Effects of 5,7-dihydroxytriptamine (5,7-DHT) on circadian locomotor activity of the blow fly, Calliphora vicina

Effects of 5,7-dihydroxytriptamine (5,7-DHT) on circadian locomotor activity of the blow fly, *Calliphora vicina*

Bronislaw Cymborowski

Warsaw University, Department of Invertebrate Physiology, 1 Miecznikowa Str., 02-096 Warszawa, Poland
bron@biol.uw.edu.pl

Abstract

The biogenic amine serotonin (5-HT) is a neuromodulator in both vertebrates and invertebrates. It has been shown that serotonin, apart from its distinct effects on behavior, also plays a morphoregulatory role during the ontogeny of the insect's nervous system. The role of serotonin in modulating circadian locomotor activity of the blow fly, *Calliphora vicina* was explored. Injection of a specific neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), into the hemolymph appeared to significantly reduced the level of locomotor activity and lengthened the period (τ) of circadian rhythmicity. After drug injection in constant darkness flies continued with their free-running rhythm of a locomotor activity, depending on the time of 5,7-DHT injection. This compound causes phase delay when administered in the early subjective day, and phase advance in the late subjective day. This effect is the opposite of the phase response curve obtained for 5-HT injections. This suggests that 5-HT might act as an entraining agent *via* the output pathway by feedback to clock neurons in the brain. Some of the injected insects regained their normal level of activity after a few days. These findings suggest a potential role for serotonin as modulator of circadian rhythms in insect including regulation of the level of locomotor activity.

Abbreviation:
5-HT   5-hydroxytryptamine, serotonin
5,7-DHT   5,7-dihydroxytryptamine

Introduction

5-hydroxytryptamine (5-HT), also known as serotonin, is one of the major biogenic amines widely distributed in the insect central nervous system (Evans, 1980; Nässel, DR and Cantera, R 1985), and is recognized as a neuromodulator and neurotransmitter (Cymborowski, 1970; Ali, 1997; Cymborowski, 1998). In the central nervous system of insects, serotonin is expressed by a set of neurons that are easily identifiable (Strambi et al., 1989; Seidel and Bicker, 1996). In addition, serotonin may play a morphoregulatory role during the ontogeny of the nervous system (Seidel and Bicker, 1996).

It has also reported that serotonin plays important roles in the circadian system of insects. There is a distinct correlation between the 5-HT level in the brain and the intensity of locomotor activity of cricket, *Acheta domesticus*. (Muszynska-Pytel and Cymborowski, 1978a; Muszynska-Pytel and Cymborowski, 1978b). A high level of serotonin in the hemolymph was found just after lights-off when locomotor activity was highest. Injection of 5-HT into the optic lobe of the cockroach shifts the circadian locomotor activity rhythms in a phase dependent manner (Eskin et al., 1982; Page, 1987) In the cricket, *Grillus bimaculatus*, sensitivity to the light stimuli of visual interneurons in the optic lobe is highest when

the 5-HT content is also high. (Tomioka et al.,1993). A more detailed study has shown that injection of 5-HT into the optic lobe of the house fly (*Musca domestica*) mimics the daily changes in the axonal size of the first-order large monopolar cell L1. (Pyza and Meinertzhagen, 1996). Recently it was found that there is a distinct correlation between locomotor activity rhythms and the size of two classes of interneurons, L1 and L2 in optic lobe of the blow fly, *Calliphora vicina* (Pyza and Cymborowski, 2001).

Apart from these studies, there are still a number of questions about how the serotoninergic system is involved in the circadian rhythms in insects. It seems that a very good approach to this issue would be to reduce 5-HT content within the nervous system using the specific neurotoxin 5,7-hydroxytryamine (5,7-DHT) that causes selective degeneration of serotoninergic neurons both in vertebrates and in invertebrates (Morin and Blanchard, 1991; Dugar et al., 1998; Sinhababu and Borchard, 1988; Pyza and Meinertzhagen, 1996). For example, the periods (tau) of wheel running activity in mice can be changed depending on serotonin levels in their brains. The role of serotonin in the suprachiasmatic nucleus was measured during wheel-open and wheel-locked conditions (Mistlberger et al. 1998). Mice exhibited a significant lengthening of period within 3 weeks when running wheels were

locked. Immunocytohistochemical investigation confirmed 5-HT depletion in the suprachiasmatic nucleus. Birthelmer et al. (2002) found that 5,7-DHT lesions enhance, and serotoninergic grafts normalize, the evoked overflow of acetylchcholine in rat hippocampal slices of the brain.

To examine this further, the effects of injections of the neurotoxin 5,7-DHT on the circadian rhythms of locomotor activity in the blowfly were investigated. Our previous evidence, suggested that serotonin may play a role in the generation of circadian rhythmicity of behavior as well as of accompanying cellular processes, for example, in the daily changes in axonal size of some (L1 and L2) optic lobe interneurons. The approach used here was to investigate the role of serotonin in circadian rhythmicity by injection of 5,7-DHT. Changes in the flies's circadian rhythm of locomotor activity were examined, especially by investigation of changes in: (1) the overall level of the flies's locomotor activity (2) the period (τ), and (3) phase of the rhythm, depending on the time of injection.

Materials and Methods

Flies

The strain of the blow fly, *Calliphora vicina*, used in this study was collected in Warsaw. Maintenance of the stock culture was done as previously described by Saunders (1987). Flies were LD 18:6 h at 26 ± 0.5 °C. Flies used for each experiment came from the same larval culture, emerged at the same time, and were thus all of the same age.

Recording of locomotor activity

Adult locomotor activity of the blow fly was recorded as described by Cymborowski et al. (1996) in 9-cm Petri dishes, with the recording device comprised an infrared light beam (5 mm diameter) passing vertically through one side of the dish onto a phototransistor. Sugar solution was provided through cotton wool wicks. Dishes were placed on a wooden platform that provided support for the infrared emitters and detectors. The whole assembly was then placed in a light-tight wooden box held at 26 ± 0.5 °C. Experimental flies were exposed to constant dark conditions.

Drug injection

All experiments were performed on adult flies of both sexes within 2-4 days of their emergence. The test protocol for all experiments was identical. At the beginning of the experiment, free-running locomotor activity was monitored under constant dark conditions. After short CO_2 anaesthesia, the flies were injected with 0.5 mM of the neurotoxin 5,7-DHT (creatine-sulfate salt, Sigma, www.sigmaaldrich.com) dissolved 1µl of insect saline/ascorbic acid (0.1%) in the abdomen using a Hamilton syringe. This dosage of 5,7-DHT was used after performing a preliminary experiment using different concentrations. The higher dosages caused higher mortality, the lower ones gave unclear results for statistical analysis. In this study a special computer system was used that shows the phase of fly's rhythm, making it possible to inject the drug at different circadian times. Control flies were handled by the same procedure, but received only solvent. It should be mentioned that doses of 5,6-DHT lower than 0.3 mM had no effect on fly's

locomotor activity rhythm, therefore they served as controls as well. Each fly was used only once.

Data analysis

Activity events were registered as the number of times the infrared light beam was broken by a moving fly within successive 10-min intervals. These numbers were recorded using an IBM computer and assembled into a 'double-plotted' actogram format (Cymborowski *et al*, 1993). Subsequent periodogram analysis and calculations of period (τ) of free-running rhythms were carried out as previously described (Cymborowski et al., 1994) and was similar to that used by Sokolove and Bushell (1978). Values were analyzed by Statistica Programme and are expressed as mean ± SD. Significance was considered at P<0.05.
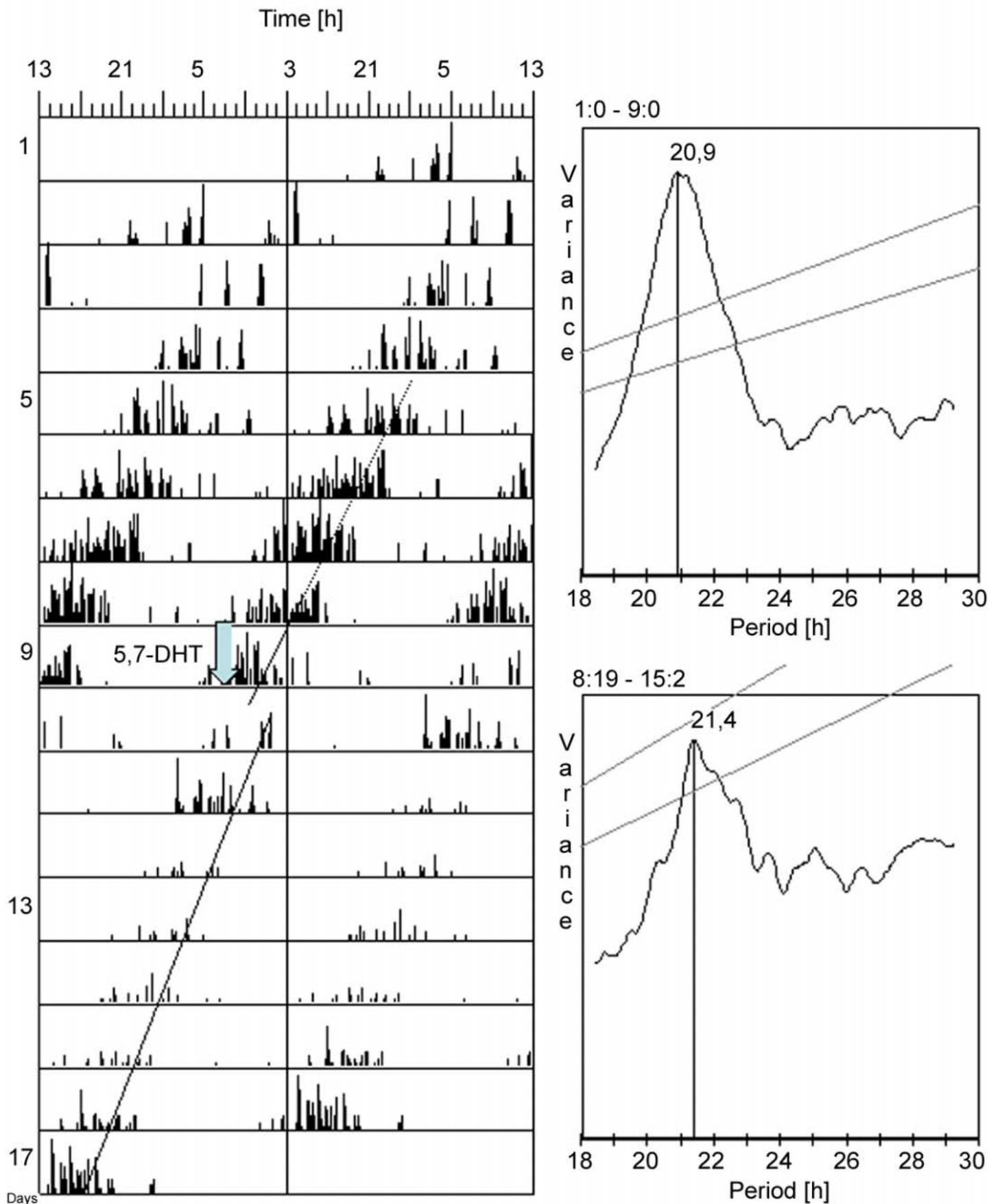
Results

Effect of 5,7-DHT injection on the level and phase of circadian locomotor activity

The distribution of free-running period for flies kept at 26 ºC in constant darkness before 5,7-DHT treatment was 22.63 ± 0.73 h for 83 flies, and showed little change over the duration of the pre-treatment recording (8-10) days. After administration of 0.5 mM of 5,7-DHT there was a substantial reduction in the level of locomotor activity of all flies investigated. Representative actograms are shown in Fig. 1-3.

Some insects injected with 0.5 mM of drugs regained their pre-injected level of locomotor activity after 2-3 days (Fig. 1). In a further study it was found that this compound causes phase shifts depending on the time of injection. It delayed the phase when 5,7-DHT was injected in the early subjective day (Fig. 1) and advanced the phase when injected in the late subjective day (Fig. 2). In about 5% out of 83 investigated animals there was a clear arrhythmicity that started on day 2 after the drug injection (Fig 3). The average activity duration (α) was 1.54 ± 0.60 movements/24h of recording comparing with drug treated flies in which this value hardly reached about 0.26 ± 0.11 movements/24 hours (Fig.4). It seems that locomotor activity rhythms can be best characterized by activity/rest (α/ρ) coefficient. This ratio is an important parameter in determining the effects of a given time cue on an insect's activity rhythm, especially in the absence of a change in τ (Pyza and Cymborowski, 2001). In control insects there were no changes in phase of the rhythms and the mean coefficient was close to about 0.32 ± 0.19, whereas after drug injection dropped down to about 0.06 ± 0.02 (p<0.001).

### Effect of drug injection on the period of circadian locomotor activity rhythm

The most pronounced effect of 5,7-DHT injection was seen in the case of period (τ) of circadian locomotor activity. All pre-treated flies had period τ shorter than 24 hours with mean of 21.80 ± 0.84 hours (Fig. 5). After drug injection all insects significantly (p<0.001) lengthened their period τ with mean of 24.70 ± 2.13 (Fig. 5). Percentages of all investigated flies having different period τ before and after drug injection are included in Fig. 6. The highest percentage (about 45%) of control flies had a period between 21-22 hours, whereas after treatment in some cases the period exceeded

Cymborowski B. 2003. Effects of 5,7-dihydroxytriptamine (5,7-DHT) on circadian locomotor activity of the blow fly, *Calliphora vicina*. 8pp. *Journal of Insect Science*, 3:14, Available online: insectscience.org/3.14

3

Figure 1. Locomotor activity rhythms (actograms) of the blow fly, *Calliphora vicina* in constant dark at 26 ℃ (double plotted). It shows unperturbated free-running activity with τ = 20.9 h in the pre-treated fly. On Day 9, 0.5 μg of 5,7-DHT in 1μl of insect saline/ascorbic acid was injected (arrow) in early subjective day. This caused phase delay by a few hours. The level of locomotor activity was greatly reduced and period was lengthened to 21.4h. The reduction in activity level started 2 days after drug injection and after 4 more days normal level of locomotor activity was regained. Periodograms for the appropriate sections of the activity records are shown alongside the actograms. Lines are drawn through activity periods for better visual inspection of the phase shifts.

4
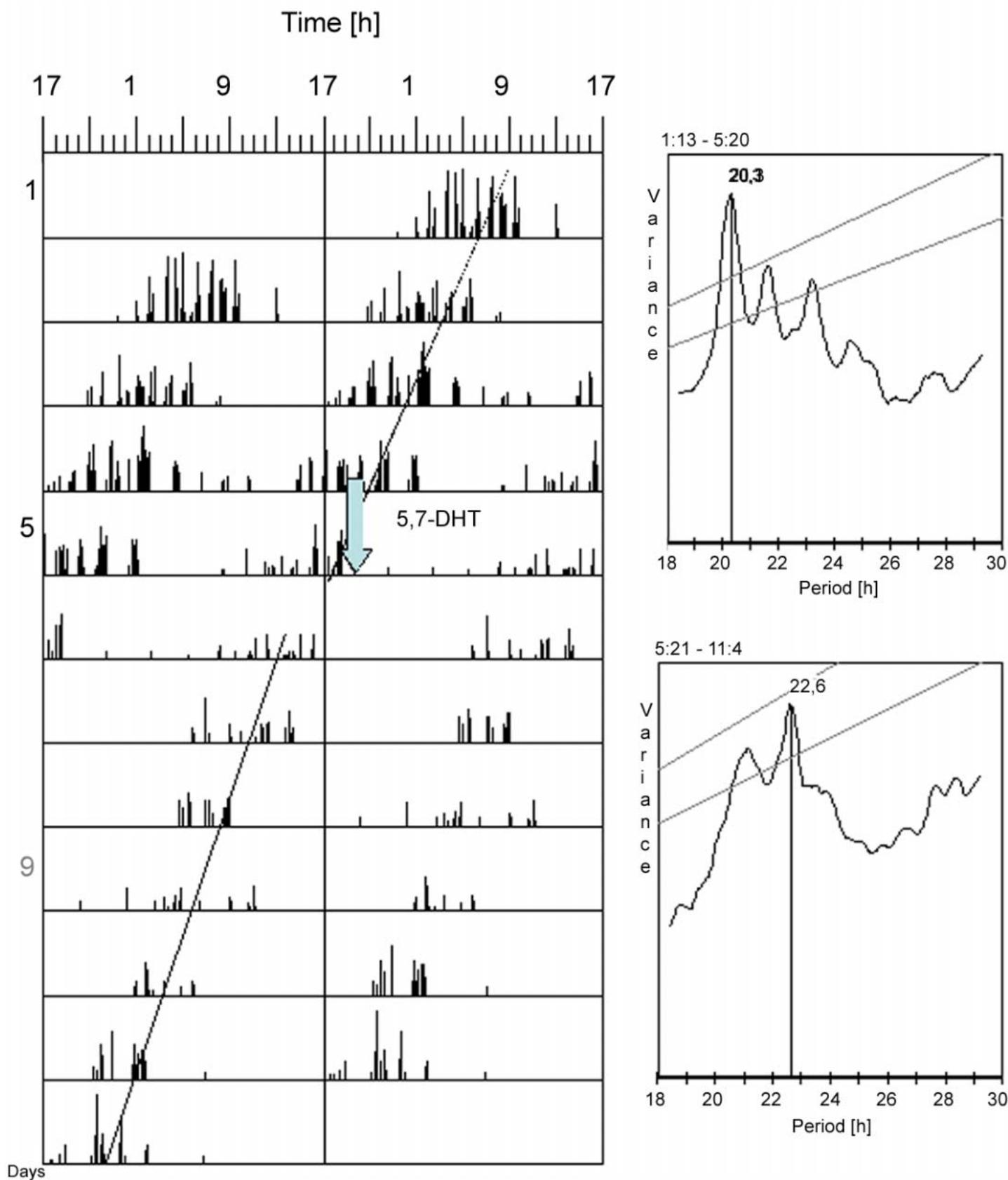
Figure 2. Locomotor activity rhythms of *Calliphora vicina* under constant dark conditions. The drug injection in the late subjective day caused a phase advance and lengthening of τ from 20.3h before treatment to 22.6 after administering the drug. Also the level of activity was very low after treatment. Further explanation see Figure 1.
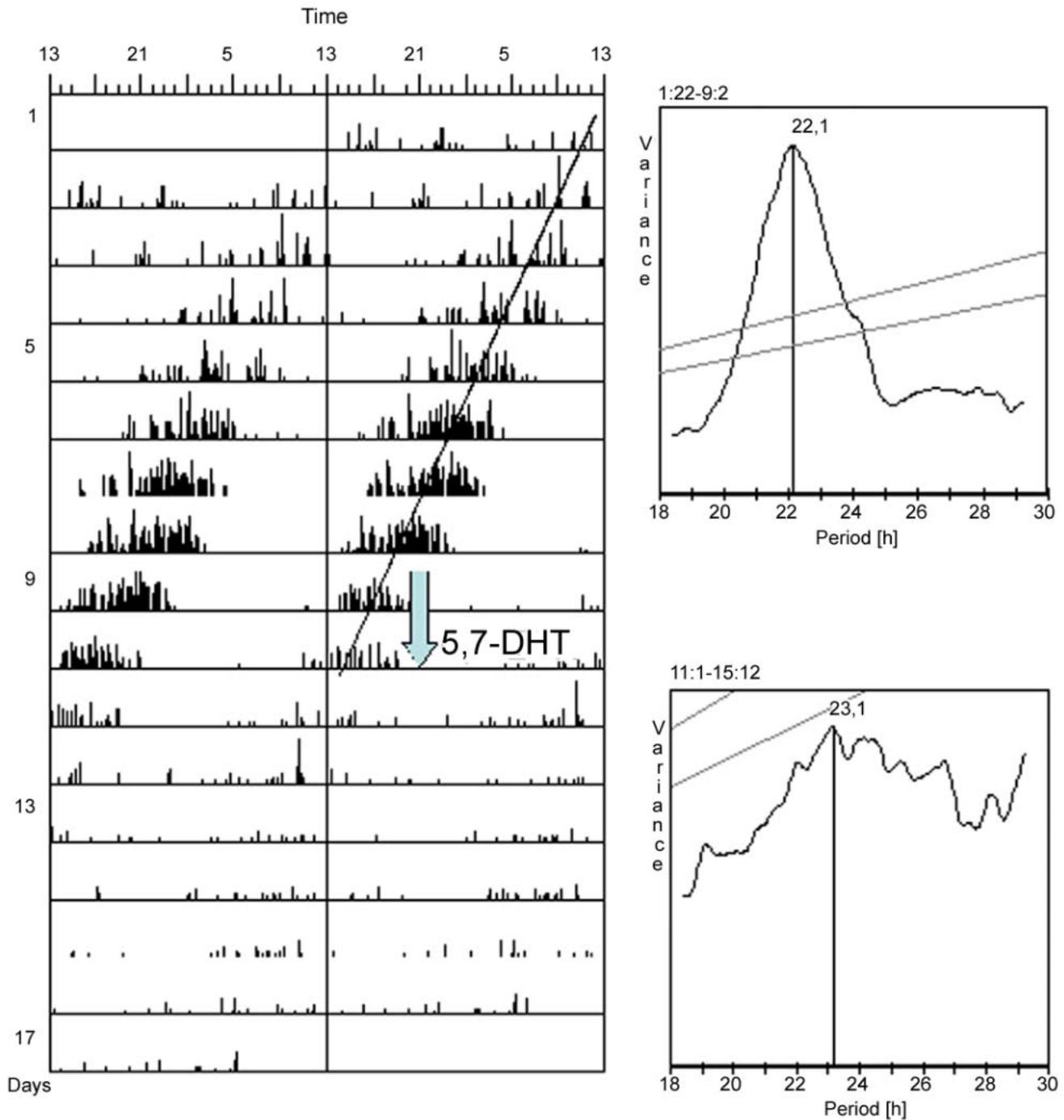
Figure 3. Actograms (double plotted) of a rhythms of the blow fly in constant darkness before and after injection of 0.5 μg of 5,7-DHT in 1 μl of insect saline/ascorbic acid on Day 10. After drug injection fly became completely arrhythmic with a substantially reduced level of locomotor activity. For further explanation see Figure 1.

even 28-29 hours (Fig. 6).

Discussion

The locomotor activity rhythm of the blow fly, *C. vicina* has been studied previously and the present study confirms the pattern of the blow fly's activity and the period of its circadian rhythm as previously reported (Cymborowski and King, 1996). A previous study also showed that a circadian clock regulating this rhythm is located in the central part of the brain because removal of

the optic lobes did not disturb this rhythm (Cymborowski et al., 1994)

Flies with 5,7-DHT injected into the abdomen showed that the locomotor activity rhythm different from that of controls injected only with solvent. Injection of the drug resulted in significant reduction of the activity level. Reduction in the level of locomotor activity in the cricket (*A. domesticus*) was also observed after injection into the hemolymph of reserpine and LSD, which depleted the nervous system of biogenic amines, including serotonin (Cymborowski, 1970). Injection of serotonin into the protocerebrum
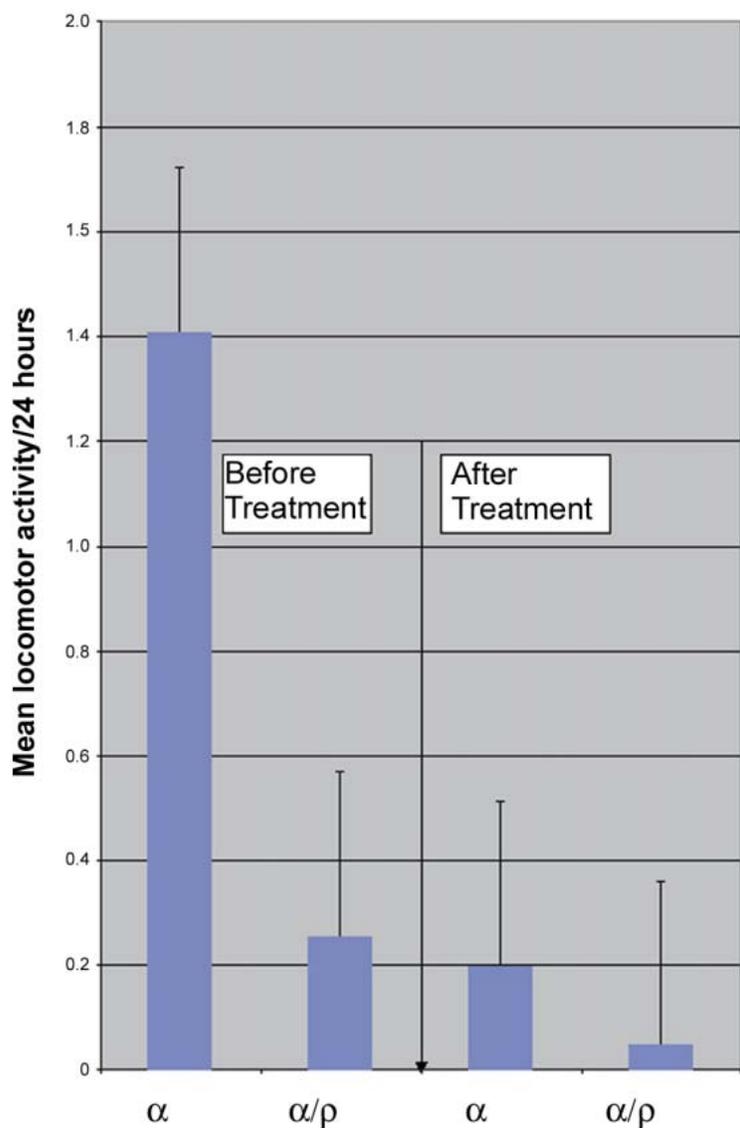
Figure 4. Mean locomotor activity (α) per 24 h of recording and coefficient (α/ρ) before injection of 5,7-DHT and after treatment with this compound. Both parameters were significantly ($p<0.001$) reduced after drug injection.

of this insect resulted in a gradual increase in locomotor activity levels (Cymborowski and Muszynska-Pytel, 1974). It is interesting that treated flies usually needed 2-3 days for the activity to decrease. Probably during this period of time, the serotonin level in the brain gradually decreases. In crickets (*G. bimaculatus*) after injection of this drug into the optic lobe the serotonin level was reduced to 50% of that in the Ringer's injected animals when examined 4 weeks after injection (Germ and Tomioka, 1998).

In addition to the behavioral and pharmacological studies (Cymborowski and Muszynska (1974), molecular correlates of behavioral state (rest and activity) in insects were identified (Greenspan *et al*, 2001). Most significantly, it was found that several genes that were upregulated during waking versus rest in *Drosophila melanogaster* correspond to genes that are upregulating during waking wersus sleep in the rat. One of the genes exhibiting state-dependent expression is an enzyme involved in the catabolism of serotonin, dopamine, and octopamine, arylalkamine acetyltransferase (aaNAT), also known as dopamine

acetyltransferase (Dat), (Maranda and Hodgetts, 1977). Its expression is 70% higher during waking and 50% higher after sleep deprivation (Shaw et al., 2000). These findings suggest a potential role for serotonin as modulator of circadian rhythms in insects including the regulation of locomotor activity.

A common property of the circadian system is its sensitivity to light pulses and various non-photic treatments. This sensitivity is best characterized by a phase response curve that plots magnitude and direction (advance or delay) of the phase shifts caused by single pulse, as a function of the phase in the circadian cycle at which the pulses are given. (Pittendrigh, 1960; Page, 1987; Cymborowski et al., 1993; Cymborowski et al., 1996). In contrast to the light pulse phase response curve, that for 5,7-DHT treatment is of "inverted" type with phase delay in the early subjective day and phase advance in the late subjective day (see Figures 1 and 2). This suggests that this drug exerts its phase control through the "output" pathway from the clock to the regulated system (overt activity) via serotonin (5-HT), a physiological factor that may act as internal Zeitgeber by feeding onto overt rhythmicity activities such as locomotion (Saunders, 2002).
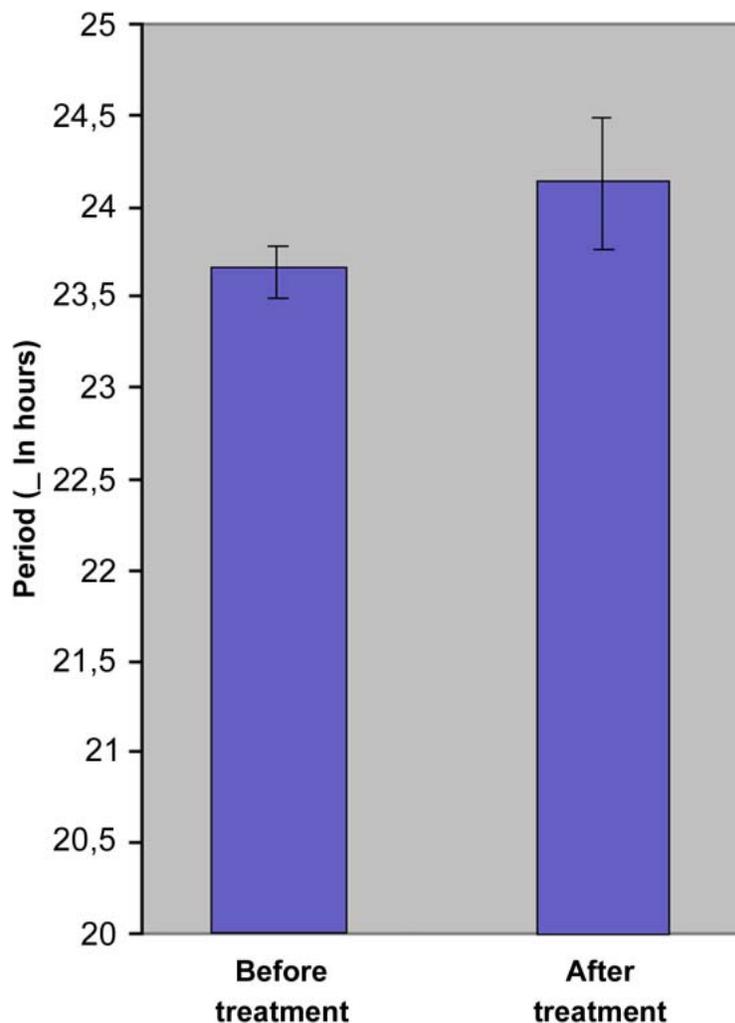
Figure 5. Mean ($\pm$ SD) length of the period (τ) in hours before and after treatment with 5,7-DHT drug. The period after treatment significantly ($p<0.001$) increased.
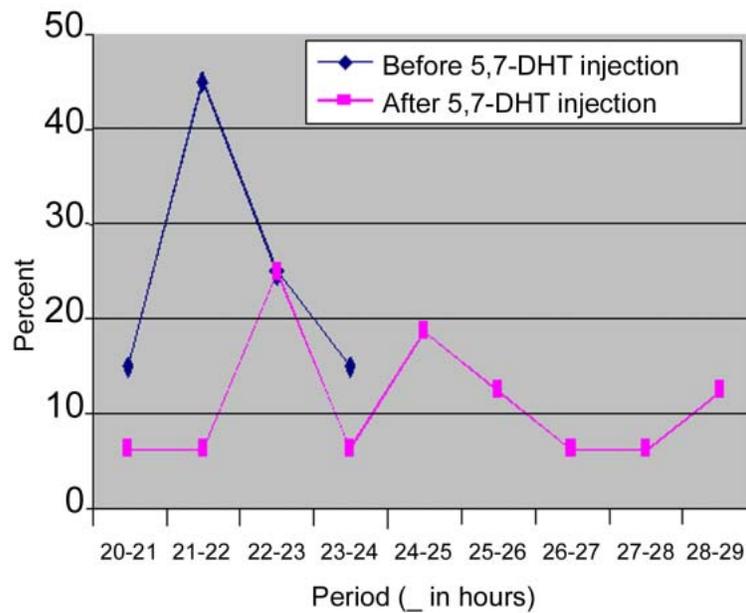
Figure 6. Cumulative percentage of all investigated flies having different period τ before (rhomboid symbols) and a after 5.7-DHT treatment (square symbols). The highest percentage (about 45%) of control flies had period τ between 21-22 hours, whereas after treatment in some cases the period exceeded 28-29 hours.

The present results also show that 5,7-DHT injection caused changes in the period (τ) of circadian locomotor activity. Changes in period after single light pulses have been described so far (Pittendrigh, 1960; Eskin et al., 1982). For example in rodents, exposure to single 15 min light pulse caused increases in τ associated with phase delay, and decreases in τ associated with phase advances (Pittendrigh and Daan, 1976). It was suggested that phase-dependent changes in τ had a functional significance in the entrainment phenomenon (Pohl, 1982). Photic entrainment in some insect species imparts a stable behavioural rhythm long after the entraining photoperiod has ended. This was the case with cockroach *Leucophaea maderae*, which strongly entrains to different photoperiods. (Page et al., 2001). For example, when cockroaches were entrained to T22 and T26 h days, the τ in constant darkness was 0.7 h shorter in T22 than in T26 h days and lasted for 40 days of recordings.

Acknowledgements

References

Ali D. 1997. The aminergic and peptidergic innervation of insect salivary glands. *Journal of Experimental Biology*. 200:1941-1949.

Birthelmer A, Schweizer T, Jeltsch H, Jackisch R, Cassel J-CH. 2002. 5,7-dihydroxtryptamine lesions enhance and serotoninergic grafts normalize the evoked overflow of acetyclcholine in rat hippocampal slices. *European Journal of Neuroscience* 16:1839-1849.

Cymborowski B. 1970. The assumed participation of 5-hydroxytiptamine in regulation of the circadian rhythm of locomotor activity in *Acheta domesticus. Comparative and General Pharmacology*. 1: 316-322.

Cymborowski B. 1998. Serotonin modulates a photic response in circadian locomotor rhythmicity of adults of the blow fly, *Calliphora vicina. Physiological Entomology* 23:25-32.

Cymborowski B, Gillanders SW, Hong S-F, Saunders DS. 1993. Phase shifts of the adult locomotor activity rhythm in *Calliphora vicina* induced by non-steroidal ecdysteroid agonist RH-5849. *Journal of Comparative Physiology*. A 172:101-108.

Cymborowski B, Hong S-F, McWatters HG, Saunders DS. 1996. S-antigen antibody partially blocks entrainment and the effect of constant light on the circadian rhythm of locomotor activity in the adult blow fly, *Calliphora vicina. Journal of Biological Rhythms* 11:68-74.

Cymborowski B, King V. 1996. Circadian regulation of *Fos*-like expression in the brain of the blow fly *Calliphora vicina. Comparative Biochemistry and Physiology* 115C:239-246.

Cymborowski B, Lewis RD, Hong S-F, Saunders DS. 1994. Circadian locomotor activity rhythms and their entrainment to light-dark cycles continue in the flies (*Calliphora vicina*) surgically deprived of their optic lobes. *Journal of Insect Physiology* 40:501-510.

Cymborowski B, Muszynska-Pytel M. 1974. The Effect of Some Psychotropic Drugs on the Circadian Rhythm of Locomotor Activity of *Acheta domesticus* L. *Journal of interdisciplinary Cycle Research* 5:362-370.

Dugar A, Patanov C, O'Callaghan JP, Lakoski JM. 1998. Immunohistochemical localization and quantification of glial fibrillary acidic protein and synaptosomal-associated protein (mol. wt 25000) in the ageing hippocampus following administration of 5,7-dihydroxytryptamine. *Neuroscience* 85:123-133.

Eskin A, Corrent G, Lin C.Y, McAdoo DJ. 1982. Mechanism for shifting the circadian rhythmicity by serotonin: involvement of cAMP. *Proceedings of the National Academy of Sciences USA* 79: 660-664.

Evans P. 1980. Biogenic amines in the insect nervous system. *Advances in Insect Physiology*. 15:317-473.

Germ M, Tomioka K. 1998. Effects of 5,7-DHT Injection into the Optic Lobe on the Circadian Locomotor Rhythm in the Cricket, *Gryllus bimaculatus. Zoological Science* 15:317-322.

Maranda B, Hodgetts R. 1977. A characterization of dopamine acetyltransferase in *Drosophila melanogaster. Insect Biochemistry* 7:33-43.

Greenspan RJ, Tononi G, Cirelli C, Shaw P. 2001. Sleep and the fruit fly. *Trends in Neuroscience* 24:142-145.

Mistlberger RE, Bossert M, Holmes M, Marchant EG. 1998. Serotonin and feedback effects of behavioral activity on

circadian rhythms in mice. *Behavioural Brain Research* 96:93-99

Morin L P, Blanchard J. 1991. Depletion of brain serotonin by 5,7-DHT modifies hamster circadian rhythm response to light. *Brain Research* 566: 173-185.

Muszynska-Pytel M, Cymborowski B. 1978a. The role of serotonin in regulation of the circadian rhythms of locomotor activity in the cricket (*Acheta domesticus* L.). I. Circadian variation in serotonin concentration in the brain and hemolymph. *Comparative Biochemistry and Physiology* 59C:13-15.

Muszynska-Pytel M, Cymborowski B. 1978b. The role of serotonin in regulation of the circadian rhythms of locomotor activity in the cricket (*Acheta domesticus* L.). II. Distribution of serotonin and variation in different brain structure. *Comparative Biochemistry and Physiolog* 59C:17-20.

Nässel DR, Cantera R. 1986. Mapping of serotonin-immunoreactive neurons in the larval nervous system of the flies *Calliphora erythrocephala* and *Sarcophaga bullata.* A comparison with ventral ganglia in adult animals. *Cell and Tissue Research* 239: 423-434.

Page T L. 1987. Serotonin phase-shifts the circadian rhythm of locomotor activity in the cockroach. *Journal of Biological Rhythms* 2:23-34.

Page T L, Mans C, Tomioka K. 2001. History dependence of circadian pacemaker period in the cockroach. *Journal of Insect Physiology* 47:1085-1093.

Pittendrigh C S. 1960. Circadian rhythms and circadian organization of the living systems. *Cold Spring Harbor Symposium on Quantitative Biology* 25:159-182.

Pittendrigh C S, Daan S. 1976. A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. *Journal of Comparative Physiology* 106:223-252.

Pohl H. 1982. Characteristics and variability in entrainment of circadian rhythms to light in diurnal rodents. In: Aschoff J, Daan S, Groos GA, editors. *Vertebrate Circadian Systems.* Springer, pp 339-346. Berlin.

Pyza E, Cymborowski B. 2001. Circadian rhythms in behaviour and in the visual system of the blow fly, *Calliphora vicina. Journal of Insect Physiology* 47: 897-904.

Pyza E, Meinertzhagen IA. 1996. Neurotransmiters regulate rhythmic size changes amongst cells in the fly's optic lobe. *Journal of Comparative Physiology* A 178: 33-45.

Saunders DS. 1987. Maternal influence on the incidence and duration of larval diapause in *Calliphora vicina. Physiological Entomology* 12: 331-338.

Saunders DS. 2002. *Insect Clocks,* 3rd edition. Elsevier

Seidel C, Bicker G. 1996. The developmental expression of serotonin-immunoreactivity in the brain of the pupal honeybee." *Tissue and Cell* 28: 663-672.

Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. 2000. Correlates of sleep and waking in *Drosophila melanogaster. Science* 287: 183-187.

Sinhababu AK, Borchardt RT. 1988. Molecular mechanism of biological action of the serotonergic neurotoxin 5,7-dihydroxytryptamine. *Neurochemistry International* 12: 273-284.

Sokolove PG, Bushell WN. 1978. The chi square periodogram: its utility for analysis of circadian rhythms. *Journal of Theoretical Biology* 72: 131-160.

Strambi C, Bennis N, Renucci M, Charpin P, Augier R, Strambi A, Cymborowski B, Puizillout J-J. 1989. Serotonin-Immunoreactive Neurons in the Cerebral Complex of *Acheta domesticus.* Experimental Study in Normal and Drug Treated Insects. Zoologischer Jarbucher Abteilung fur Algemeine Zoologie und Physiologie der Tiere. 93: 353-374.

Tomioka K, Ideka M, Nagao T, Tamotsu S. 1993. Involvement of serotonin in the circadian rhythm of an insect visual system. *Naturwissenschaften* 80:137-139.