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ORIGINAL ARTICLE

Circadian entrainment by light and host in the Chagas disease vector, *Triatoma infestans*

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Triatoma infestans (Reduviidae: Triatominae, ''kissing bug'') is the main insect vector of *Trypanosoma cruzi*, the causative agent of Chagas disease, a chronic trypanosomiasis infecting 10 million people world-wide. This hematophagous bug feeds on diurnal and nocturnal species during each host's quiescent time. As the hosts are also its major predators, kissing bugs are subjected to dual selective pressures from a single source. Therefore, synchronization of feeding with the host's behavior is critical to the insects' survival. We show that nonphotic signals linked to the host eclipse the role of light and dark as the primary circadian *zeitgeber* for these bugs, although light still strongly inhibits locomotor behavior directly. In nature, this combination provides the insect with great flexibility in organizing physiology and behavior: anticipating a quiescent host or avoiding its potential predation while remaining directly responsive to immediate environmental conditions. Manipulation of nonphotic entrainment could be a useful chronobiotic tool in the control of Chagas disease.

Keywords: Circadian rhythms, kissing bugs, nonphotic synchronization, photic synchronization, *Triatoma infestans*, triatominae

INTRODUCTION

Chagas has become the most important parasitic disease of the Americas (Hotez et al., 2008), specifically in the southern cone of South America. Nearly 10 million individuals are infected, and the annual economic burden has been placed at over $US 7 billion. Total projected healthcare cost for these infected individuals alone is estimated at nearly $25 billion, and the life cost at nearly 30 million disability-adjusted life years (Lee et al., 2013).

Triatominae, the insect vectors of *Trypanosoma cruzi*, are known to feed on diverse hosts, including various mammals and birds, and other insects. A few Triatominae species are restricted almost exclusively to human dwellings, inhabiting crevices in walls and roofs, while feeding mainly on domestic animals, and humans (Gürtler et al., 1997). Other reports have described triatomines feeding on sylvatic nocturnal and crepuscular animals including bats (Thomas et al., 2007), wild guinea-pig (*Galea musteloides*), mice (*Calomys callosus*) and occasionally domestic cats (Bermudez et al., 1993; Gürtler et al., 2007, 2009). In the laboratory, some species also adapt well to artificial chicken feeding during the day time (Amelotti et al., 2010; Szumlewicz, 1975).

Triatominae species show marked temporal organization in their behavior. They are inactive during the day and usually found in a quiescent state, aggregated inside their refuges in close contact with other members of the population (Lorenzo Figueiras & Lazzari, 2000). At night, they are active, searching for food, mating opportunities and oviposition sites (Ampleford & Davey, 1989; Lazzari, 1991, 1992; Lorenzo & Lazzari, 1998; Lorenzo Figueiras et al., 1994).

The reliable and precise external day–night cycle is the identified synchronizer of circadian rhythms in the majority of species studied across the phylogenetic range (Johnson et al., 2004), but in heterothermic and parasitic species (e.g. kissing bugs) that rely on other rhythmic species as food sources, other environmental cycles may turn out to be relevant *Zeitgebers* (Mrosovsky

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et al., 1989). Rhythmic behavior in the laboratory has been described for *Triatoma infestans*; however, inconsistencies are apparent in the descriptive data. Settembrini (1984) described unimodal patterns of locomotor behavior, with circadian periods >24 h; whereas Lazzari (1992) described bimodal patterns with circadian periods <24 h. Long-term continuous activity monitoring and analysis of individual insects in different entrainment conditions has not been conducted.

As one of the main vectors of *T. cruzi, T. infestans* is a hematophagous bug that takes blood meals during the quiescent times of species that have diverse activity allocations during day or night; we predicted that the bugs would exhibit strong temporal flexibility, reflecting an ability to anticipate the availability of hosts with widely different temporal patterns of behavior. Therefore, we tested the hypothesis that the circadian system of these bugs is adapted for entrainment by nonphotic mechanisms that are responsive to the activity cycles of the current host.

Using rocking-actimeters, we analyzed long-term continuous individual locomotor activity rhythms of male adult *T. infestans* in four different conditions: (a) 24-h photic light cycle (LD12:12); (b) constant dark (DD); (c) daily scheduled nonphotic stimulus in DD; and (d) daily nonphotic stimulus in LD. Results indicate that while responsive to light and dark, *T. infestans* is also highly sensitive to disturbances and other signals that might indicate the presence of a potential host or predator, and that nonphotic mechanisms could have a relevant role for entrainment in this species.

MATERIALS AND METHODS

Insects

Animals were adult (less than 20 days after their last molting) males from our laboratory colony (mean weight at the beginning of monitoring: 165 ± 22.84 mg). Insects were reared in a controlled environment under a light:dark cycle 12:12 h (LD; lights on at 07:00 h), constant temperature ($25 \pm 1\,^{\circ}C$) and humidity ($70 \pm 5\%$ RH). The Centro Regional de Investigaciones Científicas y Transferencia Tecnológica colony originated from nymphs provided by the *Coordinación Nacional de Control de Vectores (Punilla, Córdoba)*. In our Institute, these animals had gone through at least five generations without any external genetic input. In the colony, insects are fed every 15 days with live chickens (*Gallus* sp) as part of the routine maintenance procedure. The procedure for maintenance of the chickens was approved and authorized by the Experimental Ethics Committee (*Consejo de Ética en Investigación*) of the Public Health State Ministry of the La Rioja Province, Argentina (*Ministerio de Salud Pública de la Provincia de La Rioja*), permission # 892 and meets the Ethical Standards outlined in Portaluppi et al. (2010).

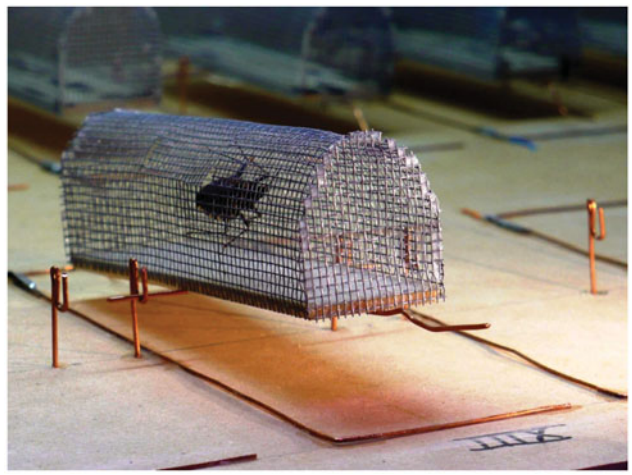

Figure 1. Individual actimeter used to monitor locomotor activity in kissing bugs.

Activity monitoring system

Activity was recorded by means of home-made rocking-actimeters, similar in many aspects, to the ones used by Lazzari (1992). The actimeters (weight 4 g) consisted of a rectangle base of thin light balsa wood (3×10 cm) on top of which a semi cylindrical nylon netting (3.5 cm high) was attached on the whole perimeter (Figure 1). A 6-cm copper wire was located across the width of the base at the longitudinal midpoint, so that each cage-like unit was able to pivot on two lateral cradle-like copper supports. In the rectangle wood base was another copper wire (15 cm long) that could make contact with an external wire to complete an electric circuit according to the pivoting position of the cage. As the insect inside walked across the midline of the cage, the actimeter would slightly re-balance (as a seesaw) closing an electric circuit at each inclination. Each electric contact was registered as a pulse of activity in the monitoring computer system.

Locomotor activity was recorded continuously using Vital-View (Phillips-Respironics, Bend, OR), which was programmed to register activity of each insect continuously and summed in 5-minute intervals. Actimeters were placed in light-tight ventilated wooden cabinets ($60 \times 40 \times 45$ cm) in a room with controlled temperature. In experiments I, III and IV, 16 recently fed insects were placed individually in the actimeters in one cabinet. In experiment II, two of these isolating cabinets were used and consequently 32 animals (16/cabinet).

Light inside the cabinets during the light phase of the LD cycles was provided by a 15-W, 0.42-m-long fluorescent bulb (Cool Day Light OSRAM L) controlled by a mechanical timer (TBCin, Zhejiang, China). Light intensity varied from 300 to 400 lux at the level of the actimeters. Light intensity was measured with a digital Luximeter (Model TM-201, Tenmars Electronics CO., Ltd., Taipei, Taiwan). Temperature inside the boxes was controlled with a HOBO (*data loggers U10/003 Onset Computer Corporation, Bourne, MA*), which registered

ambient temperature and humidity every 15-minute interval. Once the experiment started, the box was closed and not opened unless extremely necessary; this was to avoid possible effects of the researchers as a nonphotic host stimulus. If control was necessary in the dark phase of the LD cycle, or in constant dark condition, a red head-light was used.

Feeding procedure

Feeding consisted of taking each insect out of its individual actimeter and placing it in a plastic jar (7.0 cm diameter, 6.0 cm high) with a mesh covered top. The top of each jar was attached to the abdomen of a chicken for a 15-minute interval or until feeding was interrupted spontaneously. Insects were returned to their original actimeter immediately after the blood meal to resume activity recording.

Nonphotic pulse

A live chicken was placed in a card board box (45 cm long $\times$ 46 cm wide $\times$ 49 cm height) connected in series with the ventilation system of the cabinet housing the kissing-bugs. A fan located in the output of the chicken box assured a good air connection between the boxes. The nonphotic stimuli consisted in placing the hen in the box at the same time each day and the fan switched on. Exposure lasted for 1 h, after which the ventilation apparatus was disconnected from the kissing bug box. In experiment III, a control disturbance procedure was used without the presence of the chicken.

Experimental procedures

Four experiments were performed, each using a different set of animals. In each experiment, animals were fed immediately prior to the start of behavioral recording.

Experiment I

Photic entrainment and free running period. Animals were exposed sequentially to three lighting regimes: (1) light–dark cycle (LD; 12:12 h) for 10 days; (2) constant dark (DD) for 18 days; and (3) reestablishment of the previous LD cycle for another 19 days. Before the second LD routine, insects had the opportunity to feed.

Experiment II

Nonphotic entrainment (''chicken pulse''). The first 19 days were lost due to technical problems in the monitoring system, consequently the data showed starts with 19-day starved animals in a LD cycle. Animals were exposed to two lighting regimes: (1) LD (light–dark cycle) 12:12 h for 34 days, to ensure comparable photic entrainment to experiment I. On day 23, the animals were taken out from the actimeters for feeding opportunity. (2) DD for 32 days. After 10 days in theses conditions, the kissing bugs were exposed to a 1-hour daily proximity of a live chicken, always at the same time, during 12 consecutive days, after which DD continued.

Experiment III

Nonphotic entrainment (disturbance). As the bugs are reactive to physical disturbance, we included a full control experiment to mimic the conditions of experiment II but without the presence of a live food source. Sequential conditions were the same as in experiment II except that the initial entrainment in LD was recorded for only 12 days.

Experiment IV

Photic–nonphotic interaction. Animals were exposed to LD conditions for 19 days followed by 16 days in DD. A nonphotic cycle was imposed for the last 13 days in LD, consisting of the daily proximity of a chicken in the middle of the light phase. Thus, all animals were exposed simultaneously to the 24-hour LD cycle with a daily 1-hour nonphotic stimulus.

Data analysis

Graphical output (actograms) and rhythm analysis were carried out using *El Temps* software (A. Díez-Noguera, Universitat de Barcelona, Barcelona, Spain). Analysis of actograms was done by visual inspection by two independent observers assisted by the ''point-and-read'' tools of *El Temps*. Determination of daily activity onset was required to calculate phase angle of entrainment (ψ) with respect to the LD cycle (ψ_{OL}) or nonphotic stimulation (ψ_{ONP}) as well as determination of free-running period in constant conditions. The definition of onset used was the first bout of activity of at least 60 minutes of intense activity after a previous 60-min or longer interval of inactivity. Importantly, this definition had to be in accordance with a group pattern, that is, this should occur for a minimum of three consecutive days and in a coherent way (showing a 24-h period related to synchronization or a consistently increasing or decreasing period in case of constant conditions).

ψ_{OL} was defined as the number of hours that activity onset either preceded dark onset (positive ψ) or followed it (negative ψ). Similarly ψ_{ONP} was defined as the number of hours that activity onset preceded or followed the beginning of a nonphotic stimulation. Period in DD (τ_{DD}) was estimated using the original Chi-square periodogram analysis described by Sokolove & Bushell (1978) with a global risk level of $p<0.05$. We used long-term recordings of activity, during which various parameters were measured. Unfortunately, not all subjects provide usable information for each measurement. Therefore, we end up having different number of animals for each feature that is analyzed. In the figures, we placed most of the animals that had the most complete actograms. Data were expressed in mean values $\pm$ standard error. When comparing two groups, paired or unpaired *t*-tests were used using excel.

RESULTS

Experiment I (entrainment and freerun)

Of 10 animals with complete records, eight animals clearly synchronized to the first LD cycle, while activity of two animals (10 and 14) was too low to determine phase angle (Figure 2). Phase relationship observed during entrainment was always negative ($\psi_{OL} = -0.8 \pm 0.2$ h; Table 1). Locomotor activity occurred mainly in the dark, and activity was highest during the early part of the scotophase with durations ranging from 1.2 h (animal #5) to 7.7 h (animal #15) and a mean of 3.8 ± 0.7 h.

A different determination of ψ_{OL} was made using the subsequent free-run in DD. In this experiment, all animals exhibited $\tau_{DD} > 24$ h (Table 1). Because τ_{DD} consistently lengthened during the first week in DD, only the first few days reflect the phase of the rhythm that existed during the prior entrainment. Therefore, ψ_{OL} was calculated this time using eye-fitted lines through activity onsets during the first four full days in DD, which were extrapolated to the final day in the preceding LD cycle. Using this method, ψ_{OL} was positive ($\psi_{OL} = +0.5 \pm 0.5$ h; Table 1), although activity onset was not expressed overtly in the LD locomotor behavior due to masking of activity by light. Upon return to the LD cycle on day 27, ψ_{OL} resumed a negative value (-0.06 ± 0.02 h).

Experiment II (nonphotic entrainment)

During the LD cycle, the amount of activity measured four days previous to feeding was significantly greater than in experiment I (measured 4 days previous to DD; 14604.9 ± 3641.9 vs 1190.5 ± 532.7 bouts of activity; $p = 0.0163$) and ψ_{OL} was more positive (-0.35 ± 0.05 vs -0.8 ± 0.2 h, $p = 0.0458$; Figures 2 and 3), due to the fact that animals had not been fed prior to recording (total of 38 fasting days). Activity levels decreased abruptly after feeding (14604.9 ± 3641.9 vs 4719.3 ± 1165.7; $p = 0.0007$) and ψ_{OL} (-0.4 ± 0.3) becomes more similar to experiment I ($p = 0.1754$; Table 1 and Figure 3). τ_{DD} (26.5 ± 0.4 h) was comparable to the τ_{DD} in experiment I (Table 1).

After 10 days in DD, regular daily proximity of a potential food source (live chicken) was provided for 12 days. Three different patterns of response were observed. Nine animals entrained to the nonphotic pulses with a negative phase relationship (ψ_{ONP}) (Table 1 and Figure 3; e.g. animals #7 and #20); activity followed the stimulus. Five animals entrained with a positive phase relationship ($+\psi_{ONP}$) (Table 1 and Figure 3; e.g. animals #11 and #26); activity preceded the stimulus. Finally, for two animals, the nonphotic pulse fell during the subjective night and these did not entrain (Figure 3; animals #3 and #21).

In addition, for nine animals where τ_{DD} could be determined both before and after the NP pulse, the period was shortened significantly ($p = 0.0076$; $\tau_{after\ NP} = 24.6 \pm 0.3$ h) after nonphotic entrainment (Table 1).

Experiment III (nonphotic disturbance)

To address the nature of the signals that might be instrumental in nonphotic entrainment, a control experiment was performed wherein the bugs were exposed to the action of food presentation but without the chicken. Various signals linked to food sources (including human) attract these insects (Barrozo & Lazzari, 2004; Barrozo et al., 2004a,b) so might be effective agents of entrainment. In addition, nonphotic signals that produce behavioral arousal have been shown to produce clock resetting in other species (Mrosovsky et al., 1989). These bugs had been fed immediately prior to being introduced into the recording apparatus, which may explain the low initial level of activity and delayed ψ in the LD cycle, both of which were similar to experiment I (Figure 2). In DD, 9 of 13 animals free-ran with $\tau_{DD} > 24$ h, and only these animals synchronized with the daily nonphotic ''disturbance''. Six of the nine animals synchronized with a negative phase angle (Figure 4; e.g. animals #11 and #16), and three animals entrained with a positive phase angle (Figure 4; e.g. animals #9 and #10). For the others, three animals with $\tau_{DD} < 24$ h (Figure 4; e.g. animal # 6), and one with $\tau = 24$ h (Figure 4, animal #15) did not entrain.

Experiment IV

When two different cycles were simultaneously administered, activity was always concentrated in the dark. Activity onset in LD began abruptly at the beginning of the dark phase, suggesting a strong masking effect of light. This was confirmed by the following free running behavior in DD. Based on the initial behavioral pattern in DD, ψ_{OL} was positive with respect to dark onset by more than 2 h (Table 1 and Figure 5). Furthermore, the activity onset was also associated with the timing of the nonphotic stimulus. With respect to the nonphotic pulse, ψ_{ONP} was negative by just over 2 h (Table 1). This was not statistically different (-232.9 ± 22.3 minutes, $n = 13$ vs -173.1 ± 32.4, $n = 14$, $p = 0.1422$) from entrainment of the subgroup of animals, from experiments II and III, which entrained with a negative phase relationship to the nonphotic pulse presented in DD.

τ_{DD} for this group was significantly shorter than in experiment I, where only the photic *zeitgeber* was present (27.09 ± 0.4, $n = 10$ vs 24.8 ± 0.4, $n = 13$, $p = 0.000004$). In eight animals, τ_{DD} was equal to or shorter than 24 h, while τ_{DD} was longer than 24 h only in five animals.

DISCUSSION

The ability to anticipate important daily environmental conditions or events is thought to provide a selective advantage which has driven the evolution of circadian clocks that are synchronized or entrained by regular

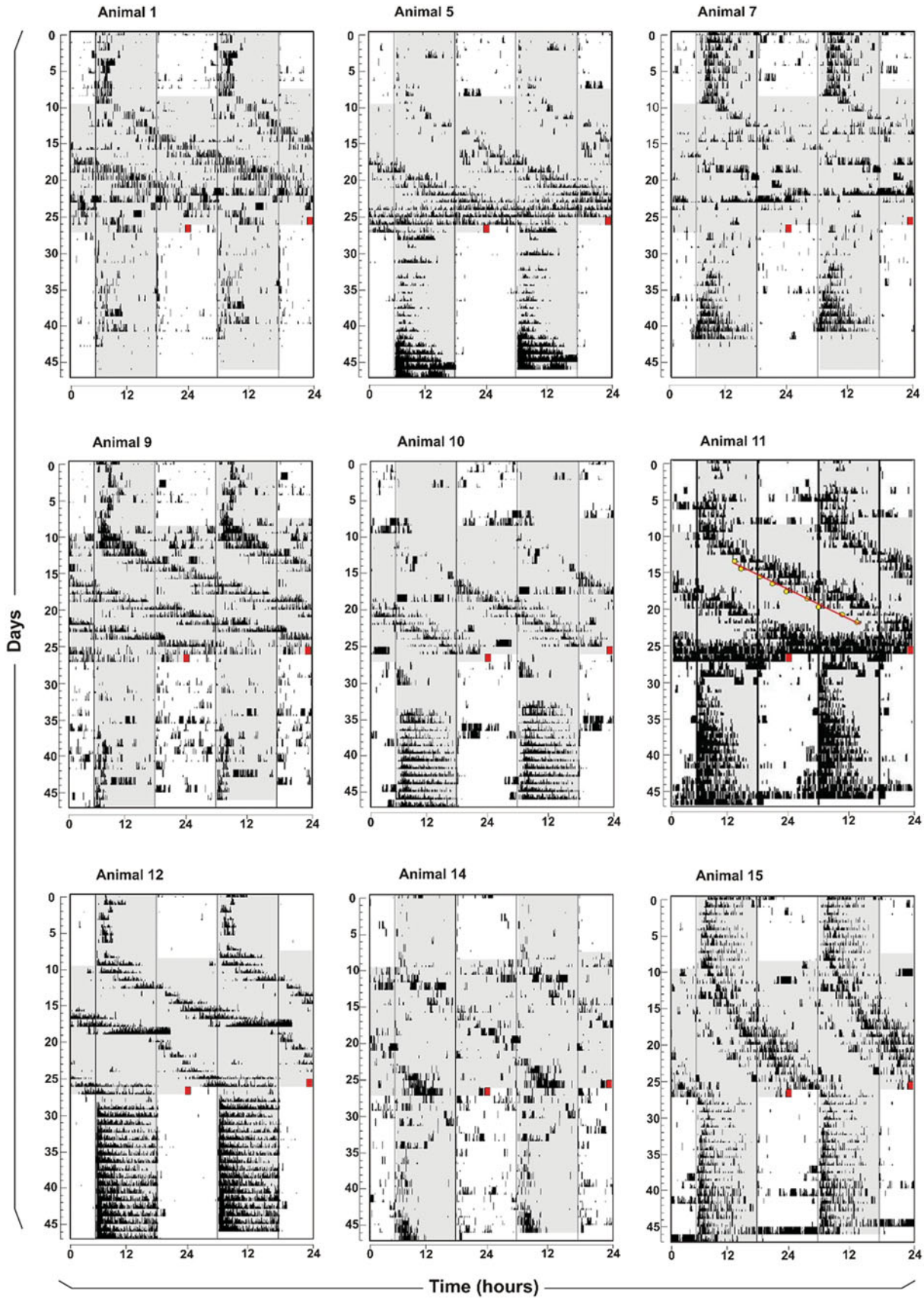

Figure 2. Kissing bug activity during photic treatment. Double-plotted actograms of nine kissing bugs submitted to a standard LD 12:12 h cycle (days 0–9), constant darkness (days 9–27) and reinstating of the LD cycle for 19 more days. Grey background indicates lights off. Squares at the end of the constant-dark interval, indicate feeding time. Onset determination is exemplified during DD in animal #11, where onsets were marked and an eye-fitted line was placed.

Table 1. Descriptive statistics in hours and comparison of experiments.

	Experiment 1 LD/DD	Experiment 2 Nonphotic	Experiment 3 Nonphotic control	Experiment 4 LD + nonphotic
ψ_{OL}	-0.8 ± 0.2 (8)	-0.4 ± 0.3 (20)[a]	-0.8 ± 0.6 (13)	$+0.04 \pm 0.03$ (13)
ψ_{OL}*	$+0.5 \pm 0.5$ (10)	$+1.5 \pm 0.2$ (20)	$+0.5 \pm 0.3$ (14)	$+3.0 \pm 0.4$ (13)
$\psi_{ONP(-)}$	–	-2.5 ± 0.5 (9)	-2.4 ± 0.8 (6)	–
$\psi_{ONP(+)}$	–	$+8.3 \pm 1.3$ (5)	$+10.9 \pm 2.7$ (3)	–
$\psi_{ONP(LD)}$	–	–	–	-2.4 ± 1.3 (8)
τ_{DD} (after LD)	27.09 ± 0.3 (10)	26.5 ± 0.4 (9)	24.6 ± 0.2 (4)	–
τ_{DD} (after NP)	–	24.6 ± 0.3 (9)	24.5 ± 0.3 (4)	–
τ_{DD} (after LD/NP)	–	–	–	24.8 ± 0.4 (13)

[a] = value for post feeding interval prior to DD; N values are indicated in parentheses.
OL = onset-light; ONP = onset-nonphotic.
ψ_{OL} = phase angle of entrainment based on direct measurement in LD12:12.
ψ_{OL}* = phase angle extrapolated from freerun in DD following LD12:12.
$\psi_{ONP(-)}$ = phase angle of entrainment to a nonphotic pulse in DD (activity follows pulse).
$\psi_{ONP(+)}$ = phase angle of entrainment to a nonphotic pulse in DD (activity precedes pulse).
$\psi_{ONP(LD)}$ = phase angle extrapolated from freerun in DD following LD12:12.
τ_{DD} = free running period in DD following entrainment to LD12:12 or LD + NP.
– = does not apply.

environmental cycles (De Coursey, 1964; Pittendrigh, 1993; Pittendrigh & Daan, 1976). A commonly held assertion in chronobiology is that the main *zeitgeber* for circadian systems in nature is the reliable cycle of sunrise and sunset, and most research on entrainment mechanisms has focused on the issues of circadian responses to light. Much less attention has been paid to the influences of nonphotic signals that can also reset circadian rhythms. This bias can be attributed to the fact that in nature (1) there is less temporal precision offered by daily nonphotic conditions or events compared with the light/dark cycle; (2) important events may occur only occasionally or sporadically; and (3) important nonphotic stimuli (e.g. rhythmic food availability) may entrain peripheral oscillators rather than the central clock that is entrained by light.

Whereas the LD cycle seems to have a universal role as a *zeitgeber* across species, non-photic synchronizers appear to be crucial under certain circumstances (e.g. food restriction in hungry animals), or in certain roles (e.g. social entrainment; Klerman et al., 1998; Mistlberger & Skene, 2004; Hut et al., 1999), or interspecies interactions (Barrozo et al., 2004b; Saunders, 2002). The parasite–host relationship represents a circumstance where light–dark cycling may mean less to a parasitic organism than the presence and behavior of its host.

Our data demonstrate that in *T. infestans*, nonphotic (arousal) signals can entrain circadian rhythms in DD and also in the presence of a strong LD cycle. The angle of entrainment to the nonphotic stimulus in LD was the same as the one adopted by the majority of animals tested in DD. Notably, nonphotic entrainment in DD was bimodal with a small group of animals adopting a different phase angle, but based on the published nonphotic phase response curves (PRCs) from other species, there likely will be at least two points in the circadian cycle where entrainment might occur (Figure 6). However, only one of these points will allow stable entrainment in DD as it would fall on a point in the PRC with a negative slope (Pavlidis, 1967; and see Pittendrigh, 1993 for discussion). Therefore, the shape of the hypothetical PRC shown in Figure 6 is sufficient to explain the two angles of nonphotic entrainment in DD. Moreover, when photic and nonphotic cycles were administered simultaneously (experiment 4), all of the animals entrained to the nonphotic stimulus with the same phase angle as the majority in DD. This suggests a predominance of nonphotic entrainment mechanisms over direct photic effects.

The traditional model of circadian entrainment where light is the primary *zeitgeber* and nonphotic responses are frequently short lived adjustments for local (short term) exigencies, may not apply to the current situation. Such a model may be useful for predator–prey and consumer–producer relationships where the anticipation of rewards and risks involves both long-term probabilities requiring entrainment to a light entrainable circadian clock, as well as short-term probabilities requiring the use of a continuously consulted clock (see Pittendrigh, 1958), and/or an oscillator ensemble that can be set quickly to various times through experience (see Ralph et al., 2013). However, this could be modified in parasite–host relationships where the host becomes both the most necessary as well as the most dangerous factor in the parasite's environment. The definition of parasite means that the host gains nothing from it, and may employ various defense mechanisms to get rid of it. Therefore, the survival and reproduction of many parasites, ectoparasites in particular, rely most heavily on the ability to anticipate the behavior of specific individual hosts. Thus, in parasites such as the triatomines, a selective pressure could exist for nonphotic mechanisms to become a stronger agent of entrainment (Barrozo et al., 2004b).

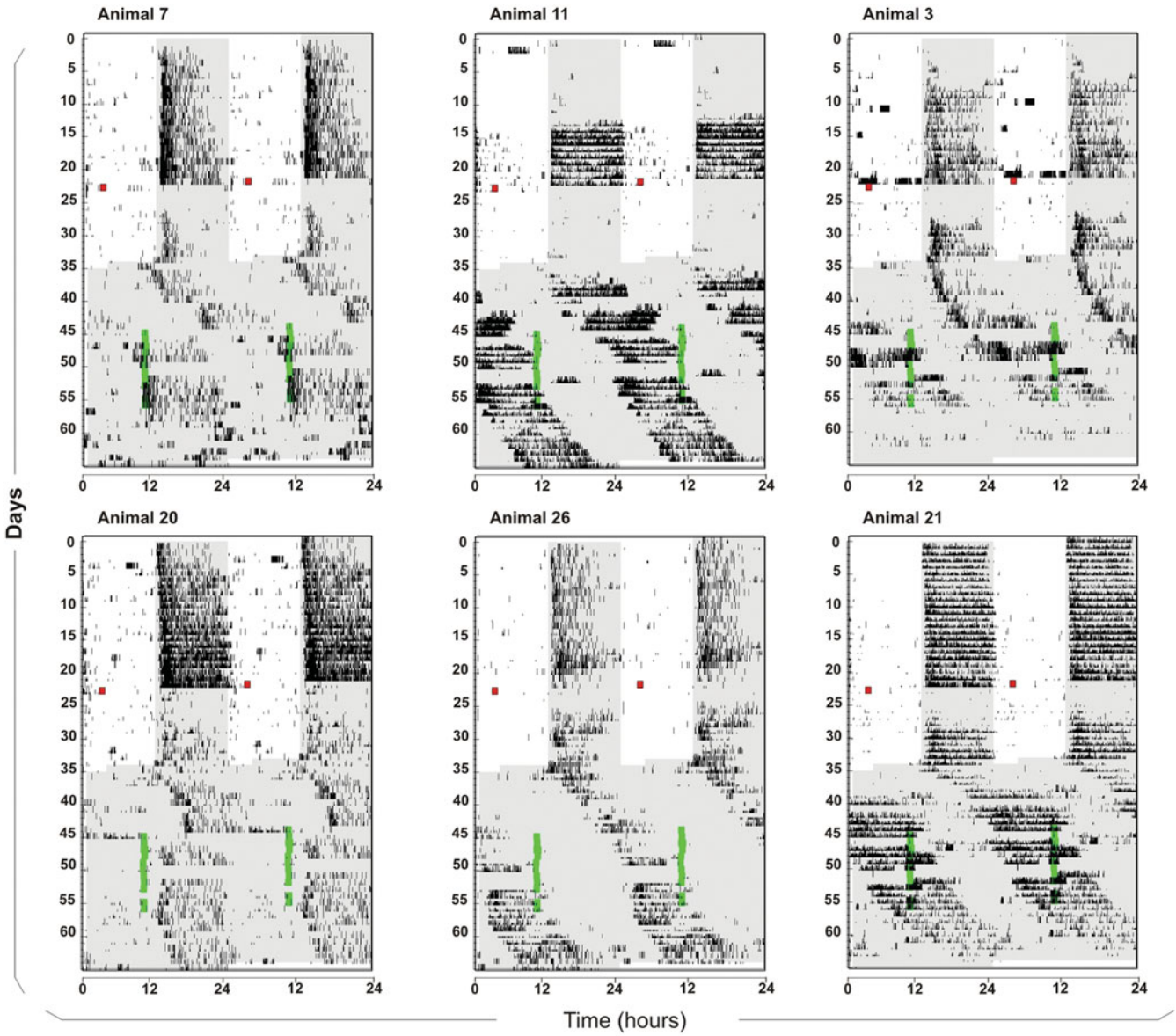

Figure 3. Kissing bug activity during photic and nonphotic (chicken pulse) cycles. Double-plotted actograms of six animals of experiment II submitted to a standard LD 12:12 h cycle (days 0–34) and then constant darkness (days 35–66) during which a 1-hour daily chicken pulse for 12 days occurred (vertical rectangles; days 45–56). Two animals entrained with a negative phase relationship (left panel), another two entrained with a positive phase relationship (middle panel) and animals in which entrainment did not occur (right panel). Grey background indicates dark phase. Squares during light phase, before decreased activity level, indicate feeding time on day 23.

The present findings support this model. It is apparent from all four of the experiments that the light cycle can be a *zeitgeber* for the circadian system of *T. infestans*. However, regular (daily) nonphotic stimuli also entrained the rhythms as shown in experiments II and III. Four significant characteristics of the nonphotic entrainment pattern are noteworthy:

(1) There were two distinct stable phase relationships established between the nonphotic stimulus and the circadian clocks. One of these occurred with the activity of the animal preceding the pulse (11 animals), and the other, more common relationship, had the animals' activity onsets lagging the stimulus by about 2 h (20 animals).
(2) The nonphotic pulses produced stable entrainment by causing significant phase advances.
(3) Nonphotic entrainment also tended to produce a shortening of τ_{DD}.
(4) Nonphotic entrainment failed when either (a) the stimulus fell during the animals' subjective night (active time) or (b) if τ_{DD} were already $\leq$24 h.

This pattern of entrainment can be explained most simply with a nonphotic PRC comprising phase advance responses during the animals' inactive time (subjective day), and either delays or no phase shift responses if the stimuli fall in the subjective night. This model is shown graphically in Figure 6. The hypothetical PRC depicted is similar in shape to PRCs that have been derived for circadian responses to arousal

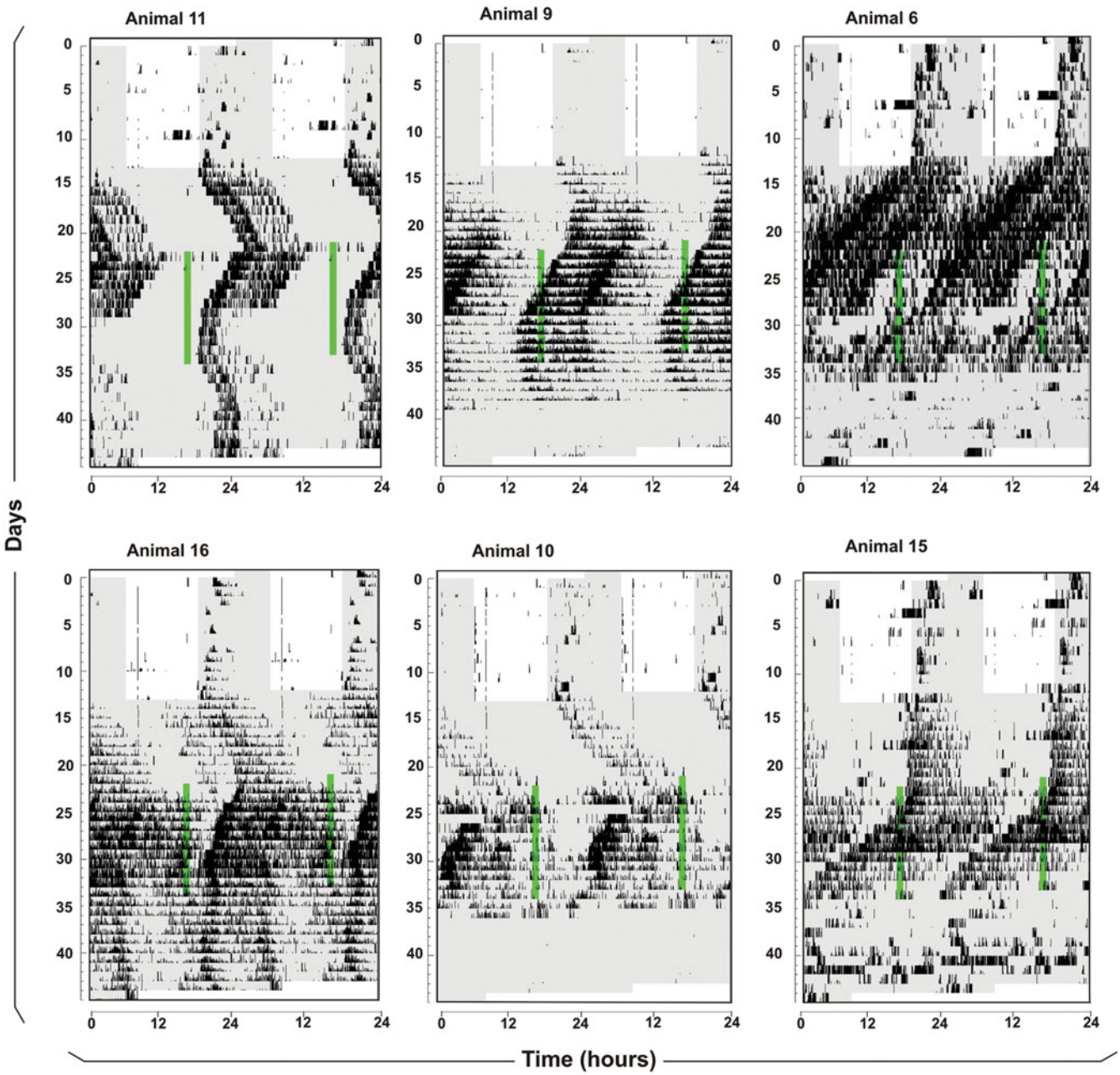

Figure 4. Kissing bug activity during photic and nonphotic (disturbance) cycles. Double-plotted actograms of six animals of experiment III submitted to a standard LD 12:12 h cycle (days 0–13) and then constant darkness (days 14–45) during which a 1-hour daily disturbance pulse for 11 days occurred (vertical rectangles; days 23–34). Animals entrained with either a negative phase-angle (left panel), positive phase-angle (middle panel) or did not synchronize (right panel), to the nonphotic stimulus (without a chicken). Grey background indicates dark phase.

described previously for rodents [e.g. Cain et al., 2004; Koletar et al., 2011; Mrosovsky et al., 1989). Although the exact shape would need to be verified using single stimuli presented in free-running conditions, the entrainment responses reported here are consistent with this hypothetical PRC, and suggest a mechanism by which the insects would be able to synchronize their foraging behaviors with the presence and rhythmic movement of prospective hosts. Arousal in this case could be caused by numerous factors, including chemical detection of the host–predator as a food source, as well as detection of the host's movement as a potential threat.

Triatoma infestans feeds on resting animals, so it is expected that the bug would be active when the host is inactive. In our experiments, nonphotically entrained bugs become active in about 2 h after the end of a pulse. The activity appears to represent anticipation of a quiescent host, as it persists from this phase with a circadian frequency after the pulses have ceased. However, while feeding is an obvious motivating factor that might be linked to locomotor activity or foraging

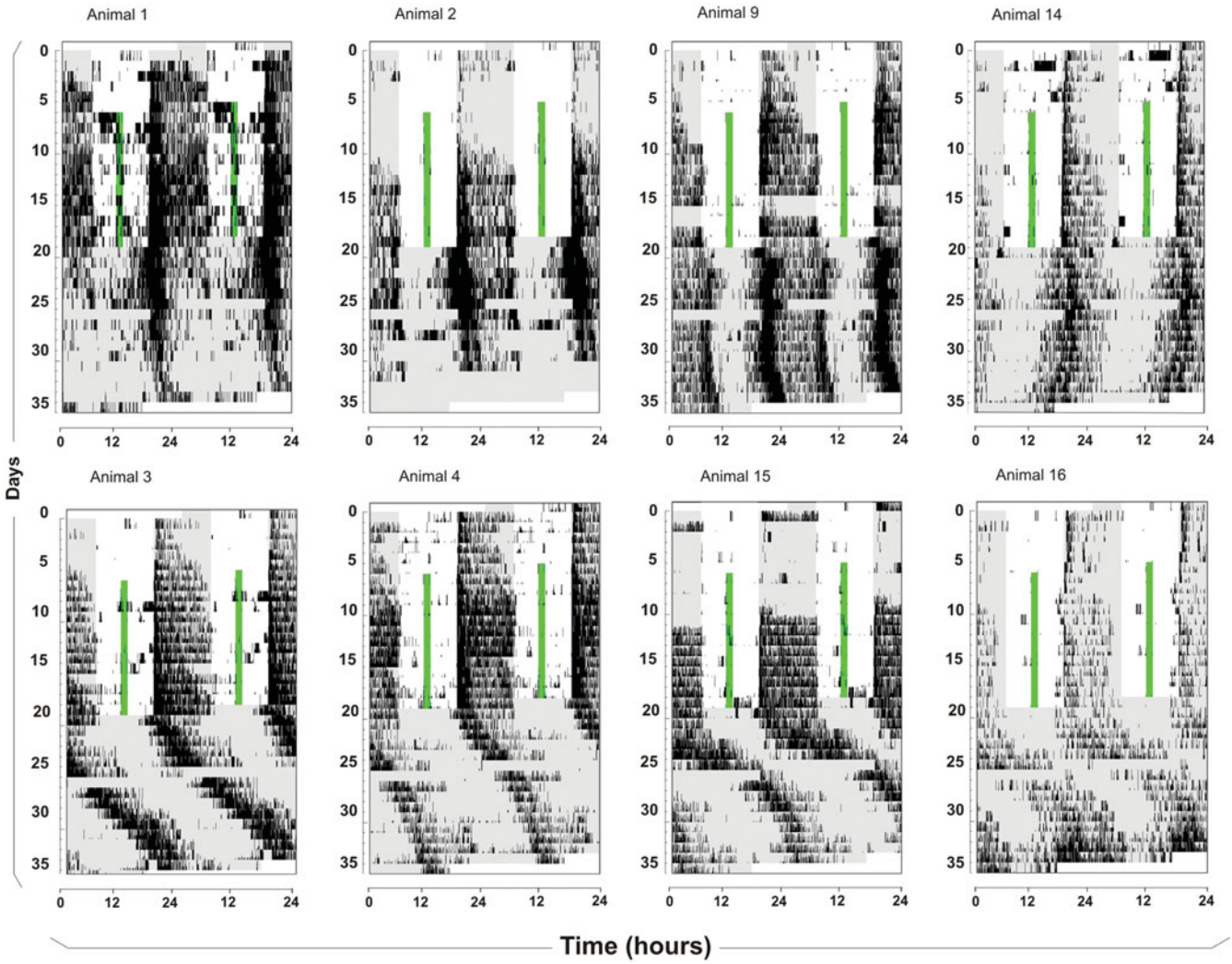

Figure 5. Kissing bug activity with simultaneous photic and nonphotic cycles. Double-plotted actograms of eight representative animals of experiment IV submitted simultaneously to photic and non-photic cycles. A standard LD 12:12 h cycle (days 0–11) during which a 1-hour daily disturbance pulse for 13 days occurred (vertical rectangles; days 7–19). Afterwards, constant darkness from days 19 to 35. Four animals showed a shorten free-run (top panel) and four animals a long free-run (lower panel). Grey background indicates dark phase.

behavior, animals in experiment 3 were entrained by disturbance alone. Therefore, some caution should be exercised in the interpretation of the animals' motivation, as escape to safety could be a factor also. It should be recognized, however, that neither disturbance nor the presence of food caused an acute change in the animals' locomotor behavior in any of the experiments.

In addition, in nature the animals will be exposed to both photic and nonphotic agents. Consequently experiment IV might be closer to situations that animals might encounter in nature. Two significant changes occurred when nonphotic pulses were applied during LD. First, ψ_{OL} was significantly advanced compared with entrainment in LD alone. Second, the free run in DD was significantly shorter than after entrainment to LD alone. Notably, τ_{DD} was significantly shortened by 12 days of nonphotic entrainment, which may help explain why period was shorter following LD + NP versus LD alone.

It is also important to recognize that although ψ_{OL} was significantly advanced in LD when the daytime nonphotic pulse was present, the relationship of activity to the nonphotic pulse ($\psi_{ONP(LD)}$) during LD + NP was not different from the relationship found in DD ($\psi_{ONP(A)}$), meaning that the timing of activity when the two *zeitgebers* are present together can be attributed to the nonphotic pulse, while light appears to have little influence on the phase of the underlying circadian oscillator. Therefore, circadian entrainment in the presence of the nonphotic stimulation during the day can be attributed mainly to nonphotic phase shifts and effects on the underlying circadian period.

This is not to say that light has no effect. On the contrary, the actograms presented in Figure 5 (experiment IV) demonstrate a clear masking effect of light that is represented by the difference in ψ_{OL} measured from activity during versus following entrainment (Table 1). Masking effects have been well studied in mammalian models of photic entrainment (for review, see Mrosovsky 1999) and contribute significantly to the temporal pattern of behavior during entrainment.

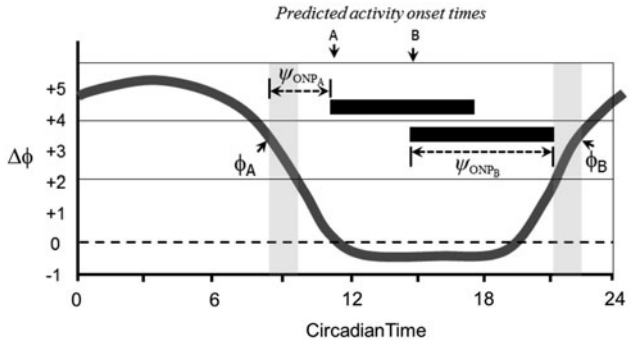

Figure 6. Hypothetical model of nonphotic entrainment. Entrainment characteristics of kissing bugs presented with daily nonphotic stimuli can be explained using the classic mammalian nonphotic PRCs (grey curve). Such PRCs include predominantly phase advance regions in the subjective day (CT0-12), with little or no delays (Reebs & Mrosovsky, 1989; Koletar et al., 2011). The hypothetical PRC includes two points (marked Φ_A and Φ_B) at which nonphotic phase shifts are sufficient for entrainment. It is assumed that the response is integrated over the entire stimulus exposure which is indicated by the shaded vertical bars. The timing of locomotor activity relative to Φ_A and Φ_B (indicated by horizontal black bars) and their phase relationships with the nonphotic stimuli (ψ_{ONPA} and $\psi_{ONPB)}$ reflect the empirical data. Point Φ_A is predicted to allow stable entrainment while point Φ_B allows sufficient potential for phase shifts but not entrainment (see text).

Locomotor records indicate that the natural behavior of the bugs is nocturnal, and very little locomotor activity is exhibited during the daytime. It seems likely that nonphotic entrainment could work in concert with photic masking as a mechanism for allowing animals to feed on multiple species, while restricting the animals' locomotor activities to times of day when the risk of predation would be lowest.

Overall, some of the general patterns of behavior observed in our experiments reflect and expand upon similar findings reported by others. Two different authors, Settembrini (1984) and Lazzari (1992), used the same type of rocking actimeters to measure the locomotor activity pattern of *T. infestans* reporting them as 5–10 day-long activity profiles. Settembrini observed unimodal patterns of activity that began with the onset of dark and $\tau_{DD} > 24$ h (24 h, 50 minutes), whereas Lazzari observed bimodal patterns of activity with peaks at the early scotophase and beginning of the photophase. These two peaks were ascribed to food and refuge search at dawn and dusk, respectively (Lorenzo Figueiras et al., 1994; Lorenzo & Lazzari, 1998; Johnson et al., 2004). In those experiments, activity remained bimodal in DD, with $\tau_{DD} < 24$ h (23 h, 50 min and 22 h, 22 min). Although our data are mostly consistent with Settembrini (1984), but with the average τ_{DD} even longer in our animals, we did obtain some examples that reflect the patterns reported by Lazzari (1992). Interestingly, in our experiments bimodality as described by Lazzari (1992) was associated with a shorter τ_{DD} that followed entrainment to a nonphotic stimulus (cf. Figure 4; animals #9, #11, and #16; Figure 5, animal #9).

Taken together, these findings emphasize the critical importance of nonphotic *zeitgebers* in the regulation of circadian rhythmicity and entrainment in *T. infestans*. Triatomines have a very high sensibility, capable of detecting diverse stimuli produced by their hosts. Moreover, this sensibility has been shown to express a rhythmic component (Barrozo & Lazzari, 2004; Barrozo et al., 2004a,b; Minoli & Lazzari, 2003). It seems reasonable that the bugs would entrain so that their activity is coincident with the inactivity of the host, regardless of whether the host is nocturnal or diurnal. In this way, the bugs can parasitize both diurnal organisms (e.g. human hosts and domestic animals) as well as nocturnal as needed. Our experiments demonstrate that diurnal entrainment in *T. infestans* involves (1) a relevant role for nonphotic factors in the entrainment of the circadian clock responsible for the anticipation of food availability and predation; and (2) an important role for light in the direct suppression of locomotor behavior in an important hematophagous parasite. The results demonstrate a heretofore unexplored model of nonphotic entrainment in nature, driven by the primary focus of the host as the parasites' source of food as well as the most dangerous attribute of its environment. Moreover, vector control as an approach to the eradication of Chagas disease may benefit from chronobiotic approaches that rely on an understanding of circadian regulation in this kissing bug.

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DECLARATION OF INTEREST

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