Daily variations in oxygen consumption, antioxidant defenses, and lipid peroxidation in the gills and hepatopancreas of an estuarine crab

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Abstract: This study identified daily variations in oxygen consumption, antioxidant-defense system, and lipoperoxidation in the gills and hepatopancreas of the crab *Chasmagnathus granulata* (Dana, 1851) (Decapoda, Brachyura). In gills, oxygen consumption was higher in the early afternoon and in the middle of the night ($p < 0.05$). Lipoperoxidation level and non-proteic sulfhydryl content were higher in the middle of the night ($p < 0.05$). The enzyme glutathione-*S*-transferase showed lower activity at the end of photophase and higher values during the night ($p < 0.05$). The enzyme catalase did not show daily variation in activity ($p > 0.05$). Oxygen consumption in the hepatopancreas showed a similar profile to that in gills, i.e., higher values in the early afternoon and the middle of the night. Glutathione-*S*-transferase activity and lipoperoxidation levels showed significant variation, with lower values during the afternoon and peaks in the middle of the night ($p < 0.05$). Catalase activity was significantly higher ($p < 0.05$) in the middle of the night. The non-proteic sulfhydryl content remained fairly constant ($p > 0.05$). The results showed daily variations in aerobic metabolism of the gills and hepatopancreas of *C. granulata*, with concomitant oxidative damage (lipoperoxidation), but with differences between tissues. Whereas in the gills the defense system focused on catalase and non-proteic sulfhydryl, in the hepatopancreas other non-enzymatic components and other antioxidant enzymes besides catalase and glutathione-*S*-transferase might be involved.

Résumé : Nous avons observé une variation journalière de la consommation d'oxygène, du système de défense antioxydant et de la lipoperoxydation dans les branchies et l'hépatopancréas du crabe *Chasmagnathus granulata* (Dana, 1851) (Decapoda, Brachyura). Dans les branchies, la consommation d'oxygène est plus élevé au début de l'après-midi et au milieu de la nuit ($p < 0{,}05$). La lipoperoxydation et la concentration de composés sulfhydriles non protéiques sont plus élevées au milieu de la nuit ($p < 0{,}05$). L'enzyme glutathion-*S*-transférase a une activité plus faible à la fin de la photophase et une activité plus forte durant la nuit ($p < 0{,}05$). L'enzyme catalase ne subit pas de variation journalière d'activité ($p > 0{,}05$). Dans l'hépatopancréas, la consommation d'oxygène suit un profil semblable à celui des branchies, i.e., des valeurs accrues au début de l'après-midi et le milieu de la nuit. L'activité de la glutathion-*S*-transférase et l'importance de la lipoperoxydation montrent des variations significatives, avec des valeurs basses en après-midi et des maximums au milieu de la nuit ($p < 0.05$). L'activité de la catalase est significativement plus élevée ($p < 0{,}05$) au milieu de la nuit. Les concentrations de composés sulfhydriles non protéiques demeurent relativement constantes ($p > 0{,}05$). Nos résultats démontrent l'existence d'une variation journalière du métabolisme aérobie des branchies et de l'hépatopancréas chez *C. granulata* accompagnée de dommages oxydatifs (lipoperoxydation) qui varient selon le tissu. Alors que dans les branchies, le système de défense se concentre sur la catalase et la concentration de composés sulfhydriles non protéiques, d'autres composantes non enzymatiques et d'autres enzymes antioxydants peuvent être impliqués dans l'hépatopancréas en plus de la catalase et de la glutathion-*S*-transférase.

[Traduit par la Rédaction]

Introduction

Organisms with aerobic metabolism have to deal with the so-called oxygen paradox, since at the time when the oxygen molecule is needed to produce energy for the living organism, it also generates intermediate compounds, the reactive oxygen species (ROS), which can react with proteins, lipids, and DNA (Halliwell and Gutteridge 1999; Regoli et al. 2000). Some of the more common ROS include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2{\cdot}^-$), and the hydroxyl radical ($HO\cdot$), the last being the most reactive (Halliwell and Gutteridge 1999).

Owing to the progressive oxidative characteristics of the atmosphere, a number of organisms are prone to acquire antioxidant defenses in order to intercept and degrade the ROS generated during oxidative phosphorylation and other physiological processes. Besides this defense, non-enzymatic antioxidant defenses also exist, such as the vitamins E and C, β-carotenes, and tripeptides like glutathione (GSH). This last

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substance is an important HO· scavenger and also serves as a cosubstrate of antioxidant enzymes (Storey 1996; Gavin and Sies 2001). The enzymatic antioxidant defenses are represented by enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Storey 1996; Halliwell and Gutteridge 1999; Dröge 2002). Other enzymes, like glutathione reductase and glutathione-*S*-transferase (GST), aid in protective and repair processes (Leaver and George 1998).

Several physiological processes can induce daily variations in aerobic metabolism. For example, specific dynamic action alters oxygen consumption during the absorptive step in the digestive cycle. In the brachyuran crabs *Ocypode quadrata* (Fabr., 1787) and *Cardisoma guanhumi* Latr., 1825 it was established that a peak in oxygen consumption occurs 8 h after a single meal (Burggren et al. 1993). Also, it is known that physiological parameters like oxygen consumption and locomotor activity vary rhythmically, which should imply alterations in aerobic metabolism and, presumably, in ROS production. Among crustacean species, Rosas et al. (1992) reported that in the portunids *Callinectes similis* Williams, 1966 and *Portunus spinicarpus* (Stimpson, 1871) and the shrimp *Farfantepenaeus aztecus* (Ives, 1891), oxygen consumption and hemolymphatic glucose levels were higher at night, a result that the authors related to the greater locomotor activity recorded during this period. Other authors, such as Crear and Forteath (2000), observed that in the prawn *Jasus edwardsii*, oxygen consumption was augmented at night. In the estuarine crab *Chasmagnathus granulata* (Dana, 1851) (Decapoda, Brachyura), a circadian rhythm of locomotor activity, with a peak during the night, was reported (Pereyra et al. 1996).

Taking into account the references cited above, it could be postulated that variations in tissue aerobic metabolism should impose variations in ROS generation. ROS fluctuations could be associated with variations in antioxidant-defense systems and (or) oxidative stress if the defenses are overwhelmed by ROS production. Oxidative stress is defined as an imbalance between the concentrations of pro-oxidants and antioxidants, favouring the first, with the potential to generate lipid peroxides (LPO) and oxidized proteins and cause DNA damage (Escobar et al. 1996; Halliwell and Gutteridge 1999).

Studies in vertebrate species have shown that, for example, GPx activity and LPO levels in rat liver are higher during the night (Baydas et al. 2002). Diaz-Muños et al. (1985) observed augmented LPO levels in rat cerebral cortex at night, paralleled by a lowering of CAT activity and higher SOD activity. To our knowledge, only Fanjul-Moles et al.'s (2003) study has analyzed daily variations in the antioxidant-defense system in crustaceans, but only in species of Cambariidae (*Procambarus clarkii* (Girard, 1852) and *Procambarus digueti* (Bouvier, 1897)). In view of the daily variations in several physiological parameters that can directly or indirectly influence aerobic metabolism, as well as the scarcity of information on this topic in crustacean species, the objective of this work was to verify the existence of daily variations in oxygen consumption, the antioxidant-defense system, and LPO levels (oxidative damage) in tissues with a high level of aerobic metabolism, such as the gills and hepatopancreas of *C. granulata*. This species is a typical estuarine crab, exposed to fluctuations in several environmental factors, including salinity, temperature, photoperiod, and dissolved oxygen concentration, among others (D'Incao et al. 1992). In its feeding habits, *C. granulata* is considered to be a generalistthat uses detritivorous and opportunistic strategies. As great percentage of the energetic flux in estuaries follows the detritivore food web, *C. granulata* is presumed to play an important role in energetic transfer in this environment (D'Incao et al. 1990).

Material and methods

Adult male *C. granulata* at stage C or early stage D of the intermoult cycle (Drach and Tchernigovtzeff 1967) were collected near the city of Rio Grande in southern Brazil and transported to the laboratory. Organisms were acclimated for 30 days at 20‰ salinity, 20 °C, and a fixed photoperiod of 12 h light : 12 h dark (lights on at 0700 and off at 1900) with a light intensity of 500 lx. Crabs were fed ad libitum 3 times a week with ground beef.

Two days before of the beginning of the experiment animals were transferred to nine glass aquaria (25.5 cm × 32.0 cm × 14.0 cm). Each aquarium contained five crabs. Every 3 h during a 24-h period (9 time points) all the crabs in one aquarium were weighed, immediately sacrificed, and dissected to obtain the tissues (gills and hepatopancreas). Subsamples of each tissue were extracted to measure oxygen consumption, antioxidant-enzyme activity, non-proteic sulfhydryl (NP-SH) groups, and LPO level.

Oxygen consumption was measured using small pieces of hepatopancreas (50 ± 5 mg) or three gill arches collected as described above. Gills or hepatopancreas were incubated at 20 °C in saline solution (10 mmol $MgCl_2$/L, 355 mmol NaCl/L, 16.6 mmol $CaCl_2$/L, 5 mmol H_3BO_3/L, 10 mmol $KHCO_3$/L, 8 mmol $Na_3C_6H_5O_7 \cdot 2H_2O$/L; pH adjusted to 7.6). The protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 1 mmol/L; Sigma) was added during the hepatopancreas incubation, since preliminary experiments showed that consumption by the hepatopancreas was inhibited when no PMSF was added. The oxygen concentration in the saline solution was measured at time zero and after 20 min of incubation using a portable oxymeter. Oxygen consumption was expressed in milligrams of oxygen per gram wet mass of tissue per hour.

For antioxidant-enzyme analysis, gills and hepatopancreas were homogenized (1:10 and 1:4 *w*/*v*, respectively) in a cold (4 °C) buffer solution containing Tris base (20 mmol/L), EDTA (1 mmol/L), dithiothreitol (1 mmol/L, Sigma), sucrose (500 mmol/L), KCl (150 mmol/L), and PMSF (0.1 mmol/L), with pH adjusted to 7.6. Homogenates were centrifuged at 9000*g* (4 °C) for 30 min and the supernatants were then employed as antioxidant-enzyme sources. All enzymatic determinations were done at least in duplicate.

CAT and GST activities were determined using the spectrophotometric methods described by Beutler (1975) and Habig and Jakoby (1981), respectively. Specific enzyme activity was calculated considering the total protein content in the homogenates. All results are expressed in enzyme units. One CAT unit represents the amount of enzyme needed to degrade 1 μmol of $H_2O_2 \cdot min^{-1} \cdot (mg\ total\ protein)^{-1}$ (Merck) present in the homogenates at 30 °C and pH 8.0. One GST

unit is the amount of enzyme necessary to conjugate 1 μmol of 1-chloro-2,4-dinitrobenzene·min^{-1}·(mg total protein)$^{-1}$ (Sigma) 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. Protein content in homogenates was determined using a commercial reagent kit (Doles Reagents Ltd., Goiânia, Goiás, Brazil), based on the Biuret reagent (550 nm).

The method employed for the measurement of NP-SH content was based on Sedlak and Lindsay (1968). Gills and hepatopancreas were homogeinized (1:4 and 1:9 *w*/*v*, respectively) in 0.02 mol EDTA/L. 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 (Sigma). 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.

The methodology for determining LPO levels was based on Hermes-Lima et al. (1995) and Monserrat et al. (2003). Gills and hepatopancreas were homogenized (1:9 *w*/*v*) in cold methanol and centrifuged at 1000*g* (4 °C) for 10 min and the pellet was discarded. For LPO measurements, $FeSO_4$ (1 mmol/L), H_2SO_4 (0.25 mol/L), xylenol orange (1 mmol/L, Sigma), and MilliQ water were sequentially added. Samples (30 μL for gills and 20 μL for hepatopancreas) or methanol (blanks) were added and incubated for 90 min (gills) or 75 min (hepatopancreas). After that, absorbance (550 nm) was determined using a microplate reader. Cumene hydroperoxide (CHP; Sigma) was employed as a standard. LPO values were expressed in CHP equivalents per gram of wet mass.

Data were analyzed through ANOVA followed by an a posteriori means comparison (Newman–Keuls test, $\alpha = 0.05$). Previously, ANOVA assumptions were verified and transformations applied if a lack of normality and (or) variance homogeneity were detected (Zar 1984).

Results

A similar profile of daily variations in oxygen consumption was observed for both gills and hepatopancreas (Fig. 1). In the gills, two peaks ($F_{[8,36]} = 7.47$, $p = 0.00001$) in oxygen consumption were observed, one at the beginning of the afternoon (0.38 ± 0.04 mg O_2·g^{-1}·h^{-1} (mean ± SE)) and another in the middle of the night (0.51 ± 0.10 mg O_2·g^{-1}·h^{-1}) (comparison with oxygen consumption at 24 h: $p = 0.0002$ and $p = 0.0001$, respectively). In the hepatopancreas, peaks were recorded ($F_{[8,36]} = 19.18$, $p = 0.00001$) at the same time points as in the gills, with values of 0.34 ± 0.02 and 0.39 ± 0.01 mg O_2·g^{-1}·h^{-1} (comparison with values at 24 h: $p = 0.0002$ and $p = 0.0001$, respectively).

Concerning CAT activity in the gills, no statistical differences ($F_{[8,26]} = 0.92$, $p > 0.05$) were observed during the 24-h period (Fig. 2*a*). However, CAT activity in the hepatopancreas was higher ($F_{[8,21]} = 12.35$, $p = 0.000002$) at night (2.33 ± 0.36 U CAT·(mg protein)$^{-1}$, where 1 U ≈ 16.67 nkat) when compared with the basal value at 12 h ($p = 0.0002$) (Fig. 2*b*). Interestingly, CAT activity in the gills was almost 10 times higher than that recorded for the hepatopancreas. For GST, a similar pattern was observed for both tissues (Fig. 3). In the gills the lowest ($F_{[8,32]} = 2.66$, $p = 0.02$) GST activity during the 24-h experimental period was recorded at the end of the afternoon (0.06 ± 0.01 U GST·(mg protein)$^{-1}$; comparison with the peak: $p = 0.046$). In the hepatopancreas, the lowest ($F_{[8,30]} = 3.84$, $p = 0.003$) value was 0.10 ± 0.02 U GST·(mg protein)$^{-1}$; comparison with respect to the peak: $p = 0.024$).

The NP-SH content augmented significantly ($F_{[8,34]} = 3.31$, $p = 0.01$) in the gills during the nocturnal period (0.02 ± 0.01 μmol NP-SH·(mg protein)$^{-1}$) (comparison with the basal value at 12 h: $p = 0.045$, which corresponds to the highest oxygen consumption; Fig. 4*a*). Concerning the hepatopancreas, no statistical differences ($F_{[8,28]} = 1.01$, $p > 0.05$) were observed in the same variable during the whole experimental period (Fig. 4*b*).

Finally, LPO levels varied significantly ($p < 0.05$) in both tissues during the experimental period. In the gills, the highest ($F_{[8,31]} = 3.79$, $p = 0.003$) LPO levels were detected at the beginning of the afternoon (355.95 ± 73.68 nmol CHP·g^{-1}) and

Fig. 1. Daily variations in oxygen consumption in the gills (*a*) and hepatopancreas (*b*) of the estuarine crab *Chasmagnathus granulata*. Each point represents the mean ± 1 SE ($n = 5$). The hours when tissues were sampled and oxygen consumption was determined are indicated on the *x* axis. The open and solid bars indicate light and dark phases, respectively. Values accompanied by the same letter are not statistically different ($p > 0.05$).

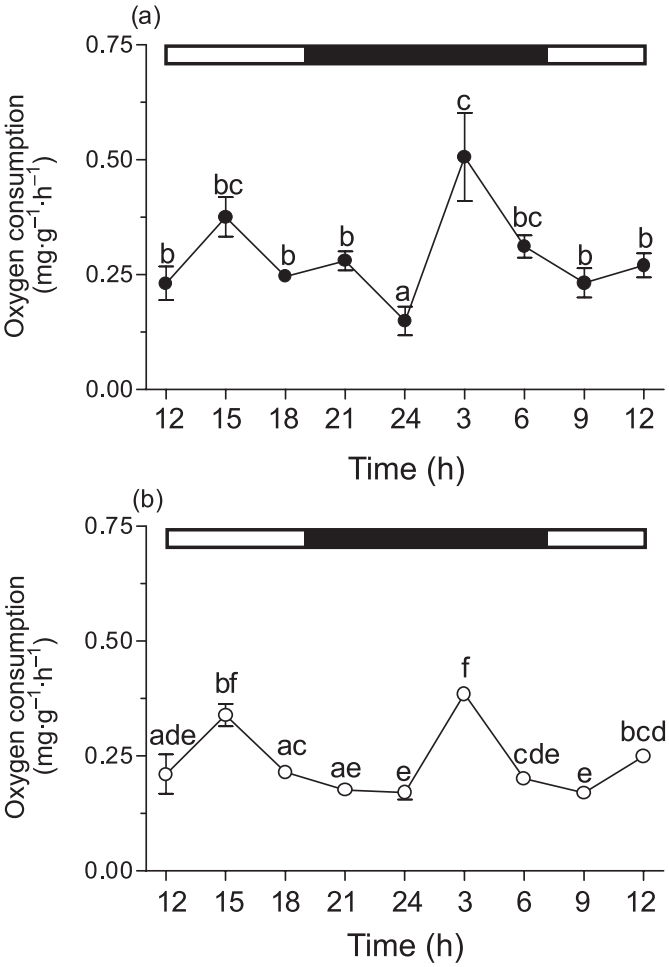


Fig. 2. Daily variations in catalase (CAT) activity in the gills (*a*) and hepatopancreas (*b*) of *C. granulata*. Each point represents the mean ± 1 SE (n = 3–5). The hours when tissues were sampled and CAT activity was determined are indicated on the x axis. One CAT unit represents the amount of enzyme 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. The open and solid bars indicate light and dark phases, respectively. No statistical differences ($p > 0.05$) were detected in the gills during the 24-h period of analysis. In *b*, values accompanied by the same letter are not statistically different ($p > 0.05$).

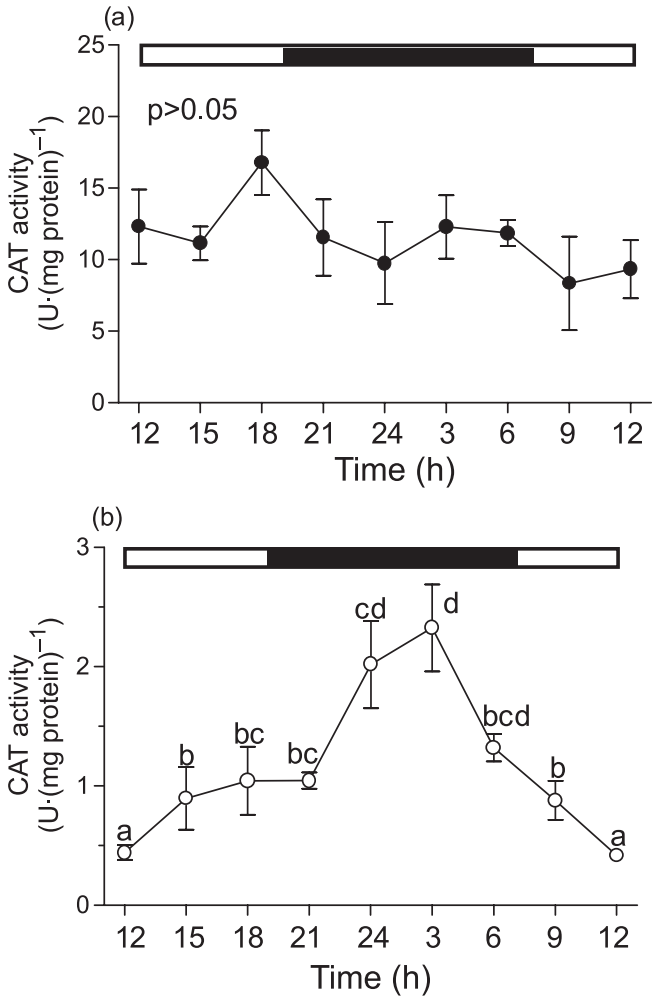
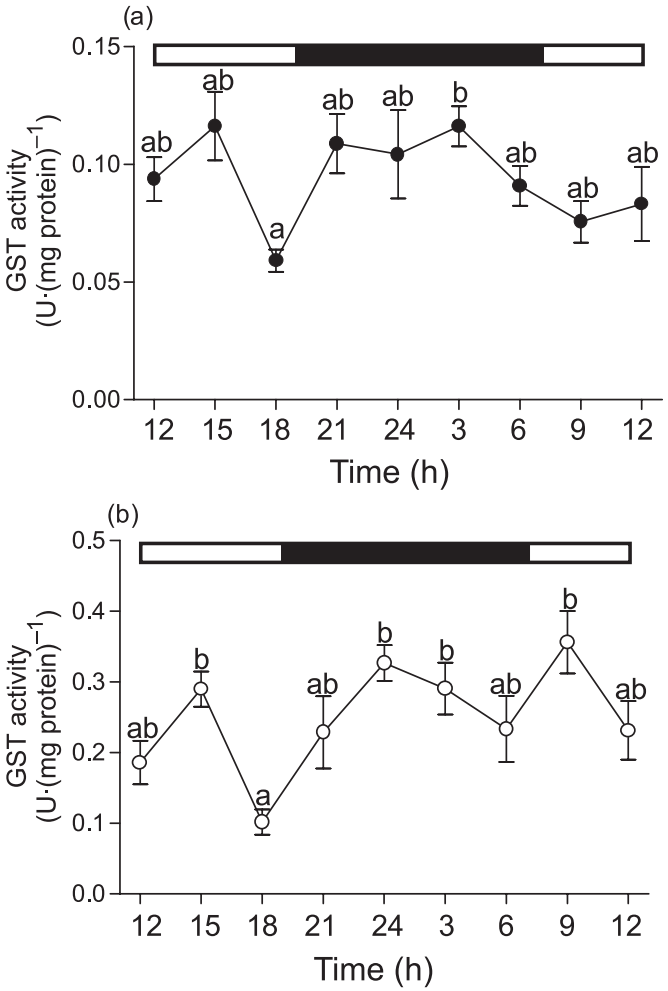


Fig. 3. Daily variations in glutathione-*S*-tranferase (GST) activity in the gills (*a*) and hepatopancreas (*b*) of *C. granulata*. Each point represents the mean ± 1 SE (n = 3–5). The hours when tissues were sampled and GST activity was determined are indicated on the x axis. One GST unit is the amount 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. The open and solid bars indicate light and dark phases, respectively. Values accompanied by the same letter are not statistically different ($p > 0.05$).



lower values during the rest of the experimental period (p = 0.005) (Fig. 5*a*). In the hepatopancreas, the lowest ($F_{[8,30]}$ = 2.52, p = 0.03) LPO levels were observed at the beginning of the afternoon (32.84 ± 6.13 nmol CHP·g^{-1}) and higher LPO levels during the nocturnal period (p = 0.023) (Fig. 5*b*).

Discussion

The oxygen-consumption data show that the crab *C. granulata* exhibits daily variation in the aerobic metabolism of the gills and hepatopancreas. For both tissues the period of highest oxygen consumption is nighttime, followed by a another peak with lower values at the beginning of the afternoon. Oxygen consumption in the gills and hepatopancreas follows the circadian rhythm of locomotor activity verified in this species (Pereyra et al. 1996), which generally induces variation in whole-animal aerobic metabolism. Variations in daily aerobic metabolism in other crustacean species have been previously reported, with higher oxygen consumption also recorded at night (Rosas et al. 1992; Crear and Forteath 2000).

Considering that increases in aerobic metabolism should elevate ROS production (Yan and Sohal 2000), variation in the antioxidant-defense system is expected to occur. If this counteractive response does not exist, or occurs to a limited extent, daily variation in oxidative stress should be evident. The observed LPO levels indicate that, in fact, daily variation in oxidative stress occurs, but there are differences between the gills and hepatopancreas. In the gills, the greatest damage was recorded at the beginning of the afternoon, whereas in the hepatopancreas maximum levels were re-

Fig. 4. Daily variations in non-proteic sulfhydryl (NP-SH) content in the gills (*a*) and hepatopancreas (*b*) of *C. granulata*. Each point represents the mean ± 1 SE (n = 3–5). The hours when tissues were sampled and NP-SH content was determined are indicated the on the x axis. The open and solid bars indicate light and dark phases, respectively. In *a*, values accompanied by the same letter are not statistically different ($p > 0.05$). No statistical differences ($p > 0.05$) were detected in the hepatopancreas during the 24-h period of analysis.

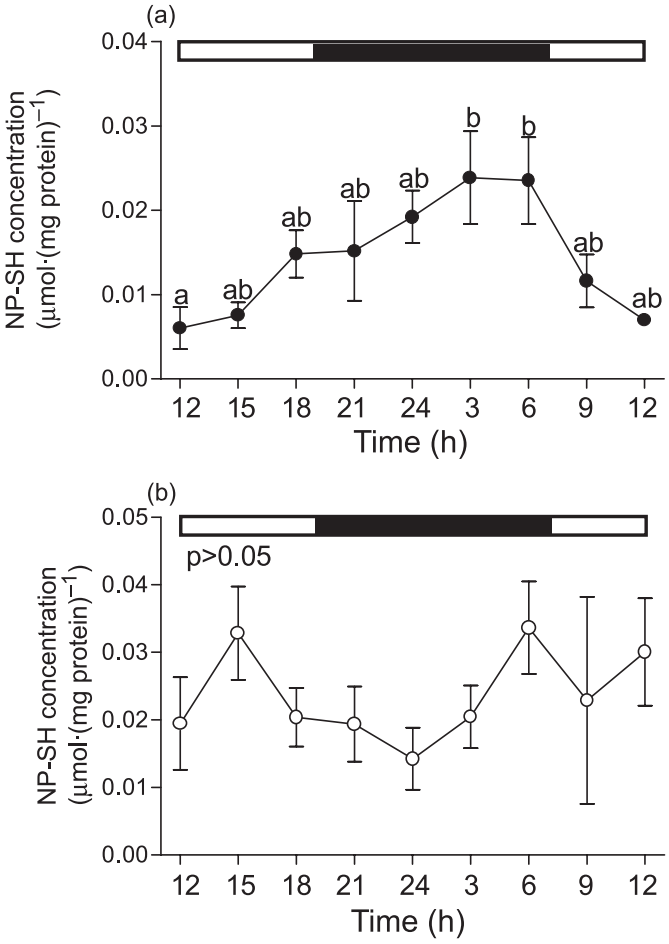
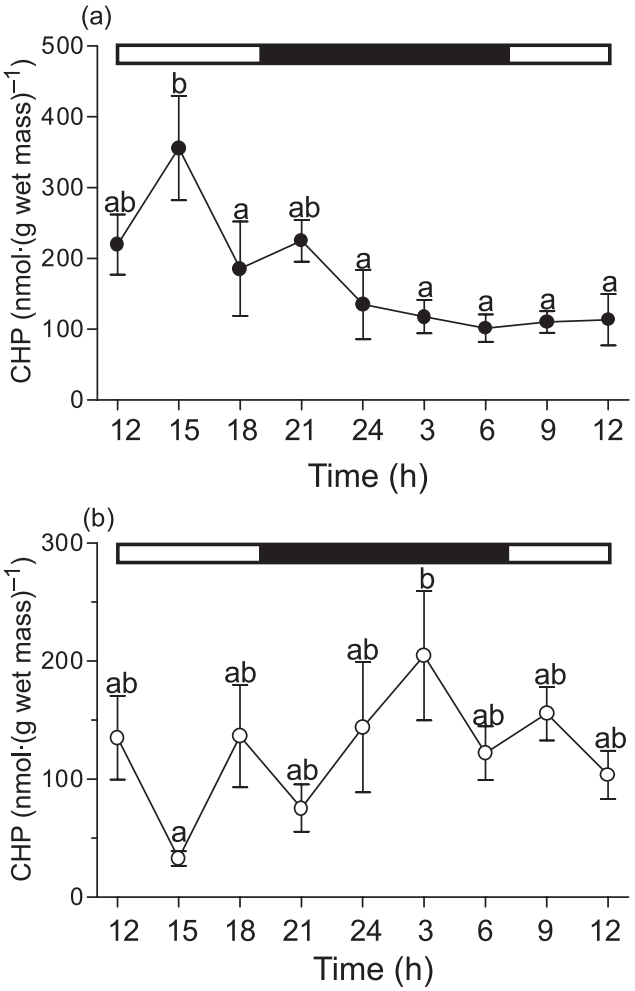


Fig. 5. Daily variations in lipid peroxide (LPO) content in the gills (*a*) and hepatopancreas (*b*) of *C. granulata*. Each point represents the mean ± 1 SE (n = 3–5). The hours when tissues were sampled and LPO content was determined are indicated on the x axis. LPO content is expressed in cumene hydroperoxide (CHP) equivalents per gram of wet mass. The open and solid bars indicate light and dark phases, respectively. Values accompanied by the same letter are not statistically different ($p > 0.05$).



corded at night. This daily variation in LPO content corroborates previous findings in vertebrate species. Dias-Muños et al. (1985) and Baydas et al. (2002) found that under laboratory conditions, daily variation in LPO levels could be correlated with daily variation in aerobic metabolism of rat cerebral cortex and liver.

As daily LPO variations did not occur simultaneously with the observed daily variation in aerobic metabolism in either tissue, it is inferred that the antioxidant-defense system also varied throughout the experimental period, although the variations were insufficient to prevent oxidative damage. It seems that the antioxidant-defense strategies employed by gill and hepatopancreas tissues are different. In the gills a high level of CAT activity was observed, which remained unchanged throughout the experimental period. As oxygen diffuses through the gill cells, the rate of generation of ROS in this tissue is presumed to be higher. A possible strategy to counteract this problem is to develop a permanently high level of CAT activity, favouring the degradation of H_2O_2, a molecule known to be a precursor of the highly reactive hydroxyl radical (Dröge 2002). In the hepatopancreas this strategy is not observed. CAT activity is much lower than in the gills, but daily variation was observed, with a peak of enzyme activity at night, which corresponds to the period of higher oxygen consumption in this tissue.

With respect to GST activity, the daily variations were similar in both tissues. GST is a phase II enzyme catalyzing GSH conjugation with several molecules, including LPO, and this is considered to ameliorate the oxidative damage (Storey 1996). According to our results, this enzyme showed lower activity at the end of the afternoon, which fits with the lower oxygen consumption observed during this period. Higher GST activity was verified during the nocturnal period, a result that again correlates well with the higher oxygen consumption observed in both tissues.

Fanjul-Moles et al. (2003) analyzed, under laboratory conditions, daily variations in activity of glutathione reductase

and GPx in hemolymph and hepatopancreas of the prawns *P. clarkii* and *P. digueti*, and observed higher activity of both enzymes at night. Diaz-Muños et al. (1985) reported, in rat cerebral cortex, higher activity of SOD during the nocturnal period. Also, Baydas et al. (2002) recorded higher GPx activity in rat liver, a result possibly related to the higher rate of metabolism observed in the same period.

In the gills, the highest concentration of NP-SH groups was observed during the nocturnal period, and was paralleled by higher oxygen consumption, which in turn should generate more ROS. In this way, augmented antioxidant levels should counterbalance this effect, as evidenced by the low LPO levels observed during this period. In the hepatopancreas no daily variations in NP-SH groups were verified, suggesting the existence of other non-enzymatic antioxidants, like flavonoids or β-carotenes, molecules known to be in high concentrations in this tissue (Sagi et al. 1995; Vershinin 1996). Also, other antioxidant enzymes not analyzed in the present work, such as GPx and SOD, might be involved. Other studies have reported daily variations in GSH levels, in both invertebrate and vertebrate species. Fanjul-Moles et al. (2003) and Diaz-Muños et al. (1985) found higher GSH levels during the day in the hemolymph and hepatopancreas of *P. clarkii* and *P. digueti* and in rat cerebral cortex, which indicates that this tripeptide acts as an antioxidant against the ROS generated during this period, whereas antioxidant enzymes are preferentially employed at night; this result is similar to the observations in the present work concerning CAT and GST activities.

As previously mentioned, LPO levels were higher when NP-SH concentration were lower (gills) or remained unchanged in spite of high CAT and GST activities (hepatopancreas). This indicates the importance of sulfhydryl groups in the prevention of the formation of LPO, at least in the gills, especially during a period when oxygen consumption is high. Previous unpublished results from our laboratory have shown that when gills are incubated for 120 min with H_2O_2 (0.1 mol/L), at noon (the period of lowest NP-SH concentration according to the present results) NP-SH levels are statistically higher ($p < 0.05$) than those in a control group (0.014 ± 0.002 and 0.006 ± 0.001 μmol NP-SH·(mg protein)$^{-1}$, respectively). Again this result indicates the importance of NP-SH in intercepting ROS, or in conjugating reactions catalyzed by GST or GPx, when GSH is required as co-substrate.

Overall, the results obtained show that the estuarine crab *C. granulata* exhibits daily variations in the aerobic metabolism of the gills and hepatopancreas. Differential responses by the antioxidant defenses were recorded between the analyzed tissues (gills and hepatoancreas), suggesting the employment of different strategies. In the gills, the antioxidant system should depend on high and constant CAT activity and on the presence of NP-SH groups. In the hepatopancreas, other antioxidants (both enzymatic and non-enzymatic) could be involved. The recorded daily variations in the antioxidant defenses are probably related to the observed variations in oxygen consumption, since the period of highest GST activity and NP-SH concentration in the gills and the highest CAT and GST activities in the hepatopancreas are correlated with the period of highest oxygen consumption in these tissues.

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