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Primary Research Paper

Automated recording of vertical negative phototactic behaviour in *Daphnia magna* Straus (Crustacea)

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Abstract

Diurnal vertical migration is a well-known phenomenon in the circadian activity rhythms of zooplankton. Our goal was to test whether negative phototaxis in *Daphnia magna* clone BEAK (provoked by artificially induced light stress, alternating light and dark phases in 2 h intervals), and its interference with the endogenous rhythm of diurnal vertical migration, can be automatically registered with a biomonitor. For the first time the vertical swimming behaviour of *D. magna* was recorded quantitatively based on non-optical data recording in a fully automated biotest system, the Multispecies Freshwater Biomonitor in a new experimental setup consisting of a column of three recording units (3-level chambers). Circadian vertical migration was clearly recorded in the 3-level chambers and the rhythm was more clear with 5 than with 1 organism per chamber. The organisms clearly responded to induced light stress with negative phototaxis, however best in larger chambers. The artificially induced rhythm was influenced by the endogenous rhythm. This approach may facilitate long-term observations of vertical swimming activity of zooplankton in the future.

Introduction

Due to the key position *Daphnia* spp. (Crustacea, Cladocera) occupies as a zooplanktonic grazer and prey species in many lentic food webs, its large size, ease of culture, ability of parthenogenetic reproduction and sensitivity to pollutants, it has been widely accepted as a standard toxicity test species in aquatic ecotoxicology (e.g. Persoone & Janssens, 1993). Moreover, *Daphnia* is also used as a surveillance organism to monitor online changes in water quality in large rivers and drinking water supplies (review in Gerhardt, 1999). The rationale of online biomonitoring is the expectation of a change of behaviour when the animal is exposed to pollution pulses, resulting in an alarm, as part of a biological early warning system (BEWS). Until now several single species biomonitors using daphnids have been developed: e.g. the 'Dynamic Waterflea biotest' (Knie, 1978) is based on the registration of the number of interruptions of infrared lightbeams by swimming waterfleas. An adaptation of this test consisted of inducing animals issued from a positive phototactic clone to swim up and down between two light sources which alternatively switched on and off. Another biomonitor specifically designed to assess vertical swimming, e.g. as a function of exposure to ultraviolet B light, is the Vertiquant, consisting of 6 IR sensors distributed along a 16 cm high glass column (Vareschi et al., 1999). Several biomonitoring techniques register the movements of waterfleas with digital image video and image processing (e.g. van Hoof et al., 1994; Blübaum-Gronau and Hoffmann, 1997; Baillieul & Scheunders, 1998). One important disadvantage of

optical techniques is the inability to register movements in darkness or turbid water, hence forcing the investigator to keep the animals constantly in light, creating a possible additional stress or artefactual behaviour. The Multispecies Freshwater Biomonitor (MFB), used in the present study, records automatically the behaviour of aquatic organisms and is based on quadrupole impedance conversion technology, a non-optical method useful in e.g. turbid waters or at night (Gerhardt et al., 1994). The MFB has already been used to record the swimming behaviour of *D. magna* in surface water (Gerhardt et al. 2003) and Acid Mine Drainage (Gerhardt et al., 2005b).

One behavioural feature in *Daphnia* which deserves special attention is its ability to react to light by swimming towards the source of light (positive phototaxis) or away from it (negative phototaxis), where phototaxis is defined as an oriented reaction to a light stimulus (Ringelberg, 1987, 1999). In daphnids it is known to depend on a multitude of factors, such as genotype (De Meester, 1991), pH of the water (Van Uytvanck & De Meester, 1990), food quality and quantity (Hamza & Ruggiu, 2000), water temperature (Calaban & Makarewicz, 1982), or the presence of infochemicals (Weber & Van Noordwijk, 2002). Such behavioural plasticity is a special case of phenotypic plasticity (Komers, 1997) and allows for a wide range of adaptation to environmental fluctuations (Boriss & Gabriel, 1998). Phototaxis can be interpreted as an underlaying behavioural mechanism for vertical migration (Van Gool, 1997). Diel vertical migration (DVM) is a special case of depth selection behaviour by active swimming in response to light changes, which has been observed in numerous freshwater and marine zooplankton species (Cushing, 1951). As explained by Ringelberg (1999), DVM is induced by a relative change in light intensity. The normal response in *Daphnia* consists of negative phototaxis during the day, resulting in deeper residence depths. This is explained by the generally accepted predator avoidance hypothesis, stating that during the night zooplankton usually migrates upwards for grazing, because the predation pressure from visual hunters like fish is reduced (Lampert, 1993). Monitoring DVM might be in the future an ecologically relevant parameter for pollution detection in BEWS. This could be done with devices which are not dependent on artificial visible light sources, such as the Vertiquant (Vareschi et al., 1999) or the MFB (Gerhardt et al., 1994). Therefore, the MFB was tested with special recording chambers, designed for measuring behaviour in three vertically arranged heights. Given the fact that individual *Daphnia* might react differently from *Daphnia* swimming in groups (Ordemann et al., 2003), several combinations of chamber sizes and densities were tested as well. A negatively phototactic clone was used in order to obtain vertical movements induced by alternating periods of light.

Our goal was to test whether phototactic behaviour (1) could be recorded with the non-optical MFB, (2) whether manipulation with an artificially induced photoperiod regime would be registered as well and (3) whether this phototactic behaviour would be density- or volume- dependent.

Materials and methods

Multispecies Freshwater Biomonitor (MFB)

The MFB is an automated biomonitor system based on frequent semicontinuous and quantitative registration (trace length 240 sec., interval 6 min.) of the behavioural pattern of all kinds of aquatic and benthic organisms (Gerhardt et al., 1994, 1998; Gerhardt, 1999, 2000). The measurement unit is the test chamber, here made of an acryl glass cylinder, which can be sealed on both ends with screw-rings covered with nylon gaze (mesh size: 0.25 mm). Two pairs of stainless steel electrode plates are attached at the inner walls of the chamber, one pair generating a high frequency alternating current (100 kHz), the other, non-current carrying pair of electrodes is arranged at an angle of 90 degrees and serves as sensor of impedance changes caused by the organisms movements in its medium. The signals can be distinguished according to their amplitudes and frequency, for example locomotion is characterised by high amplitudes and low, irregular (0.5–2.5 Hz) frequencies, which are analysed by discrete fast Fourier analysis. The swimming activity of *D. magna* (3–5 mm in size) could be assigned by simultaneous MFB-recording and visual observation to a signal frequency of ≤ 0.5 Hz, predominantly performed by the antennae. The

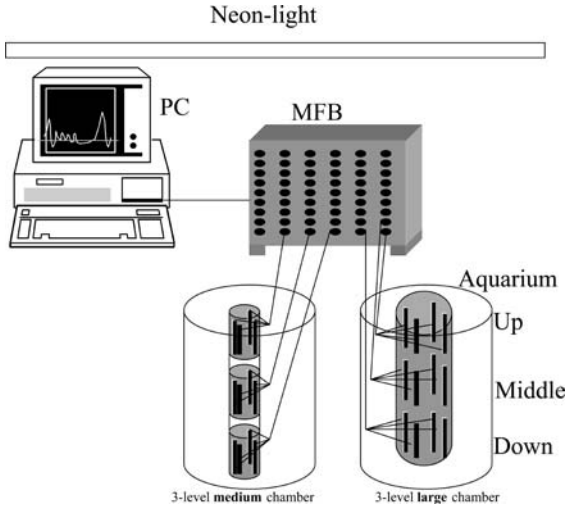

Figure 1. Experimental setup with the "Multipecies Freshwater Biomonitor" (MFB) and the different types of chambers.

organisms were placed individually or in groups (see further) in the test chambers, because many planktonic organisms live in swarm and collective behaviour might be different from the behaviour of a single organism (Ordemann et al., 2003). In order to calibrate the signal/noise ratio for movements of *Daphnia* in the MFB-chambers, three chambers with different volumes (2.2, 2.9, 5.6 cm^3) were tested with a single *Daphnia*. Whereas signal amplitude was dependent on the size, i.e. the volume of the test chambers (Spearman Rank $R > 0.65$, $p < 0.05$), the frequencies generated by these signals (Fast Fourier Transformation) did not differ between the chambers. The behavioural MFB-data are always expressed as 'time spent on specific frequencies', and thus will not be affected by chamber volume, as long as the volume of the chambers remains within a critical range allowing for minimal detection of signals above background noise. Therefore we selected two types of chambers with volume of 31.4 and 70.7 cm^3, where signals of single organisms could safely be detected. Moreover, previous calibration (unpubl.) has shown that different numbers of organisms (e.g. *Tubifex*: 0.05, 0.10, 0.20 g) in the same chamber volume had no significant effect on FFT data (Anova, $p > 0.1$).

Test species and culture

The culture conditions were specifically set up to provide a sufficient number of adult animals for our short-term experiments in a cost- and time-effective way. *Daphnia magna* Straus 1820, originating from the clone 'BEAK', issued from a laboratory culture, was cultured in 3 replicates in 1 l glassbeakers filled with commercial dechlorinated drinking water, 5 ml seaweed extract (Pereira et al., 1999), with additional aeration and fed with dried *Chlorella vulgaris* (0.12 g C/l). Each week, water was renewed, new extract and food added. The cultures were kept at room temperature, illuminated with 2 standard daylight neonlights 1 m above the culture (set on 12 h light/ 12 h dark photoperiod with the intensity of 18 Watt). The adults for each experiment were taken by random sampling equally from the triplicate culture tanks.

Experimental setup

Dechlorinated artificial water of drinking water quality (pH = 7.3, Ca^{2+} = 91 mg/l, Mg^{2+} = 19.9 mg/l, Na^{+} = 7.3 mg/l, HCO_3^- = 258 mg/l, SO_4^{2-} = 105 mg/l) was the medium for the 24–48 h experiments with adults of *D. magna* (3–5 mm). The experiments were performed at room temperature (20 ± 2 °C) under static conditions with aerated water, using two different sizes of test chambers and arrays of electrodes, i.e. recording fields: (1) 3-level medium chambers (Length = 10 cm, Diameter = 2 cm, Volume = 31.4 cm^3) consisting of three small chambers glued together, hence having three vertically arranged electrode levels and (2) 3-level large chambers (L = 10 cm, D = 3 cm, V = 70.7 cm^3) (Fig. 1). The respective electrode levels were connected to different channels in the Multispecies Freshwater Biomonitor, thus allowing to record the behaviour of the animals in three different vertical heights (DOWN, MIDDLE, UP) (Fig. 1).

The experimental design is summarised in Table 1. Prior to recording with the MFB, the animals got an acclimation phase of 20 min. Photoperiod was simulated by connecting two 18 Watt daylight neon lights placed 1 m above the experimental setup to a timer without dimming. Two photoperiod regimes were tested: (1) "diurnal photoperiod" (exposure during 48 h), consisting of 12 h darkness overlapping with natural night time and 12 h light overlapping with the natural day time and (2) "induced photoperiod" (exposure during 24 h), consisting of alternating periods of light and dark phases of 2 h. For the diurnal photoperiod we

Table 1. Experimental design with *Daphnia magna* in dependence of chamber size and organism density

Type and Nr. Of experiments	Levels and size of chamber	Nr. of animals per chamber	Nr. of replicate chambers	Total *N* animals
Diurnal				
2	3-level medium	1	5	10
2	3-level medium	5	5	50
2	3-level large	5	5	50
Induced				
2	3-level medium	5	5	50
2	3-level large	5	5	50

tested respectively 3-level medium chambers with respectively 1 and 5 animals/chamber and 3-level large chambers with 5 animals/chamber. For the induced photoperiod, all tests were performed with 5 animals/chamber. All experiments were repeated two times (Table 1), each time with new animals. Differences between replicated experiments were not significant (one-way anova, $p > 0.05$). Animals were not fed during the experiments and the whole experimental setup was isolated from natural day light with black plastic sheets.

Statistics

Survival data (%), arcsine $((x/100)^{0.5})$ transformed, proved to be normally distributed (Kolmogorov–Smirnov goodness of fit test) and to have homogenous variances (Levene's test). Statistical comparisons of survival between different treatments (animal density, chamber types) were performed with two-tailed two-way ANOVA's.

Every 10 min., the MFB recorded traces of 4 min. giving the percentage of time spent on swimming by respectively 1 (individual behaviour) or 5 (collective behaviour) animals. Each electrode level (Fig. 1) generated quantitative behavioural data. Prior to analysis we made sure that zero values indicating death at the end of a time series were excluded. On the other hand, behavioural activity tended to be clustered around 70–95% time spent on activity, generating a distribution curve asymmetrically skewed towards high values, typical for the incessant activity of planktonic organisms. Therefore, the non-parametric Mann–Whitney *U*-test ($p < 0.05$) was used to compare different treatments, replicates or experimental conditions.

Given the regular periodicity of the % time of swimming in DOWN, a sinus-function was empirically fitted to the data from DOWN-level in the experiments with induced photoperiod according to the following general formula:

$$\mathrm{SWIM}_{\mathrm{norm,t}} = \alpha + [\sin(t - \theta/\beta)]/\gamma$$

whereby $\mathrm{SWIM}_{\mathrm{norm,t}}$ = % time spent on swimming at time t/mean of treatment (is a normalisation of the behavioural data around 1), t = time (hour expressed in decimals), α = correction constant to adjust the sinus curve according to the position of the behavioural data on the Y-axis, θ = correction constant to adjust the sinus curve according to the position of the behavioural data on the X-axis, β = correction factor to adjust the periodicity of the sinus curve according to the observed negative phototaxis, γ = correction factor to adjust the amplitude of the sinus to the observed minima and maxima of the behavioural data.

The sinus curves represent the predicted behavioural data according to the hypothesis of negative phototaxis. The data from the artificially induced photoperiod experiments were matched against the sinus curves with paired *t*-tests for the entire exposure periods, in order to test whether the circadian endogenous rhythm (expected to lower the activity in DOWN during the night) had an influence upon the manipulated two-hour phototactic rhythmicity.

Results

Survival

The survival after 24 h ranged between 60 and 92% and was not significantly different between the different treatments, such as chamber volume

and organism density (Table 2). However, after 48 h, survival decreased significantly with increasing number of animals per chamber ($F_{2,30} = 4.26$, $p = 0.02$) (Table 2). Survival under diurnal photoperiod did not differ from survival under induced photoperiod.

Swimming behaviour in different chamber sizes and animal densities

Under diurnal photoperiod, time spent on swimming behaviour of groups (5 animals) of daphnids in the medium 3-level chambers was consistently higher than in the large 3-level chambers, irrespective of day time, or vertical level (range of $Z = -2.12$ to -18.60, $p < 0.05$). In all experiments with induced photoperiod the time spent on swimming was higher in the medium 3-level chambers compared to the large 3-level chambers for the respective test condition (level, dark/light phase) (range of $Z = -2.38$ to -14.40, $p < 0.05$). Moreover, the recorded % of time spent on swimming generally increased with increasing number of animals per chamber ($1 < 5$, range of $Z = -2.59$ to -20.79, $p < 0.05$), irrespectively of vertical level.

Swimming behaviour and diurnal photoperiod

During day time, *D. magna* spent more time on swimming at the bottom of the 3-level chambers (DOWN) than at night, which was confirmed in all experiments without exceptions and independently of chamber size and organism density (Fig. 2) (Mann–Whitney *U*-test, range of $Z = -2.96$ to -21.25, $p < 0.05$). However, during the night, *D. magna* migrated to the uppermost level of the 3-level chambers (UP), which was confirmed in all experiments (Mann–Whitney *U*-test, range of $Z = -2.14$ to -15.20, $p < 0.05$) (Fig. 2). These data demonstrate diel vertical movements, even on a small scale (10 cm vertical height). The activity in MIDDLE showed no clear tendency, due to its intermediary position.

Swimming behaviour and manipulated photoperiod

Phototactic responses to alternating changes of light could clearly be recorded in the 3-level large chambers and less clearly in the 3-level medium chambers. The most clear results were recorded in the DOWN levels, i.e. at the bottom of the chambers: during light phases *D. magna* spent most time on the bottom (medium chambers: $Z = -4.79$, $p < 0.05$; large chambers: $Z = -13.90$, $p < 0.05$), whereas in dark phases activity increased in the uppermost (UP) level (3-level medium chambers: $Z = -5.08$, $p < 0.05$). Although this rhythm was clear during the real day time, it was weakened during the real night time (Fig. 3). The swimming in the 3-level large chambers in the UP and MIDDLE levels were less affected by light changes.

During light phases, the time spent on swimming of collectives of 5 *Daphnia magna* in all vertical levels of 3-level medium chambers was higher when exposed to diurnal photoperiod compared to induced photoperiod (range of $Z = -3.30$ to -10.86, $p < 0.05$). During dark phases, the contrary prevailed in DOWN and MIDDLE (range of $Z = -3.74$ to -9.43, $p < 0.05$).

The sinus functions of the predicted fits for the induced photoperiod are given in Figure 3. The best fit was obtained for the day time in the 3-level large chambers, where paired *t*-tests were not significant. During night, the sinus fit significantly scored higher than the data (paired *t*-test, $t = -5.92$, $p < 0.05$), showing the influence of

Table 2. Survival of *Daphnia magna* clone BEAK in the different experiments after 24 h and after 48 h

Photoperiod	Nr. Animals per chamber	Total *N* animals	Chamber size (Nr. of levels)	24 h-SURV. % Mean ± Std. Dv.	48 h-SURV. % Mean ± Std. Dv.
Diurnal	1	10	medium (3)	88.9 ± 33.3	80.0 ± 44.7
	5	50	medium (3)	66.0 ± 37.8	46.0 ± 44.3
	5	50	large (3)	86.0 ± 21.2	54.0 ± 28.4
Induced	5	50	medium (3)	60.0 ± 33.2	– –
	5	50	large (3)	68.0 ± 29.3	– –

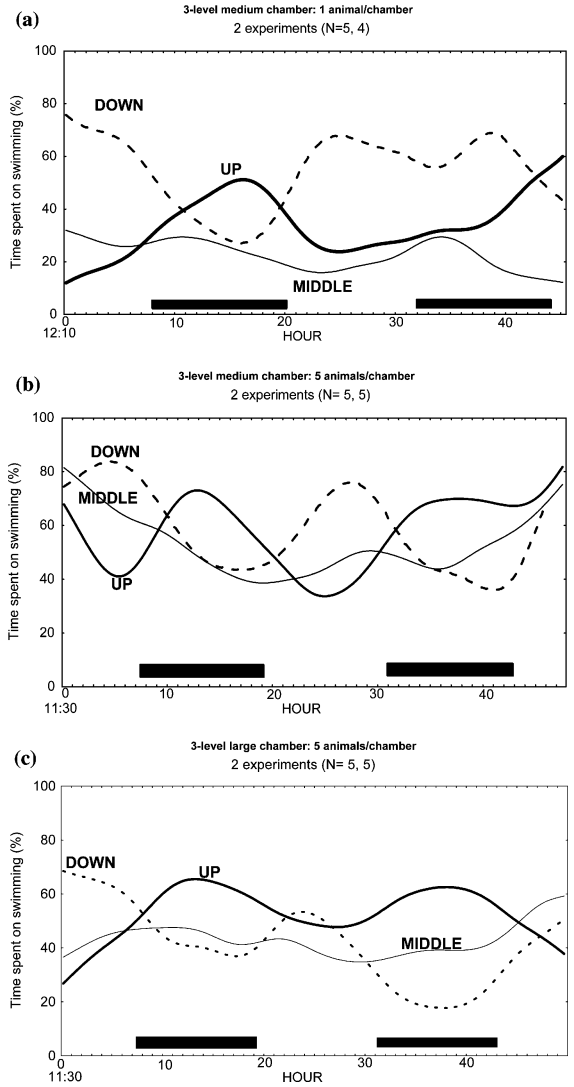

Figure 2. Diurnal activity patterns (means, least square fit) of *D. magna* clone Beak recorded by the "Multispecies Freshwater Biomonitor" in 3-level chambers under a photoperiod regime of 12 h night/12 h day. Nights are indicated by black bars. (a), 1 animal/3-level medium chamber ($N=2\times8$ chambers), (b), 5 animals/3-level medium chamber ($N=2\times5$ chambers), (c), 5 animals/3-level large chamber ($N=2\times5$ chambers). In all experiments the range of variation of the mean percentage of time spent on swimming between individual MFB-chambers was $\pm$SD = 2.8–45 and $\pm$SE = 1.2–30. The number of organisms per chamber did not significantly affect the variation of the recorded swimming behaviour.

endogenous rhythmicity. For the 3-level medium chambers, the sinus fit differed from the data for most of the time (paired *t*-test: day: $t=2.26$, $p=0.03$; night: $t=-2.36$, $p=0.02$).

Discussion

The use of the Multispecies Freshwater Biomonitor (MFB), equipped with vertically arranged recording chambers containing *Daphnia magna*, achieved the goal of the study: the online recording of negative phototactic behaviour of a zooplanktonic species. Manipulation of the endogenous diel circadian rhythmicity by changing the light regime resulted in altered behaviour, clearly registered by the MFB, especially in larger chambers. The non-optical nature of the MFB opens perspectives for research on the behaviour of plankton at night and in turbid waters. Moreover, automatic continuous recording of diel vertical swimming rhythmicity might find applications in biological early warning systems such as online biomonitoring.

Survival of *D. magna* decreased with increasing organism density. A number of 5 animals per chamber of a volume of between 30 and 70 cm^3 can only be accepted for short-term experiments. However, a volume of only 30 cm^3 was applied for long-term experiments in the dynamic waterflea assay with 20 daphnids per chamber (Knie, 1978), a volume of 45 cm^3 was applied in the Behaviour Quant biomonitor with 10 daphnids per chamber (Blühbaum-Gronau et al., 2000) and a volume of 112,5 cm^3 in a flat (1.5 cm in depth) chamber with 25 daphnids has been used by Baillieul & Scheunders (1998). These comparisons show that even smaller volumes and higher organism densities have been used for long-term biomonitoring of behaviour. In a previous experiment with continuous flow-through of Rhine water, which contained natural particles as food for *D. magna*, 5 organisms were used in small single level chambers of 9 cm^3 for short- and long-term monitoring with a survival of 70% over 20 days of exposure (Gerhardt et al., 2003). This indicates that the lack of space might be of less importance for survival in case of continuous supply of food and oxygen. Optimal culture conditions according to international standards (APHA, 1989) are expected to lower the mortalities observed in the present study. The relatively high mortalities in the present study are probably due to the suboptimal culture conditions, but were not decisive for interpreting the results, as still enough surviving individuals provided the necessary behavioural signals to distinguish the different vertical levels and chamber treatments.

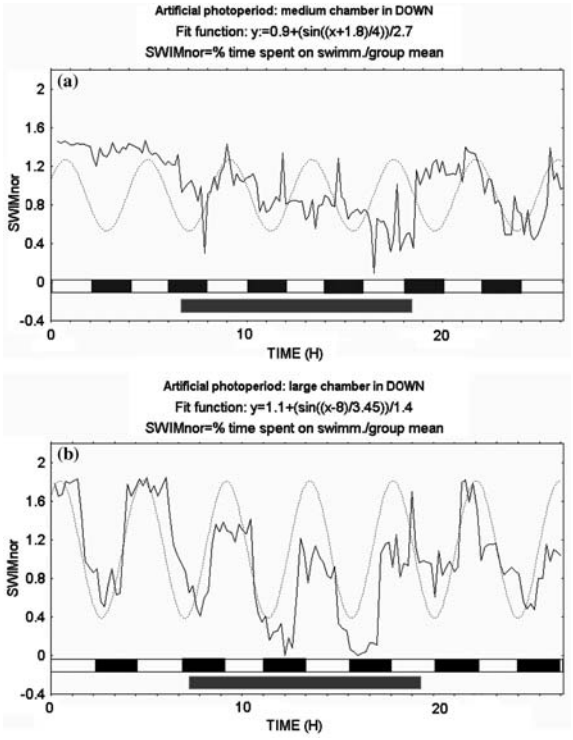

Figure 3. Activity patterns (means normalised to 1) of *D. magna* clone Beak recorded by the "Multispecies Freshwater Biomonitor" in DOWN level of (a) 3-level medium chambers ($N=2\times5$ chambers) and (b), 3-level large chambers ($N=2\times5$ chambers), each containing five animals, under an induced photoperiod regime of 2 h dark (black bars)/2 h light. The real night time is indicated by a grey bar. Dotted lines represent the predicted sinusoïd fits.

Recording diel vertical migration (DVM), the use of a group of 5 organisms improved the results in the current study. We assume that DVM might be a "collective" behaviour, i.e. the movements of one organism might stimulate the others to join. Ordemann et al. (2003) induced a swarming behaviour in *Daphnia*, by increasing the animal density. This finding confirms the present results. Given this, and the fact that the rhythmicity of the animals was less pronounced in single individuals than in collective ones, we can exclude the argument that the different signals obtained by the MFB with single specimens or collectives of five animals are due to different signal/noise ratio. Our findings are not due to changes in signal quality in relation to the chamber size. As shown by previous calibration of the test chambers, the number of animals does not affect the quality of signal frequencies. Moreover, we see no contradiction of stimulation of individual activity by other animals and the fact that they all belong to the same clone. Each animal might be genetically programmed to behave differently in different density situations.

The minimum space requirements for DVM in *D. magna* was 10 cm height and 30 cm^3 volume. The use of chambers containing three vertically arranged levels over a distance of 10 cm produced clear circadian curves with a negative phototactic swimming pattern in a size range which is currently used in other online BEWS. This pattern appeared to be very robust, irrespective of chamber size. The fact that the DOWN curve did not tend to zero when the UP curve was at its maximum, indicates that at any time, some animals were present in all three levels.

Circadian rhythms are driven by endogenous clocks, entrained by non- specific responses to chemicals and other exogenous factors, which are regulated by a complex visual system, sometimes in combination with serotonin and have been found in many aquatic animals (Dell' Omo, 2002). Similarly, increased locomotory activity during the night has been recorded with the MFB in the trichopteran *Hydropsyche angustipennis* (Gerhardt, 2000), the mayfly *Choroterpes picteti* (Gerhardt et al., 2005a), the crustacean amphipode *Gammarus pulex* (Gerhardt et al. 1998) and the freshwater shrimp *Atyaephyra desmaresti* (Janssens de Bisthoven et al., in press).

In the present study, the negative phototactic pattern could be induced under frequently changing manipulated photoperiods, hence illustrating the inherent underlaying basic phototactic reflex. In both 3-level medium and large chambers, the swimming in the DOWN levels showed rhythmicity as a function of artificially induced light phases with a period of ±4 h. As would be expected from a negative phototactic population, the average activity at the bottom during the light phases was higher than during the dark phases. In both types of chambers, the amplitude of the movement waves remained constant over time, however the maxima decreased during night time, even though the 2 h light phases continued to alternate with the two hours dark phases throughout the night.

Both types of responses, the phototactic reflex and the circadian rhythm seem to overlap in the night, therefore maxima within DOWN levels were weaker due to interference. Moreover, the phototactic activity was much more clear in the large chambers than in the medium chambers, showing that volume might play an important role for vertical migration.

The reaction time of *Daphnia magna* as well as the swimming speed for DVM was found by Van Gool (1997) to depend on the intensity of the changes in light and was also affected by circadian and annual rhythms (Whitman & Miller, 1982). Direct comparisons of laboratory studies with field studies should be taken cautiously, as the light intensity is likely to be much stronger in the field (De Meester, pers. comm.).

Phototaxis has recently been used in toxicological tests. *D. magna* responded with decreased phototaxis to 0.06 mg Cd/l (Michels et al., 2000). Gerhardt et al. (2005b) observed an amplification of circadian rhythmicity in *Daphnia magna* clone BEAK when exposed during 48 h to acid mine drainage, supporting that this behaviour might be appropriate for a sensitive sublethal behavioural toxicity assay (Dell' Omo, 2002) and online-biomonitoring of pollution peaks (Gerhardt, 1999, Gerhardt et al., 2005b). Moreover, phototaxis recorded in the laboratory in chambers (25 cm height, 5 cm diameter) was a good predictor of phototactic behaviour verified in outdoor experimental containers (55 cm depth), thus showing a good up-scaling and field validation (Michels et al., 2000). Whitman & Miller (1982) demonstrated that Naphthalene had a negative effect on negative phototaxis of *Daphnia magna*.

Obviously, the results obtained in the present study are specific to one specific clone of *Daphnia*. We don't exclude the possibility of obtaining different ranges of interference between endogenous rhythms and artificially induced rhythms, or even different types of phototaxis when using other clones or natural populations containing several clonal lineages.

Although the chamber sizes in the present study were similar to the ones used in other *Daphnia* biomonitors, our data would support the use of larger chambers with additional recording levels in order to (1) avoid mortality due to density stress and (2) better record diel vertical migration.

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