A CIRCADIAN RHYTHM IN THE LOCOMOTOR BEHAVIOUR OF THE GIANT GARDEN SLUG *LIMAX MAXIMUS*

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SUMMARY

The locomotor activity of the garden slug *Limax maximus* was examined for components of circadian rhythmicity. Behavioural (running wheel) studies clearly demonstrated that the activity satisfies the principal criteria of circadian rhythmicity. In constant darkness at a constant temperature, the locomotor activity freeran with a period of about 24 h (range 23.6-24.6 h). The rhythm was also expressed in constant light with a period for individual slugs that tended to be shorter in LL than in DD. The period of the rhythm was temperature compensated (11.5-21.5 °C) with a $Q_{10}$ approximately equal to 1.00. The locomotor rhythm could be entrained to 24 h LD cycles such that the circadian activity peak occurred during the dark. The phase angle between the onset of activity and lights-off was not fixed, but was a function of the photoperiod of the entraining light cycle.

INTRODUCTION

There have been relatively few investigations of circadian behaviour in molluscs. The locomotor activity of *Aplysia californica* has been reported to be diurnal in a 24 h light cycle (LD 12:12) and weakly rhythmic in constant darkness (Kupfermann, 1967; Strumwasser, 1971; Block & Lickey, 1973). Jacklet (1972), however, suggested that *A. californica* lacked a distinct activity rhythm in constant darkness (DD) and that, under constant conditions, the expression of clear periodicity was dependent on constant illumination (LL) of moderate intensity (greater than 40 lux).

Newell (1966) has described the nocturnal behaviour of the slug *Agriolimax reticulatus* in natural LD cycles and Dainton (1954) has shown that this species is rhythmic in DD when exposed to 24 h temperature cycles. Clear evidence of rhythmic locomotor activity under constant conditions has been provided only in the case of the slug *Arion ater*: Lewis (1969) showed examples of long-term activity data obtained from two slugs in which clear locomotor rhythms of less than 24 h were expressed for 3 and 5 weeks in DD at constant temperature.

The present study describes a circadian activity rhythm in the giant garden slug *Limax maximus*, preliminary to development of a preparation in which to study the
neurophysiological basis of circadian behaviour. We have found that *L. maximus* possesses a number of characteristics that recommend it as a model system for this purpose.

**METHODS**

Specimens of the giant garden slug *Limax maximus* were collected locally (Kentucky and Maryland) and maintained in the laboratory under artificial days (LD 12:12, i.e. 12 h of light alternating with 12 h of darkness, LD 14:10, or LD 16:8). They were housed in separate groups of 6–10 animals and fed a diet of carrot, potato and lab chow (Wayne Lab-Blox, Allied Mills Inc., Chicago). Colony containers were cleaned and the food replenished weekly.

Slugs were collected in the late fall of 1974 and from early spring through late summer of 1975. Experiments were conducted on freshly collected animals, animals that had been kept in the laboratory for 2–4 weeks following collections, animals that had been reared from eggs under long days (LD 14:10), and animals that had been raised and maintained on 'short' days (LD 12:12) for periods exceeding 1 month.

The locomotor activity of individual *Limax* specimens was monitored using methods similar to those developed for studying insect activity (Roberts, 1960; Nishitsutsuji-Uwo & Pittendrigh, 1968; Sokolove, 1975 a). Each animal was placed in an enclosed ‘running wheel’ constructed from a plastic tub with a tight-sealing lid (Fig. 1). Switch closures that occurred each \( \frac{1}{4} \) wheel revolution were recorded as pen-deflexions.
on a channel of a 20-channel Esterline Angus event recorder at a chart speed of 0.75 in/h (5.29 x 10⁻⁴ cm/s).

Running wheels were housed in a constant-temperature chamber that was light-tight. Chamber lighting was controlled by an external 24 h timer. Both incandescent and fluorescent light sources were employed in the course of this study. Light sources were not water-jacketed, but temperature fluctuations never exceeded ±0.5 °C and were found to be independent of the light-cycle.

Humidity was maintained between 90% and 100% R.H. for the duration of an experiment regardless of temperature. Although slugs were not given food while in the running wheels, survival in the wheels was almost 100% for runs of up to 4 weeks. Animals were weighed both before and after a run. No substantial weight loss was found under these conditions. Indeed, some animals showed a weight gain even though starved for a prolonged time. This was probably the result of water uptake during the course of a run and/or ingestion of the filter paper lining the wheels. Most animals used in these studies weighed between 4 and 10 g.

When not entrained to a 24 h light-cycle, slugs typically showed a locomotor rhythm with a period of about (but usually not exactly) 24 h. In order to estimate the period length, individual pen deflexions were hand-counted from event-recorder charts. Counting was truncated at a density of 20 events/h when overlap of successive pen deflexions did not allow the counting of individual events. A ‘periodogram’ (Enright, 1965) was used to analyse the data for estimates of circadian periods in the range of 14–34 h. Hourly intervals of data were averaged and 0.1 h increments in the trial period were used. When a prominent peak was found in the periodogram, its mode was taken as the ‘best’ estimate of the free-running period. Although there is not yet a rigorous theoretical basis for assessing the accuracy of this estimate, experience with mock data suggests that for runs of 5 days or more (a criterion exceeded by all experiments reported here), the estimate will rarely differ from the ‘true’ period by more than ±0.3 h (Sokolove, unpublished). Peaks were considered prominent if their mode values were at least 3 times the maximum excursion about the trend found in randomized periodogram plots of the same data (see Fig. 7C). Peaks that were obviously present but failed to meet this criterion were considered ‘low’ (mode values given in parentheses, Tables 1 and 2).

RESULTS

(A) Light-dark cycles

It has been shown that periodic changes of the light regimen (LD cycles) can control (entrain) the phase and period of many behavioural rhythms (Aschoff, 1960; Bünning, 1973). The locomotor activity of Limax maximus in activity wheels was examined for entrainment in 24 h LD cycles. Animals were placed in the wheels at constant temperature (20 °C) in an LD 12:12 cycle. Fig. 2A shows the average locomotor activity for ten recently collected animals over 7 days of LD 12:12. Animals become active soon after the light–dark transition. Maximum activity occurs within 2 h and continues until 3 h before the next dark–light transition. The onset of activity is considerably sharper than the offset which occurs over the course of 4 h. The total activity time is 9–10 h. The increase in the standard error of the mean activity during the dark portion of the cycle reflects the variation in the level of activity between...
individuals. Generally, animals that had been recently collected from the field exhibited a higher level of locomotor activity in the wheels than those maintained for long periods in the laboratory culture. There is very little variability in the timing of the onset of locomotor activity as shown by the very small standard errors during the quiescent portion.

Although most slugs exhibited only a single burst of activity beginning at lights-off, several showed a bimodal pattern. There is an indication of this type of activity in the activity plot in Fig. 2A. The animal from which data in Fig. 3 were obtained exhibited the bimodal pattern of activity. It became active 0.5–1 h after dark and remained active for 2–3 h. After a quiet period of about 3 h, there was another low-amplitude burst of activity lasting 1–2 h. The animal was relatively quiet for the remainder of the dark period and during the subsequent light period. Animals collected from the field immediately before the experiment showed a bimodal activity pattern more frequently than those which had been maintained in the laboratory for several months.

About 24% of the animals in LD 12:12 (10 out of 42) exhibited a ‘lights-on’ burst of activity beginning 0.5–1 h after the dark–light transition (Fig. 2A). The amplitude and duration of this burst was variable. It usually disappeared after 3–5 days. The lights-on burst was more pronounced in animals which had been collected in the fall of 1974 and maintained for approximately 3 months in the laboratory at 12 °C, LD 12:12, before being run in the spring of 1975 (Fig. 4).

Some animals were run under various photoperiods to examine the effect of the length of the light period on the phase and duration of the locomotor activity. Fig. 2B is a graph of the mean activity for nine *Limax* over 1 h intervals in LD 16:8. The onset of activity precedes the light dark transition by 2 h. The onset is not as sharp as
Fig. 3. Entrainment of the locomotor activity by LD cycles. Subsequent 24 h intervals of the activity records from the event recorder are arranged under one another. Pen deflexions indicate activity. The bars at the bottom of the figure designate the lighting schedule for the indicated days. The shaded area corresponds to the dark portion of the LD cycle. A slug that had been maintained on LD 14:10 was exposed to LD 12:12 (lights on 08.00 to 20.00) for 15 days. The slug began activity approximately one hour after lights-off (21.00) and continued until four hours before lights-on (04.00). On day 16 the LD cycle was delayed by 6 h (lights on 14.00–02.00). The slug was now active from 03.00 (1 h after lights-off) to 10.00 (4 h before lights-on).

Fig. 4. The 'lights-on' activity burst. A slug that had been maintained on LD 14:10 was exposed to LD 12:12 (lights on 08.00–20.00). Circadian locomotor activity began at 22.00 and continued to 07.00. Beginning at 08.30 there was a burst of intense activity lasting about 5 h.
Fig. 5. Entrainment of the locomotor activity of LD 16:8. A slug that had been maintained on LD 14:10 was exposed to LD 16:8 (lights-on 04.00–12.00) for 11 days. Locomotion began several hours before dark and continued until lights-on. When released into DD on day 12, the locomotor rhythm persisted with T less than 24 h.

Fig. 6. Phase shift of the free-running rhythm by LD cycles. On days 14–31 the slug was exposed to a light cycle that resulted in a delay of about 8 h in its DD locomotor rhythm. See text for details. Periodogram analysis of the data from the two DD free-runs gave T estimates of 23.6 h (days 1–13) and 23.95 h (days 32–38).
it is in LD 12:12. Maximum activity is reached by lights-off and maintained for 4–5 h. It is somewhat lower than the level of activity seen in LD 12:12. The low level of activity characteristic of the light period is reached by the dark–light transition. The duration of the activity time in LD 16:8 is increased to approximately 12 h. The phase, amplitude and duration of activity have all been altered by the change in the LD cycle.

The endogenous component of rhythmic locomotor activity was studied in an animal exposed to LD 16:8 for 11 days before being placed in constant darkness (DD) (Fig. 5). While in LD the locomotor activity was phased to the light–dark transition. In DD the time of the peak activity began to drift relative to the LD peak with a period of less than 24 h. Thus the activity peak seen in darkness in LD cycles represents a true circadian peak.

The ability of LD cycles to entrain the normal rhythm was examined by rephasing of the DD free-run (Fig. 6). Slugs (n = 12) were kept first on a light-cycle of LD 16:8 with the dark period beginning at 20.00. The animals were then tested on running wheels in DD at 20 °C (2 weeks). All expressed a free-running locomotor rhythm which initially coincided with the dark period of the prior light-cycle. After 2 weeks the slugs were removed from the wheels, placed in individual containers with fresh food, and exposed to an LD 16:8 light-cycle with the dark period beginning at 00.00. After 18 days the slugs were removed and again allowed to free-run in DD at 20 °C for 8 days. In the second DD free-run all animals expressed a locomotor rhythm that had been phase-delayed by 8 h; in all 12 cases the activity peaks now coincided (at least initially) with the projected dark period of the second light-cycle. This reset or phase shift of the free-running rhythm demonstrates that the circadian oscillators of the slugs had become entrained to the new LD regime. It is highly unlikely that the shift in timing of the peak represented continued free-running of the rhythm rather than re-entrainment to the new light-cycle. First, the 8 h delay was found in every animal and it is most improbable that each of them was free-running with a period (T) equal to 24-4 h during the 18-day re-entrainment period. Secondly, periods were estimated in each case for the 2-week free-run in DD, and in seven animals the period was less than 24 h (range: 23-6–23-9 h). If these animals had continued to free-run with the same period during the 2-5 weeks when they were not on running wheels, there would have been an apparent phase advance in the rhythm rather than a delay.

The dynamics of entrainment were examined in separate experiments in which the phase of the locomotor activity of slugs was altered by changing the phase of the LD cycle while continuing to monitor activity. Phase shifts generally did not occur immediately, but only after several days of transient activity cycles in which the phase of the locomotor activity gradually moved to its original phase-relationship with respect to the new LD cycle. Fig. 3 illustrates a 6 h phase delay in an animal which had been entrained to LD 12:12 at 20 °C. On the 16th day when the light–dark transition did not occur at the expected time, the normally occurring activity at 21.00 was suppressed. The slug became active 20 min after the delayed onset of darkness. Over the next several days the onset of locomotion drifted relative to the new dusk, before re-entraining with a negative phase angle of approximately 1 h (i.e. beginning 1 h after the L to D transition), the same phase relationship it had before the phase shift. A
In constant darkness (DD) at 20 °C individual *Limax* expressed a rhythm in locomotor activity usually with a free-running period ($\tau$) of slightly less than 24.0 h. A typical example of one animal is shown in Fig. 7A. A periodogram constructed from these data shows a distinct peak with its maximum at 23.6 h (Fig. 7B). When the same data are ordered randomly, no peak is found in the periodogram and variations in amplitude with test period are nowhere as great as the peak in the original periodogram (Fig. 7C). Table 1 gives the $\tau$ estimates for 14 *Limax* run in DD at 20 °C and also for eight of this group run in DD at 12 °C in a separate experiment. In each case the animal was run for at least 10 days and the $\tau$ for each run was derived by periodogram analysis of the data. The average $\tau$ for the group at 20 °C was found to be 24.0 ± 0.1 h. For the smaller sample run at 12 °C the average was slightly lower; 23.7 ± 0.1 h.

Most of the slugs in these two experiments were drawn from a population raised in the laboratory from eggs laid by slugs captured locally in late autumn of 1974 (animals PS 47–58). Similar results were obtained from animals collected locally in the spring and early summer of 1975 (animals PS 69–76, Table 2). In these two groups a majority of the animals expressed a clearly defined free-running rhythm when tested in DD.
 Slug circadian rhythm

Table 1. Free-running periods (τ) of Limax maximus tested in DD at 20 and 12 °C in separate experiments

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>20 °C</th>
<th>12 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS 30B</td>
<td>24·6</td>
<td>—</td>
</tr>
<tr>
<td>PS 41</td>
<td>24·3</td>
<td>—</td>
</tr>
<tr>
<td>PS 47</td>
<td>24·0</td>
<td>(27·4)</td>
</tr>
<tr>
<td>PS 48</td>
<td>23·8</td>
<td>23·8</td>
</tr>
<tr>
<td>PS 49</td>
<td>24·0</td>
<td>(23·7)</td>
</tr>
<tr>
<td>PS 50</td>
<td>23·6</td>
<td>23·6</td>
</tr>
<tr>
<td>PS 51</td>
<td>24·2</td>
<td>23·4</td>
</tr>
<tr>
<td>PS 52</td>
<td>23·9</td>
<td>23·93†</td>
</tr>
<tr>
<td>PS 53</td>
<td>24·1</td>
<td>23·7</td>
</tr>
<tr>
<td>PS 54</td>
<td>24·0</td>
<td>23·7</td>
</tr>
<tr>
<td>PS 55</td>
<td>23·6</td>
<td>23·8</td>
</tr>
<tr>
<td>PS 56</td>
<td>23·7</td>
<td>—</td>
</tr>
<tr>
<td>PS 57</td>
<td>23·8</td>
<td>23·8</td>
</tr>
<tr>
<td>PS 58</td>
<td>23·8</td>
<td>—</td>
</tr>
<tr>
<td>Mean ± s.e.m.</td>
<td>24·0 ± 0·1</td>
<td>23·7 ± 0·1</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>8</td>
</tr>
</tbody>
</table>

* Values in parentheses are cases in which a periodogram peak was present but low. These were omitted in calculating mean and standard error.
† The periodogram peak spanned the range 23·9–24·0.

under our experimental conditions. In contrast, only about 10% of the slugs collected in late autumn or maintained in the laboratory under LD 12:12 for 3 months, expressed a clear locomotor rhythm when tested in DD (4 out of 36). Frequently, general levels of activity were too low to draw any conclusion about the presence or absence of a rhythm. In some cases, however, even with a respectable level of locomotor activity no clear periodicity could be detected either in the activity data, or in the corresponding periodogram.

(C) Variation in τ upon a 10 °C temperature shift

One group of slugs was run at two different temperatures in non-sequential experiments separated by about a month (Table 1; see previous section). A comparison of average τ for the group run at 20 °C with that of the sub-set run at 12 °C shows no significant difference at the 0·02 level (Student's two-tailed t test; d.f. = 20). When mean τ's are compared for only the sample of eight animals run at both temperatures they do not differ significantly at the 0·10 level. This result suggests that, as in the case of other circadian rhythms, the period of the Limax locomotor rhythm is compensated for changes in temperature (Q10 ~ 1·0).

In order to determine Q10 values for individual animals a separate experiment was carried out in which slugs were subjected to an abrupt 10 °C temperature rise (11·5/21·5 °C) in the course of a single run in DD (Fig. 8). The results are tabulated in Table 2. Approximately half of the animals were laboratory-reared and had been used in earlier experiments (animals PS 47–58). The remainder (animals PS 69–76) were freshly collected in April and May of 1975. Of the 18 animals in the sample, 16 expressed a clear rhythm both before and after the temperature shift. In 12 cases τ was longer (the rhythm slower) at the higher temperature. Individually calculated
Fig. 8. Effect of a 10 °C temperature shift. The slug was first allowed to free-run in DD at 11.5 °C. After 5 days a clear locomotory rhythm developed with a period of less than 24 h ($\tau$ (11.5 °C) = 23.5 h). On day 12 the temperature was raised to 21.5 °C. The slug became somewhat less active, but the period of the rhythm clearly changed to greater than 24 h ($\tau$ (21.5 °C) = 24.15 h).

Table 2. Free-running periods ($\tau$'s) and $Q_{10}$'s of Limax maximus before and after a 10 °C temperature shift from 11.5 to 21.5 °C

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>11.5 °C</th>
<th>21.5 °C</th>
<th>$Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS 47</td>
<td>23.6</td>
<td>23.95</td>
<td>0.99</td>
</tr>
<tr>
<td>PS 49</td>
<td>23.0</td>
<td>23.6</td>
<td>0.98</td>
</tr>
<tr>
<td>PS 50</td>
<td>23.3</td>
<td>24.15</td>
<td>0.96</td>
</tr>
<tr>
<td>PS 51</td>
<td>23.4</td>
<td>24.6</td>
<td>0.99</td>
</tr>
<tr>
<td>PS 52</td>
<td>—</td>
<td>(24.3)</td>
<td>—</td>
</tr>
<tr>
<td>PS 53</td>
<td>22.95</td>
<td>23.9</td>
<td>0.96</td>
</tr>
<tr>
<td>PS 54</td>
<td>(23.4)</td>
<td>24.5</td>
<td>—</td>
</tr>
<tr>
<td>PS 55</td>
<td>23.4</td>
<td>25.3</td>
<td>0.93</td>
</tr>
<tr>
<td>PS 56</td>
<td>23.4</td>
<td>23.4</td>
<td>1.00</td>
</tr>
<tr>
<td>PS 58</td>
<td>23.4</td>
<td>23.7</td>
<td>0.99</td>
</tr>
<tr>
<td>PS 69</td>
<td>23.95</td>
<td>23.15</td>
<td>1.04</td>
</tr>
<tr>
<td>PS 70</td>
<td>24.3</td>
<td>24.3</td>
<td>0.99</td>
</tr>
<tr>
<td>PS 71</td>
<td>23.5</td>
<td>23.9</td>
<td>0.98</td>
</tr>
<tr>
<td>PS 72</td>
<td>24.0</td>
<td>24.45</td>
<td>0.98</td>
</tr>
<tr>
<td>PS 73</td>
<td>23.6</td>
<td>23.95</td>
<td>0.99</td>
</tr>
<tr>
<td>PS 74</td>
<td>24.2</td>
<td>23.6</td>
<td>1.03</td>
</tr>
<tr>
<td>PS 75</td>
<td>24.3</td>
<td>23.6</td>
<td>1.02</td>
</tr>
<tr>
<td>PS 76</td>
<td>23.3</td>
<td>24.15</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Mean ± S.E.M. 23.5 ± 0.1 24.0 ± 0.1 0.99 ± 0.01

$Q_{10}$'s range from 0.93 to 1.04. The average for the sample was slightly less than unity: mean $Q_{10} ± S.E.M. = 0.99 ± 0.01$.

In no case did we note a phase shift in the rhythm following the 10 °C temperature step, and all animals that exhibited a rhythm appeared to be in steady state within a single cycle following the temperature step. Since the post-step free-run was of relatively short duration, it is possible that transients in $\tau$ might have been detected had there been a longer post-step free-run. However, we note that when individual $Q_{10}$'s are calculated for the eight animals exhibiting steady-state free-runs in non-
Fig. 9. Free-running periods in LL and DD. This slug was removed from LD 16:8 (darkness beginning at 04.00) and placed in a running wheel on day 0. A free-running rhythm was found in LL (intensity about 250 lux in this wheel) with a period of less than 24 h (T = 23.6 h; days 1-10). On day 11 the lights were turned off at 06.00. The level of activity in DD became slightly lower, but a clear rhythm was still evident with a period longer than that observed in LL (T = 24.0 h; days 12-23).

Table 3. Free-running periods (τ's) of Limax maximus in constant light (LL; 200-500 lux) followed by constant darkness (DD) at 15 °C.

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>LL</th>
<th>DD</th>
<th>Δτ* (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS 100</td>
<td>23.95</td>
<td>23.95</td>
<td>0.0</td>
</tr>
<tr>
<td>PS 101</td>
<td>24.5</td>
<td>(23.95)</td>
<td>(+ 0.5)</td>
</tr>
<tr>
<td>PS 102</td>
<td>24.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS 103</td>
<td>24.2</td>
<td>23.9</td>
<td>+ 0.3</td>
</tr>
<tr>
<td>PS 104</td>
<td>23.8</td>
<td>(24.5)</td>
<td>(-0.7)</td>
</tr>
<tr>
<td>PS 105</td>
<td>23.95</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS 106</td>
<td>24.2</td>
<td>24.4</td>
<td>-0.2</td>
</tr>
<tr>
<td>PS 107</td>
<td>24.0</td>
<td>(24.2)</td>
<td>(-0.2)</td>
</tr>
<tr>
<td>PS 109</td>
<td>23.0</td>
<td>23.6</td>
<td>-0.6</td>
</tr>
<tr>
<td>PS 110</td>
<td>23.6</td>
<td>24.0</td>
<td>-0.4</td>
</tr>
<tr>
<td>PS 111</td>
<td>23.7</td>
<td>24.0</td>
<td>-0.3</td>
</tr>
<tr>
<td>PS 113</td>
<td>23.8</td>
<td>24.3</td>
<td>-0.5</td>
</tr>
<tr>
<td>PS 115</td>
<td>23.95</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS 116</td>
<td>23.95</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PS 118</td>
<td>23.6</td>
<td>24.0</td>
<td>-0.4</td>
</tr>
<tr>
<td>PS 119</td>
<td>24.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>24.1 ±0.2</td>
<td>24.2 ±0.1</td>
<td>-0.3 ±0.1</td>
</tr>
</tbody>
</table>

* Δτ = τLL - τDD; Δτ < 0 implies τDD > τLL
sequential runs at 20 and 12 °C (Table 1), the mean $Q_{10}$ is also found to be slightly less than unity (0.99 ± 0.01). It therefore seems clear that, in this species, the locomotor circadian rhythm is temperature compensated ($Q_{10} \approx 1.0$) and a significant number of individuals appear to have $Q_{10}$'s of slightly less than 1.0 in the range tested.

(D) Locomotor activity in constant light

The ability of Limax to express a locomotor rhythm was also tested under conditions of constant light. An array of six 25 W incandescent bulbs provided the illumination; light intensity ranged between about 200 and 500 lux, depending upon the position of the running-wheel in the isolation chamber. Animals were freshly collected and maintained in LD 16:8 for 1–2 weeks before the experiment. At 15 °C the majority of animals (17 out of 20) exhibited a clear locomotor rhythm in LL (Fig. 9). In 3 of the 20, activity was low-level and irregular with no evidence of rhythmicity: no peak was present in the periodograms. One slug was clearly periodic, but with a non-stationary period (see following section). The mean period length in LL was 24.1 ± 0.2 h (Table 3). No significant correlation was found between period length and light intensity.

When released into DD, 11 of the 16 slugs showed evidence of a locomotor rhythm although in 3 animals the peak present in the periodogram was low. In the remaining 5 cases no peak was present. In no case was an animal clearly rhythmic in DD after failing to express a rhythm in LL. That is, animals that were inactive and/or arrhythmic in LL did not express a rhythm when released into DD. Thus, it is unlikely that arrhythmic behaviour in such cases was the result of exposure to LL conditions.

In comparing the free-running behaviour of slugs in LL and DD conditions, two results are evident. First, at 15 °C, slugs in LL tend to be somewhat more active than following their release into DD. Secondly, the free-running period in LL tends to be shorter than that found in DD. The example shown in Fig. 9 is typical in both respects. As tabulated in the rightmost column of Table 3, the period in DD was greater than the period in LL for 6 of the 8 cases where periodic behaviour was clear.
under both conditions. When data from all 8 slugs were averaged, the mean difference between the periods expressed in DD and LL was found to be about 18 min.

In separate experiments run at 20 or 22 °C the majority of animals failed to express a locomotor rhythm in LL at a variety of intensities (75, 700 and 1100 lux). In some cases, these animals were also run in DD following a short (4 days) exposure to LD 12:12. 3 of 4 slugs were found to be capable of rhythmic activity in DD, although it had not been expressed in the earlier LL free-run. At present we cannot account for the failure to observe a locomotor rhythm in LL at the higher temperatures.

(E) Stability of the Limax rhythm

Experience to date suggests that in Limax, as in other circadian systems (see Discussion), the circadian period of individual animals may not always be constant. ‘Nonstationarity’ of the Limax locomotory rhythm during a single run can be manifested primarily in two ways:

1. An abrupt change in the free-running period (seen thus far only in LL).
2. A change in phase of the rhythm relative to the LD cycle during entrainment.

An example of the former is shown in Fig. 10. This slug was one of the group of 20 animals described in the previous section as having been exposed first to LL and then to DD. During the first 5 days in LL, T was 23.8 h, but increased abruptly to 24.5 h on day 6 continuing to day 10. When released into DD on day 11, the level of activity dropped precipitously and therefore no rhythm was observed. A careful check of all records showed no enviromental changes (e.g. a shift in temperature or light intensity) that might easily account for the sudden period change during the LL free-run. Other animals in the same run showed no evidence of a similar change in period (cf. Fig. 9). We cannot yet account for this observation, nor can we as yet specify under what conditions slugs might generally be expected to show the phenomenon. Similar changes in T (lengthening of the period in LL) have been seen in other individuals during shorter LL free-runs and at higher light intensities (Sokolove, unpublished observations). Abrupt changes in period have not been found in DD free-runs.

The other non-stationarity phenomenon, drift in phase angle difference during LD entrainment, has been observed in nearly all slugs entrained for 1 week or longer to LD 12:12 or LD 16:8. When observed it resembles a ‘wobble’ in the activity rhythm: activity onsets occur slightly earlier for a few days and then slightly later for the next few, so that onsets may drift back and forth by as much as an hour over the course of 1-2 weeks (see Fig. 3, days 16-30).

Finally, it should be noted that in some animals the DD free-running period may change gradually over time. This is not yet certain, due to the lack of single extended free-runs (4 or more weeks). However, in one experiment (described earlier) circadian activity was assayed twice in DD at 20 °C with the assays separated by a 2.5 week period (see Fig. 6). In only 3 of the 12 slugs thus treated were the periodogram estimates of T identical for both free-runs. In seven cases the second T-estimate was smaller than the first by an average of 0.5 h (range 0.3-0.7 h) and in two cases (e.g. Fig. 6) it was larger by about 0.6 h (0.35 and 0.9 h).
The giant garden slug *Limax maximus* has an endogenous circadian oscillator whose output is expressed as a locomotor rhythm. The activity of individual animals can be readily monitored for up to 4 weeks in running wheels. The major properties of the locomotor rhythm are as follows:

1. The rhythm can be entrained and phase-shifted by 24 h LD cycles.
2. When entrained to LD cycles, the bulk of activity occurs in the dark, but the phase angle between the activity peak and the onset of darkness varies with the photoperiod of the entraining regime.
3. The rhythm can be expressed in constant light of moderate intensity as well as in constant darkness. Although not studied at different intensities, we note that the period of the rhythm of individuals tends to be shorter in LL than in DD. This suggests that *Limax* may be an exception to that part of the Circadian Rule which states that the period of nocturnal animals tends to lengthen with increasing light intensity (Aschoff, 1960).
4. The rhythm is temperature-compensated (over the approximate range 10–20°C) with a $Q_{10}$ that is often, although not invariably, slightly less than unity.

Other investigators have reported evidence suggesting circadian behaviour in terrestrial molluscs, but characterization of the rhythm in those cases was incomplete or lacking. In a study of the effect of temperature on locomotion of *Agriolimax reticulatus*, Dainton (1954) concluded that the normal activity rhythm was driven by the daily temperature cycle with nocturnal activity being triggered by the evening temperature drop. Evidence was cited which suggested that slugs were rhythmic in DD in 24 h temperature cycles, but that rhythmicity was lost after 4–5 days in DD at constant temperature (18°C). In *Arion ater*, Lewis (1969) reported an endogenous locomotor rhythm that in one individual lasted 5 weeks in DD (10°C). These slugs were reported not to entrain to an 18:5 h light cycle (LD 9:9:5), nor to follow a 6 h phase advance in a 24 h light cycle (LD 9:15). The effects of different temperatures or of altered phase relations during entrainment to different light cycles were not examined in either *Agriolimax reticulatus* or *Arion ater*.

A circadian locomotor rhythm has also been examined in the marine mollusc *Aplysia*. Various methods, including a partially submerged running-wheel (Jacklet, 1972), a computerized TV tracking system (Strumwasser, 1974) and various mechanical ‘lever-movement’ devices (Kupfermann, 1967; Block & Lickey, 1973) have been employed to assay locomotor activity. When the rhythm is assayed under conditions of constant darkness or in constant dim red light, activity peaks are not particularly well defined. The indistinct nature of the *Aplysia* rhythm in DD (cf. Jacklet, 1972) may, in fact, account for the conflicting reports concerning the persistence of the free-running rhythm following removal of the eyes (Block & Lickey, 1973; Strumwasser, 1974). Neither temperature compensation nor phase relationships during entrainment to different light-cycles has been studied in the case of the *Aplysia* locomotor rhythm. Evidence supporting the Circadian Rule in this diurnally active mollusc (shortening of the period with increasing light intensity together with an increase in the activity:rest-time ratio) has been reported by Jacklet (1972).
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The 'post-dawn' activity peak in Limax

Although it has been shown that the activity peak representing the entrained circadian rhythm is normally restricted to the dark portion of an entraining LD cycle, it is also true that in many slugs one finds a prominent 'lights-on' burst of activity during the first few days of LD. As noted earlier, this lights-on burst was most frequently found in animals maintained in the laboratory under LD 12:12 for extended periods, and tended to be absent or less pronounced in freshly captured slugs when tested in LD 12:12. The difference in the behaviour of 'old' and 'new' animals is not fully understood. It is also unclear why the lights-on burst is usually lost after only a few LD cycles. However, it seems very likely that, as in other nocturnally active animals (cf. Roberts, 1960; Page & Larimer, 1972), the lights-on burst represents an exogenously driven response rather than an entrained rhythm.

The lights-on burst observed in these experiments may reflect a light-avoidance escape response or an expression of post-dawn homing behaviour. Although the high level of post-dawn activity (occasionally more intense than the nocturnal activity peak) suggests that the escape response predominates, it is possible that homing behaviour, which normally occurs at the end of nocturnal activity (Gelperin, 1974), may also be involved. The loss of the post-dawn burst after a few days in the running wheel might, in fact, reflect acceptance of the wheel as the home site.

The effects of temperature on slugs

Temperature compensation of the frequency of biological rhythms has long been stressed as an essential feature of circadian oscillators (Pittendrigh, 1960). Thus, it is not surprising to find that the Limax locomotor rhythm has a $Q_{10}$ of about 1.0. Even the finding that many of the individuals tested showed a $Q_{10}$ of less than unity is not remarkable, since other rhythms have been described with a $Q_{10}$ of less than 1.0 over at least some portion of the temperature range examined (see, for example, Hastings & Sweeney, 1957; Caldarola & Pittendrigh, 1974). Whether the frequency of the Limax rhythm is a monotonically decreasing function of temperature cannot be determined from the present data. However, an interesting comparison can be made between the temperature dependence of the locomotor rhythm and that of the pedal wave rhythm in the Limax foot.

Both the frequency of pedal waves and the velocity of forward movement tend to be reduced when the temperature is lowered. By examining pedal waves of animals crawling at equal velocities up a glass plate, Crozier & Pilz (1924) were able to establish a $Q_{10}$ of 2.1 for the pedal wave frequency over the range 11-21 °C. This value is considerably higher than that obtained in the case of the circadian rhythm. The difference suggests that the physiological mechanisms responsible for compensating the behaviourally expressed circadian rhythm are not shared by nervous-system elements that organize the locomotor behaviour itself.

Segal (1961) has reviewed the temperature dependence of other measures such as heart rate, growth, and $O_2$ consumption, in a variety of molluscs including a related species, Limax flavus. In general, all measures are increased at higher temperatures whether or not animals are allowed time to acclimate. For example, $O_2$ consumption in a slug acclimated at 10 °C is more than doubled when the temperature is raised to
20 °C. When re-acclimated at 22 °C, O₂ consumption is still nearly 1.8-fold greater than the rate that had been found at 10 °C. Thus, the metabolic rate of *L. flavus*, although compensated to some degree by acclimating mechanisms, is in no way as efficiently compensated as is the *L. maximus* locomotor rhythm.

**Non-stationarity of the Limax rhythm**

The most striking examples of non-stationarity of the *Limax* circadian system were found in a few individuals which showed an abrupt and apparently spontaneous increase in period in LL. No reason for such changes can yet be suggested. Similarly abrupt changes in period were never observed in single DD runs. However, there is evidence which suggests that the DD period is also somewhat labile. In slugs assayed at different times, but under presumably identical conditions, the free-running period is often found to be shorter in the later assay. Whether this is due to physiological changes occurring in the animals or is a result of possibly different conditions (as yet undetermined) prior to each assay, cannot be specified without further investigation.

Lability of τ is not unique to the *Limax* locomotor rhythm. Spontaneous abrupt changes have been noted in lizards (Hoffman, 1960), Arctic ground squirrels (Swade & Pittendrigh, 1967), bats (Rawson, 1960), and mice (Pittendrigh, 1968). In a systematic study of sparrow rhythmicity, Eskin (1971) described both sudden and gradual changes in τ as well as after-effects following photo-entrainment in various LD cycles. Of particular interest is the observation that in birds tested twice following entrainment to the same LD cycle, τ of the second free-run was generally shorter than that of the first free-run. Although the significance of this observation is not clear, it is perhaps noteworthy that the *Limax* system appears to behave in a similar fashion.

The non-stationarity of the phase angle difference during LD entrainment might be attributed to the 'weakness' of the entraining signal, but thorough studies have not been carried out to determine whether such phase instability would be eliminated in higher intensity LD cycles. It is also possible that phase instability is due entirely to long-term transients associated with entrainment to a new light cycle. Although phase angle instability can be found for up to 4 weeks after the beginning of entrainment, it should be noted that long-term phase angle transients have been found to persist for a month or more in sparrows (Eskin, 1971).

**Limax as a system for the study of circadian behaviour**

The role of the nervous system in the organization and maintenance of circadian rhythms of behaviour has not yet been completely determined for any animal. In some cases surgical manipulations coupled with behavioural assays have yielded clear evidence of CNS involvement in the generation of circadian oscillations (Nishiitsutsuji-Uwo & Pittendrigh, 1968; Truman, 1972; Loher, 1972, 1974; Stephan & Zucker, 1972; Roberts, 1974; Sokolove & Loher, 1975; Sokolove, 1975b). However, similar techniques have shown that in sparrows the timing of circadian behaviour appears to be controlled by an endocrine clock in the pineal that requires no direct neural connexions with the CNS (Zimmerman & Menaker, 1975). Whether the *Limax* locomotor rhythm will ultimately prove to be mediated by neural or hormonal mechanisms cannot be predicted. Regardless of the specific mechanisms involved, we believe it may prove possible to study, perhaps at the single cell level, the role of the
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CNS in the generation of rhythmicity, and/or the means whereby clock output modulates the locomotor behaviour of Limax. The CNS of Limax is well suited to cellular neurophysiological and anatomical studies (MacKay & Gelperin, 1972; Chang, Gelperin & Johnson, 1974; Prior & Gelperin, 1976; Gelperin & Forsythe, 1976). Sensory inputs (Eakin & Brandenburger, 1975; Donnelly, 1976) and motor output (Prior & Gelperin, 1974) relevant to circadian locomotion can be analysed. The ubiquitous occurrence of Limax (Quick, 1961; Chichester & Getz, 1973) and ease of obtaining developmental stages (Forsythe, 1977) are also of advantage. Our initial studies suggest that the behavioural rhythm of Limax is sufficiently robust to warrant the continued investigation of this system.

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