CIRCADIAN RHYTHMS OF LOCOMOTOR ACTIVITY IN THE NILE TILAPIA *OREOCHROMIS NILOTICUS*

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ABSTRACT

The Nile tilapia behavioural rhythms were investigated to better characterize its circadian system. To do so, the locomotor activity patterns of both male and female tilapia reared under a 12: 12-h light-dark (LD) cycle were studied, as well as the existence of endogenous rhythmicity under free-running conditions (DD and 45-min LD pulses) in males. When exposed to an LD cycle, the daily pattern of activity differed between individuals: some fish were diurnal, some nocturnal and a few displayed an arrhythmic pattern. This variability would be typical of the plastic circadian system of fish and reproductive events clearly affected the behavioural rhythms of female tilapia, a mouthbrooder teleost species. Under DD, 50% (6 out of 12) of male fish showed circadian rhythms with an average period (*tau*) of 24.1 ± 0.2 h whereas under the 45-min LD pulses 58% (7 out of 12) of fish exhibited free-running activity rhythms and *tau* was 23.9 ± 0.5 h. However, interestingly in this case activity was always confined to the dark phase. Furthermore, when the LD cycle was reversed a third of the experimental fish showed gradual resynchronization to the new phase, taking 7-10 days to be completely re-entrained. Taken altogether these results suggest the existence of an endogenous circadian oscillator that controls the expression of locomotor activity rhythms in the Nile tilapia, although its anatomical localization remains unknown.

Keywords: Circadian rhythms, locomotor activity, Nile tilapia, photoperiod.

1. INTRODUCTION

The geophysical cycles created by the movements of Earth are responsible for the recurrent changes in daily light-dark (LD) cycles experienced by living organisms. Under such cycles the animals have developed behavioural and physiological mechanisms to anticipate predictable changes, optimizing biological processes (Daan, 1981; Godman, 2001; Kumar, 1997). Environmental changes act as synchronizers, which,entrain biological rhythms in their periodicity, amplitude and phase (Aschoff, 1981; Rensing & Ruoff, 2002). As previously suggested, several conditions have to be met for an environmental factor to be considered as a real synchronizer (zeitgeber): once the animal is exposed to the external factor there must be a stable phase relationship between the rhythm and the zeitgeber and once the latter is suppressed the biological rhythm should free-run from the phase previously determined by the zeitgeber (Moore-Ede *et al.*, 1982). In vertebrates, biological rhythms have been classified according to their periodicity (ultradian, circadian, infradian). However, to date work has mainly focused on rhythms related to daily environmental changes (i.e. circadian rhythms).

Under an LD cycle fish show daily patterns of locomotor activity that can be classified into several types: diurnal, nocturnal, crepuscular and combination of them (Eriksson, 1978; Herrero *et al.*, 2003; Schulz and Leuchtenberger, 2006). However, in some teleosts species the characterisation of the daily pattern of activity is not straightforward. Thus, different individuals within the same species can show a great variability in their daily activity patterns (Helfman, 1993).

To investigate the existence of endogenous control of the behavioural rhythms in fish, constant environmental conditions are usually used (e.g. constant darkness, DD or constant illumination LL) or ultradian pulses (very short light-dark photocycles)

(Eriksson and van Veen, 1980; Sánchez-Vázquez *et al*., 1995a; Sánchez-Vázquez *et al.*, 1996; Reebs, 2002). Animals exposed to such conditions lose external entrainment and their circadian rhythms can free-run, with an approximate period (*tau*) of 24 hours in the case of circadian rhythms (Aschoff, 1981; Edmunds, 1988). Such rhythms are self-sustained during at least some days after the animal is separated from its external zeitgeber, thus indicating its endogenous control (Aschoff, 1960; Iigo and Tabata, 1996; Sánchez-Vázquez *et al.*, 1995a). In mammals these circadian rhythms can be sustained under constant conditions for up to a month, whereas in teleost fish they usually disappear after a few days (Iigo and Tabata, 1996; Naruse and Oishi, 1994; Nishi, 1990). The demonstration of endogenous timing mechanisms can also be tested by phase-shifting the zeitgeber and monitor the display of transient cycles of activity (Reebs, 2002).

The Nile tilapia (*Oreochromis niloticus*) is a tropical freshwater species that inhabits a variety of shallow freshwater environments and is one of the most commercially important species for the world wide aquaculture industry (El-Sayed & Kawanna, 2007). Previous investigations have proposed that this species could be endowed with a circadian system in which the pineal gland would not be light sensitive and might lack an independent circadian pacemaker, as the daily melatonin rhythm was abolished after bilateral ophtalmectomy (Migaud *et al.*, 2007). However, the regulatory mechanism of behavioural daily rhythms in tilapia has not been yet investigated.

Due to the reproductive strategy of Nile tilapia (mouth-breeding), this study mainly focused on male fish to avoid spawning to interfere with activity rhythms. The objectives of this work were 1) to characterize the daily locomotor rhythms of male tilapia reared under an LD cycle, 2) to elucidate the existence of endogenous control of male tilapia activity rhythms by investigating circadian rhythmicity under constant

photic conditions (DD) or ultradian pulses and studying resynchronization after phase-shifting the LD cycle and 3) to confirm the influence of spawning on the daily locomotor rhythms in female tilapia.

2. MATERIALS AND METHODS

2.1 Animals and housing

The present study was carried out in the tropical fresh-water aquarium of the Institute of Aquaculture at the University of Stirling (UK). A total of 12 males and 11 females Nile tilapia from the same stock (15 months old, 150g mean body weight) red strain bred in the facilities were housed individually in rectangular 114 L glass tanks (54cm x 46cm x 46cm) within light proof compartments. Each tank was provided with a constant flow of well aerated freshwater and the temperature of the water was kept constant during the experiment at 27 ºC.

Animals were acclimatized to a 12:12 h LD cycle for two weeks before the start of the experiments. Lighting was provided by a 60 W pearl bulb (Lampways Triple Plus, UK) placed above tanks. Light intensity during the photophase was 0.6 $W \cdot cm^{-2}$ (equivalent to approximately 100 lux) and photoperiod was controlled by a timer.

All experiments were carried out in accordance with the Animal (Scientific Procedures) Act 1986 UK under the approval of the local ethical review board and in accordance with the international ethical standards of the journal (Portaluppi et al., 2008).

2.2. Activity monitoring

To record the locomotor activity each aquarium was equipped with one infrared photocell (E3Z-D67, OMRON, China) placed in the centre of the front glass wall. Previously, the position of the photocell had been optimised. Photocells were connected to a motherboard (USB-1024HLS, Measurement Computing, USA) plugged to a

computer. Every time a fish interrupted the infrared light beam it produced an output signal that was recorded and stored in 10 minute bins using a specialized software (DIO98USB, University of Murcia, Spain).

2.3. Experimental design

In a first experiment 12 male tilapia were reared under a 12:12 h LD cycle for 2 weeks to characterize their daily activity rhythms before exposing them to DD conditions for 3 weeks in order to study circadian rhythmicity. Thereafter, fish were resynchronized to the LD cycle for 2 weeks and exposed to an ultradian LD cycle (45:45 min LD) for 2 additional weeks. Subsequently, the 12:12 h LD was restored again for 2 weeks and finally reversed (lights on at 20:00 h and off at 08:00 h) to characterize the resynchronization to the new photocycle phase.

In a second experimental phase 11 female tilapia were exposed to a 12:12 h LD cycle for 3 weeks, with lights on at 08:00 h and off at 20:00 h. Throughout the experiment spawning events were recorded to determine their potential effects on locomotor activity.

In both experiments, fish were fed at random times during the photophase every 2 days to avoid feeding from acting as a potential synchronizer.

2.4. Data analysis

Locomotor activity records were stored in a computer and analyzed using a software package for chronobiological studies (El Temps®, Prof. A. Díez-Noguera, University of Barcelona). In order to define whether individual fish were diurnal, nocturnal or arrythmic during the LD phase, statistical differences between mean diurnal and nocturnal activity were analyzed by t-Student test using Microsoft Excel (statistically significant threshold of $P<0.05$). When no statistical difference between diurnal and nocturnal activities was observed for a given fish, it was classified as

arrhythmic. When a statistical difference was found the fish was either defined as diurnal or nocturnal depending on when locomotor activity occurred the most (day or night). The period length (*tau*) of free-running rhythms was determined by Sokolove-Bushell periodogram analysis at a confidence level of 95%, for which El Temps was also used.

3. RESULTS

3.1. Daily and circadian rhythms of locomotor activity in male tilapia

Male tilapia showed inter-individual differences when kept under an LD cycle (Table I): 4 out of 12 fish were mostly diurnal (Fig. 1A), with an average locomotor activity of 70.3 ± 7.4 % occurring during the photophase, 5 were mainly nocturnal (Fig. 1B), with an average activity of 64.4 ± 4.5 % displayed during the scotophase and 3 were defined as arrhythmic (Fig. 1C).

Under both DD and ultradian pulses the appearance of free-running rhythms differed between fish, some of them showed self-sustained rhythms once the external 12:12 h LD cycle was removed whereas others were arrhythmic during the first few days and then started to show endogenous activity rhythms.

Under DD, 6 out of 12 male tilapia exhibited self-sustained circadian rhythms with locomotor activity free-running with an average *tau* of 24.1 ± 0.2 h, ranging from 23.5 to 25.2 h (Table II). Figure 2 shows two representative fish with locomotor free-running rhythms longer than 24 h. There was no prevalence of fish with diurnal or nocturnal activity under an LD cycle displaying rhythmicity during the DD phase (2 were diurnal, 3 nocturnal and 1 arrythmic).

When fish were exposed to 45-min LD pulses, 7 out of 12 male tilapia showed circadian rhythmicity. Locomotor activity free ran with an average *tau* of 23.9 ± 0.5 h,

ranging from 21.0 to 24.7 h (Table II). Locomotor activity from two experimental fish is presented in Fig. 3. The activity rhythms of the first fish started to free-run 6 days after the LD cycle was removed and showed circadian rhythms with *tau* shorter than 24 h (Fig. 3A). On the contrary, locomotor activity of the second fish free-ran once the fish was exposed to the 45-min LD pulses and *tau* was longer than 24 h (Fig. 3B). Underlying the free-running rhythms, when ultradian pulses were applied, locomotor activity of tilapia was mostly confined to the dark phase (Fig.4). Interestingly, this was observed in both diurnal and nocturnal tilapia, when reared under a 12:12 h LD cycle.

Finally, the daily LD cycle was reversed in the last experimental phase to investigate how fish resynchronized after the zeitgeber phase-shifting. Three out of 12 fish changed immediately the phase of their locomotor activity rhythm, so that it coincided with the new phase of the photocycle (Fig. 5A). However, 4 experimental tilapia showed a gradual resynchronization to the new phase, as transient cycles of activity were observed. The day following the shift from LD to DL, fish were active at the same time as before the phase-shifting but day after day they started to be active earlier and slowly resynchronized to the new phase (Fig. 5B). Other tilapia did not resynchronize at all, e.g. one diurnal individual became arrhythmic after the LD cycle was reversed but after a few days was active during the same phase as before the shifting, although then it corresponded to the scotophase (Fig. 5C).

3.2. Effect of spawning on the daily rhythms of locomotor activity in female Nile tilapia

When female tilapia were reared under an LD cycle, the daily distribution of activity showed high variability among individuals: 5 out of 11 fish displayed a mostly diurnal locomotor activity pattern (with 74.4 ± 6.3 % of their activity occurring during the photophase), 1 tilapia showed nocturnal rhythmicity (68.0% of activity displayed at

night) and 5 fish were arrhythmic throughout the experiment (data not shown). Ten out of eleven individuals spawned during the experiment, four of them twice. The reproductive activity clearly affected the daily locomotor rhythm in nine of the experimental fish. Among the four individuals which displayed activity rhythms (either diurnal or nocturnal), two showed a reduction in activity from the egg incubation stage until few days later (Fig. 6A), one reduced its activity prior spawning and then became arrhythmic (Fig. 6B) and the last one showed both a reduction in activity and arrhythmicity from 3-4 days prior spawning and during egg incubation. All remaining individuals (5), which were initially arrhythmic, remained so and showed a reduction of activity during and several days following egg incubation.

4. DISCUSSION

This study provides interesting preliminary results on locomotor activity rhythms in the Nile tilapia (*O. niloticus*).

Male and female tilapia showed large inter-individual variations of daily activity distribution when reared under an LD cycle. From a total of 23 fish used in our experiments, 9 were mostly diurnal, 6 nocturnal and 8 were arrhythmic. In males, 9 out of 12 fish exhibited daily rhythms of locomotor activity (45% of which were diurnal) whereas in females only 6 out of 11 fish showed rhythmicity in their activity (83% were diurnal). The present results are in contrast to the general knowledge that has defined the Nile tilapia as a diurnal species and differences between males and females could either be sex-dependent or due to inter-individual variations.

The variability of behavioural patterns observed in the present study is in accordance with previously published data obtained in other fish species. Indeed, when goldfish (*Carassius auratus*) were exposed to an LD 12:12 h cycle, the daily rhythm of

locomotor activity also differed between individual fish: some were diurnal and others were nocturnal (Sánchez-Vázquez *et al.*, 1996). Furthermore, sea bass (*Dicentrarchus labrax*) showing diurnal feeding rhythms could be turned into nocturnal by restricting food availability to the night period (Sánchez-Vázquez *et al.*, 1995a). In sharpsnout seabream (*Diplodus puntazzo*) spontaneous shifts from diurnal to nocturnal patterns of locomotor activity and *vice versa* were reported, as well as the existence of phase independence between locomotor and feeding rhythms (Vera *et al.*, 2006). In contrast, other teleost species show clear daily rhythms of locomotor activity, such as the zebrafish and the tropical fish *Halichoeres chrysus* which mostly confine their activity to the photophase under an LD cycle (Gerkema *et al.*, 2000; Hurd *et al.*, 1998) whereas the tench (*Tinca tinca*) shows a strictly nocturnal pattern, even under extremely short photoperiods (e.g. LD 22: 2 h) (Herrero *et al.*, 2003). Therefore, it has been generally accepted that activity patterns in fish show a strong plasticity (Ali, 1992; Madrid *et al.*, 2001; Reebs, 2002). Freshwater teleosts have been proposed to have a more flexible circadian system than marine fish, probably due to the relative instability of their environment (Reebs, 2002). Indeed, in the current study, large inter-individual variability of the tilipia behavioural patterns was observed. This could suggest that the circadian clocks in tilapia might not be as robust as seen in other vertebrate species, since they do not always maintain activity to a particular phase of the LD cycle (Gallistel, 1990; Reebs, 2002). Interestingly, the fact that some fish showed nocturnal locomotor activity rhythms raises the question whether these individuals would feed during the night if they were given the chance. The study of daily feeding rhythms in this species would be certainly an interesting subject to pursue.

Circadian rhythmicity in locomotor activity of tilapia reared under DD and ultradian light pulses was observed in our study, suggesting the existence of a circadian

clock in this species that regulates its activity. Under DD, 50% of the fish exhibited self-sustained activity rhythms and 58% under a 45:45 min LD cycle. In tench 41% of experimental fish showed circadian rhythmicity under DD (Herrero *et al.*, 2003) and in sharpsnout sea bream only a third of the individuals did (Vera *et al.*, 2006). In mammals, however, the ratio of animals showing circadian rhythmicity under constant conditions is usually higher and the self-sustained rhythms can persist longer (Benstaali *et al.*, 2001). Some tilapia showed free-running rhythms immediately after the removal of the LD 12:12 h cycle whereas others were arrhythmic during the first few days under constant photic conditions and then showed free running locomotor activity. This was also observed in zebrafish when transferred from a daily LD cycle to DD (Hurd *et al.*, 1998). The period length also differed among individuals and although some fish showed circadian rhythms both under DD and 45:45 min LD others only did under one of the two experimental conditions. Similar results were obtained when goldfish and zebrafish circadian activity rhythms were studied (Iigo and Tabata, 1996; Hurd *et al.*, 1998), supporting that the circadian system of teleosts could be considered as a multiphotoreceptor and multioscillator system and that the coupling between the different oscillators may differ between individuals within the same species depending on the physiological and environmental conditions (Iigo *et al.*, 1994; Kavaliers, 1980; Kavaliers, 1981; Pittendrigh, 1974; Tabata, 1992; Underwood, 1994).

Interestingly, when tilapia were exposed to ultradian light pulses we observed that underlying to the expression of circadian rhythms the locomotor activity was confined to the dark phase of the 45:45 min LD cycle, in both fish displaying a diurnal and nocturnal pattern during the 12:12 h LD cycle. This could be due to the splitting of the circadian rhythm into two components, so that the unimodal pattern would become bimodal (Earnest, 1982; Pittendrigh, 1974). However, the existence of negative light

masking could also explain this phenomenon (Mrosovsky, 1999). Masking involves the passive influence of the environmental factors on the behavioural patterns without affecting the endogenous oscillators (Aschoff, 1981) and its effect was observed in goldfish locomotor and feeding rhythms when exposed to ultradian pulses (Sánchez-Vázquez *et al.*, 1996). Another way to investigate the existence of a circadian clock is to phase-shift the zeitgeber. In the present study when the LD cycle was suddenly reversed to DL a third of the experimental fish showed a gradual re-entrainment to the new phase, taking 7-10 days to resynchronize completely. This has also been reported in the hagfish *Eptatretus burger* (Ooka-Souda *et al.*, 1985) and golden shiners *Notemigonus crysoleucas* (Laguë and Reebs, 2000) although most of fish species studied to date usually displayed only one day of transient activity (Godin, 1981; Nelson and Jhonson, 1970; Sánchez-Vázquez *et al.*, 1995a; Tabata *et al.*, 1989).

Previous studies have reported that tilapia might lack an independent circadian pacemaker as after bilateral ophtalmectomy the day-night melatonin rhythm was suppressed (Migaud *et al.*, 2007) although when fish were exposed to DD, a strong circadian melatonin rhythm was maintained for 18 days (Martínez-Chávez *et al.*, 2008), suggesting the existence of endogenous oscillators in this species. Our study supported this hypothesis as endogenous activity rhythms were observed under constant conditions (DD) and ultradian pulses, as well as the existence of gradual resynchronization after phase-shifting the LD cycle.

Finally, a clear effect of spawning was observed on the daily rhythms of activity in female tilapia, characterised by an important reduction of activity before, during and/or following spawning and causing arrhythmicity in some cases. Tilapia is a mouthbrooder species able to spawn multiple times throughout the year. When females do not have males to fertilise their eggs they hold the unfertilised eggs in their mouth

for 1-2 days and then spit them out (El-Sayed, 2006). Previous investigations in cardinalfish (*Apogon fragilis* and *Apogon leptachanthus*) suggested that mouthbrooding can increase the rate of oxygen consumption, reduced the ability of fish to take up oxygen and impaired the capacity for sustained aerobic swimming (Östlund-Nilsson and Nilsson, 2004). These results could explain the change of activity patterns observed in female tilapia from the present study. The fact that reproductive events also caused arrhythmicity in some tilapia a few days before and after mouthbrooding agrees with previous observations in other species that during the spawning season activity is displayed throughout the day (Helfman, 1981).

To conclude, the present study suggests that tilapia is endowed with a flexible circadian system, which endogenously controls the expression of behavioural rhythmic patterns, although further investigations will be needed to elucidate the mechanisms at work.

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Table I: Percentage of activity displayed during the photophase by male tilapia under a 12:12 h LD cycle and ultradian pulses. D: diurnal; N: nocturnal; A: arrhythmic.

Fish	% Activity during photophase (12:12 h LD)	% Activity during photophase (45:45 min LD)
1	58; D	26
2	83; D	21
3	21; N	18
4	45; A	19
5	57; D	42
6	42; N	23
7	43; N	48
8	42; N	22
9	63; A	36
10	48; A	22
11	28; N	18
12	83; D	18

Table II: Free-runnig rhythms period (*Tau*) in male tilapia under DD and ultradian pulses. NS: non significative.

Fish	*Tau* under DD (h)	*Tau* under ultradian pulses (h)
1	NS	24.4
2	23.8	NS
3	24.8	25.2
4	24.5	21.0
5	24.2	24.5
6	NS	24.7
7	23.8	NS
8	NS	NS
9	NS	NS
10	NS	NS
11	23.5	23.5
12	NS	24.2

FIGURE LEGENDS:

Figure 1: Average diel profile of locomotor activity from three male tilapia reared under a 12: 12 h LD cycle, showing a diurnal (A), nocturnal (B) and arrhythmic (C) pattern. The white and black bars at the top of each graph indicate the light and dark periods, respectively. Data represent the mean (continuous line) + S.E.M. (dotted line) of one fish during two weeks.

Figure 2: Locomotor actograms (upper graphs) and their corresponding periodogram analysis (down) from two male tilapia subjected to DD conditions. The period of the free-running rhythm (*tau*) is indicated above the periodograms. Actograms are double-plotted for better visualization.

Figure 3: Locomotor actograms (left graphs) and their corresponding periodogram analysis (right) from two male tilapia exposed to ultradian light pulses (45: 45 min LD). The period of the free-running rhythm (*tau*) is indicated above the periodograms.

Figure 4: Average diel profile of tilapia locomotor activity under a 45-min LD cycle. Data represent the mean (continuous line) + S.E.M. (dotted line) of twelve male tilapia during two weeks.

Figure 5: Locomotor actograms from three male tilapia exposed to different subsequent photoperiods along the experiment: 12:12 h LD, DD, 45:45 min LD and DL.

Figure 6: Locomotor actograms (left) and average diel profile activity (right) under a 12:12 h LD cycle from a nocturnal (A) and a diurnal (B) female tilapia showing spawning events during the experiment. Waveforms represent the mean (continuous lines) + S.E.M. (dotted lines) of one fish during the experiment.

Figure 1

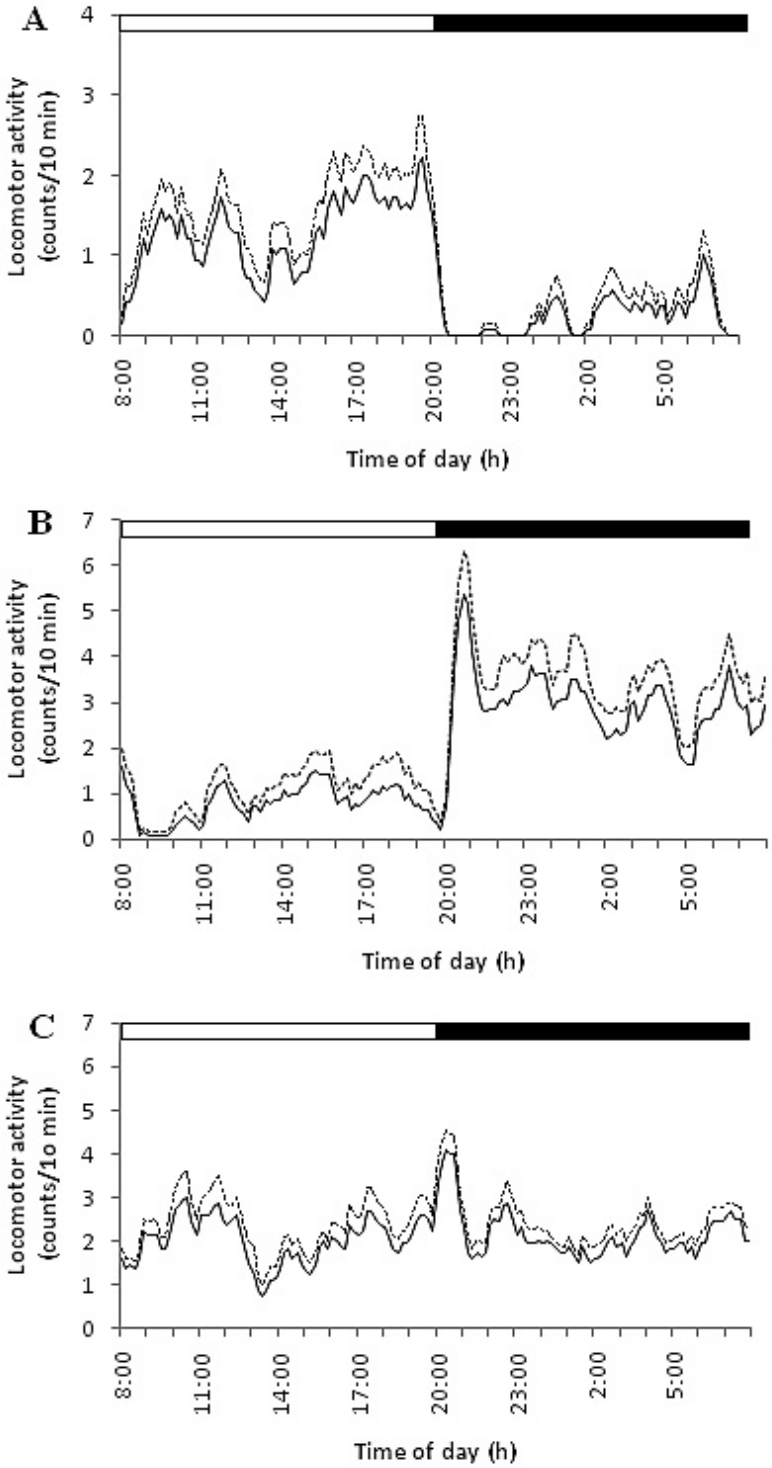

A
Locomotor activity (counts/10 min)
0
1
2
3
4
8:00
11:00
14:00
17:00
20:00
23:00
2:00
5:00
Time of day (h)
B
Locomotor activity (counts/10 min)
0
1
2
3
4
5
6
7
8:00
11:00
14:00
17:00
20:00
23:00
2:00
5:00
Time of day (h)
C
Locomotor activity (counts/1o min)
0
1
2
3
4
5
6
7
8:00
11:00
14:00
17:00
20:00
23:00
2:00
5:00
Time of day (h)

Figure 2

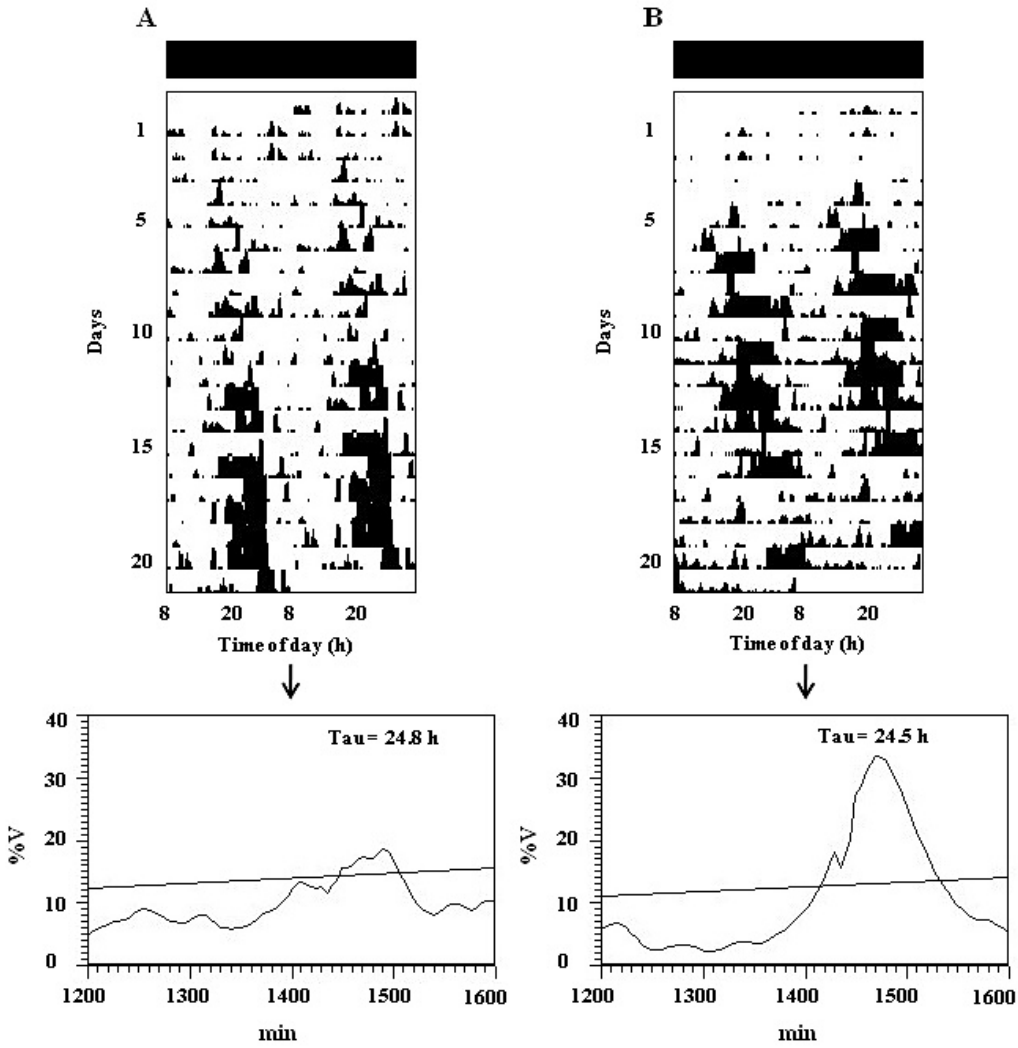

A
B
Days
1
5
10
15
20
8
20
8
20
Time of day (h)
Tau = 24.8 h
Tau = 24.5 h
%V
0
10
20
30
40
1200
1300
1400
1500
1600
min

Figure 3

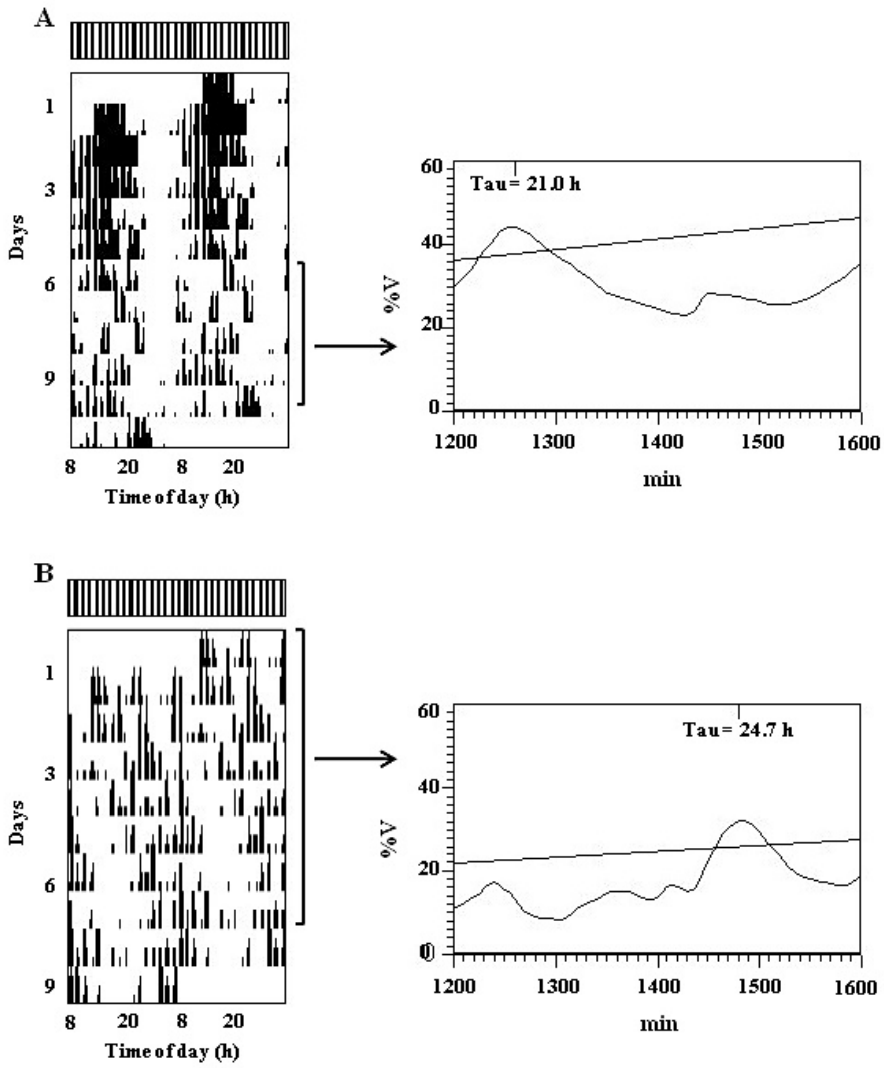

A
1
3
6
9
Days
8
20
8
20
Time of day (h)
60
40
20
0
%V
Tau = 21.0 h
1200
1300
1400
1500
1600
min
B
1
3
6
9
Days
8
20
8
20
Time of day (h)
60
40
20
0
%V
Tau = 24.7 h
1200
1300
1400
1500
1600
min

Figure 4

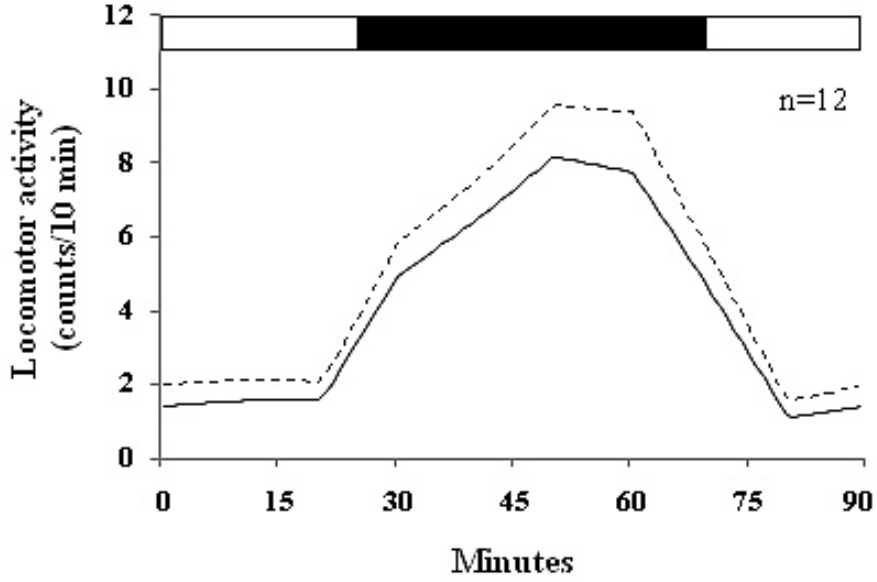

12
10
8
6
4
2
0
n=12
Locomotor activity
(counts/10 min)
0
15
30
45
60
75
90
Minutes

Figure 5

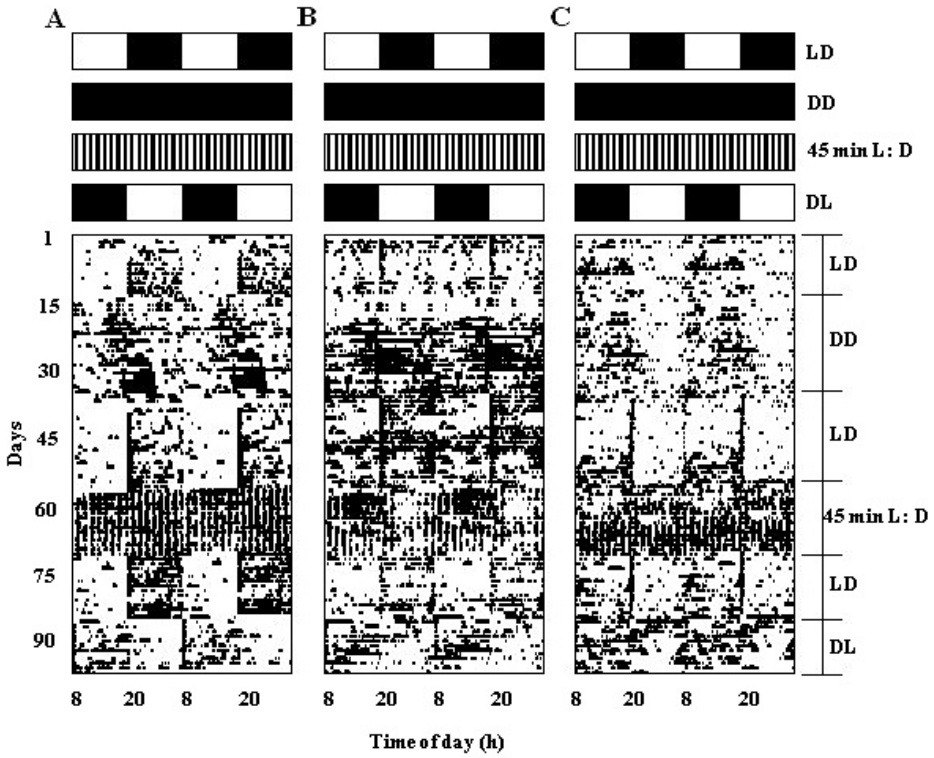
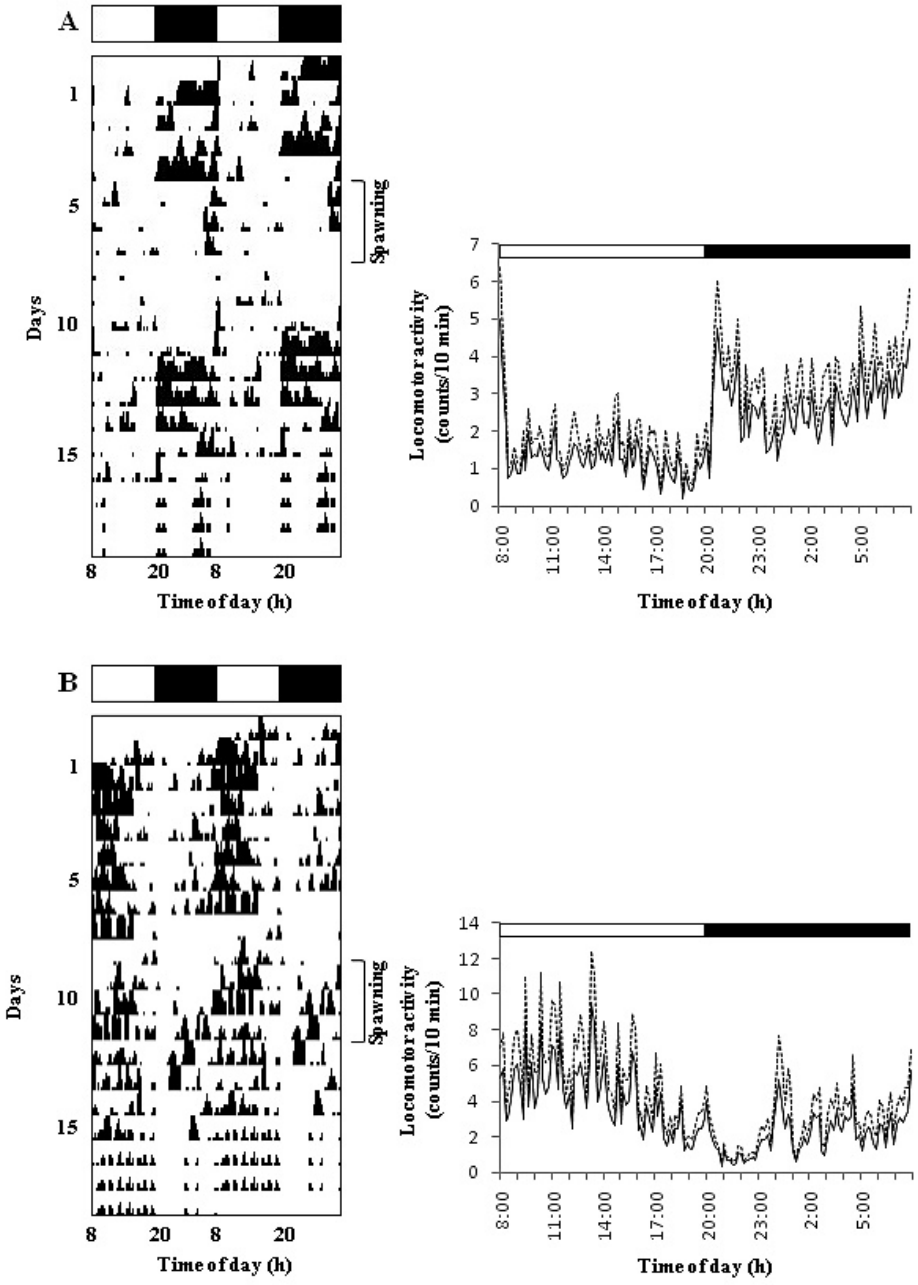

A
B
C
LD
DD
45 min L: D
DL
1
15
30
45
60
75
90
Days
LD
DD
LD
45 min L: D
LD
DL
8
20
8
20
8
20
8
20
8
20
8
20
Time of day (h)

Figure 6


A
1
5
10
15
Days
Spawning
8
20
8
20
Time of day (h)
Locomotor activity (counts/10 min)
0
1
2
3
4
5
6
7
8:00
11:00
14:00
17:00
20:00
23:00
2:00
5:00
Time of day (h)
B
1
5
10
15
Days
Spawning
8
20
8
20
Time of day (h)
Locomotor activity (counts/10 min)
0
2
4
6
8
10
12
14
8:00
11:00
14:00
17:00
20:00
23:00
2:00
5:00
Time of day (h)