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Aspects of Entrainment of CHH Cell Activity and Hemolymph Glucose Levels in Crayfish*

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Abstract. We investigated the effects of several experimental conditions, such as constant darkness, light/dark phase-shift, covered eyes, eyestalks and rostral regions, and optic tract sectioning, on the entrainment of daily blood glucose rhythmicity in the crayfish. Hemolymph glucose determination over a 24 h period and a morphometrical study on the secretory activity of the Crustacean Hyperglycemic Hormone (CHH)-producing cells in the eyestalk using immunocytochemistry were carried out. Our results indicate that *Astacus leptodactylus* exhibits an endogenous circadian blood glucose rhythm entrained by the light/dark schedule.

The light stimuli that control the rhythm are not detected by the compound eyes nor by the caudal photoreceptor but most probably by a photoreceptor located elsewhere in the eyestalk. After disruption of the neural connection between the optic lobes and the cerebral ganglion, blood glucose rhythmicity persists, which indicates that the biological clock of the blood glucose rhythm is located within the optic lobes.

Introduction

The Crustacean Hyperglycemic Hormone (CHH)-producing system of the crayfish *Astacus leptodactylus* consists of a number of neurosecretory perikarya located on the rostral latero-ventral side of the medulla terminalis ganglionic X-organ (MTGX). The axons of these cells pass through the medulla terminalis (MT) and terminate in the sinus gland. The location and morphology of this cell system has been described in detail (for *Astacus leptodactylus* see Van Herp and Van Buggenum, 1979; Gorgels-Kallen and Van Herp, 1981; Gorgels-Kallen and Voorter, 1984; for other decapod species see Jaros and Keller, 1979; Gorgels-Kallen *et al.*, 1982; Van Herp *et al.*, 1984).

Under constant light/dark conditions, the CHH cell system of *Astacus leptodactylus* reveals a daily rhythmicity in the synthetic activity of the perikarya, transport of CHH-material to the sinus gland, and release of CHH into the hemolymph which results in a 24 h rhythm of blood glucose level (Gorgels-Kallen and Voorter, 1985). Similar results are described for the prawn *Palaemon serratus* (Van Herp *et al.*, 1984). Diurnal rhythmicity of hemolymph glucose content is also described for the crayfish *Orconectes limosus* (Hamann, 1974) and for the freshwater field crab *Oziotelphusa senex senex* (Reddy *et al.*, 1981). Hamann (1974) examined the day/night rhythmicity of blood glucose content of *Orconectes limosus* under various conditions. His findings signified the importance of light signals as entraining stimuli to maintain blood glucose rhythmicity and indicated the presence of an endogenous pacemaker. Furthermore, we recently described the presence of synaptic input on the ramifications of CHH axons in the MT neuropileum (Gorgels-Kallen, 1985). All the above mentioned findings point to the presence of a system controlling the entrainment of CHH metabolism.

The present work was undertaken in an effort to obtain additional information on the role of the prevailing environmental light/dark conditions in the entrainment of daily rhythmicity of CHH cell activity and, as a consequence, the hemolymph glucose level in *Astacus leptodactylus*. Based on Hamann's (1974) experiments, the daily blood glucose rhythm was examined under various conditions in order to study its exogenous or endogenous character and to learn more about the location of photo-

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receptor(s) and oscillator(s) involved in modulation. Furthermore, since we are immunocytochemically able to determine the secretory activity of individual CHH cells (Gorgels-Kallen and Voorter, 1984, 1985), we investigated the effect of the experimental conditions on the cellular activity of the CHH cell system.

Materials and Methods

Animals and blood sampling

Crayfish of the species *Astacus leptodactylus* (Nordmann), were imported from Turkey via a commercial dealer and kept in the laboratory in running tap water (13–15°C), and fed weekly with pieces of meat or fish. Except when otherwise stated, animals were kept under light/dark conditions (LD 12:12, light on 8.00 am). Experiments were performed with adult female and male crayfish of equal size and in intermolt stage. Blood samples, obtained from 5 to 10 animals per sample time, were collected over a 24 h period at regular intervals. 100 μl of hemolymph was drawn into a calibrated capillary pipet which was inserted between the coxa and the basis of the left cheliped. Samples were taken in duplicate from each animal and frozen immediately. The blood glucose level was determined using the Gluco-Quant Test Combination (Boehringer Mannheim GmbH).

Experimental conditions

Constant darkness. Crayfish were kept in constant darkness (code DD). Blood samples were taken at the start of the experiment and 6, 11, and 35 days after the onset of DD conditions.

Phase-shift. Crayfish kept under normal LD conditions were exposed to a 12 h phase-shift (code PS) accomplished by lengthening the light period with 12 hours at the onset of the experiment. Hemolymph samples were taken at the start of the experiment and 6, 12, and 18 days after introduction of the phase-shift.

Covered retinas and rostral regions. Several experiments were performed covering different regions of the eyestalk and the rostrum. Either both retinas (code RR), both eyestalks (code EE), or the rostral cephalic region including the eyestalks (code CR) were painted using a black textile dye (Silka; Talens, Apeldoorn, The Netherlands) which formed a completely opaque, water-resistant coverage. Before painting blood samples were taken. The experimental conditions were maintained for 35 days, then hemolymph was again sampled.

Optic tract operation. Between the hard exoskeleton of the eyestalk and rostrum there is a softer region enabling articulation of the eyestalk. In order to disrupt the neural connection between the optic ganglia and CHH cell system with the cerebral ganglion, a fine pointed cauterization needle or a microdissection scalpel was pushed through the soft region to section the optic tract locally (code NO). Blood samples were taken before the start of the experiment and 35 days after operation. At the end of the experiment, crayfish were sacrificed and the eyestalks were microscopically examined to check the success of the optic tract section, the condition of the isolated ganglia and the normal course of the blood circulation.

Light microscopy

From animals kept under the above described experimental and control conditions, eyestalks were ablated at 12.00 am and fixed in Bouin Hollande fluid (24 h) containing 10% of a saturated aqueous solution of sublimate. The fixed material was embedded in Paraplast (57°C). The immunocytochemical staining was based on the peroxidase-antiperoxidase (PAP)-method (Sternberger, 1974) and was performed on 7 μm sections with an overnight incubation at 4°C for the anti-CHH. The primary antiserum was raised in rabbits against a purified hyperglycemic fraction derived from eyestalks of *Astacus leptodactylus* (for further details of the purification of the antigen and the production of the antiserum see Gorgels-Kallen and Van Herp, 1981). The procedure of the immunostaining followed Van Herp and Van Buggenum (1979), with the primary antiserum applied in a dilution series. For each experiment the optimal dilution of the primary antiserum proved to be 1/150, which conforms to the optimal dilution used for eyestalks from animals kept under normal LD and laboratory conditions. The specificity of the immunostaining was tested as described previously (Gorgels-Kallen and Van Herp, 1981).

The secretory cell stages of individual CHH cells were determined on the basis of the observed differences in staining intensity which enables the arbitrary division of the immunoreactive cells into three categories: +, ++, and +++, representing the cells with least intense, moderately intense, and most intense immunostaining, respectively. In a previous study, morphometric analyses of the cells at both the light and electron microscopic levels led to the following characterization of the three cell stages: + cells show the weakest immunoreaction which is correlated to the low content of CHH granules in their cytoplasm. The cells possess a large nucleus which points to a high level of mRNA production. ++ Cells show an intermediate immunoreaction. In their cytoplasm the content of granules is increased. These cells possess the largest nuclear and cytoplasmic volumes, which indicate high synthetic activity. +++ Cells show an intense immunoreaction which is correlated with the largest numerical density of CHH granules in their cytoplasm. The synthetic level of these cells is low as documented by

their small cytoplasmic and nuclear volumes. Moreover, the CHH granules show a lower electron density and an increased diameter as compared with those of + and ++ cells. These facts point to the occurrence of a maturation process, indicating that the secretory granules are youngest in the + cells and oldest in the +++ cells, which indicates different degrees of synthetic activity of the three distinguished cell stages. For a detailed description of the morphology and the results of a morphometric study of the CHH cell stages we refer to our previous study (Gorgels-Kallen and Voorter, 1984) and to our study on the secretory dynamics of the CHH cells in the course of the day/night cycle (Gorgels-Kallen and Voorter, 1985). The numbers of +, ++, and +++ cells were counted in the left eyestalks from three specimens per experimental or control condition. In addition, the cytoplasmic and nuclear volumes of five of each of the +, ++, and +++ cells, per eyestalk, were calculated according to Weibel's (1979) method and as described in detail previously (for illustration see Fig. 3; Gorgels-Kallen and Voorter, 1984, 1985).

Results

Hemolymph glucose rhythmicity

Control experiments. Figure 1a represents the results of the glucose determination in the hemolymph for all specimens used in the DD and PS experiment, determined during normal 12 h light and 12 h dark conditions. The blood glucose values show the usual rhythmicity: around 4 hours after the onset of the dark period the glucose level doubles compared to the level found in the daytime.

Constant darkness. Figures 1b–d show the blood glucose levels over a 24 h period, measured after 6 (Fig. 1b), 11 (Fig. 1c), and 35 days (Fig. 1d) under constant darkness conditions. After 6 days absence of the light stimulus, the circadian hemolymph glucose pattern still persists, although the maximum decreases around midnight. After 11 days the normal rhythm is tempered: yet, at midnight an increased variation of the mean glucose level can still be seen. Constant darkness for 35 days results in total absence of any daily rhythm. Furthermore, the data show that removing the periodic changes in light intensity leads to consistently low glucose levels during the whole 24 h period.

Phase-shift. Figures 1e–g show the hemolymph glucose values over a 24 h period measured 6 (Fig. 1e), 12 (Fig. 1f), and 18 days (Fig. 1g) after the introduction of a 12 h shift in the normal LD pattern, by lengthening one light period by 12 hours. Six days after the phase-shift blood glucose rhythmicity is disturbed. The graph shows three zones with an increase followed by a decrease in blood glucose content and increased variation in the

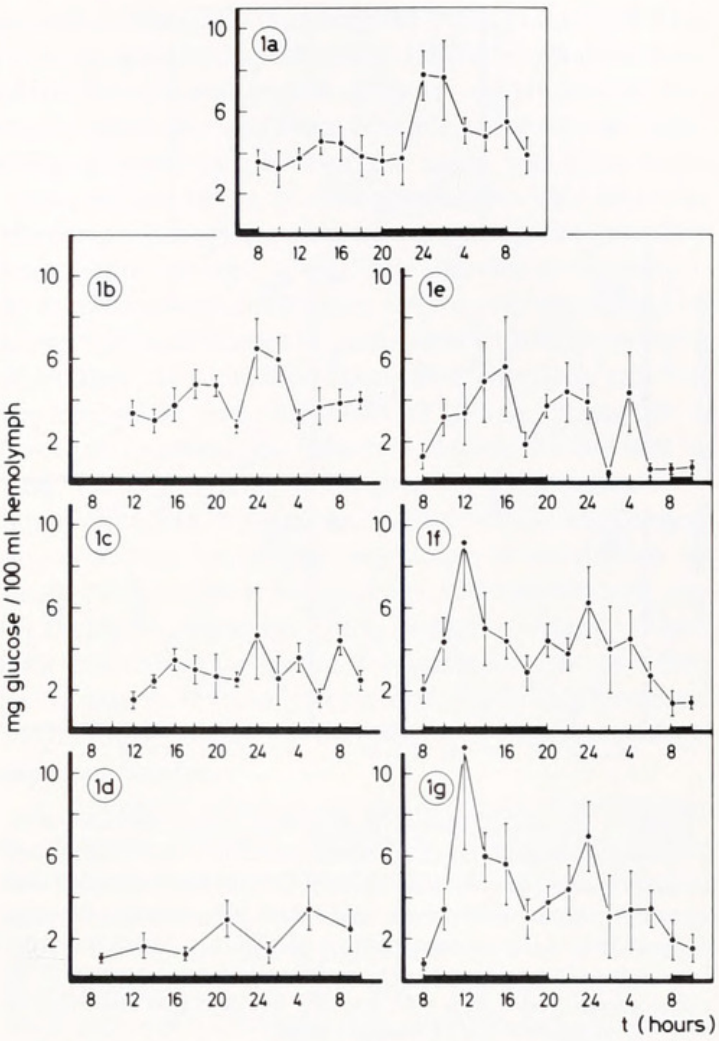

Figure 1. (a–g) Daily hemolymph glucose pattern: (a) under normal light/dark conditions (LD 12:12; n = 10); (b) after 6 days constant darkness (n = 5); (c) after 11 days constant darkness (n = 5); (d) after 35 days constant darkness (n = 5); (e) six days after a 12 h phase shift (n = 5); (f) twelve days after a 12 h phase-shift (n = 5); (g) eighteen days after a 12 h phase-shift (n = 5). Means ± SEM.

mean glucose values for most sample points. After 12 days the normal circadian blood glucose pattern reappears; the maximum in glucose level is found at 12 a.m., 4 hours after the onset of the "new" dark period. However, during the light period at 12 pm a second smaller peak is seen. This glucose pattern persists and even becomes more pronounced 18 days after the start of the experiment. At 12 am, the new midnight, the mean glucose level increases firmly whereas at 12 pm, the former midnight, a second smaller, although distinct rise in blood glucose content is found.

Painted eyestalks and rostral regions. Prevention of light perception via the ommatidia (light percepting units forming the compound eye) does not abolish circadian rhythmicity in the hemolymph glucose content (Fig. 2b). During the dark period the glucose level still doubles. The amplitude and mean glucose levels during the total 24 h period are closely comparable to the con-

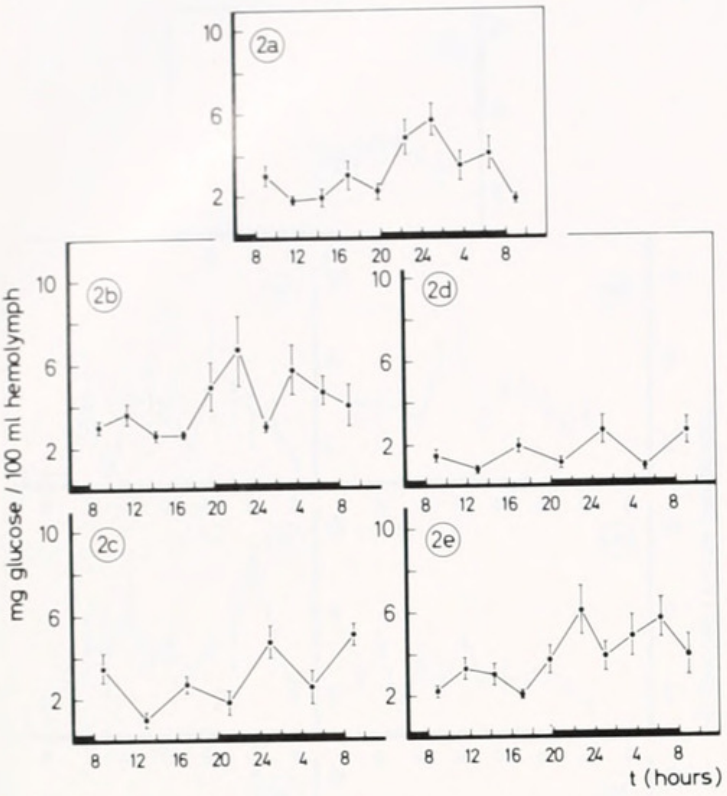

Figure 2. (a–e) Daily hemolymph glucose pattern after several experimental conditions: (a) under normal light/dark conditions before the start of the experiment (LD 12:12; n = 5); (b) thirty-five days after covering of the compound eyes (code RR; n = 5); (c) thirty-five days after covering of the whole eyestalks (code EE; n = 5); (d) thirty-five days after covering of the whole eyestalks and the rostral region of the cephalothorax (code CR; n = 5); (e) thirty-five days after section of the optic tract (code NO; n = 5). Means ± SEM.

trol values obtained from these specimens before starting the experiments (Fig. 2a). Yet, some influence must be noticed. The nocturnal rise in glucose is found immediately at the start of the dark period and the mean values show increased variation. After painting the whole eyestalks, an increased glucose level during the dark period is still seen, although the amplitude is very reduced (Fig. 2c). Covering the whole eyestalks including the rostral part of the cephalothorax leads to a complete absence of circadian blood glucose rhythmicity and during the whole 24 h period the glucose levels consistently stay very low (Fig. 2d).

Optic tract operation. Disruption of the connection between the eyestalk and the cerebral ganglion by sectioning the optic nerve does not interfere with the persistance of a daily rhythmicity in hemolymph glucose content (Fig. 2e).

Secretory activity of the CHH cells

We investigated the effect of a change in environmental conditions on the secretory activity of the CHH cells. Animals that exhibit both a change (DD experiment), and no change (RR experiment) in hemolymph glucose rhythmicity were used. The results of this morphometric investigation are presented in Table I. Animals kept in constant darkness have a ratio between the number of +, ++, and +++ cells which differs from the ratio found for crayfish kept under control conditions. The number of +++ cells increases accompanied by a decrease in the number of ++ cells. The morphometric data reveal an increase in the cellular volume of the +++ cells. Immunocytochemical staining of the eyestalks of the RR animals reveals that both the proportion of +, ++, and +++ cells, and their cellular and nuclear volumes, are similar to those of the control animals. Disruption of the optic nerve produces a ratio of the +, ++, and +++ cells different to the ratio found for control animals. The number of + cells increases firmly, accompanied by a decrease in ++ cells. The morphometric results reveal increased cellular and nuclear volumes of the + cells. Furthermore, the overall conditions of these operated eyestalks were normal: blood circulation was not affected and no signs of degenerating eyestalk tissues were observed, nor regeneration of the sectioned optic nerve.

Discussion

Blood glucose levels found in *Astacus leptodactylus* after exposing the animals to constant dark conditions reveal that the normal 24 h rhythm persists for many cycles. The rise in blood glucose content around midnight remains clearly distinguishable after 6 days of constant darkness; after 11 days the rhythm is tempered, but the high variation in the mean glucose content at 12 pm still indicates a masked presence of the nocturnal peak. Introduction of a 12 h phase-shift results in a very slow adaptation of the 24 h rhythm to the newly imposed light schedule. Six days after the onset of the phase-shift, the normal blood glucose rhythm is still disturbed. This disturbance is expressed as: (a) a high variation in the mean glucose levels of most sample points, and (b) three periods with an increase followed by a decrease. This result corresponds to the description of the so-called transient phase, *i.e.*, a temporary loss of synchrony among the various units involved in a particular circadian rhythm, which results in frequency beats modulating the free-running period (Pavlidis, 1973). Twelve days after introduction of the phase-shift, the normal circadian blood glucose rhythm is restored, adapted to the new light/dark scheme, *i.e.*, around 4 hours after the onset of the dark period a firm blood glucose peak is detected. However, even 18 days after the phase-shift the "old" glucose peak still can be seen.

In his introduction of the symposium "Biological Clocks" (Cold Spring Harbor, 1960) Aschoff underlines the importance of the establishment of the free-running

Table I

Morphometric data on the CHH-producing cells of the crayfish, Astacus leptodactylus, *determined after control and experimental conditions. Mean (±SEM)*

Experimental condition	Cell stage + Number of cells/eyestalk	+ Cellular volume ($\times 10^3\ \mu m^3$)	+ Nuclear volume ($\times 10^3\ \mu m^3$)	++ Number of cells/eyestalk	++ Cellular volume ($\times 10^3\ \mu m^3$)	++ Nuclear volume ($\times 10^3\ \mu m^3$)	+++ Number of cells/eyestalk	+++ Cellular volume ($\times 10^3\ \mu m^3$)	+++ Nuclear volume ($\times 10^3\ \mu m^3$)
Control	9 (±2)	30.7 (±3)	5.2 (±0.4)	19 (±1)	35.3 (±3)	6 (±0.6)	6 (±1)	22.8 (±2)	3.3 (±0.2)
	(n1 = 3)	(n2 = 15)	(n2 = 15)	(n1 = 3)	(n2 = 15)	(n2 = 15)	(n1 = 3)	(n2 = 15)	(n2 = 15)
DD	11 (±2)	34.2 (±3)	4.5 (±0.3)	13 (±1)[s]	38.4 (±2)	5.2 (±0.7)	13 (±2)[s]	28.3 (±1)[s]	4 (±0.2)[s]
	(n1 = 5)	(n2 = 25)	(n2 = 25)	(n1 = 5)	(n2 = 25)	(n2 = 25)	(n1 = 5)	(n2 = 25)	(n2 = 25)
RR	10 (±2)	32.3 (±2)	5.6 (±0.5)	19 (±3)	39.2 (±3)	5.7 (±0.4)	8 (±2)	22.4 (±2)	3.4 (±0.3)
	(n1 = 3)	(n2 = 15)	(n2 = 15)	(n1 = 3)	(n2 = 15)	(n2 = 15)	(n1 = 3)	(n2 = 15)	(n2 = 15)
NO	21 (±4)[s]	42 (±4)[s]	4.2 (±0.4)[s]	6 (±2)[s]	32.2 (±2)	4 (±0.3)[s]	8 (±2)	24.4 (±2)	3.3 (±1)
	(n1 = 5)	(n2 = 25)	(n2 = 25)	(n1 = 5)	(n2 = 25)	(n2 = 25)	(n1 = 5)	(n2 = 25)	(n2 = 25)

Control: Results obtained from eyestalks of crayfish kept under normal light/dark schedule (LD 12:12).
DD: Results obtained from eyestalks of crayfish kept under constant darkness for 35 days.
RR:Results obtained from eyestalks of crayfish kept under normal light/dark schedule, however, with covered compound eyes for 35 days.
NO: Results obtained from eyestalks of crayfish kept under normal light/dark schedule and 35 days after disruption of the connection between the cerebral and optic ganglia.
n1 = number of measured eyestalks from different animals.
n2 = number of cells counted: 5 cells per cell stage per eyestalk.
s = significantly different from control data; Student's *t*-test, $P < 0.05$.

period of individual organisms in order to establish a periodicity as an endogenous one. However, establishment of the free-running period of the blood glucose rhythm in crayfish in this way is not possible, since repeated sampling of hemolymph from the same specimen leads, within several hours, to hyperglycemia caused by stress. Hamann's (1974) method of measuring the circadian hemolymph glucose levels from individual crayfish via an extracorporeal circulatory system only succeeded for a few cycles and cannot be performed with large numbers of animals. As such, blood glucose rhythmicity can only be depicted by sampling different groups of crayfish as described in this paper. However, despite the fact that we were not able to determine one of the major characteristics of circadian rhythm, we believe that the above mentioned results, *i.e.,* (a) the persistance of the rhythm for many cycles without external periodic light stimuli, and (b) the slow adaptation of the rhythm to changed light/dark conditions, allow us to postulate that the daily blood glucose level in *Astacus leptodactylus* is generated within the organism and therefore may be called endogenous or circadian.

Furthermore, since prolonged exposure of the animals to constant darkness eliminates hemolymph glucose rhythmicity and changing the external light stimuli produces entrainment, our data show the final indispensability of the prevailing light/dark cycle as a Zeitgeber in entrainment of the daily blood glucose rhythm. The importance of an external light stimulus can also be seen from the results on the determination of the secretory activity of the CHH cells after 35 days DD conditions: an increased number of enlarged +++ cells and a decreased number of actively producing ++ cells is found. These data point to a reduced production of CHH and increased storage in the perikarya.

Covering both retinas does not abolish reception of a light entraining signal, as blood glucose rhythmicity still persists. This is also supported by the results of the determination of secretory activity of the CHH cells: the resultant data are closely comparable to those of control animals. Painting both eyestalks results in a very faint nocturnal blood glucose increase and covering both eyestalks and the rostral region of the cephalothorax leads to a complete disappearance of rhythmicity and a consistently low glucose level during the whole 24 h period: the resulting hemolymph glucose graph is closely comparable to the graph obtained after constant DD conditions. Comparable experiments with the crayfish *Orconectes limosus,* performed by Hamann (1974), show the same blood glucose patterns. Therefore it appears that the eyestalks (optic lobes) have a dominant role in perception of light, but the presence of an involved photoreceptor in the rostral region of the cephalothorax cannot be excluded. The effect of disruption of the optic tract further

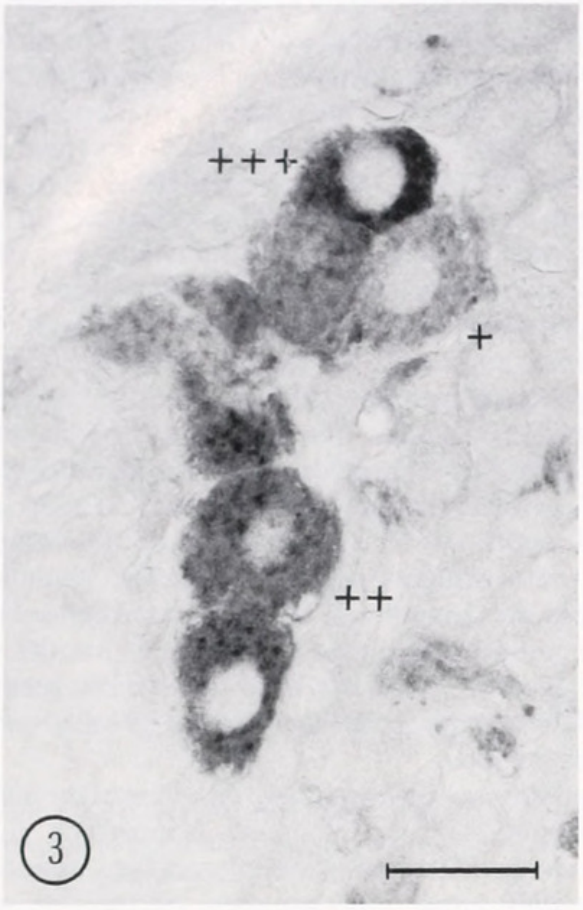

Figure 3. PAP-staining of CHH-producing cells in the MTGX, illustrating +, ++, and +++ cells. Bar represents 50 nm. + cells: mean cell volume 32.5×10^3 μm^3, mean nuclear volume 5.5×10^3 μm^3; ++ cells: mean cell volume 38.8×10^3 μm^3, mean nuclear volume 5.6×10^3 μm^3; +++ cells: mean cell volume 27.8×10^3 μm^3, mean nuclear volume 4.1×10^3 μm^3. (Morphometrical data from Gorgels-Kallen and Voorter, 1984).

supports the importance of the entraining function of the eyestalk: daily rhythmicity of the hemolymph glucose level is not affected after disturbance of the neural connection between the cerebral and optic ganglia. This result points to the presence of a pacemaker or biological clock of the glucose rhythm located in the optic lobes, although hormonal modulation from a pacemaker located elsewhere cannot be ruled out. Indeed, such hormonal influence is not disturbed by the optic tract operation, since microscopic investigation of these eyestalks did not reveal any irregularities concerning blood circulation or the condition of eyestalk structures. However, the secretory activity of the CHH cells of these NO animals does not show the same picture as found in control animals. Yet, as the blood glucose rhythmicity is comparable to control animals, it could be that CHH release is modulated by an oscillator located in the eyestalk. However, that regulation of CHH synthesis is more complex and (also) affected by a pacemaker located elsewhere. Another possibility might be that the CHH cells not only produce a hyperglycemic factor but also other hormonally active substances. Synthesis of hyperglycemic hormone might be affected by optic nerve section, while the synthesis of other hormones might go on undisturbed. Such an effect can be visualized immunocytochemically. Our results exclude any regulatory effect caused by the caudal photoreceptor described for the first time in crayfish by Prosser (1934).

Our data are supported by the work of Page and Larimer (1972), who studied the entrainment of the circadian locomotor activity in the crayfish *Procambarus clarkii*. They found that removal of the caudal photoreceptor, removal of the ommatidia of both eyes, or removal of both the ommatidia and the lamina ganglionaris, did not effect entrainment of rhythmicity in locomotion. Comparable results were also obtained by Pollard and Larimer (1977) regarding circadian rhythmicity of the heart rate in *Procambarus clarkii*. Page and Larimer (1976) demonstrated the existence of a photoreceptor in the brain in the same species. In contrast with these findings are data by Glantz *et al.* (1983), who intracellularly recorded the electrical activity of neurosecretory cells in the eyestalk induced by illumination of retinal fields. Fuentes-Pardo and Inclán-Rubio (1987) recently described the participation of the caudal photoreceptor in synchronizing the circadian locomotor rhythm in *Procambarus bouvieri*. Williams (1985) proposed, after his evaluation of the impact of optic tract section on the locomotor activity of the shore crab *Carcinus maenas*, the presence of a presumptive neural clock in the cerebral ganglion involved in regulation of locomotor rhythm.

In conclusion, we postulate that *Astacus leptodactylus* exhibits an endogenous circadian hemolymph glucose rhythm entrained by the prevailing light/dark schedule. Neither the retinas nor the caudal photoreceptor represent the main modulating receptors for blood glucose level and synthetic activity of the CHH cells. The present results indicate that the eyestalks possess the major receptors for the entraining light stimulus and also contain the oscillatory center for the blood glucose rhythm.

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