the aerobic gill metabolism in *N. granulata*.

In conclusion, although melatonin was not capable of modulating NP-SH in the gills of *N. granulate,* it, nevertheless, seemed capable of depressing gill activity in this crab by decreasing TPRSC and the aerobic metabolism.

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