Is the rhythm of vertical migration of *Daphnia longispina* circadian or simply nycthemeral ?

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From an ecological viewpoint, diel vertical migration (DVM) of zooplankton is an adapted response to environmental factors. DVM coincides with changes in light intensity, but persists also under constant illumination conditions. This fact suggests the existence of an internal clock. In *Daphnia longispina*, it could be located in the eye. Indeed, the spatial distribution of the pigments containing in the eye ommatidia exhibits significant changes over time when *Daphnia* are kept under light/dark (LD) conditions. These variations coincide with displacements of the organisms. Furthermore, it is observed that the blue/dark conditions, which inhibit DVM, also stop changes in the distribution of pigments in the ommatidia. The modifications of the eye observed under LD white light represent either the internal clock or its manifestation.

Keywords : migration, *Daphnia longispina*, rhythm, eye, pigments.

Introduction

Diel vertical migration (DVM) behaviour of zooplankton has found continuous interest in recent years. The number of published papers on this topic has increased since the phenomenon was first described (Loeb 1893). *Daphnia* as well as other small crustacean zooplankton exhibit DVM. Typically, the population sinks as day light rises, spends the day deep in the lake, and then moves upward toward the surface in the evening, as sun light fades (Cushing 1951, Pourriot & Meybeck 1995, Fiksen 1997). Most of the papers dealing with DVM suggest that numerous abiotic and biotic factors explain DVM behaviour. The evidence generally presented supports the view that a phototactic reaction to relative changes in light intensity is at the basis of DVM. This phototaxis is enhanced by fish kairomones (Gliwicz 1986, Dodson 1990, Dodson et al. 1997, Leibold 1990, Ringelberg 1991 a, b, Bollens et al. 1992, Lampert & Loose 1992, Haney 1993, Lampert 1993, Loose 1993 a, b, Loose et al. 1993, Ringelberg & Flik 1994, Spaak et al. 2000, Von Elert & Pohnert 2000) and influenced by temperature (Gerritsen 1982), food and individual size (Angeli et al. 1995, Worthington 1931). Ringelberg (1980) even classified the factors regulating DVM into two categories. On the one hand, the endogenous factors, also named proximate causes, are related to an organism's evolutionary strategy. On the other hand, exogenous factors, also named ultimate factors, influence DVM.

Apart from the above general results, the question of whether DVM is synchronised on the nycthemeral cycle has yet to be fully understood. In the laboratory, DVM is often observed under alternating light/dark conditions (denoted by LD in the chronobiology nomenclature). Moreover several authors reported, under constant conditions of white light (LL), the persistence of the swimming activity (Esterly 1917, Harris 1963, Ringelberg & Servaas 1971, Beauchaud 1987). Thus, DVM still occurs *in situ* in the absence of an appropriate light signal (Berthon & Buisson 1984). Harris (1963) even found an internal clock with a 24-hour cycle in *Daphnia magna* under DD (complete dark) conditions. These facts suggest that the rhythm is circadian, i.e., controlled by an internal oscillator.

The aim of the present study was to investigate the physiological changes that appear in *Daphnia* during DVM. We tried to understand the mechanisms that may induce DVM. We were not concerned with the ecological consequences, but only with the underlying process of DVM. We therefore investigated this issue by studying photoreceptors of daphnids in particular. We previously reported that there were significant variations of the pigment distributions around the rhabdom of *Daphnia longispina* when they were measured before and after dusk, and that the same phenomenon was observed when animals were kept under constant LL conditions (Cellier 1997, Cellier et al. 1998). The significant variations observed under LD may be due to the light factor, but their persistence under LL suggests the intervention of an internal clock which is synchronised with the nycthemeral alternance. The eye modifications might represent either such a clock or its manifestation.

In this study, we monitored changes in the distribution of pigments within the eye of *Daphnia longispina* at various epochs of the nycthemer, and observed its structure under LD white light conditions. Since blue light inhibits DVM (Beauchaud 1987, Berthon 1988, Berthon & Brousse 1995), a concomitant experiment was done under blue LD light conditions since it is important to know whether inhibition of DVM by blue light also induces a recess of structural variations of the eye. If this was the case, it would confirm a link between DVM and structural variations.

Material and methods

Biological material

Zooplankton species were taken with a 88 μm plankton net from the hypereutrophic Grangent reservoir (Loire, France), where migrations of crustaceans were previously observed *in situ* (Berthon & Buisson 1984, Berthon 1988). They were immerged in lake water in large containers and brought to the laboratory. In the laboratory, daphnids (*Daphnia longispina*) and lake water were immediately poured into the columns.

Experimental design

Daphnids were dispatched into two altuglass columns (3x0.25m) placed in an air-conditioned room. The whole experimental apparatus was described in details by Brousse (1990), Berthon & Brousse (1995) and Cellier et al. (1998). The two columns were illuminated with a cold light generator (Schott KL 1500) using an incandescent lamp of 150 Watts placed on the top of the column. Illumination intensity at the top of the column was 58 $W.m^2$ in white light and 2.5 $W.m^2$ in blue light. An isolating chamber allowed access to the experimental room without modifying the experimental conditions. The temperature of the water was held constant at 23°C with a pH around 7.3 - 7.4. Water sampling did not modify the experimental conditions. In order to observe the animals during the dark phase, we placed a dim red light. Cladocerans, as well as many invertebrates, are insensitive to this type of light (Berthon & Brousse 1995).

Protocol

Animals were acclimated during 48 hours to an alternating light/dark regimen similar to the outdoors one. They were then put under LD conditions, with 14 hours of white or blue light, and 10 hours of dark light (LD 14 : 10). Dawn and dusk were fixed at 7.00 and 21.00. Samplings of Daphnids from the columns were performed with a small container , filled with Seltz water and linked to an air free pump (KNF), at the following times : 17.00, 20.00, 22.00, 1.00, 4.00, 6.00, 8.00 and 11.00. In the sequel, we will refer to these sampling epochs as hour groups. There were at least five animals per sample. After a series of histological manipulations, animals were imbedded in Spurr. Polymerisation was achieved in 24 hours at 60°C. We then realised 400 nm cuts in the sagittal plan of the eye (ultramicrotome Sorval Porter-Blum MT 6000). This thickness corresponds to an average value for the diameter of a pigment seed.

Analysis

Cuts were observed under a photonic microscope and the views were treated with an image analysis software package (Lamba logiciel 2005). The eye of daphnids is composed of ommatidia. Each ommatidium is made of a crystalin and a rhabdom surrounded by pigments (Fig. 1), and a nervous fibre. For the analysis, each ommatidium was naturally divided into three zones. The first one, at the outer part of the eye, is delimited by the crystalin. The other two parts surround the rhabdom, zone 2 is around the upper part and zone 3 around the lower part. A quick examination of an ommatidium cut reveals an area stained by a series of spots corresponding to pigments. Because counting every pigment is hard to perform, we simply measured pigmentary surfaces on the basis of coloration differences. These surfaces will be denoted by X_1, X_2 and X_3 for pigmentation in zone 1, 2 and 3 respectively. All the areas over which pigments were analysed in each zone were quite similar. Each observation on an om-
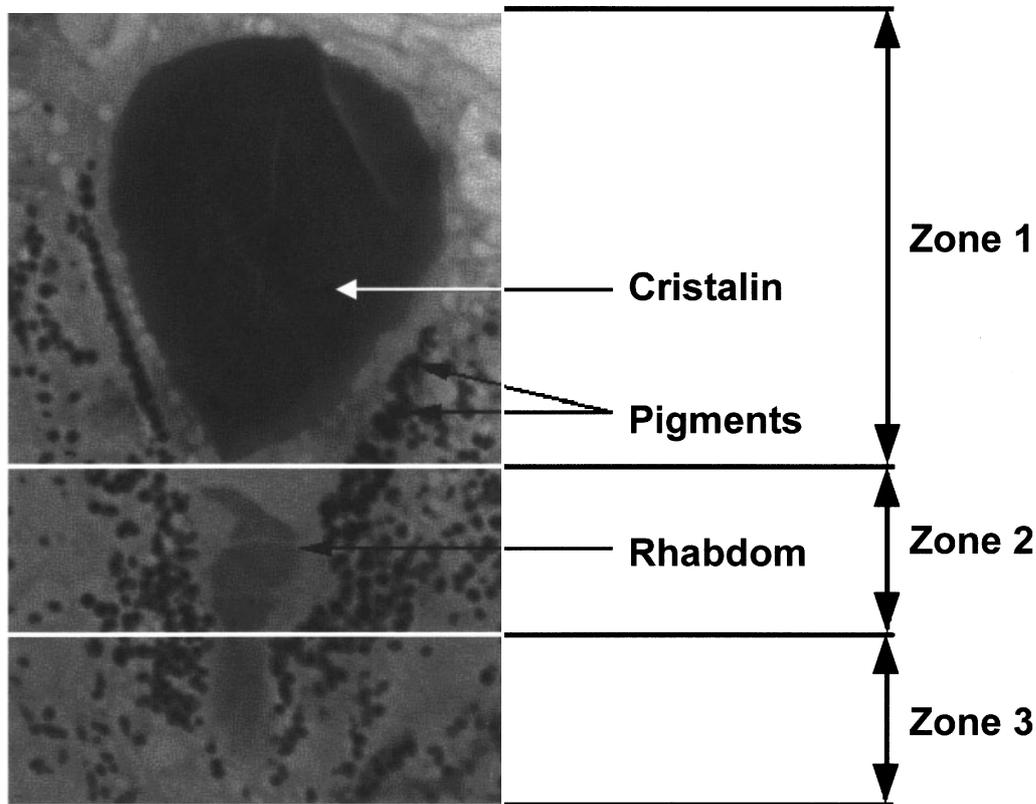
Fig. 1 . Transversal section of a *Daphnia longispina* ommatidium. Zones 1-3 : see text.

matidium was therefore represented by a vector $X = (X_1, X_2, X_3)$ of R^3.

Statistical analysis was based on the ? Wilks statistic of the MANOVA and post-hoc pairwise comparisons. The measurement scale used in the analysis was the square root of the pigmentary areas. Indeed, examination of normality and homoscedasticity by various standard tools (histograms, Kolmogorov-Smirnov statistic, correlation between mean and variance and Box's test) showed that a variance stabilising transform was necessary.

Results

There was no interaction between light and hour groups (Table 1). Changes in pigmentation were uniform throughout the various hour groups as we moved from one type of light to another. The type of light (white or blue) had a significant effect on pigmentation (Fig. 2). Consistently, pigmentation under LD white was larger than under LD blue ($P < 10^{-4}$). Also, there was a significant hour groups effect ($P = 0.0054$). Since effect of time on pigmentation was our primary concern, we restrained our analysis to each type of light separately in the sequel. The lack of interaction allowed such separate analyses. Therefore, two one-way MANOVAs were performed (Table 2).

Blue light inhibited displacements (Table 2) of the pigments ($P = 0.106$) and this was the case in all three zones ($P = 0.3806, 0.2638$ and 0.6688 for X_1, X_2 and X_3 respectively). However, a graphical representation (Fig. 3) of the data shows an increase, though not significant, of pigmentation between 11:00 and 14:00.

White light (Table 2, Fig. 4) had a significant effect on pigmentation ($P = 0.006$), particularly for zone 3 ($P = 0.0035$).

Pairwise post-hoc comparisons (Table 3) lead to the following comments. Three distinct gatherings of hour groups were revealed. The first one corresponded to 11:00 ; it was significantly different from almost all

Fig. 2 . Changes in the pigmentation surface under white (grey bars) and blue light (dark bars) in zones 1 (a), 2 (b) and 3(c), and mean values of pigmented areas in the three zones under the two light conditions (d).

Table 1. Results of the two-way Manova with light type (blue and white) and hour groups (with nine levels) as factors. ns = non significant.

Source	Wilks statistic Λ	P
Light	0.6702	$<10^{-4}$
Hours Group	0.6983	0.0054
Interaction	0.7525	ns

Table 2. Results of one-way Manova and Anova. In both analyses the factor is hour groups. ns = non significant.

BLUE LIGHT		WHITE LIGHT	
MANOVA			
Wilks statistic Λ = 0.4901		Wilks statistic Λ = 0.5243	
P = 0.1106 (ns)		P = 0.0006	
ANOVA for the three zones			
Zone 1	P = 0.3806	Zone 1	P = 0.3236
Zone 2	P = 0.2638	Zone 2	P = 0.1563
Zone 3	P = 0.6688	Zone 3	P = 0.0035

Table 3. Results of post-hoc pairwise comparisons among the various hour groups under white light. ns = non significant.

	4:00	6:00	8:00	11:00	14:00	17:00	20:00	22:00
1:00	ns	ns	ns	0.0235	ns	ns	ns	ns
4:00		ns	ns	0.0212	ns	0.0073	0.0105	0.0005
6:00			ns	ns	ns	0.0150	0.0227	0.0006
8:00				0.0091	ns	ns	n.s.	0.0345
11:00					ns	0.0204	0.0212	0.0002
14:00						ns	n.s.	ns
17:00							n.s.	ns
20:00								ns

other groups (except hour groups 6:00 and 14:00). Hour groups 17:00, 20:00 and 22:00 were alike and differed significantly from the cluster made of groups 4:00, 6:00 and 8:00. Hour groups 1:00 and 14:00 did not differ from any other group (except for a difference observed between 1:00 and 11:00). Again, these differences were more distinctly observed in zone 3.

Discussion and conclusion

In situ, Daphnids residing near the surface water can very well perceive changes in light intensity (at sunrise). On the other hand, because of the light fading off along a vertical gradient (law of Lambert-Beer in Devaux 1976) it is quite unrealistic for daphnids residing in deep water layers to perceive the light signal that will trigger their upward migration.

Waterman (1974 in Ringelberg 1995) even observed DVM of chaetognaths at 1400-1700 m depth during the day. Therefore, we assume that the incoming light synchronises vertical migration at sunrise and that the displacement cycle begins with downward swimming of *Daphnia* towards deep water. Under such an assumption, the upward movement supposes in turn a biological process (an internal oscillator). Such a process is related to the photoperiod since it leads to a rerise towards surface level around sunset. We can advance the hypothesis that an oscillator is used as a relay between the light seen at dawn and the upward movement at dusk. Light is usually recognised to be the most important zeitgeber (Rusak & Zucher 1979). If light is the synchroniser of the rhythm observed in *Daphnia*, it can only act at dawn.

Two approaches are available for searching for an internal component in an organism. Either, a rhythmic phenomenon still occurs in this organism placed under constant conditions, or structural / hormonal variations are observed in correlation with one rhythm in this same organism. Structural variations of the eye between day and night were observed in many Cladocerans species (Debaisieux 1944, Nilsson & Odselius 1981, Land 1996, Meyer-Rochow 2001). Merely the state of eyes at light and dark were described, nothing about structural variation over time was reported. Accoring to Debaissieux (1944), results varied according to species (e. g., *Artemia salina*, *Chiroaphalus diaphanus*). This author asserted that pigments play a protector role against light. Cellier et al. (1998) showed that there was a variation in the pigmentation in *Daphnia* among zones. We can qualify this analysis as a local one since merely two hour groups were considered. In this paper, a more comprehensive approach was adopted. We considered several measuring times and tried to have a finer idea of how pigmentation evolves.

Pigment distribution under blue light (Fig. 3) was not the same as the one observed under white light illumination (Fig. 4). Under the latter condition, the graphic representation revealed two periods of substantial increase in pigmentation : one running from 8:00 to 11:00 and one from 22:00 to 1:00, i. e., after the tur-
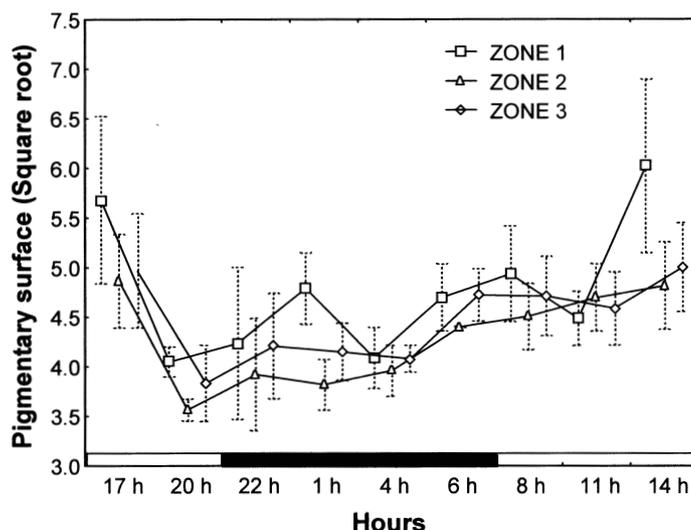

Fig. 3 . Diel changes in the pigmentary areas in the three zones under blue light.

ning on and the turning off of the light respectively. These phenomena (Fig. 4) coincide with the movements of daphnids during DVM (Worthington 1931). At dawn, the augmentation of the pigment quantity could be explained by the direct reaction of the apposition eye type to light, as suggested by Debaissieux (1944). But at dusk, the significant difference in pigments distribution could not be explained only by light variations. It would also be logical to observe a steady decrease in pigmentation.

These results indicate that the modifications of the eye structure may be due to an internal mechanism, and not solely to light. These pigment variations are synchronised with the illumination occurring at dawn, and would thus generate the first pigmentation peak. Because organisms cannot perceive the disappearance of the day-light in deep water, the beginning of their swimming upward as well as the second pigmentation peak, can only be a coincidence and not a behaviour correlated with light. Photoperiod is probably involved with this coincidence. Also, a synchroniser will be in phase with a cycle starting at dawn. As it is often the case, such a circadian rhythm, under natural conditions, should be a nycthemeral one.

Effects of blue light may be more important since it penetrates much deeper (Lampert 1989). Therefore, under this light, the results of the experiments suggest that there is an inhibition of pigments displacement in *Daphnia*. Under blue light, the presence of a peak bet-ween 11:00 and 14:00 (for zone 1) might have two explanations. Either it represents the persistence, not so strong, of the rhythm observed under natural conditions, or it is a completely random phenomenon. The first hypothesis would mean the existence of a correlation between the inhibition of the pigment variation and the recess of the upward movement. However, the rhythm was not really maintained under LD blue. In fact, DVM is inhibited under LD blue and daphnids remain in deep water (Beauchaud 1987, Brousse & Berthon 1995). It is really the upward movement which does not occur. Also, the eye seems to be an oscillator or in relation with it. Though, we cannot definitely assert that there is a causal relationship between DVM and pigment displacement, because of the «absence» of an apparent connection. But significant pigment variation, which is inhibited by blue light, indicates that the eye structure is correlated with DVM and may therefore control their rhythm. Moreover, structural variations around dusk, observed under L.D. conditions, are even observed under (LL) constant conditions (Cellier et al. 1998, Cellier-Michel 2001).

Our results raise several questions for future work. We used an on-off light stimulus to influence *Daphnia* behaviour. Does a gradual change in light intensity cause the same changes ? We observed animals for only 24 h, as they swam in a 3x0.25 meter altuglass column. It would be interesting to confirm changes of the eye structure in daphnids taken directly *in situ*. We
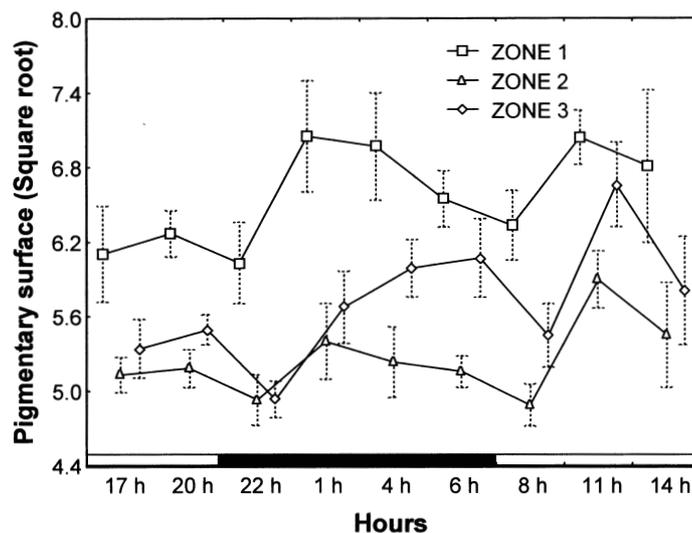

Fig. 4 . Diel changes in the pigmentary areas in the three zones under white light.

would also see if variations observed in laboratory are a good representation of the natural process, or if they are altered by the bias induced by artificial light. In order to verify a causality between pigment displacement and DVM, a similar experience must be done under constant light conditions, complete LL or DD. Genotypic polymorphism often plays a role in DVM explanations (De Meester 1993). Our work was based on *Daphnia longispina*. Do other *Daphnia* species show the same response ? Bollens et al. (1995) affirmed that the exact cues eliciting DVM in zooplankton vary between species. There is clearly much more to know about the relationship between individual swimming and pigment eye repartition in *Daphnia*. As Ringelberg reported «after more than 150 years of field observation and 100 years of laboratory research, diel migrations remain an enigma» (Huntley 1985 in Ringelberg et al. 1991).

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