

NONPARAMETRIC EFFECTS OF MONOCHROMATIC LIGHT ON THE ACTIVITY RHYTHM OF JUVENILE CRAYFISH

Manuel Miranda-Anaya and Maria Luisa Fanjul-Moles*

L. Neurofisiología Comparada, Ap. Postal 70-371, Coyoacán,
C. P. 04510, D. F. Mexico

ABSTRACT

The current study was carried out to test the influence of blue and red monochromatic light upon the motor activity rhythm of juvenile crayfish as well as determine whether this effect involves extraretinal photoreception. Two groups of 46 juvenile instars were used: (1) intact control animals and (2) animals lacking retina and lamina ganglionaris. All animals were individually monitored with a motor activity recording system for 30 days. For the first 10 days the animals were maintained and kept in constant darkness (DD) and then submitted to 24-h skeleton photoperiod cycles (SP) consisting of 30 min red or blue light signals calibrated to the same irradiance (25 Wm^{-2}) during the next 10 days. Afterwards, they were left in DD for the last 10 days of the experiment. Activity was quantitatively and qualitatively analyzed. Results show that all control intact animals synchronized to blue or red light exhibited shift advances or delays. These results indicate that both circadian responses to monochromatic light investigated in this study are mediated by extraretinal photoreceptors. (*Chronobiology International*, 14(1), 25–34, 1997)

Key Words: Circadian rhythm—Crayfish—Locomotor activity—Skeleton photoperiod—Monochromatic light.

Submitted July 17, 1996; returned for revision August 27, 1996; accepted September 26, 1996.

*To whom all correspondence should be sent.

INTRODUCTION

Light has been recognized as the primary entraining agent for circadian rhythms, but relatively few studies have examined the effect of its spectral composition on circadian processes (1). In some uncel organisms as well as invertebrates a detailed action spectrum for phase advances and delays has been measured (2–4). In crayfish, the identity of photoreceptors that transduce light input to the circadian pacemaker has not been elucidated. Some authors have proposed that photoreceptors that entrain the locomotor and electroretinographic (ERG) amplitude rhythms in adult crayfish reside in the supracerebral ganglion (5) and both rhythms can be entrained by extraretinal pathways. Other studies on the development of crayfish circadian system (6–8) have proposed the existence of two kinds of circadian photoreceptors participating in the ERG amplitude rhythm resetting, a short and a long wavelength photoreceptor system, that could interact with the pacemaker system by means of retinal and extraretinal inputs. This study was carried out to test the influence of the different wavelengths on the synchronization of the motor activity rhythm in juvenile crayfish as well as to determine whether this effect involves extraretinal photoreception. When maintained in 12:12 LD cycles under complete photoperiod, crayfish exhibits masking effects on the activity rhythm, making the synchronization process difficult to evaluate (9). Symmetrical skeleton photoperiods (SP) can successfully entrain the locomotor activity rhythm of some animals in the same way as the corresponding complete photoperiod. (10). Hence the motor activity rhythm of unrestrained, intact, and retina and lamina ganglionaris-deprived juvenile crayfish was recorded to evaluate their ability to entrain to monochromatic red and blue SP. The results show the crayfish is able to synchronize to red and blue monochromatic SP even in the absence of the retina and lamina ganglionaris.

MATERIALS AND METHODS

Animals and Procedure

Forty-six young crayfish *Procambarus clarkii* instars, 16–20 weeks old, were used. All were born in our laboratory from field-collected animals, acclimatized to laboratory conditions, and mated here. Animals were divided into two groups: (1) intact control animals and (2) animals with retina and lamina ablation. During the experiment all organisms were placed individually in small double-compartment aquaria made of black acrylic plastic. One end of the aquarium was sealed by glueing a piece of wax containing fish and vegetables serving as food during the experiment. One of the compartments was a tunnel simulating a burrow and the other was a wide chamber (9). Temperature was kept constant at $20 \pm 1^\circ\text{C}$. Light pulses were provided by an optic fiber (Dolan-Jenner mod 170.2) placed 15 cm from the chamber and controlled by a programmable timer. Light intensity was adjusted by neutral density filters; monochromatic light was provided by Kodak Wratten narrow band interference filters with peak transmittance wavelengths of 460 and 640 nm. The intensity of the stimulus was calibrated with respect to the crayfish position using a Li-Cor quantum-radiometer-photometer with a Li-Cor pyranometer, mod PY-17197. Irradiance was fixed at a value of 25 watts m^{-2} . All the crayfish were placed in light-dark cycles (LD 12:12) from eclosion to the beginning of the experiment. The experiment always started at 15:00 h, during the photophase of the cycle. Each crayfish was transferred to its home cage and placed in complete darkness during

the first 10 days of the experiment. From the eleventh to twentieth day of the experiment, the crayfish were submitted to a 30 min blue or red light SP simulating a complete LD 12 : 12 photoperiod (lights on at 07:00 and 19:00 h). On the twentieth day of stimulation, the lights were turned off and the animals were maintained in darkness (DD) for a minimum of 10 days.

Data Recording and Analysis

Animals were individually monitored with a motor activity recording system as previously described (9). The activity during the 24-h periods was conventionally double-plotted as actograms (Tau program, Minimitter Co., Inc.). Analysis of the motor activity was based on the occurrence of major bursts of movement. To define activity peaks, the levels of locomotor activity had to be larger than the mean \pm standard deviation; i.e., small movements such as from antennae were filtered out. The motor rhythm of crayfish is not very prominent and sometimes not present (11). Hence, quantitative statistical methods had to be used to determine if a rhythm existed or not and to estimate its parameters. To determine the presence or lack of rhythmic patterns objectively, the data of 10 consecutive days were plotted in 20-min bins and analyzed according to Sokolove and Bushnell's (12) chi-square periodogram at 10–30-h intervals. All animals showing significant periodograms, i.e., spikes above a confidence interval of $p < 0.01$, were considered rhythmic. Period differences between free running in preentrained and postentrained conditions of the two groups of animals were tested using a nonparametric Kolmogorov-Smirnov test. The phase reference point for measuring the possible advances or delays induced by the light pulses was either the maximal peak of activity (mode) or the onset of activity. Activity time was ascertained from the moment when the amplitude of a cycle reached 50% of its maximum until it had returned to this value. Hence, circadian time (CT) 12 denotes the onset of activity. To estimate this phase reference point, we calculated the onset of activity from the estimated rhythm in each 24-h period. This was projected to the first recording day by means of a regression line calculated by the least-squares method in which the day in the sequence was the independent variable and the onset of activity was the dependent variable. The slope of the regression line was used to verify the period value (13). To determine the rhythm phase under LD, the onset of activity, occurring relative to whether the lights were on or off, was chosen as a reference point. Average phases for both groups of animals under blue or red SP were determined using Rao's and Rayleigh circular statistic tests for nonrandomness (14).

Surgery and Histology

Surgical lesions were performed on animals anesthetized with ice. Under a dissecting microscope the retina and lamina ganglionaris of the eye complex were bilaterally removed using a razor knife. The wounds were sealed with small amounts of melted wax and the animal was left to recover before continuing the experiment. At the end of the experiment, the eyestalk was dissected to verify the surgical lesion. Afterwards, it was fixed in formaldehyde, included in paraplast, and cut with a microtome. Slices were dyed with toluidine blue and observed and photographed using a Nikkon Labophot 2 light microscope.

RESULTS

Figure 1 shows the effect of blue light upon the locomotor activity of two juvenile crayfish: (a) intact and (b) retina-ablated. a) The actogram and the corresponding periodograms show a clear unimodal circadian rhythm that depicts a significant period of 24.6 h ($p < 0.01$) under DD. This rhythm changes to a bimodal pattern during the exposure to the blue SP cycle. The rhythm synchronizes to this cycle throughout a phase jump advancing 2.3 h, changing to a bimodal pattern and shortening to $\tau = 23.8$ h. The periodogram depicts a significant peak at 12 h. This bimodal pattern changes to an unimodal one during the last 10 recording days in darkness. Under these postentrainment conditions, the free-running rhythm lengthens, $\tau = 24.8$ h. b) The actogram shows the effect of blue light on the activity of one animal lacking retina and lamina ganglionaris. The unimodal circadian rhythm shown by this animal under the DD condition ($\tau = 25.1$ h)

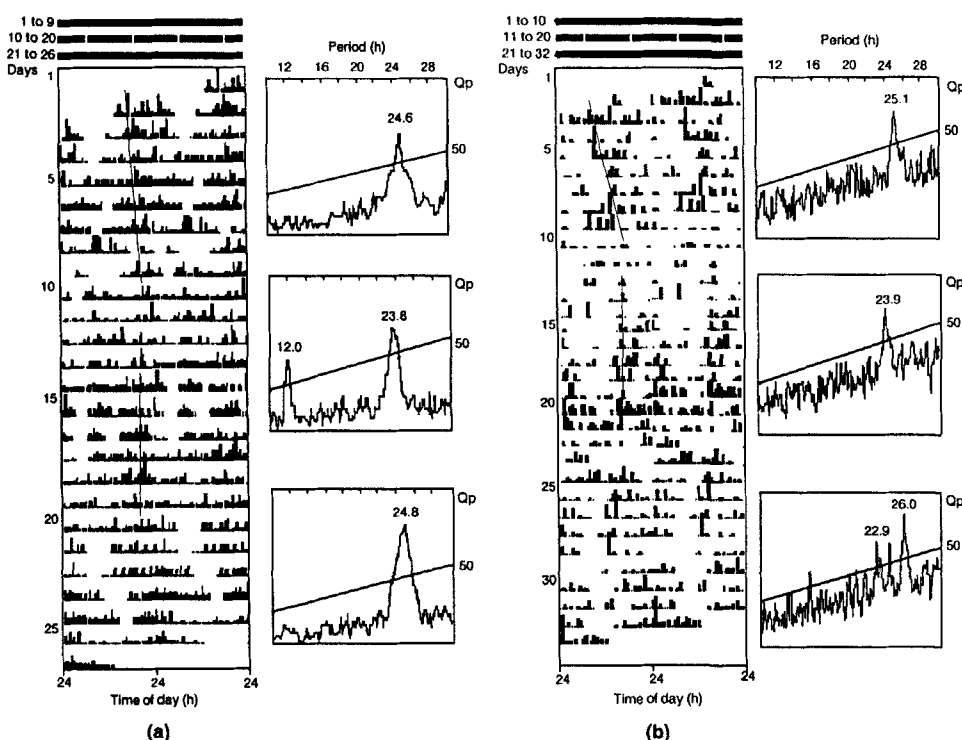


FIGURE 1. Locomotor activity rhythms of two juvenile crayfish that were each maintained in DD for 10 days and afterwards exposed to blue SP (two 30-min pulses of 460 nm and 25 W m⁻², at 07:00 and 19:00 h). Data are presented in the double-plotted format with the activity of successive days stacked vertically. (a) Intact crayfish, (b) retina-ablated crayfish. (Left) Actogram for 10 days of recording in DD, SP, and DD again. (Right) Corresponding periodograms for 20-min bins of the activity record computed for 10 days under each condition. In (a) 19:00 h pulsed light occurring at CT 16 in the 10th day seems to evoke a phase advance. (b) 19:00 h pulsed light occurring at CT 22 produces a phase advance. Regression line periods are in (a) 24.4 and 23.8 h and in (b) 25.2 and 24.01 h, respectively. See text for further explanation.

persists in the SP cycles after some days of apparent arrhythmia. The rhythm synchronizes to SP after 2 days of apparent lack of rhythmicity advancing 2 h and shortening its period value ($\tau = 23.9$ h). During the DD postentrainment condition this animal's rhythm seems to fragment with emergence of three circadian peaks and one significant ultradian peak.

Figure 2 shows the effect of the red SP cycles on the activity rhythm of two young crayfish: (a) intact and (b) retina-ablated. Figure 2a depicts a clear and statistically significant unimodal rhythm in DD as shown in the corresponding periodogram. This rhythm seems to synchronize to the red SP cycles after 5 days of transients, with a phase delay of 9.0 h, showing the absolute peak about 20:00 h and a second peak at the 07:00 h lights-on time. Postentrainment DD shows the control phase and disappearance of the lights-on peak. Under this condition, the rhythm shortens its period value to 23.5 h. During the 35 days of recording, some changes in the activity level were observed as previously described for this rhythm during development (9). In Figure 2b, the actogram

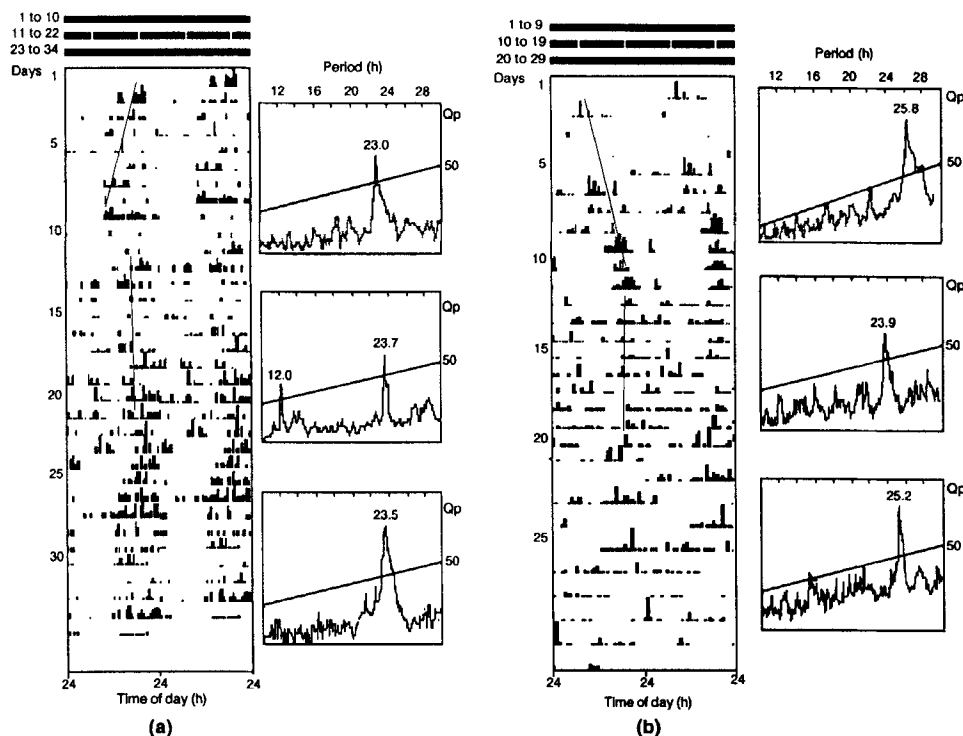


FIGURE 2. Locomotor activity record and its associate periodograms of two juvenile crayfish—(a) intact and (b) retina-ablated—that were exposed to red SP (two 30-min pulses of 640 nm and 25 W m^{-2}). Note the “lights-on” peak during the entrainment condition in the intact crayfish is not present in retina-deprived crayfish. Note the rhythm splitting as well as the change of the rhythm period value during the DD postentrainment. In (a) 19:00 h pulse light occurring at CT 14 during the 11th day evokes a phase delay. (b) 19:00 h light pulse occurring at CT 13 produces an advance. Regression line periods are in (a) 22.8 and 24.1 h, and in (b) 25.3 and 23.88 h, respectively. See text for details.

depicts the circadian unimodal activity rhythm of a young animal lacking retina and lamina. This rhythm is able to synchronize to the red SP cycle by means of a phase advance of about 1.83 h and by displaying an unimodal circadian rhythm ($\tau = 23.9$ h.). Afterwards, during the DD postentrainment, this rhythm lengthens to $\tau = 25.2$ h, and several ultradian bouts emerge; only one of these peaks shows a significant value in the periodogram.

Based on the rhythmicity criterion established, i.e., at the $p < 0.01$ level in the periodogram, 90% ($n = 22$) of all the intact animals showed a statistically significant circadian activity rhythm; 80% ($n = 17$) of the animals lacking retina and lamina showed a statistically significant circadian rhythm. The period of the rhythm showed great variability in both intact and retina-ablated animals. Generally, two kinds of young crayfish were found, a group exhibiting a shorter τ than 24.0 h and another one having a longer τ of over 24.0 h. All animals showed free-running activity rhythms that entrained to blue or red SP by means of phase advances or delays. Figure 3 shows the mean of the rhythm's period value in DD, SP, and postentrainment DD conditions in animals with

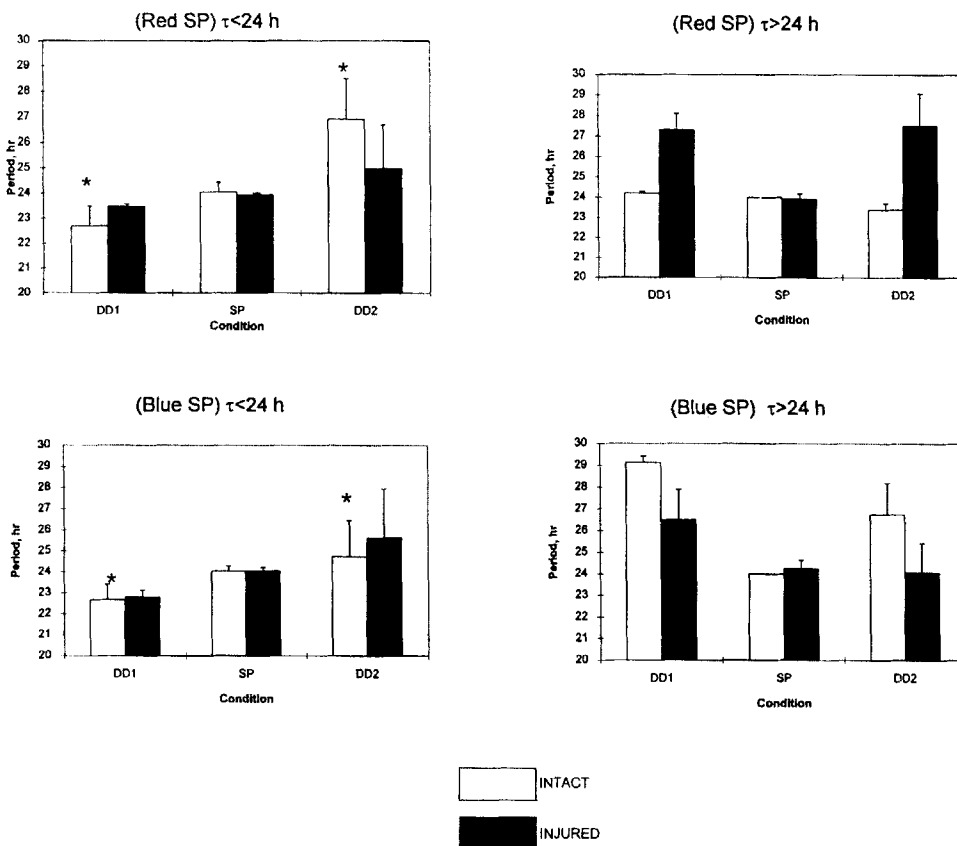


FIGURE 3. Changes in the free-running rhythm period value after rhythm entrainment to blue and red SP in intact and retina-ablated juvenile crayfish. Note all crayfish of both intact and deprived retina groups entrain to blue and red SP. Each bar indicates the average and the vertical lines standard deviation. * $p < 0.05$. See text for explanation.

short and long period values submitted to blue and red SP cycles. In general, intact animals had a shorter period than retina-ablated ones when recorded under DD prior to the SP cycles. Both groups of animals synchronized equally well to blue or red SP cycles. Considerable lengthening or shortening of the rhythm period value during the postentrainment DD condition can be observed in both groups of animals. Statistical testing showed significant differences ($p < 0.05$) between the rhythm's free-running period in intact animals before and after being submitted to red and blue SP. Both groups of animals under blue or red light conditions showed no significant differences in the number of transients required to synchronize to the blue or red SP.

The distribution of phases among the animals belonging to both groups, intact and retina-ablated resetting to blue and red SP, is shown in Figure 4. Groups 1 and 2 recorded under blue SP and group 1 recorded under red SP exhibited resetting to the morning and evening pulse. Rao's test revealed a significant ($p < 0.01$) nonrandom bimodal clustering in these groups. Group 2 recorded under red SP shows an unimodal resetting to the evening pulse; Rao's test demonstrated a statistical significant unimodal nonrandom distribution. Rayleigh's test indicated a nonsignificant unimodal distribution in all the cases ($p > 0.05$).

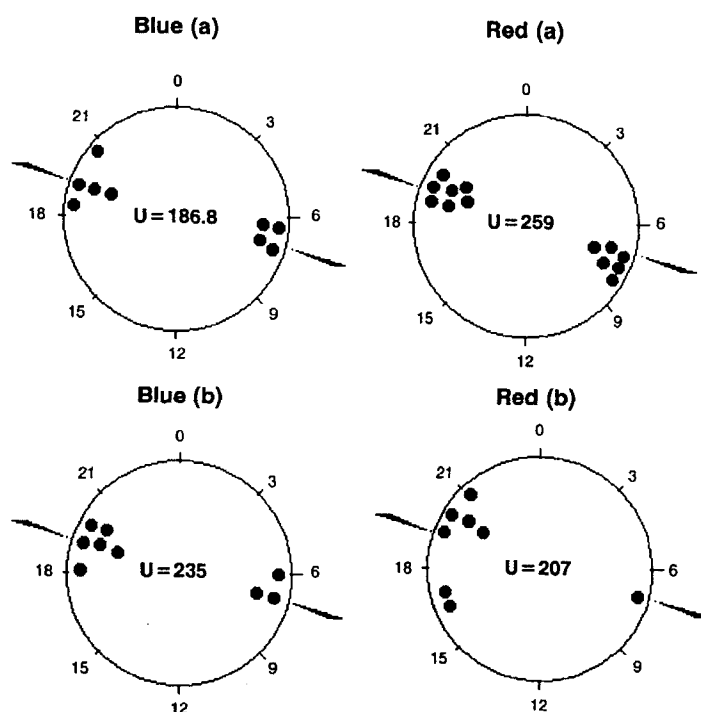


FIGURE 4. Estimated phases of the activity rhythm for the two groups of crayfish recorded under blue or red SP. The times of the two pulses forming the SP are indicated by filled arrows. Bimodal and unimodal Rao's test and unimodal Rayleigh's test showed $p < 0.01$ and $p > 0.05$, respectively. See text for explanation.

DISCUSSION

These results show that the activity rhythm of young *P. clarkii* can be synchronized with skeleton photoperiod regimes simulating a complete photoperiod of LD 12:12. The discrete entrainment involves abrupt phase shifts of the pacemaker caused by the two pulses defining the skeleton photoperiod which allows the circadian responses to the light, including the delay and advance shifts involved in rhythm resetting, to be observed (15). When animals were placed under red or blue light, the locomotor activity of intact animals exhibited a bimodal activity rhythm with two peaks—the shorter duration one immediately resetting at the first pulse of light (07:00 h) and the second longer burst of activity occurring near the second pulse in the evening (Figs. 1a and 2a). The shorter burst of activity seems similar to the “lights-on” peak that has been described for adult crayfish and which has been proven to be exogenously driven (5,11,16). This peak seems clearer when recorded under red light, appearing at each cycle. When the crayfish is recorded under blue light, this first burst of activity waxes and wanes throughout the different recording days (Fig. 1a). Removal of ommatidia and lamina abolishes this peak (Figs. 1b and 2b) in adult crayfish as reported by some authors (11). The aforementioned could indicate the influence of retinal photoreceptors with a differential spectral sensitivity during development upon the walking leg activity responsible for this peak (17). The longer burst of activity corresponds to the endogenously driven “lights-off” peak of the adult crayfish (18). This longer burst of activity synchronizes to both blue and red monochromatic light even in the absence of retina and lamina indicating the presence of extra-retinal circadian photoreceptors that are able to detect short and long wavelengths.

Both in the intact and retina-ablated animals, long and short-wavelength monochromatic light pulses evoked phase advances or delays on the rhythm according to when the light occurred during the subjective day or night of the free-running rhythm. The phase-response curve of the circadian oscillator controlling the locomotor activity in the restrained adult crayfish seems to show a type 0 curve, displaying a large fraction of delays from 20 to 12 h CT and a peak advance at about CT 17 h (19). During the first day of the entrainment, morning or evening pulses during the free-running rhythm originated either a phase advance or delay based on the animal's CT (Figs. 1 and 2). Afterwards, the successive phase shifts caused by the two light pulses stabilized the rhythm during entrainment as it was proposed elsewhere for other nocturnal animals (15).

Both in intact and retina-ablated animals, no differences in the number of transients required to synchronize to the LD cycle were found; nor were phase relations between the light pulse and the rhythm phase disturbed. The afore-mentioned findings indicate that the synchronization mechanism to light irradiance is extraretinal. Although synchronization persists in animals lacking retina and lamina, the level of activity increases suggesting that retina and lamina could be involved in its regulation. Comparison of the free-running period value of control and experimental animals previous to entraining with that calculated in the postentrainment DD reveals aftereffects in τ resulting from phase shifts. These aftereffects are more frequent in the intact groups, suggesting that this phenomenon could be associated with the retina. The bimodal pattern shown by the intact and retina-ablated animals under blue light as well as the intact crayfish under red light could indicate bistability (10). These groups of animals are able to exhibit two behavioral patterns, some young crayfish selecting the first and the others the second dark interval in which to express their main activity. This phenomenon suggests the need to carry out new experiments to investigate the strategies used by this nocturnal species

to adapt to a changing photoperiod. The results of this study indicate that both circadian responses to monochromatic light examined herein are mediated by extraretinal photoreceptors, as has been proposed for other invertebrates (20). Further studies must investigate the characteristics and location of this photoreceptor, its influence on other parameters of this rhythm, as well as its relation to the circadian photoreceptors proposed for the ERG amplitude rhythm during crayfish development.

ACKNOWLEDGMENTS

We are very grateful to Dr. Michael H. Smolensky for his advice and editorial help. We are grateful too to Dr. Raúl Aguilar Roblero and Dra. Margarita Chavez Cano for their critical commentaries and suggestions to this work and Jesús Sáenz for his technical support. This work is part of doctoral thesis of Manuel Miranda Anaya. Research was supported by PADEP UNAM 03001, CONACyT 0112P-N, and DGAPA fellow.

REFERENCES

1. Hastings WJ, Rusak B, Boulos Z. Circadian rhythms: the physiology of biological timing. In: Prosser CL, ed. *Neural and integrative animal physiology*. New York: Wiley-Liss, 1992:435–546.
2. Klemm E, Ninneman H. Detailed action spectrum for the delay shift in pupae emergence of *Drosophila pseudoobscura*. *Photochem Photobiol* 1976; 24:369.
3. Frank KD, Zimmerman WF. Action spectra for phase shifts of a circadian rhythm in *Drosophila*. *Science* 1969; 163:688.
4. Morse DJ, Hastings W, Roenber T. Different phase responses of the circadian oscillators in *Gonyaulax*. *J Biol Rhythms* 1994; 9(3-4):263–74.
5. Page TL, Larimer JL. Extraretinal photoreception in entrainment of crustacean circadian rhythms. *Photochem Photobiol* 1976; 23:245–51.
6. Fanjul-Moles ML, Miranda-Anaya M, Fuentes-Pardo B. Effect of monochromatic light upon the ERG circadian rhythm during ontogeny in crayfish *Procambarus clarkii*. *Comp Biochem Physiol* 1992; 102A(1):99–106.
7. Fanjul-Moles ML, Miranda-Anaya M, Bernal-Moreno JA. Effect of the light quality on the phase response curve of the ERG circadian rhythm in juvenile instars of crayfish *Procambarus clarkii*. *Soc Neurosci Abstr* 1993; 19:1616 (abstract 662.23).
8. Bernal-Moreno JA, Miranda-Anaya M, Fanjul-Moles ML. Phase shifting the ERG amplitude circadian rhythm of juvenile crayfish by caudal monochromatic illumination. *Biol Rhythm Res*. 1996; 27 (in press).
9. Fanjul-Moles ML, Miranda-Anaya M. Circadian locomotor activity rhythm during ontogeny in crayfish *Procambarus clarkii*. *Chronobiol Int* 1996; 13(1):15–26.
10. Pittendrigh CS. Circadian systems: entrainment. In: Aschoff J, ed. *Handbook of behavioral neurobiology*. 4. *Biological Rhythms*. New York: Plenum Press, 1981: 95–124.
11. Page TL, Larimer JL. Entrainment of the circadian locomotor activity rhythm in crayfish. The role of eyes and caudal photoreceptor. *J Comp Physiol* 1972; 78: 107–20.

12. Sokolove PG, Bushell WN. The Chi-square periodogram: its utility for analysis of circadian rhythms. *J Theor Biol* 1978; 72:131–60.
13. Enright JT. Data analysis. In: Ashoff J, ed. *Handbook of behavioral neurobiology. 4. Biological Rhythms*. New York: Plenum Press, 1981:21–38.
14. Batschelet E. *Circular statistics in biology*. New York: Academic Press, 1981.
15. Pittendrigh CS, Daan S. A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as a clock. *J Comp Physiol* 1976; 106:291–331.
16. Chapple WD. Light and the movement of crayfish. Master's degree thesis, Syracuse University, 1960.
17. Fanjul-Moles ML, Fuentes-Pardo B. Spectral sensitivity in the course of ontogeny of crayfish *Procambarus clarkii*. *Comp Biochem Physiol* 1988; 91A:61–6.
18. Page TL, Larimer JL. Neural control of circadian rhythmicity in the crayfish. I. The locomotor activity rhythm. *J Comp Physiol* 1975; 97:50–80.
19. Viccon-Pale J, Fuentes Pardo B. Synchronization by light of the circadian rhythm of motor activity in the crayfish. *Biol Rhythm Res* 1994; 25:267–76.
20. Page TL. Extraretinal photoreception in entrainment and photoperiodism in invertebrates. *Experientia* 1982; 38:1007–13.