INFLUENCE OF LIGHT INTENSITY ON PLASMA MELATONIN AND LOCOMOTOR ACTIVITY RHYTHMS IN TENCH

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ABSTRACT

Melatonin production by the pineal organ is influenced by light intensity, as has been described in most vertebrate species, in which melatonin is considered a synchronizer of circadian rhythms. In the case of tench, strict nocturnal activity rhythms have been described although the role of melatonin has not been clarified. In this study we investigated daily activity and melatonin rhythms under 12:12 light-dark (LD) conditions with two different light intensities (58.6 and 1091μW/cm^2), and the effect of one hour broad spectrum white light pulses of different intensities (3.3, 5.3, 10.5, 1091.4 μW/cm^2) applied at mid darkness (MD) on nocturnal circulating melatonin. The results showed that plasma melatonin in tench under LD 12:12 and high light conditions displayed a rhythmic variation, where values at MD (255.8 ± 65.9 pg/ml) were higher than at mid light (ML) (70.7 ± 31.9 pg/ml). Such a difference between MD and ML values was reduced in animals exposed to LD 12:12 and low light intensity. The application of one hour light pulses at MD lowered plasma melatonin to 111.6 ± 3.2 pg/ml (in the 3.3-10.5 μW/cm^2 range) and to 61.8 ± 18.3 pg/ml (with the 1091.4 μW/cm^2 light pulse) and totally suppressed nocturnal locomotor activity. These results showed that melatonin rhythms persisted in tench exposed to low light intensity although the amplitude of the rhythm is affected. In addition, it was observed that light pulses applied at MD affected plasma melatonin content and locomotor activity. Such a low threshold suggests that the melatonin system is capable of transducing light even

under dim conditions, which may be used by this nocturnal fish to synchronize to weak night light signals (e.g. moonlight cycles).

Key words: *Tinca tinca*, light intensity, melatonin, locomotor activity, light pulse.

INTRODUCTION

Circadian rhythms in animals are entrained by external factors known as zeitgebers, with light reported as the main zeitgeber (Aschoff, 1981). Light can further affect circadian rhythms and mask information from internal clocks, promoting direct changes in animal activity levels (Mrosovsky, 1999). In fish, daily locomotor and feeding patterns depend on the light, temperature and availability of food (Eriksson, 1978).
In teleosts, light information is transduced by the pineal organ, which is a photosensory organ containing photoreceptor cells (pinealocytes), neurons and ependimal interstitial cells (Ekström and Meissl, 1997). Pinealocytes were early thought to be the source of melatonin synthesis (Oksche and Kirschstein, 1967; Rüdeberg, 1968) and this was confirmed by later studies (Falcón et al., 1981; Vivien-Roels et al., 1981; van Veen et al., 1984; Östholm et al., 1987; Ekström and Meissl, 1990). A generally accepted feature concerning melatonin synthesis in all vertebrates is that pineal melatonin is regulated by the environmental light-dark cycle, peaking during the scotophase and releasing the lowest levels during the photophase. Such melatonin synthesis by the pineal organ is directly related to plasma melatonin levels (Gern et al., 1978; Kezuka et al., 1988; Falcón et al., 1989; Zachmann et al., 1992; Iigo et al., 1995; Randall et al.,

1995). When cultured *in vitro* in continuous darkness, pineal organs of most teleosts release melatonin following a circadian rhythm, for at least a few cycles. This implies that there is a circadian oscillator in the pineal organ that controls melatonin synthesis (Ekström and Meissl, 1997). In addition, light exerts a direct action on melatonin production, suppresing the endogenous rhythm while a light pulse during the scotophase inhibits melatonin production (Falcón et al., 1987; Bolliet et al., 1995; Cahill, 1996). Indeed, the more intense the light pulse at mid-darkness (MD) the lower the level of plasma melatonin in sea bass (Bayarri et al., 2002) and brook trout (Zachmann et al., 1992). The light threshold required to modify melatonin contents was established at 6μW/cm^2 in the sea bass (Bayarri et al., 2002).

It has been suggested that the circadian clock of freshwater fish shows more plasticity than its marine counterpart, which implies that freshwater fish might modify their activity pattern, even changing their nocturnal behaviour to diurnal if needed and *viceversa* (Helfman, 1981; Cook and Bergersen, 1988; Baras, 1995; Naruse and Oishi, 1996). However, recent studies have concluded that circadian activity rhythms in tench are driven by an internal circadian clock, which is, however, strongly influenced by light. Curiously, when light intensity was dramatically decreased during the photophase (down to 0.3 lux), tench remained strictly nocturnal (Herrero et al., 2003). However, the role of melatonin on light transduction under such an LD cycle with low light intensity is unknown.

The objective of the present work was to confirm the existence of plasma melatonin rhythms under low light conditions in tench and investigate the effect on plasma melatonin and locomotor activity of exposure at MD to one hour light pulses of different light intensities: 1091, 10.5, 5.3 and 3.3 μW/cm^2 (1300, 3, 1 and 0.3 lux) .

MATERIALS AND METHODS

Animals and Housing

This research consisted of three experiments. To determine the existence of a daily plasma melatonin rhythm under low light conditions (exp. 1) and the effect of one hour light pulses on melatonin at MD (exp. 2), ninety-two tench of mean body weight 938 ± 22 g were used, while to study the influence of a light pulse at MD on locomotor activity at MD (exp. 3) seven fishes of 100 ± 7 g were recorded. The animals were kept in GRP (Glass Reinforced Polyester) tanks in an open water circuit, located in a local research institute (Centro Vegas del Guadiana, Badajoz, Spain). To record activity rhythms, animals from the same research institute were transferred to an isolated laboratory and placed in seven 60 L. glass aquaria, where water was recirculated and filtered by means of a skimmer and a biological filter.

Experimental Design

Experiment 1: daily plasma melatonin rhythm under low light conditions.

Prior to sampling, the fish were maintained in light-tight tanks isolated from external environmental light for two weeks, thus allowing the animals to acclimate. A white fluorescent tube (GRO-LUX, 40 W, Germany) was placed over each tank, providing a final broad spectrum white light. Fluorescent tubes provided a final white light although their spectrum showed major peaks at 438, 471, 547 and 658 nm..

The light intensity during daytime was on average 58.6 $\mu W/cm^2$ (20 lux) at the water surface. To obtain the desired light intensity, lamps were covered with aluminium foil with holes punched in it. To avoid external light, each tank was covered with a black vinyl sheet. The light/dark (LD) cycle was programmed by an electronic timer and set at 12 h light: 12 h dark (LD 12:12), lights coming on at 0700 and switching off at 1900 hours.

For melatonin analysis, blood samples were taken every three hours. During the light phase, sampling was carried out in the same light intensity conditions that fish had experienced for the previous two weeks, whereas during the dark phase a dim red light was used, and fish heads were covered with aluminium foil. Before withdrawal of blood, each fish was anaesthetized with water containing 0.5 ml/l of 2-phenoxiethanol. As soon as the fish lost their equilibrium, they were weighed and blood was taken from the caudal vessel using 2 ml heparinized syringes (TERUMO, Leuven, Belgium). The blood was put into polypropylene Eppendorf tubes, and after centrifugation, plasma samples were frozen on solid CO_2 and finally stored at - 80º C until analysis.

Experiment 2: effect of one hour light pulses of different intensities at MD on nocturnal melatonin production.

The animals were reared for two weeks under a 12:12 LD cycle, with an average light intensity during the daytime of 1091 $\mu W/cm^2$ (1300 lux) at the water surface.

Before sampling, 32 fish were divided into four groups. Each group was exposed at MD to a one hour light pulse of different intensity (1091, 10.5, 5.3 or 3.3 $\mu W/cm^2$). In addition, two more groups were sampled as controls, one at mid-light (ML) and another at MD. Six tench were used in each of these latter groups.

Special care was taken to ensure that the spectral composition of light remained equal irrespective of intensity. To this end, decreasing light intensity was adjusted by covering the fluorescent tubes with aluminium foil and punching holes in it until the desired light intensity was reached. The spectral composition of the different intensity lights is represented in Fig.1. Note that the spectrum remained equal at all light intensities tested. Blood samples were taken after each light pulse at MD, the intensity of the lights used during sampling being equal to the intensity used for the light pulses. Withdrawal of blood took less than ten minutes for each group. The control groups were sampled at ML, during the light phase, and at MD with no pulse. The sampling procedure was the same as described in Experiment 1.

Experiment 3: influence of one hour light pulse of low intensity applied at MD on locomotor activity.

Activity rhythms in tench reared in seven 60 L. glass aquaria isolated from external conditions were registered by means of infrared photocells (OMROM E3S-AD62, Japan) placed in each aquarium. Light was provided by a white fluorescent tube (GRO-LUX, 40 W, Germany) placed over each aquarium with an average intensity of 1091 $\mu W/cm^2$ at the water surface. Animals were exposed to a 12:12 LD cycle during the first thirteen days and from day 14 onwards a one hour light pulse of 3.3 $\mu W/cm^2$ was applied at MD.

Melatonin Analysis.

Melatonin was determined using a commercial radioimmune assay (RIA) kit (IBL, Hamburg, Germany). Intra-assay coefficient of variation (CV) was: 8.1-8.5%, and inter-assay CV was: 14.8-15.0%. The limit of detection (LD) of the kit was <3.5 pg/ml. Samples were thawed and 200 µl of each one was placed in polystyrene tubes. Enzyme solution was added and the mixture centrifuged and incubated for 2 hours at room temperature, after which ^{125}I tracer and antiserum solution were added. The tubes were then submitted to centrifugation and incubation for 36-48 hours at room temperature. The precipitating reagent was added and the tubes centrifugated again. Finally, supernatants were removed and tube radioactivity was measured in the γ scintillation counter (WALLAC 1470 Automatic Gamma Counter, Perkin Elmer).

Data Analysis.

To test the intensity and the spectral features of light, a spectroradiometer (Analytical Spectral Devices ASD. Inc FieldSpec® Handheld) was used. Data were further processed to obtain graphics of spectral composition. Locomotor activity records (exp. 3) were stored in a computer and analysed using a computer program (EL TEMPS® Antoni Díez Noguera, University of Barcelona). Excel and SPSS were used for data analysis. The statistical differences between mean melatonin levels in Experiments 1 and 2 were analysed by one-way analysis of variance (ANOVA) followed by Duncan´s test, with $P < 0.05$ taken as the statistically significant threshold. Statistical differences between activity levels at MD before and during the application of a light pulse in Experiment 3 were analysed by t-Student test, with $P < 0.05$ taken as the statistically significant threshold.

RESULTS

Experiment 1

Under LD 12:12 (both high and low intensities) plasma melatonin peaked at night. Tench exposed to high light conditions exhibited significant differences between day (ML) and night (MD), the lowest levels being recorded during the day. Plasma melatonin values at ML in tench under high light intensity and under low light intensity were 70.7 ± 31.9 pg/ml and 21.29 ± 11.1 pg/ml, respectively. At MD the plasma melatonin content of animals exposed to a high light intensity was 255.8 ± 65.9 pg/ml and of those under low light intensity was 178.1 ± 53.8 pg/ml. It was observed that differences between ML and MD values were greater in the animals exposed to a higher light intensity during the photophase, although no statistically significant differences were found. Plasma melatonin levels in tench under LD 12:12 with low light intensity showed a rhythmic variation. Plasma melatonin levels started rising at the beginning of the scotophase, peaking at MD. When lights came on, plasma melatonin dropped. Considering daily variations, statistically significant differences between ML and MD levels were found (Fig. 2).

Experiment 2

When a one hour light pulse was applied at MD, plasma melatonin significantly dropped to ML levels (ANOVA, P=0.025) at all the intensities tested (1091, 10.5, 5.3 and 3.3 $\mu W/cm^2$). In the range of 3.3 to10.5 $\mu W/cm^2$, melatonin dropped to around 111 pg/ml, whereas the 1091 $\mu W/cm^2$ light pulse further decreased plasma melatonin values

down to 62 pg/ml, although no significant differences between these last values were found (Fig. 3).

Experiment 3

Locomotor activity rhythms in tench reared under LD 12:12 (L=1091 : D=0 $\mu W/cm^2$) displayed an almost strict nocturnal behaviour, 97.9% of activity occurring during the scotophase. The total average daily counts for all the tench remained equal during LD and LD + MD pulse phases, around 1850 counts/day. However in some individuals, when light pulses were applied the daily activity counts increased. In all cases, during the pulses at MD locomotor activity dropped (t-Student, P=0.047) (Fig.4).
The average diel profile of locomotor activity showed a square wave with an activity peak occurring at the beginning of the dark phase, while before lights came on another increase was observed. This latter peak could constitute an anticipation of the light phase. When a 3.3 $\mu W/cm^2$ light pulse was applied at MD the nocturnal locomotor activity of fish dramatically dropped; however when the lights were switched off again tench resumed their activity until the photophase started (Fig. 5).

DISCUSSION

In the present study, we found rhythmic variations in plasma melatonin in tench under both high (L=1091 $\mu W/cm^2$) and low light intensities (L=58.6 $\mu W/cm^2$). Previous experiments carried out in our laboratory indicated the capacity of the tench pineal organ cultured *in vitro* to secrete melatonin, with high titters during the scotophase and low levels during the photophase (unpublished data). Plasma melatonin levels at ML

and MD in tench exposed to LD with high light intensity were close to those observed by other authors (Rebollar et al., 1999). However, differences between day and night plasma melatonin in animals under LD with low light intensity were lower than those obtained in fish exposed to LD with high light intensity. Previous studies have revealed that Atlantic salmon responded to moderate increases in dark phase illumination by varying the production of melatonin (Porter et al., 2001). In that study, Atlantic salmon were exposed to different light intensities during the dark phase (ambient, 1 lx, 20 lx, 100 lx, 400 lx), whereas during the photophase all animals were under the same natural light conditions, it being observed that the amplitude of melatonin rhythms decreased with increasing light intensity. Thus, while the duration of raised melatonin synthesis in fish is dictated by the prevailing photoperiod, the amplitude is thought to be influenced by other environmental factors such as light intensity (Gern and Greenhouse, 1988; Meyer and Millam, 1991) . Furthermore, in other vertebrates, e.g. the Japanese quail, dim light may be considered as day or as night, depending on the associated light intensity, its plasma melatonin levels being higher during the phase of lower light intensity and the amplitude of the rhythm significantly decreased compared to that observed in a normal LD cycle (Max and Menaker, 1992).

The level of melatonin synthesis is directly related to the level of ambient illumination (Zachmann et al., 1992; Meissl and Yáñez, 1996; Jarmak et al., 1998; Iigo et al., 2003), which may explain the lower amplitude of the melatonin rhythm in tench when daytime light intensity was low. Fish that were exposed to a higher light intensity showed higher differences in plasma melatonin levels at ML and MD. Recently, it has been concluded that melatonin may be used as an indicator of photoreception and melatonin production may be closely associated with light (Brainard et al., 1984).

The drop in plasma melatonin when a one hour light pulse was applied at MD is consistent with data obtained for other species. Brook trout (Zachmann et al., 1992) and sea bass (Bayarri et al., 2002) plasma melatonin shows great sensitivity to light, with light thresholds of about 20 lux and 10 lux, respectively. In tench, however, melatonin showed greater sensitivity to light, so that a light pulse intensity of 3.3 $\mu W/cm^2$ (0.3 lux) led to plasma melatonin dropping to levels close to those obtained at ML. Of note is the fact that this low intensity is near to that of full moon light, and it suggests that the pineal gland of this nocturnal species may be able to detect moonlight. Plasma melatonin could therefore show variations during the lunar cycle, which may synchronise the reproductive status of tench, as has been suggested in other species (Tarlow et al., 2003).

In addition to light intensity, the spectral composition of light has been reported to be important in suppressing melatonin production in several species. In sea bass, light of shorter wavelengths (blue) appeared to be more effective at suppressing melatonin production at night than that of longer wavelengths (red), although the latter may affect plasma melatonin over threshold intensities (Bayarri et al., 2002). In our study, this effect was minimized by using different light intensities while keeping the same spectral composition.

Previous studies revealed that activity rhythms in tench show an average nocturnal activity of over 97%. When the photoperiod was reversed (from LD 12:12 to DL 12:12) resynchronization to the new LD cycle was fast and nocturnal activity remained. Furthermore, under LD cycles of decreasing light intensities (L=300, 30, 3 and 0.3 lx) animals maintained activity adjusted to the dark phase (Herrero et al., 2003). Our present results indicate that under such a low intensity LD cycle, melatonin rhythms remained, thus transducing light information.

The change in the activity pattern of tench observed in exp. 3, when a light pulse was applied at MD, supports the above findings, as light had a strong masking effect on tench activity rhythms, blocking their expression (Herrero et al., 2003). This result suggested that tench exhibited a strictly nocturnal behaviour, even when animals were exposed to strong changes in light intensity and when the length of the dark phase was dramatically reduced, meaning that the circadian response in tench is strongly influence by light.

In conclusion, the main results of our research showed that tench exhibited a daily plasma melatonin rhythm under a LD cycle with low light intensity, while exposure to a one hour light pulse of different intensities at MD in the range 1091 to 3.3 $\mu W/cm^2$ (1300 to 0.3 lux) depressed plasma melatonin levels, which suggests that melatonin rhythms may be able to transduce weak light signals such as moonlight, and synchronise lunar rhythms.

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FIGURE LEGENDS

Figure 1. Spectral composition of the experimental lights (GRO-LUX, 40 W, Germany) with 1091 μW/cm^2 (continuous line) and 3.3 μW/cm^2 (dotted line). Spectral composition is expressed as irradiance (μW/cm^2) in the range of the visual spectrum. Irradiance was measured by spectroradiometer (Analytical Spectral Devices FieldSpec® Handheld).

Figure 2. Daily plasma melatonin rhythm in tench under a LD 12:12 cycle. Light intensity during the photophase was 58.6 μW/cm^2 and 0 μW/cm^2 during the scotophase. The white and black bars at the bottom indicate light and darkness, respectively. The values are the mean ± S.E.M. The different letters indicate statistically significant differences among sampling points (ANOVA, Duncan´s test, $P<0.05$). Numbers in brackets indicate the number of replicates per group.

Figure 3. Influence of 1 hour light pulse of increasing intensities (3.3, 5.3, 10.5, 1091 μW/cm^2) on plasma melatonin. Light pulses were applied at MD. ML and MD samples were used as controls. The values are the mean ± S.E.M. The different letters indicate statistically differences between groups (ANOVA, Duncan´s test, $P<0.05$). Numbers in brackets indicate the number of replicates per group.

Figure 4. Actogram from a representative tench before and after the application of a light pulse at MD. The abscisa represents the time of day, while the ordinate represents the days. Actogram is double-plotted for better visualization. The white and black bars at the top indicate light and darkness, respectively.

Figure 5. Average diel profile of locomotor activity of tench under 12:12 LD cycle (A) and during exposure to a 3.3 μW/cm^2 light pulse applied at MD (B). The white and black bars at the bottom indicate light and darkness, respectively. N=6.

FIGURE 1

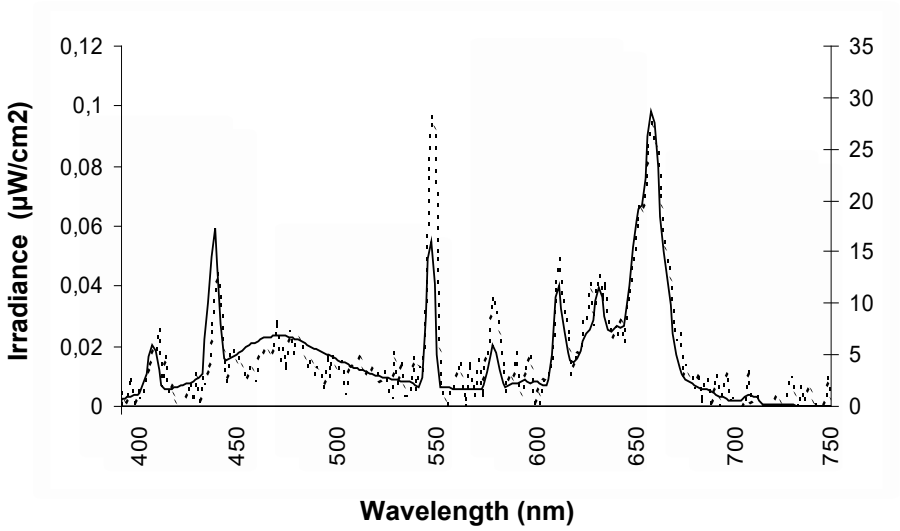

Irradiance (µW/cm2)
0,12
0,1
0,08
0,06
0,04
0,02
0
35
30
25
20
15
10
5
0
400
450
500
550
600
650
700
750
Wavelength (nm)

FIGURE 2

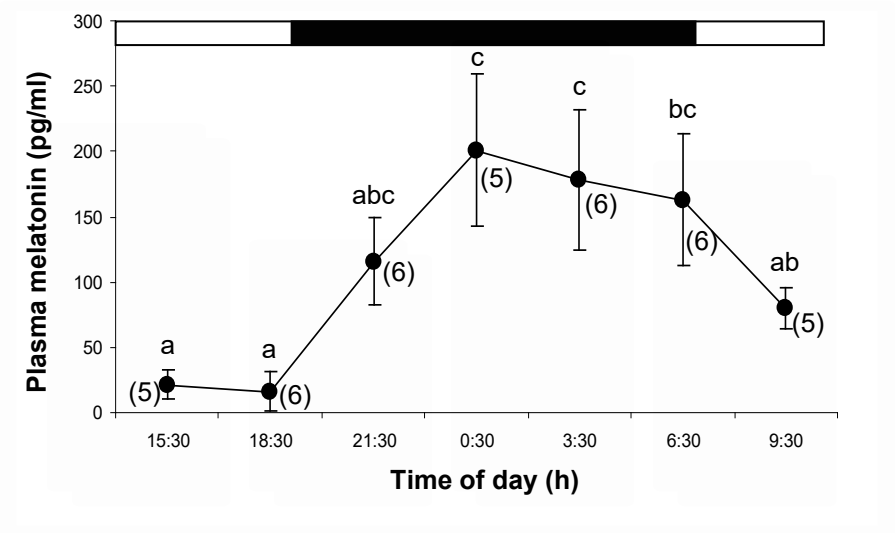

Plasma melatonin (pg/ml)
300
250
200
150
100
50
0
a
a
abc
c
c
bc
ab
(5)
(6)
(6)
(5)
(6)
(6)
(5)
15:30
18:30
21:30
0:30
3:30
6:30
9:30
Time of day (h)

FIGURE 3

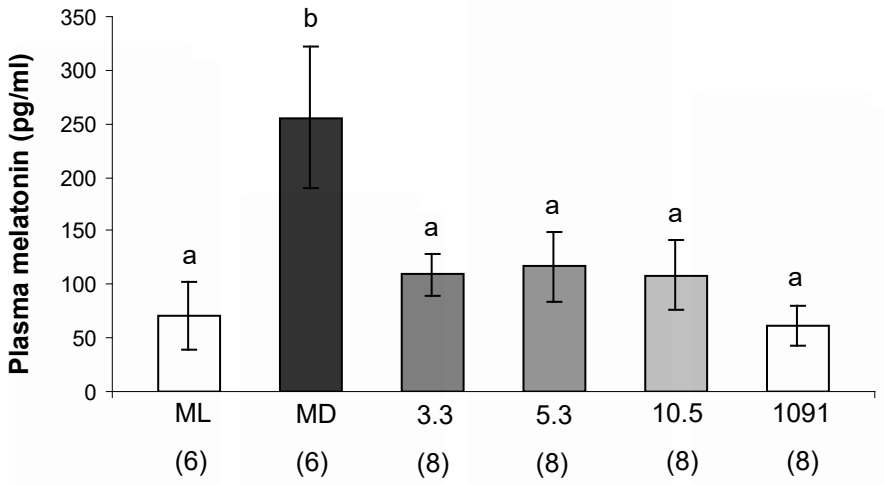

Plasma melatonin (pg/ml)
350
300
250
200
150
100
50
0
a
b
a
a
a
a
ML
(6)
MD
(6)
3.3
(8)
5.3
(8)
10.5
(8)
1091
(8)

FIGURE 4

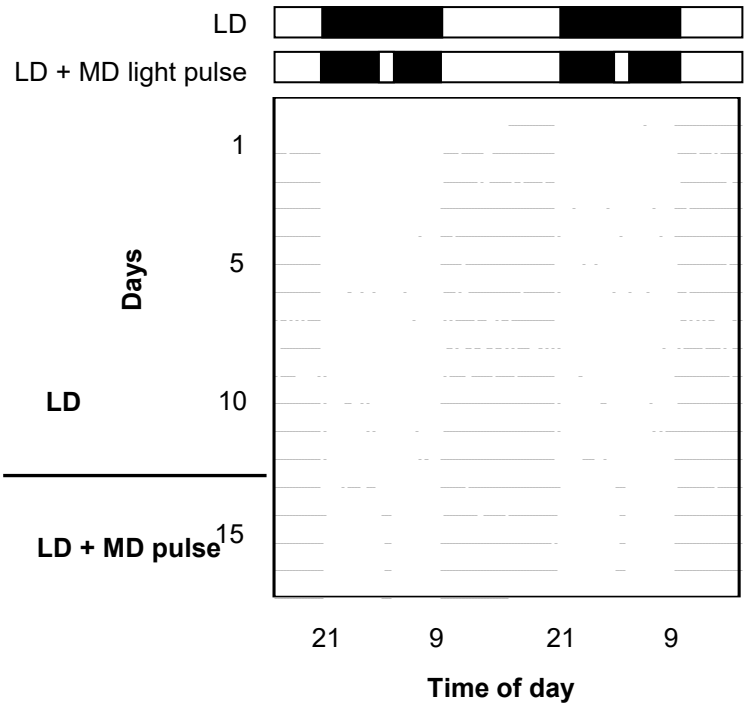
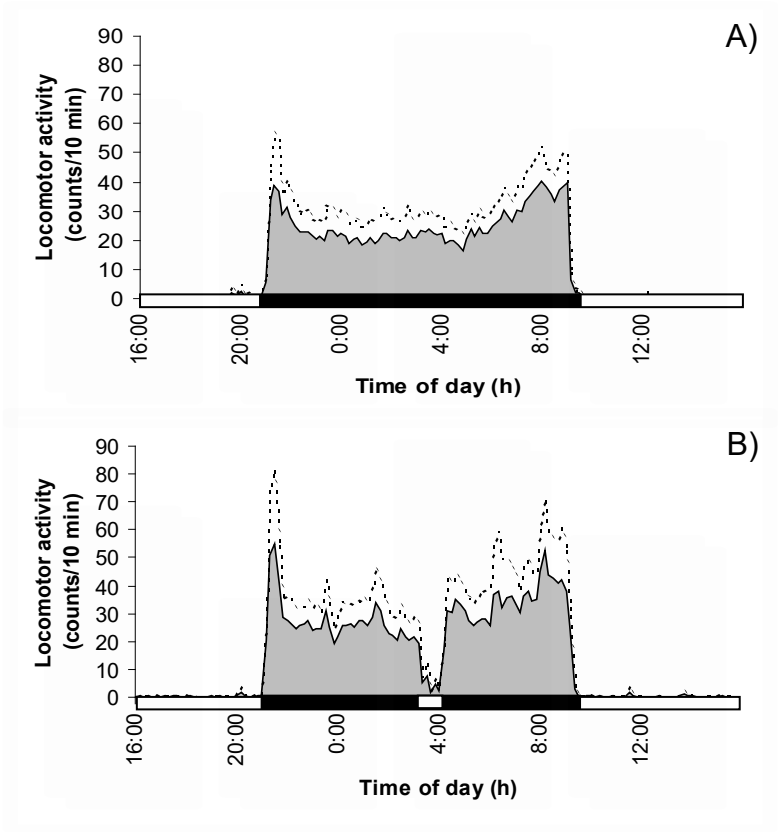

LD
LD + MD light pulse
1
5
10
15
Days
LD
LD + MD pulse
21
9
21
9
Time of day

FIGURE 5


A)
Locomotor activity (counts/10 min)
90
80
70
60
50
40
30
20
10
0
16:00
20:00
0:00
4:00
8:00
12:00
Time of day (h)
B)
Locomotor activity (counts/10 min)
90
80
70
60
50
40
30
20
10
0
16:00
20:00
0:00
4:00
8:00
12:00
Time of day (h)