

# Inbreeding Affects Locomotor Activity in *Drosophila melanogaster* at Different Ages

Tommaso Manenti · Cino Pertoldi · Neda Nasiri · Mads Fristrup Schou · Anders Kjærsgaard · Sandro Cavicchi · Volker Loeschcke

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**Abstract** The ability to move is essential for many behavioural traits closely related to fitness. Here we studied the effect of inbreeding on locomotor activity (LA) of *Drosophila melanogaster* at different ages under both dark and light regimes. We expected to find a decreased LA in inbred lines compared to control lines. We also predicted an increased differentiation between lines due to inbreeding. LA was higher in the dark compared to the light regime for both inbred and outbred control lines. As expected, inbreeding increased phenotypic variance in LA, with some inbred lines showing higher and some lower LA than control lines. Moreover, age per se did not affect LA neither in

control nor in inbred lines, while we found a strong line by age interaction between inbred lines. Interestingly, inbreeding changed the daily activity pattern of the flies: these patterns were consistent across all control lines but were lost in some inbred lines. The departure in the daily pattern of LA in inbred lines may contribute to the inbreeding depression observed in inbred natural populations.

**Keywords** Inbreeding depression · Circadian rhythm · Daily activity · Light:dark regime · Ageing

## Introduction

Locomotor activity (LA) can be defined as the active movement by an animal (e.g. Bahrndorff et al. 2012). LA is closely associated with many fitness traits (Wisco et al. 1997) such as the ability to locate food (Kristensen et al. 2008b), courtship and mating activities (Partridge et al. 1987; Long and Rice 2007), colonizing new habitats, escaping predators (Dingemanse and Reale 2005) and coping with stressful conditions (Loeschcke and Hoffmann 2007; Kjærsgaard et al. 2010; Overgaard et al. 2010).

LA responses to regular environmental changes are coordinated by an endogenous circadian clock system (Martin et al. 1999). This internal clock is important for organisms as it facilitates the optimization of behavioural activities at the appropriate time of day (Panda et al. 2002). The circadian clock plays an important role not only for daily cycles of light and dark but also for seasonal fluctuations in diurnal rhythms (Stoleru et al. 2004). Different approaches have been used to study LA. Pittendrigh (1954) counted fly emergence in intervals over the course of the day in order to quantify LA in different *Drosophila* species. In the early 1970s, LA of *Drosophila melanogaster* was assayed by

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T. Manenti (✉) · C. Pertoldi · N. Nasiri · M. F. Schou · A. Kjærsgaard · S. Cavicchi · V. Loeschcke  
Department of Bioscience, Genetics, Ecology and Evolution,  
Aarhus University, Ny Munkegade 114-116, Buildg. 1540,  
8000 Aarhus C, Denmark  
e-mail: tommaso@biology.au.dk

C. Pertoldi  
Department of Biotechnology, Chemistry and Environmental  
Engineering - Section of Biology and Environmental Science,  
Aalborg University, Sohngårdsholmsvej 57, 9000 Aalborg,  
Denmark

C. Pertoldi  
Aalborg Zoo, Aalborg, Denmark

A. Kjærsgaard  
Institute of Evolutionary Biology and Environmental Studies,  
University of Zurich, Winterthurerstrasse 190, 8057 Zurich,  
Switzerland

testing clock period mutants (Konopka and Benzer 1971). In the late 1990s a larger interest in LA arose from the discovery of the conserved nervous system development between insects and mammals (Hirth and Reichert 1999). Some genes that control LA in *Drosophila* may therefore play a role in human neurodegenerative diseases. For example, some genes that affect LA in *Drosophila* and mice (Tunnicliff et al. 1969) have been shown to play a role in Parkinson's disease (Olanow and Tatton 1999; Chen et al. 2005). The identification and mapping of genes associated with the circadian clock in behavioural mutants of *D. melanogaster* (*Period*, *Timeless*, *Clock*, *Cycle*, *Double time*, *Shaggy*, *Casein Kinases 2alpha and beta*) (Young and Kay 2001; Allada 2003; Blau 2003) have further revealed that even complex behavioural traits are under genetic control (Young and Kay 2001; Allada 2003; Blau 2003) and that a large number of pleiotropic genes exhibit correlated transcriptional responses to multiple behaviours (Jordan et al. 2006). Furthermore, Jordan et al. (2006) found a strong response of *D. melanogaster* to selection for both high and low LA, indicating a presence of genetic variation.

Inbreeding results in increased homozygosity (Whitlock and Fowler 1999), leading to the expression of deleterious recessive alleles and thus a decrease in individual fitness (Charlesworth and Willis 2009). Inbreeding can increase environmental sensitivity (Armbruster and Reed 2005; Fox and Reed 2011), sterility and decrease mating success (Sharp 1984), as well as lead to decreased productivity and increased developmental time (Frankham 1995; Bijlsma et al. 2000; Hill and Mackay 2004; Armbruster and Reed 2005; Kristensen et al. 2008a). Increasing environmental stress has been shown to increase inbreeding depression (Fox and Reed 2011; Reed et al. 2012) and one study found inbreeding to decrease adaptive phenotypic plasticity (Auld and Relyea 2010). However, despite these well-known effects of long-term natural stressors on inbred organisms, it is unknown how inbreeding affects the ability to track and respond to daily periodic changes in the environment. A commonly investigated behavioural response to the daily cycle is LA, however, few studies have focussed on the relationship between LA and inbreeding. Jordan et al. (2007) studied LA in inbred lines of *D. melanogaster* and identified several pleiotropic candidate genes, including *Drop* and *Turtle*, to play an important role in establishing neural circuits and coordinate the activity of flies.

Behavioural responses to environmental stimuli are important throughout the life of most organisms, but they may, however, not be constant with increasing age as ageing in general results in decreased LA in *D. melanogaster* (Lebourg and Lints 1984). Lebourg (1987) also showed that age influences the daily fluctuation of LA in relation to the light regime in *D. melanogaster*. Therefore, any effect of inbreeding on the behavioural response of LA to the daily cycle may be influenced by age.

This study compares LA of control and inbred lines of *D. melanogaster* at different ages (3, 10 and 17 days) under light and dark regimes. We aimed to investigate how inbreeding affects absolute LA and the daily pattern of LA. In addition, including flies of different ages allowed us to identify possible interactions between age and LA. We did not find any decrease in the total LA due to inbreeding. We found an increased diversification between inbred lines and a genotype by age interaction. Interestingly, the main inbreeding effect manifested itself in the daily pattern of LA.

## Materials and methods

### Line establishment

Lines were established from a laboratory population of *D. melanogaster*, founded in 2010 by 589 wild-caught females (for details on the laboratory population see Schou et al. 2014). Two generations after population establishment, three control lines (A, B, C) and six inbred lines (5, 6, 7, 9, 10, 11) were established. To generate each of the control lines, 250 virgin females and 250 virgin males were randomly sampled from the founder population, while only five virgin females and five males were used to establish the inbred lines. The lines were maintained under constant population sizes and with a 1:1 sex ratio for 25 generations, by only allowing 500 or 10 flies respectively to contribute to the next generation. During the 25 generations the flies were reared at a standard agar-sugar-yeast-oatmeal medium (Leeds medium) and maintained at a daily thermal and light regime following a Gaussian distribution. The night temperature was  $23.5 \pm 0.5$  °C and the daily midday temperature peak was at  $27.5 \pm 0.5$  °C, resulting in a mean daily temperature of  $24.8 \pm 0.5$  °C.

The experiment was performed at generation 25 where the inbreeding coefficient of the inbred lines was close to 0.70 (Crow and Kimura 1972).

### Phenotypic assessments

Experimental flies were density controlled during development by allowing 30 females and 30 males from each line to lay eggs for 12 h on spoons containing Leeds medium. 40 eggs were placed into each of several 7 mL vials with fresh Leeds medium and maintained under 12:12 L:D and at  $25 \pm 1$  °C. To avoid effects of CO<sub>2</sub> anaesthesia (Nicolas and Sillans 1989), emerging male flies were collected without CO<sub>2</sub> anaesthesia and maintained in separate vials. Due to the space restriction in the LA monitor, we choose only to assess males in order to not decrease the statistical power of our analyses. Furthermore, the link

between fitness and male LA is easier to interpret because males need to find, track and court females, which, on the other hand, may show lower LA levels to avoid attention from males and to direct resources to egg-production. At 3, 10 and 17 days of age, 14 randomly selected males from each line were used to assess LA rhythms in a *Drosophila* Activity Monitor system (DAM, Trikinetics, Waltham, MA, USA) (Suh and Jackson 2007; Catterson et al. 2010), which scores the number of times a fly interrupts an infrared light beam in the middle of a small glass vial (diameter = 0.5 cm, length = 5 cm). Every vial contained one male fly, allowing us to determine the LA of individual flies. Data were recorded every 5 s over a 24 h period. We used four monitors allowing simultaneous automated measurement of 128 samples. To avoid the impact of starvation and desiccation during the 24 h of testing we provided an agar-based food source in one end of the vial, which was sealed with Parafilm to prevent the food from drying out. The other end of the vial was closed with a cotton stopper to facilitate air circulation. The vials were placed horizontally in the monitor with the cotton stoppers on the same side. The recordings were performed under standard conditions 12:12 L:D and at  $25 \pm 1$  °C.

#### Statistical analysis

The data, originally consisting of 24 h of measurements, were split into two groups of 10 h, with the exclusion of data from the first and the last hour of both of the light and dark regimes. These data-points were not used because *Drosophila* LA is highly sensible to both dark-to-light and light-to-dark transitions, as well as to mechanical disturbances during transfer to the monitor and the incubator (Allada and Chung 2010).

The effect of inbreeding on the total amount of LA was analysed by calculating the sum of recorded scores of each single fly under light and dark regime. A two-way ANOVA was performed to test the effects of inbreeding and age on LA. The data were log-transformed to meet the assumptions of normality and homogeneity of variances. The sources of variation tested were: (a) comparison between inbred and control lines (inbreeding effect) and (b) an age effect. As LA was strongly affected by light and dark regime (see below) the analysis was performed separately for the dark and light regime. To assess the influence of inbreeding on the daily pattern of LA fluctuations (c), the LA data of each individual (*i*) during the 20 h were grouped in 5 min intervals, resulting in 240 time intervals for each age tested (Fig. 1). These values were subsequently transformed by a cumulative function (CLA), where each value of LA of an individual at a specific time interval was added to the sum of LA of the same individual at all previous time intervals ( $CLA_{i,t}$ ). To make the

cumulative LA among lines comparable, it was individually normalized by dividing each value of cumulative LA by the value of cumulative LA at time 240 ( $CLA_{i,240}$ ).

$$CLA_{i,t} = \sum_{t=1}^{240} LA_{i,t}$$

$$NCLA_{i,t} = \frac{CLA_{i,t}}{CLA_{i,240}}$$

This allowed us to compare the pattern of LA between lines that had different total LA. For example, a line that is mainly light active has a flat normalized cumulative LA (NCLA) during the dark regime and increases NCLA in the light regime, a straight line indicates a constant LA over 24 h, while a sinuous curve indicates high variation of LA during the 24 h. The NCLA showed a starting value of 0 at time 1 and a value of 1 at time 240 (Fig. 2) in all lines. Differences in the daily pattern of LA between lines were tested by one-way ANOVA at the time corresponding to the dark to light transition.

Two different linear models were compared at each age. In the first model all three control lines were kept separately while in the second model the three control lines were pooled together into one line.

We used R statistical software for all analyses (version 3.0.2) (Gui 2013). Age was treated as a fixed effect, and line as a random effect. We used the package “Lme4” to test the mixed model ANOVA (Douglas et al. 2013).

## Results

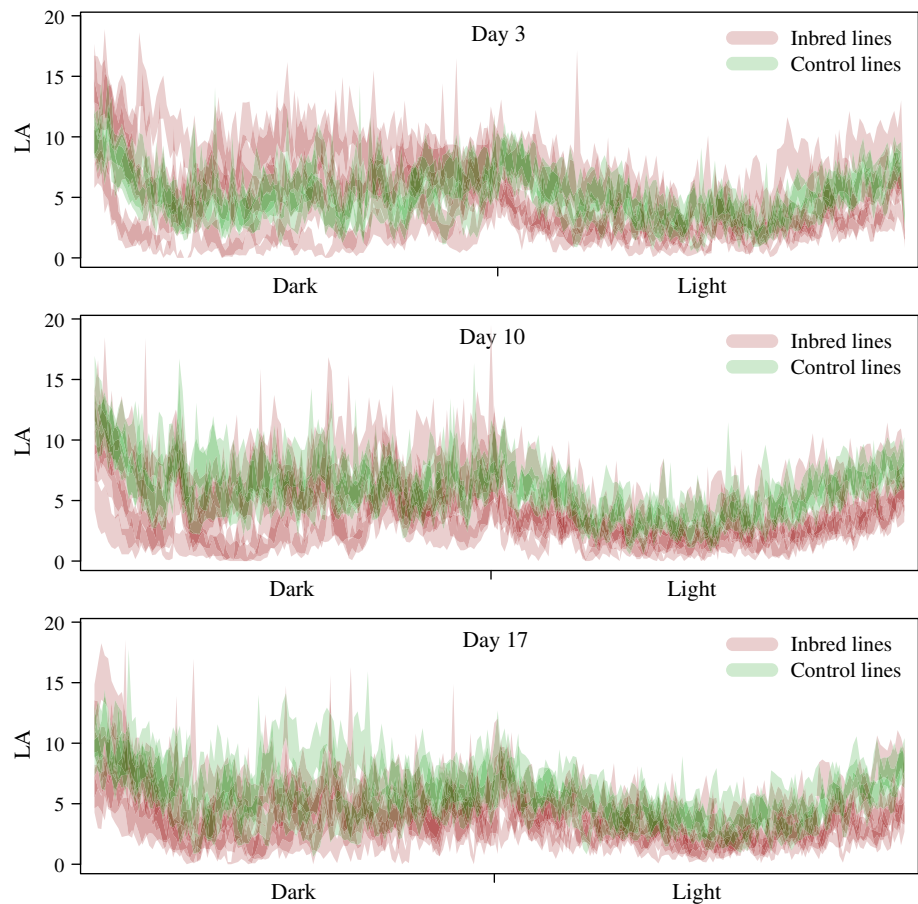
### Inbreeding

Both the inbred and control lines showed increased LA during the dark compared to the light regimes at each age class (age 3-day old,  $F_{(1,119)} = 125.6$ ,  $P < 0.001$ ; age 10-day,  $F_{(1,113)} = 153.15$ ,  $P < 0.001$ ; age 17-day old,  $F_{(1,97)} = 98.4$ ,  $P < 0.001$ ). Therefore, all subsequent analyses were performed separately for dark and light regimes. During the dark regime, neither of the ages in the inbred nor the control lines were different. Looking at the LA between control and inbred lines separately, control lines showed a similar LA, while a significant difference was detected between inbred lines (Table 1). During the light regime, control lines showed higher LA than inbred lines, with the exception of day 3 (Table 1, Fig. 3). Similar to the dark regime, differences between lines were found only between inbred lines and not between control lines at all ages (Table 1, Supplementary Table 1).

### Ageing

Similar results were obtained for the dark and light regimes as the flies aged: the total LA did not change significantly

**Fig. 1** Daily locomotor activity (LA) at age 3-day old, 10-day old and 17-day old under light (Light) and dark (Dark) regimes. Control lines are represented in *green* and the inbred lines in *red*. The area covered by a line represents the standard error of the mean. The colours are transparent, such that overlap between error bars is visualized by a darker colour. The LA data of each individual during the 20 h was grouped in five minutes intervals, resulting in 240 time intervals for each age. Each line counts approximately 15 samples (Color figure online)



with advancing age in both control and inbred lines (Table 2). The LA of control lines was similar between different age classes. Inbred lines showed a significant line by age interaction for both dark and light regimes. Inbred lines were different at all ages and in both light and dark regime (Table 2, Supplementary Table 1).

#### Daily pattern of LA

Flies generally showed two peaks of activity at the light-to-dark and dark-to-light switch (Fig. 1). The linear model with the three control lines kept separately did not differ from the linear model with the three control lines pooled together at each time interval (Fig. 1). Therefore, subsequent analyses were performed with the pooled control lines.

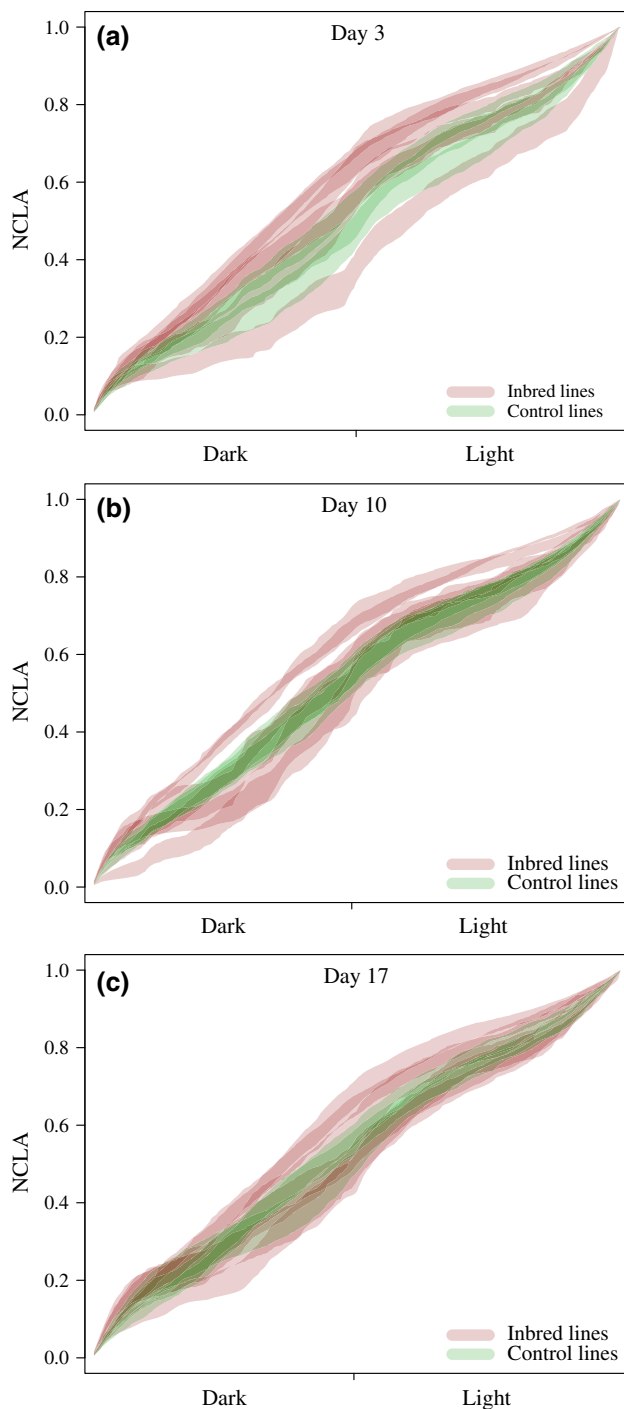
Inbreeding affected the daily pattern of LA at each age class (age 3:  $F_{(6,119)} = 9.2$ ,  $P = 2.6 e^{-8}$ ; age 10:  $F_{(6,113)} = 3.2$ ,  $P = 0.007$ ; age 17:  $F_{(6,97)} = 7.5$ ,  $P = 2.0 e^{-7}$ ) (Fig. 2). Tukey test showed that, at 3 days of age, inbred lines 5, 6 and 9 showed a different NCLA compared to control lines ( $P = 0.01$ ,  $P < 0.001$ ,  $P = 0.02$ , respectively). At 10 and 17 days of age, only the inbred line 9

was different from controls ( $P = 0.04$  and  $P = 0.6 e^{-3}$ , respectively) (Fig. 2, Supplementary Figs. 1–3).

#### Discussion

Despite though locomotor activity (LA) being a heritable trait correlated with fitness (Partridge et al. 1987; Dingemans and Reale 2005), and although inbreeding is common in natural populations (Crnokrak and Roff 1999), not many studies have investigated the inbreeding effect on LA (Fernandez et al. 1999; Jordan et al. 2007). In this paper we explored the effects of inbreeding on LA at different age classes as well as how inbreeding affects the daily activity pattern of flies.

When inbred lines were compared to control lines using the total LA, significant differences were observed only at 10 and 17 days of age in the light regime. This was not in agreement with our expectations that inbreeding leads to a decrease in LA at all ages (Frankham 1995; Falconer and Mackay 1996; Roff 1998). Inbreeding did not result in reduced LA but in an increased line differentiation. One should notice that there were also inbred lines that had a



**Fig. 2** Daily locomotor activity after cumulative transformation (NCLA) at age 3-day old (a), 10-day old (b) and 17-day old (c) under light (L) and dark (D) regimes. Control lines are represented in green and the inbred lines in red. The area covered by a line represents the standard error of the mean. The colours are transparent, such that overlap between error bars is visualized by a darker colour. The LA data described in Fig. 1 were subsequently transformed by a cumulative function (CLA) and normalized (NCLA) (Color figure online)

significantly higher LA than control lines at 3 days of age (Fig. 3). Unlike most fitness related traits such as productivity and viability, LA can be assumed to have an intermediate phenotypic optimum if the level of LA is adaptive. Thus a lower or a higher LA can be considered as inbreeding depression, and in essence any departure from the control value can be interpreted as inbreeding depression.

LA of many organisms is governed by daily cycles of light and dark regimes (Nash et al. 2002). *Drosophila* reacts to light-to-dark and dark-to-light transitions with increasing LA (Fig. 1). Flies had been kept under the same light regime, as used during this study, for almost a year before this experiment was initiated. We can therefore assume that the samples were not very stressed by the transitions in the light cycles, a feature that surely can alter LA (Wheeler et al. 1993). We found that *D. melanogaster* lines kept at constant  $25 \pm 1$  °C were most active at night. For this reason, an effect of inbreeding was expected mainly during this regime. Contrary to this expectation, differences between control and inbred lines were found only in the light regime, and at ages 10 and 17 days. *Drosophila* shows various behaviours associated with specific photoperiods (Helfrich-Forster 2000), e.g. the early morning peak of eclosion (Pittendrigh 1954). So, inbreeding might influence some behavioural responses that are specific or exclusive for the light regime.

Theories of ageing predict a decreasing activity and metabolic rate with ageing (Fukagawa et al. 1990; Fernandez et al. 1999). Ageing did not affect the total LA of neither control nor inbred lines, but a significant interaction of age by inbred line was observed. Fernandez et al. (1999) investigated LA throughout life (more than 40 days) of 5 inbred lines of *D. melanogaster*. They detected an age-dependent decline in LA, however this trend was only observed in 2 out of 5 inbred lines. The difference in the results between the two studies is likely to be found in the difference in power as well as the fact that we tested flies from 3 to 17 days of age. In this limited time period flies could not reach the seniority stage, characterized by high mortality rate. Therefore, a strong age effect might not be expected during the investigated time period.

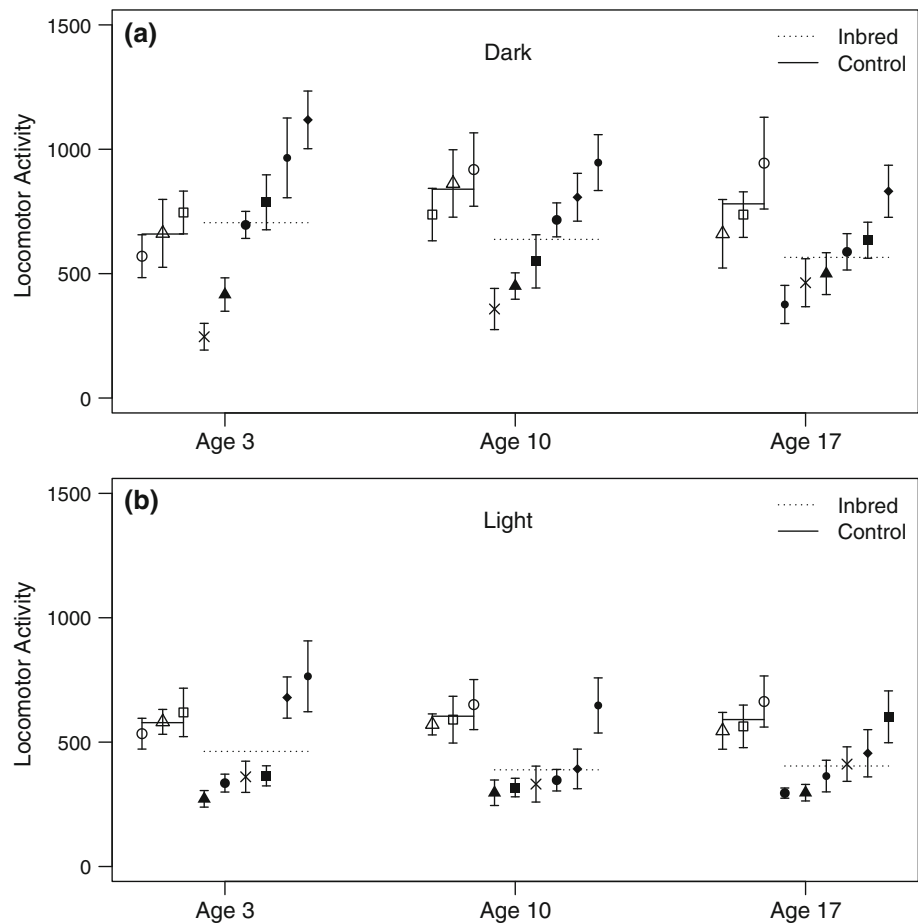
Even though age did not affect total LA within inbred and control lines, an interesting age by line interaction was seen in inbred lines. This confirms the genetic differentiation between inbred lines, as a consequence of strong genetic drift, which resulted in different effects of age on the genetic control of LA: some inbred lines showed decreased or increased LA with age, while the LA of the control lines did not seem to be affected by age.

**Table 1** ANOVA (mixed model) analysis for LA at three different ages (3, 10 and 17-day old)

Source of var	3 (days)		10 (days)		17 (days)	
	F	P(F)	F	P(F)	F	P(F)
<b>Dark regime</b>						
Between inbred/control	0.04 <sub>(1,108)</sub>	0.84	2.17 <sub>(1,103)</sub>	0.18	3.17 <sub>(1,96)</sub>	0.12
Within control	0.69 <sub>(2,39)</sub>	0.5	0.51 <sub>(2,39)</sub>	0.6	1.08 <sub>(2,37)</sub>	0.35
Within inbred	10.44 <sub>(5,68)</sub>	<0.001	6.22 <sub>(1,63)</sub>	<0.001	3.83 <sub>(1,58)</sub>	<0.001
<b>Light regime</b>						
Between inbred/control	0.92 <sub>(1,108)</sub>	0.37	7.55 <sub>(1,103)</sub>	0.02	5.9 <sub>(1,96)</sub>	0.04
Within control	0.35 <sub>(2,39)</sub>	0.71	0.25 <sub>(2,39)</sub>	0.78	0.51 <sub>(2,37)</sub>	0.6
Within inbred	7.2 <sub>(5,68)</sub>	<0.001	3.36 <sub>(1,63)</sub>	<0.001	2.39	0.04

Degrees of freedom are given within parentheses  
*F* F-ratio, *P*(*F*) probability associated with F-ratio

**Fig. 3** LA ( $\pm$ SEM) at 3, 10 and 17 days of age, under dark (a) and light (b) regimes. Open symbol: control line, closed symbol: inbred line. The solid and dotted lines represent mean LA for control or inbred lines, respectively



A strong inbreeding effect on the daily pattern of LA was observed (Fig. 1, Fig. 2). Control lines showed approximately the same patterns of LA over 24 h. The daily pattern of LA, quantified by the NCLA, at each age class was similar in the three control lines (Supplementary Figs. 1–3). However, in some inbred lines (lines 5, 6, 9) we detected a changed daily pattern of LA (Supplementary Figs. 1–3). They seem to have lost the ability to track the time of the day and display behaviour accordingly. The

cause of this alteration is not easy to identify because of the complexity of circadian pathways (Allada and Chung 2010). It might be due to changes in the mechanism used by flies to collect information from the environment, e.g. the photoreceptor neurons in the compound eyes of flies (Zerr et al. 1990; Ewer et al. 1992) or in brain pacemaker neurons (Lu et al. 2008). Assuming that the daily pattern of LA shown by the control lines represents adaptive behaviour, any departure from this pattern will be maladaptive as

**Table 2** ANOVA analysis of ageing effect on LA

Source of var	Dark regime			Light regime	
	DF	F	P(F)	F	P(F)
<b>Control</b>					
Between lines	2,115	1.49	0.23	0.14	0.87
Between ages	2,115	0.75	0.48	0.13	0.88
Lines × age	4,115	0.31	0.5	0.4	0.81
<b>Inbred</b>					
Between lines	5,219	15.655	<0.001	3.88	<0.001
Between ages	2,219	0.26	0.77	1.7	0.19
Lines × ages	10,219	3.35	<0.001	2.11	0.02

DF degrees of freedom, F F-ratio, P(F) probability associated with F-ratio

important behavioural responses such as stress avoidance, locating food and predator avoidance can be impaired. The departure in the daily pattern of LA observed in multiple inbred lines in this study exemplifies how inbreeding can result in a changed daily pattern of LA, which can, eventually, contribute to the inbreeding depression observed in inbred natural populations.

In conclusion, inbreeding led to an increased diversification between inbred lines and to a genotype by age interaction. Inbreeding changed the behaviour of *D. melanogaster*, affecting the daily pattern of LA. Future investigations should repeat the LA comparison between inbred and control lines during the whole lifespan of flies to test if inbreeding effects on LA change at older age.

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**Conflict of Interest** Tommaso Manenti, Cino Pertoldi, Neda Nasiri, Mads Fristrup Schou, Anders Kjærsgaard, Sandro Cavicchi, and Volker Loeschcke declare that they have no conflict of interest.

**Human and Animal Rights** All institutional and national guidelines for the care and use of laboratory animals were followed.

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