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Temperature compensation of circasemilunar timing in the intertidal insect *Clunio**

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Summary. In cultures of a subtropical population of the one-hour midge *Clunio tsushimensis*, semilunar rhythms of emergence with a period of 15 days can be entrained by using artificial moonlight cycles of 30 days in otherwise invariant 24-h light-dark cycles (0.3 lux over four successive nights every 30 days of LD 12:12). After changing to an invariant photoperiod (LD 12:12 without the moonlight programme) or even to continuous darkness, freerunning semilunar rhythms were observed for up to 3 months using cultures of a mixed age structure containing all larval instars. The mean period was 14.2 days at 19 °C, i.e. clearly shorter than under entraining conditions (14.7 days in nature, 15.0 days with the artificial zeitgeber). In the range 14°–24 °C (corresponding to the mean seawater temperatures at the place of origin in winter and summer) there was only slight temperature dependence. The Q_{10} of the circasemilunar period, however, was not significantly different from 1.0. In continuous darkness the freerunning period was about 15.2 days. Both experiments provide supporting evidence for the existence of a temperature-compensated circasemilunar oscillator acting as an endogenous clock mechanism controlling the timing of imaginal disc formation and pupation in the intertidal chironomid.

Introduction

In ectothermic animals, and algae and plants, the daily rhythms of metabolic events and behavioural activities freerun under constant laboratory conditions; their period of about 24 h is stable over a relatively wide range of temperatures. This was first demonstrated in *Drosophila pseudoobscura* by Pittendrigh (1954) and in *Gonyaulax polyedra* by Sweeney and Hastings (1960). In conclusion, this temperature compensation or homeostasis of the freerunning period τ is considered an essential property of the underlying physiological timing mechanism, called the circadian oscillator (Bünning 1958; Pittendrigh 1954, 1981; Pittendrigh and Caldarola 1973). From an ecological point of view, this temperature independence of the circadian period is a prerequisite for reliable timing within the real day because the homeostasis of τ results in a stable phase relationship between the circadian rhythm and the 24-h day-night zeitgeber cycle.

With regard to long-term biological rhythms that correlate with environmental cycles such as semimonthly spring tide cycles, lunar months or seasons, it has been repeatedly assumed that temperature compensation is also an integral property of the underlying circalunar or circannual oscillators (see Pittendrigh 1981). For circannual rhythms, convincing results were presented as early as 1959 for the beetle *Anthrenus* (Blake 1959). For circalunar rhythms, however, strong evidence for this theory has until recently been lacking (Neumann 1981). For the study of lunar rhythms of intertidal animals, appropriate laboratory stocks have become available during the past few years so the influence of temperature on freerunning lunar rhythms could also be tested. In the lunar-monthly reproductive rhythm of the polychaete *Typosyllis prolifera* (mean freerunning period 31 days), Franke (1985) demonstrated Q_{10} coefficients of 1.04 for a 10 °C increase in the temperature range 15°–25 °C, and this during an invariant 24-h light-dark cylce with 16 h light (LD 16:8). Corresponding results on semilunar (i.e. lunar-semimonthly) rhythms have up to now only been

* Dedicated to Prof. Colin S. Pittendrigh on the occasion of his 70th birthday, in recognition of his leading and stimulating contributions in the field of biological timing systems

mentioned in reviews (Neumann 1985, 1986). The detailed data are presented in this paper. In addition, a freerunning experiment in conditions of constant darkness instead of invariant 24-h light-dark cycles was conducted to determine whether the circasemilunar clock mechanism is based on some kind of mechanism for counting about 15 photoperiodic cycles or on a real long-term oscillation of about a fortnight. The subject of the study is the marine chironomid *Clunio*, which is characterized by a semilunar rhythm of metamorphosis and emergence occurring within synchronized populations.

Material and methods

The laboratory stock of *Clunio tsushimensis* Tokunaga was derived from egg masses collected in 1980 near to the Shimoda Marine Research Centre on the Pacific coast of the Izu peninsula, Japan. The semilunar pattern of emergence and reproduction of the local population was described by Oka and Hashimoto (1959). The experiments were conducted in temperature- and light-controlled rooms using the breeding methods employed for the European species *Clunio marinus* (Neumann 1966).

The generation time of the four larval instars and the pupal stage in *C. tsushimensis* lasted for several months, this being considerably longer than in both the field population under the Shimoda summer conditions (6 weeks; Oka and Hashimoto 1959) and the stocks of *Clunio marinus* (range 40–100 days between specimens at 20 °C; Neumann 1966). This lenghtening of the development in the *C. tsushimensis* larvae perhaps results from special nutritional requirements which could not be met by the feed utilised in the laboratory at Cologne (blue-green algae, a few diatoms, and mainly fine nettle powder). However, the long generation time gave an excellent opportunity for observing freerunning semilunar rhythms of emergence for up to at least 3 months by releasing synchronized mass cultures of a mixed age structure into conditions with an invariant photoperiod or even into continuous darkness.

The standard cultivation conditions for the *C. tsushimensis* stock were a 24-h light-dark cycle with 16 h light (LD 16:8) at 19 °C (climatically controlled chambers with fluorescent light tubes, about 1000 lux during the light period; daily temperature fluctuation in the seawater medium less than ± 0.5 °C). The semilunar rhythm was evoked by artificial moonlight of about 0.3 lux presented every 30 days over four successive nights.

Synchronized mass cultures were then exposed to three different temperatures (14°, 19° and 24 °C) under LD 12:12 conditions without moonlight during the subsequent months (the pretreatment of each mass culture was for at least 60 days under the conditions mentioned previously). 14 °C is in the range of the mean winter seawater temperatures of the place of origin, and 24 °C corresponds to the seawater temperature during July and August (Oka and Hashimoto 1959). At each of the three temperatures, two cultures were studied. The number of females and males emerging was counted daily. The specimens of the parallel cultures in each experiment were pooled because the emergence patterns were very similar within the range of the clear-cut peaks of the entrained as well as the freerunning semilunar rhythms. In this way, some temporal fluctuations (caused by the age distribution of the larval populations varying with time) could be balanced between both cultures. In other words, the two cultures were considered as parts of one and the same mass culture. The three experiments were repeated once with new mass cultures (serie II). The important results of both series (I, II) are summarized in Table 2. The repeat experiment demonstrated the freerunning rhythms best of all; the results are shown in Fig. 1 and Table 1.

In an additional freerunning experiment (Fig. 2), semilunar periodic cultures grown at 19 °C (artificial moonlight programme for at least two 30-day cycles, LD 12:12) were exposed to continuous darkness for up to 3 months. This experiment only succeeded in cultures with a small substrate layer and with little food; under these conditions the larvae were not impaired by the decomposition of the algae and the deficit of oxygen. The emergence of the midges was checked daily using a torch with red light of long wavelength (Schott filter RG 5, light only above 640 nm).

Results

When mass cultures of *Clunio tsushimensis* were bred under invariant conditions (e.g. LD 12:12, 19 °C), the rate of emergence of midges was more or less uniform (Neumann 1985, control in Fig. 3, p. 168). Increasing or decreasing numbers within one culture corresponded to variations in the age structure of the population, and occurred without any regularity or synchrony with other cultures. However, strong synchrony of a semilunar emergence rhythm could always be evoked by an artificial 30-day moonlight programme which was independent of the natural lunar month (*C. marinus*, Normandy stock, Neumann 1966; *C. tsushimensis*, Neumann 1985). The rhythm is characterized by a clear phase relationship between the peaks of emergence and the days with illuminated night (Fig. 1, days 30–60).

In the present experiments (Fig. 1), some cultures were exposed to an abrupt temperature drop, others to an abrupt increase, both occurring after the last moonlight treatment. Independent of these different temperature treatments, the semilunar emergence patterns of the synchronized cultures freerun with at least five regular peaks over $2\,^1/_2$ months. The details can best be evaluated by using the medians of the peaks (Table 1), at least during that part of the free running experiment where there are intervals of no emergence or clear minima between the separate peaks (F_1–F_5). Thereafter, the emergence pattern changed to being more or less irregular, presumably because the offspring of the culture had not been exposed to the entraining moonlight 3 or more months previously.

After the change to the lower and higher temperatures a small but significant transient temperature-induced effect occurred with regard to the first emergence peak only; this was delayed by about $1\,^1/_2$ days at 14 °C and advanced by about 3 days

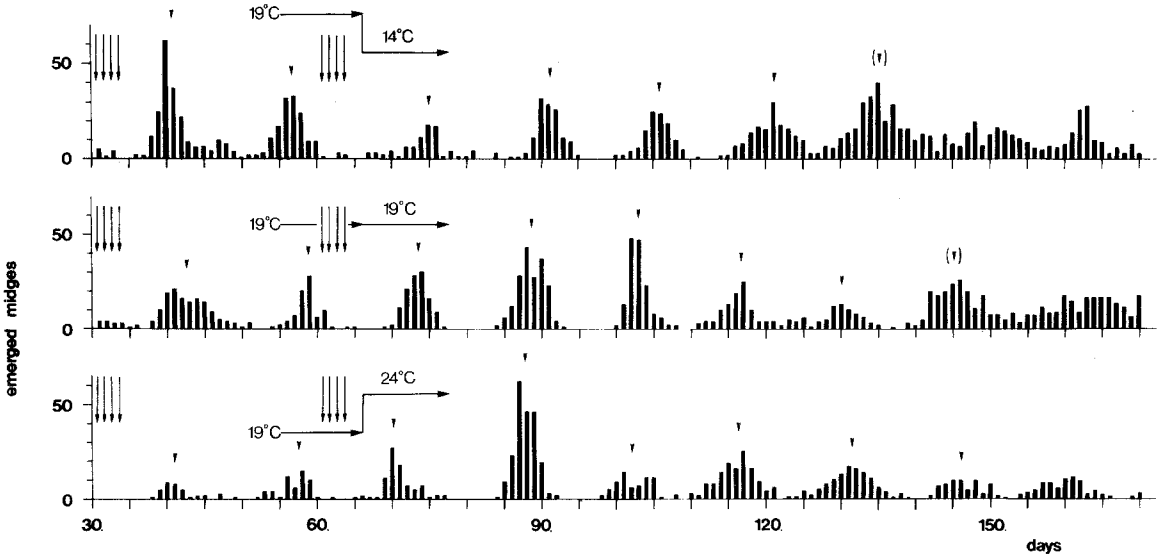

Fig. 1. Freerunning semilunar emergence rhythms of males of *Clunio tsushimensis* at three different temperatures during LD 12:12 conditions. The lunar-semimonthly synchronisation of the three experimental groups occurred at 19 °C during days 1–64 by exposure to artificial moonlight every 30 days during nights 1–4, 31–34, and 61–64 (long arrows). Recording of the number of midges emerging per day started on day 30 when the synchronisation became manifest. On day 66, groups 1 (above) and 3 (below) were transferred to 14° and 24 °C, respectively. Arrowheads, medians of the individual emergence peaks. Arrowheads in brackets, estimate of medians with overlapping cohorts

Table 1. Medians and 95% confidence intervals (CI) of the emergence peaks in the freerunning semilunar rhythm shown in Fig. 1

		Z_2	F_1	F_2	F_3	F_4	F_5
14 °C	$\tilde{x}$	56.7	74.8	91.0	105.6	120.9	(134.9)
	95% CI	56.5–57.1	74.1–75.4	90.6–91.4	105.2–106.1	120.5–121.4	134.3–135.2
	n	142	82	128	112	158	256
19 °C	$\tilde{x}$	58.7	73.4	88.5	102.8	116.6	130.0
	95% CI	58.3–59.1	73.0–73.8	88.2–89.1	102.5–103.1	116.1–117.1	129.3–130.8
	n	79	119	183	151	111	70
24 °C	$\tilde{x}$	57.4	70.6	87.7	102.0	116.2	131.4
	95% CI	56.3–58.0	70.2–71.2	87.4–88.0	101.1–103.9	115.5–116.8	130.7–132.1
	n	55	82	211	66	130	117

Z_2, second peak with moonlight zeitgeber (days 54–60); F_1–F_5, peaks after the last moonlight stimulus (days 61–64); n, number of male midges per emergence peak.

at 24 °C (compare F_1 medians, Table 1). In none of the following peaks and intervals similar temperature modifications of the freerunning semilunar rhythm could be detected; the range of intervals observed was very similar for all three temperatures. The best correspondence was found between the second and fourth peak, which were the most critical for evaluating the freerunning semilunar rhythm (see Discussion). Here, the intervals between the medians ranged only between 13.8 and 15.4 days.

Table 2 summarizes the results of all the freerunning data, based on the median calculations and their 95% confidence intervals (compare Table 1). Both sexes are represented separately because the emergence rhythm of the females was sometimes delayed by about 1 day in comparison to the males. Thus, the interval data are neither burdened by this sex-specific property nor by the varying ratio of the numbers of males and females. A Q_{10} calculation reveals the low influence of temperature on the period of the freerunning semilunar rhythm. For this purpose, a set of Q_{10} values was calculated by the limits of the circasemilunar periods (Table 2) for each of the temperature intervals 14°–19 °C, 19°–24 °C, and 14°–24 °C. The corresponding means of the Q_{10} sets and the 95% confidence limits were not different from 1.0 in most cases (e.g. males of series II, $Q_{10} = 1.03 \pm 0.18$ between 19° and 24 °C, $Q_{10} = 1.18 \pm 0.20$ between 14°–24 °C; similar values for all temperature intervals of series I). Only for the interval 14°–19 °C

Table 2. Ranges of the circasemilunar period between peaks F_2 and F_3 and between F_3 and F_4 of the freerunning semilunar rhythm in the two experimental series (I and II)

			F_2–F_3	F_3–F_4
14 °C	I	♂♂	14.2–15.4	14.0–15.2
		♀♀	14.0–17.1	12.8–16.2
	II	♂♂	13.8–15.5	14.4–16.2
		♀♀	13.9–15.8	14.4–16.1
19 °C	I	♂♂	13.4–14.3	14.0–15.8
		♀♀	13.3–14.3	13.9–15.6
	II	♂♂	13.4–14.9	13.0–14.6
		♀♀	13.2–14.4	12.9–15.2
24 °C	I	♂♂	13.5–14.7	12.7–14.1
		♀♀	13.5–14.6	13.2–14.5
	II	♂♂	13.1–16.5	11.6–15.7
		♀♀	13.8–15.2	14.0–15.9

The upper and lower limits of each range were calculated by the 95% confidence intervals of the medians, as shown in Table 1 for the males of series II

in series II was the Q_{10} slightly higher than 1.0 (males, $Q_{10}=1.16\pm0.11$; females, Q_{10} 1.19 ± 0.12).

Freerunning lunar rhythms for marine organisms have generally been recorded in breeding conditions with an invariant photoperiod (Hauenschild 1960; Bünning and Müller 1961; Neumann 1966, 1981; Franke 1985; Fig. 1 this paper). Thus, it was an unsolved problem whether the circalunar oscillator underlying the lunar-semimonthly or lunar-monthly timing integrates some kind of counting mechanism for successive photoperiodic cycles – about 15 days in semilunar rhythms, and about 30 days in lunar-monthly rhythms. Figure 2 shows freerunning circasemilunar rhythms in continuous darkness. The main properties of the rhythm are the same as in the LD 12:12 control (Fig. 1, middle): a concentration of emerged midges occurred about every 2 weeks for at least 2 months, and the mean period was 15.2 days on the basis of the median calculations (7 intervals, min. 13.0 days, max. 16.9 days). The numbers are not as convincing as with an invariant photoperiod; this was most probably a result of deterioration of the breeding conditions (decay of algae in the substrate), but may also have been a consequence of impaired development under conditions with disturbed circadian organisation of the specimens and no light stimulation. However, the semilunar rhythm persists, which means that the period of the circasemilunar timing mechanism of each larvae is independent of any photoperiod information from the environment.

Discussion

In insects, the properties of those timing mechanisms controlling developmental steps such as pupation or emergence can only be analysed at the level of synchronized laboratory populations because these events occur only once in the life time of each individual. However, from the freerunning rhythms of pupation and emergence one can easily determine the period of the endogenous timing system which autonomously controls both waiting intervals in each specimen and temporal gates for overt developmental processes.

The circadian clock system triggers the emergence of the pharate imago from the pupal skin (Pittendrigh 1954; Truman 1972). This is also true for *Clunio marinus* (Neumann 1966). The semilunar timing system of the chironomid *Clunio* switches two steps during the metamorphosis of

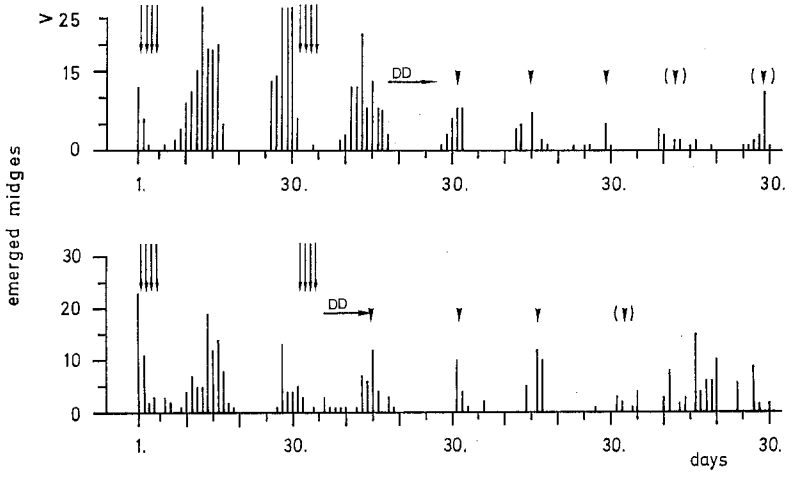

Fig. 2. Freerunning emergence pattern of *Clunio tsushimensis* in continuous darkness (DD) after pretreatment for semilunar periodic synchronisation (long arrows, artificial moonlight; LD 12:12, 19 °C). Results of two independent experiments are shown

the larva every 2 weeks: firstly, a distinct period in early imaginal disc formation during the last larval instar (about stage 3 out of nine in L IV; stages according to Wülker and Götz 1968), and secondly about 2 weeks later pupation. After a pupation period of 3–6 days (in the temperature range 14°–24 °C), a semilunar rhythm of emergence occurs in the population (Krüger and Neumann 1983; Neumann 1986). One may conclude that the freerunning circasemilunar rhythm of emergence demonstrates the existence of a circasemilunar oscillator.

The peaks of the freerunning semilunar rhythm correspond to successive cohorts of different physiological ages starting the metamorphosis from stage L IV-3 at approximately semimonthly intervals. The first two peaks after the last moonlight period in Fig. 1 (peaks F_1 and F_2 in Tables 1, 2) correspond to the two semimonthly peaks which occurred during the standard moonlight entrainment (in Fig. 1 between days 30 and 60). Given that, the first semilunar switching point occurs about $2\,^1/_2$ weeks before emergence (see above), it may be concluded that only the subsequent freerunning peaks represent the real freerunning rhythm (peaks F_3–F_5 in Tables 1, 2). They convincingly demonstrate that the circasemilunar timing system initiates the final metamorphosis within a small temporal gate of a few days about every 2 weeks. The first semilunar synchronisation during imaginal disc formation (step 1) is thereby stabilised by the second semilunar synchronisation during pupation about 15 days later.

Because of the foregoing considerations and because desynchronisation begins at about peak 5, the peaks F_3 and F_4 and the periods both between F_2 and F_3 and between F_3 and F_4 are the most critical ones for evaluating the freerunning semilunar rhythm (Table 2). Therefore, only these values have been used for calculating the circasemilunar periods and Q_{10} coefficient (p. 673, 674). As tested by statistical methods (95% confidence limits), the Q_{10} was not different from 1.0 in most cases. However, if the Q_{10} is calculated on the basis of the intervals observed between the medians of the emergence peaks (F_2, F_3 and F_4) only, then a weak tendency is indicated for the Q_{10} being different at different ambient temperatures (1.12 in the range 14°–19 °C, 1.00 in the range 19°–24 °C). In any case, the Q_{10} was clearly within the range cited for freerunning circadian rhythms (0.8–1.2; Sweeney and Hastings 1960; Aschoff 1979).

In continuous darkness, the accuracy of the freerunning semilunar rhythm was not as high as with the invariant photoperiod; the amplitude (given by the number of emerged specimens) was decreased. There might be two main reasons for this: (a) deterioration of the breeding conditions with increasing mortality at all physiological stages; and (b) the occurrence of some kind of developmental dormancy during larval growth. However, the mean period in continuous darkness was 14.6 days, a little longer than the 14.2 days in the LD 12:12 cycles, and both were somewhat shorter than the mean periods of the entrained semilunar rhythms (15.0 days in the laboratory, 14.7 days in the field).

Finally, the period of freerunning semilunar rhythms of *C. tsushimensis* is relatively close to the period under synchronized field conditions. Furthermore, its period is nearly temperature compensated in the absence of temporal cues. As demonstrated in this species for lunar-semimonthly rhythms and in *Typosyllis* for lunar-monthly rhythms, the temperature compensation seems to be a fundamental property of the physiological oscillators of rhythms selected for timing in correlation to the main environmental cycles.

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