

Preliminary evidences of circadian fan activity rhythm in *Sabella spallanzanii* (Gmelin, 1791) (Polychaeta: Sabellidae)

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SUMMARY: The fan activity rhythm of *Sabella spallanzanii* (Gmelin, 1791) and its entrainment capability to light were studied. Animals were tested under constant darkness (DD) followed by two consecutive 24 h light-darkness regimes: a first 11 h light period (LD) and a second 9 h light period, with its phase inverted (DL). An infrared analogical video-camera took shots each 30 s. A number of pictures with open fan were counted every 15 min. In DD a weak free-running periodicity in the circadian range was found, thus reinforcing the matching of the 24 h period under study in both photoperiod regimes. A nocturnal activity was characterised with a consistent anticipation to light_{OFF} (i.e. entrainment). Moreover, this phase of entrainment differed between DL and LD. The presence of endogenous activity rhythm with a variable phase angle of entrainment is a distinctive feature of circadian pacemakers.

Keywords: *Sabella spallanzanii*, fan activity, circadian rhythms, entrainment.

RESUMEN: EVIDENCIAS PRELIMINARES SOBRE EL RITMO CIRCADIANO EN LA APERTURA DEL ABANICO DE *SABELLA SPALLANZANII* (GMELIN, 1791) (POLYCHAETA: SABELLIDAE). – En este trabajo, se estudiaron el ritmo de apertura del abanico del poliqueto *Sabella spallanzanii* (Gmelin, 1791) y su capacidad de encarrilamiento a la luz. Los animales fueron expuestos bajo oscuridad constante (DD) seguida por dos regímenes consecutivos de luz-oscuridad de 24 h, el primero compuesto por 11 h de luz (LD), y el segundo por 9 h de luz y con su fase invertida (DL). Dicha actividad se midió utilizando fotogramas tomados cada 30 s, mediante una videocámara analógica provista de iluminación infrarroja. Los fotogramas mostrando el abanico abierto fueron sumados cada 15 min. En DD, se detectó una periodicidad débil, en curso libre de rango circadiano en todos los animales. El ritmo se mostró más robusto y asumió un valor de 24 h bajo los dos fotoperíodos empleados. Se evidenció una actividad nocturna con anticipación (encarrilamiento) al apagado de la luz. Además, esta fase de encarrilamiento fue diferente entre LD y DL. La presencia de un ritmo de actividad endógeno con una fase de encarrilamiento variable, sugiere la presencia de un oscilador circadiano.

Palabras clave: *Sabella spallanzanii*, actividad del abanico, ritmos circadianos, encarrilamiento.

INTRODUCTION

The Mediterranean polychaete *Sabella spallanzanii* (Gmelin, 1791) is a large filter-feeder exhibiting

a behavioural activity of fan opening and retraction, mainly related to feeding upon suspended particles and respiration (e.g. Riisgård and Ivarsson, 1990; Giangrande, 1991). To date, little is known on the reg-

ulation of this behaviour with respect to environmental cycles. In polychaetes, the interplay of endogenous activity rhythms and light-darkness cycles was postulated as a basis for the regulation of seasonal growth and reproduction (Olive, 1984). In *Sabella* spp., a synchronous spawning between sexes was characterised in winter (Giangrande and Petraroli, 1994; Giangrande *et al.*, 2000; Currie *et al.*, 2000).

In marine environments, photic and tidal cycles are the most important time cues (i.e. zeitgebers) responsible for the entrainment of organisms' rhythms over 24 h (e.g. Naylor, 1985; 2005). An important feature of the light-darkness cycle as a zeitgeber is the duration of its photophase whose variations produce different phase angles of entrainment (Aschoff, 1981). The entrainment of daily cycles in behaviour and physiology to different photoperiod lengths is of importance for polychaetes to regulate growth, reproduction and behaviour accordingly to seasons (e.g. Schiedges, 1979; Garwood and Olive, 1982; Last *et al.*, 1999; Last *et al.*, 2000; Giangrande *et al.*, 2000; Last and Olive, 2004). In this study, the occurrence of an endogenous fan activity rhythm and its entrainment capability were studied in *S. spallanzanii* kept under laboratory constant darkness and in different artificial photoperiod regimes.

MATERIAL AND METHODS

Experimental setup and data collection

A total of 14 individuals with an average tube length of 21.5 cm (± 0.1 mm precision) were used in this study. Animals were collected in October at a depth of 5–10 m off Formia, (Italy, the Tyrrhenian Sea: 41°15'14"N, 13°36'14"E). After capture, animals were immediately transferred into an aquarium (40×40 cm surface; 30 cm depth) set in a light-proof room. There, animals were placed vertically avoiding reciprocal contact within a plastic vial rack positioned on the aquarium floor. The appropriate circulation and filtration of seawater was supplied at a constant temperature ($19 \pm 0.1^\circ\text{C}$) and salinity (35) by an external pump for the entire duration of the experiment.

The animals' activity was recorded by a b/w Urmet Sideltronix EX 1090/105 analogical video camera. This was connected via cable to a PC endowed with the DT3155 Frame Grabber (Costa *et al.*, 2006). A digital frame was acquired every 30 s.

Frames were classified based on fan posture: 0 - fan retracted (i.e. inactivity); 1 - fan extruded and opened (i.e. activity). All frames referring to the same animal were classified by the same observer in order to prevent interpersonal subjectivity.

Fourteen animals were submitted to DD treatment for 8 days. Out of these animals, ten were then exposed to two consecutive stages of 6 days each of light-darkness (LD; 11:13 h) and a 12 h phase-inverted stage of darkness-light (DL; 9:15 h). The DD stage allowed a reliable estimation of the rhythm's spontaneous periodicity (τ) in absence of the consolidating effect of the zeitgeber on behavioural rhythms of invertebrates (see Saunders, 2002). This stage also allowed them to recover from the stress of capture and become acclimatised to laboratory conditions (e.g. Aguzzi *et al.*, 2005). The light-darkness stages were used to assess the phase of the fan activity rhythm with respect to that of the light cycle and the entrainment capability under critical conditions (i.e. the 12 h phase inversion).

The photophase was obtained by superimposing two white-light neon lamps of different intensity (i.e. of ~ 100 and ~ 300 lux at animal location, respectively). Their ON and OFF timings were temporally shifted 30 min simulating gradual dusk and dawn transitions in order to avoid unknown damaging effects to the animals' photoreceptor system. For the LD stage, the light_{ON} timings were 06:30 (the 100 lamp) and 07:00 (the 300 lamp). The light_{OFF} timings were 17:00 (the 300 lamp) and 17:30 (the 100 lamp). Phase-inversion was achieved by extending for 12 additional hours the photophase of the last LD cycle. For the DL stage, the light_{ON} timings were 17:00 (the 100 lamp) and 17:30 (the 300 lamp). The light_{OFF} timings were 07:30 (the 300 lamp) and 08:00 (the 100 lamp). The photophase length considered for the analyses of entrainment was always defined by the ON-OFF timings of the 100 lamp.

The experiment lasted only 20 days since animals were not fed during tests in order to prevent the possible entrainment effects of the feeding schedule (Fernández de Miguel and Aréchiga, 1994). Additionally, these are fragile organisms and their prolonged maintenance in laboratory conditions results in a high rate of mortality (Costa, unpub. obs.).

Data processing

Behavioural data of invertebrates show high variability over short time intervals, so their pro-

cessing is necessary prior to time series analysis (e.g. Dowse and Ringo, 1994; Saunders, 2002). Time series of behavioural events every 30 s were summed per 15 min. All data sets were then filtered with a three-step moving average (time window of 45 min) in order to remove persisting high frequency noise (Dowse and Ringo, 1994; Levine *et al.*, 2002). This filtering frequency is consistently lower than that characterising any ultradian and circadian rhythm of ecological interest in the marine context (e.g. Levine *et al.*, 2002; Aguzzi *et al.*, 2004). This procedure is usually done at greater time intervals (i.e. T/2) in order to eliminate the reactive peaks that mask the endogenous control of activity at light-darkness transitions (Canal-Corretger *et al.*, 2001, 2003). All data sets were represented in the time domain by means of actograms.

Periodogram analysis

The periodicity at each stage (DD, LD and DL) was tested by a periodogram analysis on corresponding segments of the time series. A regressive periodogram (Clemfuss and Clopton, 1993) was used to screen data sets between 10 and 26 h thus detecting both circatidal and circadian rhythms. In periodogram plots, the highest significant ($p < 0.05$) peak represented the maximum percentage of total data variance explained by the dominant periodicity (Canal-Corretger *et al.*, 2001). That peak value was chosen for period attribution.

Phase angle analysis

The entrainment of behavioural rhythms under LD and DL was studied by computing a phase angle value between the rhythm and the darkness phase over consecutive days. Time series were divided into sections corresponding to LD and DL stages. Each of these sections was then subdivided into 24 h segments. The phase of the activity rhythm was assessed for each segment by computing an average from all its values (e.g. Hammond and Naylor, 1977; Aguzzi *et al.*, 2004). That average was represented as a horizontal line in the 24 h plot. Values above it constituted the active phase of the rhythm. This was delimited by an onset and an offset that were temporally defined by activity values that remained above and below the horizontal line, respectively, for a minimum of 3 h. The timing in $\text{light}_{\text{OFF}}$ was subtracted from timings of activity onset and offset,

giving corresponding values of phase angle in minutes (i.e. $\Psi_{\text{onset}} = \text{onset} - \text{light}_{\text{OFF}}$; $\Psi_{\text{offset}} = \text{offset} - \text{light}_{\text{OFF}}$). Thus, negative phase angles denoted an advance of the rhythm phase with respect to the one of the zeitgeber. For each photoperiod treatment, Ψ_{onset} and Ψ_{offset} of all animals were averaged at corresponding days. Mean values were graphically represented along with vertical lines depicting timings in $\text{light}_{\text{OFF}}$ and light_{ON} .

The difference in the magnitude of phase angle of entrainment depending on the light regime was also estimated. To do this, a waveform was computed for LD and DL stages by averaging 24 h data segments at corresponding 15 min. For each animal, Ψ_{onset} and Ψ_{offset} were calculated as detailed above. Resulting values were then averaged for each photoperiod regime. Mean phase angle values (\pm sd) for LD and DL were represented in the same plot considering time 0 (the black vertical line) as the reference for both light_{ON} and $\text{light}_{\text{OFF}}$. A single line was chosen in order to compare the response of fan activity rhythms at each photoperiod treatment independently from different photophase lengths.

A linear regression analysis was performed to assess the occurrence of transient changes of the rhythm phase over consecutive days at each photoperiod treatment. Student's t-test for dependent samples was used to find significant differences in phase angles in LD and DL. The normal distributions and homogeneity of variances of previously defined variables (rhythm onset and offset; Ψ_{onset} and Ψ_{offset}) for the chosen categories (i.e. days and stages) were proven (Kolmogorov-Smirnoff test, $p > 0.1$; Levene test, $p > 0.05$, respectively) prior to parametric statistical analysis.

RESULTS

Sabella spallanzanii expressed a measurable fan activity rhythm under constant darkness (DD) and consecutive stages in different photoperiod regimes (LD, DL). All animals survived without feeding over the whole test period with the exception of one that died during the first day in DL.

Periodogram Analysis

Figure 1 shows a double-plotted actogram depicting the fan activity rhythm of one animal (A) over the different stages of the experiment as an

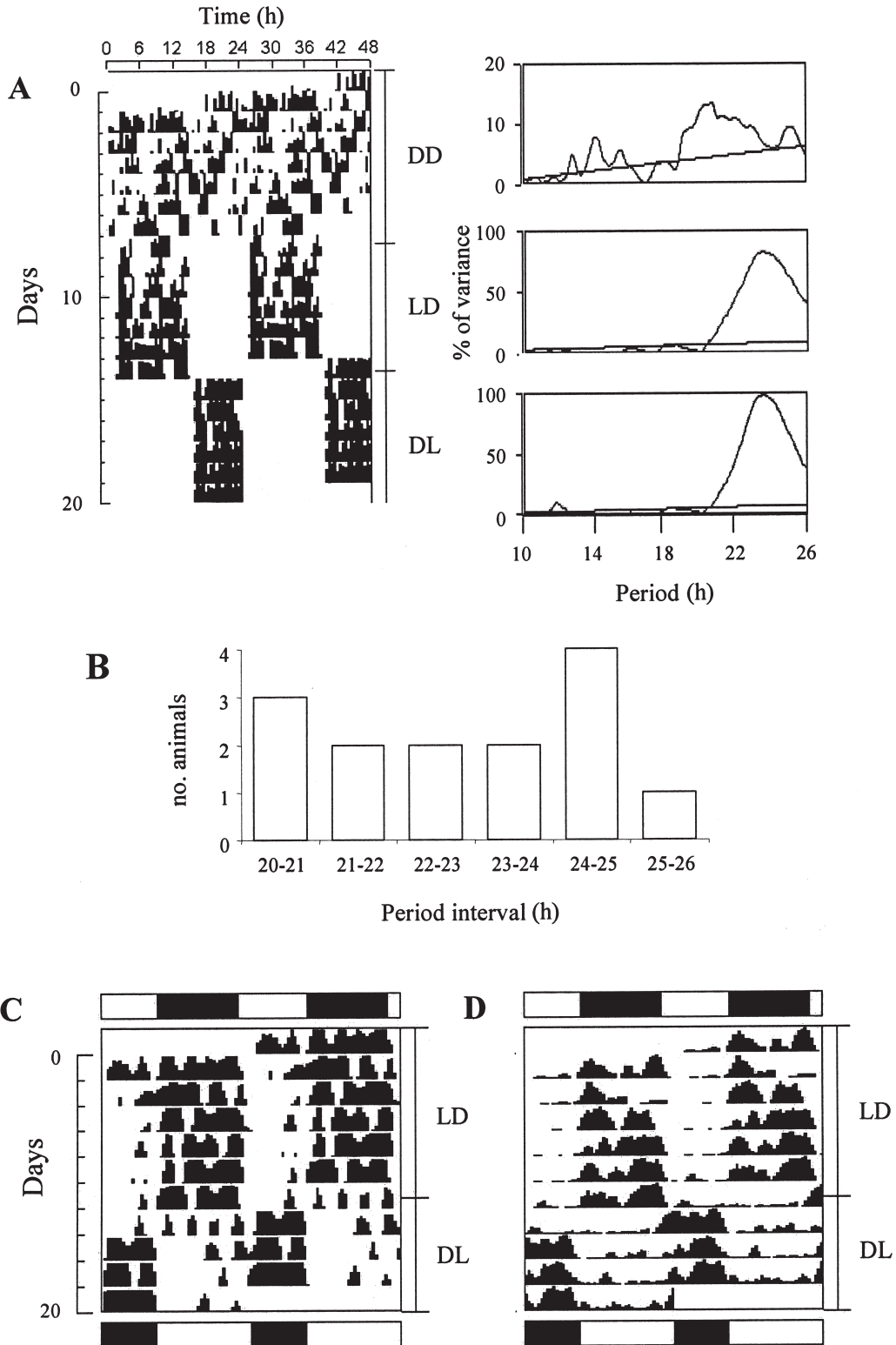


FIG. 1. – The fan activity rhythm of *Sabella spallanzanii* during consecutive experimental stages in different photoperiod treatments. The double-plotted actogram of a representative animal (A) is shown as an example of fan rhythmicity during DD, LD and DL. The periodogram plots (left-hand side) show significant circadian periodicity in each stage. In both LD and DL, the activity is always locked to the scotophase. The distribution of the individual periods recorded in DD (B) is also presented. They are all in the circadian range. The enlarged sections of actograms (the whole LD stage plus the first 3 days in DL) depict the timing shift of activity in response to photoperiod inversion for two different animals (C, D). Upper and lower black and white horizontal bars account for LD and DL cycles, respectively.

example. In DD, this animal required few days to show a discernible activity pattern. Although activity bouts were distributed over a wide temporal window, a major component was visible. The free running periodicity was 20.5 h in this animal. At this stage, all other individuals also manifested weak circadian rhythms with different strengths, their periodicity being lower than 24 h in most cases (mean and sd: $\tau = 22.9 \pm 0.2$ h; variance = $17.7 \pm 0.7\%$, $N = 14$). In Figure 1B the distribution of individual free-running periods is presented as a description of the variation in the sample. When animals were transferred to LD, periodogram analysis detected a change in the periodicity along with an increase in its strength. In all cases activity rhythms almost matched the 24 h period (mean and sd: period = 24.1 ± 0.1 h; variance = $84.4 \pm 4.3\%$, $N = 10$). The rhythmicity was very stable at that time, showing a marked nocturnal phase, and very little activity took place in the photophase. When the animals were transferred to DL (inverted), the detected periods also matched the 24 h (mean and sd: period = 24.1 ± 0.1 h; variance = $77 \pm 7.5\%$, $N = 10$). The activity was still locked in darkness, and was low during the day. The clustering of activity bouts was more pronounced in this stage than in the previous one, in agreement with the decrease in the night duration.

Focusing on the transition between LD and DL treatments (Fig. 1C, D), the animals suddenly shifted their activity phase of 12 h. However, some of them (20%) showed a disrupted pattern consisting in regular bouts of activity (from 3 to 5) expressed in the subjective day (e.g. Fig. 1C). These were gradually coupled back into a major nocturnal cluster over the next cycles. Conversely, the other animals (80%) displayed a sudden inversion of their phase without showing daily bouts in the subjective day (e.g. Fig. 1D).

Phase angle analysis

Figure 2 shows the results of the phase angle analysis over consecutive days for the LD and DL treatments. During the first day in LD (Fig. 2A), the rhythm showed a delay in its phase for both the onset and the offset with respect to the last day in DD (i.e. day 0). During the following days, Ψ_{onset} remained advanced with respect to $\text{light}_{\text{OFF}}$, showing a gradual phase adjustment. Phase angles were nearly zero at days 4 and 5, re-establishing a negative value during day 6. On the contrary, Ψ_{offset} was nearly zero for the entire stage because the activity

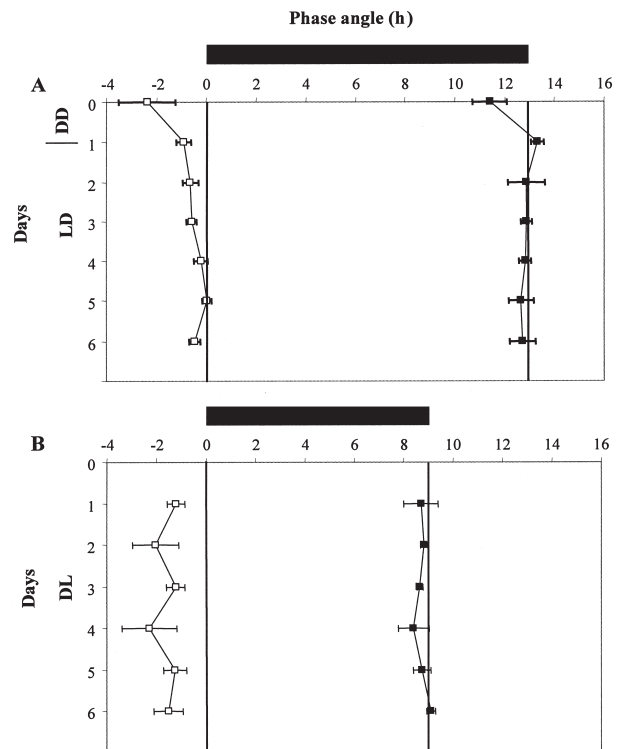


FIG. 2. – The phase angle of entrainment of *Sabella spallanzanii* fan activity rhythm over consecutive days of each photoperiod regime. Mean phase angles (\pm sd) as Ψ_{onset} (\square) and Ψ_{offset} (\blacksquare) are reported over consecutive cycles of LD (A) and DL (B). In Fig. 2A, the first point refers to the activity phase onset recorded during the last day of DD treatment (day 0). This phase was computed by projecting during DD the expected $\text{light}_{\text{OFF}}$ timing of LD. Black vertical lines indicate $\text{light}_{\text{OFF}}$ and light_{ON} timings; top horizontal black bar shows the scotophase length ($D = 13$ h for the LD stage, $D = 9$ h for the DL stage).

ceased in coincidence with light_{ON} (e.g., Fig. 1A). A significant linear regression of Ψ_{onset} from day 1 to 6 revealed transient phase changes from DD to LD ($F_{1,52} = 4.7$, $R = 0.3$, $p < 0.05$). On the other hand, a non-significant regression was found for the Ψ_{offset} over the corresponding temporal window ($F_{1,52} = 0.98$, $R = 0.14$, $p > 0.1$).

During the following exposure of animals to DL (Fig. 2B) a distinct dynamic was observed. The Ψ_{onset} still held negative values as in the previous stage (Fig. 2A) due to the persistent anticipation of activity onset to $\text{light}_{\text{OFF}}$ along days, but transient changes in the rhythm's phase across days were not significant in linear regression analysis ($F_{1,42} = 0.01$, $R = 0.02$, $p > 0.1$). Ψ_{offset} values were close to 0 for the entire stage since activity was nearly coincident with light_{ON} . Also, a non-significant regression output was found for Ψ_{offset} values ($F_{1,42} = 0.17$, $R = 0.06$, $p > 0.1$).

In Figure 3, a different response of the rhythm phase in each photoperiod regime was measured. The anticipation at Ψ_{onset} was greater during DL

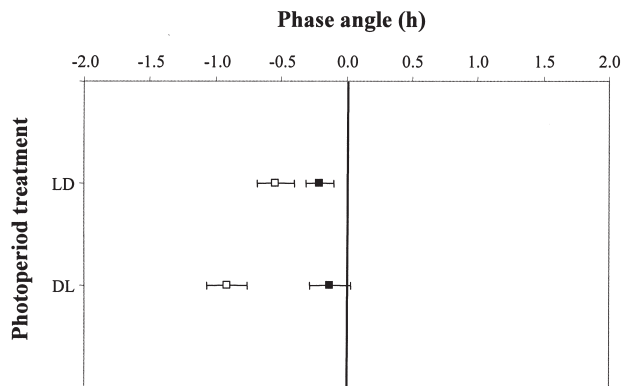


FIG. 3. – The difference in the magnitude of phase angles of entrainment in the fan activity rhythm of *Sabella spallanzanii* depending upon the photoperiod regime. This was assessed by reporting mean (\pm sd) Ψ_{onset} (\square) and Ψ_{offset} (\blacksquare) recorded in all animals during LD and DL. The black line represents $\text{light}_{\text{OFF}}$ and light_{ON} timings. The onset of rhythm appeared in advance to light_{ON} in both LD and DL. The advance observed at DL was greater than that observed in LD. The offset was always coincident with light_{ON} in both stages.

than LD (Ψ_{onset} mean and sd: LD = -32 ± 8 min; DL = -55 ± 9 min; t-test for dependent samples: $t_{0.05[7]} = 2.31$, $p < 0.05$). Furthermore, no significant difference was detected for activity offset in LD and DL since it remained close to light_{ON} in both photoperiod regimes (Ψ_{offset} mean and sd: LD = -13 ± 7 min; DL = -7 ± 9 min; t-test for dependent samples: $t_{0.05[7]} = -0.7$, $p > 0.1$).

DISCUSSION

In this study, *Sabella spallanzanii* held in constant darkness (DD) showed a weak circadian rhythmicity lower than 24 h in the majority of cases. This rhythmicity was visible in actograms after a few days of acclimation to laboratory conditions. In invertebrates, weakness in the detected circadian regulation is due to the splitting of activity peaks into submultiple units when animals are transferred to the laboratory (e.g. Dowse and Palmer, 1990; Warman and Naylor, 1995; Palmer, 2000; Aguzzi *et al.*, 2005). The animals showed a measurable fan activity rhythm in both photoperiod treatments. The measurement of fan activity rhythm under different light-darkness regimes (LD and DL) revealed that activity is always nocturnal and subjected to entrainment, and that the light-darkness cycle is necessary to compact behavioural bouts into a single coherent output (Dowse *et al.*, 1988).

The modulation of fan activity rhythm was tested under different photoperiod regimes. Firstly, the

activity onset was always measured in anticipation to light_{ON} (i.e. the negative phase angles; Fig. 2). Secondly, transient changes in activity onset were consistently recorded when animals were transferred from DD to LD (Fig. 2). Thirdly, different magnitudes in the phase angle were measured in different photoperiod treatments (Fig. 3). Considering all these data, an active process of entrainment may take place: a biological clock is gradually adjusting its period and phase of oscillation to the imposed zeitgeber cycle (Aschoff, 1981; Roenneberg *et al.*, 2003). For nocturnal species, an optimal strategy of entrainment occurs when the free-running periodicity (in DD) is lower than 24 h (Pittendrigh, 1981a). In agreement with this, a pacemaker oscillating faster than 24 h entrains in advance to its zeitgeber (Aschoff, 1981). This may determine the negative phase angle observed in this study.

The inversion of the light-darkness cycle (DL) suddenly shifted the phase of the activity rhythm of 12 h (Fig. 1A). Some animals showed a transient splitting of circadian peaks of activity into subcomponents during the photophase (e.g. Fig. 1C). Negative phase angles were still recorded at rhythm onset in DL, the magnitude of this advance being greater than that recorded during LD (Fig. 3). For *Sabella* a phase response curve is still missing, but the recorded 180° shift in the activity phase at LD-DL transition is coherent with data on entrainment of several invertebrate species (e.g. Wiedenmann, 1977; Peterson, 1980; Saunders, 2002). Also, some interindividual dispersion occurred in phase angles on days 2 and 4 of the DL stage (Fig. 2B) due to sudden phase advances in some animals. This suggests that entrainment is unstable under that photoperiod condition or that more days of acclimation to it are needed in order to acquire stability.

The detection of a systematic anticipation of activity onset to $\text{light}_{\text{OFF}}$ indicates an active process of entrainment. Although this phenomenon was reported here, only few transient changes were observed when a photoperiod inversion (the LD-DL transition) was imposed. This indicates a direct response of the rhythm to light presence in terms of an inhibition of activity (i.e. the negative masking). The reactivity of the rhythmic output to light increases the difficulty in studying the entrainment of *Sabella* under laboratory conditions and suggests precaution when manipulations of photoperiod are planned.

Considering major effects of dusk on the pacemaker of most nocturnal species (Pittendrigh,

1981a,b), a modulation of entrainment upon the day length in *Sabella* should be dependent on the phase relationship between this portion of the photoperiod and the onset of activity. In polychaetes, dusk and dawn activities are probably controlled by an endogenous mechanism and an exogenous response to light, respectively (Last and Olive, 2004). In hamster (e.g. Pittendrigh and Daan, 1976) and fruit fly (e.g. Helfrich-Forster, 2000) the entrainment is controlled by two oscillators whose components show a 12 h shift in their phases of functioning at day-night transitions. As a nocturnal species, *Sabella* may be sensitive to light-darkness transitions, therefore being susceptible to entrainment only at dusk (Pittendrigh, 1981a).

In conclusion, the weak free-running periodicity observed in constant darkness and the measured features of the entrainment suggest the presence of a weak oscillator controlling the fan activity rhythms in *Sabella*.

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