VERSITA

Biologia **64**/1: 187—191, 2009
Section Cellular and Molecular Biology
DOI: 10.2478/s11756-009-0016-8

Melatonin does not affect the black pigment migration in the crab *Neohelice granulata*

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Abstract: N-acetyl-5-methoxytryptamine or melatonin is a multifunctional molecule. The main physiological function, at least in vertebrates, is to transduce to the animal the photoperiodic information and regulate rhythmic parameters. But studies have also observed the action of this molecule on pigment migration in ectothermic vertebrates. Thus the aim of this paper was to investigate *in vivo* and *in vitro* the influence of melatonin on the pigment migration in melanophores of the crab *Neohelice granulata*. Injections of melatonin (2×10^{-9} moles $\cdot$ crab^{-1}) at 07:00 h or 19:00 h did not affect ($p > 0.05$) the circadian pigment migration of the melanophores in constant darkness. Additionally no significant pigment migration ($p > 0.05$) was verified in normal and eyestalkless crabs injected with melatonin (10^{-10}-10^{-7} moles $\cdot$ crab^{-1}) during the day or night. In the *in vitro* assay, the response of melanophores to the pigment-dispersing hormone in eyestalkless crabs injected with melatonin (2×10^{-9} moles $\cdot$ crab^{-1}) 1 and 12 hours before the observations did not differ ($p > 0.05$) from the control group (injected with physiological solution). These results suggest that melatonin does not act as a signaling factor for pigment dispersion or aggregation in the melanophores of *N. granulata*.

Key words: *Neohelice granulata*; melatonin; pigment migration; melanophores; crustacean; pigment-dispersing hormone; color change.

Abbreviations: CCAP, crustacean cardioactive peptide; DRC, dose response curve; DD, constant darkness; MCH, melanin-concentrating hormone; α-MSH, α-melanocyte-stimulating hormone; β-PDH, β-pigment dispersion hormone; RPCH, red-pigment-concentrating hormone.

Introduction

Physiological color change is characterized by a fast (from minutes to some hours) tegument lightening or darkening due to a pigment migration within pigment cells, called chromatophores. Depending on the color of the pigment or the internal structure, the chromatophores may be divided in melanophores (black or brown), erythrophores (red), xanthophores (yellow), leucophores (white) and iridophores (iridescent). The movements of these pigments can directly be regulated by environmental factors like light intensity or temperature, or indirectly, with hormones involvement (Fujii 2000; Rao 2001).

In vertebrates the α-melanocyte-stimulating hormone (α-MSH) is the most relevant hormone in pigment dispersion (Castrucci et al. 1984; Fujii & Oshima 1986; Hruby et al. 1987; Rollag et al. 1989; Visconti & Castrucci 1993; Filadelfi & Castrucci 1994). The melanin-concentrating hormone (MCH) is the antagonist of α-MSH, evoking pigment aggregation (Fujii & Oshima 1994). Other substances like cathecoleamines, prolactin, endothelins and melatonin (Fujii et al. 1993; Fujii & Oshima 1994; Filadelfi & Castrucci 1996; Camargo et al. 1999; Fujii 2000) have been demonstrated to influence the color change.

The β-pigment-dispersing hormone (β-PDH) and red-pigment-concentrating hormone (RPCH) are the major substances secreted from the X-organ/sinus gland neuroendocrine complex in the eyestalk of crustaceans, with the main action in pigment migration. Hence their names, β-PDH promotes pigment dispersion in all types of chromatophores and RPCH promotes pigment aggregation, in erythrophores in brachyuran and isopod species, and in leucophores, melanophores and xantophores in others species (Josefsson 1975; Skorkowski & Biegniewska 1981; Yang et al. 1999). Besides β-PDH and RPCH, other molecules like the crustacean cardioactive peptide (CCAP) and again the indoleamine melatonin have been shown to influ-

ence color change in some species (Nery et al. 1999; Granato et al. 2004).

In the 1950s, Lerner et al. (1958) isolated and characterized melatonin from the pineal gland, demonstrating a lightening effect on amphibian melanocytes. Although melatonin acts in color changes, the main effect of this molecule (at least in mammals) is to inform the photoperiod changes, regulating a variety of physiological parameters such as body temperature, locomotor activity, reproduction, and others (Underwood 1981; Mayer et al. 1997; Lutterschmidt et al. 2003). Melatonin is not exclusively from mammals, being present in almost all groups of animals, from superior vertebrates to unicellular organisms. In vertebrates and in the majority of studies conducted in non-vertebrates, melatonin is produced in a circadian-rhythm manner with a peak during night (Reiter 1991; Vivien-Roels & Pévet 1993).

As mentioned before, melatonin has a pronounced action in color changes. This molecule has the ability to promote pigment aggregation in ectothermic vertebrate chromatophores (Kavaliers et al. 1980; Binkley et al. 1987, 1988; Binkley 1988; Filadelfi & Castrucci 1994). In crustaceans, the involvement of melatonin in pigment translocation is almost unknown. Only the study conducted by Nery et al. (1999) reported the effect of melatonin in decreasing the aggregating response to RPCH in erythrophores of *Macrobrachium potiuna*.

Granato et al. (2004) reported a circadian rhythm of pigment translocation in the melanophores of the crab *Neohelice granulata*, being dispersed during the daytime and aggregated during the nighttime, and this circadian rhythm is dependent on the eyestalk. Moreover the authors verified in both *in vivo* and *in vitro* assays a higher responsiveness of melanophores to β-PDH during the day period when compared to the night period. The authors suggested that this rhythm might be regulated by β-PDH synthesis and/or release from eyestalks and an endogenous rhythm of responsiveness of melanophores to β-PDH.

Thus the aim of this article was to verify whether melatonin is involved in the circadian rhythm of pigment migration in the melanophores of the crab *N. granulata* and whether this molecule acts as a signaling factor for color change.

Material and methods

Recently, Sakai et al. (2006) reviewed the external and gastric morphology of the species of crabs that fit in the Vanurinae family, and concluded that the crab *Chasmagnathus granulata/granulatus*, found in the South Latin America, should be relocated to the genera *Neohelice*, thus being renamed as *Neohelice granulata*. Adult males *N. granulata*, weighing 8.95 ± 0.31 g (mean ± S.E.M) were collected near Rio Grande City, Brazil, and transported to the laboratory. The crabs were acclimated in tanks for 30 days, temperature of 20 °C, salinity of 20 and fixed photoperiod of 12L:12D (lights on at 07:00 h). The animals were fed *ad libitum* with ground beef 3 times a week.

Melatonin and β-PDH were purchased from Sigma (USA). The stock solutions were made in ethanol (melatonin) and 5% acetic acid (β-PDH). For each solution, the final dilution was made in physiological saline, and the final concentration of the non-aqueous solvents never exceeded 1%. The physiological saline contained: 10 mM $MgCl_2$, 355 mM NaCl, 16.6 mM $CaCl_2$, 5 mM H_3BO_3, 10 mM $KHCO_3$, 8 mM $Na_3C_6H_5O_7{\cdot}2H_2O$; pH adjusted to 7.6.

To verify the effect of melatonin in the circadian pigment migration, 40 animals were divided into 4 groups ($n = 10$) and submitted to constant darkness (DD) photoperiod for 25 days. In the 13th day the crabs were injected with 100 μL of melatonin (2×10^{-5} M) or physiological saline at 07:00 h or 19:00 h in the base of the 4th or 5th pair of the walking legs. In the days 2–4/9–11 (before injections) and 16–18/23–25 (after injections), crabs were analyzed every 1 hour to verify the degree of pigment dispersion in the melanophores in the meropodite of the 3rd pair of maxillipedes. The animals were not fed during this period. Pigment dispersion was assessed using the Hogben & Slome (1931) index, which defines stage 1 as that with full pigment aggregation, stage 5 that with full pigment dispersion, stages 2, 3, and 4 being intermediate conditions. The rhythm of pigment dispersion was represented by chromograms.

In the time-course dispersing response of melanophores to melatonin, intact and eyestalkless crabs were injected with 100 μL of melatonin (10^{-6}-10^{-3} M) or physiological saline (control) in the base of the 4th or 5th pair of legs during the day period (between 10:00 h and 16:00 h) or night period (between 22:00 h and 03:00 h). The degree of pigment dispersion (Hogben & Slome 1931) was evaluated *in vivo* before (time zero) injections and 30, 60, 90, 120, 150 and 180 minutes after injections.

In the *in vitro* assays, eyestalkless crabs were injected with 100 μL of melatonin (2×10^{-5} M) at 12:00 h in the base of the 4th or 5th pair of walking legs, and 1 and 12 hours later the meropodite of the 3rd pair of maxillipedes was collected and incubated in physiological saline for 30 min before the experiments. The pieces were then taped to a glass cover slip, which was turned upside down and mounted in a perfusion chamber, as described by Britto et al. (1990). Then the maxillipedes received increasing concentrations of β-PDH (10^{-11}–10^{-5} M) for 20 min each one. The cumulative dose response curves (DRCs) were determined as percentages of the maximum response of the length of melanophores fitted to a sigmoid curve by nonlinear regression. The DRCs were compared using Two-way ANOVA followed by Newman-Keuls test ($\alpha = 0.05$).

The chromograms were done attributing different colors to the pigment dispersion index, white color between 1 and 2 index, gray color between 2.1 and 3.9 index, and black color between 4 and 5 index. The differences among treatments were analyzed visually.

In the time-course dispersing response of melanophores were compared using One-way ANOVA non-parametric ($\alpha = 0.05$).

Results

The indoleamine melatonin injected (2×10^{-9} moles$\cdot$ crab^{-1}) in crabs maintained in DD photoperiod in the subject day (07:00 h) or night (19:00 h) did not promote a clear phase shift in the period of the circadian rhythm of pigment migration in the melanophores of *Neohelice granulata* (Fig. 1).

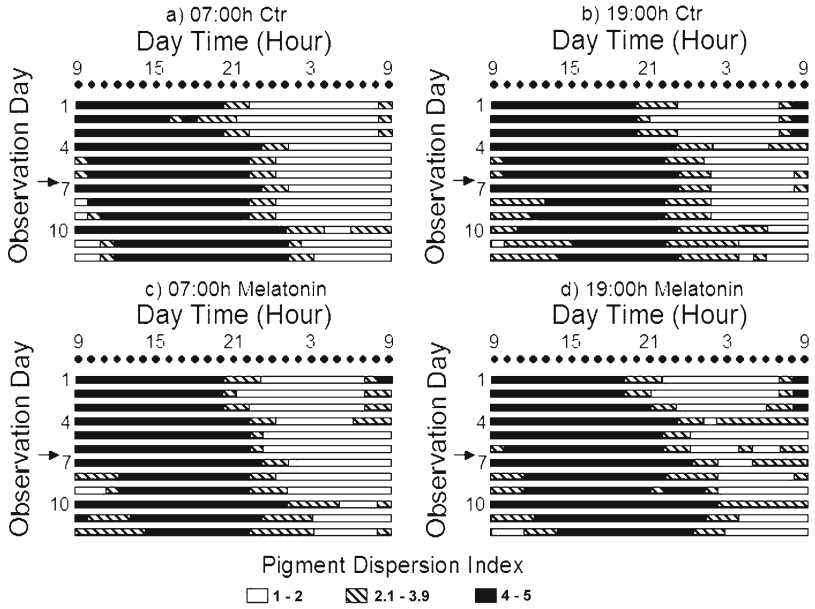

Fig. 1. Chromograms of pigment migration in melanophores of *Neohelice granulata* kept in constant darkness (DD) for 25 days. (a) Crabs injected with 100 μL of physiological saline (control group) at 07:00 h in the 13th day of experiment (7th observation day). (b) Crabs injected with 100 μL of physiological saline (control group) at 19:00 h in the 13th day of experiment (7th observation day). (c) Crabs injected with 100 μL of melatonin (2×10^{-5} M) at 07:00 h in the 13th day of experiment (7th observation day). (d) Crabs injected with 100 μL of melatonin (2×10^{-5} M) at 19:00 h in the 13th day of experiment (7th observation day). The arrows indicate the day of the injections.

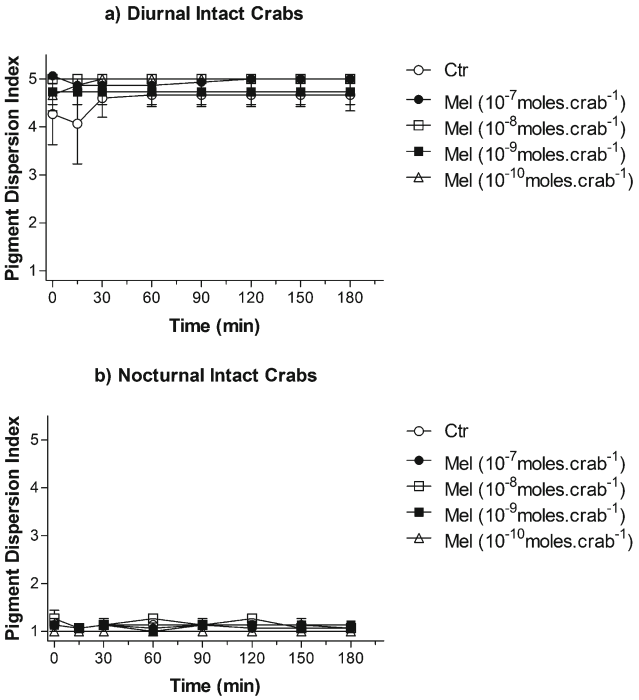

Fig. 2. Time-course dispersing response of melanophores of intact *Neohelice granulata* injected with 100 μL of physiological saline (control group) or melatonin (10^{-10}–10^{-7} moles $\cdot$ crab^{-1}) during the (a) day period (between 10:00 h and 16:00 h) or (b) night period (22:00 h and 03:00 h). Each point represents the mean $\pm$ S.E.M. of pigment dispersion index at the time noted (n = 10–20).

In the time-course dispersing response of melanophores of *N. granulata* to injections of melatonin (10^{-10}–10^{-7} moles $\cdot$ crab^{-1}) in the day or night period, in both intact and eyestalkless crabs no significant differences ($p > 0.05$) from the control group (injected with physiological solution) were observed (Figs 2,3). The melanophores of diurnal intact crabs stayed dispersed (Fig. 2a) during all of the time-course and in nocturnal intact crabs the melanophores stayed in an aggregate form (Fig. 2b). In diurnal and nocturnal eyestalkless animals, the melanophores of control or treatments groups stayed in an aggregating form during all of the time of observation (Fig. 3a,b).

In the *in vitro* assay no significant differences ($p > 0.05$) were verified in the dose-response of the melanophores to the β-PDH in eyestalkless crabs injected with melatonin (10^{-11} moles $\cdot$ crab^{-1}) 1 and 12 hours before the observations (Fig. 4a,b).

Discussion

Melatonin is a multifunction molecule, but the marked effect on pigment migration in amphibians, reptilians and fishes was the first function attributed to this molecule (Lerner et al. 1958). In amphibians and reptilians this hormone promotes skin lightening, probably because of an inhibition of adenyl cyclase and/or a decrease in response of pigmentary agonistics, such as α-MSH (Binkley et al. 1987, 1988; Filadelfi & Castrucci 1996). The study conducted by Fujji (1961) in the goby *Chasmichthys gulosus* was the first evidence of melatonin's melanophore-aggregating effect in fishes. Since then, many studies have verified the lightening effect of this molecule on chromatophores in this group of animals (Fujii 2000). However, Visconti & Castrucci (1993) reported that the melanophores of the lungfish *Lepidosiren paradoxa* are weakly responsive to melatonin.

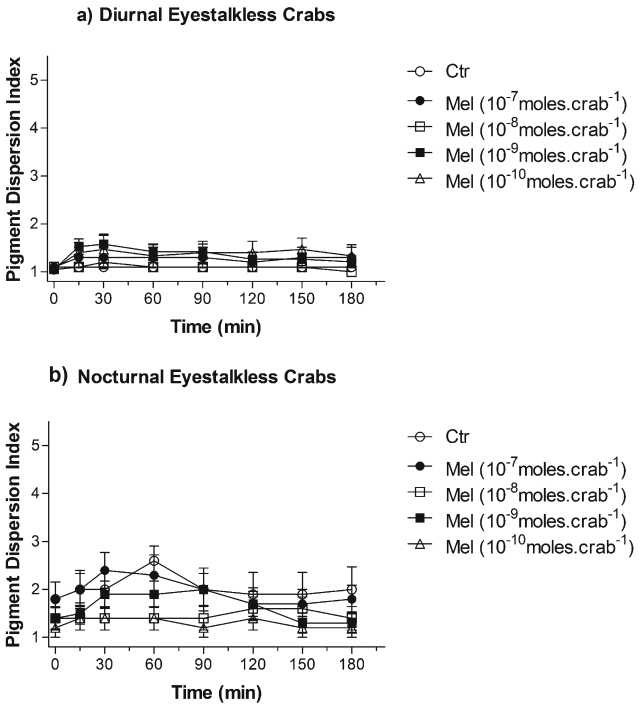

Fig. 3. Time-course dispersing response of melanophores of eyestalkless *Neohelice granulata* injected with 100 μL of physiological saline (control group) or melatonin (10^{-10}–10^{-7} moles $\cdot$ crab^{-1}) during the (a) day period (between 10:00 h and 16:00 h) or (b) night period (22:00 h and 03:00 h). Each point represents the mean $\pm$ S.E.M. of pigment dispersion index at the time noted (n = 10–20).

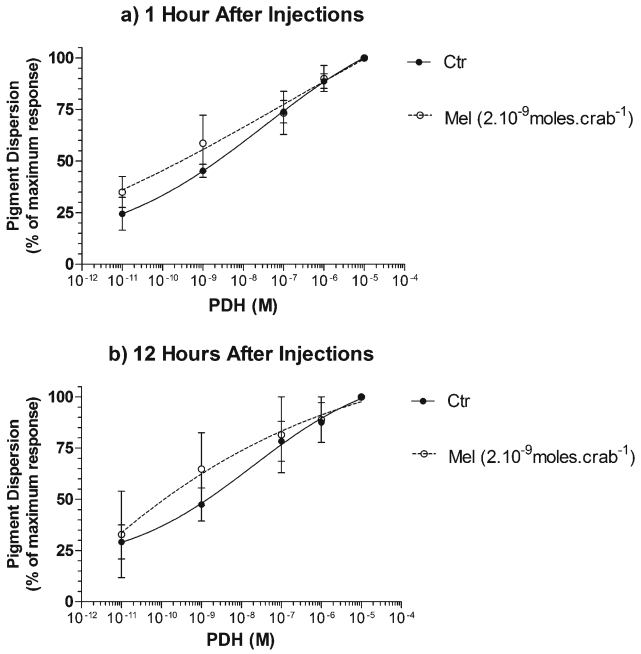

Fig. 4. *In vitro* dose-response curve for PDH of melanophores of eyestalkless *Neohelice granulata* 1 hour (a) or 12 hours (b) after injections of 100 μL of physiological saline (control group) or melatonin (2×10^{-9} moles $\cdot$ crab^{-1}). Each point represents the mean $\pm$ S.E.M. dispersing response at the concentration noted (n = 4–5).

The same authors also verified that this indoleamine did not have any effect on pigment migration in the melanophores of the elasmobranch *Potamotrygon reticulata*. Moreover, an autodesensitizing action and the lack of response to pigment aggregation/dispersion agonistics were verified in the chromatophores of *Xenopus laevis* tadpoles (Rollag & Lynch 1993), *Labrus ossifagus* (Märtensson & Andersson 1996), *Synbranchus marmoratus*, *Rana pipens*, *Bufo ictericus* and *Anolis carolinensis* (Filadelfi & Castrucci 1994). Thus the effect of melatonin on pigment migration may vary according to the species analyzed.

In crustaceans, few studies have been conducted to verify the role of melatonin. Balzer et al. (1997) verified an increase of period and amplitude in the circadian rhythm of eletroretinogram in *Procambarus clarkii* when injected with melatonin. In this same species, Tilden et al. (2003) reported that injections of melatonin increased the synaptic transmission in the neuromuscular junctions. In the fiddler crab *Uca pugilator*, melatonin increased the rate of limb regeneration (Tilden et al. 1997) and caused a phase delay of the circadian rhythm of glucose and lactate in the haemolymph (Tilden et al. 2001).

Concerning the effect of melatonin on tegument pigment migration, only one study was conducted. Nery et al. (1999) observed that this indoleamine did not induce *per se* pigment aggregation or dispersion in the erythrophores of the freshwater shrimp *Macrobrachium potiuna*, but decreased the erythrophore response to the RPCH. *Neohelice granulata* has a circadian rhythm of black pigment aggregation/dispersion similar to many crustaceans (Fingerman 1955; Powell 1962a,b, 1966; Shibley 1968; Thurman 1988), with black pigment dispersion at day period and black pigment aggregation at night period. In both periods melatonin was not capable of inducing any significant pigment translocation in intact and eyestalkless crabs (Figs 2,3). This circadian rhythm of black pigments is dependent on a circadian rhythm of β-PDH synthesis and/or release and on a circadian rhythm of melanophore response to β-PDH (Granato et al. 2004). Then the next step was to test the hypotheses that melatonin is capable of modulating the circadian rhythm of pigment migration and/or the responsiveness of the melanophores to β-PDH. The results showed that injections of this molecule did not affect the circadian rhythm of black pigment dispersion neither in photophase nor in scotophase (Fig. 1). Differently to erythrophores of *M. potiuna*, melatonin was not capable to change significantly the responsiveness of melanophores to β-PDH in *N. granulata* (Fig. 4).

This suggests that melatonin does not have a signaling function for black pigment aggregation/dispersion in *N. granulata*. We have not ruled out the possibility that melatonin affect pigment migrations in others chromatophores types. Further studies in other crustaceans species and also different chromatophores types are necessary to confirm the absence of melatonin effect in the color change regulation.

Acknowledgements

Brazilian agencies FINEP, CNPq, CAPES and FAPERGS supported this work. FEM was fellow by CAPES, MAG was fellow by CNPq and BPC was fellow by FAPERGS.

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Received February 2, 2008
Accepted August 27, 2008