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Circadian rhythm of pigment migration induced by chromatrophorotropins in melanophores of the crab *Chasmagnathus granulata*

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Abstract

The circadian rhythm of black pigment migration of melanophores of the crab *Chasmagnathus granulata* and the variation in responsiveness of these cells to pigment-dispersing hormone (β-PDH), crustacean cardioactive peptide (CCAP), and red pigment-concentrating hormone (RPCH) were investigated. Melanophores of *C. granulata* possess an endogenous circadian rhythm of pigment migration, with black pigments staying more dispersed during the day period and more aggregated during the night period. This rhythm seems to be largely dependent on an endogenous release of neurohormones from eyestalks, and to a lesser extent on a primary response to illumination. β-PDH was the most potent PDH isoform to induce pigment dispersion in both in vivo (EC_{50} = 0.4 pmol/animal) and in vitro (EC_{50} = 0.18 μM) assays. CCAP also induced pigment dispersion in vivo and in vitro assays (EC_{50} = 12 μM), but it was less potent than β-PDH. In vivo, RPCH induced a low and nondose-dependent pigment aggregation, while in vitro, it had no effect on pigment migration. The responsiveness of melanophores of *C. granulata* to β-PDH was significantly higher during the day period when compared to the night period in both assays, in vitro and in vivo. These results suggest that the endogenous circadian rhythm of black pigment migration is dependent on both endogenous circadian rhythm of β-PDH synthesis and/or release from eyestalks and on an endogenous rhythm of responsiveness of melanophores to β-PDH.

Keywords: *Chasmagnathus granulata*; CCAP; Circadian rhythm; Crab; Melanophore; PDH; Pigment migration; RPCH

1. Introduction

Many authors have verified that crustaceans, mainly fiddler crabs, show a circadian rhythm of physiological color change (Brown, 1950; Brown et al., 1953; Hines, 1954; Barnwell, 1963; Thurman, 1988). However, few studies were performed to verify if this rhythm is an intrinsic response of chromathophores, or if it is dependent on oscillators localized in other tissues. Early experiments studying the origin of diurnal rhythms of physiological color change have indicated that eyestalk ablation abolished this rhythm (Kalmus, 1938a,b). Further ablation experiments, however, rendered controversial results. Circadian rhythm of pigment migration of melanophores of the fiddler crab (*Uca pugilator*) persisted after eyestalk ablation (Webb et al., 1954; Fingerman and Yamamoto, 1967). In addition, these authors also verified that this rhythm persisted for 48 h in autotomized legs. Since those reports, no study has been developed with other crustacean species in order to clarify this point.

Physiological color change occurs by one of two basic mechanisms: a primary or a secondary response. Primary responses are pigment migration inside chromathophores independent of hormone control, generally elicited by illumination or temperature. Secondary responses occur through indirect routes involving hormones.

To date, two hormones affecting color change, released mainly from the X-organ/sinus gland neuroendocrine complex, are well known: the pigment-dispersing hormone (PDH) and the red pigment-concentrating hormone (RPCH). PDH exists in several forms in different species of crustaceans. All isoforms are octadecapeptides and exhibit pigment-dispersing activity in all types of chromatophores investigated so far. The first isoform to be characterized was the α-PDH from eyestalks of the shrimp *Pandalus*

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borealis (Fernlund, 1976), after which β-PDH was characterized in the crab *U. pugilator* (Rao et al., 1985). RPCH was initially isolated from the eyestalk of the shrimp *P. borealis* and identified due to its red pigment-aggregating activity (Fernlund and Josefsson, 1972). The RPCH is an octapeptide structurally related to the insect peptides of the adipokinetic hormone (AKH) family (Gäde, 1990), constituting a family of arthropod neuropeptides with diverse functions. Unlike PDH, RPCHs from various decapod crustaceans show identical structures (Gaus et al., 1990), suggesting that it has been conserved during evolution. Besides its concentrating effect on red pigment, RPCH has been reported to induce pigment aggregation in other types of chromatophores, such as leucophores in *P. borealis* (Josefsson, 1975) and melanophores and xanthophores in *Crangon* sp. (Skorkowski and Biegniewska, 1981), and *Penaeus japonicus* (Yang et al., 1999). However, the RPCH pigment-aggregating effect cannot be generalized to all types of crustacean chromatophores, especially melanophores. Lack of RPCH effect on melanophores was reported in the isopods *Ligia occidentalis* (Rao and Hackett, 1973) and *L. exotica* (Tuma et al., 1993) and the crabs *U. pugilator* (Fingerman, 1973) and *Uca rapax* (Tuma et al., 1993).

Despite the fact that the function of PDH and RPCH in the regulation of pigment migration has been well established, some reports suggest that other agonists may also play a role in this regulation. In the last decade, Gaus and Stive (1992) verified that crustacean cardioactive peptide (CCAP) increases the electroretinogram amplitude in *Orconectes limosus*, similarly to PDH. More recently, Nery et al. (1999) verified the capacity of CCAP to induce pigment dispersion in erythrophores of the freshwater shrimp *Macrobrachium potiuna*. However, to our knowledge, no additional investigations have been carried out to confirm such effects in other crustacean species.

In this paper, we describe the circadian rhythm of pigment migration in the melanophores of the grapsid crab *Chasmagnathus granulata* and verify the occurrence of variations in responsiveness to PDH, RPCH, and CCAP between day and night periods.

2. Materials and methods

Adult males of the crab *C. granulata*, weighing 9.83 ± 0.23 g (mean ± S.E.M.), in stage C or early D of the molting cycle, were collected in salt marshes near the city of Rio Grande (southern Brazil). The crabs were kept in tanks with salt water at 20‰ salinity, 22 ± 2 °C and photoperiod LD 12:12, for at least 7 days before the assays. During this period, the crabs were fed ground beef regularly.

Hormones RPCH and CCAP were purchased from Bachem Bioscience (USA), α-PDH from University of Kentucky (USA), the isoform $Nle^{4,15}$ α-PDH was kindly furnished by Dr. K.R. Rao (University of West Florida, USA). The hormone β-PDH was purchased from Bachem Bioscience, referred to here as β-PDHbac, and from Sigma (USA), abbreviated as β-PDHsig. The stock solutions were made in water (RPCH) and 5% acetic acid (β-PDH, α-PDH, $Nle^{4,15}$ α-PDH, and CCAP). For each solution, the final dilution was made in physiological saline, and the final concentration of the nonaqueous solvents never exceeded 1%. The physiological saline contained, in mmol l^{-1}: $MgCl_2$, 0.01; NaCl, 0.355; $CaCl_2$, 0.016; H_3BO_3, 0.005; $KHCO_3$, 0.010; $Na_3C_6H_5O_7$, 0.008; pH 7.6.

To verify the occurrence of circadian rhythm, normal (nonablated) and eyestalkless crabs maintained on white background were observed over 72 h under different photoperiods: 12:12 (LD), constant darkness (DD), and constant light (LL). Illumination was at 500 lx (cool white lamps). Crabs were ablated 24 h before the beginning of observations. For each photoperiod condition, crabs were analyzed every 2 h to verify the degree of pigment dispersion in the melanophores. Crabs were not fed during this period. Pigment dispersion was assessed using the Hogben and Slome (1931) index, which defines stage 1 as that with full pigment aggregation, stage 5 that with full dispersion, stages 2, 3, and 4 being intermediate conditions. The region chosen for observation was the meropodite of the third pair of maxillipedes, due to the fact that this region has a thin and light colored exoskeleton, thus facilitating observation.

In the in vivo assays, eyestalkless crabs were injected with different doses (0.0001 up to 300 pmol/animal) of the β-PDHbac, α-PDH, $Nle^{4,15}$ α-PDH and CCAP and physiological saline (control). On the other hand, normal crabs were injected with different doses of RPCH (0.0001 pmol/animal up to 30.0 μmol/animal) or physiological saline. With the hormones β-PDHsig and CCAP, in vivo assays were performed during the day period (between 10:00 and 16:00) and night period (between 22:00 and 03:00). The degree of pigment aggregation or dispersion was evaluated before (time zero) and 15, 30, 60, 90, 120, 150, 180, 210, and 240 min after injections. From these results, the standard integrated response (SIR), as described by Fingerman et al. (1967), was calculated in order to determine dose-response curves (DRCs).

In the in vitro assays, the meropodite of the third pair of maxillipedes was 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). This preparation received increasing concentrations of CCAP (1.0 nM up to 30 μM), β-PDHsig (1.0 nM up to 10 μM), or RPCH (10 nM up to 10 μM). With the hormone β-PDHsig, the in vitro assay was also performed during the day period (between 10:00 and 16:00) and night period (between 22:00 and 03:00). The cumulative DRCs to CCAP, β-PDHsig and RPCH were determined as follows: the physiological saline was replaced with a single agonist concentration, and the dispersion or aggregating response evaluated under the light microscope with the aid of an ocular micrometer. After a maximal response to a concen-

tration of the agonist was determined, the response was calculated as the percentage change in the apparent length of a previously chosen melanophore process. No saline rinses were done between applications of the various hormone concentrations.

The period and robustness of the rhythm was determined using Lomb–Scargle periodogram procedure (Ruf, 1999). In this procedure, rhythm robustness is expressed as the PN statistic, which reflects the strength or regularity of a rhythm independent of its amplitude. For data sets of 3 days with 2-h resolution, a perfect wave (such as a mathematically generated cosine wave) produces a PN value of 34. Robustness values above 15% maximal robustness are statistically significant at the 0.05 level, and values above 18% maximal robustness are statistically significant at the 0.01 level. For computation of the acrophase, mesor, and amplitude of the rhythm, cosine waves were fitted to the average rhythm, and the time corresponding to the peak of the best-fitting cosine wave was taken as the acrophase of rhythm (Nelson et al., 1979). Comparisons among period, mesor, amplitude, and acrophase of different photoperiods were performed based on 95% confidence intervals. The EC_{50}s (effective agonist concentration for a half-maximal response) to the hormones tested were calculated from DRCs fitted to a sigmoid curve by nonlinear regression. The DRCs were compared using a two-way ANOVA followed by the Student Newman-Keuls (SNK) test. The differences were considered significant when $p < 0.05$.

3. Results

The melanophores of *C. granulata* submitted to LD photoperiod showed a pronounced pigment migration during the experiment (Fig. 1A) with a robust daily rhythmicity of 23.9 h (Table 1). The black pigment in the melanophore is more dispersed throughout the light phase of the LD cycle and more aggregated during the dark phase. The acrophase of this rhythm was reached at 12.7 h of the day period. When the crabs were submitted to DD conditions, the circadian rhythm of black pigment migration persisted (Fig. 1B). However, there was a small increase in the mesor of the rhythm from 2.4 in LD to 2.8 in DD conditions (Table 1). The amplitude and acrophase of the rhythm was not different in LD conditions. When the crabs were submitted to LL conditions, the circadian rhythm of black pigment migration also persisted, but there was a huge increase in the mesor and acrophase (Fig. 1C and Table 1). In the first cycle of LL conditions, the rhythm was apparently altered. In the following cycles, the rhythm was more clearly reestablished, but with the black pigments staying more dispersed during the correspondent night period than the day period.

Twenty-four hours after ablation of the eyestalks, black pigments in the melanophores became more aggregated. In eyestalkless crabs maintained in LD photoperiod, a circadian rhythm with a period of 22 h persisted, but with an amplitude of 0.1 and mesor of 1.1 (Fig. 1A and Table 1). However, eyestalkless crabs maintained in LL and DD did not show circadian rhythm of black pigment migration in their melanophores. In eyestalkless crabs maintained in LL, the black pigment showed a constant oscillation, while in eyestalkless crabs maintained in DD, they were almost stabilized in stage 1 (Fig. 1B, C).

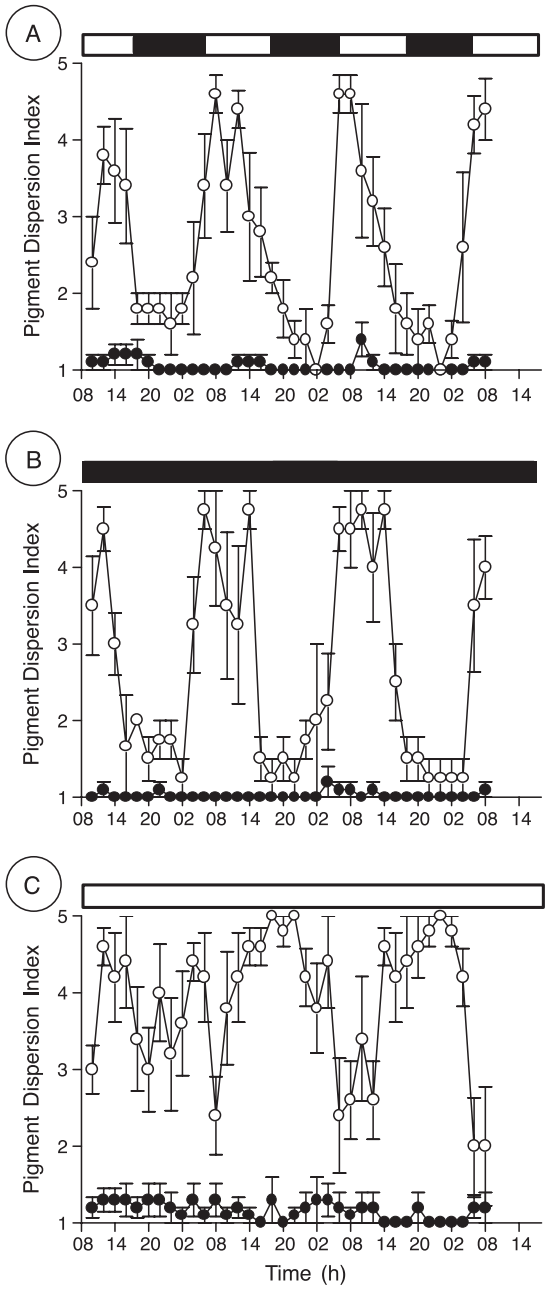

Fig. 1. Black pigment migration of melanophores of normal (nonablated; empty circle) and eyestalkless (full circle) crabs (*C. granulata*) maintained under different photoperiod conditions over 3 days. (A) LD conditions, (B) DD conditions, and (C) LL conditions. The white and black blocks at the top of each graph represent the duration of the light and dark phase of the cycle. Each point represents the mean (± S.E.M.) of pigment dispersion index at the time noted ($n = 10$).

Table 1
Rhythmic parameters of pigment migration of melanophores of normal (nonablated) crabs (*C. granulata)* submitted to 12:12 photoperiod (LD), constant darkness (DD), and constant light (LL), and eyestalkless crabs submitted to LD photoperiod (EL)

	LD	DD	LL	EL
Mesor	2.4 (2.35–2.45)	2.8 (2.43–2.97)*	3.9 (3.74–4.42)*	1.1 (1.01–1.15)*
Amplitude	1.6 (0.92–2.21)	1.7 (1.69–1.71)	1.4 (1.20–1.60)	0.1 (0.1–0.24)*
Acrophase (h)	12.7 (12.2–13.3)	11.7 (10.9–12.5)	20.2 (19.2–21.3)*	13.2 (11.9–14.5)
Period (h)	23.9	24.9	25.9	22.0
Robustness (%)	38.3	38.3	26.5	20.6

The values in the parentheses are 95% confidence intervals.
* Denotes values significantly different ($p<0.05$) of LD group.

In the in vivo assay, all PDH isoforms tested (α-PDH, β-PDH, and Nle^{4,15} α-PDH) induced pigment dispersion in a dose-dependent manner (Fig. 2). The EC_{50} (with 95% confidence interval) for α-PDH, Nle^{4,15} α-PDH and β-PDH was 11.1 (6.2–19.9), 1.5 (1.1–2.2), and 0.4 (0.2–0.9) pmol/crab, respectively. β-PDH was the most potent isoform of PDH for pigment dispersion in *C. granulata* melanophores ($p<0.05$). When β-PDH was injected into the crabs during the night period, the DRC was significantly ($p<0.05$) shifted to the right (approximately twofold; Fig. 3). In the in vitro assay, the DRC for β-PDH in experiments performed during the night was also significantly ($p<0.05$) shifted to the right (approximately 180-fold; Fig. 4). The EC_{50} (with 95% confidence interval) for β-PDH during day and night periods was 1.54 (0.81–2.92) nM and 0.28 (0.12–0.66) μM, respectively.

The neuropeptide CCAP also induced pigment dispersion in *C. granulata* melanophores. Similar to β-PDH, the maximal dispersion response occurred after 60 min, returning to their initial stage 210 min after cessation of dispersing stimulus (Fig. 5). However, comparing the CCAP-induced pigment dispersion to that obtained with β-PDH, it can be observed that CCAP is significantly ($p<0.05$) less potent than β-PDH as dispersing agonist (Fig. 6). There was no significant ($p>0.05$) difference between the DRC for CCAP in experiments performed during the day and night. In the in vitro assay, CCAP also induced pigment dispersion in a dose-dependent manner (Fig. 7). The EC_{50} of CCAP in the in vivo and in vitro assays was not calculated because maximal pigment dispersion was not achieved.

The RPCH, up to 30 μmol/animal, induced only a small pigment aggregation in *C. granulata* melanophores in vivo, and in addition, RPCH did not induce any pigment aggregation in the in vitro assay in either period (data not shown).

4. Discussion

The black pigment in the melanophores of *C. granulata* shows a circadian rhythm of pigment migration. The existence of a circadian rhythm in the black pigment migration in melanophores of crustaceans was established many years ago. However, concerning crabs, the study of circadian rhythm has been almost restricted to the genus *Uca* (for review, see Thurman, 1988). Besides *Uca* species, the circadian rhythm of black pigment migration was also described in *Callinectes sapidus* (Fingerman, 1955), *Carcinus maenas* (Powell, 1962a,b, 1966), and *Cancer magister* (Shibley, 1968). In the latter, this rhythm was also not persistent under constant conditions, as observed in *Uca thayeri* and *Uca subcylindrica* (Barnwell, 1963; Thurman, 1990). The circadian rhythm of black pigment migration in melanophores of *C. granulata* was persistent under both

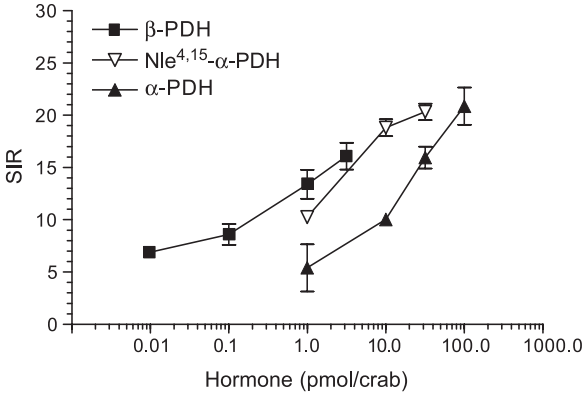

Fig. 2. In vivo dose-response curves for α-PDH, Nle^{4,15} α-PDH, and β-PDH of melanophores of the crab *C. granulata*. Each point represents the mean (± S.E.M.) dispersing response, measured as standard integrated response (SIR), at the concentration noted ($n=15$).

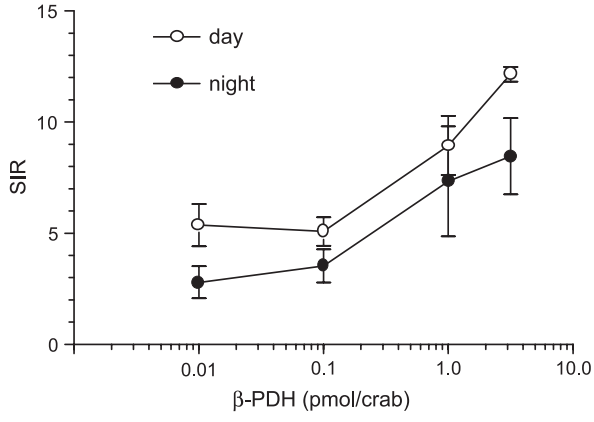

Fig. 3. In vivo dose-response curve for β-PDH of melanophores of the crab *C. granulata* in experiments performed during the day and night. Each point represents the mean (± S.E.M.) dispersing response, measured as standard integrated response (SIR), at the concentration noted ($n=15$).

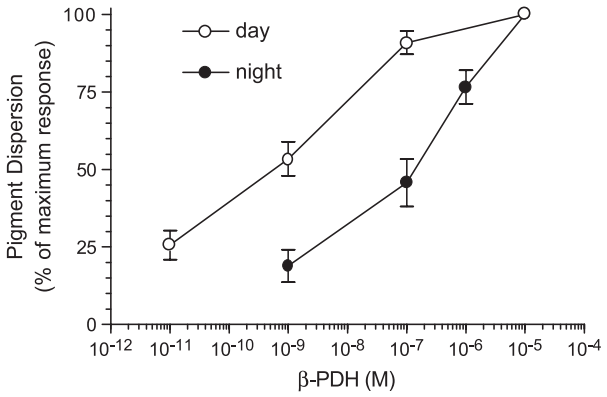

Fig. 4. In vitro dose-response curve for β-PDH of melanophores of the crab *C. granulata* in experiments performed during the day and night. Each point represents the mean (± S.E.M.) dispersing response at the concentration noted ($n = 6$).

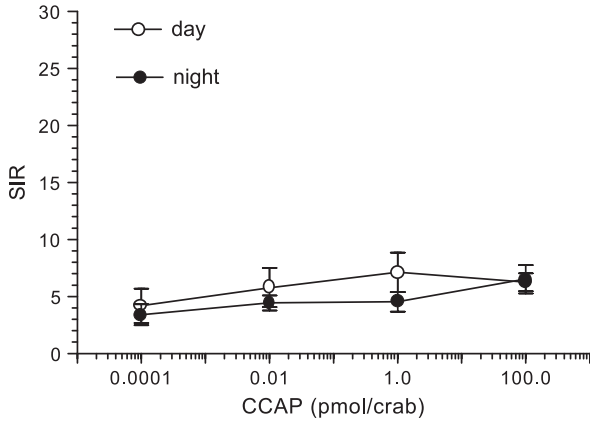

Fig. 6. In vivo dose-response curve for CCAP of melanophores of the crab *C. granulata* in experiments performed during the day and night. Each point represents the mean (± S.E.M.) dispersing response, measured as standard integrated response (SIR) to the concentration noted ($n = 15$).

constant conditions and is therefore considered endogenous, as it is in the majority of crabs.

The origin of endogenous rhythms in several physiological functions has been attributed to cyclic changes in synthesis and/or release of neurohormones from the X-organ/sinus gland complex in the eyestalk. In fact, the early experiments of Kalmus (1938a,b) with the crayfish *Potamobius astacus* demonstrated that after bilateral eyestalk ablation, the circadian rhythm of pigment movements in the chromatophores was abolished. However, further experiments rendered controversial results. Webb et al. (1954) and Fingerman and Yamamoto (1967) showed the persistence of pigment migration rhythm in eyestalkless *U. pugilator*, but Powell (1966) reported loss of circadian rhythm in *C. maenas*. In the crab *C. granulata*, when the eyestalks were removed, the rhythm of black pigment migration in animals maintained under LD conditions persisted, but it was lost in animals maintained in DD or LL. These results point out that the endogenous rhythmicity is at least dependent on neurohormones released from the X-organ/sinus gland neuroendocrine complex.

To nonablated crabs maintained under LL conditions, the mesor, which is the midline of an estimated statistic of rhythm, increased, probably due to the black pigments scarcely remaining below stage 2 of the pigment dispersion index. This fact suggests that illumination can induce black pigment dispersion, partially overriding the nocturnal pigment aggregation. In this sense, the rhythm of low amplitude of eyestalkless crabs maintained under LD, and the absence of this rhythm in eyestalkless crabs maintained under DD and LL, can be due also to the presence of light, indicating that melanophores of *C. granulata* possess a primary response to illumination besides a secondary response mediated by neurohormones from the eyestalks.

Although circadian variation in the eyestalk content of neurohormones and neurotransmitters involved in the regulation of pigment migration in crustaceans have been described (Rodriguez-Sosa et al., 1994; Castanon-Cervantes et al., 1999), no study has been made to verify if concurrent changes in the responsiveness of chromatophores also occur.

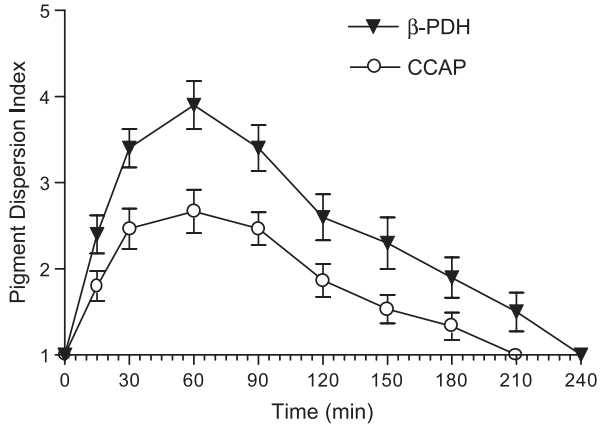

Fig. 5. Time-course dispersing response of melanophores of the crab *C. granulata* to β-PDH and CCAP (1.0 pmol/animal), as determined in in vivo assays. Each point represents the mean (± S.E.M.) of pigment dispersion index at the time noted ($n = 15$).

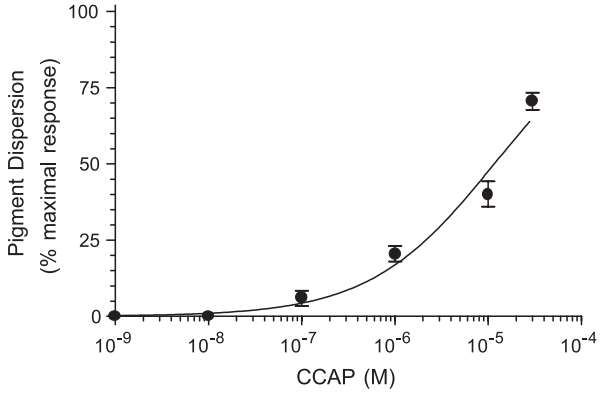

Fig. 7. In vitro dose-response curve for CCAP of melanophores of the crab *C. granulata*. Each point represents the mean (± S.E.M.) dispersing response at the concentration noted ($n = 4$).

Our next step was thus to investigate which hormones could be involved in the induction of black pigment migration in *C. granulata* and if melanophores have a circadian variation of response to these hormones. As previously mentioned, three hormones have been identified to regulate the pigment migration in Crustacea: PDH, RPCH, and CCAP. The PDHs of crustaceans have been grouped into two subfamilies: α-PDH and β-PDH. β-PDH is considered the major isoform of PDH in crabs (Rao, 2001). Rao et al. (1985) reported that β-PDH was about 20 times more potent than α-PDH when tested on melanophores of *U. pugilator*, and that in this crab β-PDH was the only isoform isolated and identified. Although α-PDH has been isolated and identified only in pandalid species, Britto et al. (1990) and Tuma et al. (1993) working with erythrophores of *M. potiuna* and *Macrobrachium acanturus*, respectively, observed that α-PDH was more potent than β-PDH. In addition, Riehm and Rao (1987) working with synthetic isoforms of α-PDH verified that the $Nle^{4,15}$ α-PDH was more potent than α-PDH in those species where β-PDH had been demonstrated to occur. Nery et al. (1999) also verified that α-PDH is more potent than $Nle^{4,15}$ α-PDH in *M. potiuna* erythrophores. Results from this study indicate that the main dispersing hormone in *C. granulata* is probably an isoform of β-PDH, because this isoform was about 14 times more potent than $Nle^{4,15}$ α-PDH and 27 times more potent than α-PDH. In addition, the responsiveness of melanophores of *C. granulata* to β-PDH is significantly different in both in vitro and in vivo assays when comparing day and night periods. This fact suggests that the endogenous circadian rhythm of black pigment migration is dependent on both the endogenous circadian rhythm of β-PDH synthesis and/or release from eyestalks and the endogenous rhythm of responsiveness of melanophores to β-PDH. Variation in chromatophore responsiveness associated with chromatophorotropin content in the eyestalk has also been verified in the crayfish *Procambarus clarkii* (Rodriguez-Sosa et al., 1997). The responsiveness of *P. clarkii* erythrophores to RPCH and the RPCH content in the eyestalk were smaller during the winter months.

Besides PDH, crustacean cardioactive peptide (CCAP) could be another agonist for pigment migration. Nery et al. (1999), studying the possible effect of CCAP in pigment translocation in the erythrophores of the freshwater shrimp *M. potiuna*, has also shown that CCAP induces pigment dispersion. However, our results show that β-PDH is approximately 100 times more potent than CCAP. Similar potency difference was also observed in *M. potiuna* erythrophores (Nery et al., 1999). CCAP circulating levels are unknown, but the concentrations (10^{-7} up to 3.0×10^{-5} M) necessary to induce pigment dispersion in *C. granulata* melanophores were supposedly above those expected for a blood-borne agonist. However, it is possible that lower concentrations alter the dispersing response to PDH. In fact, 10^{-8} and 10^{-7} M CCAP effectively decrease the aggregating response to RPCH in *M. potiuna* erythrophores (Nery et al., 1999). Therefore, although the expected blood concentration could not induce per se pigment migration, it may be sufficient to modulate the melanophores sensitivity to classical chromatophorotropins.

In conclusion, melanophores of *C. granulata* have an endogenous circadian rhythm of pigment migration, but also have a primary response to illumination, which could amplify the black pigment migration. In addition, this rhythm is dependent in part on a variation in responsiveness to β-PDH besides a probable variation in the PDH content of the eyestalk.

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