Circadian Regulation of Bioluminescence in the Prey-Luring Glowworm, *Arachnocampa flava*

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Abstract The glowworms of New Zealand and Australia are bioluminescent fly larvae that generate light to attract prey into their webs. Some species inhabit the constant darkness of caves as well as the dim, natural photophase of rainforests. Given the diversity of light regimens experienced by glowworms in their natural environment, true circadian rhythmicity of light output could be present. Consequently the light emission characteristics of the Australian subtropical species *Arachnocampa flava*, both in their natural rainforest habitat and in artificial conditions in the laboratory, were established. Larvae were taken from rainforest and kept alive in individual containers. When placed in constant darkness (DD) in the laboratory they maintained free-running, cyclical light output for at least 28 days, indicating that light output is regulated by an endogenous rhythm. The characteristics of the light emission changed in DD: individuals showed an increase in the time spent glowing per day and a reduction in the maximum light output. Most individuals show a free-running period greater than 24 h. Manipulation of the photophase and exposure to skeleton photoperiods showed that light acts as both a masking and an entraining agent and suggests that the underlying circadian rhythm is sinusoidal in the absence of light-based masking. Manipulation of thermoperiod in DD showed that temperature cycles are an alternative entraining agent. Exposure to a period of daily feeding in DD failed to entrain the rhythm in the laboratory. The endogenous regulation of luminescence poses questions about periodicity and synchronization of bioluminescence in cave glowworms.

Key words temperature, rainforest, Diptera, photophase, diurnal thermal cycle

Bioluminescence is a rare phenomenon in insects, being restricted to the fireflies (Coleoptera), some click beetles (Coleoptera), a small number of flies (Diptera; Harvey, 1952), and Collembola (Lloyd, 1978). The use of light to attract prey into a web is even more unusual, being confirmed in larvae of *Orfelia fultoni*, found in North America's Appalachian Mountains, and in larvae of *Arachnocampa*, commonly known as glowworms, found in Australia and New Zealand (Meyer-Rochow, 2007; Sivinski, 1998). In *Arachnocampa*, light is produced in modified, terminal cells of the malpighian tubules (Wheeler and Williams, 1915), the insects' excretory system. The cells are greatly enlarged and possess a dense cytoplasm with many mitochondria. They are innervated by neurons containing neurosecretory and neurotransmitter vesicles (Green, 1979). From ligation experiments, Gatenby (1959) concluded that neural

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connection between the terminal abdominal ganglion and the light-emitting cells is necessary to repress light output, speculating that nervous control prevents air diffusing from the tracheal reflector to the luminescent cells. A requirement for O_2 was demonstrated by a dimming response after evacuating air, and exposure to anesthetics such as CO_2 or ether causes intense glowing (Lee, 1976). The light-producing chemical reaction uses ATP, a luciferin and a luciferase (Viviani et al., 2002).

Colonies of larvae are generally found in rainforest embankments (epigean populations) or on the walls of wet caves (troglophilic populations). Individuals are confined to a snare that they enlarge as they grow. Each snare is made up of a horizontal mucous tube suspended from the substrate. Larvae produce a web of silk threads dotted with mucus droplets that hang from lines suspending the tube. When prey items, usually flying insects, are caught in the web, the larva hauls up the thread and consumes the prey (Broadley and Stringer, 2001).

The ecology of glowworms poses questions about the mechanisms the larvae use to regulate light output and their associated energy budgeting. It is well known that epigean glowworms switch on only at night and light output is essentially continuous through the night (Meyer-Rochow and Waldvogel, 1979), although, until now, it has not been specifically measured. Cave populations of *A. luminosa* in New Zealand have been reported to glow continuously (Gatenby, 1959; Richards, 1960). Video recording of cave *A. luminosa* during a 2-h block in the afternoon and another at night indicated that the duration of prolonged glowing bouts is longer during the day and reductions in intensity or interruptions in light output were more likely to occur during the night (Ohba and Meyer-Rochow, 2004); however, phase and synchronization of individuals was not investigated.

Exposure to light causes glowworms to decrease their light intensity or to completely douse within a few minutes. Also, larvae are negatively phototactic, preferentially moving to darker environments (Meyer-Rochow and Waldvogel, 1979). The simplest conceivable rule governing light output is for bioluminescence to be de-repressed in darkness and repressed on exposure to light; however, given the diversity of light regimens experienced by glowworms in their natural environment we suspected that true circadian rhythmicity could be present. We set out to establish whether glowworms placed in DD show a continuing rhythm of bioluminescence.

MATERIALS AND METHODS

Laboratory Recording

Arachnocampa flava were collected from Springbrook National Park, Queensland. Larvae were placed into halved, inverted plastic containers (77 mm height × 70 mm diameter) with moist clay pressed into the base (Takaie, 1997). The front of the chamber was covered with clear plastic and the inverted lid was filled with water to maintain high humidity in the chamber. After 1 week of acclimation, 12 to 15 larvae in chambers were placed into a glass aquarium with free water in the base. The aquaria were placed in light-tight cabinets and a Firewire camera (Sony XCD-X710, 8 mm focal length lens) focused on the glowworms. Artificial light was produced by a desk lamp with 12-V 50-W halogen bulb connected to an electronic timer switch. Light intensity at the larvae was measured at 23 lux (DSE Q-1400 lux meter). The timing of light on and off was computer controlled using the software Indigo (version 1.6.0) by Perceptive Automation (Dallas, TX). The camera was connected to a Mac computer running Evological EvoCam (version 3.5) to grab individual frames (1024 × 768 pixels, 8 bit gray scale) at 10-min intervals. Temperature and humidity in the cabinets were recorded using Tinytag data loggers (Chichester, West Sussex, UK). Temperature was maintained at 23 ± 1 °C.

Exposure to LD, DD, and Skeleton Photoperiods

Fifteen individuals in cabinet A were exposed to 12:12 h LD for 5 days and then kept in constant darkness (DD) for 28 days. As a control, 12 individuals in cabinet B were exposed to 12:12 h LD for the same period. The light was switched on at 0600 h and off at 1800 h for all 12:12 h LD settings (all times are Australian Eastern Standard Time). For exposure to skeleton photoperiods, larvae were exposed to 7 days of DD, followed by 7 days of 1L:11D:1L:11D, followed by a further 7 days of DD.

To expose larvae to different phases of LD after DD, 12 larvae were placed in each cabinet and exposed to DD for 7 days. Then a 12:12 h LD phase was applied to cabinet B, and cabinet A was simultaneously exposed to an inverted day-night light exposure 12:12 h DL, with light on at 1800 h and off at 0600 h. After 5 days of LD or DL exposure the larvae in both cabinets were exposed to constant darkness (DD) for a further 7 days.

In these experiments larvae were fed weekly. Two *Drosophila* adults were placed in the snare of each larva at about 1800 h. Dim red-filtered white LED light from a headlamp was used for illumination during feeding. Exposure of the glowworms to light was kept to a minimum. Thus glowworms in the D:D condition were exposed to dim light for approximately 30 min at feeding time each week.

Persistent Feeding in DD

The experiment was conducted to investigate whether the provision of food acted as a cue for resetting the phase of the bioluminescence rhythm. The protocol was: 5 days unfed DD, 14 days DD fed daily, 9 days unfed DD. During the feeding exposure period, 3 *Drosophila* adults were fed to each larva (n = 13) every day starting at 1800 h as described above. Thus larvae were exposed to dim light for approximately 30 min at feeding time. After the feeding period, glowworms were unfed for an additional 8 days in DD.

Thermal Cycles in DD

To determine whether exposure to a daily thermal range could reset the phase of luminescence, 2 groups of 10 glowworms were placed in aquaria within light-tight programmable incubators for time-lapse computerized imaging as described above. In 1 experiment, both groups were initially exposed to LD 13:11 for 24 h to establish a baseline bioluminescence output followed by 4 days of DD at 23 °C to establish the free-running periods of each individual. They were then exposed to a 23 °C to 20 °C temperature square wave on a 12:12 cycle: 1 group experienced low temperature (20 °C) during the external scotophase (lowered at 1800 h and raised at 0600 h) and the other was exposed to continuous 20 °C during the same period (7 days), followed by DD at 23 °C for another 6 days. In a second experiment, 2 groups of 10 glowworms were exposed to triangular temperature waves for 10 days, after initial exposure to LD 12:12 on day 1 and DD for days 2 to 4. After the exposure to thermal cycles they were observed for another 6 days at 23 °C. The triangular thermal cycles were approximated by programming the incubator to make 10 stepwise changes each day, with 1 treatment peaking at 1800 h, the other at 0600 h. Temperatures were monitored using Tinyview or Tinytag data loggers (reading resolution, 0.02 °C or better; accuracy, ±0.4 °C).

Field Recording

Field recording of an *Arachnocampa flava* colony located in the banks of a rainforest gully with intermittently flowing stream took place in Springbrook National Park (28°12.5'S, 153°17.5'E) in August 2005 (southern hemisphere winter). A tripod-mounted digital camera (Nikon Coolpix 4500) with external 12-V power source was focused on the colony. A Harbortronics DigiSnap 2000 external programmable remote control unit was connected to the camera to capture images at 10-min intervals. Exposure was set at 8 sec, f2.8, ISO 400. Temperature and relative humidity were measured using a Tinytag data logger (reading resolution, 0.02 °C or better; accuracy, ±0.4 °C).

Light Intensity and Rhythmicity Analysis

The application "ImageJ" version 1.37d (or later versions) was used to analyze light output of individuals and groups of glowworms. Images were converted to 8-bit gray scale and a threshold level was selected to separate the luminescence signals from background noise. The intensity of each individual was calculated at each time point using the "integrated density" calculation in the "analyze particles" command. It calculates the sum of pixel values above the threshold level within any selected area. The units for light output are arbitrary and were not calibrated against standards. The data were transferred to Microsoft Excel and Canvas X (ACDSee) for graphical output. To provide a visual impression of trends, circadian double plots were produced by graphing the bioluminescence activity record in a column series.

Rhythmicity of bioluminescence was calculated using a comprehensive suite of MATLAB version 7.2.0.283 m-files (Levine et al., 2002). Autocorrelation analysis was used to determine whether rhythmic signals are present in the data and to produce a rhythmicity index (RI), an estimate of the strength of the rhythmicity (maximum = 1) based upon the value of the 3rd peak of the correlogram. The rhythmicity statistic (RS) is the ratio of the RI to the absolute value of the 95% confidence line. RS > 1 indicates the rhythm is significant. For analysis, the data were normalized, then Butterworth filtered (low- and high-pass filters set at 2 h and 72 h, respectively) to eliminate low- and high-frequency noise. Autocorrelation analysis was then used to establish whether rhythmicity is present in the data, shown graphically by a sinusoidal curve with peaks and troughs exceeding

the level set by the confidence line on the correlogram. To calculate the peak phase of light output in thermal cycle experiments, the light output of individuals or mean light output of a group was smoothed using Butterworth filtering (16 h low-pass filter) and the time of occurrence of each peak recorded using the "peakphaseplot" function (Levine et al., 2002).

RESULTS

Bioluminescence under Light-Dark Cycles (LD)

For laboratory recording, larvae were taken from rainforest and placed individually in clay-roofed chambers where they readily rebuild their snares. Larvae are sensitive to disturbance such as exposure to vibration and destruction of the snare; consequently a completely nonintrusive method of light detection using time-lapse video monitoring was developed by which the relative intensity of light output in uncalibrated units could be monitored over long periods. In the scotophase, individuals' light output varies over time and occasionally they completely douse. Time-lapse video recordings using infrared illumination (data not shown) show that activities such as addition of new silk lines, removal of old lines, feeding and defecation take place through the night as described by Broadley and Stringer (2001) for *A. luminosa.* The total light output of a group of 15 larvae over 28 days under artificial photoperiod (12:12 L/D) in phase with the external photoperiod is shown in Figure 1A. The saw-toothed characteristic of light output through the night is demonstrated by averaging the light output of the 15 larvae over 10 consecutive nights (Fig. 1B). Bioluminescence reaches a peak about 2 h after lights-off. The proportion of larvae glowing peaks at about the same time (Fig. 1B). The group's light output gradually decreases through the night, primarily due to a decrease in individuals' light emission levels because the proportion glowing decreases only slightly through the night. Glowworms show this characteristic light-emission pattern over a long term in the laboratory where individuals have been kept for many months until they pupate. The same pattern is seen in the wild. Time-lapse photography of a group of approximately 20 larvae in a rainforest gully showed a peak light emission at approximately 2200 h (Australian Eastern Standard Time), decreasing thereafter (data not shown).

Bioluminescence under Constant Darkness (DD)

At the same time as the light output of glowworms in LD 12:12 was recorded, a second group (n = 15) with identical preparation was exposed to 28 days of constant darkness. The light output of the group appears as a progressively damped series of cycles in which the peak intensity decreased and the trough increased over time (Fig. 1C). A graph of the mean daily light output of the group, averaged over the first 5 complete 24-h periods (Fig. 1D), shows that the onset and switch-off of bioluminescence was gradual compared with the glowworms exposed to LD (Fig. 1B). Both the number glowing and mean light output steadily varied in a sinusoidal pattern.

Because the damping evident in the group response over a long time span could be the result of rhythmic individuals becoming progressively out of phase in free-run, the rhythmicity of 11 individuals was analyzed in detail (1 larva ceased to glow and 3 pupated during the experiment and were not analyzed). All larvae showed detectable periodicity over the full 28-day period. Individuals' free-running periods, calculated using autocorrelation analysis (Levine et al., 2002), varied between 24.2 h and 26.0 h (mean 24.8 h). The mean RI did not vary substantially when analyzed over the 4 contiguous 7-day intervals (Table 1). As a comparison, the RI for the total 28 days and 4 consecutive 7-day intervals was calculated for the total light output of the 15 individuals exposed to LD in the parallel, control experiment (Table 1). The RI of 0.77 indicates the value achievable under reentraining conditions, compared with the RI of 0.47 for DD larvae over the same period. Visual observations of double plots show that, over time, individuals tend to decrease the maximum intensity of the light they emit each subjective day and increase the minimum, with shorter periods spent not glowing per cycle.

Weekly feeding produced a marked increase in the peak mean response curve following feeding. Nine of the 13 individuals showed an increase in daily maximum light output upon feeding at day 7 and subsequently on days 14 and 21, sufficient to produce a spike in the graph of total light output (Fig. 1C). Four showed a reduction in light output after the feeding events.

Phase Entrainment by Light-Dark Cycles

To determine whether exposure to LD entrains the rhythm, 2 groups of glowworms were exposed to

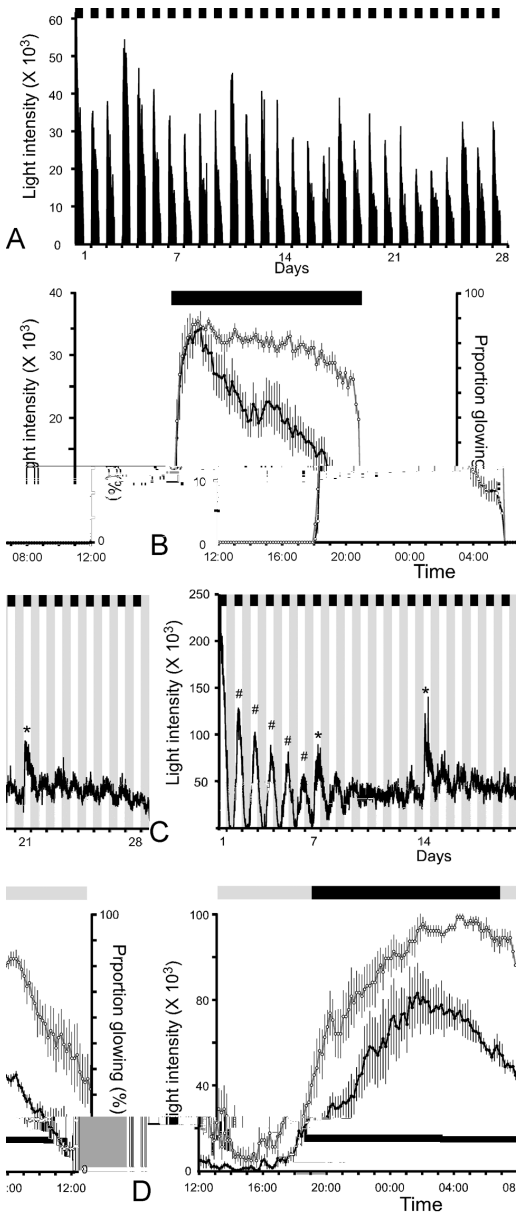

Figure 1. Glowworms in constant darkness show damping of the luminescence rhythm. (A) Total light output of 15 *A. flava* glowworms in the laboratory over 28 consecutive days under LD 12:12 conditions. Photophase is indicated by light-dark bars with dark indicating lights-off. (B) Mean light output (±SE) of 15 glowworms in the laboratory over 10 consecutive 24-h periods and the mean proportion glowing as the night progresses. Lights-off occurred at 1800 h and lights-on at 0600 h with dark bar indicating lights-off. Light intensity (filled circles) is in uncalibrated pixel-value units. Proportion glowing (unfilled circles) is a percentage (±SE). (C) Total light output of 15 glowworms over 28 days under DD conditions. Light intensity is in uncalibrated pixel-value units. Times when larvae were fed are indicated with asterisks. Gray bars are provided as a reference showing the external photophase. (D) Mean light output of 15 glowworms over 5 consecutive 24-h periods under constant darkness (corresponding to peaks under hash marks in C). Symbols same as for B.

Table 1. Free-running period (τ) and rhythmicity index of glowworm larvae exposed to different light regimens.

DD	*N*	*τ*	*Rhythmicity Index (RI; Mean ± SEM)*	*LD*	*τ*	*RI*
DD_{1-28}	11	24.8 ± 0.17	0.47 ± .03	LD_{1-28}	24.0	0.77
DD_{1-7}	11	25.1 ± 0.19	0.40 ± .04	LD_{1-7}	24.1	0.57
DD_{8-14}	11	25.1 ± 0.46	0.34 ± .04	LD_{8-14}	24.0	0.57
DD_{15-21}	11	24.9 ± 0.41	0.25 ± .04	LD_{15-21}	24.0	0.60
DD_{21-28}	11	24.7 ± 0.17	0.38 ± .04	LD_{21-28}	24.1	0.48

NOTE: For the constant darkness (DD) treatment, individuals were assessed over the full 28 days of observation (DD_{1-28}) or in 7-day blocks and the mean ± standard error was calculated. For the LD treatment the free-running period and rhythmicity index for the same periods were calculated using the total light output of 15 glowworms over 28 days.

constant darkness for 7 days, followed by 12:12 light-dark cycles for 5 days, followed by DD for 5 days (Fig. 2). In 1 group, termed LD (n = 9), the light-dark cycle was in phase with the external cycle to which the subjects were entrained before the onset of DD; in the other (n = 14), it was 180° out of phase (termed DL). The aim was to vary the relationship between the circadian phase and the exposure phase.

The group response and 2 typical individuals for each treatment are shown in Figure 2. During the initial 7 days of DD the light output followed the free-running, damped pattern revealed in the previous experiment (Fig. 1C). During exposure to LD, the light intensity pattern matched the characteristics of control LD cycles, that is, a saw-toothed pattern with an initial peak after lights-off followed by a progressive decline through the scotophase. Visual inspection of the group (Fig. 2A1, B1) and individuals (Fig. 2A2, A3, B2, B3) show that after exposure to either LD or DL, individuals resumed free-running with a phase relationship matching that established in the preceding LD photophase. The resulting entrainment indicates the circadian rhythm is sensitive to the environmental photoperiod and is reset by several days of exposure to a 12:12 LD photoperiod.

Phase Entrainment by a Skeleton Photoperiod

To assess whether skeleton photoperiods could reset the phase, a group of 15 glowworms was initially placed in DD for 7 days, then in the skeleton regimen, 1L:11D:1L:11D, with the 1-h light pulses at 0600 h (circadian time 0, CT0) and 1800 h (CT12) for 7 days, followed by 7 days of DD. During the skeleton regimen, larvae responded after the dusk pulse

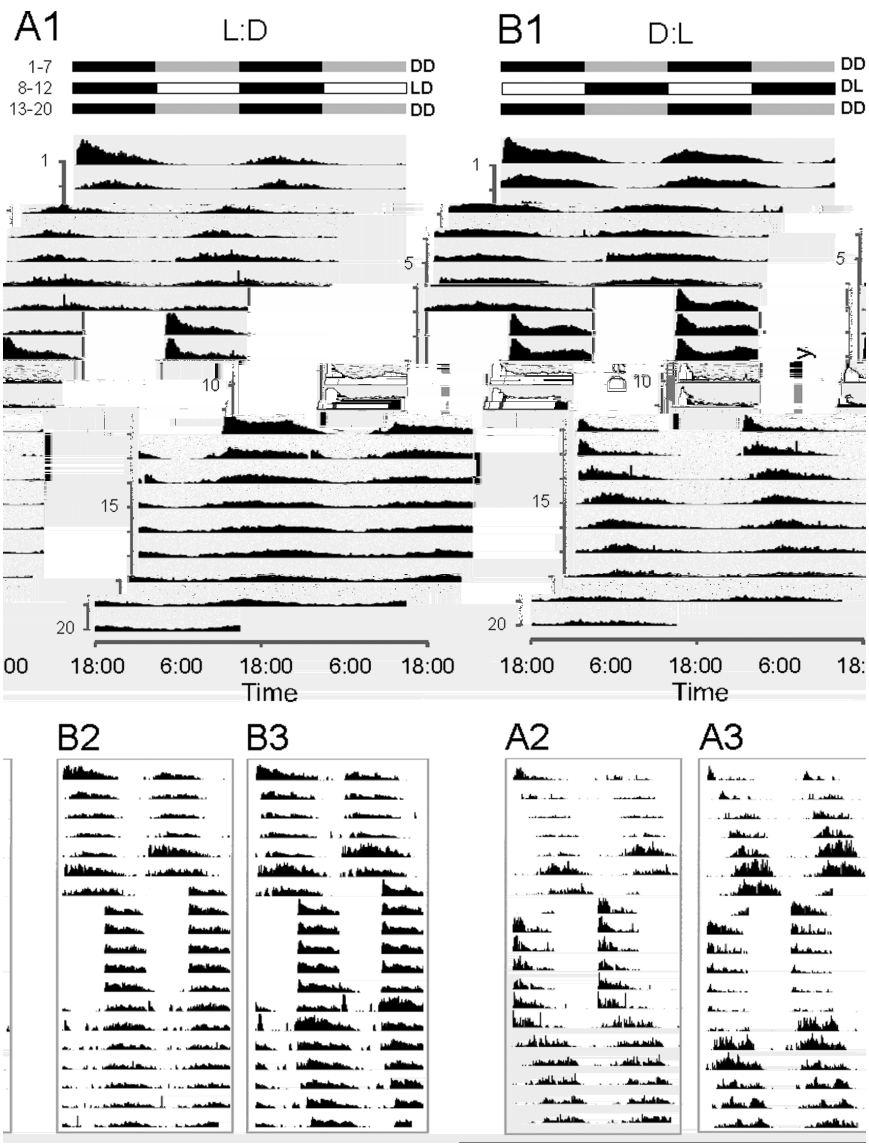

Figure 2. Exposure to light-dark cycles entrains the luminescence rhythm. Circadian double plots showing phase entrainment induced by exposure of glowworms in DD to 5 days of LD 12:12. (A1, B1) The light output of groups that are initially free-running (days 1-7) followed by 5 days of LD 12:12. In A the LD cycle matched the external photophase to which larvae were exposed before the experiment began, and in B it was 180° out of phase. In both cases, reversion to DD saw the larvae revert to free-running with an initial bioluminescence onset indicating that phase entrainment had occurred. A2, A3, and B2, B3 are plots of representative individuals exposed to the 2 light regimens. In all panels, the axes are the same as depicted in A1.

with an immediate postexposure rebound (Fig. 3), similar to the saw-toothed light-onset pattern seen in 12:12 LD conditions but not as pronounced. The dawn pulse was not followed by a rebound; instead light output was markedly reduced. Subsequently, light output increased in the late afternoon preceding the dusk light pulse but was extinguished at its onset. Compared with larvae kept in DD, the imposition of the skeleton photoperiod substantially compressed bioluminescence output into the period CT12-24 and this compression was subsequently released on reexposure to DD. The phase induced by the skeleton photoperiod matched the preexposure phase, indicating that, based on this group response, most individuals were phase entrained by the skeleton photoperiod.

Phase Entrainment by Daily Thermal Cycles

To determine the effect of thermal regimen on the luminescence cycle, *A. flava* were first exposed to DD at a constant temperature (23 °C) to allow them to free-run for several days. One group was exposed to a 3 °C 12:12 square-wave thermal cycle with the step-down to 20 °C occurring at 1800 h and step-up to 23 °C at 0600 h (Fig. 4A). Upon exposure to the thermal cycles, the luminescence period became fixed at close to 24 h (mean, 24.2 ± 0.1 h) with the peak phase occurring at 0108 h (the mean of the 7 peaks) during the cycling period. The control group, exposed to continuous 20 °C through the period when the treated group was exposed to temperature cycles, showed a continuation of free-running throughout their exposure to the reduced temperature and thereafter on reexposure to 23 °C, seen as an upward trend in the time of occurrence of the peak phase throughout the duration of the experiment (Fig. 4A).

In a second experiment, larvae were exposed to symmetrical, triangular-wave thermal cycles of periods of 24 h to more closely match the wave-form of the natural thermal cycle: 1 group was exposed to the naturally occurring phase (thermal minimum at 0600 h, maximum at 1800 h); the other 180° out of phase. Initially, both groups were exposed to DD at a

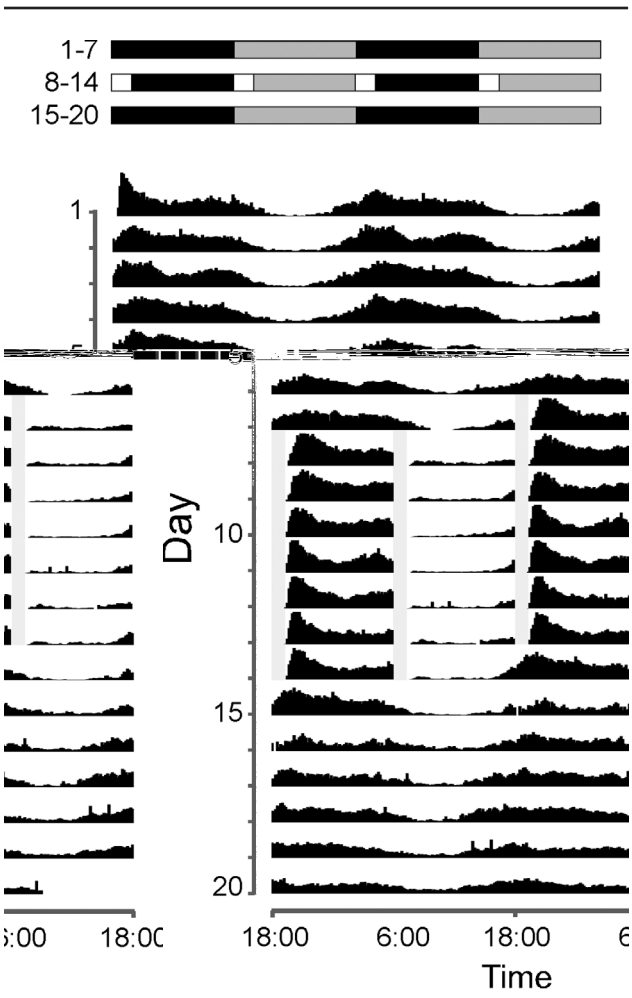

Figure 3. Skeleton photoperiods entrain the luminescence rhythm. Circadian double plots showing response of 15 *A. flava* larvae to the imposition of skeleton photoperiods. Larvae were exposed to 7 days of DD, followed by 7 days of 1L:11D:1L:11D. In the plot, gray bars indicate exposure to light.

constant 23 °C to allow them to free-run for several days. The group exposed to the natural thermal phase maintained a static period, close to 24 h, with the peak occurring at 0111 h (mean of 10 peaks; Fig. 4B, lower trace). The second group, exposed to a thermoperiod 180° out of phase, showed a long free-running period over the next 7 days, before plateauing at a 24-h cycle with the peak phase occurring at approximately 1300 h, matching the relationship to the temperature cycle seen in the companion treatment. Upon return to constant temperature, the graph of time of peak phase trends upward once again (Fig. 4B). The peak phase on day 13 in this trial is unreliable because there was an overall increase in luminescence amplitude of the group due to feeding on that day (Fig. 4C). Our interpretation is that the phase mismatch between thermoperiod and endogenous luminescence rhythm produced a number of transient cycles before the luminescence rhythm phase locked to the same point of the thermal phase seen in the companion treatment (approximately the midpoint of the falling phase).

Phase Entrainment by Feeding

To investigate whether feeding could reset the phase of individuals' rhythms, 13 glowworms were maintained in DD for 5 days to allow their free-running period to be assessed. They were then fed daily for 14 days in DD, then 8 days unfed in DD. Feeding occurred at the same time every day, and some exposure to dim red light occurred. Daily feeding accelerated the onset of pupation, with 4 of the 13 larvae pupating and ceasing to glow in the course of the experiment. The light output of the 9 consistently glowing individuals began to increase with the onset of the daily feeding regimen, reaching a maximum after 8 to 11 days, followed by a decline over the last few days (Fig. 5A, B). The decline continued after feeding terminated.

There was no obvious sign of phase entrainment due to the daily feeding regimen. Visual assessment of the individual circadian double plots shows that 5 of the 9 individuals maintained a steady free-running period consistently greater than 24 h during and after the feeding regimen (Fig. 5C: 1, 4, 5, 6, 8), indicating that the luminescence phase was unaffected by daily feeding. Of the remaining 4 individuals, 1 was arrhythmic over the last 8 days (Fig. 5C: 2) and 3 showed free-running periods close to 24 h (Fig. 5C: 3, 7, 9); hence phase resetting could not be reliably assessed. The mean free-running period of the 9 individuals that remained as larvae through the 14-day feeding regimen was 24.8 ± 0.2 h (±SE), based on autocorrelation analysis.

DISCUSSION

Glowworms Show a Persistent Rhythm of Bioluminescence in DD

A. flava larvae taken from rainforest and placed in constant darkness in the laboratory maintained rhythmic luminescence, indicating there is an endogenous component to their light output. Most

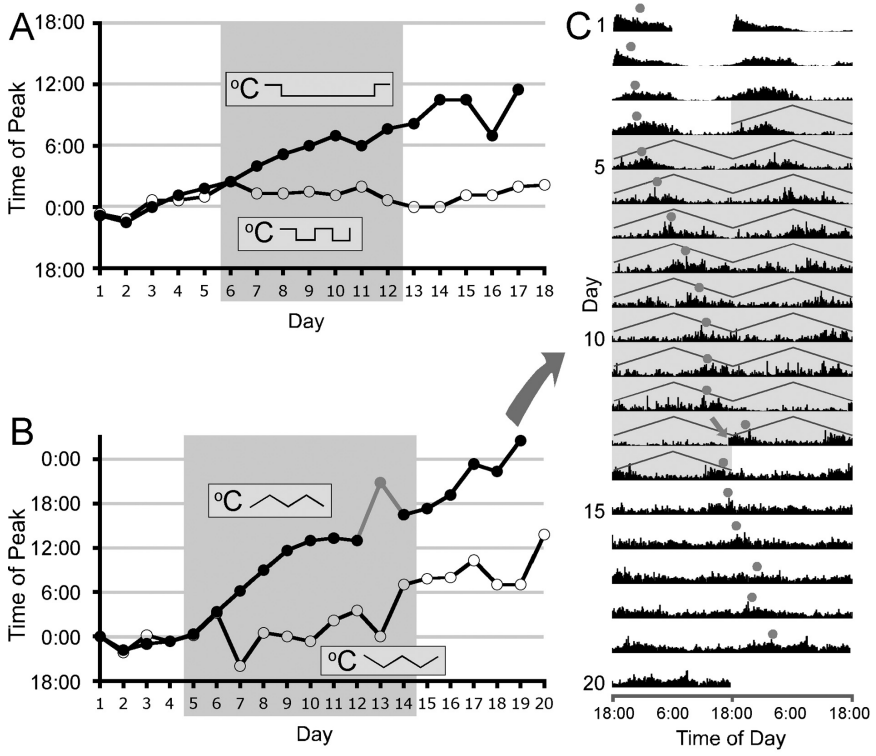

Figure 4. Temperature cycles entrain the luminescence rhythm. Glowworms were kept in constant darkness before exposure to a temperature square wave (A) or triangular wave (B). In A and B, each plotted point represents the time of day (shown on the *y* axis) of each daily peak, calculated using peak phase analysis. A flat line connecting points indicates that the glowworms have a luminescence period of 24 h, a rising line indicates a period > 24 h. The gray band indicates the period of exposure to the altered temperature regimen (days 6-12). In A (upper trace) larvae were exposed to a continuous 20 °C through days 6-12, acting as a control. Bioluminescence peak analysis showed that larvae continued to free-run with a period greater than 24 h, shown as a rising trend. In A (lower trace), larvae were exposed to a temperature square wave, varying between 23 °C and 20 °C. For the period of exposure (gray band) the larvae became entrained to a 24-h period. In B, larvae in both treatments were exposed to triangular temperature waves of a 24-h period, 180° out of phase with each other, through days 5-14 (gray band). As in A, the temperature varied between 23 °C and 20 °C. In the lower trace (open circles), the thermophase matched the photophase that the larvae were exposed to prior to the start of the experiment, that is, the temperature minimum occurred at 0600 h and the maximum at 1800 h. In the upper trace (closed circles), the thermophase was 180° out of phase with the preexposure photophase, that is, minimum at 1800 h and maximum at 0600 h. Phase advance occurred during the exposure period until a plateau level was reached with the peak occurring at about 1200 h on days 10-12 (see text for more detail). C is a circadian double plot of the upper trace of (B). The time at which the peak phase occurs is shown as a gray circle on each 2-day line. The triangular temperature wave is superimposed to provide a reference point. The time of peak becomes fixed at approximately the midpoint of the dropping temperature phase on days 10-12. An aberrant peak phase point in B (gray point and line) and C is due to an increase in light output occurring at the time of feeding (arrow).

individuals showed a free-running period greater than 24 h. The characteristics of individuals' light output changed over time in constant darkness. They developed a gradual onset and offset of glowing, resulting in an increase in the time spent glowing per 24-h period. They also showed a decrease in the daily maximum brightness and an increase in the daily minimum brightness. In terms of the curve of light output, exposure to DD produced a damped curve with a reduced daily maximum amplitude and an increased daily minimum amplitude. This was not an artifact of the experimental setup because control glowworms kept in identical conditions in a 12:12 LD light regimen continued to show a characteristic saw-toothed pattern of light output. Also, glowworms reexposed to LD cycles after many days of DD immediately readopt the saw-toothed pattern (Fig. 2).

Light as a Masking Agent

The experimental results are consistent with *A. flava* possessing an underlying sinusoidal rhythm that is masked and reset by exposure to light. We hypothesize that as dusk approaches, the propensity to glow increases but the output remains masked by ambient light. A similar masking effect of light on activity of nocturnal insects such as fireflies and cockroaches has been postulated (Dreisig, 1976, 1978; Dreisig and Nielsen, 1971). The initial peak after lights-off seen in laboratory experiments is due to release of the masking effect, resulting in the saw-toothed pattern of light output. Similarly, exposure to skeleton photoperiods showed that the dusk light pulse elicits a rebound, consistent with this pulse inhibiting light output. The dawn light pulse suppresses subsequent light output, consistent with a waning of propensity to glow around dawn.

The complete removal of masking in DD reveals the underlying rhythm. The absence of natural light cycles for reentrainment eventually leads to almost continuous glowing, but individuals' rhythmicity is maintained for at least 28 days, fluctuating around an intermediate level of light intensity.

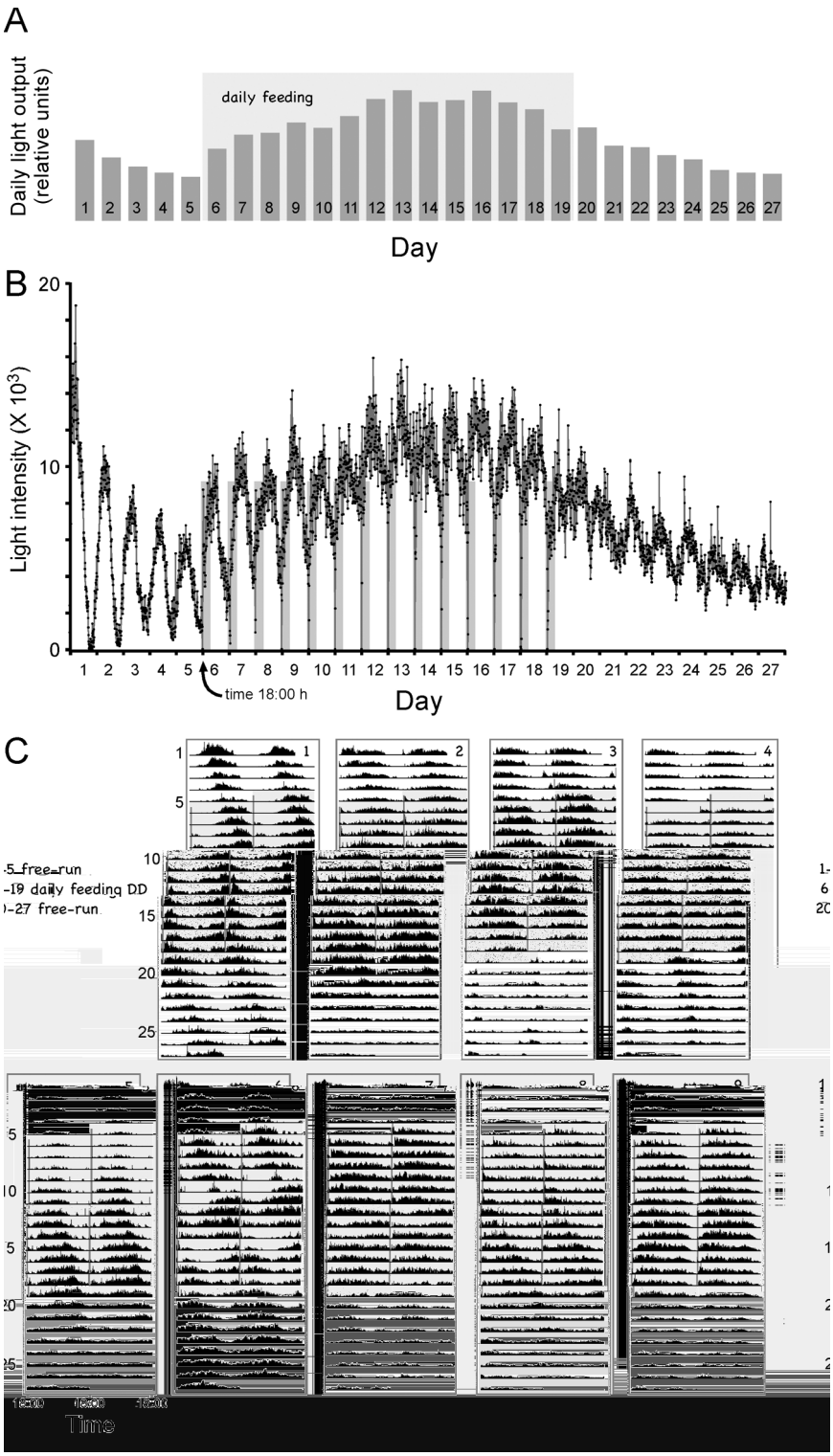

Figure 5. Daily feeding increases light output. (A) Daily light output of a group of 9 *A. flava* that were exposed to DD for days 1 to 5 and then fed daily at 1800 h over the next 14 days. Daily light output is the sum of readings for 24 h from 1800 h. (B) A plot of mean light intensity at 10-min intervals of the same 9 larvae. Feeding time is shown as a series of gray bars. In both A and B, light intensity is in uncalibrated units. (C) Circadian double plots of the light output of the 9 individuals. The 14-day feeding period is shaded gray and the actual feeding times are shown as dark gray bars. The axes shown in panel 5 apply to all plots in part C.

Amplitude damping is probably due to the maximum light output being reduced in compensation for the longer period of light production per day. There could be a limit to the energy available for light production or the metabolites luciferin or luciferase used in producing bioluminescence. In support, the time series plot of light output under DD (Fig. 1C) shows peaks at 7-day intervals associated with feeding, perhaps due to replenishment of energy stores and increased allocation of energy to light production. The elevated luminescence levels after feeding are consistent with prey attraction as the primary function of bioluminescence (Broadley and Stringer, 2001): the behavior can be interpreted as capitalizing on the presence of prey by emitting more brightly to capture even more. Daily feeding also resulted in markedly increased mean light output, followed by a decline after 10 days (Fig. 4). The decline could be a response to excessive food levels: when food is in oversupply it may be advantageous to stop bioluminescing to prevent prey items damaging the snare. Experiments currently under way on *A. flava* show that vibration stimuli produce an acute elevation of light output followed by a decay over 15 to 30 min; hence the long-term light increases in response to feeding seen in this study are more likely to be a response to increased nutrient supply than an acute arousal response.

The reason for individual variability in response to weekly feeding, some individuals increasing, others decreasing their light output, is not clear. All larvae were taken from the wild and there is no known method of in vivo staging, so it is possible that larval stage or previous nutritional history could explain the direction of the response.

Temperature

Temperature is recognized as the next most common zeitgeber in poikilotherms after light. Experimentally imposed temperature shifts have been shown to cause phase shifting in a number of insects (Saunders, 2002). In general, rises in temperature simulate lights-on and drops simulate lights-off, in accord with the diurnal thermal cycle in which the thermophase coincides with photophase and cryophase with scotophase (Beck, 1991). In nocturnally active insects, temperature steps up tend to cause phase delay while steps down cause phase advance (Dreisig, 1976, 1978; Nielsen, 1984). Here, exposure of *A. flava* to a square-wave daily thermal range of 3 °C caused phase locking of light output to the middle of the cryophase, the peak luminescence occurring around midnight. Exposure to triangular waves caused phase locking to the middle of the falling phase, also corresponding to approximately midnight in a natural hypogean thermal cycle. These experiments showed that the phase of thermally induced luminescence rhythms matches the phase of light-induced rhythms, in accord with the thermophase/photophase linkage characteristic of nocturnal insects.

CONCLUSIONS

We conclude that glowworms exhibit true circadian regulation of their light output. Light acts as both an entraining agent and a masking agent. The dominant role of light in establishing the characteristics of the light output rhythm raises questions about the rhythmicity and period of bioluminescence within caves where glowworms have never been exposed to daylight. A number of species such as *A. luminosa* from New Zealand and *A. tasmaniensis* from Tasmania, Australia, have large populations in caves as well as in rainforest. Based on laboratory analyses of *A. flava*, glowworms in caves would either be arrhythmic because they have never been exposed to photic entrainment cues, or would be rhythmic but individuals in a colony would be asynchronous because they have different free-running periods. It will be of interest to establish the rhythmicity and phase of luminescence in cave-dwelling glowworm populations. The fact that members of the genus *Arachnocampa* inhabit both photoperiodic and aphotoperiodic habitats makes them ideal for examination of the retention of circadian rhythmicity in cave environments where very few circadian cues are present.

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