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AN INFRA-RED BEAM DEVICE FOR THE STUDY OF THE MOTOR ACTIVITY RHYTHMS IN GROUNDWATER MYSIDACEA

RIASSUNTO

L’attività motoria o locomotoria è un parametro utile per comprendere il funzionamento degli orologi biologici. Gli animali cavernicoli rappresentano un eccellente modello per gli studi cronobiologici oltre che per comprendere l’evoluzione e l’adattamento alla vita sotterranea.

Le ricerche in ambito cronobiologico richiedono spesso monitoraggi molto lunghi per i quali è necessario ricorrere all’utilizzo di sistemi automatici.

L’obbiettivo primario del presente lavoro consiste nel testare, con animali vivi, il prototipo di un apparato elettronico ad infrarossi appositamente costruito per il monitoraggio dell’attività motoria di animali acquatici di piccole dimensioni. La specie utilizzata in questa ricerca è un misidaceo, *Spelaeomysis bottazzii* Caroli 1924*,* proveniente dalla Grotta di San Isidoro (Nardò – LE, Pu/507). La registrazione dell’attività motoria ha riguardato animali mantenuti singolarmente in celle di coltura da 30 ml (5x3x2 cm). L’attività motoria è stata rilevata con un sistema di 9 barriere a luce infrorossa (ciascuna barriera composta da una sorgente e un sensore alle sue estremità). I sensori erano collegati ad un circuito elettronico che inviava i segnali ad un personal computer che a sua volta li archiviava. È stata eseguita un’analisi qualitativa dell’attività motoria dei singoli animali, rappresentandola graficamente per mezzo di attogrammi. Inoltre è stata eseguita un’ analisi quantitativa esplorativa di questi primi dati sull’attività motoria dei misidacei. A tal fine tutte le serie temporali sono state analizzate applicando la trasformata discreta di Fourier (DFT).

Sono state eseguite registrazioni per un totale di 20 giorni, 24 ore su 24 (12 gg il primo animale, 4 gg il secondo ed il terzo animale). Durante tale periodo non sono stati riportati problemi di eccessivo surriscaldamento dell’acqua, la componente elettronica (in particolare quella optoelettronica) si è rivelata altamente sensibile e non ha mostrato segni di usura dovuti alle condizioni di utilizzo. Gli ani-

mali sono rimasti vivi sino alla conclusione dell'esperimento. L'ispezione visiva degli attogrammi ha evidenziato una continua e costante rilevazione dell'attività locomotoria dei misidacei.

L'analisi cronobiologia si è concentrata sul calcolo della lunghezza del periodo circadiano in *free-running*, trovando un periodo medio di 24,7 h. La DFT ha rilevato la presenza di un picco a circa 24 h in tutte le registrazioni esaminate, evidenziando inoltre una bassa potenza spettrale.

Oltre al ritmo circadiano, in tutte le registrazioni ricorrevano ritmi ultradiani nell'attività motoria.

Nonostante le repliche sperimentali non siano sufficienti a giustificare conclusioni circa i ritmi dell'attività motoria di *Speleomysis bottazzii*, i ritmi individuati e la loro differente intensità, testimoniano di un incoraggiante funzionamento dell'apparato progettato.

SUMMARY

Locomotor activity and cave animals represent excellent models for study of biological clocks. An infrared-based detection device for recording motor activity has been tested on the stigobitic *Spelaeomysis bottazzii* Caroli, 1924 (Crustacea: Mysidacea). The individual activity of mysidaceans was monitored in continuous darkness. Data were analyzed by a DFT and chi-square periodogram. The apparatus was able to read rhythms of activity, in particular a lower circadian rhythm and some ultradian ones.

INTRODUCTION

Locomotion or motor activity is an useful parameter for the study of behaviour, but also for monitoring the biological clock functions. In this studies long-term monitoring automatic detection is required.

Technology for automated detection and recording of motor activity has notably evolved; small animals and invertebrates have been monitored with ultrasound (Hinano Akaka and Houck, 1980), infra-red beams (Clarke and Smith, 1985), stabilimeters (Zeier and Tschannen, 1968), running-wheels (Roberts, 1956), capacity trasducers (Schechter *et al.,* 1963), sound detectors (Jones, 1964) and radars (Vanuytven *et al.,* 1979; Martin and Unwin, 1980; Kropveld and Chamuleau, 1993; Pasquali and Renzi 2005, Pasquali *et al.,* 2005). The infra-red beam methods have a number of advantages: they provide automatic monitoring of an animal's general motor activity in its home cage without perturbing the pattern of its normal behaviour or initiating the spurt of exploration occasioned by transfer to a novel environment; it is not intrusive, produce output suitable for direct computer analysis, and is adaptable to different conditions.

Cave dwelling animals represent an excellent model for understanding the evolution of adaptations to subterranean life, particularly as regards the biological rhythms. Circadian rhythms in motor activity have already been documented in some hypogean animals. At present, the widest array of knowledge on cave animals rhythm, particularly among the insects, originates from the experiments of Lamprecht and Weber (1979; 1983; 1991). These Authors reported the occurrence of clock-controlled circadian rhythms in motor activity of troglophilic and troglobitic species, but also added more data about random activity patterns. Recently, other Authors showed interest toward cave animals. Hoenen and Gnaspini (1999) founded in cave harvestmen (*Pachilospeleus strinatii*) a circadian rhythm in the motor activity, computed by circular statistic. Koilraj *et al*., (2000) reported unclear results on the cave-dwelling millipede *Glyphiulus cavernicolus sulu*. Some cave-dwelling millipedes showed circadian rhythms in the motor activity, while other animals are arrhythmic. Pati (2001) pointed out circadian rhythms in hypogean and epigean loach-es, but also ultradian rhythms with period in the range 10-17 hr had reported. Trajano *et al*. (2001) used fast fourier transform (FFT) analysis for studing troglobitic fishes. They reported an absence of significant circadian component in motor activity under continuous darkness, but circadian component under light-dark cycle. Most recently Pasquali *et al*. (2005) point out the study on the motor activity in *Dolichopoda* cave crickets from different environments such as natural and artificial caves. Preliminary data showed a variability of the circadian period significantly different in the two types of populations. Moreover, the spectral analysis and periodogram revealed the presence of ultradian rhythms.

In this study a home made infra-red beam device for monitoring motor activity is tested. The species investigated is the troglobitic *Spelaeomysis bottazzii* Caroli, 1924 (Crustacea: Mysidacea) never used before in these kinds of researches. Motor activity is an arbitrarily defined unit of measurement that depends on the resolution of the detection system and the objectives to be attained in a given experiment. In order to define only gross body movements as signals, and maintenance behaviours as noise, only very sophisticated and expensive activity monitors would be satisfactory.

The infra-red beam based activity monitoring herein described is a modification of the system developed from our group in studying ant behaviour (Renzi and Pasquali, 2000).We modified the original approach to adapt this infra-red beam to smaller water animals, such as the *S. bottazzii* in our experimental system.

MATERIAL AND METHODS

Animals

The species used for the present research is the mysidacean *Spelaeomysis bottazzii* Caroli, 1924 which was collected in the "Grotta di San Isidoro" (Nardò – LE, Pu/507) in the Salento Peninsula. The cave has a mean water temperature 17.5°C, pH 8, salinity 2.77‰ and electric conductivity 4500 μS/cm^2.

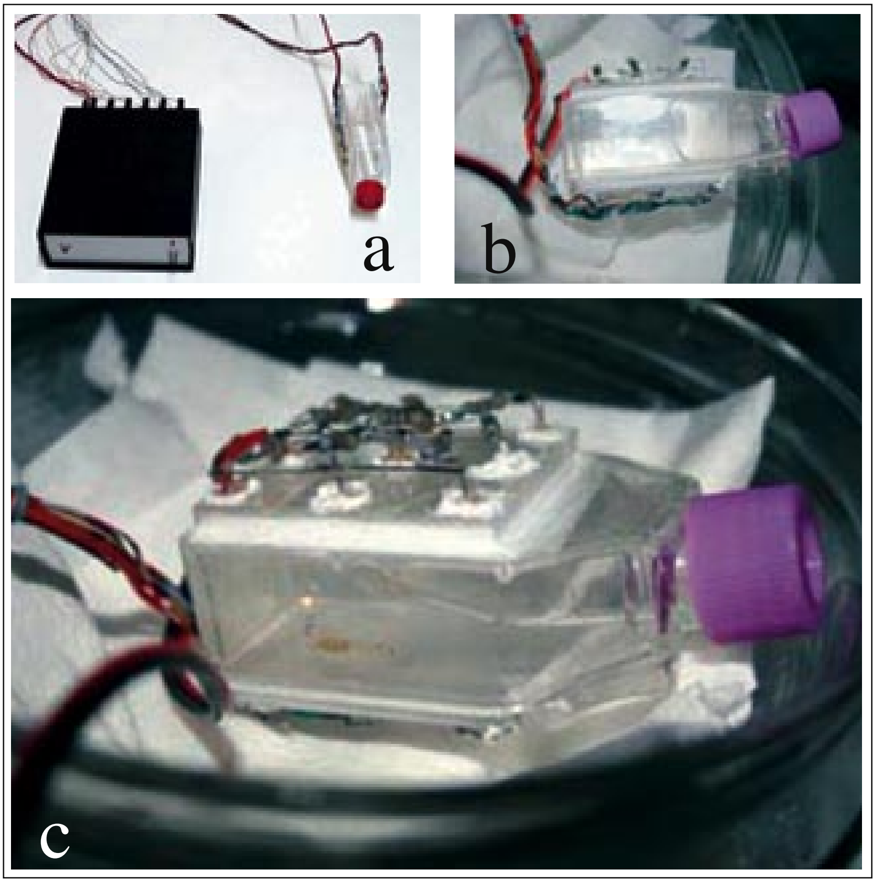

Fig. 1 - Pictures show the cell of recording and the connection to the electric circuit (a); cell view of side and position of the sensors (b). Cell and mysidacean in recording (c).

S. bottazzii is a troglobitic species, endemic to the Apulean region. Firstly collected in the anchialine system of the Zinzulusa cave (Castro, Lecce), the species was later redescribed by Pesce (1976) as well as it was also collected in other caves of the Salento Peninsula (Buco dei Diavoli, L'Abisso, Grotta dei Cervi, Grotta del Leone) and from phreatic systems of Apulia (Murge, Adriatic coast between Bari and Brindisi, Salentine Peninsula) (Inguscio *et al.*, 1999; Pesce *et al.*, 2004).

S. bottazzii is a large mysidacean (6-13 mm), euryaline and eurythermic, which can support wide light variations as well as complete darkness (Ariani, 1982).

Genetic research on the variability of the species (De Matthaeis *et al.*, 1982) pointed out that *S. bottazzii* colonized ground waters in recent time, following the Mio-Pliocenic Mediterranean regression.

Procedure

To test and quantify the activity of the nominate species a chronobiological approach was used. To this purpose, an automatic electronic device was built for the continuous monitoring of the general spontaneous motor activity of individuals *S. bottazzii*. Our analysis were based on inferential methods, used here to obtain the quantitative resolution of the features of the rhythm: amplitude and frequency.

Animals taken in the natural cave were transported in 5 l tank with ambient water to the Department of Biology, University of Salento and they were kept in a thermostatic room in the conditions utilized subsequently for the experiment: continuous darkness (DD), constant temperature at 17°±0.5°C and food *ad libitum* (tetra prima ® discus). All mysidaceans remained for more than 15 days in DD before the experiment. After this period of aclimatization, motor activity was recorded individually by an infra-red device mounted on a 30 ml box with water collected in the cave.

Infra-red device for motor activity: Electronic components and construction

Motor activity was recorded automatically by the realization of 9 barriers of infer-red beams in each cell culture box of 3 ml. Each beam/barrier had and infra-red source and an infrared sensor at the opposite extremities. The infrared sensors were connected to a personal computer (IBM compatible) which registered the number of movements (beam interruptions) in each time unit (minute).

The optoelectronics components (RS Components, Italia) were: a) Ø3 mm GaAIAs plastic infrared emitting diodes OP298B narrow irradiance pattern, λ 890 nm, ϕ 25°, max output power 4.8 mW/cm^2(TX); b) Ø3 mm NPN plastic silicon phototransistors OP598B wide receiving angle, λ 860 nm, ϕ 25° (RX). RX signal of each sensor were amplified by operational 3303, then a logic integrated circuit 7421 (positive-AND gate) sum all signals. The input signal for the I/O board (PIO-12, Keithley Instruments) was derived from a mono-stable μA 555, which forms an impulse of 15 msec +5V CC. A simple program "DAP-24", written in C language (by Micaloni, Renzi and Pasquali), read the channels of the I/O board. Parameters: sampling frequency, collected interval, length of the experiment, were easily modifiable. The number of actometers supported by the device depend only on the I/O board number of channels. Device was putted in to the thermostatic chamber at 8°C because resistor near TX, normally elevated water temperature to 30°C. But in that location water temperature was 16°C. It is important to make the followings checks: a) no interference between the sensors; b) all sensors measure the same quantity of movements; c) checking that this setting does not drift with time. We also checked that the device did not yield false signals in the absence of movement.

Data analysis

For the actograms a program written in BASIC for Machintosh was used. Software processed directly the files created from the "DAP" program. For the Fourier analysis, time series were smoothed (3 point moving average) and the linear trend was removed before analysis. Two different analyses were used: 1) power spectra were computed for each subject by Discrete Fourier Transform (DFT). Smoothed estimation of the spectra were obtained by a Parzen window. A one sample Kolmogorov-Smirnov test was used to check the random origin of the spectra (white noise). In spectra significantly different from white noise, peaks above 2.81 standard deviation ($p < 0.01$) were considered as significant (Conte *et al.*, 1995). 2) To estimate the circadian period the data with the use of the chi-square periodogram of Sokolove and Bushnell (1978) were analyzed.

RESULTS

The infra-red device recorded movements of 3 individuals of different size, from 8 mm to 13 mm (measures from head to telson extremities), housed in independent cell-culture box. Motor activity measurements were performed for a total of 20 days, device has not shown any problem and the recordings were sufficiently accurate.

Visual inspection of single actograms do not showed a rhythmic behaviour during the experiments (Fig. 1a, 2a, 3a). In the next step, for each animal, the power spectrum (Fig. 1b, 2b, 3b) and periodogram (not showed) were computed; analysis revealed significant peaks both for circadian and ultradian rhythms in motor activity in free running (with photoperiod DD).

Periodogram analysis showed the presence of a circadian rhythm, the average free-running period (τ) was 24.7 ± 1.2 h (mean ± SD, n = 3). Data on the circadian rhythm was also confirmed by the DFT, which revealed the presence of significant peaks at 24 h in all the mysidaceans with lower spectral power: 4.8 ± 1.2 (mean ± SD, n = 3).

Besides the circadian rhythms, ultradian rhythms in motor activity occurred in each individual, which we were able to point out with the spectral analysis. DFT pointed out that in all animals more significant peaks in the range 1-8 h are present. Mean spectrum showed significant peaks to 2 h, 3.3 h and 6.5 h, this latest with high spectral power (Fig. 4). The mean max power of the ultradian peaks was 6.6 ± 0.6 (mean ± SD, n = 4), higher than circadian ones.

DISCUSSION

In the present study an infrared device for evaluating troglobitic animals (*Spelaeomysis bottazzii* Caroli, 1924) motor activity has been tested.

In previous tests with other prototypes devices, size and environment of the mysidaceans were the principal problems, but also the perfect alignment of the sensors. In the present work 3 different size individuals were used. The best result was obtained with big and medium size specimens. Small size specimen did not show a similar definition but the quality of the data were equally adopted for study behaviour and/or computer analysis.

After 20 days of operation we id not measure an overheating of the water in the cell of recording. Electronic components, particularly optoelectronic components, id not show usury for working conditions and showed a good sensibility for the animal size. Visual inspection of the actograms showed a continuous and constant monitoring of the motor activity, without a visible rhythmic behaviour.

In general our device showed a good sensibility, accuracy and constancy in the measurements.

We had only 3 subjects for 20 days of recording in total. Obviously this data there are not representative of the species motor parameters, but we can consider results as encouraging in the use of the apparatus setting.

Data showed a significant circadian rhythm with lower power, this suggesting an endogenous circadian rhythm in motor activity. It is not unusual, many Authors reporting circadian rhythmicity in motor behaviour of different cave species like insect, water invertebrates, fishes (Ginet, 1960; Jegla and Poulson, 1968; Koilraj *et al.*, 2000). Probably, the circadian organization is important to the manteinance of the internal temporal organization (Oda *et al.* 2000) even in troglobitic animals.

We think interesting the similarity of present data with those obtained by Pasquali *et al.* (2005) on *Dolichopoda baccettii*. Populations from natural cave and in particular from first lake of Grotta degli Stretti (this population can be considered troglobitic), show an interesting similitude with our mysidacean: in both actograms there are not visible rhythmic behaviours and both spectra show different ultradian peaks and a circadian amplitude lower than ultradian ones (see actograms Fig. 5 and spectra Fig.6).

This is only a speculative observation but we consider deserving of further experimental observations. Realization and validations of this device is the first step for future studies.

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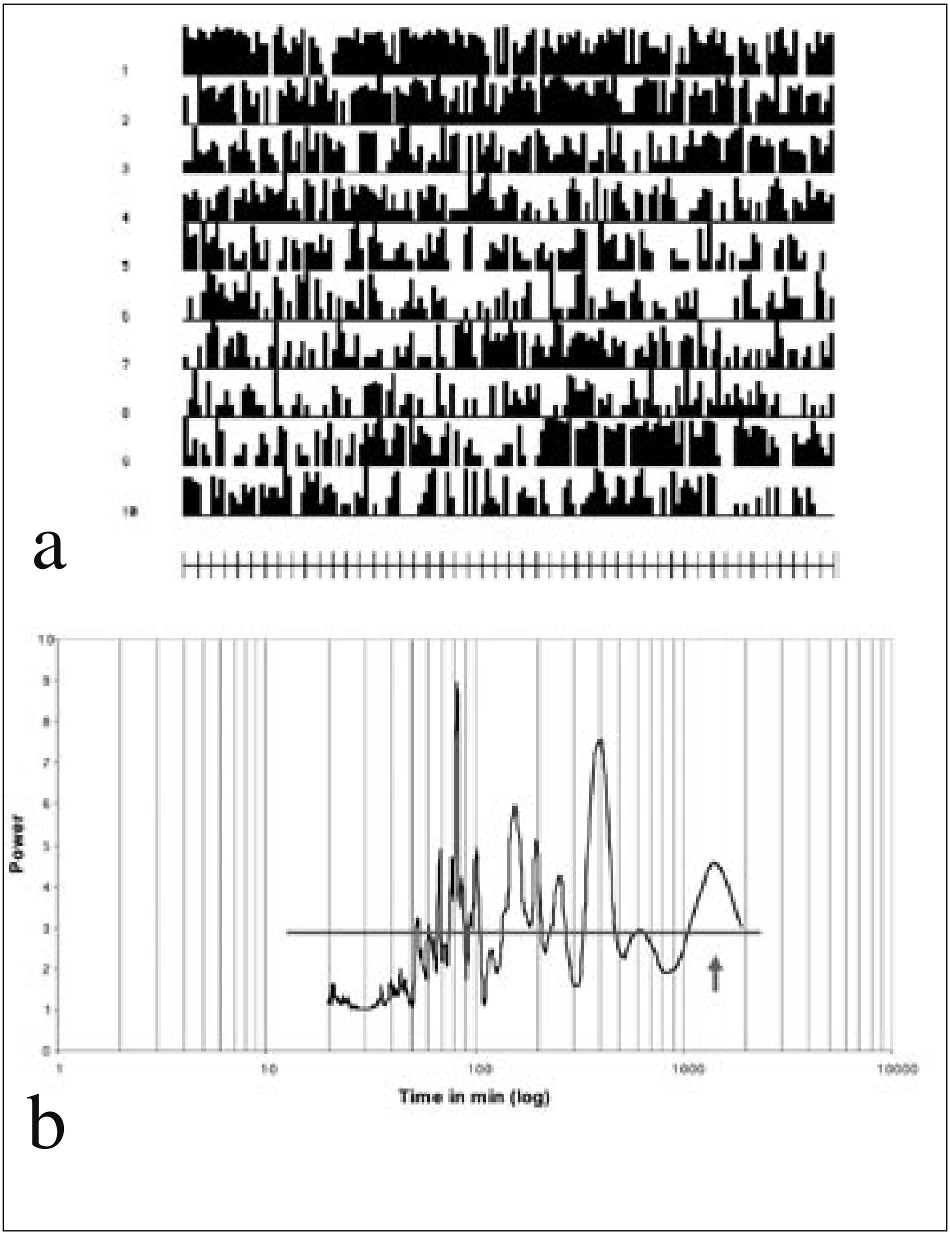

Fig. 2 - Actogram and power spectrum mysidacean #1.

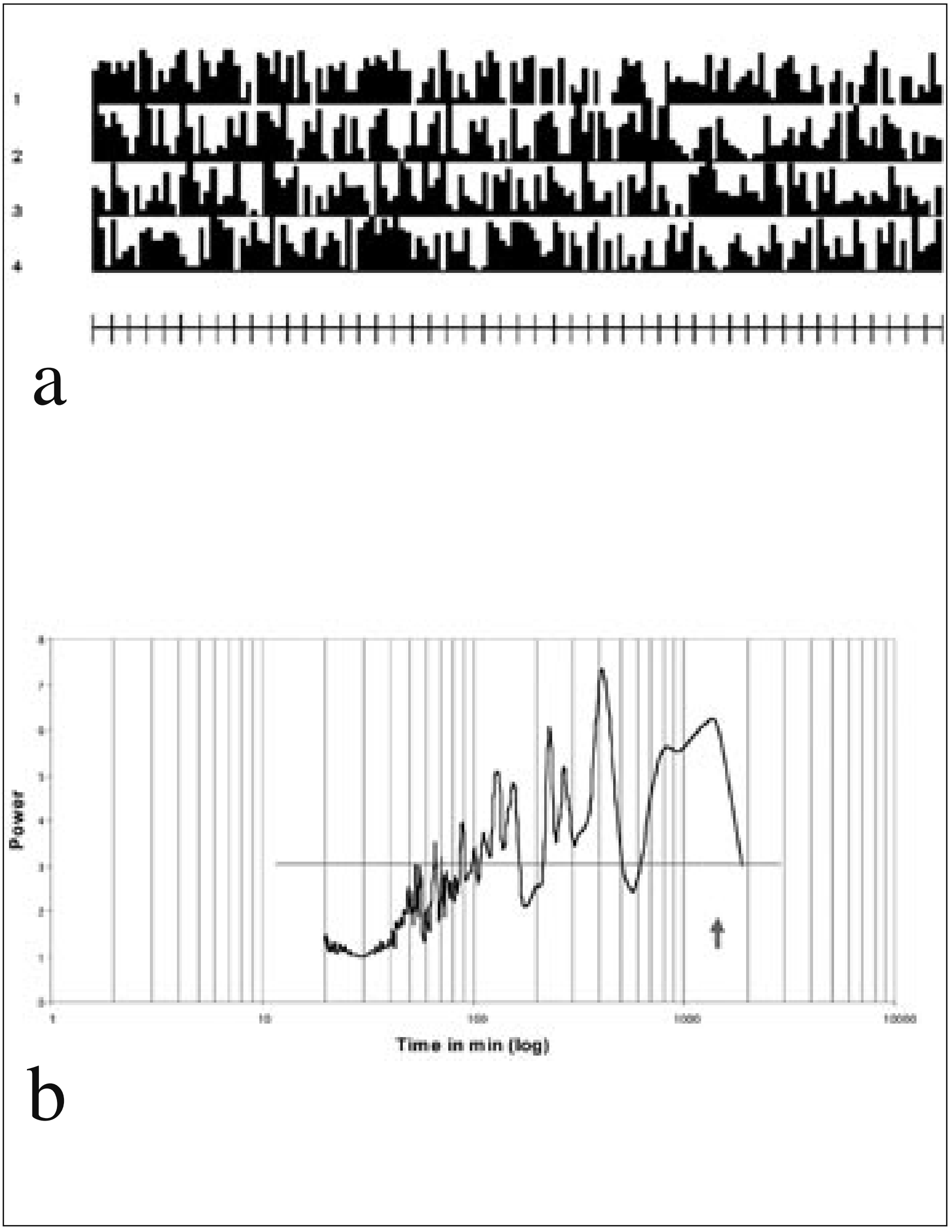

Fig. 3 - Actogram and power spectrum mysidacean #2.

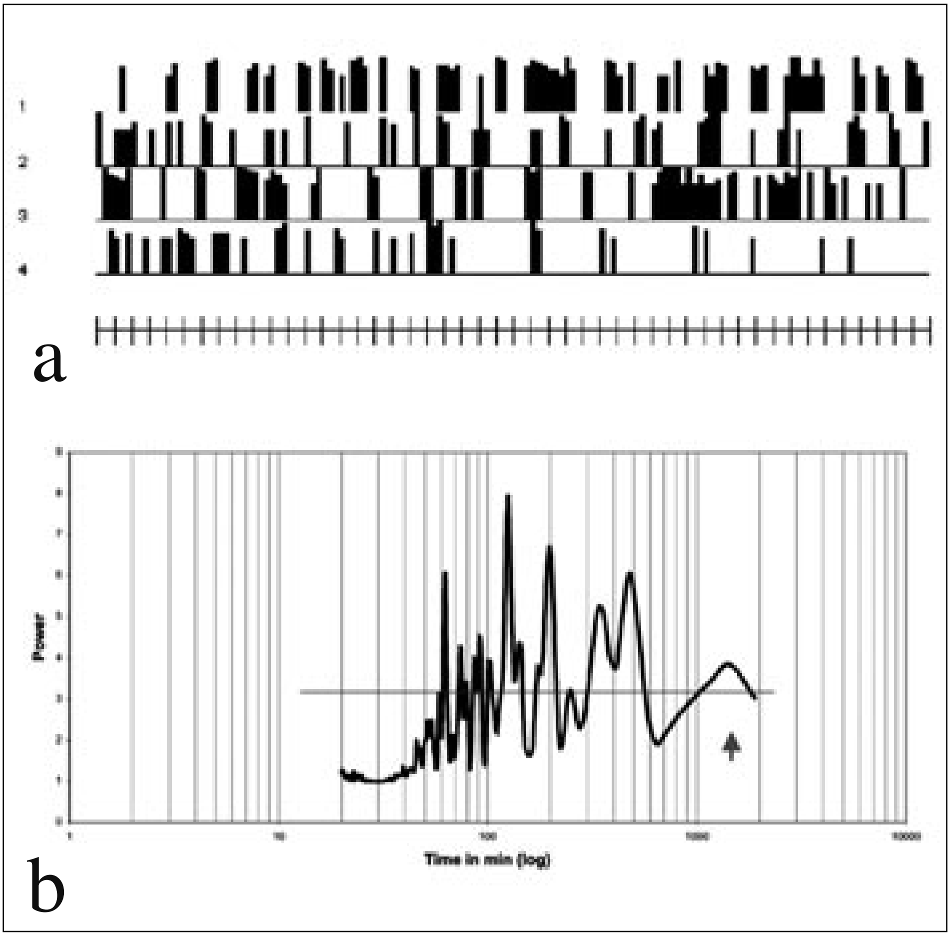

Fig. 4 - Actogram and power spectrum mysidacean #3.

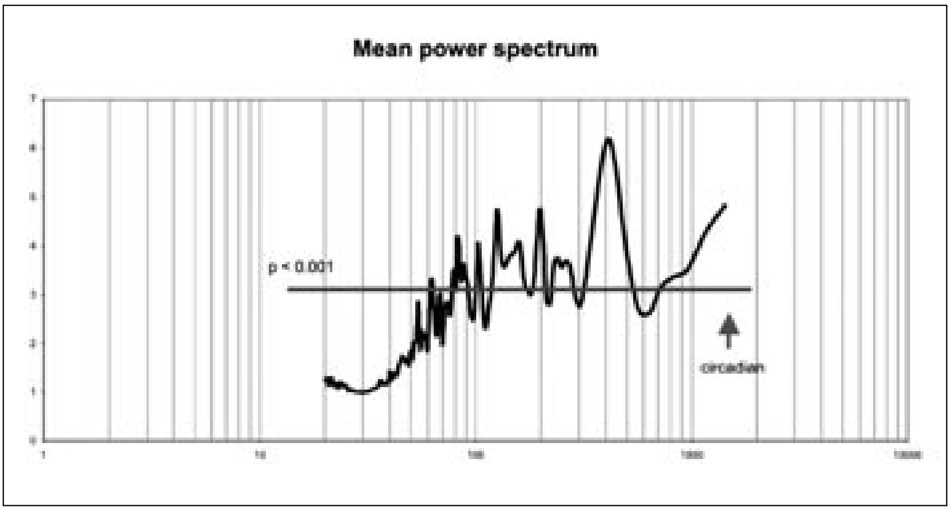

Fig. 5 - Mean power spectra of 3 mysidaceans. Power values on the y-axis, x-axis is period (in minutes) in logarithmic scale.

NOTE

The software and the electronic scheme circuit are available freeware. vittorio.pasquali@uniroma1.it

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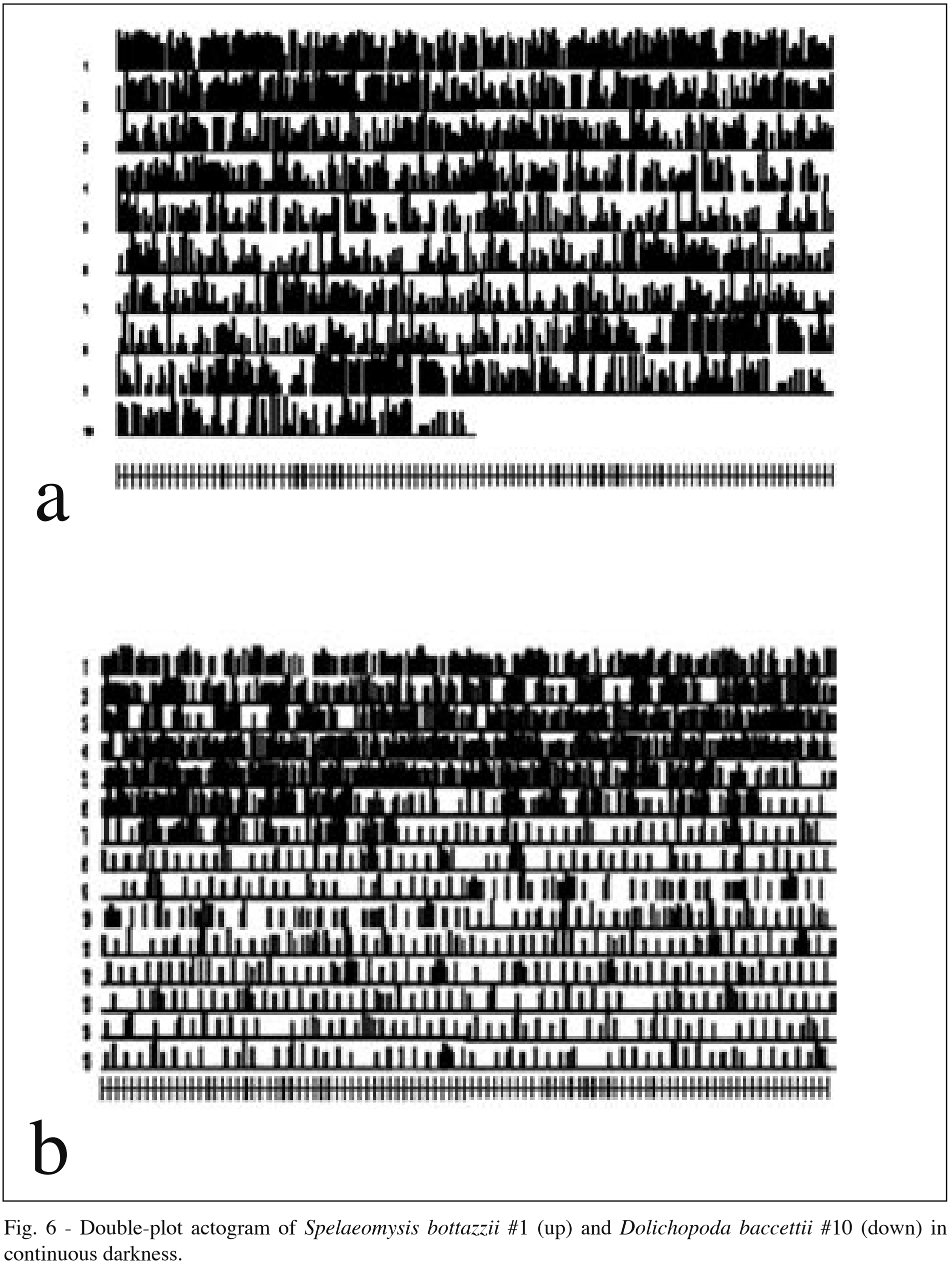

Fig. 6 - Double-plot actogram of *Spelaeomysis bottazzii* #1 (up) and *Dolichopoda baccettii* #10 (down) in continuous darkness.

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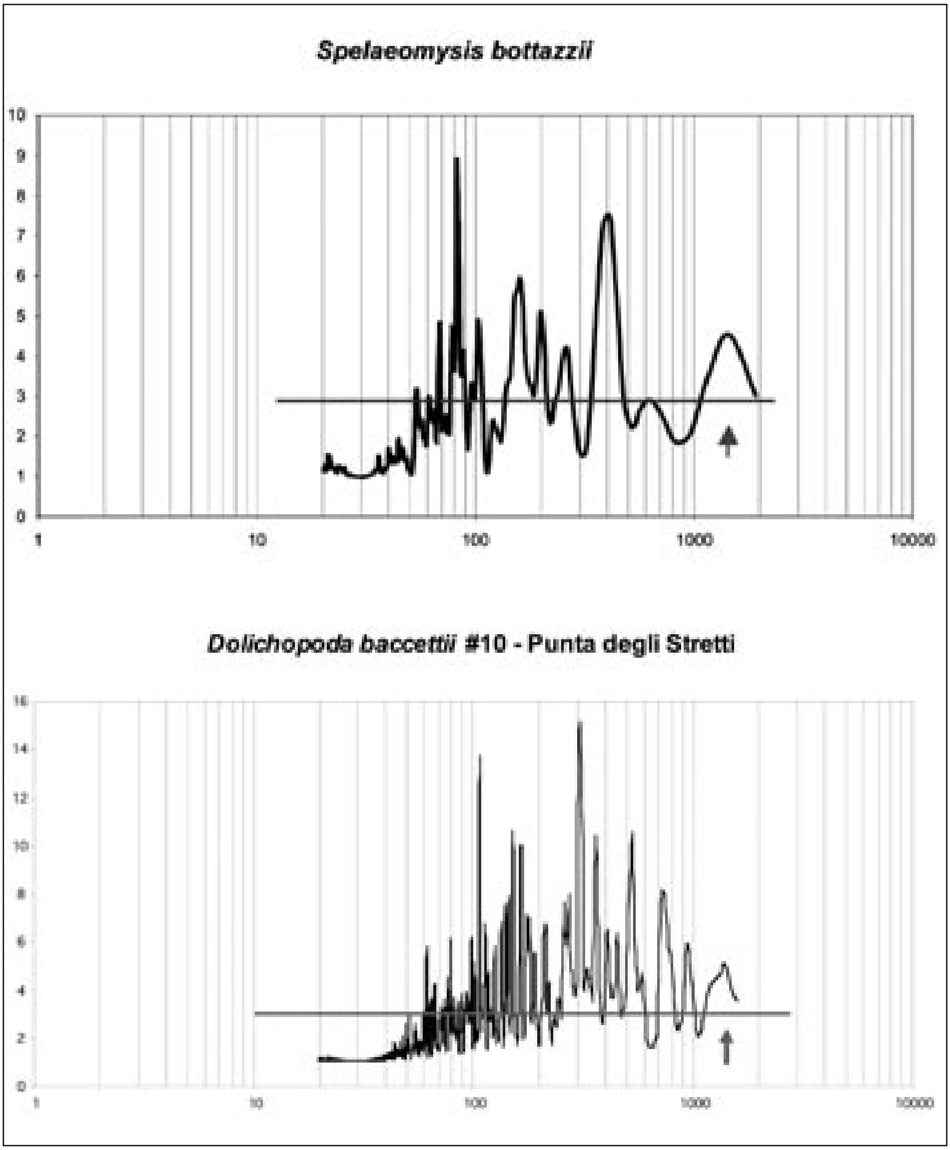

Fig. 7 - Spectral analysis of activity rhythms of *Spelaeomysis bottazzii* #1 (up) and *Dolichopoda baccettii* #10 (down). Power values on the y-axis; x-axis is period (in minutes) in logarithmic scale.