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Effect of melatonin in the antioxidant defense system in the locomotor muscles of the estuarine crab *Neohelice granulata* (Decapoda, Brachyura)

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ABSTRACT

In vertebrates, many studies verified different effects of melatonin in the antioxidant defense system (ADS). In crustaceans, few studies have been conducted to verify this possibility. We verified the melatonin effects in the crab *Neohelice granulata* using low (0.002 and 0.02 pmol/crab) and high (2.0 and 20.0 pmol/crab) melatonin dosages in short-term (0.5 h) and long-term (9.5 h) experiments. We analyzed the antioxidant capacity against peroxyl radicals (ACAP), reactive oxygen species (ROS) concentration, levels of by products of lipid peroxidation (LPO), oxygen consumption (VO_2), the activity of glutamate cysteine ligase (γ-GCL) and catalase (CAT) and glutathione content (GSH). Finally, the effects of exogenous melatonin were verified in terms of melatonin and N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) content in the muscles of *N. granulata*. In short-term experiment and low dosages, melatonin increased the VO_2, γ-GCL activity and GSH content ($p < 0.05$) and decreased melatonin content ($p < 0.05$) without effects in ROS, ACAP and LPO ($p > 0.05$). Possibly, melatonin is acting in the ADS increasing its efficiency and/or acting in mitochondrial activity and/or through signaling muscles to increase its consumption. AFMK was only detected in the eyestalk and cerebroid ganglia. In high dosages melatonin effects decreased, possibly by the desensitization of their receptors. In long-term experiment, melatonin decreased ACAP ($p < 0.05$), and CAT activity ($p < 0.05$) in low dosages. In high dosages melatonin reduced VO_2 ($p < 0.05$) and increased ACAP ($p < 0.05$), possibly stimulating others components of the ADS. In conclusion, melatonin in the locomotor muscles of *N. granulata* affects the antioxidant/pro-oxidant balance in a time and dosage dependent manner.

1. Introduction

In vertebrates melatonin is known as the product of pineal gland secretion, where its classical function is to inform the organism the day/night length, which allows a synchronization of the physiological parameters to environmental factors (Reiter, 1991). Besides the pineal gland, melatonin is also synthesized in several other tissues and organs. This indoleamine was detected in the retina (Cahill and Besharse, 1989), the Harderian gland (Djeridane and Touitou, 2001), in gastrointestinal tract (Bubenik, 2002), skin (Slominski et al., 2005) and in various parts of the nervous system (Hardeland and Poeggeler, 2007). In some of these places, melatonin levels exceed several times the levels found in the pineal gland. Although this indoleamine is synthesized in others tissues and organs, this molecule apparently does not show a pronounced rhythmicity in these places, possible not taking a role in rhythmic physiological responses (Hardeland, 2008).

Melatonin is a ubiquitous molecule present from unicellular algae to vertebrates (Bittman, 1993). In crustaceans, melatonin was reported in some tissues and organs such as hemolymph, head, eyestalk, optic ganglion and eyes (Vivien-Roels and Pevet, 1986; Withyachumnarnkul et al., 1992a,b, 1995; Agapito et al., 1995; Balzer et al., 1997; Tilden et al., 1997, 2001; Meyer-Rochow, 2001; Pape et al., 2008). Recently Maciel et al. (2008) verified a biphasic profile of melatonin content in the eyestalk of *Neohelice granulata*, with a period of 12 h in animals kept in 12L:12D and constant dark photoperiods, but with a small amplitude during the profile.

In crustaceans, some studies have been conducted to verify the role of melatonin. Balzer et al. (1997) verified the influence of melatonin in the phase delay and amplitude of the circadian rhythm of electroretinogram in *Procambarus clarkii*. In this same species, Tilden et al. (2003) reported that injections of melatonin increased the synaptic transmission in the neuromuscular junc-

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tions. In the fiddler crab *Uca pugilator*, melatonin increased the rate of limb regeneration (Tilden et al., 1997) and the influence in the phase delay of the circadian rhythm of glucose and lactate in the hemolymph (Tilden et al., 2001). Regarding the effects of melatonin in the ADS of crustaceans Maciel et al. (2009) verified that melatonin in a single dosage of 2.0 pmol/crab and 30 min after injected, induced a pronounced reduction in the total peroxyl radical scavenging capacity (TPRSC) and also in the oxygen consumption (VO_2) in gills of the crab *N. granulata*.

In vertebrates, the effects of melatonin in the antioxidant defense system (ADS)[1] has been demonstrated, but with a role not yet clearly understood (Tan et al., 2000; Reiter et al., 2003; Allegra et al., 2003; Rodriguez et al., 2004). The majority of studies verified an antioxidant function for this molecule acting through several mechanisms. Firstly, it was proposed that melatonin may act as a "*scavenger*" of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$) and nitric oxide (NO) (Reiter et al., 1996, 2002; Tan et al., 1998, 2000; Anisimov, 2003). At this point, the direct effects of melatonin were observed mainly in pharmacological concentrations. For other side, melatonin in supraphysiological and physiological concentrations may also acting in the regulation of some antioxidant enzymes (Reiter et al., 1996; Longoni et al., 1998; Liu and Ng, 2000). In addition, it has been demonstrated that melatonin may have an action in mitochondrial activity preventing the increase in ROS production and also increasing the ATP synthesis (Acuna-Castroviejo et al., 2003). Finally, for other side, some studies verified an opposite melatonin effect acting as a pro-oxidant molecule in the ADS in high dosages (Wolfer et al., 2001; Barsacchi et al., 1998; Osseni et al., 2000).

In order to better understand the effects of melatonin in the ADS in crustaceans, the purpose of this study was to verify the effects of melatonin in locomotor muscles of the crab *N. granulata* analyzing its effects at different dosages and times. Thus, initially we analyzed the effects of this indoleamine in low dosages (0.002 and 0.02 pmol $crab^{-1}$) and high dosages (2.0 and 20.0 pmol $crab^{-1}$). Furthermore, the melatonin effects were observed in short-term (0.5 h) and long-term (9.5 h) experiments. This study examined the melatonin effects in the antioxidant capacity against peroxyl radicals (ACAP), ROS concentration, oxygen consumption (VO_2), catalase and glutamate cysteine ligase (γ-GCL) activity, glutathione (GSH) content and lipoperoxidation levels (LPO). Finally, we also analyzed the melatonin effects in the locomotor muscles of *N. granulata* in terms of endogenous melatonin and AFMK concentration in this tissue.

2. Materials and methods

2.1. Animals

Adult male crabs of *N. granulata* species weighing 10.2 ± 0.2 g (mean ± SEM) where the lowest weight crab had 8.7 g and the highest had 12.1 g were collected in salt marshes around Rio grande City, Brazil. Thereafter, they were transferred to laboratory for acclimatation period. In the laboratory the were acclimated, at least, during 10 days in tanks under constant conditions of temperature (20 °C), salinity (20) and fixed photoperiod (12L:12D). They were fed *ad libitum* with ground beef 3 times a week.

2.2. Reagents

Melatonin, AFMK (N^1-acetyl-N^2-formyl-5-methoxykynuramine), ABAP (2,2′-azobis-2-methylpropionamidine dihydrochloride), H_2DCF-DA (2′,7′-dichlorofluorescein-diacetate), NDA (naphthalene-2,3-dicarboxyaldehyde), glutathione, ATP (adenosine triphosphate), glutamate, borate, serine, cysteine and CHP (Cumene hydroperoxide) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were supplied by Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Formic acid analytical grade, by Merck (Darmstadt, Germany). Water was purified with a Direct-Q UV3® (resistivity 18.2 MΩ cm) water purification system (Millipore, Bedford, MA, USA). The stock solutions for melatonin were made in ethanol. For each solution the final dilution was prepared in physiological saline, and the final concentration never exceeded 0.01% of ethanol. The physiological saline contained 10 mmol L^{-1} $MgCl_2$, 355 mmol L^{-1} NaCl, 16.6 mmol L^{-1} $CaCl_2$, 5 mmol L^{-1} H_3BO_3, 10 mmol L^{-1} $KHCO_3$ and 8 mmol L^{-1} $Na_3C_6H_5O_7{\cdot}2H_2O$ in pH adjusted to 7.6.

2.3. Experimental procedures

After the acclimatation period, the crabs ($n = 5$) for each group and time, were divided into three groups and kept over constant darkness for 4 days. At the beginning of the 3rd day, two groups of animals had their eyestalks ablated (the eyestalks were cut off with scissors and then the crabs were cauterized) over a red light and the other group kept intact (Intact group). One day later, the eyestalkless crabs were injected in the fifth pair of pereopods with (100 μL) of physiological saline (Control) or melatonin in four dosages (0.002; 0.02; 2.0 and 20.0 pmol/crab). After 30 min (0.5 h, short-term) and 570 min (9.5 h, long-term), of injection time, muscles of meropodites of the second pair of pereopods were collected and stored at −80 °C for analysis.

As positive control of the presence of AFMK a pool of 20 eyestalk and 10 supraesophageal ganglia were collected 1hour after injection of 20.0 pmol/crab melatonin. Soon after, the tissue preparation procedure was the same for muscles.

2.4. Tissue preparation

For melatonin and AFMK quantification locomotor muscles, eyestalk and supraesophageal ganglia were weighted and homogenized in 500 μL in methanol 100%. Thereafter, the extracts were centrifugated at (10,000*g*, 4 °C for 10 min) and the supernatants were collected and completed to 1 mL with methanol for LC–MS/MS analysis.

For ACAP and ROS analysis, the muscles were weighed and homogenized (1:10 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 centrifugated twice at (2000*g*, 4 °C for 20 min) and the supernatant were collected and centrifugated again (10,000*g*, 4 °C for 45 min). The supernatants of the last centrifugation were employed to analysis.

For catalase activity, muscles were weighed and homogenized (1:10 w/v) in a cold (4 °C) buffer solution containing Tris base (20 mM), EDTA (1 mM), dithiothreitol (1 mM, Sigma), KCl (150 mM), and PMSF (0.1 mM), with pH adjusted to 7.6. Homoge-

[1] *Abbreviations used:* ADS, antioxidant defense system; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; $HO^{\cdot}$, hydroxyl radical; $O_2^{\cdot -}$, radical anion superoxide; HOCL, hipoclorous acid; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); ABAP, 2,2′-azobis-2-methylpropionamidine dihydrochloride; EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHP, cumene hydroperoxide; H_2DCF-DA, 2′,7′-dichlorofluorescein-diacetate; NDA, naphthalene-2,3-dicarboxyaldehyde; NO, nitric oxide; ACAP, antioxidant competency against peroxides; AMMC, 3-acetamidomethyl-6-methoxycinnolinone; AMNK, N1-acetyl-5-methoxy-3-nitrokynuramine; 3-OHMc, cyclic 3-hydroxymelatonin; GSH, reduced glutathione; CAT, catalase; NP-SH, non-proteic sulfhydryl; SOD, superoxide dismutase; GPx, glutathione peroxidase; γ-GCL, glumatate systeine ligase; PMSF, phenylmethylsulfonyl fluoride; LPO, lipid peroxidation; AFMK, N^1-acetyl-N^2-formyl-5-metoxykinuramine; AMK, N^1-acetyl-5-metoxykinuramine; AMPc, cyclic adenosine monophosphate; ATP, adenosine triphosphate.

nates were centrifugated at (9000*g*, 4 °C for 30 min) and the supernatants were employed to analysis.

For glutamate cysteine ligase activity and glutathione content, the muscles were weighed and homogenized (1:10 w/v) in a cold (4 °C) buffer solution containing Tris–HCl (100 mM), $MgCl_2$ (5 mM) and EDTA (2 mM) with pH adjusted to 7.6. The samples were centrifugated twice at (2000*g*, 4 °C for 20 min) and the supernatant were collected and centrifugated again for (10,000*g*, 4 °C for 45 min). The supernatants of this second centrifugation were then employed to analysis.

For lipid peroxidation (LPO) measurements, muscles were weighed and homogenized (1:9 w/v) with methanol (in a cold (4 °C)) conditions and centrifugated at (1000*g*, 4 °C for 10 min). The supernatant was employed to analysis.

2.5. Oxygen consumption

The muscles of the second pair of pereopods were collected and put in bottles with 10 mL of physiological saline. Oxygen levels was measured after 30 min after bottles being closed using portable oximeter (DMO-2, Digimed) in a controlled room of 20 °C. After this procedure the muscles were weighed. The oxygen consumption was expressed in milligrams of oxygen per gram wet weight of tissue per hour.

2.6. Antioxidant capacity against peroxyl radical (ACAP) analysis

The antioxidant capacity against peroxyl radicals was measured according to the method of Amado et al. (2009). Briefly, 10 μL of the supernatants prepared for enzymatic analysis were 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 samples. In three of the six wells of each sample, 7.5 μL of 2,2′-azobis-2-methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich) were added while the same volume of ultrapure water was pipetted in the other three wells. The microplate was put into a fluorescence microplate reader (Victor 2, Perkin-Elmer), at a programmed temperature of 35 °C, at which peroxyl radicals were produced by thermal decomposition of ABAP. Immediately before the reading, 10 μL of the fluorescent probe 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA) were added to 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 wavelengths of 488 and 525 nm, for excitation and emission, respectively. The thermal decomposition of ABAP and ROS formation was monitored with readings every 5 min for 60 min. According to Regoli and Winston (1999) and Regoli (2000) non-enzymatic low-molecular-weight scavengers (GSH, ascorbic acid, uric acid, vitamin E) generally accounted for 70% of the total scavenging capacity towards peroxyl radicals. So if enzymatic inhibition occurs due to the high temperature (as crabs are poikilotherms) needed to ABAP decomposition in peroxyl radical (35 °C), the lowering of antioxidant capacity should be a minor problem. Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were calculated as area difference of FU × min in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The inverse of relative difference between ROS area with and without ABAP was considered as a measure of antioxidant capacity, with high area difference meaning high antioxidant capacity, since high fluorescence levels were obtained after adding ABAP, meaning low competence to neutralize peroxyl radicals. Note that calculating antioxidant competence as:

$$1/(\mathrm{ROS_{area}}\ \mathrm{ABAP} - \mathrm{ROS_{area}}\ \mathrm{background})/\mathrm{ROS_{area}}\ \mathrm{background}$$

2.7. ROS concentration

The supernatant were used for determination of the reactive oxygen species (Viarengo et al., 1999). For ROS detection, it was used 2′,7′-dichlorofluorescein-diacetate (H_2DCF-DA, molecular probes) that in the presence of ROS generate a fluorochrome that was detected using wave lengths of 488 and 525 nm for excitement and emission, respectively. The analysis were carried out in a fluorescence microplate reader (Victor 2, Perkin) with readings every 5 min for 60 min. Total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. ROS concentration was compared to the total protein content present in the biological sample and expressed in FU $(\mathrm{mg\ of\ protein})^{-1}$.

2.8. Lipid peroxidation

The methodology was based on Hermes-Lima et al. (1995) and Monserrat et al. (2003) named FOX based on the oxidation of Fe(II) under acidic conditions. The Fox method measure lipid peroxides, one of the main products of lipid peroxidation. For LPO measurements, $FeSO_4$ (1 mM), H_2SO_4 (0.25 M), xylenol orange (1 mM, Sigma) and MilliQ water were sequentially added. Samples (30 μL) or methanol (blanks) were added and incubated for 375 min. Thereafter, absorbance (550 nm) was determined using a microplate reader (Victor 2, Perkin), and Cumene hydroperoxide (CHP; Sigma) was employed as a standard. Lipid peroxidation (LPO) is express in cumene hydroperoxide (CHP) equivalents per gram of wet mass.

2.9. Catalase activity

Catalase (CAT) activity was analyzed following Beutler (1975), determining the initial rate of H_2O_2 (50 mM) decomposition at 240 nm. The results were expressed in CAT units, where one unit is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2 per minute and per mg of protein, at 25 °C and pH 8.0. This procedure was performed in a digital spectrophotometer (Biomatte 3). Catalase activity was compared to the total protein content present in the biological samples.

2.10. Glutamate cysteine ligase activity and glutathione content

Glutamate cysteine ligase (GCL) activity and glutathione content was analyzed following White et al. (2003). This method is based in the reaction of naphthalene-2,3-dicarboxyaldehyde (NDA) with glutathione (GSH) or γ-glutamylcysteine (γ-GC) to form cyclized product that are highly fluorescent. NDA-GSH fluorescence intensity was measured (472 ex/528 em) on fluorescence microplate reader (Victor 2, Perkin-Elmer). This assay has the advantage that baseline levels of GSH can also be measured in the same tissue sample. For GCL activity, it was prepared an GCL reaction cocktail (400 mM Tris, 40 mM ATP, 20 mM glutamate, 2.0 mM EDTA, 20 mM sodium borate, 2 mM serine, 40 mM $MgCl_2$) just prior to the beginning of the assay to prevent ATP degradation. The samples plate was kept on ice until were pipetted into the reaction plate (25 °C) at 15-s time intervals. After 5 min of preincubation, the GCL reaction was initiated by adding 50 μL of cysteine (2 mM) to each GCL activity well (cysteine was not added to the GSH-baseline wells in this time). Soon after, the plate was incu-

bated during 30 min and the reaction stopped by adding 50 μL of 5-sulfosalicylic acid (SSA, 200 mM) and then 50 μL of 2 mM cysteine was added to the GSH-baseline wells. After protein precipitation, the plate was centrifugated for 5 min at 2500 rpm and then, 20 μL aliquots of supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection (Victor 2, Perkin-Elmer). The GCL activity was expressed in nM of GCL h^{-1} (mg of $protein^{-1}$) and GSH content in nM of GSH (mg of $protein^{-1}$). GCL activity and GSH content were compared to the total protein content present in the biological sample.

2.11. Melatonin and AFMK quantification

All analysis were performed using a LC–MS/MS Waters Alliance 2695 Separations Module (Waters, Milford, USA) fitted with an autosampler, a membrane degasser and a quaternary pump. Mass spectrometry was performed on a Micromass Quattro Micro API (Waters, Milford, USA) with an electrospray (ESI) interface. The HPLC column used was an XTerra 3.5 μm particle size (50 × 3 mm ID) (Waters, Milford, MA, USA), connected to a precolumn. Analytical instrument control, data acquisition and treatment were performed by software Masslynx version 4.1, 2005 (Micromass, Waters, Milford, MA, USA). A sample volume of 20 μL was injected with an autosampler. The mobile phase was Acetonitrile:purified water (52:48, v/v), acidified with 0.1% formic acid at a constant flow of 0.4 mL min^{-1}. Ionization of the compounds was studied using ESI in the positive (PI) ionization mode. Typical interface conditions were optimized for maximum intensity of the precursor ions as follows: capillary voltage, 3.5 kV, nebulizer and desolvation (drying gas) flows were set at 550 and 50 L/h, respectively, source block and desolvation temperatures were 100 and 450 °C, respectively. Nitrogen was used as nebulizing, desolvation and cone gas, and argon was used as collision gas. The ESI polarity ionization mode was set individually for each target compound. For optimizing the mass spectrometer, direct infusion of a 1000 μg L^{-1} standard solution of each analyte was used. Full scan spectra were acquired over a mass range of 100–300 *m/z* for melatonin and AFMK, with a dwell time of 0.3 s. The detection of melatonin and AFMK were performed in the Multiple Reaction Monitoring (MRM) mode. The *m/z* transition from 233 to 174 (melatonin) and 265 to 136 (AFMK) were chosen for quantification of the compounds. Quantification of melatonin and AFMK were carried out using calibration curves obtained by repeated injections of standard solutions of known concentration. The limit of quantification is 1 ng mL^{-1} for melatonin and AFMK. The results are expressed in ng (g of wet tissue)$^{-1}$.

2.12. Statistical analysis

Statistical analysis were made by analysis of variance (ANOVA) followed by Newman–Keuls test ($\alpha = 0.05$). Normality and variance homogeneity were verified as ANOVA assumptions. Mathematical transformations were performed when necessary (Zar, 1984). The results of all experiments were expressed in percentage respect to control group, due to animals of the control group were different in short-term and long-term experiments.

3. Results

3.1. Oxygen consumption

For aerobic metabolism, verified by VO_2 in short-term experiments the value of control group was 0.12 ± 0.02 mg O_2 g^{-1} h^{-1}. Melatonin at lowest dosage of 0.002 pmol/crab significantly increased ($p < 0.05$) the VO_2 173% in relation to control group (Fig. 1A). For long-term experiments the control group was 0.27 ± 0.02 mg O_2 g^{-1} h^{-1} higher than control group of short-time experiments. For this experiment, melatonin at highest dosage of 20.0 pmol/crab significantly decrease ($p < 0.05$) the oxygen consumption in 76% (Fig. 1B).

3.2. Antioxidant capacity against peroxyl radicals (ACAP)

In short-term experiments the inverse values of relative area, that demonstrated ACAP for the control group was 0.03 ± 0.003. Melatonin in these experiment at dosage of 2.0 pmol/crab significantly decrease ($p < 0.05$) the ACAP in 48%, respect to control group (Fig. 2A). At long-term experiments the value of ACAP for the control group was 0.02 ± 0.002. In this time, melatonin had opposite effects. Firstly, at dosage of 0.02 pmol/crab melatonin significantly decrease ($p < 0.05$) the ACAP over 38%, respect to control group. On the other hand, at dosage of 20.0 pmol/crab an increase of 71% was verified (Fig. 2B).

3.3. Ros concentration and lipoperoxidation (LPO)

For ROS concentration, in short-time experiments the value of control group was 159504 ± 31927.8 FU (mg of protein)$^{-1}$. In long-term experiments the value of control group was 116667 ± 15660 FU (mg of protein)$^{-1}$. When melatonin was injected no significant effects ($p > 0.05$) was observed in both times and dosages in the locomotor muscle of *N. granulata* (Fig. 3A and B) respect to control group.

For lipoperoxidation (LPO) the control group in short-time was 145.6 ± 26.8 nmol of CHP (g of wet weight)$^{-1}$. In long-term the control group was 143.2 ± 32.5 nmol of CHP (g of wet weight)$^{-1}$. However, no significant effects ($p > 0.05$) was observed in LPO levels when melatonin was injected in both dosages and times in muscles of *N. granulata* (Fig. 4A and B).

3.4. Antioxidant components

For melatonin effects in the catalase activity in short-time experiments the control group was 0.027 ± 0.014 U CAT (mg of protein)$^{-1}$. At this time, no significant differences ($p > 0.05$) was observed when melatonin was injected in both dosages (Fig. 5A). In long-term experiments a significant decrease ($p < 0.05$) of 63% was observed at dosage of 0.002 pmol/crab respect to control group (0.022 ± 0.004 U CAT.(mg of protein)$^{-1}$) (Fig. 5B). For γ-GCL, the rate-limiting enzyme of GSH synthesis, in short-term experiments, melatonin significantly increased ($p < 0.05$) the activity of this enzyme in 145% and 126% at dosages of 0.02 and 2.0 pmol/crab, respectively, respect to control group (236.4 ± 43 nM of GSH h^{-1} (mg of protein)$^{-1}$) (Fig. 6A). In long-term experiments, the control group was 236.4 ± 43 nM of GSH h^{-1} (mg of protein)$^{-1}$. However, no significant differences ($p > 0.05$) were observed when melatonin was injected (Fig. 6B). For GSH content in short-term experiments a significant increase ($p < 0.05$) of 102% at lowest dosage of 0.002 pmol/crab was seen, respect to control group (102.7 ± 66.4 nmol de GSH (mg of protein)$^{-1}$) (Fig. 7A). In long-term experiments the values of control group was 57.3 ± 9.6 nmol de GSH (mg of protein)$^{-1}$ and no significant differences ($p > 0.05$) were observed when melatonin was injected (Fig. 7B).

3.5. Melatonin and AFMK

In the locomotor muscle of the crab *N. granulata*, high levels of melatonin were detected. For the control group the value found was 1258.58 ± 73.2 ng of melatonin (g of wet tissue)$^{-1}$. However, when we injected melatonin exogenously, we observed in short-term experiments a significant decrease ($p < 0.05$) of 40% and 42% in melatonin content at dosages of 0.002 and 0.02 pmol/crab,

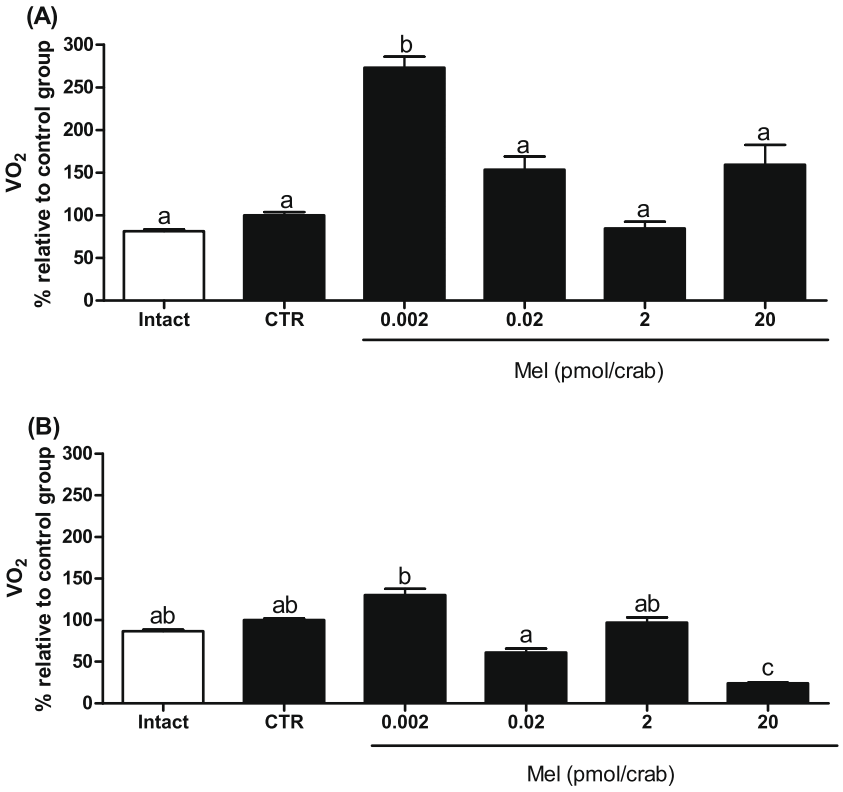

Fig. 1. Melatonin effect on aerobic metabolism measured by oxygen consumption (VO_2) in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

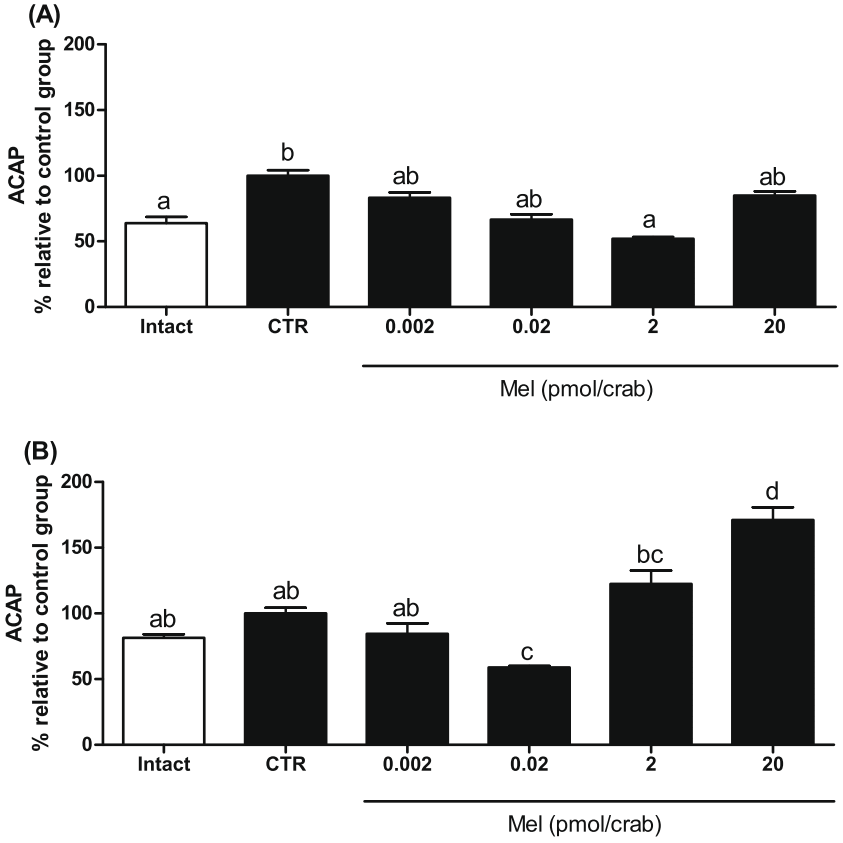

Fig. 2. Melatonin effect on antioxidant competence against peroxyl radicals (ACAP) in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

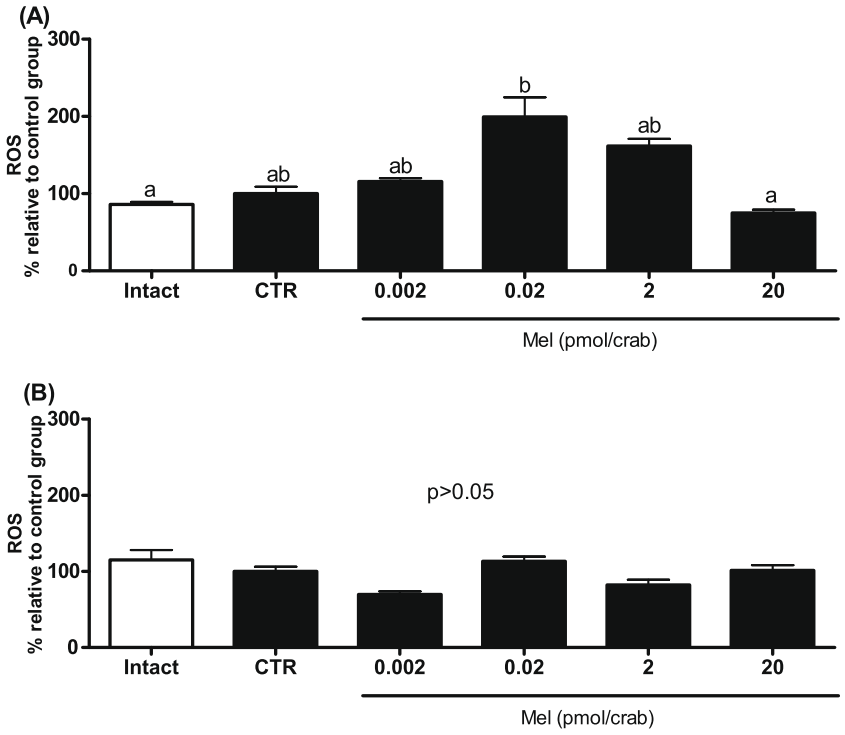

Fig. 3. Melatonin effect on ROS production in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

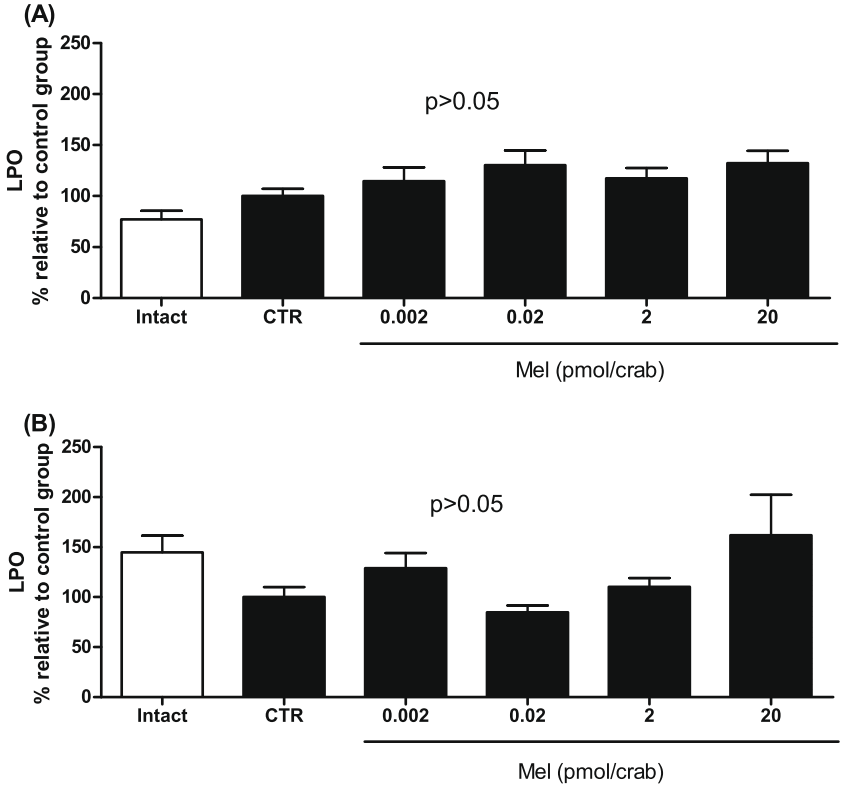

Fig. 4. Melatonin effect on lipid peroxidation (LPO) in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

respectively. (Fig. 8A). In long-term experiments the levels of control group was 960.2 ± 74.92 ng of melatonin (g of wet tissue)$^{-1}$. However, no significant differences ($p > 0.05$) were observed when melatonin were administered (Fig. 8B).

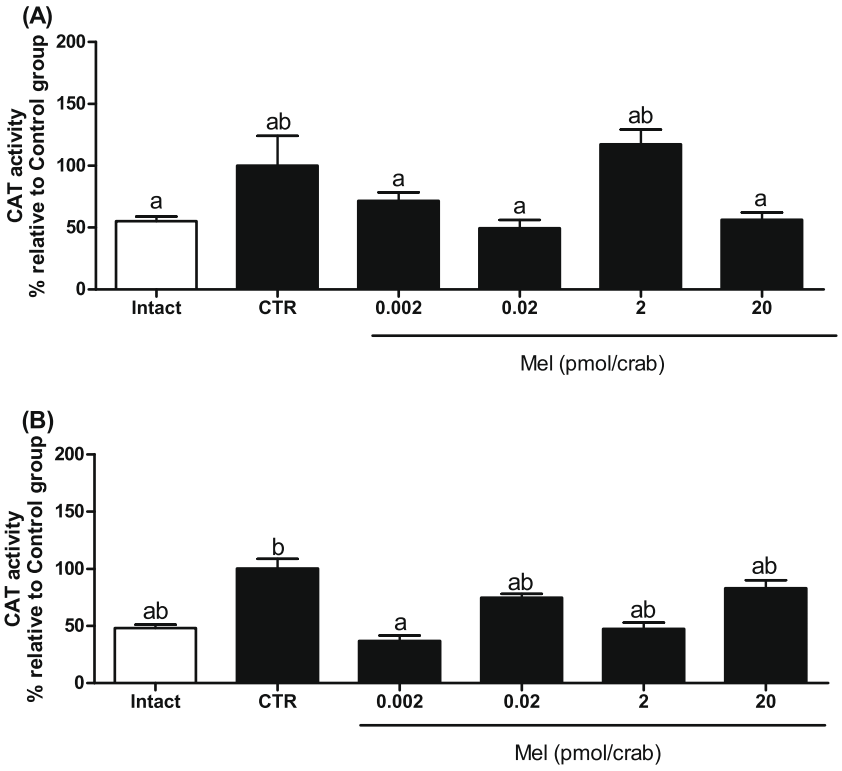

Fig. 5. Melatonin effect on catalase activity (CAT) in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

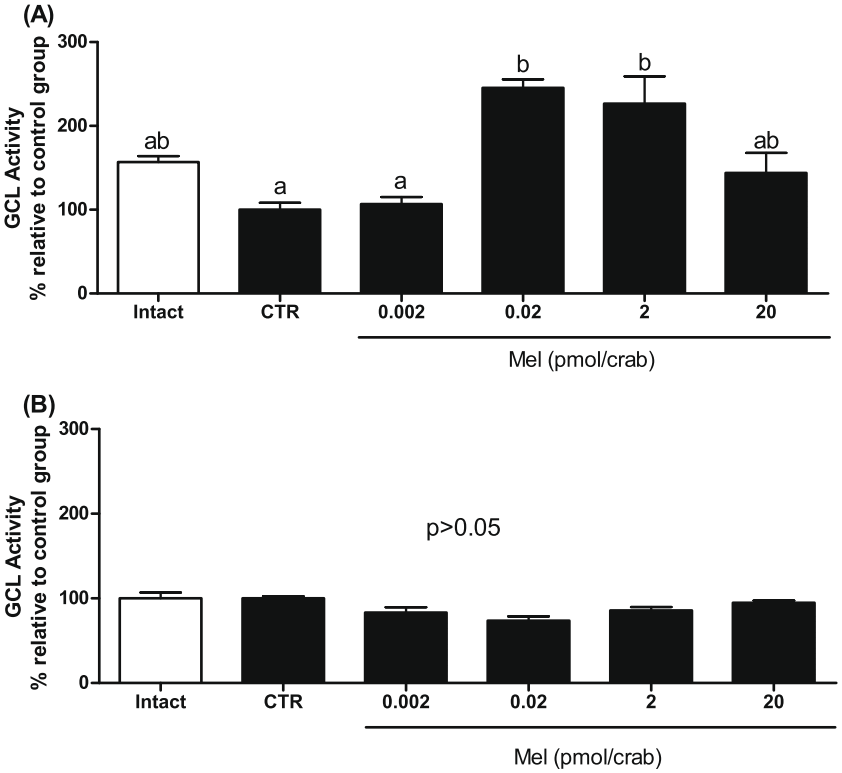

Fig. 6. Melatonin effect on glutamate cysteine ligase activity (γ-GCL) in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

For AFMK, one of the main metabolites produced when melatonin is metabolized, we failed to detect it in the muscle using the detection limit of 1 ng ml^{-1} to AFMK. However, when we analyzed eyestalk and supraesophageal ganglia, AFMK was detected. For

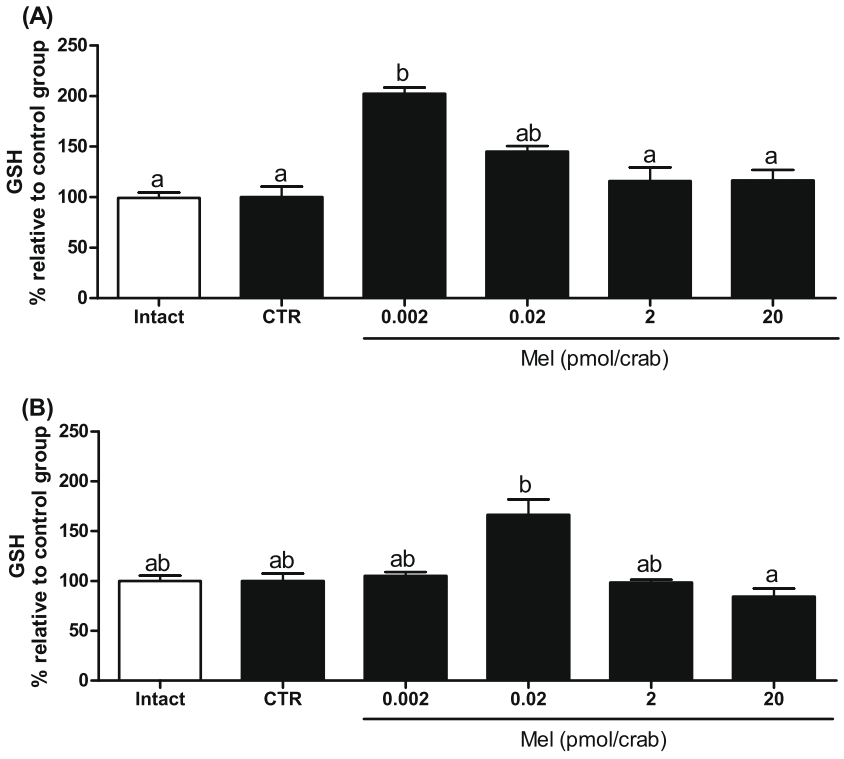

Fig. 7. Melatonin effect on glutathione content (GSH) in muscles of eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

eyestalk the value was 53.71 ± 33.19 ng of AFMK (g of wet tissue)$^{-1}$ and for supraesophageal ganglia the value was 85.72 ± 9.92 ng of AFMK (g of wet tissue)$^{-1}$.

4. Discussion

Many studies have also verified the influence of melatonin in the ADS in vertebrates acting as an antioxidant or pro-oxidant in a dosage and time-dependent manner. It has been verified that melatonin may act as a *scavenger* of ROS and RNS (Tan et al., 1993; Pieri et al., 1994; Pozo et al., 1994; Barlow-Walden et al., 1995; Cagnoli et al., 1995) mainly in pharmacological concentrations. On the other hand, in physiological and supraphysiological dosages this indoleamine may act up-regulating some antioxidant enzymes (Barlow-Walden et al., 1995; Antolin et al., 1996; Liu and Ng, 2000). In the last few years, it has been proposed that melatonin also has an action in mitochondrial activity (Acuna-Castroviejo et al., 2003). However, some studies demonstrated that melatonin may also act as a pro-oxidant molecule in the ADS mainly in high dosages (Wolfer et al., 2001).

For the crab *N. granulata*, melatonin in short-term experiments and low dosages, promoted an increase in the VO_2 of locomotor muscles (Fig. 1A). If an increase in aerobic metabolism is verified, a result that is expected is that an increase in ROS production should also occur (Storey, 1996). However, our results showed that an increase in aerobic metabolism does not lead an augment in ROS concentration (Fig. 3A) or LPO levels (Fig. 4A), indicating that melatonin in these dosages increases the aerobic metabolism without induce the oxidative stress.

The low levels in ROS concentration could be maintained by an increase in the efficiency of ADS. In this sense, our results showed that melatonin increased some antioxidant components such as γ-GCL activity leading to an increase in GSH content (Figs. 6A and 7A). Other studies verified the same effect showing that melatonin causes an increase in GSH levels, through increasing the activities of γ-GCL and/or glutathione reductase (Abe et al., 1994; Melchiorri et al., 1995; Sewerynek et al., 1995; Urata et al., 1999). Furthermore, some other antioxidant components enzymatic or non-enzymatic, not evaluated in this study, can still be stimulated by melatonin to prevent the induction of oxidative stress.

On the other side, melatonin may be acting in mitochondrial functionality. It has been demonstrated that melatonin is able to increase the efficiency of electron transport chain thereby limiting electron leakage and free radical generation (Acuna-Castroviejo et al., 2003). In rat brain and liver, melatonin increases the activities of mitochondrial respiratory complexes I and IV in a time and dosage dependent manner (Martín et al., 2000, 2002). Our results showed that melatonin increases the oxygen consumption without induce effects in the oxidative stress. So, it is also possible that melatonin may be acting in the mitochondrial activity increasing the efficiency of the electron transport chain in the muscles of *N. granulata*.

Finally, the increase in oxygen consumption, without affecting the ROS production may be also occurring due to an increase in the endogenous melatonin consumption. In the locomotor muscle of *N. granulata* high levels of melatonin were found (Fig. 8A and B). When exogenous melatonin was administered in low dosages and short-term, a reduction in the melatonin levels was detected (Fig. 8A). Knowing that melatonin provides an antioxidant function, the high levels found in the locomotor muscles might be serving as an antioxidant source, preventing an augment in ROS production by its consumption in the muscle. Hardeland (2008) suggested that extra-pineal tissues of vertebrates, where melatonin was detected in high concentrations, this indoleamine may act as antioxidant source, preventing the oxidative stress. Besides, the reduction on melatonin levels in the muscles, when exogenous melatonin was administered, could be showing that in the eyestalk of this crab, where the neuroendocrine complex X organ/sinus gland is located and melatonin is produced (Maciel et al., 2008), may be signaling to endogenous melatonin in muscles to use their stocks.

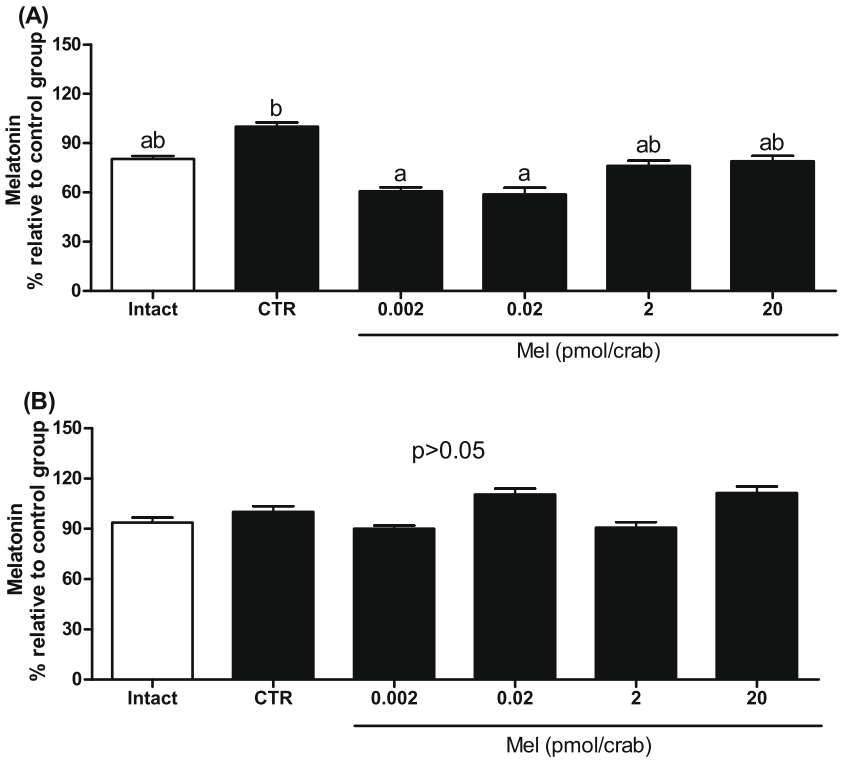

Fig. 8. Melatonin content in the muscle of intact crabs or eyestalkless crabs after 0.5 h (A) and 9.5 h (B) of melatonin injection. No injections were applied in intact crabs. Different letters represents significant differences ($p < 0.05$) between the groups. Each point represents the mean ± 1 SEM ($n = 5$).

However, if melatonin is being used and metabolized in the muscles, its metabolites should be formed. However, we do not detect this metabolite in the locomotor muscles. Then, we verified whether AFMK is produced in nervous system of *N. granulata* and detected this metabolite in this tissue. In mammals, AFMK was first detected in rat brain (Hirata et al., 1974). Recently, AFMK was also detected in the retina and serum of rats (Rozov et al., 2003), in blood and urine of mice (Harthe et al., 2003; Semak et al., 2005) and in the cerebrospinal fluid of humans with meningitis (Silva et al., 2005). Our results for *N. granulata* are in agreement with those studies performed in vertebrates showing that AFMK is one of the main metabolites detected in nervous tissues (Hirata et al., 1974). For other side, other metabolites as 3-hydroxymelatonin cyclic, 6-hydroxymelatonin, AMK and others metabolites also could be formed in this tissue. Moreover, under intense oxidative stress AFMK and all others metabolites could also be produced and detected in the locomotor muscle.

When we analyzed the effects of melatonin in high dosages we verified a pronounced decrease in ACAP values in the muscles of *N. granulata* at dosage of 2.0 pmol crab^{-1} and a gradual loss of exogenous melatonin effects in the antioxidant components. As melatonin receptors may undergo the process of desensitization when exposed to melatonin at low and high dosages (Gerdin et al., 2004a,b), it is possible that the reduction of exogenous melatonin effects in the ADS occurs by melatonin receptors desensitization. Even, if it really happens, the effects of melatonin in the ADS of *N. granulata* muscles should be mediated by receptors. Besides, Maciel et al. (2009) also verified a decrease in the ADS in gills of *N. granulata* using the same dosage. However, these authors also found a decrease in VO_2 of gills exposed to melatonin which was not found in this study.

When we analyzed the effects of melatonin in long-term and low dosages, a decrease in the TOSC (Fig. 2B) was verified. This decrease could be caused either by a decrease in ROS concentration or in the ADS components. Melatonin also not showed no effects in ROS concentration and LPO levels (Fig. 3B and 4B), similar to results of short-term experiments. In addition, when we analyzed the antioxidant components we verified a gradual loss of exogenous melatonin effects in the ADS. Melatonin in low dosages has no effect in γ-GCL activity (Fig. 6B) and GSH content (Fig. 7B). However, the catalase activity was reduced, which probably is responsible the decrease in ACAP. However, in the literature, some few reports conducted in vertebrates verified an opposite melatonin effect showing an increase in catalase activity. These studies was performed mainly in pharmacological dosages and long-term exposure (Marshall et al., 1996; Tomás-Zapico et al., 2002; Naidu et al., 2003; Esparza et al., 2005).

On the other hand, when muscles are injected with high dosages of melatonin a decrease in VO_2 (Fig. 1B) and an increase in ACAP (Fig. 2B) was verified in long-term experiment. These results are showing another melatonin effect in the ADS, different to short-term. The increase in TOSC values may be caused by an increase in the antioxidant components. However, no effect was observed in the antioxidant components analyzed. Possibly the increasing in TOSC could be due to some other components not studied in this work. In this sense, many studies have verified the influence of melatonin in the ADS, regulating some antioxidant enzymes, mainly superoxide dismutase (SOD), glutathione peroxidase (GPX-Se) and glutathione reductase (GR) (Barlow-Walden et al., 1995; Pablos et al., 1995; Kotler et al., 1998; Liu and Ng, 2000).

In conclusion, the results in this study suggest that exogenous melatonin in low dosages and short-term experiments increased aerobic metabolism and ADS by increasing the GSH content, stimulated by increase in γ-GCL activity, preventing the induction of oxidative stress. In high dosages we verified a gradual loss in melatonin effects, possibly by a desensitization of melatonin receptors. Moreover, in short-term experiments the high levels of endoge-

nous melatonin, stimulated by melatonin injections in muscles, may be contributing to reduce the oxidative stress. For other side, in long-term experiments and low dosages melatonin effect is only verified in the catalase activity. On the other side, in high dosages, melatonin increased the total oxyradical antioxidant capacity against peroxyl radicals and decreased the aerobic metabolism. These increase in ACAP could be due melatonin stimulating some other ADS components. Thus, melatonin in the locomotor muscles of *N. granulata* is acting in the oxidative balance in a time and dosage dependent manner.

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