EFFECT OF PINEALECTOMY OR ENUCLEATION FOLLOWED BY EXOGENOUS MELATONIN TREATMENT ON AIR-BREATHING ACTIVITY RHYTHM IN *CLARIAS BATRACHUS*

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Abstract

It has been aimed to examine the effect of pinealectomy or enucleation followed by exogenous melatonin treatment on air-breathing activity rhythm in Indian freshwater catfish, *Clarias batrachus*. Following 7 days of acclimation, animals (n=16) were randomly selected from the stock aquaria and were kept in specially designed glass aquaria inside the chronocubicles exposed under L: D 12:12 (lights on at 0600 hrs). Light schedule was maintained with the help of automatic timers. All the animals remain intact (IG) for first 7 days and then divided into two groups. In the first group (n=08) bilateral ocular enucleation was performed and were left for next 21 days. In the second group (n=08) pinealectomy was performed and were left for next 10 days. Then the individuals of both the groups received daily late afternoon treatment (17:00-18:00hrs) of melatonin (2µg/fish) for next 10 days. The air–breathing activity was recorded with the help of infrared photoswitches connected to a microprocessor based event recorder. The results obtained by actograms revealed that maximum no. of individuals remained entrained to the LD cycle in both intact and pinealectomized group. After melatonin treatment in the same group, the actograms depicted that air-breathing activity in five individuals was entrained atleast with the lights off time and, the greatest part of their activity was noticed during light phase of the LD regime. Further, a statistically significant 24-h rhythm in air-breathing activity was also detected in intact, pinealectomization and melatonin treatment. In the other group, all the enucleated *Clarias batrachus* were desynchronized to the LD cycle. The acrophases of the activity were located during the photophase of the LD regime in 5 out of 8 individuals. Further, 3 out of eight enucleated melatonin receiving *Clarias batrachus* apparently entrained to lights off time. In addition, 5 (Fish # 09,10, 11, 13, 15) out of 8 *Clarias batrachus* retained a statistically significant 24-h rhythm in their air-breathing activity and period (τ) of activity was prominent 24-h in Fish # 09, 11, 13, 14.

It could be concluded that in *Clarias batrachus* eyes play an important role in regulating the air-breathing activity rhythm. However, pinealectomy has been failed to modulate such rhythm under LD 12:12 regime. In addition, exogenous melatonin may cause modulation in air-breathing activity rhythm in *Clarias batrachus.*

Keywords: Pineal, blinding, catfish, circadian rhythm, enucleation, pinealectomy, air-breathing behaviour.

Introduction

A common feature of vertebrate physiology is a circadian rhythm in circulating melatonin, characterized by high levels during the night and low levels during the day (Arendt, 1995; Collin *et* *al.*, 1989). The melatonin rhythm is thought to synchronize other circadian rhythms and to modulate photoperiodic regulation of seasonal physiological rhythms (Arendt, 1995).

Melatonin is produced during the night at two major sites. One is the pineal gland, the source of circulating melatonin (Arendt, 1995; Vanecek, 1998). Circulating melatonin plays an endocrine role in seasonal and circadian physiology. The second site of melatonin synthesis is retinal photoreceptor cells, where melatonin is thought to play a paracrine role in adaptation to light and darkness (Gothilf et al., 1999).

In lower vertebrates including fishes, the pineal acts as a photo and thermoendocrine transducer which functions to synchronize internal cycle with cycles in the environment. (Underwood,1989). In teleost fish, complete melatonin rhythm generating systems, including photodetector, circadian clock and melatonin synthesis machinery, are located within individual photoreceptor cells in two sites: the pineal organ and retina. In both, light regulates daily variations in melatonin secretion by controlling the activity of arylalkylamine *N*-acetyltransferase (AANAT). (Falcon *et al*., 2003)

This study is aimed to examine the effect of pinealectomy or enucleation followed by exogenous melatonin treatment on air-breathing activity rhythm in Indian freshwater catfish, *Clarias batrachus* maintained at LD 12:12.

Materials and Methods:

Collection and care of animals

Live *Clarias batrachus* of mixed sex (40-50 g body weight) were procured locally and kept in the stock aquaria under the laboratory conditions (10 days) for proper acclimation. During the period of acclimation, water inside the aquaria was renewed every alternate day. Fishes were fed pieces of small dry fishes locally available in the market *ad libitum*. (Sahu, 2008, 2019,2020).

Surgery

Pinealectomy:

In *Clarias batrachus* the pineal lies inside a shallow concavity called pineal window, situated on the dorsal surface of the skull, covered by a thin translucent skin having sparse melonophores (Please see review Shedpure and Pati, 1995). The fish was anesthetized by keeping it on ice tray and then pineal was exposed by cutting the skin flap over the pineal window from three sides and folding it anteriorly. Thereafter, the pineal was removed by using surgical forceps under the binocular microscope (Shedpure and Pati, 1996).

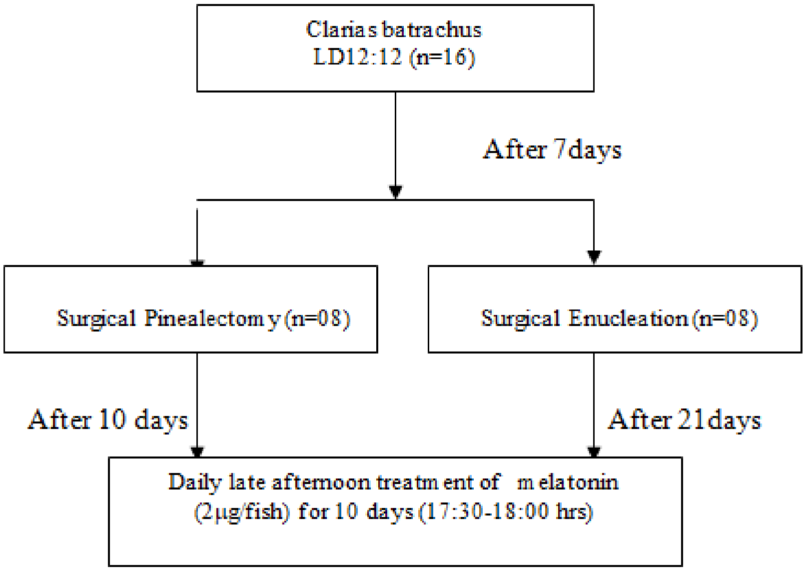
Enucleation:

The bilateral ocular enucleation was performed with the help of fine surgical instrument. After anesthetizing the animals by keeping them in ice-tray, an incision was made around the eyeball. The eyeball was pulled out carefully from the socket and the connections were cut with scissors (Chiba *et al*., 1993; Khan and Joy, 1990).

Experimental design

Following 7 days of acclimation, animals (n=16) were randomly selected from the stock aquaria and were kept in specially designed glass aquaria inside the chronocubicles exposed under L:D 12:12 (lights on at 0600 hrs). All the animals remain intact (IG) for first 7 days and then divided into two groups. In the first group (n=08) bilateral ocular enucleation was performed and were left for next 21 days. In the second group (n=08) pinealectomy was performed and were left for next 10 days. Then the individuals of both the group received daily late afternoon treatment (17:00-18:00hrs) of melatonin (2µg/fish) for next 10 days (Sahu, 2008, 2019, 2020).

The experimental protocol was as follows:



Activity recording and data collection

Each animal was kept in a specially designed glass aquaria inside the chronocubicles for recording of air-gulping/breathing activity. A porous plate was fixed just below the surface of water with a single large space, available in the centre from where the fish can come to gulp the atmospheric air. The infrared photoswitches were fixed in such way that a beam of infrared ray lies in the middle of this space. Air-gulping/breathing activity was monitored and recorded by using a PC-based event recorder (Vision Automation, Pune). This device employs the principal of IR beam interruptions to record air-gulping/breathing activity effectively. Whenever fish came to the surface for gulping air it intrupted the infrared beam. This signal was amplified and recorded by a microprocessor based event recorder. The two types of data files were prepared, viz; graphical (for obtaining actograms) and numerical (to obtain number of gulps per hour for statistical analysis) with the help of such event recorder. In addition, hourly recording of climatic variables such as water temperature, room temperature and humidity was also done simultaneously on daily time scale. Light schedule was maintained with the help of automatic timers (Sahu, 2008, 2019, 2020).

Statistical analysis

The data, expressed in number of gulps/hour and were subjected to single cosinor method at τ = 24hr (Nelson *et al*., 1979). A rhythm was characterized by three parameters, viz., the mesor (M, rhythm adjusted mean), the amplitude (A, half of the difference between the minimum and maximum of the best fitting cosine function) and the acrophase (ϕ, the time of maximum of this cosine function with local midnight as ϕ reference). The ϕ was obtained with its 95% confidence limit if a rhythm was detected with regards to the considered τ in that the amplitude differed from zero (non null amplitude test) with $P<0.05$. A power spectrum method was also employed for detecting prominent period in air-gulping/breathing activity of the fishes under LD 12:12 (De Prins *et al*., 1986) Student's *t*-test (Bruning & Kintz, 1977), ANOVA (Snedecor & Cocharan, 1994) and Duncan's Multiple range test (Duncan, 1955) were applied whenever required (Sahu, 2008, 2019, 2020).

RESULTS

16 untreated *Clarias batrachus* were entrained to LD 12:12 and considered as intact control group. After being entrained to LD 12:12, the effects of pinealectomy/enucleation on the air-breathing activity rhythm were assessed. Subsequently, effect of exogenous melatonin was also noticed.

Activity under LD 12:12

Results of actogram, cosinor rhythmometery and spectral analysis are presented in Figures 1, 2; Tables 1, 2 and Tables 3 respectively. The results obtained by actograms revealed that 14 out of 16 *Clarias batrachus* (intact under LD 12:12 regime) remained entrained to the LD cycle. Further, a statistically significant 24-h rhythm in air-breathing activity was detected for 12 (75%) individuals (Fig 3a), when all time series were subjected to cosinor analysis separately (Table 1). In most of the individuals except Fish # 02, 04, and 14, the acrophases of the activity were located during dark phase of the LD regime (Fig 4 a). However, the acrophase spread was very large (about 15.59h) the earliest being at 18.95 h in Fish # 08 and the latest at 11.54 h in Fish # 04 (Table 1). When a 12-h τ was fitted to the same sets of time series 8 out of 16 (50%) (Fig 3 b) *Clarias batrachus* have shown a statistically significant 12-h rhythm in their surfacing activity (Table 2), however, the acrophase spread was drastically reduced to 8.8h, the earliest being at 1.4 h in Fish #14 and the latest at 10.2 h in Fish # 15 (Table 1). The amplitudes of 12-h rhythm (Table 2) were increased as compared to 24-h rhythm (Table 1). In 11 out of 16 LD-acclimated *Clarias batrachus*, the prominent period was 24-h (Table 3), Fish # 01 and Fish # 03 had a τ = 21-h. The next prominent period in surfacing activity rhythm of Fish # 03 was 24-h. However, Fish # 02, Fish # 12 and Fish # 13 had τ = 14-h, τ = 19.74-h and τ = 27-h, respectively (Table 3). The mean phase angle of the peak of the air-breathing activity with reference to lights on was 17.14 (Table 4).

Pinealectomized Group

Eight *Clarias batrachus* (Fish # 01-08) were pinealectomized while entrained to LD 12:12 (Lights on 06:00 hrs). Activity records of Fish # 06 and Fish # 07 are shown in Fig.1a and b. Visual inspection of actogram revealed that five out of eight *Clarias batrachus* remained entrained to the LD cycle with greatest part of their activity during dark period (Fig.1a and b).

Results of cosinor analysis also revealed that in five out of eight (62.5%) *Clarias batrachus*, a statistically significant 24h rhythm as well as 12h rhythm in their air-breathing activity exists after pinealectomy (Table1 and 2; Fig 3 a and b). However, the acrophases of the activity were located during both light and dark phases of the LD regime (Fig 4b) within the range of 14.15 h [from 11.15 h (F#03) to 1.29h (F#01)] (Table 1). Prominent periods resulting from power spectrum analysis are shown in Table 3. Results indicated that 4 (Fish # 05, 06, 07, 08) out of eight pinealectomized *Clarias batrachus* under LD 12:12 documented a 24-h period in their air-breathing activity rhythm, whereas Fish # 01, 02, 03 and 04, had τ < 24-h. Further, the daily mean of the activity was higher in pinealectomized group as compared to intact group but statistical validation could not be obtained (Fig 5). However, in this group, difference in light and dark hour activity is less. Similarly rhythm adjusted mean i.e., mesor (Fig. 6) of the activity also increased in pinealectomized group but amplitude of the activity is decreased as compared to intact group and could not obtain statistical validation.

Group receiving melatonin treatment after pinealectomy

Ten days after pinealectomy under LD 12:12 all the 8 pinealectomized *Clarias batrachus* received the daily 2 µg intramuscular melatonin treatment in the late afternoon hours (17:30 hrs to 18:00hrs) for next ten days.

The actograms (Fig 1a and b) depicted that air-breathing activity in five individuals was entrained atleast with the lights off time. Visually, the greatest part of their activity was noticed during light phase of the LD regime. The Peak/acrophase map of the air-breathing activity also exhibits the similar result (Fig 4c).

A statistically significant 24-h rhythm in air-breathing activity was detected for most of the (75%) fishes except Fish #01 and Fish #04 by cosinor rhythmometery (Table 1; Fig 3). However, a significant 12-h rhythm was shown only by two (25%) individuals i.e., Fish #03 and Fish #07 (Table 2; Fig 3b). Further 5 out of 8 individuals have shown prominent 24-h period where as Fish #01, Fish #02 and Fish #04 have shown $\tau = 18.85$; $\tau = 17.6$ and $\tau = 16.5$, respectively (Table 3a).

The acrophases of the activity in 6 out of 8 individuals were occurred during photophase of the LD regime (Fig. 4c) and the acrophase spread was shorter (8.14h) as compared with intact and pinealectomized group. The mean phase angle of the peak of the air-breathing activity with reference to lights on was further declined as compared to both intact and pinealectomized *Clarias batrachus* (Table 4).

Enucleated Group

Eight C*larias batrachus* (Fish # 9-16) were blinded by bilateral ocular enucleation while entrained to LD 12:12 (light on 06:00 hrs). Figure 2a and b shows 2 examples of the activity records of the enucleated *Clarias batrachus*. All the enucleated *Clarias batrachus* were desynchronized to the LD cycle. The free running pattern in Fish #12 and Fish # 16 was less precise (Fig 2 a and b). Results obtained by cosinor analysis depicted a statistically significant 24-h rhythm in almost all the enucleated *Clarias batrachus* except Fish # 9 (Table 1). In addition, Fish # 10,12,13,14 have also exhibited a statistically significant 12-h rhythm in their air-breathing activity (Table 2). However, enucleation tended to shorten the period (τ) of the activity in 5 (Fish # 9, 10, 11, 13 and 14) out of 8 individuals (Table 3). The absolute τ change produced by enucleation in these 5 *Clarias batrachus* was 10.23 h. In Fish # 12 and 15 τ was prominent 24-h and in Fish #16 τ was 28.94 h.

The acrophases of the activity were located during the photophase of the LD regime in 5 out of 8 individuals (Fig. 4d). Where as, in Fish # 9, 11 and 16 the location of acrophase was during the scotophase of the LD regime (Fig. 4d). The acrophase spread was 7.16 h (earliest at 9.15 h in Fish # 12 and latest at 16.31 h in Fish #14) in these 5 *Clarias batrachus*, which is much shorter as compared with intact control individuals (Table 1). The mean phase angle of the peak of the air-breathing activity with reference to light on was also shorter as compared with intact control group with statistical validation (Table 4). Maximum daily mean activity was observed in the enucleated *Clarias batrachus* (Fig 5). Rhythm adjusted mean i.e., mesor of the activity was also higher in enucleated *Claria batrachus* (Fig 6).

Group receiving melatonin treatment after enucleation

Twenty one days after being remained in enucleated state the animals received daily late afternoon treatment of melatonin (2 µg/fish) for next ten days. The activity records obtained from Fish # 12 and Fish # 16 are shown in Fig 2a and b. 3 out of eight enucleated melatonin receiving *Clarias batrachus* apparantely entrained to lights off time. Further, 5 (Fish # 09,10, 11, 13, 15) out of 8 *Clarias batrachus* has retained a statistically significant 24-h rhythm in their air-breathing activity (Table 1) and period (τ) of activity was prominent 24-h in Fish # 09, 11, 13, 14 (Table 3). The τ of the activity was still shorter than 24-h in Fish # 10, 15 and 16 (Table 3). Further, acrophase of the activity were also located during the light phase of the LD regime in 5 out of 8 *Clarias batrachus* (Fig. 4e). However, mean phase angle of peak of the air-breathing activity with reference to light on was significantly declined as compared to intact individuals but no change was observed with enucleated individuals (Table 4).

Discussion

By circadian organization we mean the way in which the entire circadian system above the cellular level is put together physically, the principles and rules that determine the interactions among its component parts. Circadian organization extends both broadly and deeply into the physiology and behaviour of multicellular organisms (Menaker *et al*., 1997).

The influence of rhythmic physiological events on teleost behaviour is clearly documented, especially for reproduction and locomotion. Circadian rhythmicity of locomotor activity has been described in a number of teleost fishes and was related to pacemakers within the pineal gland. Extraretinal photoreception has been suggested to entrain circadian photoresponses in pinealectomized, blinded fish (Zaunreiter and Goldschmid, 2001). Many of the biological activities (Underwood, 1987; Maywood *et al.*, 1993) including air-breathing behaviour in fish (Munshi & Ghosh, 1994; Hedrick *et al.*, 1994; Srivastava *et al*., 1993; Maheshwari, 1998; Gupta, 1998; Pati *et al*., 1998; Yadu & Shedpure, 2002; Tikariha & Shedpure, 2002) have been reported to be rhythmic/cyclic in nature. In addition, it has been reported that a multifrequency rhythm (τ=24,12, 6h) occurs as a natural component of the time structure in air breathing behaviour in *Clarias batrachus* (Tikariha & Shedpure, 2002). Similarly, in the present experiment, results of cosinor analysis clearly revealed that most of the individuals exhibited 24-h rhythm in their air-breathing activity, irrespective of their state. In addition, visual analysis of the actograms depicted that in intact *Clarias batrachus* air-breathing activity is entrained with the timings of lights on/off with elevation of activity during dark period and decreased activity during light hours but no such entrainment was observed after enucleation. Resynchronization, atleast with the timing of light off was observed after treatment of melatonin in enucleated individuals. It appears that eyes help the animals in entrainment with external zietgeber. In addition, re-entrainment of rhythm after receiving melatonin treatment in enucleated group supports the earlier report that pineal is the source of circulating melatonin which plays an endocrine role in seasonal and circadian physiology where as the melatonin produced by retinal photoreceptor cells plays a paracrine role in adaptation to light and dark hours. (Gothilf *et al*.,1999). It is supported by the earlier findings viz., in lizard, *Sceloporus occidentalis* melatonin has been found to entrain the locomotor activity rhythm to a periodicity of 24-h (Hyde & Underwood, 1995). Another report on rat suggested that melatonin acts on the coupling or phase relationship between oscillators generating circadian locomotor activity rhythms (Chesworth *et al.*, 1987).

However, pinealectomy has been found to be ineffective in modulating the characteristics of 24-h variation in air-breathing activity of *Clarias batrachus* (Yadu and Shedpure, 2002). Further, it has been reported that pinealectomy does not abolish circadian rhythmicity in any teleost species tested so far (Cahill, 2002). Similarly, in the present experiment pinealectomy has failed to modulate the circadian rhythmicity in air-breathing activity of *Clarias batrachus.* It has also been reported that pinealectomy does alter the period, stability and/or amplitude of behavioural circadian rhyrhms in lake chub, *burbot* and white sucker (Kavaliers, 1979, 1980; Kavaliers and Ralph, 1980, Bertolucci *et al*., 2002). In the same way, the present experiment reveals that after pinealectomy the period of the activity was disturbed but regain of the period of activity equal to 24 h in most of the studied individuals after late afternoon melatonin treatment indicates that melatonin acts as an internal Zeitgeber (Begay *et al*., 1998; Zachman *et al*., 1992).

In fish, the pineal organ acts as a direct photoreceptor transducing light information in to neural and humoral (melatonin) signals (Srivastava, 2003). Tikariha and Shedpure in 2006 documented that photoperiod plays significant role in modulation of air-breathing activity rhythm in *Clarias batrachus* and exogenous melatonin may produce time of treatment dependent modulation of such rhythm. In the present experiment, in intact and pinealectomized, individuals air-breathing activity was increased during dark hours and decreased during light hours with most of the peaks of activity occurring during dark hours but after enucleation, most of the peaks of activity has been shifted during photophase of the LD regime. Further, daily mean and mesor of the activity was highest in the enucleated group as compared to all other groups. In pinealectomized group, after melatonin treatment, increase and decrease of the activity was observed during light and dark phase, respectively with most of the peaks of the activity located during light hours. Further, the mean phase angle of the peak with reference to lights on was declined as compared to both intact and pinealectomized *Clarias batrachus.*

Table 1- Results of cosinor analysis of air-breathing activity in *Clarias batrachus* at τ =24-h at laboratory temperature.

Group	**Animal Code**	**P^a**	**Mesor ± SE^b**	**Amplitude ± SE^c**	**Acrophase^d (95%CL)**
IG	F#01	0.778	8.42 ± 1.96	2.39	21.79
	F#02	0.09	9.24 ± 1.40	4.53	9.26
	F#03	0.0005	1.96 ± 0.50	2.64 ± 1.65	3.86 (6.52, 0.99)
	F#04	0.2754	11.81 ± 1.05	2.39	11.54
	F#05	0.001	26.66 ± 2	9.96 ± 6.76	2.25 (5.16, 23.35)
	F#06	0.001	76.34 ± 2.54	25.29 ± 8.56	0.85 (2.26, 23.45)
	F#07	0.001	7.72 ± 0.28	2.80 ± 0.95	0.60 (2.03, 23.18)
	F#08	0.001	9.72 ± 0.99	10.25 ± 3.58	18.95 (20.17, 17.70)
	F#09	<0.001	0.31 ± 0.02	0.15 ± 0.08	0.96 (3.13, 22.8)
	F#10	<0.001	0.23 ± 0.01	0.08 ± 0.05	3.83 (6.76, 0.9)
	F#11	<0.001	0.264 ± 0.022	0.27 ± 0.09	0.14 (1.29, 22.99)
	F#12	0.13	49.43 ± 2.69	7.74	20.62
	F#13	0.03	8.62 ± 0.42	1.56 ± 1.44	3.44 (8.12, 22.76)
	F#14	<0.001	26.06 ± 1.43	24.27 ± 5.06	7.63 (8.42, 6.84)
	F#15	<0.001	5.27 ± 0.24	3.62 ± 0.89	1.22 (2.19, 0.25)

	F#16	<0.001	1.32 ± 0.08	1.51 ± 0.31	0.46 (1.22, 23.69)
PxG	F#01	0.04	3.24± 0.85	3.12 ± 3.06	1.29 (5.90,20.68)
	F#02	0.7997	18.22 ± 1.51	1.06	18.12
	F#03	0.0008	5.43 ± 0.40	2.06 ±1.35	11.14 (13.77,8.11)
	F#04	0.811	41.37 ± 1.62	1.52	13.23
	F#05	0.001	12.08 ± 0.84	6.46 ± 2.94	17 (18.80, 15.36)
	F#06	0.001	98.63 ± 3.09	59.33 ± 10.97	13.27 (13.92, 12.61)
	F#07	0.9943	15.05 ± 1.20	0.16	19.71
	F#08	0.001	15.64 ± 0.87	7.54 ± 2.83	19.13 (20.78, 17.60)
PxMG	F#01	0.8954	4.76 ± 0.46	0.30	20
	F#02	0.002	13.63 ± 0.55	2.79 ± 1.96	13.35 (16.20, 10.36)
	F#03	0.001	5.88 ± 0.23	4.09 ± 0.79	19.35 (20.13, 18.58)
	F#04	0.207	21.40 ± 1.53	3.91	16.86
	F#05	0.001	22.4 ± 1.95	22.46 ± 6.92	14.02 (15.16, 12.87)
	F#06	0.001	10.48 ± 0.9	8.88 ± 3.12	11.86 (13.21, 10.46)
	F#07	0.002	6.98 ± 2.65	1.34 ± 0.94	15.37 (18.25, 12.54)
	F#08	0.001	2.39 ± 0.99	1.18 ± 0.35	14.52 (15.62, 13.41)

EG	F#09	0.7744	14.61 ± 0.69	0.6813	21.08
	F#10	0.001	25.65 ± 0.73	3.67 ± 2.47	10.07 (12.91, 7.12)
	F#11	0.025	4.58 ± 0.42	1.60 ± 1.44	20.33 (0.72, 16)
	F#12	0.0004	31.99 ± 0.82	4.94 ± 2.78	9.15 (11.52, 6.76)
	F#13	<0.001	18.31 ± 0.35	3.76 ± 1.20	11.14 (12.38, 9.87)
	F#14	<0.001	45.46 ± 1.61	14.61 ± 5.67	16.31 (17.81, 14.85)
	F#15	<0.001	6.67 ± 0.53	4.68 ± 1.81	10.95 (12.47, 9.38)
	F#16	<0.001	32.99 ± 1.57	18.15 ± 5.43	23.58 (0.73, 22.40)
EMG	F#09	0.001	0.008 ± 0.002	0.01 ± 0.009	16.58 (19.42, 13.83)
	F#10	<0.001	15.79 ± 1.57	19.46 ± 5.37	19.14 (20.23, 18.05)
	F#11	0.01	0.001 ± 0.0009	0.003 ± 0.003	17.66 (21.56, 13.88)
	F#12	0.2304	0.32 ± 0.0035	0.08	21.36
	F#13	<0.001	92.96 ± 2.36	17.36 ± 8.25	12.97 (14.82, 11.10)
	F#14	0.7487	28.43 ± 1.63	1.74	5.35
	F#15	0.01	9.01 ± 0.27	1.15 ± 0.97	12.53 (16.27,8.71)
	F#16	0.1075	22.39 ± 2.14	6.35	10.24

[a] From F test of null amplitude rejection hypothesis.

[b] Rhythm-adjusted mean of best-fitting cosine function ± 1 SE.

[c] Half of the difference between maximum and minimum of best- fitting cosine function ± 1 SE.

[d] Timing of maximum in best-fitting cosine function with 95% confidence limit.

IG = Intact group
PxG = Pinealectomized group
PxMG = Pinealectomized + melatonin treated group
EG = Enucleated group
EMG = Enucleated + pinealectomized + melatonin treated group

Table 2- Results of cosinor analysis of air-breathing activity in *Clarias batrachus* at τ =12-h at laboratory temperature.

Group	**AnimalCode**	**P^a**	**Mesor ± SE^b**	**Amplitude ± SE^c**	**Acrophase^d (95% CL)**
IG	F#01	0.8148	8.34 ± 1.96	1.74	10.81
	F#02	0.5394	9.67 ± 1.41	2.16	11.65
	F#03	0.005	1.94 ± 0.50	2.30 ± 1.74	3.98 (5.64, 2.39)
	F#04	0.1786	12.01 ± 105	2.71	11.19
	F#05	0.0008	27.54 ± 2.01	10.59 ± 6.93	6.3 (7.69, 4.95)
	F#06	0.04	76.68 ± 2.6	9.16 ± 9.02	2.8 (5.41, 0.10)
	F#07	0.198	7.78 ± 0.29	0.747	3.46
	F#08	0.6467	7.98 ± 1.00	1.30	11.4
	F#09	0.0005	108.78 ± 19.51	107.00 ± 62.96	6.25 (7.70, 4.92)
	F#10	0.09	42.64 ± 26.77	76.19	6.12
	F#11	0.02	90.18 ± 39.36	149.60	6.16 (8.31, 4.05)
	F#12	0.05	127.66 ± 39.94	132.25	6.68
	F#13	0.01	109.02 ± 42.17	160.65 ± 137.95	6.14 (8.22, 4.06)
	F#14	0.001	0.57 ± 0.10	0.58 ± 0.33	1.40 (2.59, 0.24)
	F#15	< 0.001	69.16 ± 3.10	16.85 ± 7.31	10.20 (11.06, 9.33)
	F#16	0.2733	43.35 ± 1.25	2.88	5.52
PxG	F#01	0.005	4.09 ± 0.83	3.80 ± 2.88	2.70 (4.35, 1.08)
	F#02	0.001	18.65 ± 1.12	5.61 ± 3.88	3.04 (4.50,1.60)
	F#03	0.01	5.17 ± 0.39	1.60 ± 1.37	6.86 (8.78, 4.89)
	F#04	0.155	41.53 ± 1.58	4.35	2.11
	F#05	0.1743	10.88 ± 0.81	2.12	5.25
	F#06	0.01	89.87	12.92 ± 10.82	2.81 (4.73, 0.95)
	F#07	0.002	14.84 ± 1.15	5.68 ± 3.99	6.35 (7.82, 4.84)
	F#08	0.1133	14.76 ± 0.84	2.51	9.07
PxMG	F#01	0.1224	4.71 ± 0.46	1.36	10.46

	F#02	0.2536	13.49 ± 0.55	1.29	0.59
	F#03	0.001	5.95 ± 0.23	2.44 ± 0.81	7.70 (8.36, 7.04)
	F#04	0.07	21.5 ± 1.53	4.93	5.29
	F#05	0.4869	9.95 ± 0.91	1.55	3.73
	F#06	0.4707	6.96 ± 0.26	0.46	4.77
	F#07	0.001	21.66 ± 1.96	12.71 ± 6.84	4.92 (6.01, 3.85)
	F#08	0.3521	2.35 ± 0.10	0.207	4.2
EG	F#09	0.6797	14.59 ± 0.68	0.855	7.92
	F#10	0.009	25.54 ± 0.72	3.10 ± 2.50	11.93 (13.75, 10.15)
	F#11	0.2423	31.99 ± 0.82	1.97	3.36
	F#12	0.01	4.55 ± 0.42	1.77 ± 1.48	10.94 (12.84, 9.08)
	F#13	0.02	18.09 ± 0.35	1.35 ± 1.21	11.26 (13.41, 9.16)
	F#14	0.0008	44.88 ± 1.61	8.61 ± 5.60	2.75 (4.08, 1.40)
	F#15	0.09	6.44 ± 0.53	1.61	11.89
	F#16	0.1543	34.26 ± 1.57	4.32	2.63
EMG	F#09	0.04	0.007 ± 0.002	0.01 ± 0.009	5.43 (7.98, 2.96)
	F#10	<0.001	15.58 ± 1.57	16.16 ± 5.37	7.36 (8.02, 6.70)
	F#11	0.0193	0.001 ± 0.0009	0.003 ± 0.003	5.96 (8.02, 3.98)
	F#12	0.7064	0.33 ± 0.03	0.04	1.17
	F#13	0.001	91.71 ± 2.35	12.29 ± 8.18	2.94 (4.31, 1.53)
	F#14	0.3035	28.5 ± 1.62	3.57	4.84
	F#15	0.6593	8.92 ± 0.27	0.3587	11.75
	F#16	0.0001	22.18 ± 2.12	12.74 ± 7.38	3.22 (4.38, 2.04)

Please see legend to Table-1

Table 3- Results of power spectrum analysis showing period (τ) of air-breathing activity in *Clarias batrachus*

Animal Code	IG	PxG/EG^1	PxMG/EMG^2

F#01	21	21	18.85
F#02	14	21	17.6
F#03	21 (24)	15.27	24
F#04	24	21	16.5
F#05	24	24	24
F#06	24	24	24
F#07	24	24	24
F#08	24	24	24
F#09	24	14.11	24
F#10	24	17.77	21.81
F#11	24	8.57	24
F#12	19.74	24	30
F#13	27	11.17	24
F#14	24	12.97	24
F#15	24	24	6 (12) *
F#16	24	28.94	8.27

* Second peak.

[1] F#01-08 and 09-16 are PxG and EG, respectively.

[2] F#01-08 and 09-16 are PxMG and EMG, respectively.

Please see legend to Table-1

Table 4- Results of phase angle of peak of air-breathing activity with reference to lights on in *Clarias batrachus*

Animal Code	IG	PxG/EG[1]	PxMG/EMG[2]
F#01	15.79	19.29	14
F#02	3.26	12.12	7.35
F#03	21.86	5.14	12.35
F#04	5.54	7.23	10.86
F#05	20.25	11	8.02
F#06	18.85	7.27	5.86
F#07	20.60	13.71	9.37
F#08	12.95	13.13	8.52
F#09	18.96	15.08	10.58
F#10	21.83	4.07	13.14
F#11	18.14	14.33	11.66
F#12	14.62	3.15	15.36
F#13	21.44	5.14	6.97
F#14	1.63	10.31	23.35
F#15	22.22	4.95	6.53
F#16	18.46	17.58	4.24

Please see legend to Table. 1

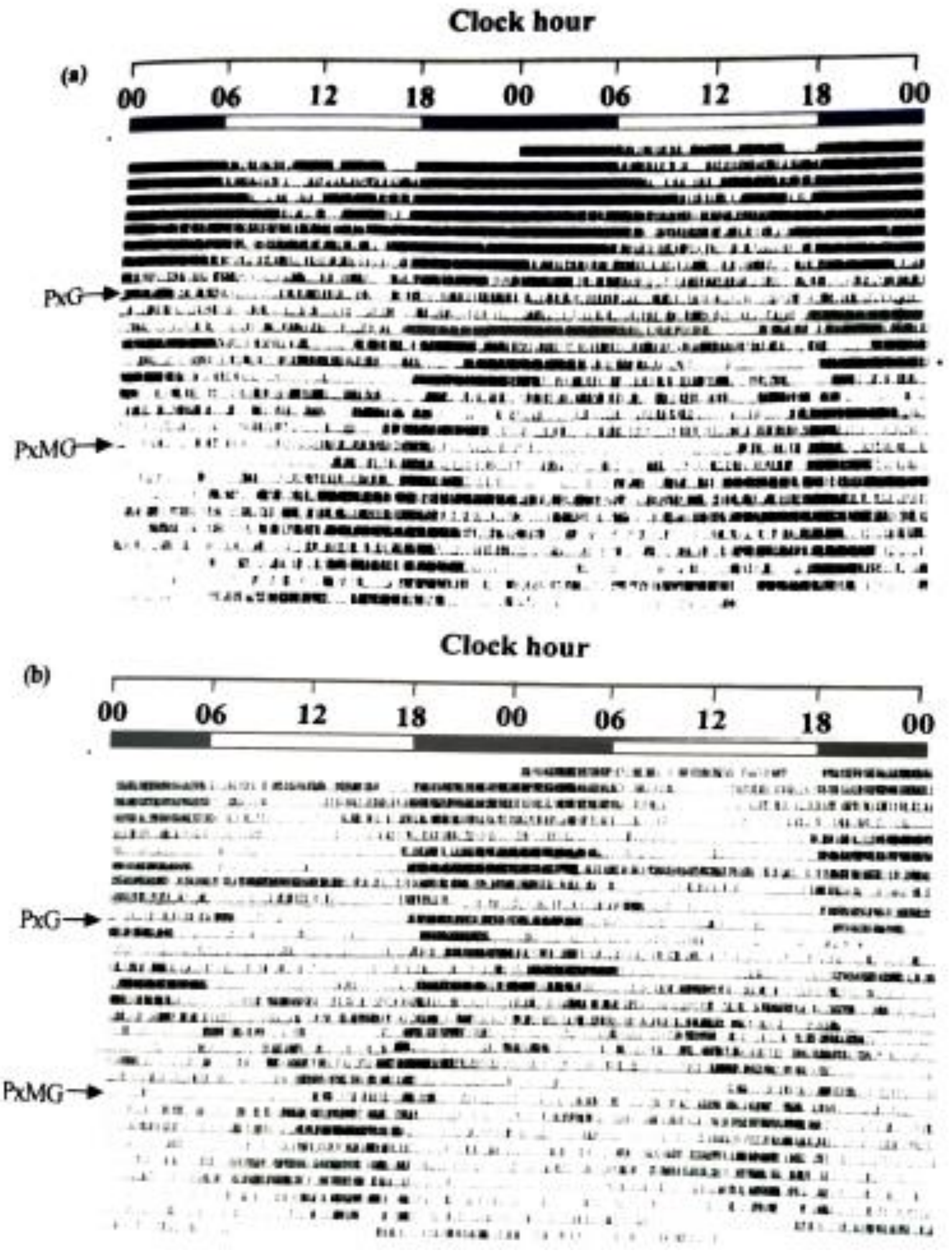


Figure 1 a & b (Fish# 6 & 7): Actogram record in double plot (48hrs) showing air-breathing activity of *Clarias batrachus* maintained under LD 12:12 regime.

PxG = Pinealectomized group; PxMG = Pinealectomized + melatonin treated group

EG = Enucleated group; EMG = Enucleated + pinealectomized + melatonin treated group.

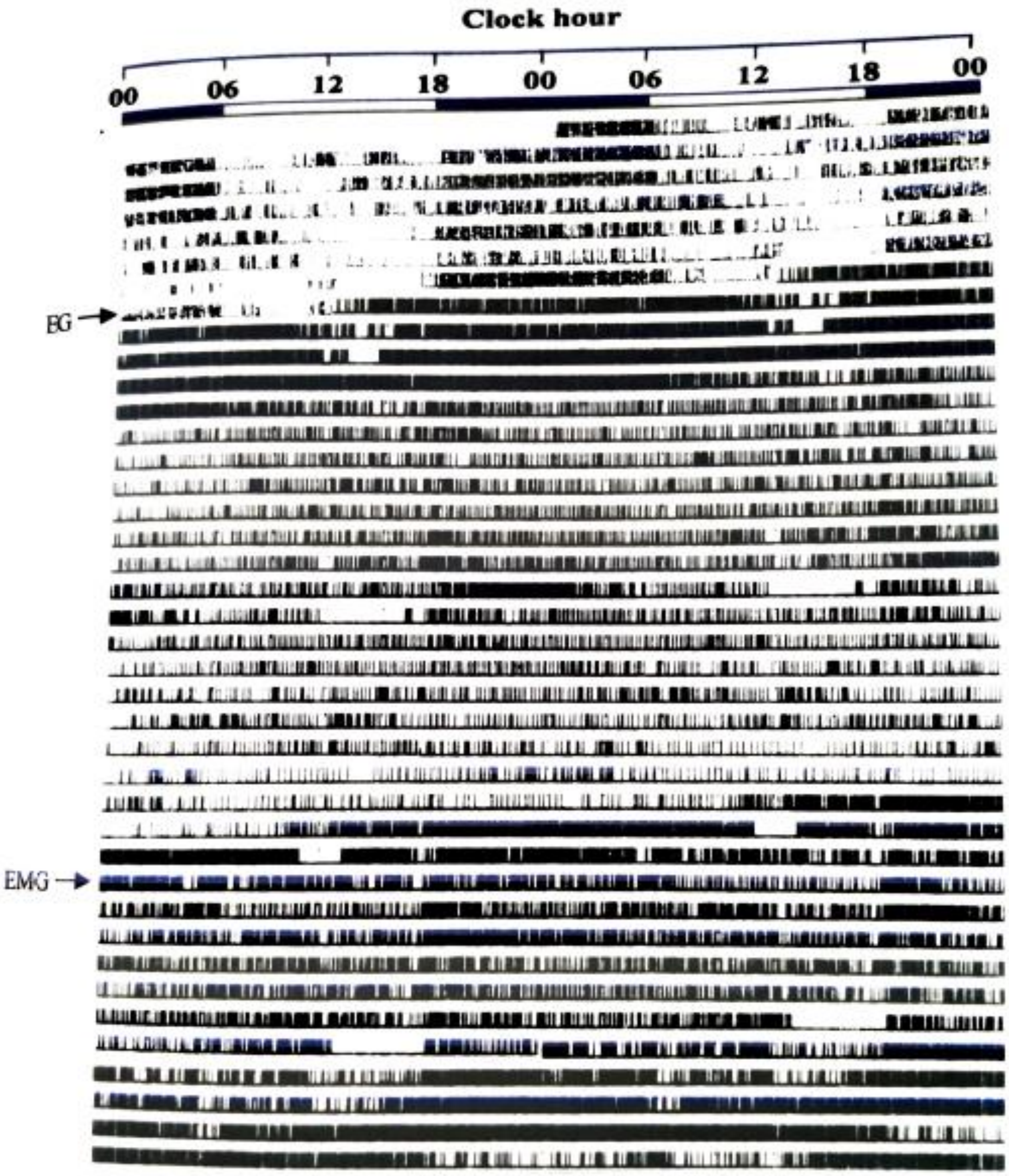


Figure 2 a (Fish#12): Actogram record in double plot (48hrs) showing air-breathing activity of *Clarias batrachus* maintained under LD 12:12 regime. EG = Enucleated group; EMG = Enucleated + pinealectomized + melatonin treated group

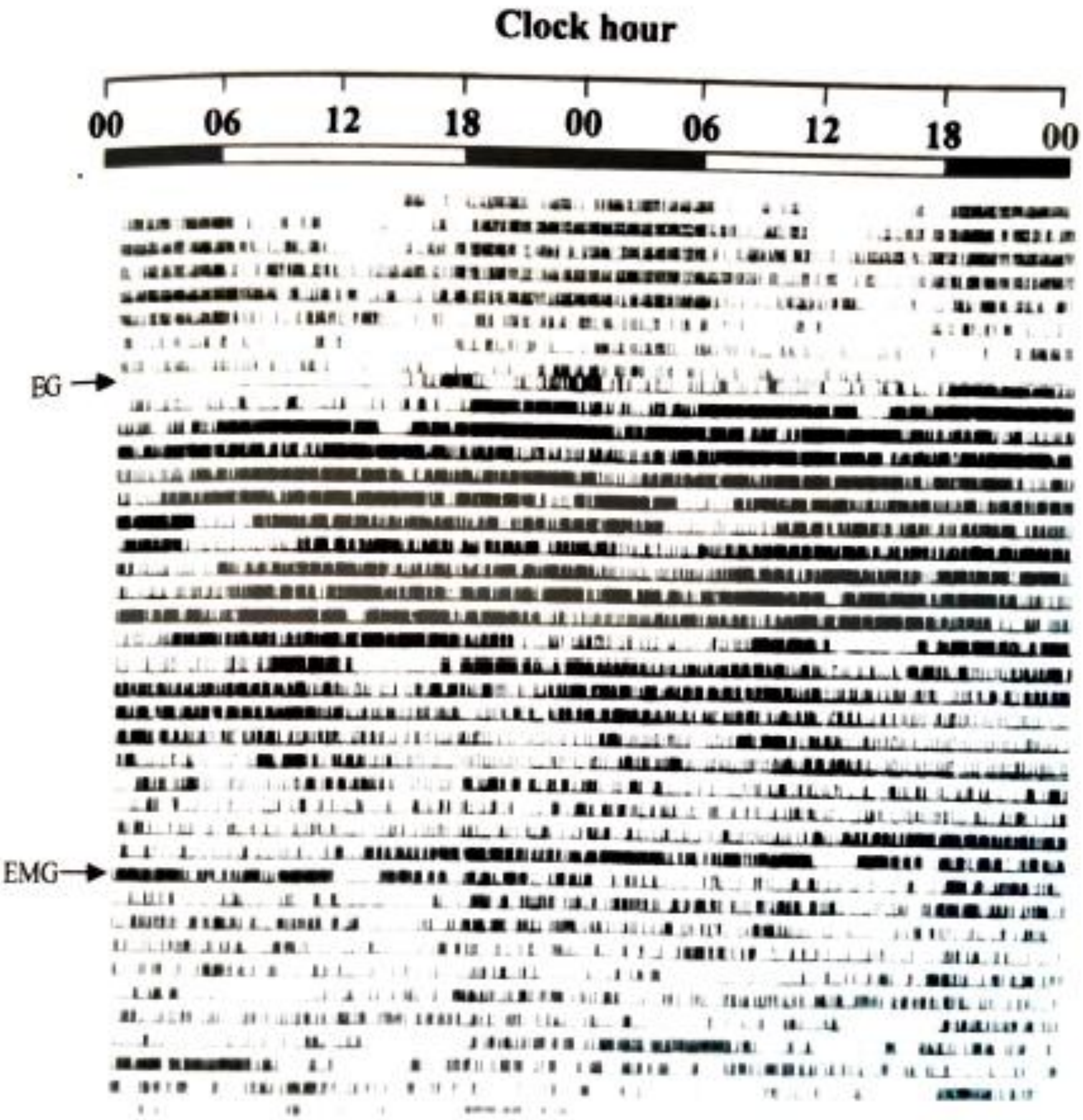


Figure 2 b (Fish#16): Actogram record in double plot (48hrs) showing air-breathing activity of *Clarias batrachus* maintained under LD 12:12 regime.

EG = Enucleated group

EMG = Enucleated + pinealectomized + melatonin treated group

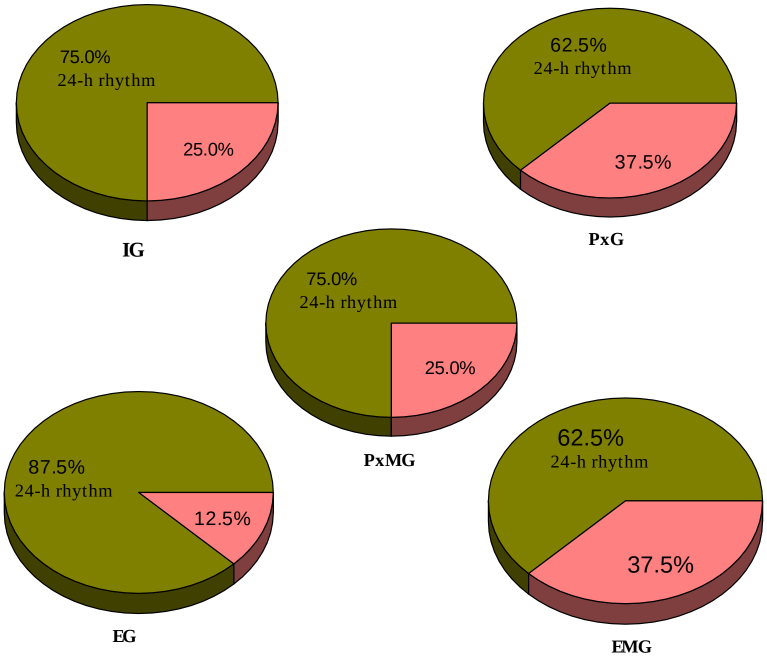


Figure 3a- Occurrence of 24-h rhythm in air-breathing activity at τ =24hr in Intact (IG), Pinealectomized (PxG), Pinealectomized + Melatonin (PxMG), Enucleated (EG), Enucleated + Melatonin treated (EMG) *Clarias batrachus* (Results based on cosinor analysis)

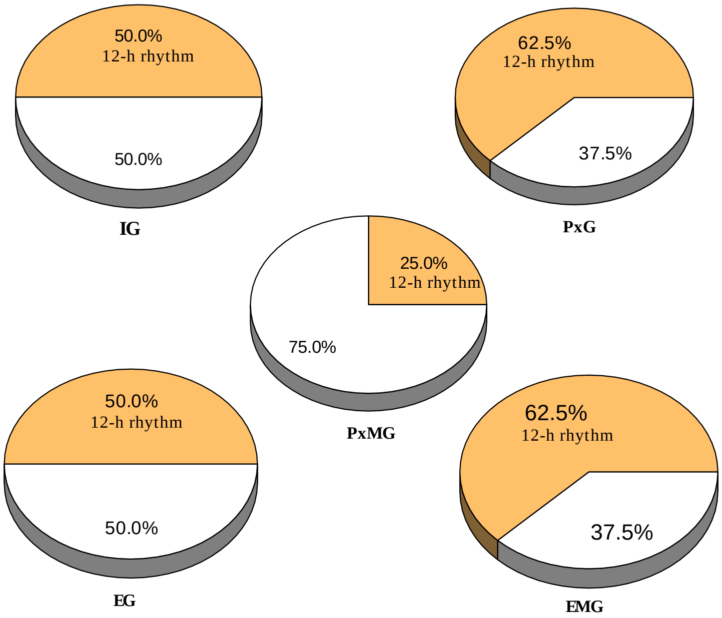


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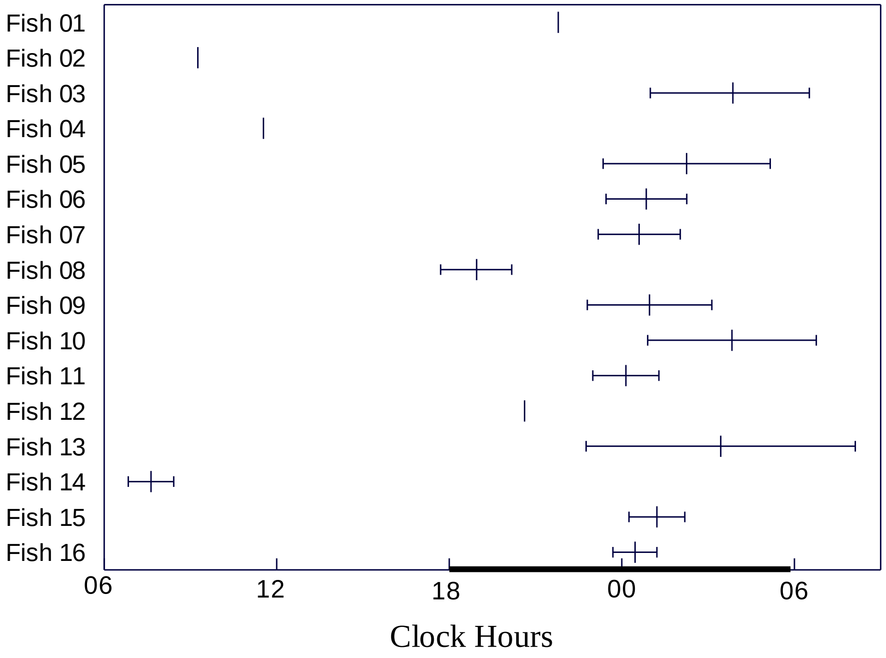
Figure 3b- Occurrence of 12-h rhythm in air-breathing activity at τ =12hr in Intact (IG), Pinealectomized (PxG), Pinealectomized + Melatonin, (PxMG) Enucleated (EG) and Enucleated + Melatonin treated (EMG) *Clarias batrachus* (Results based on Cosinor analysis)


Fish 01
Fish 02
Fish 03
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Fish 06
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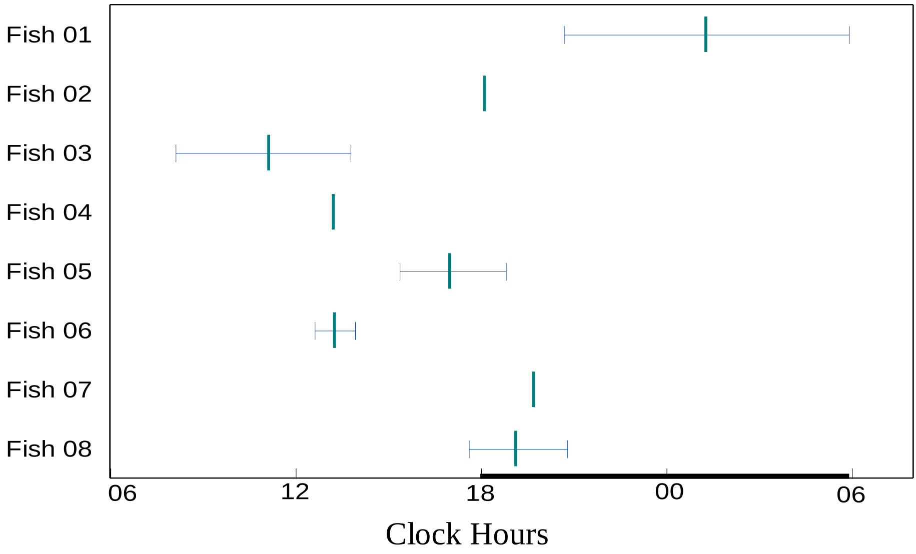
18
00
06
Clock Hours

a


Fish 01
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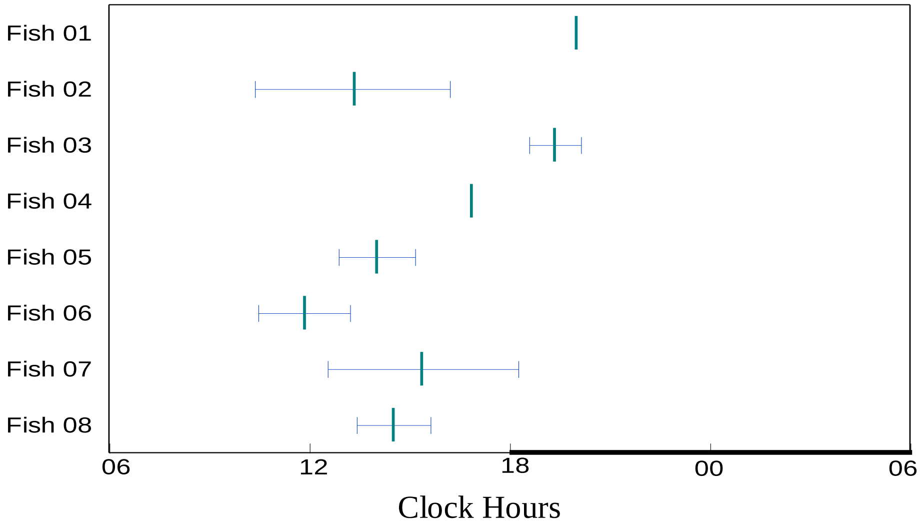
b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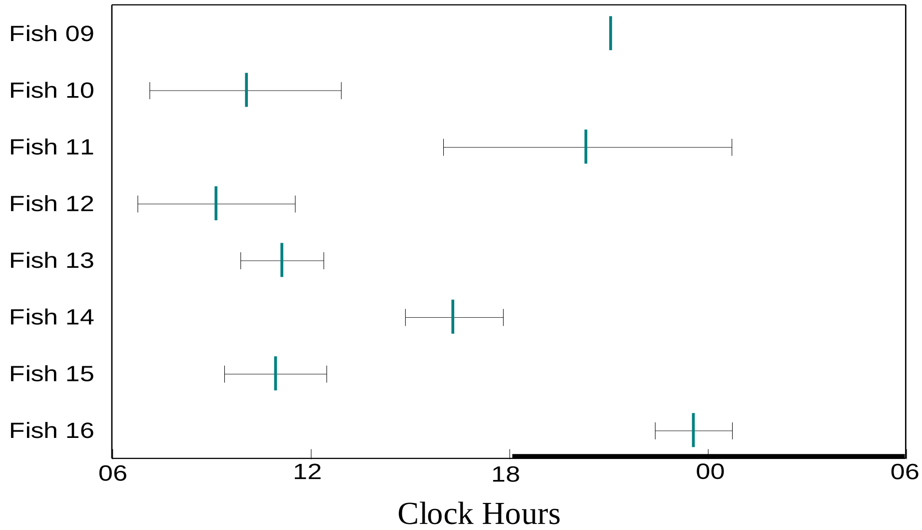
Clock Hours

c


Fish 09
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Fish 12
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Fish 14
Fish 15
Fish 16
06
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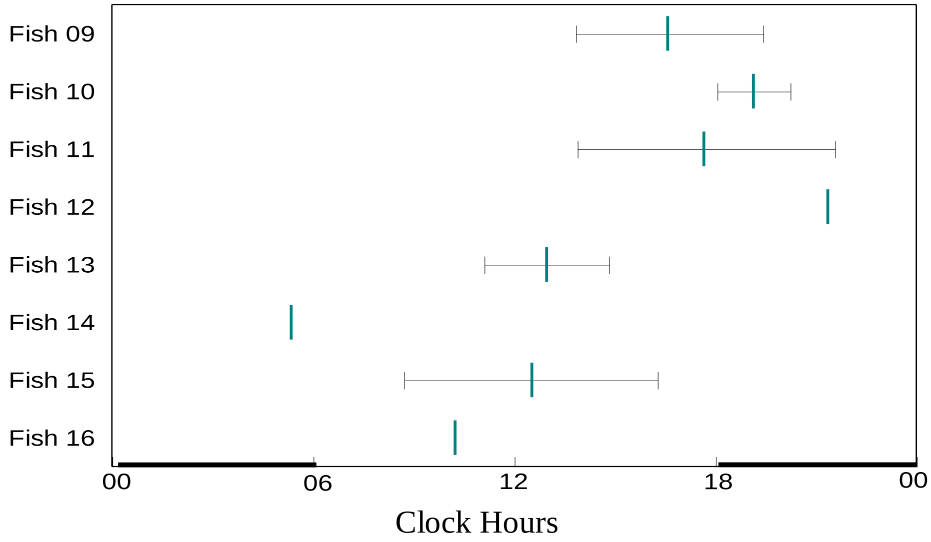
00
06
Clock Hours

d


Fish 09
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Fish 14
Fish 15
Fish 16
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12
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00
Clock Hours

e

Figure 4- Peak map for air-breathing activity of *Clarias batrachus* under LD12:12 regime (a) Intact (b) Pinealectmized (c) Pinealectmized +Melatonin treated
(d) Enucleated (e) Enucleated +Melatonin treated

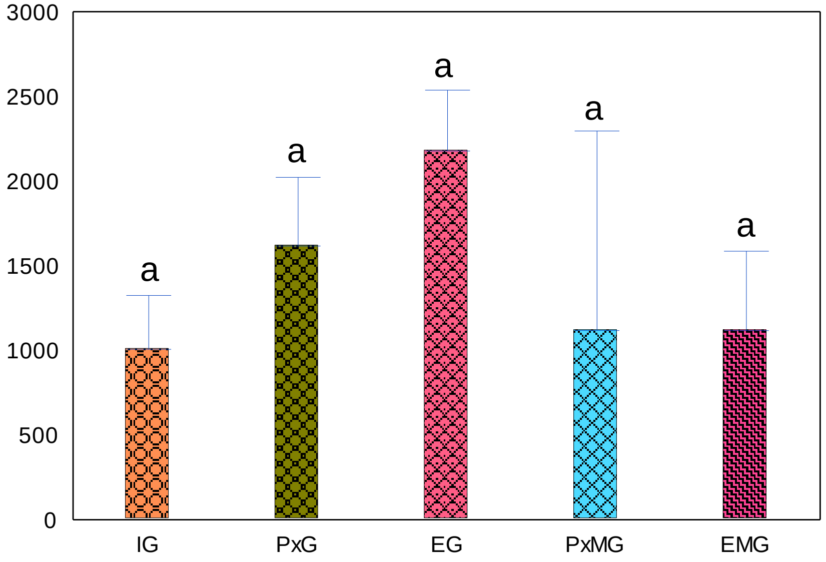

Figure 5- Daily mean of air-breathing activity in *Clarias batrachus* under LD12:12
IG = Intact, PxG = pinealectomized, PxMG = Pinealectomized + Melatonin treated,
EG = Enucleated, EMG = Enucleated +Melatonin treated

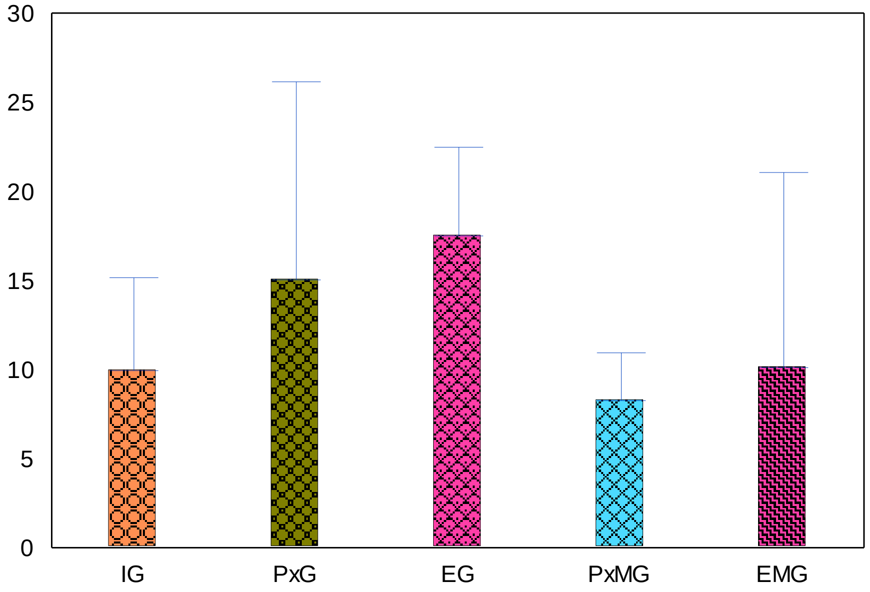

Figure 6- Mesors (rhythm-adjusted mean) of air-breathing activity in *Clarias batrachus* under LD12:12
Please see legend to Fig-5

FIGURE LEGENDS

Figure 1- a & b: Actogram record in double plot (48hrs) showing air-breathing activity of Clarias batrachus maintained under LD 12:12 regime. PxG=Pinealectomized group; PxMG = Pinealectomized+ melatonin treated group

EG = Enucleated group; EMG = Enucleated + pinealectomized + melatonin treated group.

Figure 2 a & b (Fish#16): Actogram record in double plot (48hrs) showing air-breathing activity of Clarias batrachus maintained under LD 12:12 regime.

EG = Enucleated group

EMG = Enucleated + pinealectomized + melatonin treated group

Figure 3a- Occurrence of 24-h rhythm in air-breathing activity at □ =24hr in Intact (IG), Pinealectomized (PxG), Pinealectomized + Melatonin (PxMG), Enucleated (EG), Enucleated + Melatonin treated (EMG) Clarias batrachus (Results based on cosinor analysis)

Figure 3b- Occurrence of 12-h rhythm in air-breathing activity at □ =12hr. (Please see Legend to Fig-3a)

Figure 4- Peak map for air-breathing activity of Clarias batrachus under LD12:12 regime

(a) Intact (b) Pinealectmized (c) Pinealectmized +Melatonin treated

(d) Enucleated (e) Enucleated +Melatonin treated

Figure 5- Daily mean of air-breathing activity in Clarias batrachus under LD12:12

IG = Intact, PxG = pinealectomized, PxMG = Pinealectomized + Melatonin treated, EG = Enucleated, EMG = Enucleated +Melatonin treated

Figure 6- Mesors (rhythm-adjusted mean) of air-breathing activity in Clarias batrachus Under LD12:12. (Please see legend to Fig-5)

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