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Daily variation of melatonin content in the optic lobes of the crab *Neohelice granulata*

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Abstract

Melatonin is a biogenic amine, known from almost all phyla of living organisms. In vertebrates melatonin is produced rhythmically in the pinealocytes of the pineal gland, relaying information of the environmental light/dark cycle to the organism. With regard to crustaceans only a handful of studies exist that has attempted to identify the presence and possible daily variation of this substance. We set out to investigate whether in the crab *Neohelice granulata* melatonin was produced in the optic lobes of these animals and underwent rhythmic fluctuations related to the daily light/dark cycle. Our experimental animals were divided into three groups exposed to different photoperiods: normal photoperiod (12L:12D), constant dark (DD), and constant light (LL). The optic lobes were collected every 4 hours over a 24-h period for melatonin quantification by radioimmunoassay (RIA). *N. granulata* kept under 12 L:12D and DD conditions, showed daily melatonin variations with two peaks of abundance ($p<0.05$), one during the day and another, more extensive one, at night. Under LL-conditions no significant daily variations were noticeable ($p>0.05$). These results demonstrate the presence of a daily biphasic fall and rise of melatonin in the eyestalk of *N. granulata* and suggest that continuous exposure to light inhibits the production of melatonin synthesis.

Keywords: *Neohelice granulata*; Melatonin; Crustacean; Crab; Daily variation; Circadian variation; Optic lobes; Photoperiod

1. Introduction

The rotation of the Earth and the effects of lunar gravity cause daily and seasonal changes in temperature, light intensity and tidal level, forcing living organisms to adapt their behavioral and physiological parameters to these abiotic cyclic events. It is generally agreed that early in the evolution of life forms, chemical substances like indoleamine N-acetyl-5-methoxytryptamine or melatonin evolved that could detect and monitor these rhythmic environmental changes, principally the photoperiod. Melatonin has been identified in almost all phyla of organisms, but it was in the mammalian species that the synthesis and the functions of melatonin were most keenly studied. In mammals, the main production of melatonin occurs in the pinealocytes of the pineal gland (itself an outgrowth of the diencephalons) and exhibits a peak at night. The amount of melatonin and the duration over which it is synthesized, are directly proportional to the length of the night. This has led to the definition of melatonin as a "chemical expression of darkness" (Reiter, 1987, 1991).

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Table 1
Periods of highest concentrations of the indoleamine melatonin in different species of crustaceans

Species	Tissue	Period of highest concentration	References
Carcinus maenas	Eyestalk	No variation	Vivien-Roels and Pévet, 1986
Macrobrachium rosenbergii	Optic lobes	Photophase	Withyachumnarnkul, 1992
Penaeus monodon	Optic lobes	Not studied	Withyachumnarnkul, 1995
Procambarus clarkii	Eyestalk and Haemolymph	Photophase	Agapito et al, 1995
Procambarus clarkii	Eyestalk	Scotophase	Balzer et al., 1997
Uca pugilator	Eyestalk	Photophase	Tilden et al., 1997
Astacus fluviatilis	Eye	No variation	Meyer-Rochow, 2001
Saduria entomon	Head	No variation	Meyer-Rochow, 2001

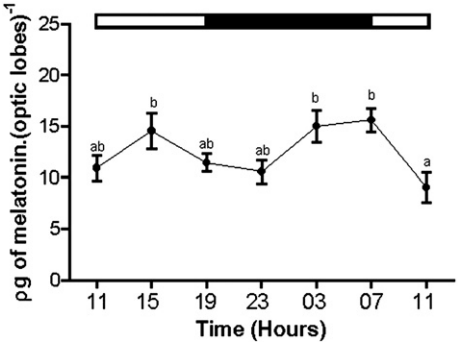

Fig. 1. Melatonin contents in the optic lobes of the crab *Neohelice granulata* under normal photoperiod (12 L:12D), monitored over a 24 hour period. Each point represents the mean±1SE (n=6–8). The white and black bars represent light and dark phases. Values accompanied by different letters are statistically significantly different (p<0.05).

The main function of melatonin seems, indeed, to inform the organism of the photoperiod. Thus, it is involved in regulating physiological parameters like body temperature, locomotor activity, reproductive cycle, color change and others (Underwood, 1981; Mayer et al., 1997; Lutterschimidt et al., 2003). More recently it could be shown that melatonin also possesses antioxidant properties, decreasing oxidative stress in tissues at risk (Reiter et al., 1996). Melatonin can influence the effectiveness of some antioxidant enzymes like superoxide dismutase and gluthathione peroxidase (Barlow-Walden et al., 1995; Liu and Ng, 2000) or act as a scavenger of reactive oxygen species such as hydroxyl radical and hydrogen peroxide (Reiter et al., 1996; Tan et al., 2000).

The first evidence of the presence of this indole in an invertebrate came from an examination of the compound eyes in the locust *Locusta migratoria,* in which the highest amounts were found at night (Vivien-Roels et al., 1984). Wetterberg et al. (1987) then reported a nighttime peak of melatonin in the brain of the fly *Musca autumnalis,* and Meyer-Rochow and Vakkuri (2002) documented winter-peaks and summer troughs of melatonin in the heads and abdomens of the honeybee *Apis mellifera*.

With regard to aquatic invertebrates the planarian worm *Dugesia dorotocephala* (Morita et al., 1987) and the unicellular alga *Gonyaulax polyedra* (Poeggeler et al., 1991) were shown to contain indoleamines. As with most other organisms studied, melatonin levels fluctuated in them over a 24-hour period, peaking during the scotophase, similar to the situation known from vertebrates. However, in crustaceans the situation is less clear (see Table 1). In the crab *Carcinus maenas* (Vivien-Roels and Pévet, 1986), the freshwater crayfish *Astacus fluviatilis*, and the isopod *Saduria entomon* (Meyer-Rochow, 2001) daily variations of melatonin production were not observed. Withyachumnarnkul et al. (1992) reported an increase of melatonin in the optic lobes of the fresh water shrimp *Macrobrachium rosembergii* during the photophase, but were unable to detect any such changes in the optic lobes of the shrimp *Penaeus monodon* (Withyachumnarnkul et al., 1995). For the crayfish *Procambarus clarkii* equally contradictory data exist: A first study indicated that melatonin production in the eyestalk and haemolymph rose during the photophase (Agapito et al., 1995), but a second study detected only a night peak of melatonin in the eyestalk of *P. clarkii* (Balzer et al., 1997). In the fiddler crab *Uca pugilator,* according to Tilden et al. (1997), melatonin synthesis in the eyestalk increased during the day. A hint that seasonal variations could be involved as well comes from data on the melatonin contents of the eyes of Finnish freshwater crayfish, monitored over several weeks at the end of summer (Meyer-Rochow, 2001).

To help the comprehension of the general pattern of melatonin fluctuation in crustaceans, here we report the profile of melatonin fluctuation in the optic ganglia of the crab *Neohelice granulata* (formely known as *Chasmagnathus granulatus* — see Sakai et al., 2006) in different photoperiods. This estuarine crab has daily variations of locomotor activity (Pereyra et al., 1996), pigment migration (Granato et al., 2004), oxygen consumption and antioxidant defense systems in gills and hepatopancreas (Maciel et al., 2004).

2. Material and methods

Adult males of the estuarine crab *Neohelice granulata*, weighing 9.81±0.27 g (mean±S.E.M) were collected during

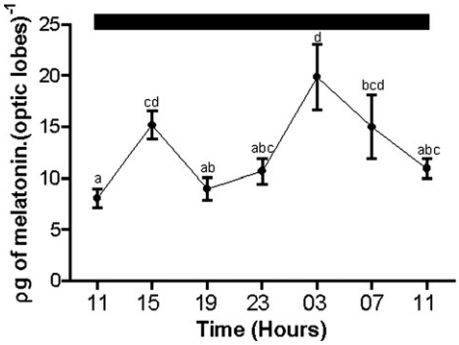

Fig. 2. Melatonin contents in the optic lobes of the crab *Neohelice granulata* under constant darkness (DD), monitored over a 24 hour period. Each point represents the mean±1SE (n=5–8). The solid black bar represents the DD photoperiod. Values accompanied by different letters are statistically significantly different (p<0.05).

the day in the salt marshes around Rio Grande City (Brazil) and transported to the laboratory. The crabs were kept for 30 days in spacious tanks under a photoperiod of 12L:12D (lights on at 07:00 h), a temperature of 20 °C, and a salinity of 20 psu before the experiments began. The animals were fed *ad libitum* with ground beef 3 times a week.

Following this acclimation period the organisms were assigned to one of three photoperiodic classes, each containing 7 glass aquaria with 8 animals per aquarium ($n=8$, *i.e.* a total of 56 per photoperiodic class). The photoperiodic conditions were: 1. normal photoperiod (12D:12 L), 2. constant darkness (DD) and 3. constant light (LL). After 10 days under these regimens, eyestalks were collected from crabs in each group every 4 h over a 24-h period. The eyes were dissected, the optic lobes separated (including the *lamina ganglionaris*, *medulla externa*, *medulla interna* and *medulla terminalis*) and frozen at −80 °C for further analyses. Specimens collected during the time of darkness, all collecting and dissecting procedures took place under dim red light.

The frozen optic lobes were then homogenized in 0.5 mL ethanol, centrifuged for 10 min (1000 ×*g*) and the supernatants were collected and lyophilized. After the samples were diluted with PBS (phosphate-buffered saline and RIA buffer (400 µL/sample)), they were sonicated for 4–5 s on an ice bath and centrifuged for 5 min (10,000 ×*g*). The supernatants were directly analyzed for melatonin (2×100 µL) as described previously using radio-iodinated melatonin as the tracer (Vakkuri et al., 1984a,b).

Statistical evaluations were based on analyses of variance (ANOVA) followed by Newman–Keuls test ($\alpha=0.05$). Normality and homogeneity variance were verified as assumptions of ANOVA (Zar, 1984).

3. Results

The optic lobes of *N. granulata* individuals, kept under a 12L:12D photoperiod, showed a significant difference ($p<0.05$) in melatonin along the 24-h period (Fig. 1). The two peaks were separated by 12 h, the daytime peak reaching 14.6±1.8 pg of melatonin (optic lobes)$^{-1}$ and the nighttime peak reaching 15.0±1.6 ρg of melatonin (optic lobes)$^{-1}$. The melatonin profile of crabs maintained under the DD photoperiod (Fig. 2), gave a similar and statistically significant result to that present in the 12 L:12D animals ($p<0.05$), but the second peak was more pronounced (15.2±1.4 versus 19.9±3.2 ρg of melatonin (optic lobes)$^{-1}$). In the LL photoperiod (Fig. 3), no significant daily variations were observed ($p>0.05$), although the increase at 15 h persisted (16.9±2.0 ρg of melatonin.(optic lobes)$^{-1}$). The night peak (03 h) that were present in crabs kept under 12 L:12D and DD photoperiods were abolished in the LL condition.

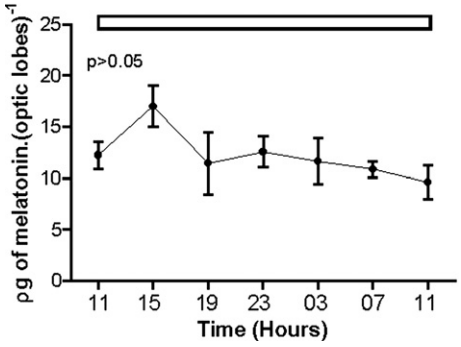

Fig. 3. Melatonin contents in the optic lobes of the crab *Neohelice granulata* under constant light (LL), monitored over a 24 hour period. Each point represents the mean±1SE ($n=4$–8). The white bar represents the LL photoperiod. No statistically significant differences ($p>0.05$) were present during the 24-hour period.

4. Discussion

Melatonin is a conserved and ubiquitous molecule, present in virtually all organisms studied to date (Hardeland and Führberg, 1996). However, as previously described the period of highest melatonin production in crustaceans is not clear. Thus, where does the crab *N. granulata* fit into all this? We know that *N. granulata* exhibits a daily variation in melatonin production under 12 L:12D and DD photoperiods. Two peaks of melatonin content were observed (Figs. 1, 2). The biphasic profile of the melatonin, with a 12 h of interval between the two peaks, resembles that known from the fiddler crab *Uca pugilator* (Tilden et al., 1997, 2001). Therefore, presently the only two crustacean species for which the presence of an endogenously controlled rhythm of melatonin synthesis could unambiguously be demonstrated are *U. pugilator* (Tilden et al., 1997, 2001) and *N. granulata* (this paper). In *N. granulata* crabs, kept under LL condition, significant melatonin cycle was not observed (Fig. 3). However, the continuous light strongly inhibited only the peak of melatonin at 03 h (observed in the other two photoperiods), remaining the increase at 15 h. Therefore, the effects of continuous illumination on melatonin levels for one phase (the subject night) in *N. granulata* were similar to those seen in most vertebrates (Lynch et al., 1981). Tilden et al. (2001), however, reported that in *U. pugilator* an increase of melatonin levels occurred under constant illumination. This suggests that light in *U. pugilator,* in contrast to that observed in most vertebrates, stimulates melatonin synthesis.

For *N. granulata* it is known that certain activities fluctuate in a daily pattern. Pereyra et al. (1996) reported a circadian rhythm of locomotor activity, showing that this species is nocturnal in behavior. Furthermore *N. granulata* exhibits a circadian rhythm of pigment migration, in which the melanosomes (*i.e.* the black granules of the integumental melanophores) are dispersed during the day and aggregated at night (Granato et al., 2004). Maciel et al. (2004) reported that daily rhythms also govern the variations in oxygen consumption and antioxidant defense system as well as lipoperoxidative processes in gills and hepatopancreas. For gills and hepatopancreas these authors confirm the presence of two peaks of oxygen consumption with a 12 hour interval between them, one in the middle of the afternoon and a second during the night. With regard to the antioxidant system, Maciel et al. (2004) observed a nocturnal peak of non-proteic sulphydryl groups (a non

enzymatic antioxidant) in the gills and another for catalase activity in the hepatopancreas. Elevated lipid peroxidation in the gills occurred at night, but with regard to the hepatopancreas in the middle of the afternoon, suggesting that variations in oxidative stress are correlated with variations in aerobic metabolism.

Melatonin can be considered a "multifunctional molecule" due to the variety of actions creditable to this indoleamine (Reiter et al., 1996). It could recently be shown that melatonin is capable of regulating some enzymes of the antioxidant system like gluthathione peroxidase and superoxide dismutase and can act as a free radical scavenger, decreasing oxidative stress such caused by lipoperoxidation, DNA damage, and protein oxidation (Tan et al., 2007). Yet, in crustaceans the role of melatonin is still not well established. The similarity profiles between aerobic metabolism (Maciel et al., 2004) and melatonin production indicate that melatonin could be involved in regulating some aspects of the antioxidant defense system, whereas variations in the aerobic metabolism should impose variations in free radical generation (Storey, 1996). We speculate that in *N. granulata* melatonin could directly increase the activity of some components of the antioxidant system at periods of higher aerobic metabolism and/or indirectly affect the system by scavenging *per se* the free radicals. Why other species of crustaceans (with the exception of *Uca pugilator,* see above) apparently lack the daily melatonin rhythm remains presently enigmatic, but it suggests that in crustaceans the melatonin/serotonin system is just one of perhaps others involved in the control of biorhythmic phenomena.

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