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Control of Flashing in Fireflies

IV. Free Run Pacemaking in a Synchronic *Pteroptyx*

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Summary. Males of the firefly *Pteroptyx cribellata* of Papua New Guinea luminesce spontaneously in two principal modes: a regular one-per-second display flash (Fig. 1A) and an irregular flicker of 3–10 peaks per second (Fig. 1B). In free run rhythmic display flashing by intact, restrained individuals, serial correlation analysis of interflash duration in successive cycles indicates that the variability of the brain-to-lantern excitation delay is negligible in comparison with the variability of the endogenous timing process (Figs. 6, 7). It is therefore possible to use the duration of the flash-to-flash interval of the intact firefly as a measure of endogenous pacemaker timing behavior. It is deduced that the cycling of the pacemaker is continuous, does not require that the animal see his own flash or even that he flash (Fig. 2A), shows intercycle independence (Fig. 5) and may phase-shift its rhythm spontaneously upon occasion (Fig. 2C). Pacemaker period is normally distributed (Fig. 3), is not correlated with flash intensity, and appears to shorten slightly if a flash is skipped (Table 3). The occurrence of spontaneous flash skipping is taken to indicate that the timing process that measures pacemaker period can cycle independently of its usual triggering of the flash-excitation message to the lantern.

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Introduction

Male fireflies of many species flash spontaneously at a regular species- and temperature-dependent frequency. This free run rhythm is timed by a pacemaker in the brain that periodically triggers a neural volley which runs down the cord to the light organ near the tip of the abdomen and there evokes a flash (Case and Buck 1963; Buck et al. 1963; Buonamici and Magni 1967; Magni 1967; Carlson 1969; Bagnoli et al. 1976; Brunelli et al. 1977).

In certain species of tropical Southeast Asia the males habitually assemble in trees in large congregations and flash rhythmically in unison, supposedly as a mating adaptation (Buck and Buck 1978). Because mass synchrony is almost unique in the animal kingdom the physiology of the behavior has attracted much attention (review: Buck and Buck 1968). In particular, since the mutual entrainment must involve phase-shifting the times of flashing of the individual fireflies, the mechanisms by which light induces this effect on the pacemaker has become a focus of interest. In a preliminary paper we reported that intact restrained males of *Pteroptyx cribellata* from New Britain could be entrained to certain rhythms of flashed electric light and we described responses to different stimulation frequencies (Hanson et al. 1971). Use of these findings in defining the timing cycle of the pacemaker implied that the brain-to-photocyte latency was constant – which had not been established. The present paper shows that the variability of the motor link in flash control is in fact negligible in comparison with that of the flash-to-flash timing process, thus validating the use of the interflash interval as a measure of pacemaker activity. We also give

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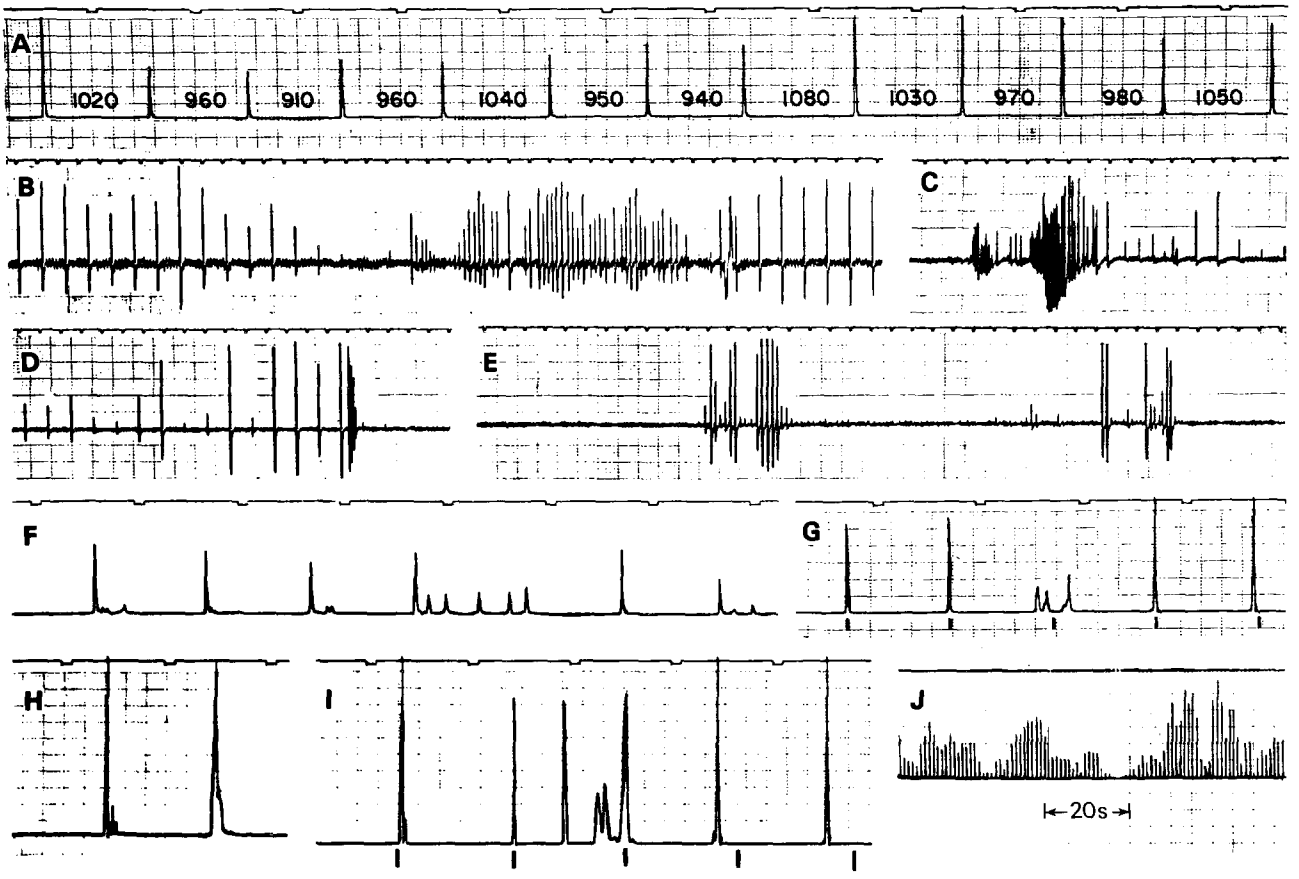

Fig. 1A–J. Free run flashing of *Pteroptyx cribellata* males free in the field (**B–E**) and under restraint (**A**, **F–J**: 'laboratory'). Time signals at top of each record are at 1 s intervals. All records read left to right. **A** Hoskins No. 8 (*H8*). Laboratory display flashing. Interflash duration shown in ms. Mean (17 cycles) = 982 ± 54 ms. **B** Field recording of rhythmic display flashing and intervening 4/s flickering of H male in flight. **C** Field recording of flicker with changing frequency, in a perched H male. **D** Field recording of rhythmic display flashing in flight, terminated by a 'flicker and land' sequence. Same male as Fig. 1C. **E** Field recording showing two bouts of flickering interrupting a quiescent period of animal in flight. Same male as Figs. 1C, D. **F** Laboratory display flashing of *H8* after 5 runs of driving, showing several types of arrhythmic luminescence. **G** Laboratory display flashing of *H8* firefly showing rhythm breakdown episode. *Marks below trace* are extrapolation of original flashing rhythm, not stimulus artifacts. **H** Laboratory display flashing of *H13* firefly showing putative afterdischarge and trailing flash shoulder. **I** Laboratory display flashing of *N2* firefly showing trailing and leading flash shoulders, sporadic interperiod flashing and a phase shift. *Marks below baseline* show extrapolation of original rhythm. **J** Laboratory display flashing of *H8* firefly, recorded at very slow chart speed, showing variability of flash intensity. Trace retouched

a comprehensive quantitative description of free run flashing modes which is essential both in defining endogenous capabilities of the pacemaker, in the present paper, and in explaining phase-shifting effects of exogenous light signals, in the following paper.

Materials and Methods

The measurements reported are from *Pteroptyx cribellata*[1] fireflies from three lowland localities on New Britain: Navanarum (N), Kerevat (K) and Cape Hoskins (H). Some records were made from free flying individuals in the field, using portable equipment, but most were made at night in a darkroom at the 1969 Alpha Helix Expedition base at Maiwara, near Alexishafen (via Madang), Papua New Guinea. The techniques used were largely those of Case and Trinkle (1968). The firefly was affixed non-injuriously to wax, ventral side up, by means of wire staples bridging the abdomen. Flashes were detected by an RCA 1P21 photomultiplier feeding into an Ampex SP300 tape recorder and chart recorders. To minimize chances of the firefly seeing his own light a 1 × 1 cm opaque shield was placed transversely at neck level or the head was inserted into a recess in a block of black plastic, the gap between body and block being closed with a mixture of graphite and petroleum jelly.

The temperature range during observation was 26–29° C. Chart traces were recorded at 25 mm/s, measured to the nearest 10 ms, and are considered reliable to within 20 ms. All means are given with standard deviations (σ), not standard errors. Student's t test was used to assess significance of difference between means. The coefficient of variation, V, was the measure of variability and the coefficient of correlation, r, the measure of association. We

1 Lampyrid firefly species often cannot be characterized adequately without knowledge of their flashing behavior in life. In spite of a thorough taxonomic study of the genus *Pteroptyx* (Ballantyne and McLean 1970) more recent field work (Lloyd 1973) suggests that further revision may be necessary. The firefly studied for this and the following paper conforms to the description of *P. cribellata* by Ballantyne and McLean. Our identifications ('H2', 'N2', etc.) will be included with specimen records in a revision now in preparation by Ballantyne and will permit change in species attribution if that proves necessary

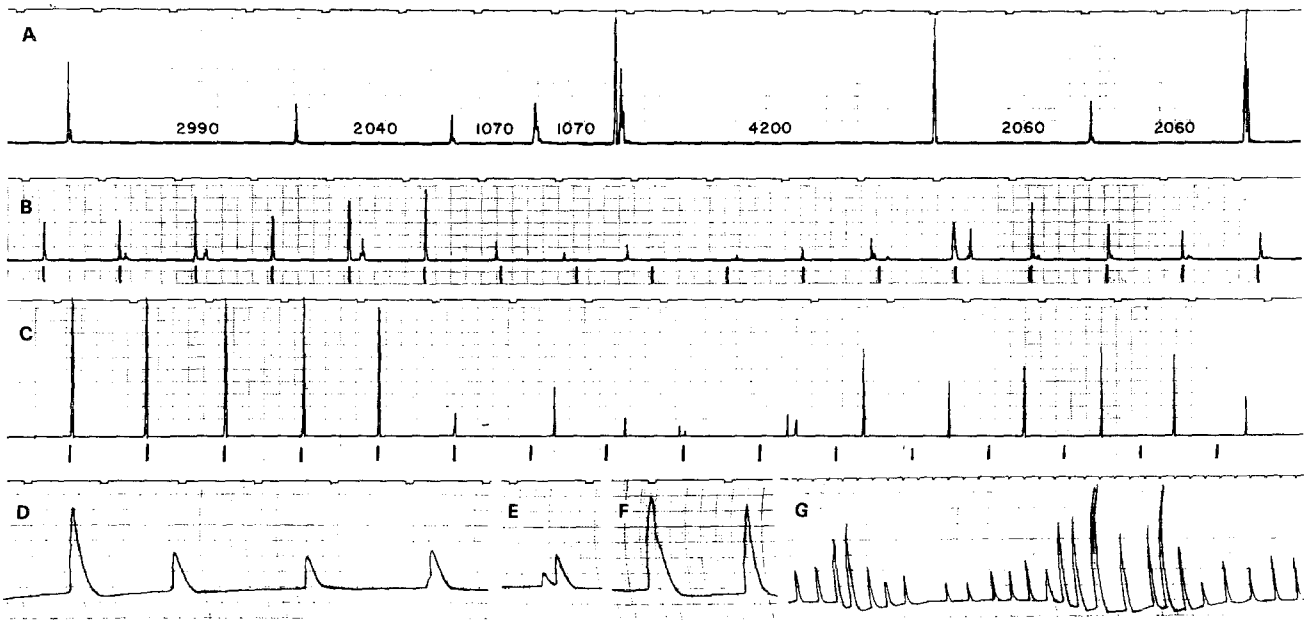

Fig. 2A–G. Irregularities in laboratory free run flashing of *Pteroptyx cribellata*. Time signals at top of each record are at 1 s intervals. **A** *H13* male, showing, successively, 3-unit, 2-unit, single, single, 4-unit, 2 unit and 2-unit interflashes. Mean of 21 single-unit (free run) periods during entire run was 1,087 ms. **B** *H8* male, following 7th bout of artificial driving. As compared with Fig. 1A, shows several small, post-flash supernumerary luminescences and temporary breakdown and recovery in the rhythm between interflashes 6 and 11. *Marks under baseline* show extrapolation of original rhythm. **C** *N2* male, showing breakdown in flashing rhythm (interflash 7) putting cadence half a cycle out of phase with original interflashes. *Marks under baseline* show extrapolation of original rhythm. **D** Hoskins female, showing greater than 200 ms flash duration and shoulder on rising phase of flash. **E** Hoskins female. Bi-partite flash. **F** Hoskins female. First flash has trailing shoulder. **G** Hoskins female. Shows irregularities in rhythm, flash intensity and flash contour. Baseline level changes are instrumental artifacts

base our analysis on the duration of the pacemaker's free run period, which is more convenient than frequency for transients and pacemaker models. The entity actually measured – the duration of the flash-to-flash interval (measured rise-to-rise or peak-to-peak) – we call the 'interflash.'

Results

1. Free Run Flashing: General

During synchronized flashing in tree swarms of *P. cribellata* many males are weaving among the branches in slow, hovering flight, while others are perched on leaves. Field records showed that individual flying males can flash for long stretches with considerable regularity; that different individuals have nearly the same average flashing frequency; and that there is no obvious difference in flashing frequency or intensity between an individual whose flashes coincide with those of other males recorded simultaneously and one who is out of phase with neighbors and so is perhaps flashing independently (details in preparation).

The *P. cribellata* male has two well-defined modes of light emission: sharp, single flashes in a reasonably regular one-per-second rhythm (Fig. 1A; Tables 1 and 2) and a flicker of much higher but inconstant frequency (3–10 flashes/s; Figs. 1B–E). The first mode, the species-specific 'display' rhythm, was the characteristic flashing mode of both synchronized and out-of-phase males in the field and of isolated, restrained animals in the laboratory, whether free-running or entrained. In the average male about 95% of the interflashes during display flashing were between 800 and 1,200 ms in duration (see below).

Flickering was seen in both flying and perched animals in the field, though relatively infrequently. It was typical of mechanically disturbed animals and was sometimes associated with taking flight or alighting (Fig. 1D; see also Lloyd 1973) although fireflies may also alight without flickering. It usually had a sharp onset and conclusion, was not infrequently preceded or followed by non-luminous intervals, and usually appeared to interrupt the one-per-second display rhythm rather than being superimposed on it (Figs. 1B, D, E). Flickering was very rare in our laboratory recordings.

Variable flash intensity was one of the most pervasive features of spontaneous luminescence, both in rhythmic display flashing and in flickering. In several long free-run display flashing series we found no cor-

relation between flash intensity and the duration of either the preceding or the succeeding interflash interval. For recording we generally chose an amplification that would detect clearly each successive flash in the major rhythm without clipping too many of the brighter flashes (e.g., Fig. 1A). However, with flash amplitude sometimes varying over a 50-fold range (Fig. 1J), it seems inevitable that occasional luminescences were lost in the baseline, resulting in a spuriously long interflash.

As in many firefly species, display flashes sometimes showed dim shoulders immediately preceding or following the main flash (Figs. 1G, H, I; 2A, D–F). Such shoulders have been ascribed to slight local asynchronies in photocyte firing times or to neural after-discharge (Buck 1966; Buck and Case 1961; Case and Buck 1963; Hanson et al. 1969).

2. *Interflash Duration: Frequency Distribution*

Since Student's t test, needed in analysing free run pacemaking, requires that interflash durations have a normal (Gaussian) frequency distribution, it is important to ascertain whether this requirement is fulfilled. To amass testable populations of interflashes from each firefly we pooled several series of 20–50 free run cycles that also served as controls for exogenous driving of these same animals (following paper).

In unselected measurements none of the pooled raw interflash data demonstrated a reasonable probability of Gaussian distribution by chi-square analysis. The departures from normality involved skewing due to excess short periods and disproportionate numbers of periods in the largest duration class interval. In data for individual males (e.g., Figs. 3B, C and D) and, in exaggerated form, in a pool of over 900 raw interflash durations from four males (Fig. 3A), it is evident that each central peak and the corresponding prolonged, low-frequency, short-duration and long-duration tails could not all belong to a single Gaussian distribution.

By truncation statistics, probably the best approach to frequency distributions in which tail inhomogeneity is suspected, the interflash durations from fireflies H2, N2 and H8 yielded chi-square values strongly supporting Gaussian distributions for the central 73–85% of the measurements. The ranges of the truncations are indicated by the horizontal black bars in Figs. 3B, C and D and the numerical details are given in the figure legend. These results indicate that some raw interflash data were inhomogeneous, consisting of a predominant, normally-distributed, population peaking at a duration of about 1,000 ms and extending to about 145 ms to either side (3σ), plus both shorter and longer interflashes not from the same population. Bounds of 800 and 1,200 ms were therefore used for test samples.

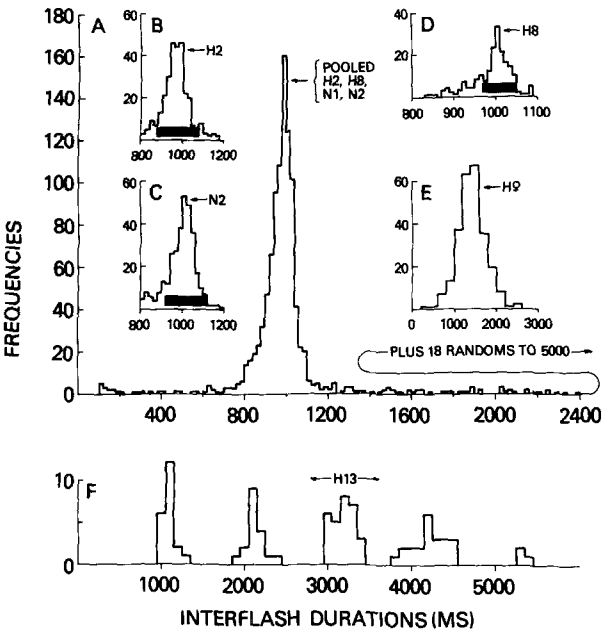

Fig. 3A–F. Frequency-distribution histograms of free run flashing. *Black horizontal bars* in **B**, **C** and **D** indicate truncation sample ranges. Note that scales are not the same in all figures. **A** Pooled data for *H2, H8, N1* and *N2* male fireflies showing distribution of about 900 interflashes with durations in 800–1,200 ms range, normalized to 980 ms mean, and random durations of about 150 additional shorter and longer interflashes. **B** Main rhythm interflashes of *H2* male. Mean interflash duration = 972 ± 63 ms (n = 297). χ^2 = 4.1 for 249 interflashes between 870 and 1,050 ms in 9 frequency classes; probability = 0.66 for 6 degrees of freedom. Theoretical mean and 3σ tails were 971 and ±144 ms. **C** Main rhythm interflashes of *H8* male. Mean interflash duration = 985 ± 52 ms (n = 195). χ^2 = 5.4 for 123 interflashes between 970 and 1,040 ms in 8 classes; probability = 0.37 for 5 degrees of freedom. Mean and 3σ tails were 1,004 and ±67 ms. **D** Interflashes of *N2* male. Mean interflash duration = 997 ± 69 (n = 325). χ^2 = 6.1 for 275 interflashes between 940 and 1,120 ms in 10 classes; probability = 0.53 for 7 degrees of freedom. Mean and 3σ tails were 1,015 and ±143 ms. **E** Hoskins female. Mean interflash duration = 1,497 ± 340 ms (n = 255). χ^2 = 5.8 for 250 interflashes between 400 and 2,400 ms in ten frequency classes; probability = 0.56 for 7 degrees of freedom. **F** *H13* male, showing single and multiple-unit interflashes indicating persistence of pacemaker timing during flash skipping. Data from record totalling about 250 single cycle equivalents

3. *Types of Arrhythmia*

Though we relied on 800–1,200 ms interflashes in defining normal pacemaker behavior during free run display flashing, study of overly short and overly long interflashes revealed several further facets of luminescence control and spontaneous pacemaker behavior. These included luminescences seemingly independent of the prime one-per-second display rhythm, pacemaker cycling independent of flashing, period short-

Table 1. Interflash durations during 8 runs of laboratory free run flashing that were alternated with 7 bouts of rhythmic artificial driving (not shown). *P. cribellata* male H8. 27 °C. Range, mean and s.d. in ms. Overall mean = 985 ± 52 ms (n = 195). Series pairs b and d, b and e, and e and g differ in mean interflash duration at or below the 5% level. Serial correlation coefficients (last column) are, with one exception, statistically non-significant

Series	Total cycles	Noisy interflashes	Range	M σ	V (%)	r[a]
a	17	0	880–1,080	982 ± 54	5.5	0.05
b	22	0	880–1,040	968 ± 40	4.1	0.33
c	18	2	840–1,120	976 ± 40	4.1	0.54[b]
d	24	1	950–1,080	994 ± 31	3.1	0.20
e	13	7	990–1,040	1,008 ± 16	1.6	0.44
f	39	19	840–1,080	987 ± 58	5.9	0.03
g	24	10	890–1,040	985 ± 38	3.9	0.21
h	38	26	800–1,080	987 ± 69	7.0	0.06

[a] nth interflash vs. $(n+1)^{th}$
[b] Significant at the 5% level. Attributed to temporary drifting of period duration

Table 2. Representative free run statistics

Firefly	Cycles	Range	M σ	V (%)
H2[a,c]	55	775–1,160	965 ± 90	9
H2[b]	64	800–1,200	969 ± 63	6
H3	25	1,020–1,100	1,071 ± 18	1
N1	30	870–1,120	1,049 ± 73	6
N2[c,d]	87	800–1,190	1,009 ± 71	7
N2[c,d,e]	136	780–1,170	990 ± 78	7
K1[c]	54	780–1,180	961 ± 58	6
K1[f]	22	900– 980	957 ± 22	2
K2[c]	22	930–1,120	1,075 ± 47	4
H13[g]	21	960–1,280	1,087 ± 59	5
H9[h]	255	350–2,550	1,497 ± 40	22

[a] Interflashes 1–62, Fig. 4B
[b] Interflashes 145–210, Fig. 4B
[c] Excluding interflashes outside 200 ms of mean
[d] Including noisy cycles
[e] Interflashes 1–193, Fig. 4C
[f] Unbroken run from series above
[g] Skippy animal. Data are for all unit interflashes occurring during the equivalent of 271 rhythm periods
[h] Female. Several series pooled

enings possibly correlated with absence of luminescence, drifting of interflash duration, spontaneous phase shifting of the flashing rhythm and individual differences between fireflies.

a) Photic Noise. The truncation results (Sect. 2) did not in themselves prove that 800–1,200 ms interflashes differ qualitatively from those shorter or longer but certain interflashes suggested flash control independent of the display rhythm. These comprised shorter-than-normal intervals produced by flashes, usually dim, that occurred within what would otherwise have been normal free run periods (e.g., Figs. 1F, 1I, 2B). Frequently these flashlets occurred within the first 200 ms after a flash of the principal display rhythm. A high proportion of all interflashes shorter than 800 ms could be combined with contiguous interflashes to form an interval totalling about 1 s. We therefore considered the adventitious flashes analogous to neural noise and classified free run interflashes as noisy if extra luminescences occurred between flashes that delimited an interval of normal one-per-second free run duration that was in phase with others in a display rhythm (filled points, Fig. 4). The extra flashes apparently did not interfere in any major way with the timing of flashes occurring at the normal free run frequency (Table 1).

b) Flash Skipping. Another category of non-standard interflashes consisted of intervals whose duration approximated a multiple of the free run period, as if flashes had failed to occur at the times expected from the preceding rhythm. Single skips (interflashes of twice the free run period) were not uncommon during the free run flashing of several animals but firefly H13 was unusual in exhibiting enough multiple skips to permit quantitative analysis (Fig. 2A). When the frequencies of all 93 interflash durations in the lengthy H13 record were plotted it was evident that all interflashes were multiples of the unit cycle of about 1,100 ms (Fig. 3F). These data therefore lead to the important conclusion that the pacemaker can continue to cycle in the absence of flashing.[2] Whenever the firefly did flash, in other words, the flash coincided closely with a forward projection of the original rhythm, as if free run timing intervals were continuing to be measured off.

Measurements of interflashes produced by flash skipping suggest a consistent and cumulative decrease in duration of about 4% per skip (Table 3). Although variance was too large to permit confirming the small duration differences statistically we provisionally accept a cycle-shortening effect of flash skipping because of its consistency, because comparable interflash shortening was seen also in a 1,100 cycle record of H13 recorded at 1 mm/s and because highly signifi-

2 Given the usual variability of flash intensity it was never possible to be certain that a flash had actually been skipped rather than simply being buried in the baseline because of low signal/noise, but since the record had many well amplified flashes we concluded that most of the apparent gaps did indeed mark failures of lantern excitation. This conclusion was further supported by the fact that skips occurred during photic driving of this same firefly (see following paper) and by the fact that any spurious skips due to undetected flashes would dilute, rather than augment, a shortening effect due to true skips

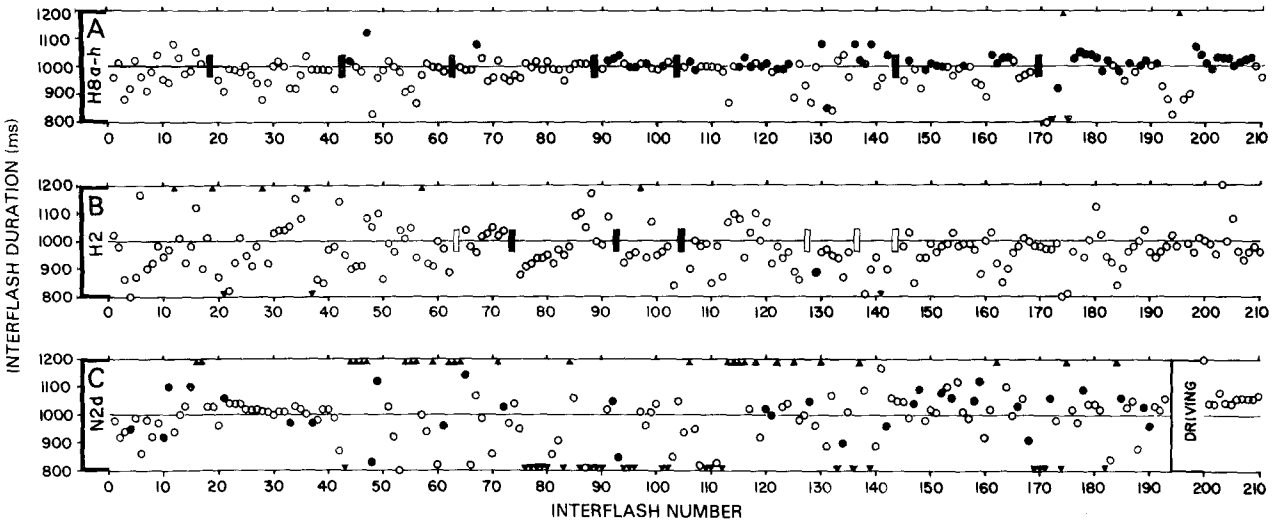

Fig. 4. Successive interflashes in free run flashing of three *P. cribellata* males (*H8, H2, N2*). *Cross-blocks* on 1,000 ms abscissa mark omissions of rest intervals (*hollow blocks*) or intervals of artificial driving (*solid blocks*). *Hollow circles* are quiet interflashes (no luminescence between flashes of major rhythm). *Solid circles* are noisy interflashes. *Triangles* along 800 and 1,200 ms boundaries mark times of occurrence of out-of-range interflashes. The artificial entrainment series which began near the end of the *N2* record (Fig. 4C) was driven at 1,056 ms and the response continued for 34 additional cycles with all interflash durations within the limits of 1,010–1,090 ms

Table 3. Durations (ms) of unit free run interflashes and of double, triple and quadruple duration interflashes caused by 1, 2 and 3 consecutive skipped flashes. Firefly H13. Predicted durations = 2×, 3× and 4× the mean unit measured free run period

Range	No.	Measured mean interflash	Predicted interflash	Predicted minus observed	Unit Δ
1,000–1,300	21	1,087 ± 59			
1,900–2,400	18	2,133 ± 117	2,174	41	41
3,000–3,400	29	3,181 ± 131	3,261	80	40
3,800–4,500	21	4,220 ± 191	4,348	128	43

cant shortening during free run skipping was established in the firefly *Luciola pupilla* (report in preparation).

c) Phase Shifting. The rhythm of display flashing was sometimes frame-shifted permanently by a single overly long or overly short interflash. In Fig. 1I, for instance, premature occurrence of the next-to-last flash caused about a quarter-cycle phase advance in the rhythm with respect to the cadence prior to the short interval (compare sub-baseline tick marks). Figure 2C illustrates an example of phase retardation that occurred when the 7th flash was delayed about 300 ms. In this instance several cycles of irregular flashing then ensued but when rhythm was fully reestablished it was out of phase with the initial cadence by almost half a cycle. The phase shift in these instances was thus not associated with a change in interflash duration that persisted after the perturbation was over. Interflash duration did sometimes drift over a span of several to many cycles, causing a corresponding slow phase shifting, but only temporarily and in minor degree (e.g., Fig. 4B, interflashes 75–90).

Small phase shifts were likely to be compensated by chance shifts in the other direction so that the rhythm changed only slightly in absolute time. The interval between flashes 6 and 13 in Fig. 2B may represent such a perturbation (compare main display rhythm flashes with tick marks below baseline, representing the extrapolation of the initial rhythm).

d) Ranges of Individual Arrhythmias. The diagrammatic cycle-by-cycle flashing records of fireflies H8, H2 and N2 in Fig. 4 illustrate additonal irregularities encountered during spontaneous laboratory flashing:

Differences in variability of interflash duration in the same firefly at different times (e.g., interflashes 68–113 of Fig. 4A vs. preceding and succeeding series; interflashes 18–42 of Fig. 4C vs. preceding and succeeding series).

Differences in ratio of noisy to quiet interflashes in the flashing of a given firefly at different times (Fig. 4A, first 90 cycles vs. subsequent 120; see also Table 1, 3d column) or between individuals (Figs. 4A, B, C).

Differences between or within individual records in relation to numbers and magnitudes of out-of-range interflashes (Fig. 4, marginal triangles). Firefly N2 (Fig. 4C) showed a far higher proportion of overly long or overly short interflashes than either fireflies H8 (which had no out-of-range interflashes in its first 170 cycles – see Fig. 4A) or H2 (Fig. 4B). Further, N2 exhibited a flurry of longer-than-1,200 ms interflashes at certain times (Fig. 4C, interflashes 44–72

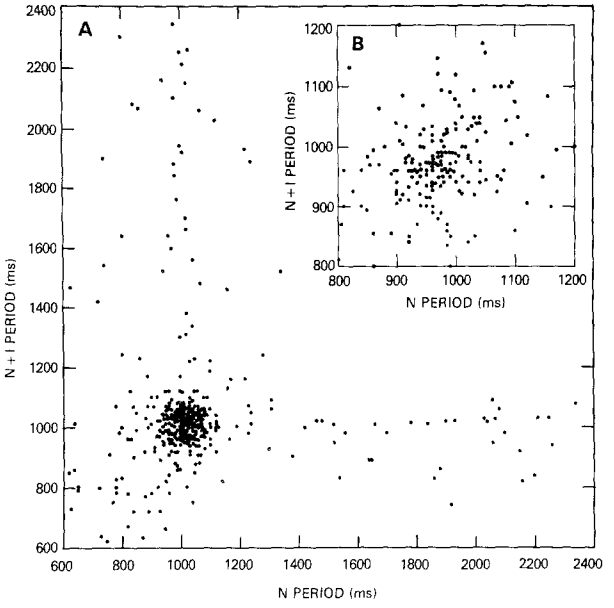

Fig. 5. **A** *N2* male. Joint-interval histogram of all interflashes up to 2,400 ms, showing independence of n and $n+1$ cycle durations. 310 cycles. The distribution indicates overlong cycles paired with cycles of free run duration. Slight spread along 45° axis due to small temporary drifting of period duration. **B** *H8* male. Joint-interval histogram for 192 interflashes (Fig. 4A) showing independence of n and $n+1$ cycle durations and indications of slight drifting of period

and 113–130) and shorter-than-800 ms interflashes at others (interflashes 76–112).

Differences in interflash durations at different times in the same firefly, sometimes occurring as a slow drift and sometimes within a few cycles. In the third series for firefly H2 (Fig. 4B, interflashes 74–92) the mean of the first 10 interflashes (934 ± 30 ms) is highly significantly different from that of the next seven ($1{,}075 \pm 59$ ms). Tables 1 and 2 also illustrate fluctuations of mean interflash durations in other individuals. This sort of short term variation thus cautions against conclusions based on small samples.

4. Inter-Cycle Relations

An important clue to pacemaker mechanisms is how the durations of successive cycles are related to each other. The very existence of rhythm shows that a timing mechanism is cycling but the rhythm can reflect a variety of mechanisms. If for example, the period of the endogenous pacemaker were corrected cycle-by-cycle by comparison with an invariant reference oscillator, an overlong interflash should be followed by a compensatingly overshort interflash, and an overshort by an overlong. If the control mechanism itself changed gradually in rate of cycling, as occurs, for example, with changes in ambient temperature, it would be expected that longer than average interflashes would show an association with longer contiguous interflashes and shorter with shorter. If, as a third alternative, there were no correlation between durations of successive timing cycles, a still different oscillator mechanism would be indicated.

Inter-cycle correlation was tested by computation and by joint-interval histograms relating the duration of each interflash (n) to the duration of the immediately succeeding ($n+1$). These data show that the durations of successive interflashes during free run flashing are mutually independent. Thus in Fig. 5 neither firefly H8 nor firefly N2 showed any indication of long-short or short-long period associations distributed at right angles to a 45° line, as would occur if there were immediate compensating alternations in the durations of successive pacemaker cycles. The N2 firefly (Fig. 5A), with a much larger number of long interflashes than firefly H8, produced a boomerang-shaped distribution of interflash sequences, showing that overlong interflashes were almost always paired with interflashes of free run duration. This reinforces the conclusion that interflashes in the one-per-second display rhythm are independent of those longer than 1,200 ms. First order serial correlation coefficients for interflash durations from typical regular flashing series of fireflies H2 and N2 were, respectively, 0.38 and 0.09. Correlation coefficients for 7 runs on firefly H8 were similarly non-significant (Table 1, last column). These results therefore support cycle-by-cycle independence of interflash durations – that is, independence of successive endogenous timing cycles during spontaneous flashing.

5. Timing and Motor Contributions to Interflash Variance

In pursuing our objective of deriving pacemaker behavior from the times of occurrence of flashes produced by the intact firefly, our working hypothesis is that interflash duration duplicates pacemaker period. However, the one second flash-to-flash interval that is measured is actually a combination of two independent delays: that of the timing process in the brain and the delay required for the chain of output or motor processes leading to flash production. These latter processes, which include the conductional delays in the nerve cord and peripheral lantern nerves plus the transductional delay for exciting chemiluminescence in the photocytes, total about 200 ms (Hanson et al. 1971), about one-fifth of the total interflash. Thus only if the variability of the output is very small compared with that of the endogenous timing process can interflash duration be a valid measure of pacemaker period.

The relative contributions of pacemaker and output processes to the variability of interflash duration can be estimated by means of a statistical analysis first applied by Hagiwara (1949, 1950) to human motor unit rhythmicity and generalized and refined by

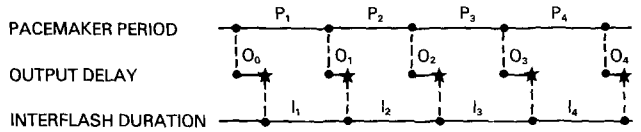

Fig. 6. Model for use in cumulating variance. Variations in time of occurrence of flash (*) and of duration of pacemaker cycle (*P*) alter the duration of the interflash (*I*)

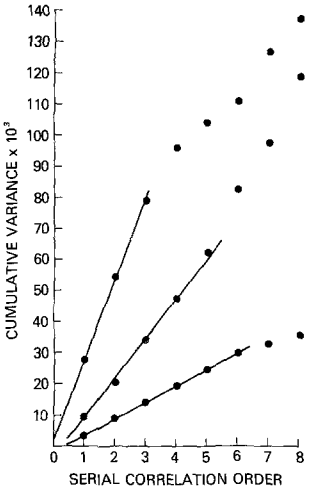

Fig. 7. Cumulative interflash variance with successive cycle increments during 3 series of free run flashing by firefly *H2*. Timing cycle-by-cycle variance is proportional to slope; motor output variance to one-half the Y intercept. The latter is clearly negligible compared with the former

Mets (1975). A diagram of the timing processes involved in regulating flash production is given in Fig. 6. The pacemaker cycle (P_n) is shown as timing from the triggering of one flash output process (O_n) to the triggering of the next (O_{n+1}). Interflash duration, I_n, equals the corresponding pacemaker cycle duration plus the duration of the triggered output process, minus the duration of the output process triggered in the preceding cycle: $I_n = P_n + O_n - O_{n-1}$.

Assuming that successive pacemaker and output durations are statistically independent variables, the variance of the interflash interval (V_I) has contributions from the variance of the pacemaker period (V_P) plus contributions from the variance (V_O) of each of the two outputs producing the flashes that begin and end the interflash. Since the variance of the sums of independent variables is equal to the sum of their individual variances, $V_I = V_P + 2V_O$. If we sum two adjacent interflashes, for example I_2 and I_3 (Fig. 6), the duration of the combined interval equals $P_2 + P_3 - O_1 + O_3$, since O_2 is contributed first additively, then subtractively, and cancels out. For a series of interflashes, therefore, only the first and last output delays will affect mean interflash duration and, if the series is long enough, should have negligible effect.

The variance of a double interval (V_{2I} receives contributions from two pacemaker cycles and two output processes: $V_{2I} = 2V_P + 2V_O$. In general, the sum of n successive interflash intervals has variance $V_{nI} = nV_P + 2V_O$. A plot of V_{nI} against n should give a straight line whose slope is V_P and which intercepts the variance axis at $2V_O$. In this way the relative contributions of V_P and V_O to V_I can be determined. In practice, correlations among successive intervals due to other causes (e.g. drift in period or phase shifts) can cause deviation from linearity. This analysis is therefore only usable with regular flash series.

Firefly H2 provided three regular and unbroken flashing series of about 60 cycles each from which the variances of successive compound interflashes were calculated through the 8th order ($I_1 + I_2$, $I_2 + I_3 \ldots I_{n-1} + I_n$; $I_1 + I_2 + I_3$, $I_2 + I_3 + I_4 \ldots I_{n-2} + I_{n-1} + I_n$ etc.). Though the three series differed in variance, all agreed in exhibiting serial linearity of variance with increasing compound interflash duration (Fig. 7). This linearity confirms the independence of pacemaker cycle and output. The fact that the $2V_O$ intercepts were all close to zero shows that practically all the variability was in the endogenous timing process. Thus cycle-by-cycle variations in the observed interflash interval directly reflect cycle-by-cycle fluctuations in the pacemaker period itself, as required by our working hypothesis.

6. Free Run Flashing by the Female

Because *Pteroptyx* females do not participate in the males' synchronic chorus in nature (Buck and Buck 1966, 1978; Lloyd 1973) and because females of many species of fireflies do not flash spontaneously, we were surprised to find that some restrained females of *P. cribellata* did flash spontaneously albeit less regularly than the males. Sequential records of successive long-duration, often double, flashes of one female are given in Figs. 2D–G and the frequency distribution of her interflashes is indicated in Fig. 3E. It is noteworthy that the period of the female was 50% longer than that of the male and that the frequency distribution of interflash durations was Gaussian over its full range.

Discussion

The night-long display of tree swarms of *Pteroptyx* fireflies gives the impression that the rhythmic flashing is incessant. This impression was supported by the present study of individuals of *P. cribellata*, which shows that both in the field and in the laboratory single males often flashed without break for hundreds of consecutive cycles. The rhythm was remarkably

steady. Most animals maintained a mean period of about one second, with coefficients of variation of 2–5% and cycle duration limits between 800 and 1,200 ms (Tables 1, 2). There was never any lasting departure from the one-per-second rhythm. Even when an occasional flash was skipped the evidence was strong that the underlying flash-timing pacemaker continued to cycle (Figs. 2A, 3F). The persistence of regular flashing in laboratory animals, and its immediate resumption after being overridden during intervals of rhythmic driving by artificial light (Fig. 4A), seem remarkable in view of the severe restraint, the absence of the visual reinforcement available in normal swarms from the synchronized flashing of neighbors and the abnormal, ventrum-up position of the subject animals (Methods). The dominance of the flash-timing oscillator is seen also in the continued rhythmic flashing of specimens in spiders' webs (Buck and Buck 1978).

In addition to the predominant one-per-second rhythmic flashing, also characteristic of the display flashing of free males in the field, a variety of irregular luminescences were emitted by restrained males upon occasion (Sect. 3). There was indication that the frequency of some of these arrhythmias increased with repeated exposure to the rhythmic flashes of artificial light used in exogenous driving (following paper), for example in firefly H8 in the series shown in Fig. 4A (filled circles), and in the much-used firefly N2. However, all these types of flashing were also seen in free males under field conditions as elements of the natural repertory of light emission too infrequent for quantitative appraisal.

Not all interpolated and irregular luminescences were necessarily under direct control of the one-per-second pacemaker. For example, rare episodes of rapid flashing (Fig. 1F, 4th interflash; Fig. 1I, 2d interflash) may have corresponded to the flicker mode of emission which was fairly common in the field. Likewise, the occasional dim shoulders on main flashes (Figs. 1G, H, I) and some of the sporadic flashlets which we call photic noise (Sect. 3) might have been due to secondary local lantern excitation or to ganglionic afterdischarge (e.g., Buck 1966; Buck and Case 1961; Case and Buck 1963; Hanson et al. 1969). The ephemeral nature of some spontaneous luminescence is suggested by the dramatic regularization of flashing sometimes induced by exogenous photic driving (end of N2 record, Fig. 4C). Sporadic emissions and noise caution against the seductive notion that every modulation of field luminescence must have functional or evolutionary significance.

The apparent slight shortening of the interflash when a flash was skipped (Table 3) must remain unexplained pending new data, particularly neural recordings. The skipping, however, indicates that the cycling of the neural pacemaker and the triggering of a flash excitation volley are independent events. A reasonable surmise is that the flash is triggered by a state of the oscillator which is normally very close to the level at which recycling is triggered. In the following paper a model is considered which could explain shortening of the pacemaker period when a flash is skipped.

The spontaneous flashing of the female, itself unexpected, had a radically different period from that of the male (Figs. 2D–G, 3E; Table 2) thus arguing against the possibility that the same pacemaker may be used for control of flash frequency in the male and for frequency recognition in the female, as in certain crickets (Hoy et al. 1977).

The evidence that the conductional and neuroeffector delay included in the flash-to-flash interval of the *P. cribellata* male is essentially constant (Sect. 5) made it possible to refer many of the findings about the timing of spontaneous flashing directly to the endogenous cycle of the cephalic pacemaker. Data from display flashing in the laboratory thus enabled us to deduce that the control of free run flashing depends on a timing mechanism that runs continuously (Sect. 3b), does not require visual feedback (Methods)[3], is independent of the intensity of the flash (Sect. 2)[4], or even of its occurrence (Sect. 3b), undergoes occasional spontaneous phase-shifting (Sect. 3c) and whose periods are independent in duration, cycle-by-cycle, and hence not subject to additional regulation by a reference oscillator (Sect. 4).

Validation of flash timing in intact fireflies as a source of information about the underlying pacemaker, and the observation that imposed rhythmic photic signals can entrain flashing (e.g., Fig. 4C), open the way for a thorough exploration of the pacemaker by application of controlled light pulses at various times in the flashing cycle. The following paper describes such a study, leading to a tentative model of the flash-timing mechanism.

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3 This conclusion would of course be vitiated by the existence of direct photic conduction from lantern to eye through body tissues, or by peripheral neural feedback

4 Since free fireflies in the field vary the intensity of their flashes our present interpretation of variable flash intensity is that the lantern does not fire maximally in each flash. Hanson et al. (1969) found, in *Photuris*, that the number of emission units participating in a spontaneous flash might vary, as if flashing in relays

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