RESEARCH ARTICLE

Circadian clocks and life-history related traits: is pupation height affected by circadian organization in *Drosophila melanogaster*?

DHANASHREE A. PARANJPE[1], D. ANITHA[1], VIJAY KUMAR SHARMA[1] and AMITABH JOSHI[2]*

[1]*Chronobiology Laboratory,* [2]*Evolutionary Biology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, P.O. Box 6436, Jakkur, Bangalore 560 064, India*

Abstract

In *D. melanogaster*, the observation of greater pupation height under constant darkness than under constant light has been explained by the hypothesis that light has an inhibitory effect on larval wandering behaviour, preventing larvae from crawling higher up the walls of culture vials prior to pupation. If this is the only role of light in affecting pupation height, then various light : dark regimes would be predicted to yield pupation heights intermediate between those seen in constant light and constant darkness. We tested this hypothesis by measuring pupation height under various light : dark regimes in four laboratory populations of *Drosophila melanogaster*. Pupation height was the greatest in constant darkness, intermediate in constant light, and the least in a light / dark regime of LD 14:14 h. The results clearly suggest that there is more to light regime effects on pupation height than mere behavioural inhibition of wandering larvae, and that circadian organization may play some role in determining pupation height, although the details of this role are not yet clear. We briefly discuss these results in the context of the possible involvement of circadian clocks in life-history evolution.

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Introduction

Most organisms studied possess biological chronometers in the form of circadian clocks (Zordan *et al*. 2000), and some observations suggest that circadian clocks play an important role in determining the timing of key ontogenetic and reproduction related events that constitute the life-history of an organism (reviewed in Prasad and Joshi 2003). For example, the period of eclosion rhythm in mutants at the *per* locus in *Drosophila melanogaster* shows parallel differences with pre-adult development time, with short period mutants developing faster, and long period mutants slower, compared to wild type flies (Kyriakou *et al*. 1990). Similarly, the phase of mating rhythm, and the free-running period of locomotor activity rhythm, appear to change as correlated responses to selection for development time or lifespan and late life fertility in the melon fly *Bactocera cucurbitae* (Miyatake 1997, 2002). Light regime has also been shown to affect pre-adult development time (Sheeba *et al*. 1999), lifetime fecundity (Sheeba *et al*. 2000), and adult lifespan in *D. melanogaster* (Pittendrigh and Minis 1972; Klarsfeld and Rouyer 1998; Sheeba *et al*. 2000), as well as lifespan in blowflies (von Saint-Paul and Aschoff 1978). Yet, other lines of evidence indicate that the involvement of circadian clocks in determining life-history related trait phenotypes may be more subtle than often thought (Prasad and Joshi 2003). Circadian clocks, for instance, are expected to show fairly strong temperature compensation (Pittendrigh 1960), whereas life-stage duration is markedly affected by temperature in ectotherms, including *Drosophila* (David *et al*. 1983), suggesting that life-stage duration is unlikely to be directly determined by circadian clock period.

*For correspondence. E-mail: ajoshi@jncasr.ac.in.

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Pupation height, the height above the food medium at which *Drosophila* larvae in laboratory culture vials pupate, is genetically variable (Markow 1979; Bauer 1984), sensitive to various environmental factors like texture of food (De Souza *et al*. 1970), temperature, relative humidity and larval density (Sokal *et al*. 1960; Joshi and Mueller 1993), and is known to change as a correlated response to selection on a variety of life-history traits (Mueller and Sweet 1986; Joshi and Mueller 1996; Chippindale *et al*. 1997; Prasad *et al*. 2001). It has also been suggested that pupation height in *Drosophila*, especially in tall vials (20–25 cm height), is a character that reflects energy expended by larvae during the post-feeding wandering phase (Chippindale *et al*. 1997). This notion is supported by the finding that pupation height tends to decrease in populations selected for rapid development, as part of a syndrome of reduced energy expenditure in the pre-adult stages (Chippindale *et al*. 1997; Prasad *et al*. 2001).

Earlier studies on light regime effects on pupation behaviour in *D. melanogaster* have shown that *D. melanogaster* larvae tend to pupate at higher levels above the medium under constant darkness (DD) than under constant light (LL) (Markow 1979; but see also Schnebel and Grossfield 1986), and when given a choice between illuminated and dark sites, prefer to pupate in dark sites (Rizki and Davis 1953; Markow 1981; Manning and Markow 1981). These findings led to the view that light has an inhibitory behavioural effect on the wandering of post-feeding larvae and that the greater pupation height of *D. melanogaster* populations in the dark is perhaps an adaptation enabling larvae to avoid bright places with enhanced risk of heat stress, desiccation or predation, as compared to more shaded places (Markow 1979; Manning and Markow 1981; Schnebel and Grossfield 1986). If this view is indeed correct, it leads to the prediction that pupation height of *D. melanogaster* under different light / dark (LD regimes) should be intermediate between that in LL and DD conditions. In any LD regime, depending on the phase at which larvae begin wandering, they will experience either light or darkness for some period of time. Thus, at most, the pupation height in a LD regime could equal that in either DD or LL, but should not be more extreme that in these two aperiodic conditions. On the other hand, if circadian clocks are involved in some way in gating or otherwise affecting the initiation of wandering or pupariation, or the duration of wandering, pupation height could be affected by LD regimes in many ways, potentially differing from the prediction made above. In *D. melanogaster*, it is not clear if pupariation is under circadian control (Bakker and Nelissen 1963; Pittendrigh and Skopik 1970), although circadian control of pupariation has been demonstrated in a few species of scaptodrosophilids (Rensing and Hardeland 1967; Eeken 1974). We tested the hypothesis that the difference in pupation height in LL and DD is simply a consequence of a behavioural inhibition of larvae from climbing high under light by measuring pupation height in four populations of *D. melanogaster* under LL, DD, and three different LD regimes.

Materials and methods

We used four laboratory populations of *D. melanogaster* (LL 1–4) that have been maintained in a cubicle at 25°C (± 1°C), under constant light of intensity ~100 lux and constant humidity, on 21-day discrete generation cycle, at moderate larval densities of ~60–80 larvae per 8 dram vial (9.0 cm height × 2.4 cm diameter) containing banana-jaggery food medium. The origin and maintenance of these populations has been described in detail earlier (Sheeba *et al*. 2000).

From the running culture of each population, eggs laid over 2 h window on banana-jaggery food medium were collected for the assay. Exactly 30 eggs each were dispensed into 8 dram vials containing ~6 ml food. Forty such vials were set-up for each population. Eight vials of each population were introduced into each of five light regimes: continuous light (LL), continuous darkness (DD), light / dark (LD) cycles of 10:10 h (*T20*), 12:12 h (*T24*), 14:14 h (*T28*). Thus, a total of 160 vials were set-up (8 vials × 4 populations × 5 light regimes). The vials were introduced in the respective light regimes at 20:00 h, when the lights went off in all light : dark regimes, whereas in the case of the two constant light regimes, the vials were introduced into DD or LL at a time corresponding to the lights-off in the periodic light regimes.

Fluorescent white light of intensity ~100 lux was used for the light phase, whereas the dark phase consisted of a red light (l >650 nm) for ease of observation and handling of vials. Red light with wavelengths >650nm is considered to be 'safe light' in dark phase as these wavelengths of light are not perceived by the *Drosophila* circadian system (Chandrashekaran *et al*. 1973). Temperature and relative humidity in all light regimes was continuously monitored using a thermo-hygrograph (Quartz Precision Thermo-Hygrograph, Isuzu Seisakusho Co, LTD). Temperature was maintained at 25°C (± 1°C) and relative humidity at ~70% (± 5%).

After all the individuals eclosed, pupation height was measured as the distance from the surface of food medium to the point between the anterior spiracles of the pupa, with any pupa touching the surface of the medium being given a pupation height of zero (Mueller and Sweet 1986). Individual pupation heights were used as data for a mixed model nested analysis of variance (ANOVA) in which replicate populations were treated as random blocks, light regime as a fixed factor crossed with block, and vial as a random factor nested within the light regime × block interaction. Multiple comparisons among mean pupation height in different light regimes were done using Fisher's LSD test. All

analyses were implemented on STATISTICA™ for Windows Release 5.0 B (StatSoft, 1995).

Results and discussion

Light regime had a significant effect on pupation height (table 1), with the least mean pupation height being observed in *T28*, and the greatest mean pupation height being seen in DD (figure 1). The mean pupation height of populations did not differ significantly between DD and *T20*, between *T24* and *T28*, and between *T20* and LL respectively (figure 1). Mean pupation height in LL was significantly greater than that in either *T24* or *T28*, and significantly lower than that in DD (figure 1). While our observation that pupation height in LL was less than that in DD confirms earlier observations (Markow 1979), the fact that pupation height in *T24* and *T28* is significantly less than that in LL clearly suggests that there is more to light regime effects on pupation height than just a behavioural inhibition of pupation height by light. If light suppresses the tendency of larvae to climb high up the walls of vials for pupation, it is hard to see why pupation height in *T24* and *T28* should be less than that in LL. At most, depending on the phase of initiation of wandering relative to lights on or off, larvae in *T24* and *T28* could experience light throughout their wandering period and, consequently, would be expected to have pupation heights similar to those seen under LL. If larvae in *T24* and *T28* experienced darkness for part of their wandering stage, one would expect pupation height in these regimes to be intermediate between that seen in LL and DD.

At the very least, it is clear that these results cannot be explained by the behavioural inhibition of pupation height by light, as suggested by Markow (1979). There is clearly some more complicated effect of light regime on pupation behaviour at work here, and there may well be some involvement of the circadian organization in determining pupation height. What exact form this involvement takes is, as yet, not clear, and would probably require not only more studies but also a better understanding of the genetic control of the initiation of wandering and pupariation, and the length of the wandering phase. In *D. melanogaster*, larvae attain a very critical developmental stage marked by a small ecdysone pulse, early in the third instar, and a commitment to metamorphosis is made at this point (Berreur *et al*. 1979). The attainment of this critical developmental stage of 'no return' appears to be correlated with the attainment of a certain critical size / weight (Bakker 1959; Robertson 1963). Late in the third instar, a large ecdysone pulse sets the stage for pupariation, which occurs about 5 h after the pulse; another ecdysone pulse about 10 h after pupariation finally sets into motion a cascade of events leading to pupa formation (White *et al*. 1997, 1999). Studies on lepidopterans indicate that the timing of the prepupariation hormonal pulse is determined by the clearing of juvenile hormone from the hemolymph, and further subjected to circadian gating, yielding a circadian rhythm in pupariation (Davidowitz *et al*. 2003), but it is not clear if this is so in *D. melanogaster* (Pittendrigh and Skopik 1970). It has also been shown recently that the downregulation of *Drosophila* neuropeptide F seems to play a role in determining the transition from feeding to wandering in later third instar larvae (Wu *et al*. 2003). The role of hormonal influences on the regulation of this gene is, however, not yet known. Incidentally, it is known that the eclosion rhythm in the populations used in this study entrains to *T20*, *T24* and *T28*, and that its free-running period in DD is 22.85 h (Paranjpe *et al*. 2003), indicating an inverse

Table 1. Results of analysis of variance on pupation height in the four LL populations in the five different aperiodic and periodic light regime. The four replicate populations were treated as random blocks, crossed with light regime as a fixed factor. Vial was treated as a random factor nested in the light regime × block interaction.

Effect	df	MS	*F*	*p*
Light Regime	4	54.63	19.18	<0.001
Block	3	36.38	21.08	<0.001
Vial	140	1.73	1.52	<0.001
Light Regime × Block	12	2.85	1.65	0.085
Error	4110	1.13		

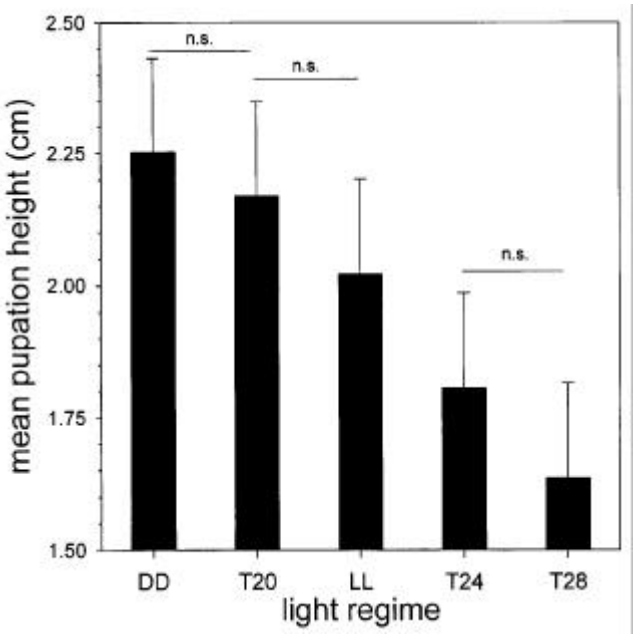

Figure 1. Mean pupation heights of the four LL populations in different periodic and aperiodic light regimes. The error bars are 95% confidence intervals based on the appropriate denominator mean square term in the mixed model nested ANOVA and can, therefore, be used for visual hypothesis testing. Horizontal bars span light regimes in which the mean pupation heights did not significantly differ (n.s.: not significant at $P = 0.05$).

relationship between the period of the eclosion rhythm and pupation height. The functional significance of this relationship, if any, is not yet clear to us. Overall, our results indicate that light / dark regime and circadian organization may impinge upon the complex of hormonal and genetic regulation that results in the initiation of wandering and pupariation in a subtle way, suggesting that further investigation of light regime / circadian clock effects on pupation need to be done.

Our results also underscore the importance of incorporating circadian organization explicitly into our thinking about life-history evolution (Prasad and Joshi 2003). Laboratory studies of life-history evolution in *D. melanogaster* have been variously conducted under LL, LD 12:12 h, LD 16:8 h, and sometimes even under fluctuating LD regimes wherein the timing of lights on and off is a function of when people enter or leave the laboratory. It is now clear that light regime affects most major life-history traits in *D. melanogaster*, including development time, fecundity and lifespan (Sheeba *et al*. 1999, 2000), and given that over 100 genes involved in a variety of functions are transcribed in a circadian manner in *D. melanogaster* (McDonald and Rosbash 2001), it is likely that many life-history related traits will also turn out to be affected by light regime. Very often, inconsistencies across laboratories have been observed in correlated responses to selection on life-history traits in *D. melanogaster*, and these have typically been discussed in the context of differences in assay conditions and initial genetic composition of populations used in the different studies (Ackermann *et al*. 2001). However, laboratories often differ in the light regime used in selection experiments, and it is possible that the differences in light regime also contribute to some of the observed discrepancies between laboratories (Prasad and Joshi 2003).

In the specific case of pupation height in populations of *D. melanogaster* selected for adaptation to larval crowding, in one study selected populations evolved greater pupation height than controls (Mueller and Sweet 1986), whereas in another study crowding adapted populations did evolve greater pupation height than controls early in the selection, but the difference became insignificant later on (Joshi and Mueller 1996). The amelioration of the pupation height difference between selected and control populations in only one of these studies was explained in terms of differences between the studies in the culture container and the consistency of food (Joshi and Mueller 1996). However, another difference between these studies was that one was carried out under LD 16:8 h (Mueller and Sweet 1986) whereas the other was conducted under LL (Joshi and Mueller 1996). In view of the effect of light regime on pupation height reported in this paper, it is possible that light regime differentially affected the phenotypic expression of underlying genetic variation for pupation height in the two studies, contributing to the different evolutionary dynamics observed. In conclusion, we would like to stress that our results support the view that life-history evolution studies need to take greater cognizance of the ubiquity of circadian phenomena in living systems, and their sensitivity to the photic environment.

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