

SPONTANEOUS LOCOMOTOR ACTIVITY OF *DROSOPHILA SUBOBSCURA* UNDER CONTROLLED LABORATORY CONDITIONS

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Abstract - The aim of this research was to examine the temporal patterns of spontaneous locomotor activity in *Drosophila subobscura* virgin females and males from isofemale lines collected from beech and oak forests and maintained in controlled laboratory conditions for five full-sib inbreeding generations. The flies were monitored in a circular open field arena for 30 min in the morning (8:00 AM, 9:00 AM, 10:00 AM, 11:00 AM). The distance travelled and the mobility of the flies decreased along with the registration time, whereby the slowest decreasing was observed at 8:00 AM. Females showed a greater range of variability in both parameters compared to males. In addition, there was a greater range of variability in the locomotor activities of flies from the oak forest compared to the beech. These findings could be attributed to their specific genetic background.

Key words: Distance travelled, mobility time, beech, oak, *Drosophila subobscura*

INTRODUCTION

Organisms live in complex environments, varying in space and time, and consequently their behavior is subject to modulation by various abiotic and biotic factors. The adaptive advantage of organisms in variable environments depends on behavioral and physiological functions. Locomotor activity is an important trait of animal behavior since it is implicated either directly or indirectly in everyday life activities. It facilitates animals to disperse, to compete and find food, to mate, avoid any danger, save territory and to respond to different stress situations (Mayr, 1963; Dingemanse et al., 2010). Locomotor activity *per se* is a complex type of behavior (Martin, 2004) and thus it is important to classify the different aspects of movement, such as distance travelled, mobility time and speed. The rapid progress of technology and software

for the observation, registration and analysis of animal behavior could give us the opportunity to detect these particular aspects of motor activity. Götz (1980) developed Buridan's paradigm video scanning device that is used for the automatic tracking of a walking fly during free choice between two visual landmarks. Many papers deal with the different parameters of locomotor activity, such as walking activity and speed, straightness of walk, and time course of activity (Götz, 1989; Strauss et al., 1992; Strauss and Heisenberg, 1993; Martin and Ollo, 1996; Strauss and Pichler, 1998; Serway et al., 2009).

Drosophila is one of the most applicable and very useful model-systems to investigate the behavior in invertebrates. In an early study, spontaneous walking activity in an open field apparatus was used to select the active/inactive wild type strains of *Dro-*

sophila (Connolly, 1966) and later for single gene mutants with aberrant locomotor activity (Meehan and Wilson, 1987; Burnet et al., 1988; Wang et al., 1997; Pendleton et al., 2002; Lehmann and Cierotzki, 2010). The locomotor activity of *Drosophila* flies has also been extensively analyzed with respect to circadian rhythms (Hall, 1995; Joshi et al., 1999; Sheeba et al., 2002; Klarsfeld et al., 2003; Rosato and Kyriacou, 2006; Lu et al., 2008; Bahn et al., 2009; Allada and Chung, 2010). Stoleru et al. (2005) showed by behavioral and molecular assays that in *Drosophila* there are two separated neuronal circuits that with an oscillator-coupling mechanism provide morning and evening locomotor activity. These neuronal structures have different sensitivity to light and temperature and their functionality is coupled to each other while these two factors are constant (Lear et al., 2009).

Drosophila subobscura has not often been an object in studies about locomotor activity. It is well known that *D. subobscura* exhibits a rich polymorphism in most of their chromosomes that is associated with changeable environmental factors (Kenig et al., 2010).

They also show obvious geographical, habitat-related and diurnal variations (Krimbas, 1993; Zivanovic et al., 1995; Orenco and Prevosti, 1996; Andjelkovic et al., 2003; Balanya et al., 2004; Savkovic et al., 2004), as well as having a significant fluctuation in effective population size across seasons (Stamenković-Radak et al., 2008). All these facts together motivate the analysis of spontaneous locomotor activity, as a fitness component of *D. subobscura*.

The aim of the present research was to examine the temporal patterns of the morning spontaneous locomotor activity of *D. subobscura* females and males originating from isofemale lines and maintained in controlled laboratory conditions for five full-sib inbreeding generations. Spontaneous locomotion or reactivity was perceived as observable activity exhibited by a fly movement not specifically activated by external stimuli (Martin, 2003).

Moreover, in this kind of movement the influences of the internal factors, such as circadian rhythm, food or drink deprivation, age, sex, strain etc., are involved.

The flies used in this study were collected from two ecologically different habitats and two parameters of locomotor activity, distance travelled and mobility were monitored in a new environment, a circular open field arena, in four morning time intervals. The contribution of this study is to reveal the general pattern of morning activity, as well as possible differences in the locomotor activity of females and males collected from the beech and oak forests.

MATERIALS AND METHODS

Drosophila stocks

The flies, *D. subobscura*, were collected from two localities on the Serbian mountain Goč: beech (*Abieto-Fagetum*, N 43°33'28.43" and E 20°45'10.96") and oak (*Fraxineto-Quercetum*, N 5 43°32'57.38" and E 20°40'2.32") forests. The air-distance between these two localities is approximately 6.9 km at different expositions. They have quite distinct microclimate conditions. The beech forest has more stable environmental factors that are reflected in high humidity, with dense vegetation coverage and feeble daylight. In contrast, the oak forest has changeable environmental factors during the day. It is slightly warmer with lower humidity, has sparse trees and therefore robust daylight.

The experimental isofemale (IF) lines (David et al., 2005) were derived each from a single gravid female collected in the wild. There were five IF lines from each forest. They were maintained for five full-sib inbreeding generations at 19°C, humidity 60%, on a standard cornmeal medium (9% sugar, 10% cornmeal, 2% agar, 2% yeast, nipagin dissolved in 96% ethanol) in a 12h:12h light:dark cycle (lights turned on at 6:00 AM) and at 300 lux illumination. The flies were collected on the first day after eclosion and females were separated from males under CO₂ anesthesia.

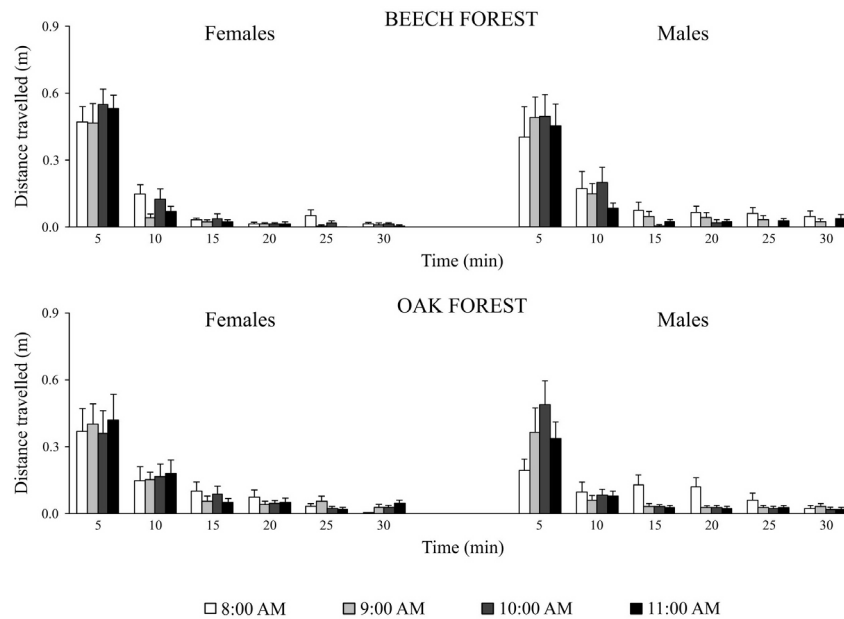


Fig. 1. Distance travelled of *D. subobscura* flies from the beech and oak forests in a circular open field arena. The results are presents as mean \pm SEM (n= 11-13)

Locomotor activity monitoring

The locomotor activity of *Drosophila* flies was monitored for 30 min in a circular open field arena in the morning (8:00 AM, 9:00 AM, 10:00 AM and 11:00 AM). The progeny of each IF line were mixed. From the pooled IF lines of each locality, about 30 randomly chosen flies per sex were tested at each morning hour. Different flies were released anew each hour for measurement. Single 3-5 day-old *D. subobscura* virgin females and males were released separately to move in empty plastic Petri dish (35 mm diameter, 10 mm high to maximally limit vertical and flying movement) directly before measurement. The locomotor activity of seven flies was simultaneously videotaped with a camera (Microsoft LifeCam VX600) positioned above the dishes. All experiments were performed in an isolated room at 19°C and 300 lux illumination. ANY-maze software (v.4.73, Stoelting Co., Wood Dale, Illinois, USA) was used to analyze the distance travelled (the total distance in meters that an animal travelled during the test) and mobility (the amount of time in seconds the animal was mobile during the test), as a measure of locomotor activity.

Statistical analysis

The results are presented as the means \pm SEM of the distances travelled and mobility for 30 min, in 5-min time intervals. The normal distribution of data was assessed using the Shapiro-Wilk test. The observed values were transformed to logarithms for distance travelled and square roots for mobility in order to stabilize variance. The method used to analyze the measured parameters of locomotor activity was repeated ANOVA, followed by a *post hoc* LSD test, where appropriate. The following factors were taken into account as possible sources of variability in the monitored locomotor activity: 1) locality (beech and oak); 2) sex (female and male); 3) morning hours (8:00 AM, 9:00 AM, 10:00 AM and 11:00 AM); 4) 5 min time interval (as repeated factor) and 5) first and second level interactions.

A statistical test that yields more information about differences in locomotor activity during the observation period is simple linear regression. All statistical analysis was performed using Statistics 5.0 software (Copyright StatSoft, Inc. 1995).

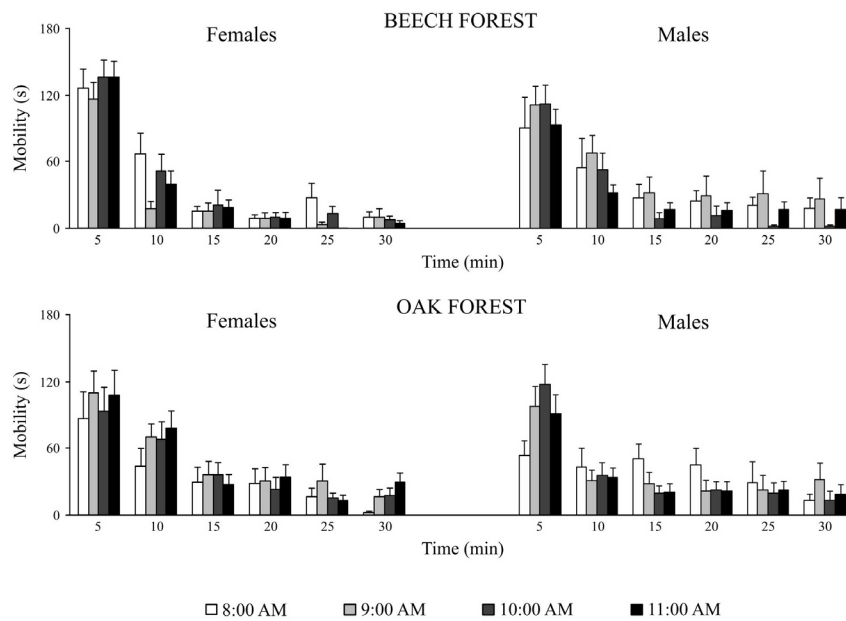


Fig. 2. Mobility of *D. subobscura* flies from the beech and oak forests in a circular open field arena. The results are presents as mean \pm SEM (n= 11-13)

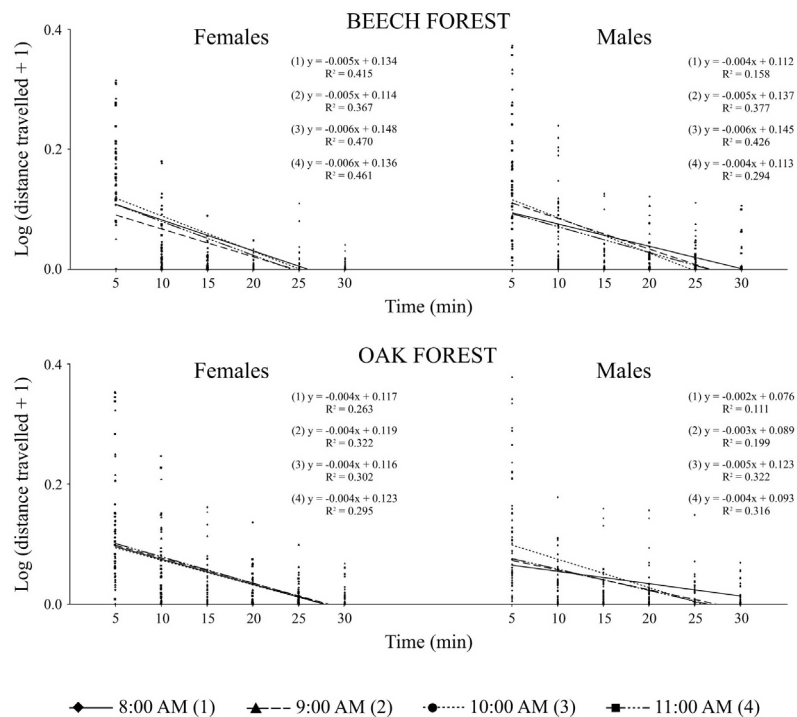


Fig. 3. Regression lines with equations and coefficients of determination for distance travelled of *D. subobscura* flies the beech and oak forests

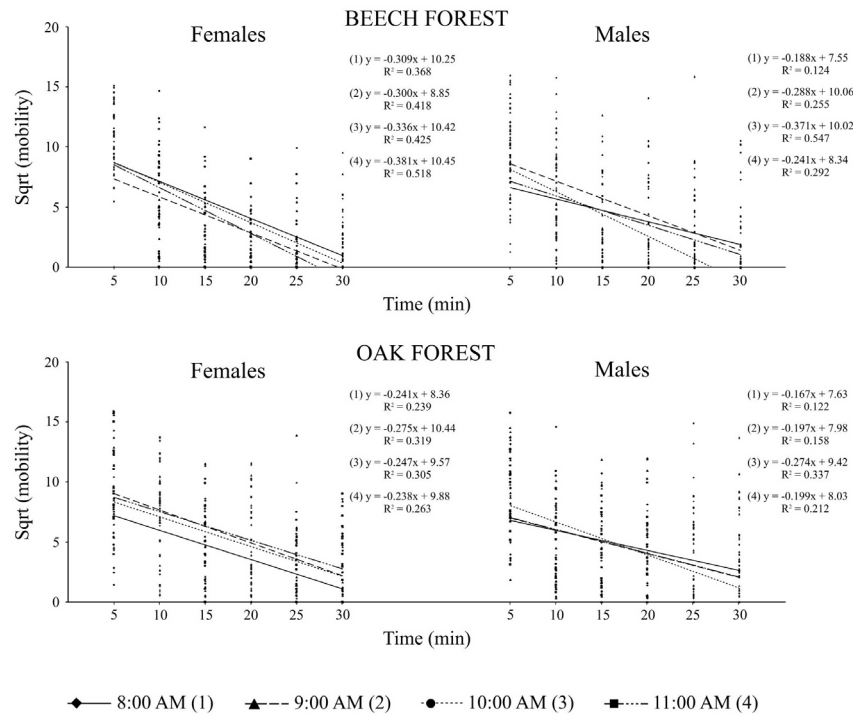


Fig. 4. Regression lines with equations and coefficients of determination for mobility of *D. subobscura* flies the beech and oak forests

RESULTS

Mean values of distance travelled and mobility during the whole period of locomotor activity monitoring are presented in Table 1. Females and males from both localities had similar mean values of distance travelled and mobility. The range of these values was 0.5-0.8 m for distance travelled and 172-294 s for mobility.

The results of repeated ANOVA (Table 2) showed significantly greater mobility in flies from the oak forest. At each morning hour, significantly, the highest mean values of distance travelled and mobility of *D. subobscura* females and males from the examined localities were observed in the first 5 min time interval (Figs. 1 and 2). Their locomotor activity rapidly decreased in the first 10 min and maintained a low level until the end of monitoring.

Regression analysis and analysis of variances of the regression lines of distance travelled and mobility

of the *D. subobscura* females and males from both localities were performed. Regression lines with equations and coefficients of determination are presented in Figs. 3 and 4.

At each morning hour, simple linear regression analysis (Table 3) showed a significant decreasing in distance travelled and mobility during the whole period of locomotor activity monitoring. The highest values of the examined parameters were observed in the first 5 min time interval for both sexes from each locality. The percentage of the total variation in distance travelled and mobility, that is measured and showed by the fitted regression, was defined with a coefficient of determination (R^2), which represents a measure of the strength of the straight line relationship. The total variation in distance travelled of females from the beech forest was in the range of 37-47%, while that of females from the oak forest was 26-32%. A similar range of variability was found for the mobility of females from the beech (37-52%) and oak (24-32%) forests. The distance travelled and

Table 1. Distance travelled and mobility of *D. subobscura* females and males from the beech and oak forests during the whole period of locomotor activity monitoring

		DISTANCE TRAVELLED (m)				MOBILITY (s)			
		8:00 AM	9:00 AM	10:00 AM	11:00 AM	8:00 AM	9:00 AM	10:00 AM	11:00 AM
BEECH	Females	0.73 ± 0.11	0.55 ± 0.10	0.75 ± 0.10	0.65 ± 0.08	254 ± 36	172 ± 25	240 ± 33	208 ± 28
	Males	0.81 ± 0.25	0.78 ± 0.14	0.73 ± 0.15	0.65 ± 0.10	233 ± 70	293 ± 75	185 ± 30	189 ± 26
OAK	Females	0.73 ± 0.19	0.74 ± 0.13	0.71 ± 0.18	0.77 ± 0.17	209 ± 60	294 ± 40	253 ± 42	290 ± 38
	Males	0.63 ± 0.15	0.54 ± 0.13	0.68 ± 0.13	0.51 ± 0.11	233 ± 64	231 ± 60	226 ± 44	206 ± 39

The results are presented as mean ± SEM (n = 11-13).

mobility of males from the beech forest had a wide range of variability: 16-43% and 12-55%, respectively. In contrast, the locomotor activity of males from the oak forest had a narrow range of variability: 11-32% for distance travelled and 12-34% for mobility. Distance travelled and mobility of each sex from the beech and oak forests were more variable at 8:00 AM and 9:00 AM.

The slopes (Fig. 3) at 8:00 AM and 9:00 AM indicated that the mean distance travelled by the females from the beech forest significantly decreased by 0.005 m, and at 10:00 AM and 11:00 AM by 0.006 m in each successive 5-min time interval. The mean distance travelled by the females from the oak forest significantly decreased by 0.004 m in each successive 5 min time interval at all of the morning hours. Males from the beech forest significantly decreased their mean distance travelled by 0.003 m at 8:00 AM, 0.005 m at 9:00 AM and 10:00 AM, and 0.004 m at 11:00 AM in each successive 5 min time interval. The males from the oak forest displayed a significant decrease in the mean distance travelled by 0.002 m at 8:00 AM, 0.003 m at 9:00 AM and 11:00 AM, and 0.004 m at 10:00 AM in each successive 5 min time interval. The slopes at 8:00 AM, 9:00 AM and 10:00 AM indicated that the mean mobility of the females from the beech forest significantly decreased by 0.3 s, and at 11:00 AM by 0.4 s in each successive 5 min time interval. Females from the oak forest significantly decreased their mean mobility by 0.2 s at 8:00 AM, 10:00 AM and 11:00 AM, and by 0.3 s at 9:00 AM in each suc-

cessive 5-min time interval. The slopes for the males from both localities showed similar trends, and the males from the beech forest significantly decreased their mean mobility at 8:00 AM and 11:00 AM by 0.2 s, and by 0.3 s and 0.4 s at 9:00 AM and 10:00 AM, respectively, in each successive 5-min time interval. The mean mobility of the males from the oak forest significantly decreased by 0.2 s at 8:00 AM, 9:00 AM and 11:00 AM, and by 0.3 s at 10:00 AM in each successive 5-min time interval.

DISCUSSION

The open field test is very useful for the analysis of the exploratory behavior and general activity of different organisms, including *Drosophila*, in controlled conditions and after some genetic and pharmacological manipulations (Hay, 1980; Liu et al., 2007; Valente et al., 2007).

In the present study, *D. subobscura* flies showed a characteristic pattern of morning spontaneous locomotor activity in a circular open field arena. It involved the highest value in the first 5 min, followed by a rapid decrease in the next 10 min and then maintaining the same level until the end of monitoring. Initial hyperactivity could be simply explained by novelty-induced exploration (Liu et al., 2007). The authors suggest a crucial role of Kurtz nonvisual arrestin in the nervous system in this initial phase and dopamine in the later phase of fly activity in the open field.

Table 2. Repeated ANOVA analysis

DISTANCE TRAVELLED			
	df	MS	F
Locality	1	0.001	0.159
Sex	1	0.002	0.438
Morning hour	3	0.002	0.427
Locality × Sex	1	0.010	2.400
Locality × Morning hour	3	0.000	0.070
Sex × Morning hour	3	0.001	0.229
Locality × Sex × Morning hour	3	0.002	0.542
ERROR	175	0.004	
5-min time interval	5	0.518	251.332 ***
Locality × 5-min time interval	5	0.016	7.956 ***
Sex × 5-min time interval	5	0.003	1.355
Morning hour × 5-min time interval	15	0.004	2.012 *
Locality × Sex × 5-min time interval	5	0.004	1.843
Locality × Morning hour × 5-min time interval	15	0.001	0.527
Sex × Morning hour × 5-min time interval	15	0.002	0.927
Locality × Sex × Morning hour × 5-min time interval	15	0.001	0.492
MOBILITY			
	df	MS	F
Locality	1	134.247	4.481 *
Sex	1	21.265	0.710
Morning hour	3	2.873	0.096
Locality × Sex	1	27.362	0.913
Locality × Morning hour	3	23.037	0.769
Sex × Morning hour	3	13.910	0.464
Locality × Sex × Morning hour	3	51.202	1.709
ERROR	175	29.959	
5-min time interval	5	1466.898	191.647 ***
Locality × 5-min time interval	5	46.856	6.122 ***
Sex × 5-min time interval	5	14.097	1.842
Morning hour × 5-min time interval	15	12.944	1.691 *
Locality × Sex × 5-min time interval	5	18.399	2.404 *
Locality × Morning hour × 5-min time interval	15	4.450	0.581
Sex × Morning hour × 5-min time interval	15	8.725	1.140
Locality × Sex × Morning hour × 5-min time interval	15	7.277	0.951

*p<0.05 and ***p<0.001

Table 3. The significance of the regression lines

			FEMALES			MALES		
			df	MS	F	df	MS	F
BEECH	Distance travelled	8:00 AM	70	0.14	49.56 ***	70	0.07	13.18 ***
		9:00 AM	64	0.10	37.03 ***	70	0.14	42.31 ***
		10:00 AM	64	0.17	56.64 ***	70	0.18	51.95 ***
		11:00 AM	70	0.17	59.84 ***	76	0.10	31.57 ***
	Mobility	8:00 AM	70	500.58	40.77 ***	70	185.48	9.94 **
		9:00 AM	64	433.10	45.99 ***	70	434.46	24.01 ***
		10:00 AM	64	543.10	47.24 ***	70	723.48	84.56 ***
		11:00 AM	70	763.39	75.22 ***	76	331.50	31.37 ***
OAK	Distance travelled	8:00 AM	70	0.09	25.03 ***	70	0.02	8.70 **
		9:00 AM	70	0.09	33.24 ***	76	0.06	18.82 ***
		10:00 AM	70	0.09	30.33 ***	76	0.13	36.13 ***
		11:00 AM	64	0.09	26.81 ***	64	0.06	29.56 ***
	Mobility	8:00 AM	70	304.38	21.95 ***	70	145.92	9.72 **
		9:00 AM	70	396.15	32.72 ***	76	220.98	14.23 ***
		10:00 AM	70	319.85	30.76 ***	76	428.26	38.65 ***
		11:00 AM	64	271.51	22.87 ***	64	190.02	17.25 ***

p<0.01 and *p<0.001

It is known that species from the *Drosophila* genus have two diurnal peaks in locomotor activity at dawn and dusk. The *Drosophila* brain consists of two physically separated but functionally coupled neuronal clocks that control that type of behavior (Helfrich-Förster, 2005; Umezaki and Tomioka, 2008). Neurons in the ventral lateral and dorsal lateral part of the brain have a role in the control of morning and evening activity, respectively (Stoleru et al., 2004).

The results of this study indicated that the spontaneous locomotor activity of *D. subobscura* flies was even more different in the morning hours, namely the slowest decrease in activity was registered at 8:00 AM. Therefore, we propose that the observed peculiarities in the morning activity of flies could be attributed to diurnal variations of neuronal activity in the part of brain responsible for its control. Moreo-

ver, 8:00 AM could be considered as the period of the most prominent activity of neurons involved in control of exploratory behavior and locomotor activity. In further behavioral studies, this morning hour could be more appropriate for an estimation of possible hypo- and hyperlocomotion effects induced by different treatments.

These results also suggest that the duration from starting exposure to light is important to spontaneous locomotor activity in *D. subobscura*. Furthermore, our registration of locomotor activity started 2 h after lights were turned on and the males showed the slowest decreasing of activity earlier than the females. In our study the activity for both virgin females and males was monitored and differences between them were detected but showed no statistical significance. Sexual differences in lo-

comotor behavior have been reported previously (Helfrich-Forster, 2000; Shaw et al., 2000) and the brain area that controls these differences has been mapped (Belgacem and Martin, 2002). In constant laboratory conditions (12:12h light:dark) males have a peak in locomotor activity around the time when the light is turned on and when it is turned off. Females, both virgin and mated, show a peak of locomotor activity around 1 h after lights-on and stay active longer than males. During the day the locomotor activity of virgin females is more similar to males compared to the evident robust locomotor activity of mated females.

Changes in gene activity, biochemistry, physiology and behavior were found in all eukaryotes and some cyanobacteria through the cycle of days and nights. The circadian rhythm is fully endogenous and persists even when organisms are placed in constant light or dark conditions. The circadian clock can be reset by external cues, of which light is one of the most important (Hirota and Fukada, 2004; Dubruille and Emery, 2008). When an animal is exposed to light, there is activation of the genes with which their promoters are connected by the protein transcription factors responsible for synchronizing the circadian clock. The existence of a light-dependent switch mechanism(s) enables better separating of the active periods of the fly at dawn and dusk, and may help the animal to adapt to seasonal changes in day length (Picot et al., 2007). Zhang et al. (2010) revealed a new mechanism(s) that integrates light and temperature inputs to control locomotor rhythms and which may contribute to a better plasticity of circadian behavior.

Locomotor activity is a good indicator of the overall physiological and biochemical state of an individual (Fleury et al., 2000; Gibert et al., 2001), as well as an important component of fitness (Gilchrist et al., 1997). Type of habitat, the natural environment in which an organism lives, or the physical environment that surrounds a population, are crucial for the prediction of different organism activity. In the present study some variations in the morning activity of *D. subobscura* females and males from the beech

and oak forests were revealed. A greater range of variability in distance travelled and mobility of flies from the oak forest compared to flies from the beech forest was observed. In each locality, females showed a greater range of variability in locomotor activity. These results obtained on flies maintained in controlled laboratory conditions for five full-sib inbreeding generations, namely under optimal environmental conditions, indicated possible differences in the genetic background of the flies collected from two ecologically different habitats (beech and oak forests). In previous analyses of the inversion polymorphism, it was shown that these populations have a different frequency of some gene arrangements (Andjelkovic et al., 2003; Stamenkovic-Radak et al., 2008; Jelic et al., 2009). Different gene arrangements can carry various alleles at specific genetic loci that are differently favored in diverse environmental conditions. Various combinations of alleles could be related to gene loci that are responsible for various physiological and biochemical responses. This means that five full-sib inbreeding generations have fixed some homozygote combinations and coadaptive complexes, which could be essential for adaptation to different environments.

In conclusion, these findings point out the importance of behavior monitoring at exactly defined periods of the day. Observed differences in the locomotor activities of flies from beech and oak forests, maintained in controlled laboratory conditions, could be connected to their genetic background and natural habitat.

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